INFLUENCE OF PROBIOTIC ORGANISMS ON PROTEOLYTIC PATTERN, RELEASE OF BIOACTIVE COMPOUNDS AND SENSORY ATTRIBUTES OF CHEDDAR CHEESE

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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B. Appl. Sc. (Hons) (Food Sci. Tech.)

2007

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Dedicated to the '*Ong Family*'

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I. Abstract

The overall objective was to study the influence of probiotic organisms on the proteolytic pattern, sensory evaluation and release of bioactive peptides in Cheddar cheeses. The study consists of three main parts. The first part of the study aimed at development of Cheddar cheeses with the addition of probiotic organisms. Three batches of Cheddar cheeses including a control cheese with starter lactococci only (Batch 1M) and two probiotic cheeses with starter lactococci and probiotic organisms including *Bifidobacterium longum* 1941, *Lactobacillus casei* 279 and *Lb. acidophilus* 4962 (Batch 2M) or *B. animalis* subsp. *lactis* LAFTI®B94, *Lb. casei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 (Batch 3M) were made. In a separate experiment, seven batches of Cheddar cheeses including a control cheese with starter lactococci only (Batch 1) and six probiotic cheeses with starter lactococci and probiotic *B. longum* 1941 (Batch 2), *B. animalis* subsp. *lactis* LAFTI®B94 (Batch 3), *Lb. casei* 279 (Batch 4), *Lb. casei* LAFTI®L26 (Batch 5), *Lb. acidophilus* 4962 (Batch 6) or *Lb. acidophilus* LAFTI®L10 (Batch 7) were made. All cheeses were made in triplicate and ripened at 4°C for 24 wk to study the influence of the probiotic adjuncts on proteolytic patterns and organic acid profiles of the cheeses.

The probiotic bacteria were added as an adjunct together with the starter lactococci and no alteration in the cheese-making procedure was necessary for incorporation of probiotic organisms into Cheddar cheese. The probiotic adjuncts survived at high levels at the end of cheese-making process (8.0 - 9.0 $\log_{10}$ cfu g^{-1}). They were also able to maintain viability at > 7.5 $\log_{10}$ cfu g^{-1} at the end of ripening at 4°C for 24 wk. The counts of lactococci in the cheeses decreased by one to two logs at the end of ripening, but counts were not significantly different ($P > 0.05$) between the control and probiotic cheeses. Addition of probiotic organisms did not alter the composition of cheeses (fat, protein, moisture, salt contents), but acetic acid concentration in probiotic cheeses (Batches 2M & 3M) was significantly higher than that of the control cheese (Batch 1M). Assessment of proteolysis during ripening showed that the concentrations of trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) in probiotic cheeses (Batches 2M & 3M) were significantly higher ($P < 0.05$) than those of the control cheeses (Batch 1M). Hydrolysis of α_{s1}-casein (CN) in the cheeses was 19.28, 46.99 and 63.42 after 24 wk of ripening in Batch 1M, Batch 2M and Batch 3M, respectively. The results show that Cheddar cheeses can be an effective vehicle for delivery of probiotic organisms to the consumer.

Each probiotic organisms influenced the organic acid profiles and the proteolytic pattern of Cheddar cheese in different ways. Acetic acid concentration in probiotic cheeses with *B. longum* 1941, *B. animalis* subsp. *lactis* LAFTI®B94, *Lb. casei* 279 or *Lb. casei* LAFTI®L26 (Batches 2-5) was higher as compared to other cheeses. Cheeses made with the addition of *Lb. casei* 279 (Batch 4) and *Lb. casei* LAFTI®L26 (Batch 5) showed higher level of α_{s1}-CN and β-CN hydrolysis when compared to other cheeses. Although *Bifidobacterium* sp. was found to be weakly proteolytic, cheeses with the addition of that species had the highest concentration of PTA-SN.

The sensory properties of the probiotic Cheddar cheeses were assessed after ripening for 9 months at 4°C. Probiotic cheeses except those with *Lb. acidophilus* 4962 (Batch 6) were found to be significantly different ($P < 0.05$) from the control cheeses made without any probiotic organism (Batch 1). The acceptability scores among the cheeses, however, were not significantly different except for that with *Lb. casei* 279 (Batch 4). Acceptability scores of cheese with *Lb. casei* 279 (Batch 4) was significantly lower ($P < 0.05$) than that of the control cheese with bitterness and sour-acid taste as the major defects. Although concentration of acetic acid in probiotic cheeses was higher than the control cheese, there was no significant correlation between the sensory scores of vinegary and the acetic acid concentration ($P > 0.05$). Scores of vinegary also did not influence the acceptability of the cheeses ($P > 0.05$). Increase proteolysis in probiotic cheeses did not influence the scores of Cheddary attribute ($P > 0.05$). There were positive correlations ($P < 0.05$) between the scores of bitterness and the level of water-soluble nitrogen. The sensory results showed that some of the probiotic microorganims used in this study can be applied successfully in Cheddar cheeses with acceptable sensory profiles.

The increase in proteolysis in cheeses with the addition of probiotic organisms indicated that more peptides were released into the probiotic cheeses. The angiotensin converting enzyme (ACE)-inhibitory activity of the water soluble extract (WSE) of cheeses was determined during ripening at 4°C. The IC_{50} (concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity) was the lowest after 24 wk of ripening in the probiotic cheeses (0.20 - 0.29 mg mL^{-1}) compared to 36 wk for cheeses without any probiotic (0.28 – 0.31 mg mL^{-1}). Cheeses made with *Lb. casei* 279 (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5) with the highest degree of proteolysis and a control cheese (Batch 1) were selected for isolation and purification of bioactive peptides. Water soluble extracts of each cheese were subjected to several stages of chromatographic fractionation. Inhibitory activity found in the crude fractions ranged from 0.1 to 2.0 mg mL^{-1}. Fractions with the highest activity were purified using a second stage chromatography. Various ACE-

inhibitory peptides corresponding to the α_{s1}-casein [(f 1-6), (f 1-7), (f 1-9), (f 24-32) and (f 102-110)] and β-casein [(f 47-52) and (f 193-209)] were identified. Our results suggested that ACE inhibition in Cheddar cheeses was dependent on proteolysis to a certain extent. Probiotics *Lb. casei* 279 or *Lb. casei* LAFTI®L26 used in this study have the potential to improve the ACE-inhibitory activity of Cheddar cheeses.

The second part of the study investigated the influence of ripening temperatures at 4 and 8°C on the viability of probiotic organisms, composition of cheeses, production of organic acids, proteolytic pattern, sensory characteristics and ACE-inhibitory activity of Cheddar cheeses. Seven batches of Cheddar cheeses were made in triplicate as in part I (Batches 1-7). The cheeses were divided into two equal portions and assigned to ripening at 4 and 8°C for 24 wk. The moisture content and pH of cheeses decreased significantly after ripening for 24 wk, depending on the probiotic adjuncts and ripening temperature used ($P < 0.05$). Ripening at 8°C accelerated the loss of starter lactococci as compared to 4°C. The counts of starter lactococci in cheeses produced with *B. animalis* LAFTI®B94 subsp. *lactis*, *Lb. casei* LAFTI®L26 or *Lb. acidophilus* 4962 ripened at 8ºC were significantly lower than that ripened at 4ºC ($P < 0.05$) at 24 wk. Probiotic organisms remained viable at the end of 24 wk and their viability was not affected by the ripening temperatures ($P > 0.05$). There were significant effects of the type of probiotic organisms used, ripening time, ripening temperatures and their interactions on the concentration of lactic, and acetic acids in the cheeses ($P < 0.05$). Lactic acid concentration was the highest in cheeses with *Lb. casei* 279 or *Lb. casei* LAFTI®L26 ripened at 8°C. The acetic acid concentration in cheeses made with *Bifidobacterium* sp. or *Lb. casei* sp. was significantly higher than that of the control cheese ($P < 0.05$). Citric, propionic and succinic acids contents of the cheeses were not significantly affected by the type of probiotic organisms or ripening temperatures used ($P > 0.05$).

Ripening at 8°C as compared to 4°C also increased the level of proteolysis of the cheeses. Product of proteolysis and organic acids released during ripening were shown to be important for the flavour of Cheddar cheeses. There were positive and significant correlations between the levels of soluble nitrogen, lactic, acetic and butyric acids, percentage hydrolysis of α_{s1}-CN and β-CN to the scores of cheddary flavour ($P < 0.05$). Scores for sour-acid and vinegary were higher in cheeses with the addition of *Bifidobacterium* sp. or *Lb. casei* 279 ripened at 8°C. The scores were positively and significantly correlated to the level of lactic, acetic and free amino acids in the cheeses ($P < 0.05$). ACE-inhibitory activity of the cheeses ripened at 4 and 8°C was maximum at 24 wk and remained relatively constant after that period. Cheeses made with the addition of *Lb.*

casei 279, *Lb. casei* LAFTI®L26 or *Lb. acidophilus* LAFTI®L10 had significantly higher ($P < 0.05$) ACE-inhibitory activity than those without any probiotic adjunct after 24 wk at 4 and 8°C. The IC_{50} of cheeses ripened at 4°C was not significantly different ($P > 0.05$) to that ripened at 8°C. The lowest value of the IC_{50} (0.13 mg mL^{-1}) and therefore the highest ACE-inhibitory activity corresponded to the cheese made with the addition of *Lb. acidophilus* LAFTI®L10 ripened at 8°C. Several ACE-inhibitory peptides from WSE of cheeses with *Lb. acidophilus* LAFTI®L10 were isolated and identified as κ-CN (f 96-102), α_{s1}-CN (f 1-9), α_{s1}-CN (f 1-7), α_{s1}-CN (f 1-6), α_{s1}-CN (f 24-32) and β-CN (f 193-209). Most of the ACE-inhibitory peptides accumulated during ripening, and as proteolysis proceeded, some of the peptides were hydrolyzed into smaller peptides. The results of the second part of the study showed that both 4 and 8°C can be used for ripening of probiotic Cheddar cheeses. Cheeses made with *Lb. acidophilus* LAFTI®L10 also had good potential for the development of Cheddar cheeses with bioactive properties. Cheeses made with *Lb. acidophilus* LAFTI®L10, however, was not the cheeses with the highest level of proteolysis.

Part 3 of the study investigated the use of elevated ripening temperature of 12°C and the addition of a highly proteolytic strain of *Lb. helveticus* to improve the proteolysis and the ACE-inhibitory activity of probiotic cheeses made with *Lb. acidophilus* LAFTI®L10 adjunct. Cheddar cheeses were made with starter lactococci (control), *Lb. acidophilus* LAFTI®L10 and starter lactococci (L10) or *Lb. acidophilus* LAFTI®L10, *Lb. helveticus* H100 and starter lactococci (H100). The counts of probiotic organisms in L10 cheeses remained at $>10^6$ cfu g^{-1} after 24 wk of ripening at 4, 8 and 12°C. Concentrations of lactic, acetic and propionic acids of the L10 and H100 cheeses were significantly higher than those of the control cheeses after 24 wk of ripening ($P < 0.05$). Proteolysis of the cheeses improved as the ripening temperature increased. WSN, TCA-SN and PTA-SN of L10 and H100 cheeses were significantly higher than those of the control cheeses ($P < 0.05$). Increase in ripening temperature from 4°C to 8 and 12°C increased the percentage of ACE-inhibition. The IC_{50} value among cheeses ripened at 4, 8 and 12°C, however, was not significantly different ($P > 0.05$). Addition of *Lb. helveticus* H100 did not further improve the proteolysis and the ACE-inhibitory activity of the probiotic cheeses. Overall results of the study showed that some probiotic organisms used in this study can be added successfully in Cheddar cheeses with acceptable organic acid, proteolysis and sensory profiles. Addition of probiotic *Lb. casei* 279, *Lb. casei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 has the potential to improve the ACE-inhibitory activity of Cheddar cheeses.

II. Certificate

Dr. Nagendra P. Shah (M.Sc., Ph.D)
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CERTIFICATE

This is to certify that the thesis entitled "Influence of Probiotic Organisms on Proteolytic Pattern, Release of Bioactive Compounds and Sensory Attributes of Cheddar Cheese" submitted by **Lydia Ong** in partial fulfillment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia
Date:

Prof. N.P. Shah
(Principal Supervisor)

III. Declaration

"I, Lydia Ong, declare that this thesis entitled "INFLUENCE OF PROBIOTIC ORGANISMS ON PROTEOLYTIC PATTERN, RELEASE OF BIOACTIVE COMPOUNDS AND SENSORY ATTRIBUTES OF CHEDDAR CHEESE" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work."

Werribee, Australia

Date: Lydia Ong

IV. Acknowledgement

My sincere thanks and gratitude go to my Principal Supervisor, Professor Nagendra P. Shah, for his guidance, valuable discussions and suggestions throughout the course of study and in preparation of manuscripts. His dedication to this work and the manner in which he efficiently dealt with problems have certainly contributed greatly to the progress of this project.

I would like to thank Dr. Anders Henriksson (DSM Food Specialties, NSW) for his assistance in providing materials for the cheese production and Dr Todor Vasiljevic for the valuable discussions and his help in statistical analysis. Many thanks are due to Mr. Dale Tomlinson (Laboratory Manager) and all laboratory technicians, Ira, Michael, Stacey, Joe, Marianne, Min for their assistance in the use of equipments, ordering chemicals and for their friendship.

I thank my fellow research students who I have worked with over the past three years including Min Tze Liong, Wun Keat Ding, Anne Lise Tang, Sudinna Hewakapuge, Shruti Harnal, Michelle Vaughn, Paul Capela, Osaana Donkor and Daniel Otieno.

I would like to express my sincere gratitude, love and respect to my father, Mr. Bendy Andries Liany and my mother, Mrs. Liong Fong Eng for their never ending love over the past 26 years. My love and thanks go to my sister, Elisa and my brothers, Budiyanto and Jansen, for their encouragement and for making my three years journey very colourful. I appreciate the great support from my flat mates and friends, Fetricia Tan, Dr. Ronny Widjaja, Ulina Mulio and Melvany Tan for taking a good care of me during my stay in Australia.

I am thankful to the Australian Research Council for providing an Australian Postgraduate Award (Industry) for this research project and I also appreciate the contribution from DSM Food Specialties Pty. Ltd. as a co-sponsor of the above scholarship.

V. List of Publications

Refereed journal publications

Ong, L., Henriksson, A., & Shah, N.P. (2006). Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus, Lb. casei, Lb. paracasei, Bifidobacterium* sp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal, 16,* 446 – 456.

Ong, L., Henriksson, A., & Shah, N.P. (2007a). Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus, Lb. paracasei, Lb. casei* and *Bifidobacterium* sp. *International Dairy Journal, 17,* 67 - 78.

Ong, L., Henriksson, A., & Shah, N.P. (2007b). Chemical analysis and sensory evaluation of Cheddar cheeses produced with *Lactobacillus acidophilus, Lb. casei, Lb. paracasei* and *Bifidobacterium* sp. *International Dairy Journal, 17,* 937 - 945.

Ong, L., Henriksson, A., & Shah, N.P. (2007c). Angiotensin converting enzyme-inhibitory activity of Cheddar cheeses made with the addition of probiotic *Lactobacillus casei* sp. *Lait, 87,* 149-165.

Ong, L., Henriksson, A., & Shah, N.P. (2007d). Probiotic Cheddar cheese Part I. Influence of ripening temperatures on survival of probiotic microorganisms, cheese composition and organic acid profiles. *International Dairy Journal* (revised manuscript submitted in April 2007).

Ong, L., Henriksson, A., & Shah, N.P. (2007e). Probiotic Cheddar cheese Part II. Influence of ripening temperatures on proteolysis and sensory characteristic of Cheddar cheeses. *International Dairy Journal* (revised manuscript submitted in April 2007).

Ong, L., Henriksson, A., & Shah, N.P. (2008a). Influence of ripening temperatures and probiotic adjuncts on the *in vitro* angiotensin converting enzyme-inhibitory activity of Cheddar cheeses. *LWT Food Science and Technology,* published online (doi:10.10161j.lwt.2007.11.026)

Ong, L. & Shah, N.P. (2008b). Influence of probiotic *Lactobacillus acidophilus* and *Lb. helveticus* on proteolysis, organic acid profiles and ACE-inhibitory activity of Cheddar cheeses ripened at 4, 8 and 12^{o}C. *Journal of Food Science,* 73, M111-120.

Chaired poster presentation

Ong, L., Shah, N.P., & Henriksson, A. (2005). Influence of probiotic adjuncts *Lactobacillus acidophilus, Lb. casei, Lb. paracasei, Bifidobacterium* sp. on proteolytic patterns and organic acid profile of Cheddar cheese. A paper presented at Institute of Food Technologist annual meetings 17-20 July 2005, New Orleans, Louisiana, USA (Abstract No.71A-39).

Chaired oral presentations

Ong, L., Shah, N.P., & Henriksson, A. (2005). Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus, Lb. casei, Lb. paracasei, Bifidobacterium* sp. and the influence of these bacteria on proteolytic patterns and production of organic acid. A paper presented at Australian Institute of Food Science and Technology 38th Annual Meeting 10 – 13 July 2005, Sydney, NSW, Australia (Abstract No. A83).

Ong, L., Shah, N.P., & Henriksson, A. (2005). Effects of incorporation of probiotic *Lactobacillus acidophilus, Lb. casei, Lb. paracasei, Bifidobacterium* spp. on proteolytic patterns and production of organic acid in Cheddar cheese. A paper presented at American Dairy Science Association Annual Meeting 24-28 July 2005, Cincinnati, Ohio, USA (Abstract No. 364). *Journal of Dairy Science, 88,* S1: 232.

Ong, L., Shah, N.P., & Henriksson, A. (2006). Antihypertensive activity of probiotic Cheddar cheeses produced with probiotic *Lactobacillus casei* or *L. paracasei.* **Award winning paper, "Manfred Kroger Graduate Paper Competition"** presented at the Institute of Food Technologist annual meetings 24-28 June 2006, Orlando, Florida, USA (Abstract No. 015-02).

Ong, L., Shah, N.P., & Henriksson, A. (2006). Isolation and purification of angiotensin-I-converting enzyme inhibitory peptides from Cheddar cheeses with the addition of probiotic *Lactobacillus casei* or *L. paracasei.* A paper presented at American Dairy Science Association Annual Meeting 9-13 July 2006, Minneapolis, Minnesota, USA (Abstract No. 16295).

Ong, L., Shah, N.P., & Henriksson, A. (2007). Influence of probiotic microorganisms and ripening conditions on proteolysis, sensory evaluation and the release of antihypertensive peptides in Cheddar cheese. A paper presented at the Australian Institute of Food Science and Technology 40th Annual Meeting 24-27 June 2007, Melbourne, Victoria, Australia (Abstract No. A53).

Ong, L., Shah, N.P., & Henriksson, A. (2007). Probiotic Cheddar cheeses delivering bioactive properties. A paper presented for the "**Malcolm Bird Award"** at the Australian Institute of Food Science and Technology 40th Annual Meeting 24-27 June 2007, Melbourne, Victoria, Australia (Abstract No. MB2).

Ong, L., Shah, N.P., & Henriksson, A. (2007). Addition of probiotic microorganisms to improve proteolysis, sensory evaluation and the release of antihypertensive peptides in Cheddar cheeses ripened at 4 and 8ºC. A paper presented at American Dairy Science Association Annual Meeting 8-12 July 2007, San Antonio, Texas, USA (Abstract No. 820).

Awards received

"Best Postgraduate Student Seminar Award 2005" in the category of advanced postgraduate students. Awarded by Victoria University, School of Molecular Sciences, 2005.

"Manfred Kroger Graduate Paper Competition Award 2006" (1st place) at the Institute of Food Technologist annual meeting, Orlando, Florida, USA, 24-28 June, 2006.

Secomb Conference and Travel Fund 2006. Monetary support awarded by Victoria University to cover a part of travel expenses to the 2006 IFT annual meetings, Orlando, Florida, USA, 24-28 June, 2006.

"Malcolm Bird Commemorative Award 2007" (1st place) at the Australian Institute of Food Science and Technology 40th Annual Meeting, Melbourne, Victoria, Australia, 24-27 June, 2007.

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IX. List of Abbreviations

ACE = angiotensin-I-converting enzyme

AOAC = Association of Official Analytical Chemists

ANOVA = analysis of variance

BSA = bovine serum albumin

CAF = chemically assisted fractionation

CFU = colony forming unit

CN = casein

Da = dalton

FAA = free amino acids

FDM = fat in dry matter

g = gram

h = hour

HHL = hippuryl-histidiyl-leucine

RP-HPLC = reverse phase-high performance liquid chromatography

IC_{50} = 50% inhibitory concentration

L = litre

LAB = lactic acid bacteria

MALDI-TOF = matrix-assisted laser desorption/ionization time-of-flight

MS = mass spectrophotometry

mg = milligram

min = minute

mL = milliliter

mm = millimeter

mo = month

MRS = DeMan Rogosa Sharpe

MW = molecular weight

NSLAB = non starter lactic acid bacteria

RSM = reconstituted skim milk

PAGE = polyacrylamide gel electrophoresis

PTA = phosphotungstic acid

s = second

SDM = salt in dry matter

SDS = sodium dodecyl sulphate

SLAB = starter lactic acid bacteria

SM = salt in moisture

SN = soluble nitrogen

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

TN = total nitrogen

WSE = water soluble extract

WSN = water soluble nitrogen

v/v = volume per volume

v/w = volume per weight

wk = week

α = alpha

β = beta

κ = kappa

µg = microgram

ºC = degree Celsius

Three and one letter abbreviations for amino acids

Arg	**R**	Arginine
Asp	**D**	Aspartic acid
Glu	**E**	Glutamic acid
Asn	**N**	Asparagine
Lys	**K**	Lysine
Gln	**Q**	Glutamine
His	**H**	Histidine
Ser	**S**	Serine
Thr	**T**	Threonine
Tyr	**Y**	Tyrosine
Gly	**G**	Glycine
Pro	**P**	Proline
Cys	**C**	Cystine
Ala	**A**	Alanine
Trp	**W**	Tryptophan
Met	**M**	Methionine
Phe	**F**	Phenylalanine
Val	**V**	Valine
Ile	**I**	Isoleucine
Leu	**L**	Leucine

1.0 Introduction to Thesis

Cheese consumption and production continue to increase over the past decades (Dairy Australia, 2006). The popularity of cheese is attributed to its great taste, the availability of new and different varieties, convenience and versatility of use, and its nutritional value. More than 300 varieties (e.g., American, Cheddar, Mozzarella, Colby) of cheese are available in various flavours and forms (e.g., chunks, slices, cubes, shredded, grated/crumbled, string/stick, spreads), and packages to meet consumers needs (Fox & McSweeney, 2004). Cheese contains a high concentration of essential nutrients, in particular high quality protein and calcium, as well as other nutrients such as phosphorus, zinc, vitamin A, riboflavin, and vitamin B12. In addition to its nutritional contribution to the diet, consumption of cheese has been demonstrated to reduce the risk of dental caries through various mechanisms (Kashket & DePaola, 2002). Ripened type cheeses, such as Cheddar, contain little or no lactose. For this reason, cheese can also be an important source of calcium and many other nutrients found in milk for lactose maldigesters or persons who have difficulty digesting lactose or milk sugar (Suarez et al., 1998).

Probiotic bacteria are defined as 'living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition' (Ross et al., 2002). A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhea, anticarcinogenic properties, reduction of blood cholesterol and improvement in immunity (Ballongue, 1993; Shah & Wu, 1999; Shah, 2000a,c). High levels of daily consumption of probiotic bacteria, however, are required to confer health benefits. For dietary cultures to be beneficial in food systems, they are expected to be viable in the food until the time of consumption and present at levels of at least 10^7 viable cells per gram or milliliter of a product (Ishibashi & Shimamura, 1993). For this reason, it is important to know changes in the numbers of viable bacteria during storage period.

A number of food products including yoghurt (Kailasapathy & Rybka, 1997), frozen fermented dairy deserts (Ravula & Shah, 1998a), spray dried milk powder (Stanton et al., 2001), cheeses (Stanton et al., 2001), ice cream (Haynes & Playne, 2002), coleslaw (Rodgers & Odongo, 2002), freeze-dried yoghurt (Capela et al., 2006) and fruit juices (Saarela et al., 2006) have been employed as delivery vehicles for probiotics to consumer. The most popular food delivery systems for probiotics have been fermented milk and

yoghurt. There are, however, numerous challenges related to the instability of some probiotic strains in these products. The condition typical of many yoghurts and other fermented milk including the low pH and the aerobic conditions of production and packaging, the presence of H_2O_2 and inhibitory substances produce by the starter bacteria may result in decreases in the count of probiotic in the final product. A few studies have shown that many commercial products have failed to successfully deliver the required level of viable cells of probiotic bacteria (Shah & Lankaputhra, 1997; Dave & Shah, 1997).

Cheeses have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to gastrointestinal (GI)-tract in that cheeses have a higher pH and a more solid consistency, where the matrix of the cheese and its relatively high fat content may offer protection to probiotic bacteria during passage through the gastrointestinal tract. Cheeses also have higher buffering capacity than yoghurt (Gardiner et al., 1999). Cheddar cheeses, however, have a long ripening time, hence development of probiotic Cheddar cheeses requires careful examination of the suitability of particular strain(s) to maintain viability throughout ripening and shelf life (Ross et al, 2002).

A number of studies have addressed development of probiotic cheeses using Canestrato Pugliese cheese (Corbo et al., 2001), Cheddar cheese (Dinakar & Mistry, 1994; Gardiner et al., 1998; Daigle et al., 1999; McBrearty et al., 2001; Phillips et al., 2006), cottage cheese (Blanchette et al., 1996), Crescenza cheese (Gobbetti et al., 1997), Fresco soft cheese (Vinderola et al., 2000), goat cheese (Gomes & Malcata, 1998), Gouda cheese (Gomes et al., 1995), Minas fresh cheese (Buriti et al., 2005a,b), semi-hard cheese (Bergamini et al., 2006) and white cheese (Kasimoglu et al., 2004). While some studies have shown adequate survival of probiotic bacteria, others have found low counts of probiotic bacteria in cheeses (Blanchette et al., 1996; McBrearty et al., 2001). These studies demonstrated that cheeses can be a potential carrier for probiotic bacteria to the consumer. The capability of probiotic organisms to survive and perform well in cheese, however, varies from strain to strain.

Six probiotic organisms have been selected for this project including *B. longum* 1941, *Lb. casei 279*, *Lb. acidophilus* 4962, *B. animalis* LAFTI®B94, *Lb. casei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10. Probiotic *B. longum* 1941, *Lb. casei 279* and *Lb. acidophilus* 4962 were obtained from Victoria University culture collection (Werribee, Vic, Australia), while LAFTI® probiotic bacteria including *B. animalis* subsp. *lactis* LAFTI®B94 (was previously classified as *B. lactis* LAFTI®B94), *Lb. casei* LAFTI®L26 (was previously classified as *Lb. paracasei* LAFTI®L26) and *Lb. acidophilus* LAFTI®L10 were provided by DSM Food Specialties (Moorebank, NSW, Australia). The probiotic organisms were

selected on the basis of their acid and bile tolerance, adhesion to intestinal cell line, anticarcinogenic properties, oxygen sensitivity, and their ability to modify gut microflora of human subjects (Lankaputhra & Shah, 1998; McIntosh, 1999; Crittenden et al., 2001; Pidcock et al., 2002; Liong & Shah, 2004). One of the broad aims of this study was to develop probiotic Cheddar cheeses using the selected probiotic organisms and to study the influence of these organisms on product quality, in particular the proteolytic pattern of the cheeses.

Proteolysis is one of the most complex biochemical events which occur during cheese ripening. Proteolysis in probiotic Cheddar cheeses is catalysed by proteinases and peptidases from several sources including indigenous enzyme from the milk, coagulant, starter lactic acid bacteria (SLAB), non-starter lactic acid bacteria (NSLAB) and probiotic adjuncts. The activities of these enzymes hydrolyze caseins (α_{s1}-, α_{s2}-, β- and κ-casein) to smaller peptides and amino acids, which contribute to flavour and texture of the cheeses (McSweeney & Sousa, 2000).

In addition to the role of these enzymes to overall proteolysis during cheese ripening, they may also contribute to a release of biologically-active peptides such as angiotensin converting enzyme (ACE)-inhibitory peptides. ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) plays a crucial role in the regulation of blood pressure and cardiovascular function. ACE converts the inactive decapeptide angiotensin-I by cleaving dipeptide from the C-terminus into angiotensin-II, a potent vasoconstrictor. Angiotensin-II is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure (Johnston, 1992). In the Kallikrein-Kinin system, ACE catalyses the degradation of bradykinin, a vasodilatory nonapeptide (Li et al., 2004). Inhibition of ACE is thus considered a useful therapeutic approach in treatment of hypertension.

The first reported competitive inhibitor of ACE is the naturally occurring peptides in snake venom (Ondetti et al., 1971). Smacchi and Gobbetti (1998) reported that peptides isolated from Italian cheeses (Crescenza, Gorgonzola, Mozzarella and Italico) were effective in reducing activity of ACE. Some ACE-inhibitory peptides have also been isolated from other cheese varieties such Parmesan (Addeo et al., 1992), Gouda (Saito et al., 2000), Cheddar (Ryahanen et al., 2001), Manchego (Gomez-Ruiz et al., 2002) and cheese prepared by commercial enzymes (Neutrase® and Debitrase®) and *Lb. casei* enzymes (amino peptidase) (Haileselassie et al., 1998). To date, only a few studies have investigated the release of ACE-inhibitory peptides in cheeses made with the addition of probiotic bacteria.

Cheddar cheese ripening is a lengthy and costly process. Hence, any decrease in ripening time, without adversely affecting flavour and texture, would be of considerable economic benefit. The biochemical reaction which generates flavour compounds or flavour precursors in cheese is accelerated by increasing the ripening temperature. Thus, the use of elevated ripening temperature can provide a technologically simple method to achieve a significant acceleration of cheese ripening (Law, 2001). The growth of most NSLAB, however, increases with higher ripening temperature and fermentation of lactose by NSLAB produces organic acid by-products such as formic, acetic, butyric and propionic acids (Fox et al., 1993). Excess of these compounds impairs the flavour balance of Cheddar cheeses. An appropriate ripening temperature is thus required for the development of probiotic Cheddar cheeses with an optimum quality.

Addition of highly proteolytic strain of lactic acid bacteria (LAB) has also been reported to improve proteolysis of Cheddar cheeses. *Lb. helveticus* added as adjunct starter was previously shown to lyse during Cheddar cheese ripening releasing its intracellular enzymes, which led to an increase in proteolysis and improved cheese flavour (Kiernan et al., 2000). The proteolytic activity of *Lb. helveticus* has also been related to the release of biologically-active peptides present in certain fermented milk products (Nakamura et al., 1995; Yamamoto et al., 1999; Seppo et al., 2002; Fuglsang et al., 2003).

The specific aims of this project were:

1. to incorporate the selected probiotic organisms in Cheddar cheeses and examine the performance of these organisms in terms of their survival during cheese-making and ripening;
2. to investigate the influence of probiotic organisms on product quality as assessed by the composition of the cheeses, the proteolytic pattern, the organic acid profiles and the sensory evaluation of the cheeses;
3. to investigate the influence of probiotic organisms on the ACE-inhibitory activity of the cheeses and to isolate and identify ACE-inhibitory peptides derived from the probiotic cheeses;
4. to study the effect of ripening temperatures on the viability of probiotic organisms, the proteolytic pattern, organic acid profiles, sensory characteristics and the ACE-inhibitory activity of the cheeses;
5. to investigate the effectiveness of *Lb. helveticus* adjunct and elevated ripening temperature to further improve the proteolysis and the ACE-inhibitory activity of the probiotic cheeses.

With respects to the content of this thesis, Chapter 2.0 contains reviews of literatures and other relevant issues affecting this field of research. Chapters 3.0 and 4.0 report on the development of probiotic Cheddar cheeses using the selected probiotic organisms (used in combination or as individual strain). The influence of the probiotic organisms on the proteolytic pattern and organic acid profiles of Cheddar cheeses is also discussed in Chapters 3.0 and 4.0. Chapter 5.0 focuses specifically on the sensory evaluation of Cheddar cheeses made with the addition of the probiotic organisms. Chapter 6.0 examines the ACE-inhibitory activity of the probiotic Cheddar cheeses. The methods developed for the isolation and purification of ACE-inhibitory peptides from probiotic Cheddar cheeses are also detailed in Chapter 6.0. Chapter 7.0 examines the influence of ripening temperatures of 4 and 8°C on the proteolytic pattern, organic acid profiles, sensory characteristics and ACE-inhibitory activity of probiotic Cheddar cheeses. Chapter 8.0 investigates methods to improve the proteolysis and the ACE-inhibitory activity of probiotic Cheddar cheeses through the use of elevated ripening temperature and addition of a highly proteolytic strain of LAB. Base on the results from Chapters 3.0 to 8.0, the conclusions of the study are summarized in Chapter 9.0. Future directions of research are discussed in Chapter 10.0 and references are listed in Chapter 11.0. Finally, Chapter 12.0 is the appendices where samples of chromatograms and other relevant data needed to support the main results of the study are included.

2.0 Literature Review

2.1. Probiotic organisms

The concept of probiotic was first introduced by Elie Metchnikoff, who observed that the consumption of fermented milk could reverse putrefactive effects of the gut microflora (Metchnikoff, 1907). The term 'probiotic' was, however, first used by Lilly and Stillwell (1965) to describe "substances secreted by one microorganism which stimulated the growth of another". Parker (1974) defined probiotic as "organisms and substances which contribute to intestinal microbial balance" and Fuller (1989) redefined probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Recently Ross et al. (2002) defined probiotic as "living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition". Section 2.1 briefly reviews the history and health benefit of probiotic bacteria including genus *Bifidobacterium* (section 2.1.1) and genus *Lactobacillus* (section 2.1.2), the selection criteria for probiotic organisms (section 2.1.3) and application of probiotic organisms in food (section 2.1.4).

2.1.1 Characteristics of genus Bifidobacterium

Bifidobacteria are normally found in the gastro intestinal (GI) tract of humans and other animals. These bacteria are Gram positive and have a rod like appearance that tend to be clubbed with branch, forming the 'y' shaped rods as shown in Figure 2.1a. The microorganism was first isolated from the faeces of breast-fed-infants and was described as *Bacillus bifidus* at the Institute Pasteur by Tissier in 1899 (Ballongue, 2004). In 1923 the first edition of Bergey's Manual of Determinative Bacteriology introduced the name *Lactobacillus bifidus* (Bergey et al., 1923), and a year later Orla-Jensen proposed an independent genus, *Bifidobacterium* (Orla-Jensens, 1924). In 1968, De Vries and Stouthamer demonstrated the presence of fructose-6-phosphate phosphoketolase (F6PPk) in bifidobacteria and the absence of aldolase and glucose-6-phosphatase dehydrogenase, two enzymes found in lactobacilli. The independent genus was later accepted in 1974 in the eight edition of Bergey's Manual of Determinative Bacteriology (Buchanan &

Gibbons, 1974). Presently there are 30 species included in the genus *Bifidobacterium,* 10 of which are from human sources and 17 from intestinal tracts of animal or rumen (Table 2.1). Of these, six species from human origins, *B. adolescentis, B. breve, B. bifidum, B. lactis, B. infantis* and *B. longum* have been used in dairy products (Boylston et al., 2004).

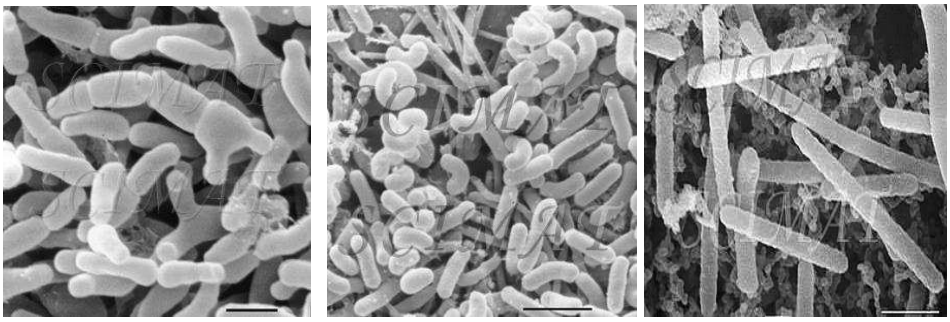


Figure 2.1. Micrograph of (a) *Bifidobacterium brevis* (bar 1 µm), (b) *Lactobacillus casei* (bar 1 µm) and (c) *Lactobacillus acidophilus* (bar 1 µm). Images are from SciMAT Photo Researchers, Inc.

Due to metabolic capacities of bifidobacteria, they are often included in the lactic acid bacteria (LAB) family, even though they are phylogenetically distinct with a high G + C (42%-67%) content and belong to the family of *Actinomycetaceae* (Klein et al., 1998). To successfully develop food product containing bifidobacteria, it is important to understand the growth characteristics of the organisms so that processing conditions can be manipulated to optimize their survival. Bifidobacteria are obligate anaerobes with an optimum growth temperature of 37°C to 41°C. Certain strains of bifidobacteria including *B. infantis, B. breve* and *B. longum* may have a mechanism by which they can avoid the toxicity of oxygen, as shown by their limited metabolic activity and production of acid under aerobic conditions (Shimamura et al., 1992). Optimum growth of bifidobacteria occurs at pH 6.5 to 7.0. The growth of bifidobacteria is inhibited below 5.0 or above 8.0 and is species and strain specific. Lankaputhra and Shah (1995) reported that under condition of typical digestion (pH 1.5-3.0), *B. longum* 1941 and *B. pseudolongum* 20097 were more tolerant to the acid conditions than the other seven strains of bifidobacteria evaluated.

It has now been conclusively demonstrated that some *Bifidobacterium* strains can survive intestinal transit and persist transiently within the colon (Fujiwara et al., 2001; Von Wright et al., 2002). The organisms used as probiotics to date appear to be safe. The therapeutic roles of bifidobacteria in animal model and in human are due to four major

mechanisms including modulation of the host immune system (Saavedra & Tschernia, 2002), resistance to infectious diseases such as against rotavirus diarrhoea and enteropathogens (Shu et al., 2001), control of inflammatory bowel disease such as Crohn's disease, ulcerative colitis and pouchitis (Marteau, 2002) and prevention of cancer (Yazakawa et al., 2001; Li et al., 2003).

Table 2.1. List of species of the genera *Bifidobacterium* and *Lactobacillus*

***Bifidobacterium* sp.**		***Lactobacillus* sp.**		
B. adolescentis	*B. indicum*	*Lb. acetotolerans*	*Lb. fermentum*	*Lb. orisa*
B. angulatum	*B. infantis*	*Lb. acidophilus*	*Lb. fructivorans*	*Lb. parabuchneri*
B. animalis	*B. lactis*	*Lb. agilis*	*Lb. fructosus*	*Lb. paracasei*
B. asteroides	*B. longum*	*Lb. alimentarius*	*Lb. gallinarum*	*Lb. pentosus*
B. bifidum	*B. magnum*	*Lb. amylophilus*	*Lb. gasseria*	*Lb. pontis*
B. boum	*B. merycicum*	*Lb. amylovorus*	*Lb. graminis*	*Lb. plantarum*
B. breve	*B. minimum*	*Lb. avarius*	*Lb. halotolerans*	*Lb. reuteria*
B. catenulatum	*B. pseudocatenulatum*	*Lb. bifermentans*	*Lb. hamsteri*	*Lb. rhamnosus*
B. choerinum	*B. pseudolongum*	*Lb. brevisa*	*Lb. helveticus*	*Lb. ruminis*
B. coryneforme	*B. pullorum*	*Lb. buchneri*	*Lb. hilgardii*	*Lb. sake*
B. dentium	*B. ruminantium*	*Lb. casei* subsp. *casei*	*Lb. jensenii*	*Lb. salivarius*
B. cuniculi	*B. saeculare*	*Lb. collinoides*	*Lb. Johnsonii*	*Lb. sanfrancisco*
B. gallicum	*B. subtile*	*Lb. confusus*	*Lb. kandleri*	*Lb. sharpeae*
B. gallinarum	*B. suis*	*Lb. coryniformis*	*Lb. kefir*	*Lb. suebicus*
B. globosuma	*B. thermophilum*	*Lb. crispatus*	*Lb. kefiranofaciens*	*Lb. vaccinostercus*
		Lb. curvatus	*Lb. malefermentans*	*Lb. vaginalis*
		Lb. delbrueckii	*Lb. mali*	*Lb. viridescens*
		Lb. farciminis	*Lb. minor*	*Lb. homohiochii*
			Lb. murinus	*Lb. intestinalis*

Adapted from Gomes and Malcata (1999)

2.1.2 Characteristics of genus Lactobacillus

At the same time as Tissier, who discovered *Bacillus bifidus*, Moro discovered a bacterium different from that of Tissier, which he identified as belonging to the genus *Lactobacillus*. Lactobacilli are in general characterized as Gram positive, non-sporeforming, non-flagellated rods or coccobacilli (Figures 2.1b & 2.1c). Some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. The growth of lactobacilli is optimum at pH 5.5-5.8 and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Axelsson, 2004). The genus is divided into three groups based on fermentation patterns including (1) homofermentative (which produces more than 85% lactic acid from glucose), (2) facultative heterofermentative (which produces only 50% lactic acid and considerable amounts of

ethanol, acetic acid and carbon dioxide) and, (3) obligate heterofermentative species (which produces DL-lactic acid, acetic acid and carbon dioxide). At present, 56 species of the genus *Lactobacillus* have been recognized (Table 2.1).

Lactobacilli are widespread in nature and many species have found application in the food industry. The therapeutic roles of some *Lactobacillus* sp. have been demonstrated. The ability of lactobacilli to convert lactose to lactic acid is used in the successful treatment of lactose intolerance. *Lactobacillus* sp. also inhibits the growth of harmful putrefactive microorganisms by lowering the pH of the intestinal environment and through the ability of some strains to produce bacteriocins and other metabolic products such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2) and diacetyl (Ouwehand & Vesterlund, 2004).

The bactericidal effect of H_2O_2 has been attributed to its strong oxidizing effect on the bacterial cell. Some of the H_2O_2- producing reactions scavenge oxygen, thereby creating anaerobic environment that is unfavourable for certain organisms. It has also been suggested that H_2O_2 production is particularly important for colonization of the urogenital tract by lactobacilli. Colonization of lactobacilli has been found to decrease the acquisition of human immune deficiency virus (HIV) infection, gonorrhoea and urinary tract infections (Fontaine et al., 1999). Formation of CO_2 creates anaerobic environment and CO_2 itself has an antimicrobial activity (Ouwehand & Vesterlund, 2004). Diacetyl on the other hand, is thought to react with the arginine-binding protein of Gram negative bacteria and thereby interfering with the utilization of this amino acid. Some strain of *Lactobacillus* such as *Lb. acidophilus, Lb. casei, Lb. helveticus, Lb. delbrueckii, Lb. lactis, Lb. plantarum, Lb. johnsonii, Lb. sake* and *Lb. curvatus* have been found to produce bacteriocins. These offer a more defined antimicrobial spectrum, ranging from only related strains to a wide variety of Gram positive and Gram negative bacteria (Ouwehand & Vesterlund, 2004).

2.1.3 Selection criteria for probiotic organisms

Probiotic organisms for human should have demonstrable health benefits and have ‘generally regarded as safe’ (GRAS) status, with a proven low risk of inducing or being associated with the etiology of disease. The probiotic organisms should preferably be of human origin. This is based on the observation that only human strains can be adhesive and colonize the human GI- tract, which is the first step in promoting colonization

resistance (Collins et al., 1998). For a strain to be used as probiotic microorganism, it must deliver a measured physiological benefit, substantiated by studies conducted in the target host (species specificity does occur, and for strains to be beneficial to a particular host, they should be isolated from the same species). The probiotic organisms must be able to survive and grow in the *in vivo* conditions of the desired site of administration thus must be able to tolerate low pH and high concentration of both conjugated and deconjugated bile acids. The probiotic organisms must be tolerated by the immune system and should not provoke the formation of antibodies against the probiotic organisms (Collins et al., 1998).

A number of probiotic organisms have been selected for this study on the basis of their acid and bile tolerance, adhesion to intestinal cell line, anticarcinogenic properties, oxygen sensitivity, and their ability to modify gut microflora of human subjects (Lankaputhra & Shah, 1998; Liong & Shah, 2004). Some of the probiotic organisms used in this study including *Bifidobacterium lactis* LAFTI®B94 (now classified as *Bifidobacterium animalis* subsp. *lactis* LAFTI®B94), *Lactobacillus paracasei* LAFTI®L26 (now classified as *Lactobacillus casei* LAFTI®L26) and *Lactobacillus acidophilus* LAFTI®L10 were provided by DSM Food Specialties (Moorebank, NSW, Australia). LAFTI® probiotic was selected based on the proven health effects, superior stability and excellent survival through the GI tract (McIntosh, 1999; Crittenden et al., 2001; Pidcock et al., 2002; Mahoney & Henriksson, 2003). LAFTI® probiotic was used to reduce intestinal tumours in male Sprague-Dawley rats (McIntosh, 1999). LAFTI® probiotics were also used to increase the safety of Hungarian salami because these cultures gave strong inhibition to both *Escherichia coli* O111 and *Listeria monocytogenes* (Pidcock et al., 2002). Recently, LAFTI®L10 and LAFTI®L26 were proven to enhance specific gut and systemic immune responses in mice (Gunaranjan et al., 2007). Other desirable effects on human host were also reported (Su et al., 2005; Clancy et al., 2006).

For successful application of probiotic in food, the probiotic used should also be technologically compatible with the food manufacturing process. In addition to that, the foods containing the probiotic bacteria must maintain the characteristic sensory attributes of the traditional food. With regard to the development of probiotic cheese, the organisms used should be culturable to high cell density for inoculation into the cheese vat and be able to retain viability and maintain its desirable characteristics during product manufacture and long ripening period.

2.1.4 Application of probiotic bacteria in foods

Growing public awareness of diet related health issues has fuelled the demand for probiotic foods. A number of food products including yoghurt (Kailasapathy & Rybka, 1997), frozen fermented dairy deserts (Ravula & Shah, 1998a), spray dried milk powder (Stanton et al., 2001), cheeses (Stanton et al., 2001), ice cream (Haynes & Playne, 2002), coleslaw (Rodgers & Odongo, 2002), freeze-dried yoghurt (Capela et al., 2006) and fruit juices (Saarela et al., 2006) have been employed as delivery vehicles for probiotic to consumer.

A number of health benefits for consuming food products containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhea, anticarcinogenic properties, reduction of blood cholesterol and improvement in immunity (Ballongue, 1993; Shah, 2000a,b; Shah & Wu, 1999). For the probiotic organisms to be of any value, they must be viable at the time of consumption. For this reason, changes in the numbers of viable bacteria during storage period should be known. It has been suggested that approximately 10^9 cfu per day of probiotic microorganisms is necessary to elicit health effects. Based on daily consumption of 100 g or mL of probiotic food, it has been suggested that a product should contain at least 10^7 cells per g or mL of a food (Ross et al., 2002), a level that was also recommended in Japan (Ishibashi & Shimamura, 1993).

Dairy foods provide ideal food system delivery of probiotic bacteria to the human gut given the suitable environment that dairy products provide to promote growth or support viability of these cultures. The most popular food delivery systems for probiotic have been fermented milk and yoghurt. There are, however, numerous challenges related to the instability of some probiotic strains in these products. The environment typical of many yoghurts and other fermented milk including the low pH and the aerobic conditions of production and packaging, the presence of H_2O_2 and inhibitory substances produced by the yoghurt bacteria may result in decreases in the count of probiotic in the final product. A few studies have shown that many commercial yoghurt have failed to successfully deliver the required level of viable cells of probiotic bacteria (Shah & Lankaputhra, 1997; Dave & Shah, 1997).

Cheeses have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to GI- tract in that cheeses tend to have a higher pH and more solid consistency where the matrix of the cheese and its relatively

high fat content may offer protection to probiotic bacteria during passage through the gastrointestinal tract. Cheese also has high buffering capacity than yoghurt (Gardiner et al., 1998). Ripened type cheeses including Cheddar cheeses, however, have long ripening times hence development of a probiotic cheese requires careful examination of the suitability of particular strain(s) to maintain viability throughout ripening and shelf life.

2.2. Cheese

The Food and Agricultural Organization of the United Nations (FAO) defines a Cheese as 'the fresh or matured product obtained by the drainage (of liquid) after the coagulation of milk, cream, skimmed or partly skimmed milk, buttermilk or a combination thereof' (FAO, 2004). Cheese manufacture is one of the classical examples of food preservation, dating from 6000-7000 BC. Preservation of the important constituents of milk is achieved via lactic acid fermentation, reduction of water activity through removal of water and addition of salt and through the secretion of antibiotics by starter and non-starter microorganisms (Fox & McSweeney, 2004). More than 500 varieties of cheeses are now produced around the world. Coagulation of casein in cheese manufacture can be achieved via iso-electric (acid) or enzymatic (rennet) coagulation. Cheeses produced by acid are usually consumed fresh and hence their production is relatively simple. Rennet cheese is almost always ripened (matured) before consumption through the action of a complex biochemical reaction. This biochemical reaction is mainly responsible for the basic textural changes that occur in the cheese curd during ripening and is also largely responsible for the basic flavour of the cheese (see section 2.5).

2.2.5 Production and consumption of cheeses

According to the Food and Agricultural Organization of the United Nations, over 18 million metric tons of cheeses were produced worldwide in 2004 (FAO, 2004). This is more than the combined yearly production of coffee beans, tea leaves, cocoa beans and tobacco. The largest producer of cheeses is the United States, accounting for 30 percent of world production, followed by Germany and France. In Australia cheeses production continues to increase over the past decades (Figure 2.2). It is a major product for the Australian dairy industry with sales of around 180,000 tonnes within Australia valued at

an estimated A$1.2 billion, and export sales of 204,700 tonnes worth more than A$835 million in 2005-2006. The biggest exporter of cheeses, by monetary value, is France. The United States, the biggest world producer of cheeses, is a marginal exporter, as most of its production is for the domestic market. In Australia, nearly 60% of Australian export of cheeses goes to Asian countries with Japan as one of the biggest Australia's overseas cheeses market, accounted for 40% of product exports, followed by Saudi Arabia and the United States (Table 2.2).

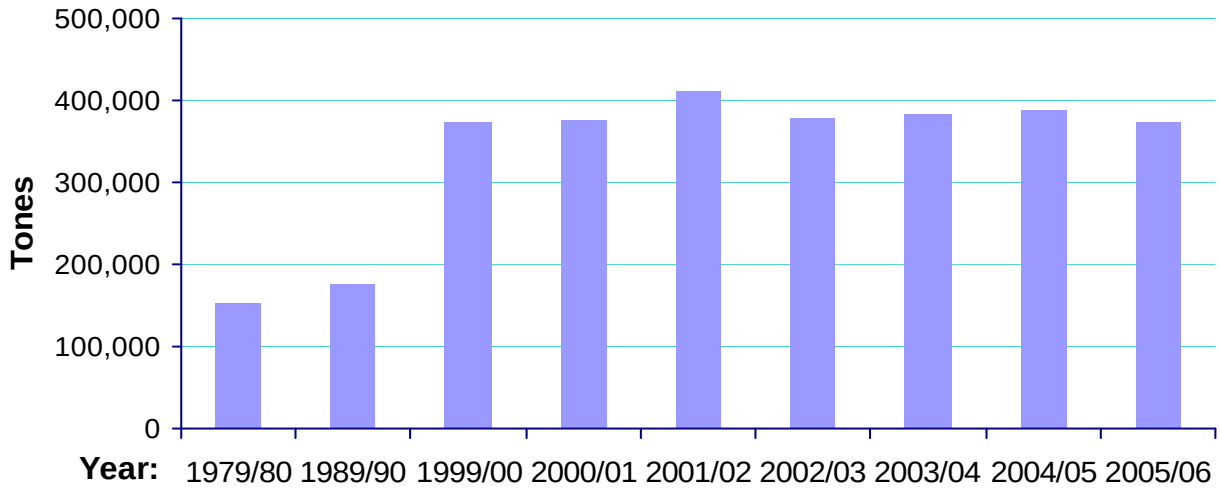


Figure 2.2. Australian total cheese production. Source: Dairy Australia (2006).

Table 2.2. Australian export of cheeses (tonnes). Source: Dairy Australia (2006).

Country	2000/01	2001/02	2002/03	2004/04	2004/05	2005/06
Asia	122,701	141,156	113,795	134,726	149,279	122,052
Middle East	25,228	26,641	24,183	25,655	29,915	36,337
Africa	7,991	9,057	10,889	9,522	7,844	9,203
Pacific	1,947	2,141	2,479	2,515	3,129	3,532
Europe	43,245	19,269	38,065	23,436	21,801	15,705
Americas	17,784	18,127	17,102	16,115	15,511	14,882
Total export	218,896	217,391	206,524	211,969	227,479	201,711

Greece is the world's largest (per capita) consumer of cheeses, with 27.3 kg eaten by the average Greek followed by France and Italy. Australia (per capita) consumption of cheese is growing from about 8.0 kg in 1985-86 to about 10.0 kg in 1995-06 and about 12.0 kg in 2005-06 (Figure 2.3). The increase in cheese consumption has put competitive

pressure on cheese producers to produce high quality cheeses more efficiently at lower costs through higher yields and reducing aging.

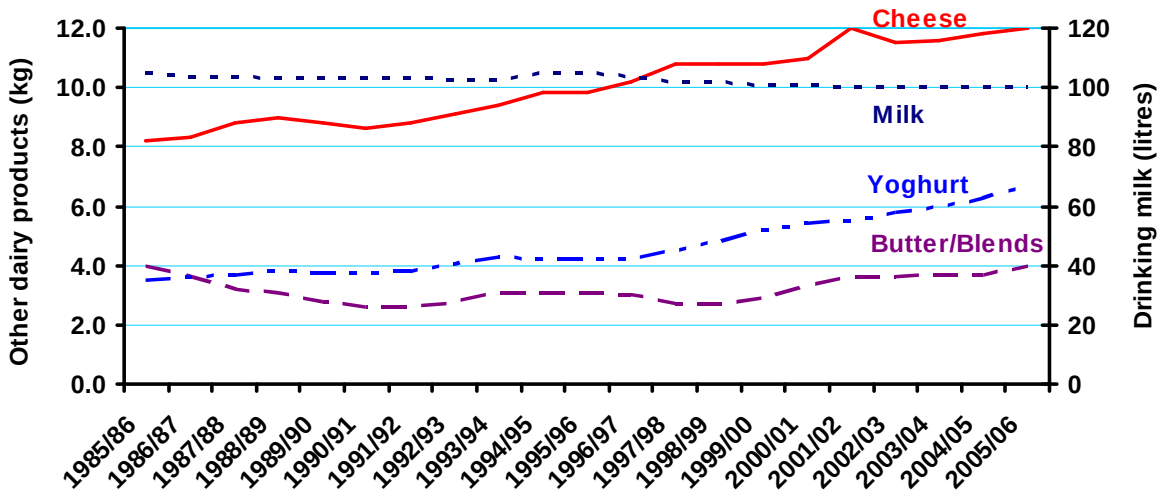


Figure 2.3. Australian per capita consumption of dairy products. Source: Dairy Australia (2006).

More than 50% of cheeses produced in Australia are Cheddar cheeses (Table 2.3). There has been a trend for producing non-Cheddar cheese type over the past six years. The non-Cheddar share of total production has increased from 43 to 49% in 2000 to 2006. Cheddar cheese, however, remained the most popular and most produced cheese in Australia.

Table 2.3. Australian cheese production by type of cheese (tonnes).

Source: Dairy Australia (2006).

Type of cheese	2000/01	2001/02	2002/03	2004/04	2004/05	2005/06
Cheddar[1]	215,047	220,329	212,811	207,795	195,887	191,693
Semi-hard[2]	84,285	91,639	83,973	88,712	90,728	76,827
Hard grating[3]	11,312	16,290	12,118	11,332	13,413	23,022
Fresh[4]	62,021	80,118	64,105	70,940	83,628	75,432
Mould[5]	3,813	3,688	5,945	4,983	4,793	5,775
Total cheese	376,477	412,063	378,952	383,762	388,449	372,749

[1]Includes: Cheddar, Cheedam, Colby, Cheshire, Gloucester, Lancashire, Leicester, Nimbin.
[2]Includes: Mozzarella, Edam, Gouda, Swiss, Emmenthal, Fontina, Havarti, Samsoe, Tilsit, Buetten, Vacherin.
[3]Includes: Parmesan, Pecorino, Romano, Bakers, Casalinga, Goya.
[4]Includes: Cottage, cream cheese, Fetta, Neufchatel, Ricotta, Fresh Pecorino, Paramagiano.
[5]Includes: Blue vein, Brie, Camembert, Quark, Stracchino, Mascarpone.

Over the past few years, there has also been an increased demand and popularity of functional food (food or ingredient that are safe to consume with added health benefit beyond inherent basic nutrition). Foods containing probiotic bacteria can be categorized as 'functional foods' and such products are gaining widespread popularity and acceptance throughout the world. Development of Cheddar cheese as a functional food will thus offer a great prospect. It will provide a potential not only to improve health status and quality of products but also to increase the range of cheeses and probiotic food products.

2.2.6 Manufacture of Cheddar cheese

Production of Cheddar cheese can be divided into two phases, manufacturing and ripening. The ripening process is largely regulated by the level of moisture, salt, pH and the cheese microflora. In turn, these components are regulated by the extent and combination of operations that is involved in cheese manufacturing. Although it is during the ripening that the characteristic flavour and texture of the individual cheese develop, the nature and quality of the finished cheese are determined to a very large extent by the manufacturing steps. For the development of probiotic cheeses, the manufacturing steps will also affect the survival of the probiotic microorganisms during production and ripening. Figure 2.4 shows the principal steps involved in the cheese manufacturing.

2.2.6.1 Pre-treatment of milk

Cheese manufacture commences with the selection of milk of high microbiological and chemical quality. Cheese milk must be free from antibiotics, which may be detrimental to the probiotic organisms, inhibit the growth of starter bacteria, delay acidification and consequently cause flavours and textural defects. Cheese milk also needs to be free from pathogens and spoilage microorganisms such as coliforms, pyschrotrophs and *Clostridium tyrobutyricum*. Contamination of milk with *Cl. tyrobutyricum* results in a defect known as 'late gas blowing' caused by anaerobic metabolism of lactate to butyrate and H_2 during ripening (Thuault et al., 1991). Cheese milk is often standardized and pasteurized. Cheese milk is standardized to give the desired values of fat and protein and also to compensate for the variations in milk composition brought by seasonal changes.

Standardization of the cheese milk to casein to fat ratio of 0.68 is necessary to achieve the desired fat in dry matter (FDM), moisture in non-fat substances (MNFS) and the balance moisture concentration of the cheese which is normally regulated by law (Kosikowski, 1977). The more fat present in the cheese milk, and therefore in the coagulum, will affect the moisture removal as the presence of fat interferes mechanically with the syneresis process.

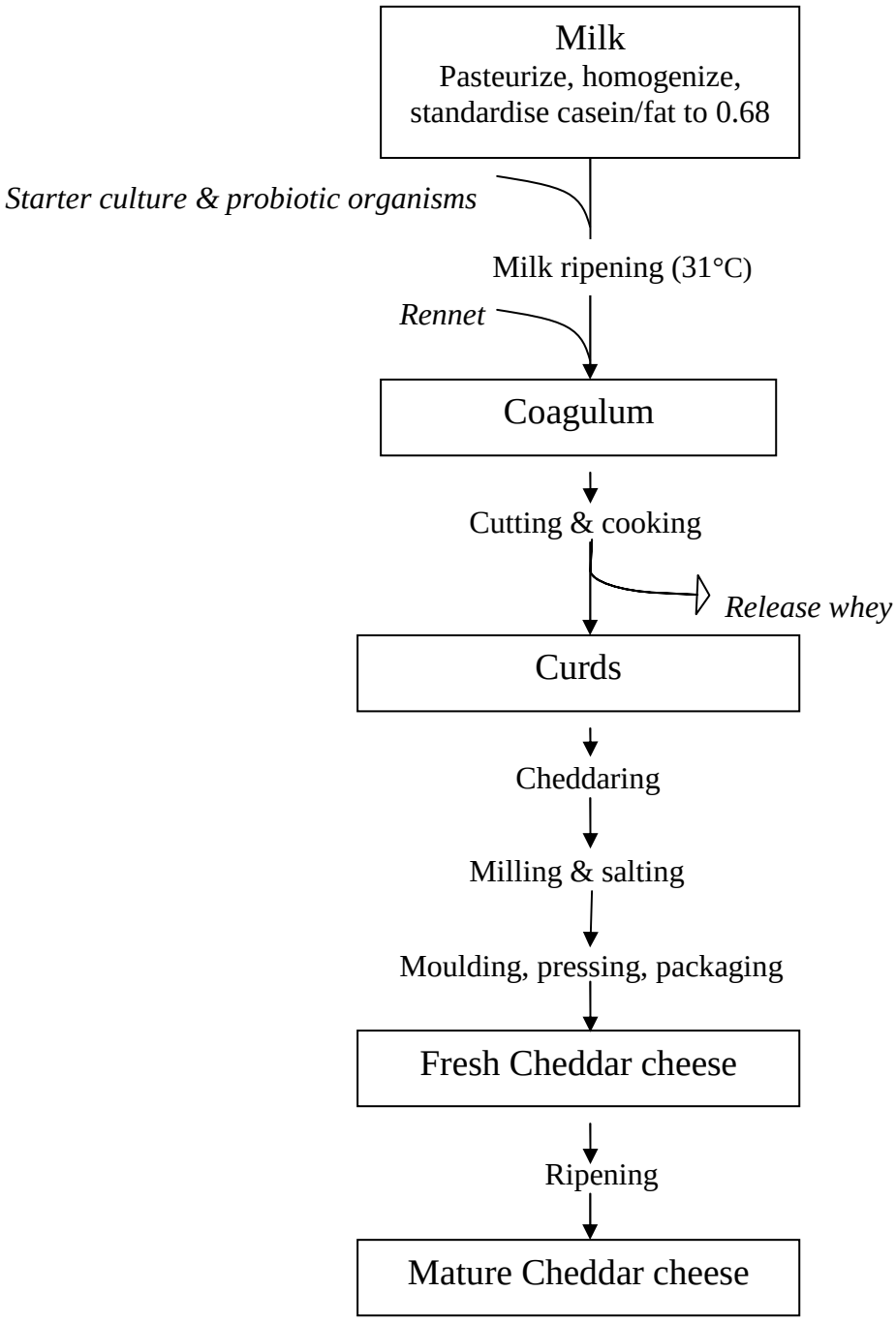


Figure 2.4. Flow diagram for the manufacture of a probiotic Cheddar cheese

Homogenization is used to reduce the size of the fat globules from 1 - 15 µm to less than 2 µm and to distribute the fat evenly in the milk. Homogenization of milk increases the interaction between fat globules and the casein in the rennet gel thus increases the rate of coagulation. Homogenization also promotes lipolysis, increases cheese yield due to improved fat and protein recovery and reduces fat separation in cheeses during storage (Fox & McSweeney, 2004).

Although raw milk is still used in industrial and farmhouse cheese making, most cheese milk is pasteurized (63°C, 30 min or 72°C, 16 s) to kill pathogens and the spoilage microorganisms. A cheese made with pasteurized milk, however, develops a less intense flavour and ripens more slowly than raw milk cheese. Heat induced changes including inactivation of milk enzymes, killing of indigenous microorganism, denaturation of whey proteins and their interaction with κ-casein could be responsible for these changes (Fox & McSweeney, 2004). The contribution of the indigenous microflora to ripening has been discussed in section 2.4.1.

2.2.6.2 Addition of starter and probiotic microorganism

Progressive acidification is necessary throughout the cheese manufacturing stage. One of the ways to achieve uniform acidification is by the addition of a culture (starter) of selected lactic acid-producing bacteria to cheese milk. The main function of starter bacteria is to produce lactic acid from lactose during cheese manufacture. By lowering the pH and competing with spoilage and pathogenic microorganisms and by producing antimicrobial compounds, starter lactococci also contribute to the microbial safety of cheeses (Parente & Cogan, 2004). Starter lactococci also contribute to cheese ripening since the enzymes released by these microorganisms are involved in proteolysis, lipolysis and conversion of amino acids to flavour compounds (Crow et al., 1995). The role of starter culture in cheese ripening is discussed in section 2.5.2.3.

The most commonly used starter bacteria for cheese include *Lactococcus lactis*, *Leuconostoc* sp., *Streptococcis thermophilus, Lactobacillus delbruecii* subsp. *bulgaricus* and *Lb. helveticus.* They are classified into two groups, the mesophilic and thermophilic starter bacteria. Thermophilic starters are used in production of Italian (Grana, Pecorino, Mozzarella) and Swiss (Emmentaler, Sbrinz, Gruyere) cheese varieties. Mesophilic starters are used in cheese varieties in which the temperature of the curd during acid

production does not exceed 40°C. For Cheddar cheese, the most commonly used starter culture consists of *Lactoccoccus lactis* subsp. *lactis* and/or *L. lactis* subsp. *cremoris*. Non-starter adjuncts including probiotic can be added together with the starter bacteria (Gardiner et al., 1998; Corbo et al., 2001, Kasimoglu et al., 2004; Buriti et al., 2005a,b; Bergamini et al., 2006). Different combination of starter bacteria and probiotic adjuncts and different mode of addition of probiotic have been used to improve the viability of the probiotic organisms in cheeses (Dinakar & Mistry, 1994; Blanchette et al., 1996; Gobbetti et al., 1997; Daigle et al., 1999; Vinderola et al., 2000) (see section 2.3).

After the addition of starter culture and probiotic adjuncts, a certain amount of time (normally 30-60 min, referred to as milk ripening) is given to the added culture to begin acid production before the rennet is added. This is necessary to ensure the culture is active before the milk is renneted. Milk ripening also allows the starter organisms to acclimatize to a new environment in preparation for rapid growth. They produce a small but critical amount of lactic acid to activate chymosin in the rennet extract and thus aid the coagulation process (Lawrence et al., 2004).

Acidification continues mainly until the end of the manufacturing process. The speed at which excess lactic acid is produced in the vat critically affects the cheese quality. A very rapid increase is not desirable because the high acid dissolves too much of the insoluble calcium phosphate into the whey. Calcium phosphate is an important buffer for maintaining satisfactory pH after salting. If it disappears early into the whey as soluble calcium lactate, a low pH will occur in the cheese at pressing. The cheese develops an intense acid flavour, weak and pasty texture (‘wet acid cheese’). Slow formation of acid preserves much calcium phosphate in its insoluble form until it is required as a soluble buffering salt in the press, which would result in cheese with good texture or ‘dry acid cheese’ (Lawrence et al., 2004).

2.2.6.3 Milk coagulation

The conversion of liquid milk to cheese curd can be achieved by the addition of milk clotting enzyme (e.g. rennet) to coagulate the milk followed by the subsequent expulsion of the whey by syneresis. In milk, κ-casein is a calcium insensitive protein which forms a protective layer around the calcium sensitive caseins (α_{s1}-, α_{s2}-, β- and γ-), resulting in stable casein micelles. In the presence of chymosin (the principal enzyme in calf rennet), milk clotting occurs in two separate phases (Figure 2.5).

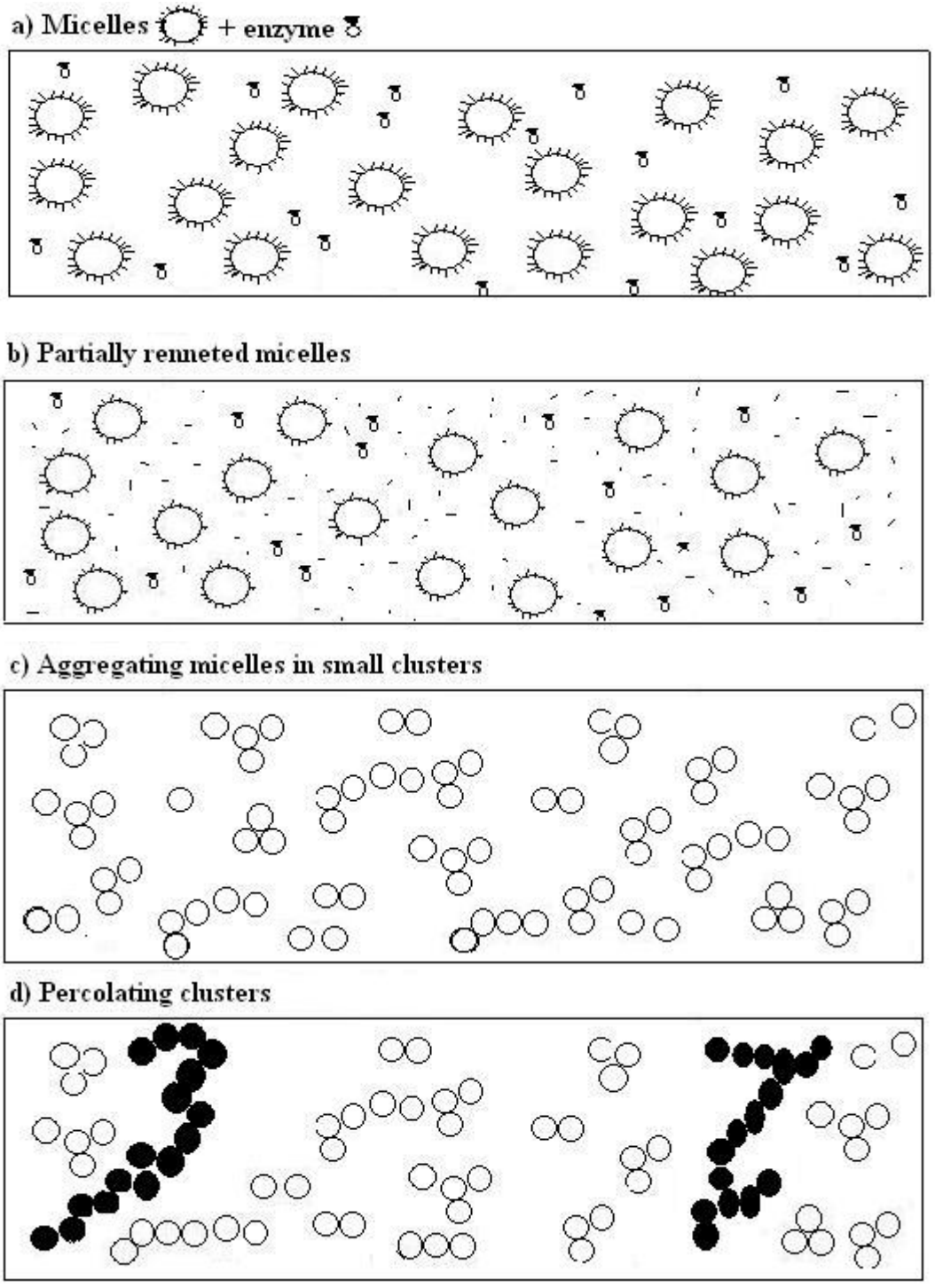


Figure 2.5. Steps involved in the enzymatic coagulation of milk.
Adapted from Horne & Banks (2004).

The first phase starts with the cleavage of κ-casein at the Phe_{105} – Met_{106} bond (Figure 2.5a), which results in the release of hydrophilic caseinomacropeptide (residues 106-109) that passes into the whey and para-κ-casein that remains bound in the casein network (Figure 2.5b). Gradual loss of caseinomacropeptide is accompanied by a decrease

in micellar zeta potential which results in destabilization of the micelle and aggregation into gel (Figures 2.5c & 2.5d) (Crabbe, 2004). The secondary phase involves the aggregation of para-κ-casein and other caseins under the influence of Ca^{2+} which leads to the gel formation.

Milk clotting is normally performed at pH 6.3-6.6 at 30-32°C. At high pH the clotting time and the curd firmness are reduced (Crabbe, 2004). The rate of milk clotting also increases with temperature, type and concentration of rennet. A wide range of clotting agent is available for cheese manufacture (Horne & Banks, 2004). Milk clotting enzyme from the stomachs of ruminants, calf and adult bovine rennets are the most widely used in cheese manufacture today. Chymosin exhibits low proteolytic acitivity but is particularly active in hydrolysis of $Phe_{105} - Met_{106}$ of κ-casein. Only those enzymes with a high ratio of milk-clotting activity to general proteolytic activity are considered suitable for cheese manufacture. A high level of non-specific proteolysis can lead to a weak gel structure, high losses of protein and fat in the whey and thus reduced cheese yield (Horne & Banks, 2004).

The release of para-κ-casein produced by rennet activity during cheese making and the release of products of proteolytic activity of the starter culture have been suggested as growth-promoting factors for the probiotic bacteria during cheese making (Boylston et al., 2004). The role of rennet in cheese ripening is discussed in section 2.5.2.2.

2.2.6.4 Cooking and cheddaring of cheese curd

Proper cutting of coagulum is necessary as improper cutting and handling of curd results in the loss of fines (small curd particles that are not recovered in the cheese). Cutting time is determined by manual testing (curd is ready to be cut if it breaks cleanly when a flat blade is inserted at 45° angle to the surface and then raised slowly). Higher temperature and low moisture varieties such as Italian hard cheese require the smaller cutting size whereas high moisture varieties like soft ripened cheese are cut to 2 cm or more. To achieve the desired moisture content in the final cheese, the best cutting size for Cheddar cheese is ~ 0.8–1.0 cm (Kosikowski, 1997). Following cutting is stirring and cooking of curd, which promotes contraction of the protein matrix, causing the curd to shrink and expel whey so firm up the curd to a state ready for texture formation, pressing and salting. A wide range of temperature maxima is required for different cheese varieties. For Cheddar cheese the best cooking temperature is 38°C with gradual increase of 1°C

every five minutes from the initial coagulum temperature of 31°C and holding the curd at 38°C for approximately 30-60 minutes or until the pH drops to 6.1-6.2 (Kosikowski, 1977). In the development of probiotic cheese, slight technological modifications were incorporated during cooking to improve the survival of probiotic in especially high heat cheese such as Canestrato Pugliese (Corbo et al., 2001) (see section 2.3).

After cooking, the curds and whey are separated. The whey is drained normally at pH of 6.1-6.2 and the curds are subjected to a process called 'cheddaring'. Cheddaring means piling and repiling blocks of warm curd (usually 38°C) in the cheese vat for about two hours during which period, lactic acid increases rapidly to a point where coliform bacteria are killed by the free hydrogen ions. As the curd blocks are repiled, their structure flattens and any holes or eyes originally present lose their identity in the deformed curd. Moisture control and the proper texture of the curd are also attained during Cheddaring. The increasing lactic acid strips some of the bound calcium from the relatively inert para-caseinate curd and transforms it into forms of para-caseinate which gives plastic properties to the resulting cheese and sufficient substrate for enzyme action, leading to proper flavour and texture development during ripening (Lawrence et al., 2004).

2.2.6.5 Milling, salting and pressing of cheese curd

Milling operation consists of mechanically cutting the cheddared curd into small pieces in order to increase the surface area of the curd and so enable more uniform salt distribution into the curd and to encourage whey drainage (Lawrence et al., 2004). The size of the curd particle affects the salt in moisture ratio of the final cheese (larger particle causes longer salt penetration). Salt is added to the curd pieces after milling. The purpose of salting is to promote further syneresis, slow down lactic acid fermentation and promote controlled ripening and flavour development. The presence of salt also prevents the growth of spoilage microorganisms and contributes toward the flavour of the cheese. Cheddar cheese has an average of 1.5% salt. Over salting may make the cheese too dry and slows ripening which leads to underdeveloped body and flavour. Normally about 2.3-3.5% of salt is added to cheese since 45% of this salt would be lost with the whey during pressing (Kosikowski, 1977). During ripening, salt diffuses throughout the cheese and so the differences in the salt content at the centre and periphery decrease with ripening time.

In the development of probiotic cheeses, salting has an impact on the growth of the probiotic bacteria as the growth and survival of these bacteria are inversely related to salt

concentration (Boyslton et al., 2004). Gomes et al. (1995) adapted the technology of Gouda cheese manufacture by varying the concentration of salt added to cheese to improve the survival of *Bifidobacterium* sp. and *Lb. acidophilus* used in that study (see section 2.3).

The curd is pressed when it is still warm (preferably < 25 °C) to prevent fat leaking from the curd if the fat solidifies. The main purpose of pressing is to form the loose curd particles into a shape which is compact enough and to expel any free whey (Lawrence et al., 2004). Pressing the curd should be gradual at first to prevent the surface layer of the cheese creating impermeable layer which will lead to moisture being retained within pockets of the cheese. Cheddar cheese is normally pressed under a vacuum of 0.17 to 0.42 MPa for 6-18 h and vacuum packed (Kosikowski, 1977). The selection of packaging material can further have a significant effect on the survival of probiotic bacteria. Packaging materials with good oxygen barriers such as polyvinylidene chloride copolymer (PVDC) (e.g. Cryovac^{TM}) and ethylene- vinyl alcohol (EVOH) have been shown to be more effective than polyethylene and polystyrene packaging materials widely used for foods in maintaining the viability of probiotic (Ishibashi & Shimamura, 1993). Cheddar cheese is then ripened at 4 to 16°C for 2 to 48 months (Kosikowski, 1977). It is during the ripening that the characteristic flavour and texture of the individual cheese develop. Biochemical reactions involved during cheese ripening is discussed in section 2.5.

2.3. Probiotic Cheddar cheese

A number of studies have addressed development of probiotic cheeses using Canestrato Pugliese cheese (Corbo et al., 2001), Cheddar cheese (Dinakar & Mistry, 1994; Gardiner et al., 1998; Daigle et al., 1999; McBrearty et al., 2001; Phillips et al., 2006), cottage cheese (Blanchette et al., 1996), Crescenza cheese (Gobbetti et al., 1998), Fresco soft cheese (Vinderola et al., 2000), goat cheese (Gomes & Malcata, 1998), Gouda cheese (Gomes et al., 1995), Minas fresh cheese (Buriti et al., 2005a,b), semi-hard cheese (Bergamini et al., 2006) and white cheese (Kasimoglu et al., 2004). Examples of probiotic cheese developments are shown in Table 2.4.

Table 2.4. Examples of probiotic cheese developments.

Cheese variety	Probiotic strain	Reference
Canestrato Pugilese cheese	*B. bifidum* Bb02, *B. longum* Bb46	Corbo et al. (2001)
Cheddar cheese	*B. bifidum* ATCC 15696	Dinakar & Mistry (1994)
	Lb. paracasei NFBC 338	Gardiner et al. (1998)
	B. infantis ATCC 27920G	Daigle et al. (1999)
	B. lactis Bb-12, *B. longum* BB536	McBrearty et al. (2001)
	Commercial strains of: *Lb. acidophilus, Bifidobacterium* sp., *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*	Phillips et al. (2006)
Cottage cheese	*B. infantis* ATCC 27920G	Blanchette et al. (1996)
Crescenza cheese	*B. bifidum, B. longum* and *B. infantis*	Gobbetti et al. (1998)
Cheese French onion dips	*Lb. acidophilus, B. animalis, Lb. paracasei* subsp. *paracasei* and *Lb. rhamnosus*	Thamaraj & Shah (2004)
Fresco cheese	*B. bifidum, B. longum, Lb. acidophilus*, *Lb. casei*	Vinderola et al. (2000)
Goat cheese	*B. lactis* and *Lb. acidophilus* (Ki)	Gomes & Malcata (1998)
Gouda cheese	*B. bifidum* (Bo), *Lb. acidophilus* (Ki)	Gomes et al. (1995)
Minas fresh cheese	*Lb. paracasei* subsp. *paracasei* LBC 82	Buriti et al. (2005a)
	Lb. acidophilus culture La-5	Buriti et al. (2005b)
Semi-hard cheese	*Lb. acidophilus*, *Lb. paracasei* subsp. *paracasei*	Bergamini et al. (2006)
White cheese	*Lb. acidophilus* 593 N	Kasimoglu et al. (2004)

Probiotic organisms can be added into cheese during manufacture, either as a starter or as adjunct to the starter culture. As a starter, probiotic cultures are often limited by its ability to produce sufficient lactic acid in milk. A large inocula and addition of growth-promoting supplements such as cysteine, yeast extract and casein hydrolysates are often required (Gomes et al., 1995; Gomes & Malcata, 1998). *B. bifidum* strain Bo (3.5% w/v) and *Lb. acidophilus* strain Ki (3.5% w/v) were used as a starter in the manufacture of Gouda cheese supplemented with milk hydrolysate. *Lb. acidophilus* strain Ki was able to grow in milk and produce acid, reaching 10^9 cfu g^{-1} during manufacture and the count decreased by two log cycles to 10^7 cfu g^{-1} after 9 wk of ripening at 13°C. *Bifidobacterium* strain Bo on the other hand, showed no growth during manufacture, but was able to remain viable at 10^8 cfu g^{-1} at the end of ripening. There was a significant effect on cheese flavour after 9 wk of ripening, possibly due to the acetic acid production by the *Bifidobacterium*. The presence of milk hydrolysate also proved unsuitable for cheese manufacture because it contributes to the development of undesirable flavours even at low levels (Gomes et al., 1995). In the same study, Gomes et al. (1995) also concluded that the survival of the probiotic bacteria was dependent on the region of the cheese and the concentration of salt. Cheese with salt contents ranging from 1.90 to 3.90% had a 55-35% survival of probiotic bacteria, with the highest survival in the centre of the cheese where oxygen and salt levels were the lowest. In another study, Gomes and Malcata (1998) recommended 0.30% (v/w) of milk hydrolysate in the production of goat cheese with *B. lactis* and *Lb. acidophilus* strain Ki. Both microorganisms remained viable for 70 days at 6°C with a level of $>10^8$ cfu g^{-1} and $>10^7$ cfu g^{-1} for *B. lactis* and *Lb. acidophilus* strain Ki, respectively. The probiotic organisms contributed significantly to ripening, especially in the formation of low molecular mass peptides and amino acids in both studies.

Addition of probiotic as adjunct to the starter culture may be a more favorable option for incorporation of probiotic in cheese. As adjunct culture, probiotic bacteria are normally added together with the starter bacteria during cheese making. In the manufacture of Argentinian Fresco cheese, probiotic cultures of *Bifidobacterium*, *Lb. acidophilus*, and *Lb. casei* were added simultaneously with the starter bacteria (*S. thermophilus* and *Lactococcus lactis*). *Bifidobacterium* sp., *Lb. acidophilus* and *Lb. casei* used in combination demonstrated satisfactory survival counts at about 10^6 cfu g^{-1} at the end of the 60-day ripening period. The probiotic strains incorporated into Fresco cheese also demonstrated good resistance in an acidic environment typical of the stomach (Vinderola et al., 2000). The *in vivo* consumption of the Argentinian Fresco cheese

containing this mixture of probiotic bacteria beneficially modulated the immune response in mice (Medici et al., 2004).

Human derived *Lb. paracasei* added into Cheddar cheese as starter adjuncts was also found to grow and proliferate to high cell numbers (>10^7 cfu g^{-1}) in cheeses at 8°C over 8 mo of ripening even when added at a relatively small inoculum (0.1% v/v) (Gardiner et al., 1998). *Lb. acidophilus* and *Lb. paracasei* subsp. *paracasei* were also used as adjunct culture in semi-hard cheese making experiments. The population of probiotic remained above 10^7 cfu g^{-1} during ripening of 60 days. Cheese with the addition of *Lb. acidophilus* also showed a significant increase in the level of low molecular weight nitrogen compounds and individual free amino acids (Bergamini et al., 2006).

While some studies have shown adequate survival of probiotic, others have found low counts of probiotic bacteria in cheeses (Blanchette et al., 1996; McBrearty et al., 2001). *B. lactis* Bb12 and *B. longum* BB636 were added as adjunct at the levels of 10^8 cfu g^{-1} of cheesemilk during manufacture. *B. lactis* Bb12 survived at high numbers (10^8 cfu g^{-1} of cheese), while the viability of *B. longum* BB536 was reduced to 10^5 cfu g^{-1} following six month of ripening. The presence of these adjunct probiotic did not adversely affect cheese composition and contributed to improved Cheddar flavour as compared to control cheese (McBrearty et al., 2001). *B. infantis* and *B. bifidum* also did not sustain high viability during storage in cottage cheese after 28 days (Blanchette et al., 1996). Different combinations of commercially available probiotic cultures (*Lb. acidophilus*, *Bifidobacterium* sp., *Lb. casei* and *Lb. rhamnosus*) were used in the production of probiotic Cheddar cheeses. All probiotic strains survived well in cheese at the level of > 10^7 cfu g^{-1} except that with *Lb. acidophilus* strains which performed poorly and the count reduced to about 10^3 cfu g^{-1} after 32 wk (Phillips et al., 2006).

A few approaches have been used to improve the survival of probiotic in cheeses including minimizing the exposure of probiotic to cheese making conditions (Dinakar & Mistry, 1994), two steps fermentation (Daigle et al., 1999), microencapsulation of probiotic strain (Gobbetti et al., 1997), modification of manufacturing condition (Corbo et al., 2001), the use of different combination of starter and probiotic (Buriti et al., 2005a,b), and modification of packaging and storage condition (Kasimoglu et al., 2004).

B. bifidum was added into Cheddar cheese as immobilized freeze-dried strains to the matrix of Cheddar cheese following cheddaring and salting (at the milling stage) to limit exposure of the culture to salt, oxygen and heat during the manufacturing process. *Bifidobacterium* remained viable for up to 24 wk at about 10^7 cfu g^{-1} of cheese without

causing any adverse effects on cheese flavour and texture or appearance (Dinakar & Mistry, 1994).

In other studies, two steps fermentation (cream fermentation and fermentation during cheese making) were used to promote the growth of *B. infantis* in Cheddar like cheese. The application of a two-stage fermentation has shown to be effective in increasing the viability of probiotic bacteria by allowing the probiotic organism to become dominant prior to the addition of the starter cultures. Since starter lactic acid bacteria produce inhibitory substances against probiotic bacteria and grow faster than them during fermentation, the viability of probiotic bacteria could be reduced (Shah, 2000b). *B. infantis* was initially grown in the cream prior to the addition of starter culture. The probiotic microorganism was shown to have survived after 84 days of ripening at 10^6 cfu g^{-1} of cheese. Improved proteolysis was also observed in the probiotic cheese indicated by the higher concentration of soluble nitrogen and percentage hydrolysis of α_{s1}- CN as compared to the control cheese (Daigle et al., 1999). Bergamini et al. (2005) also found pre-incubation to increase the population of lactobacilli in cheeses.

B. bifidum, B. longum and *B. infantis* were incorporated into Crescenza cheese as cells immobilized in calcium alginate. *B. bifidum* and *B. longum* remained viable at greater than 10^7 cfu g^{-1} in cheese but *B. infantis* died off after 14 days of ripening with no effect on cheese composition, primary proteolysis or sensory properties (Gobbetti et al., 1998). Slight technological modifications were incorporated during processing of Canestrato Pugliese cheese to improve the survival of the probiotic organism. These modifications included reducing the conditions for heating the curd in whey from 80°C for 30 s to 50°C for 2 min and holding the curd at 40°C for about 5 h to limit acidification by the lactic acid starters. *B. bifidum* Bb02, *B. longum* Bb46 remained viable at about 10^6 cfu g^{-1} after 90-day of ripening period. The composition and sensory attributes of the cheeses with the added *Bifidobacterium* was also characteristic of the traditional Canestrato Pugliese cheese (Corbo et al., 2001).

Lb. acidophilus and *Lb. paracasei* were reported to maintain their viability at 10^6 and 10^7cfu g^{-1}, respectively when added to Minas fresh cheese and stored at 5^oC for 21 days. The cheeses were reported to have a better texture and sensory profiles especially when used in combination with mesophilic type O lactic culture as compared to normal lactic culture (Buriti et al., 2005a,b). Thamaraj and Shah (2004) found bacterial interaction to significantly effect the survival of probiotic bacteria in cheese dips. Each of *Lb. acidophilus, B. animalis, Lb. paracasei* subsp. *paracasei* and *Lb. rhamnosus* showed

varied levels of antagonism. Any combination of these bacteria, however, can be used as probiotics in cheese dip.

The selection of packaging and storage condition can further have a significant impact on the survival of probiotic bacteria in cheese. Kasimoglu et al. (2004) investigated the survival of *Lb. acidophilus* during ripening of Turkish white cheese stored in vacuum or in brine at 4^{o}C for 90 days. The cheeses with *Lb. acidophilus* received a high level of proteolysis and sensory scores and with probiotic counts >10^{7} cfu g^{-1} at the end of ripening. In that study, probiotic cheese which was vacuum-packed after salting received higher sensory scores than probiotic cheese stored in brine.

Data from the development of probiotic cheeses demonstrated that Cheddar cheeses are a suitable potential carrier for probiotic bacteria to consumer. However, it is important to stress that this can be very strain dependent as many of the probiotic strains tested did not perform well in the cheese environment. Strain selection is vital to the successful development of probiotic cheese. The effect of processing conditions on the viability of the bacteria is also important. The processing conditions, cooking procedure, the aerobic environment, the impact of lactic acid starter cultures and the temperatures of ripening and storage must be evaluated in the development of probiotic cheese so that the concentration of probiotic bacteria in the final product provides a therapeutic dose to consumers (see Chapters 3 & 4). To date, very few studies have evaluated the influence of ripening temperatures on the survival of probiotic bacteria in cheeses (see Chapters 7 & 8). Growth conditions must be balanced to promote viability of probiotic bacteria without excessive growth and acid production that could adversely affect sensory attributes of cheeses (see Chapter 5). If the probiotic cheese can be manufactured with little or no alteration to the traditional cheese making technology, this would make the development of probiotic cheeses attractive for commercial production.

2.4. Cheddar cheese microflora during ripening

The microflora associated with cheese ripening is extremely diverse. They may be divided into two groups – the starter lactic acid bacteria and non-starter lactic acid bacteria (NSLAB). Starter bacteria are primarily responsible for acid production during manufacture, while the secondary microflora may not play any active role in cheese manufacture but contribute towards the ripening process. The role of starter lactic acid bacteria during cheese making has been described in section 2.2.2.2. Starter bacteria stop

growing in cheese curd soon after the end of manufacture due to the low pH, increasing NaCl concentration, low temperature and lack of fermentable carbohydrate substrate (Kosikowski, 1977). When the starter lactococci loose their viability, they release their intracellular enzyme due to autolysis (Crow et al., 1995). The role of this enzyme to proteolysis during ripening is explained in section 2.5.2.3.

2.4.1 Non-starter lactic acid bacteria

The dominant non-starter lactic acid bacteria in Cheddar cheese are homo- and heterofermentative species of lactobacilli (Broome et al., 1990; Jordan & Cogan, 1993), although there are reports on the occurrence of pediococci, micrococci and *Leuconostoc* sp. (Peterson & Marshall, 1990). Studies on non-starter lactobacilli occurring in Cheddar cheese produced in various countries consistently report the dominance of *Lb. paracasei* and *Lb. plantarum*. Other species that are frequently detected as minor components of the population include *Lb. curvatus, Lb. casei, Lb. brevis* and *Lb. rhamnosus* (Broome et al., 1990; Crow et al., 2001; Williams et al., 2002).

2.4.1.1 Sources and factors affecting the growth of NSLAB

NSLAB gain entry into the cheese either by deliberate addition as a part of the starter culture or are naturally associated with the ingredients used in cheese production. Milk withdrawn from the udder at farm level under hygienic milk conditions can routinely contain $< 10^3$ cfu mL^{-1} (Beresford & Williams, 2004). Pasteurization only kills ~ 99.9% of the bacteria found in raw milk. Other ingredients used in cheese manufacture such as rennet and salt also add to the microbial load of the cheese. Bintsis et al. (2000) reported the presence of *Lb. paracasei*, *Lb. casei* and *Lb. plantarum* in commercial brines. Cheese manufacturing conditions (milk ripening, coagulation, cooking and cheddaring at 30-40°C) facilitate the growth of these organisms. The cheese environment such as the level of salt in moisture (S/M), pH, organic acids and ripening temperature further influence the growth of these microorganisms in cheese during ripening (Shakeel-Ur-Rehman et al., 2000). NSLAB require an energy source for growth. They utilized the residual lactose in cheese, sugars derived from the glycomacropeptide of casein and the glycoproteins of the milk-fat globule membrane and products of proteolysis including peptides and amino acids (Williams & Banks, 1997). Freshly produced commercial Cheddar curd about 10^2

cfu g^{-1} non-starter *Lactobacillus*, which increases to about 10^7 within 3 mo of manufacture and remains at this level throughout the remainder of maturation (Peterson & Marshall, 1990).

2.4.1.2 NSLAB and cheese quality

Cheeses produced from raw milk develop a more intense flavour and ripen faster than those made with pasteurized milk (Beresford & Williams, 2004). A number of approaches have been used to study the role of NSLAB in cheese ripening. NSLAB were removed from raw skim milk by microfiltration (McSweeney et al., 1993). Cheeses were produced under strictly controlled microbiological condition (McSweeney et al., 1994). Shakeel-Ur-Rehman et al. (2000) inhibited the growth of NSLAB with antibiotics and low ripening temperature at 1°C. The results from these studies show that the indigenous microflora of raw milk is one of the major causes of the differences between raw and pasteurized milk cheeses.

Improvement in the hygiene of milk and the need for standardization have resulted in bland cheeses. It is becoming increasingly popular to inoculate pasteurized milk with adjunct cultures from good quality raw milk to improve the sensory properties of cheese and improve its health benefits at the same time. The addition of selected adjuncts strains of *Lactobacillus* sp. has positive influence on the quality of cheeses (Puchades et al., 1989; Broome et al., 1990; Lee et al., 1990; McSweeney et al., 1994; Drake et al., 1996; Lynch et al., 1996; Muir et al., 1996). Some adjuncts cultures were also used to accelerate ripening (Trepanier et al., 1991; El Soda et al., 2000) and suppress the growth of unwanted bacteria in cheeses (Martley & Crow, 1993). On the other hand, other studies have suggested that addition of certain *Lactobacillus* strains adversely affected flavour formation in both Gouda and Cheddar cheeses (Kleter, 1977; Puchades et al., 1989). Some NSLAB have also been implicated in the calcium D-lactate surface defect (Khalid & Marth, 1990).

2.4.2 Enumeration of starter, NSLAB and probiotic bacteria in Cheddar cheese

Considering the regulations for minimum number of probiotic bacteria in probiotic food, monitoring the survival of probiotic bacteria in probiotic Cheddar cheese is essential. Enumeration of probiotic bacteria in cheese is difficult due to the presence of

complex cheese microflora including starter LAB and NSLAB. To enumerate probiotic bacteria in such a mixed population, selective media can be employed that would allow the growth of the organisms of interest and inhibit other microorganisms found in the cheese. The selective agents that are commonly used include antibiotics, carbohydrates, salts and bile (Tharmaraj & Shah, 2003). These agents can be incorporated into the agar media and can be used in combination with correct gaseous environment and incubation temperature to allow the growth of only the desired probiotic organism. Tables 2.5 and 2.6 summarize the various culture media recommended for the enumeration of cheese starter culture, NSLAB, *Lb. acidophilus*, *Lb. casei* and *Bifidobacterium* sp.

M17 medium was initially developed by Terzaghi and Sandine (1975) for the enumeration of lactic streptococci. This media has been widely used for the selective enumeration of starter lactococci in Cheddar cheese (Gardiner et al., 1998; Daigle et al., 1999; Corbo et al., 2001; McBrearty et al., 2001). ß-Disodium glycerophosphate (GP) was one of the important components in this medium because it improved the buffering capacity and thus the growth of lactococci. When incubated aerobically, it could prevent the growth of some probiotic bacteria although some aero-tolerant probiotic strain may form pin point colonies in this medium (Gardiner et al., 1998).

Lactobacillus selective agar (LBS) has been used for the enumeration NSLAB or lactobacilli in Cheddar cheese (Gardiner et al., 1998; Daigle et al., 1999; McBrearty et al., 2001). The accompanying bacterial flora is largely suppressed by the high acetate concentration and the low pH value. The media also contain low concentrations of manganese, magnesium and iron to ensure optimal growth of lactobacilli (Rogosa et al., 1951).

The MRS medium was developed by DeMan et al. (1960) to replace the tomato juice medium and the meat extract tomato juice medium. MRS medium has been used for enrichment, cultivation and isolation of probiotic bacteria in cheese (Dinakar & Mistry, 1994; Corbo et al., 2001). The medium contains polysorbate (Tween 80), acetate, magnesium and manganese, which act as special growth factors for Lactobacilli as well as a rich nutrient base. MRS media and reinforced clostridial agar (RCA) were used as the basal media in addition to the selective agents for the selective enumeration of probiotic bacteria (Tharmaraj & Shah, 2003; Darukaradhya et al., 2006). Bifidobacteria can grow in RCA even without the addition of L-cysteine·hydrochloride. Therefore, RCA was not suitable for selective enumeration of probiotic bacteria in the presence of *Bifidobacterium* (Tharmaraj & Shah, 2003).

Table 2.5. Culture media recommended for the enumeration of *Lb. acidophilus*, *Lb. casei*, total lactobacilli and cheese starter culture

Designation	Media + supplements	Incubation conditions	References
All probiotic and lactic acid bacteria			
MRS	DeMan Rogosa Sharpe agar	2-3 days, 37°C, AnO_2	DeMan et al. (1960), Dinakar & Mistry (1994), Corbo et al. (2001), Tharmaraj & Shah (2003)
Total lactobacilli or NSLAB			
LBS	Lactobacillus selective agar	5 days, 30°C, AnO_2	Rogosa et al. (1951), Gardiner et al. (1998), Daigle et al. (1999), McBrearty et al. (2001)
Starter lactococci			
M17	M17 + supplement provided by manufacturer	2-3 days, 30°C, O_2	Terzaghi & Sandine (1975), Gardiner et al. (1998), Daigle et al. (1999), McBrearty et al. (2001), Corbo et al. (2001)
Lactobacillus acidophillus			
TGV	Tryptone glucose meat extract agar + 2% w/v NaCl	2 days, 40°C, AnO_2	Gomes et al. (1995)
MRS-Sal	MRS- salicin (0.5% w/v filter-sterilized salicin)	2-3 days, 37°C, AnO_2	Lankaputra & Shah (1996)
MRS-Sorb	MRS- sorbitol	2-3 days, 37°C, AnO_2	Lankaputra & Shah (1996), Tharmaraj & Shah (2003)
MRS-C	MRS- clindamycin - bromocresol green ($0.04g\ L^{-1}$) + clindamycin ($1\ mg\ L^{-1}$), pH 5.5	2-3 days, 37°C, AnO_2	
RCA-C	Reinforced clostridial agar - bromocresol green ($0.04g\ L^{-1}$) + clindamycin ($1\ mg\ L^{-1}$), pH 5.5	2-3 days, 37°C, AnO_2	Darukaradhya et al. (2006)
Lactobacillus casei and Lb. paracasei			
LC	*Lactobacillus casei* agar	3-4 days, 27 °C, AnO_2	Ravula & Shah (1998b)
MRS-V	MRS- vancomycine ($1\ mg\ L^{-1}$) - bromocresol green ($0.04\ g\ L^{-1}$), pH 5.5	2-3 days, 37 °C, AnO_2	Tharmaraj & Shah (2003)
RCA-V	RCA- bromocresol green ($0.04\ g\ L^{-1}$) + vancomycine ($1\ mg\ L^{-1}$), pH 5.5	2-3 days, 37 °C, AnO_2	Darukaradhya et al. (2006)

Table 2.6. Culture media recommended for the enumeration of bifidobacteria in mixed cultures

Designation	Media + supplements	Incubation conditions	Reference
MRS[1]-NNLP	MRS - NNLP (100 mg L^{-1} neomycin sulfate, 200 mg L^{-1} paromomycin sulfate, 15 mg L^{-1} nalidixic acid and 3 g L^{-1} LiCl) + 0.05% L-cysteine	3 days, 37°C, AnO_2	Laoria & Martin (1991), Dinakar & Mistry (1994), Tharmaraj & Shah (2003)
BSM[2]	MRS + mupirocin (50 µg mL^{-1}) + 0.05% L-cysteine	2-3 days, 37°C, AnO_2	Leuschner et al. (2002), Rada & Koc (2000)
TOS-NNLP	Transgalactosylated oligo saccharide- NNLP + 0.05% L-cysteine	2-3 days, 37°C, AnO_2	Wijsman et al. (1989)
L-arabinose	L-arabinose agar + 0.05% L-cysteine	2-3 days, 37°C, AnO_2	Wijsman et al. (1989)
AMC	Arroyo, Martin and Cotton agar	3-4 days, 37°C, AnO_2	Payne et al. (1999) Corbo et al. (2001)
MCA	Modified Columbia agar + 5 g L^{-1} raffinose, 0.05 % L-cysteine, 2 g L^{-1} lithium chloride and 3 g L^{-} sodium propionate. pH 5.1	3 days, 37°C, AnO_2	Roy et al. (1997) Daigle et al. (1999)
RCA[3]-AD	RCA + aniline blue (0.3 g L^{-1}) + dicloxacillin (2 mg L^{-1}), pH 7.1	2-3 days, 37°C, AnO_2	Darukaradhya et al. (2006)

[1] MRS, DeMan Rogosa Sharpe agar
[3] BSM, *Bifidobacterium* selective medium
[4] RCA, Reinforced clostridial agar

2.4.2.1 Enumeration of Lactobacillus casei

Selective media for the enumeration of *Lb. casei* in the presence of mixed cultures have been developed by several workers (Champagne et al., 1997; Ravula & Shah, 1998b; Tharmaraj & Shah, 2003; Darukaradhya et al., 2006). Champagne et al. (1997) developed a selective enumeration method for *Lb. casei* in yogurt-type fermented milks containing other probiotic bacteria based on 15°C incubation temperature and 14-d incubation time. The incubation time of 14 days, however, may not be practical for the dairy industry if the results are required in a short time. Another selective medium, *Lb. casei* (LC) agar, was developed by Ravula and Shah (1998b) for enumeration of *Lb. casei* populations from commercial yoghurts and fermented milk drinks that may contain strains of yoghurt bacteria (*S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*), *Lb. acidophilus, Bifidobacterium* sp., and *Lb. casei.* LC agar was reported to inhibit the growth of *S. thermophilus, Lb. delbrueckii* ssp. *bulgaricus, Lb. acidophilus* and *Bifidobacterium* sp., and was selective for *Lb. casei* (Ravula & Shah, 1998b; Talwalkar & Kailasapathy, 2003).

More recently, MRS-vancomycine (MRS-V) was recommended by Tharmaraj and Shah (2003, 2004), for the selective enumeration of *Lb. casei* in the presence of mixed cultures. *Lb. casei* was resistant to 1 mg vancomycine L^{-1} and grew well in MRS-V at 37^{o}C and 43^{o}C anaerobically. No other cultures tested including *S. thermophilus, Lb. delbrueckii* ssp. *bulgaricus, Lb. acidophilus, B. lactis* and *Propionibacterium* grew in this medium (Tharmaraj & Shah, 2003). Vancomycine has also been added to other basal medium such as RCA for the selective enumeration of *Lb. casei* in Cheddar cheeses (Darukaradhya et al., 2006).

2.4.2.2 Enumeration of Lactobacillus acidophilus

Lb. acidophilus was difficult to be enumerated selectively, since most of the media that supported the growth of *Lb. acidophilus* also supported the growth of other probiotic organism. Lankaputhra and Shah (1996) developed a simple method for selective enumeration of *Lb. acidophilus* in the presence of yogurt bacteria and *Bifidobacterium* sp. based on sugar fermentation patterns. Salicin (0.5 %) and sorbitol can be used for selective enumeration of *Lb. acidophilus* because this organism can utilize salicin and sorbitol (Buchanan & Gibbons, 1974). MRS-maltose can also be used for the selective

enumeration of *Lb. acidophilus* but only in the absence of *Bifidobacterium*. In the presence of the *Lb. casei*, MRS-sorbitol agar or MRS-salicin agar can be used to obtain counts of *Lb. acidophilus* and *Lb. casei*, and LC agar can be used to obtain a total count of *Lb. casei*. Then the counts of *Lb. casei* can be subtracted from the total population of *Lb. acidophilus* and *Lb. casei* enumerated with MRS-salicin or MRS-sorbitol agar (Lankaputhra & Shah, 1996).

Tharmaraj and Shah (2003) recommended the use of MRS-sorbitol for the enumeration of *Lb. acidophilus* because this media gave the highest recovery. In this medium, *Lb. casei* formed shiny, large, smooth and white colonies, while all strains of *Lb. acidophilus* tested formed rough dull, small and brownish colonies. Therefore, the counts of *Lb. acidophilus* can be obtained by counting only the small dull rough brownish colonies on MRS-sorbitol.

Other selective media for the selective enumeration of *Lb. acidophilus* in cheese include RCA or MRS agar with clindamycin and tryptone, glucose, meat extract agar (TGV) (Table 2.5). Enumeration of *Lb. acidophilus* strain Ki in Gouda cheese was performed on TGV agar with 2% w/v NaCl. The total number of *Lb. acidophilus* was determined based on morphology of the colonies on TGV-agar where *Bifidobacterium* produced pinpoint-size colonies, whereas *Lb. acidophilus* produced large colonies (Gomes et al., 1995). RCA-clindamycin (RCA-C) was reported to give a good recovery for *Lb. acidophilus* strains (Darukaradhya et al., 2006). RCA-C inhibited the growth of *Bifidbacterium* sp., *Lb. casei* and starter lactococci tested in that study. The colonies of *Lb. acidophilus* strains on this media was about 3 mm, rough, irregular, convex, granular and green in colour.

2.4.2.3 Enumeration of Bifidobacterium *sp.*

Teraguchi et al. (1978) (published in English by Laroia & Martin in 1991) first advocated the use of the selective agents neomycin, nalidixic acid, lithium chloride and paromomycin sulphate (NNLP) for the selective enumeration of bifidobacteria. Since then, NNLP added to basal media (MRS or RCA) has been used by several workers to selectively enumerate *Bifidobacterium* and inhibit the growth of other probiotic and yoghurt or cheese starter culture (Dinakar & Mistry, 1994; Tharmaraj & Shah, 2003). *Bifidobacterium* is relatively resistant to gentamicin, nalidixic acid and neomycin. Starter lactococci, *Lb. casei* and *Lb. acidophilus* on the other hand, are sensitive to these

antibiotics (Tharmaraj & Shah, 2003). A low concentration of sodium salt (lithium chloride) that was part of the NNLP mixture further prevents the growth of starter bacteria in yoghurt and in cheese. L-cysteine (0.05 to 0.1%) is normally also added to the media used for enumeration of bifidobacteria to improve recovery (Shah, 2000b). Cysteine, a sulphur-containing amino acid, could provide amino nitrogen as a growth factor while reducing the redox potential, both of which might favour the growth of anaerobic *Bifidobacterium* species. When L-cysteine was not present in the media, *Bifidobacterium* either did not grow or formed pinpoint colonies. The absence of L-cysteine can thus be used to control the growth of *Bifidobacterium* from other media.

The use of media containing the antibiotic mupirocin has also been reported (Rada & Koc, 2000; Leuschner et al., 2003) for the enumeration of *Bifidobacterium* in dairy products and in animal feeds. Rada and Koc (2000) reported that bifidobacteria are relatively resistant to mupirocin. MRS, supplemented with the reducing agent L-cysteine, is used as the basal agar medium. There are, however, ethical issues concerning the use of this antibiotic. Mupirocin is particularly useful in the treatment of MRSA, methicillin-resistant *Staphylococcus aureus*. MRSA infection is a significant threat to the health of elderly and immunodeficient patients and has become a challenge to health services in many countries. Mupirocin is also active against *Helicobacter pylori*, another bacterium that is difficult to treat using conventional antibiotic therapy. Because of concerns about the emergence of mupirocin-resistant MRSA it would be prudent to expect difficulty in obtaining this antibiotic in future (Upton et al., 2003).

Wijsman et al. (1989) used several selective enumeration methods for *Bifidobacterium* in fermented dairy products, which are considerably outnumbered by streptococci and lactobacilli. Transgalactosylated oligosaccharide (TOS) agar medium, supplemented with various concentration of NNLP was found to be the best medium for the selective enumeration of *Bifidobacterium*. The NNLP had no significant negative effect on the recovery of colonies of bifidobacteria in the medium. A minimal dilution of 10^{-4} of the NNLP mixture was suggested to prevent the growth of pinpoint colonies of streptococci and lactobacilli. There is, however, a relatively shortage of TOS component due to the fact that the manufacturer (Yakult, Tokyo, Japan) is still producing TOS in pilot amounts, prior to a full scale production process with the aim of producing TOS as a component for infant foods (Wijsman et al., 1989). The authors suggested the use of L-arabinose as a possible substitute for TOS. The arabinose agar yielded only colonies of *Bifidobacterium* sp. when enumerating product that contained the mixture of *Bifidobacterium* sp., *Lb. acidophilus* and starter *St. thermophilus*. Colonies of *St.*

thermophilus and *Lb. acidophilus* could not be detected on L-arabinose agar thus addition of NNLP was not necessary. The Bergey's Manual, however, suggested that not all *Bifidobacterium* sp. can ferment L-arabinose (Buchanan & Gibbons, 1974). Thus the effectiveness of this media has to be tested since the ability of *Bifidobacterium* sp. to ferment L-arabinose varies with strain.

A few other media such as modified Columbia agar (MCA), Arroyo, Martin and Cotton Agar (AMC) and RCA with aniline blue and dicloxacillin (RCA-AD) have also been used for the selective enumeration of *Bifidobacterium* sp. in cheeses (Table 2.6). MCA was used for the selective enumeration of *Bifidobacterium* sp. in Cheddar like cheese and in fresh cheese in the presence of starter lactococci (Daigle et al., 1999; Roy et al., 1997). No information was found about the selectivity of this media against other probiotic such as *Lb. acidophilus* and *Lb. casei.* Darukaradhya et al. (2006) compared the effectiveness of AMC and RCA-AD media for selective enumeration of *Bifidobacterium* sp. in Cheddar cheese, and found that AMC media inhibited the growth of *Lb. acidophilus* or *Lb. casei* but did not inhibit the growth of starter lactococci or NSLAB. RCA-AD, on the other hand, inhibited the growth of *Lb. acidophilus, Lb. casei* and NSLAB but it allowed the growth of starter lactococci. The presence of aniline blue (dye), however, allowed the easy distinguishing of *Bifidobacterium* colonies from starter lactococci on RCA-AD.

This review shows that several media have been suggested for isolation and differential/selective enumeration of probiotic bacteria in fermented dairy products and cheeses. The range of different media used for the detection and enumeration of probiotic bacteria in fermented foods indicates that there is no standard medium. The difficulties associated with the detection and enumeration of probiotic bacteria is caused by the strain specificity of results, the simultaneous use of different species in the product and differences found in cell recovery or colony differentiation. Many of the media developed to enumerate probiotic bacteria have failed because of the lack of recovery, lack of selectivity or differentiation among colonies (Boylston et al., 2004). The media proposed for routine analysis for differential or selective enumeration of probiotic bacteria should not be complex or time-consuming to prepare and should offer a good cell recovery for the microorganisms.

2.5. Biochemistry of cheese ripening

A series of chemical and biochemical reactions occur during Cheddar cheese ripening including glycolysis, lipolysis and most importantly proteolysis (Fox et al., 1993).

2.5.1 Glycolysis

About 98% of lactose is removed in the whey as lactose or lactate during the manufacture of Cheddar. Concentration of lactose in Cheddar cheese is ~ 1.5%. This residual lactose is metabolized rapidly, predominantly to L-lactate, mainly by starter bacteria. *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* metabolize lactose to L(+) lactic acid; the glucose moiety is metabolized via the Embden-Meyerhof (EM) pathway while galactose is metabolized via the tagatose pathway. NSLAB such as *Lactobacillus* sp. may also metabolize the residual lactose. The Gal^+ lactobacilli convert galactose via the Leloir pathway to glu-6-P and then metabolize to DL-lactate via the EM pathway (McSweeney & Fox, 2004).

The rate of fermentation of the residual lactose is greatly dependent on the salt-in-moisture (S/M) content of the curd. *L. lactis* ssp. *cremoris* is more salt sensitive than *L. lactis* ssp. *lactis*, which in turn is more sensitive than NSLAB (McSweeney & Fox, 2004). Since some NSLAB are facultative heterofermentative, the S/M concentration may determine the products of lactose fermentation during ripening. At low S/M concentration, residual lactose is converted mainly to L-lactate by the starter. At high S/M concentration, considerable amount of D-lactate is formed, partly by fermentation of lactose, partly by isomerization of L-lactate (Figure 2.6). The racemization of L-lactate causes undesirable white specks on the surface of Cheddar cheese, which may cause consumers to reject cheese as being mouldy or containing foreign bodies. Crystals growth requires nucleation centres which may be bacterial cells, or microcrystal of calcium phosphate. Low pH, high salt (causes the ion-exchange of Na^+ for Ca^{2+}) or lower ripening temperature (reduce the solubility of Ca-lactate) will favour crystal formation (Dybing et al., 1988).

Lactate can also be metabolized by LAB, depending on strain, to acetate, formate and CO_2. Thomas (1987) reported that NSLAB isolated from cheese such as *Lb. plantarum* and *Lb. brevis* can oxidize L- and D-lactate to acetate in cheese. Oxidation of lactate to acetate in cheese depends on the NSLAB population and on the availability of

oxygen, which is determined by the size of the block and the oxygen permeability of the packaging material (Thomas, 1987). Gas production by microoganisms may also occur in cheese during ripening and may be desirable (e.g., eye production in Swiss and Ducth-type cheeses) or a defect. Contamination of milk with *Cl. tyrobutyricum* results in a defect known as late gas blowing caused by anaerobic metabolism of lactate to butyrate and H_2 during ripening (Thuault et al., 1991). Cheddar cheese is not susceptible to late gas blowing mainly because it is dry-salted and has low pH (optimum growth for *Cl. tyrobutyricum* is pH 5.3 – 5.9, NaCl level of 0 – 0.6%) (McSweeney & Fox, 2004). The time lag for the salt to reach an inhibitory level throughout the cheese may, however, pose a problem. Some NSLAB such as *Lb. brevis* and *Lb. fermentum* were also able to cause late gas blowing in Cheddar cheese (Laleye et al., 1987). Late gas blowing may be avoided by minimizing spore numbers in milk by good hygiene and pasteurization.

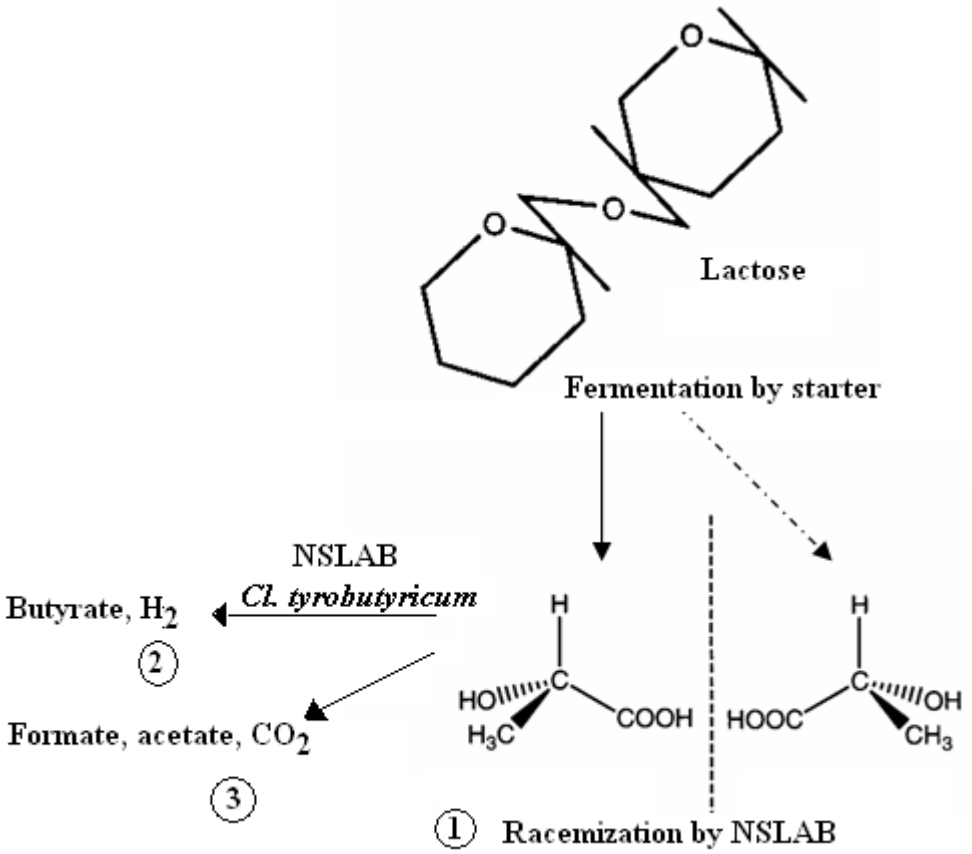


Figure 2.6. Summary of pathway in which lactose is metabolized during cheese during ripening: (1) racemization by NSLAB, (2) oxidation of lactate to formate, ethanol and acetate, and (3) anaerobic metabolism of lactate to butyrate and H_2 which leads to late gas blowing.

Acetate is usually present at high concentration in cheeses and is considered to contribute to cheese flavour, although a high concentration may cause off-flavours. Acetate may also be produced by starter and non-starter bacteria from lactose, citrate or from amino acids (Thomas, 1987). *Bifidobacterium* sp. was able to produce acetic acid as their metabolic end product. Increased acetic acid concentration in Cheddar cheeses with the addition of *Bifidobacterium* sp. has been reported (Gomes et al., 1995). In the genus *Bifidobacterium,* hexoses are degraded exclusively and specifically by the fructose-6-phosphate pathway described by Scardovi and Trovatelli (1965). The fermentation of two moles of glucose leads to three moles of acetate and two moles of lactate (Figure 2.7). The key enzyme involved in this glycolytic pathway is fructose-6-phosphate phosphoketolase (F6PPK), which is also used as a taxonomic character in identification of the genus *Bifidobacterium.*

Citrate is present in milk at a relatively low concentration (~8 mmol L^{-1}). About 94% of citrate in milk is soluble and most of it is lost in whey. Cheddar cheese contains 0.2-0.5% citrate. Citrate is metabolized by most strains of *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris* and *Lactobacillus* sp. to diacetyl, acetate, acetoin and CO_2 (Palles et al., 1998). Acetate and diacetyl produced from citrate metabolism may contribute to Cheddar cheese flavour. CO_2 production, on the other hand, is responsible for the undesirable open curd defects in Cheddar cheese. Thomas (1987) showed that citrate in Cheddar cheese decreased slowly to almost zero at 6 mo, presumably as a result of metabolism by lactobacilli which became the major component of the NSLAB flora.

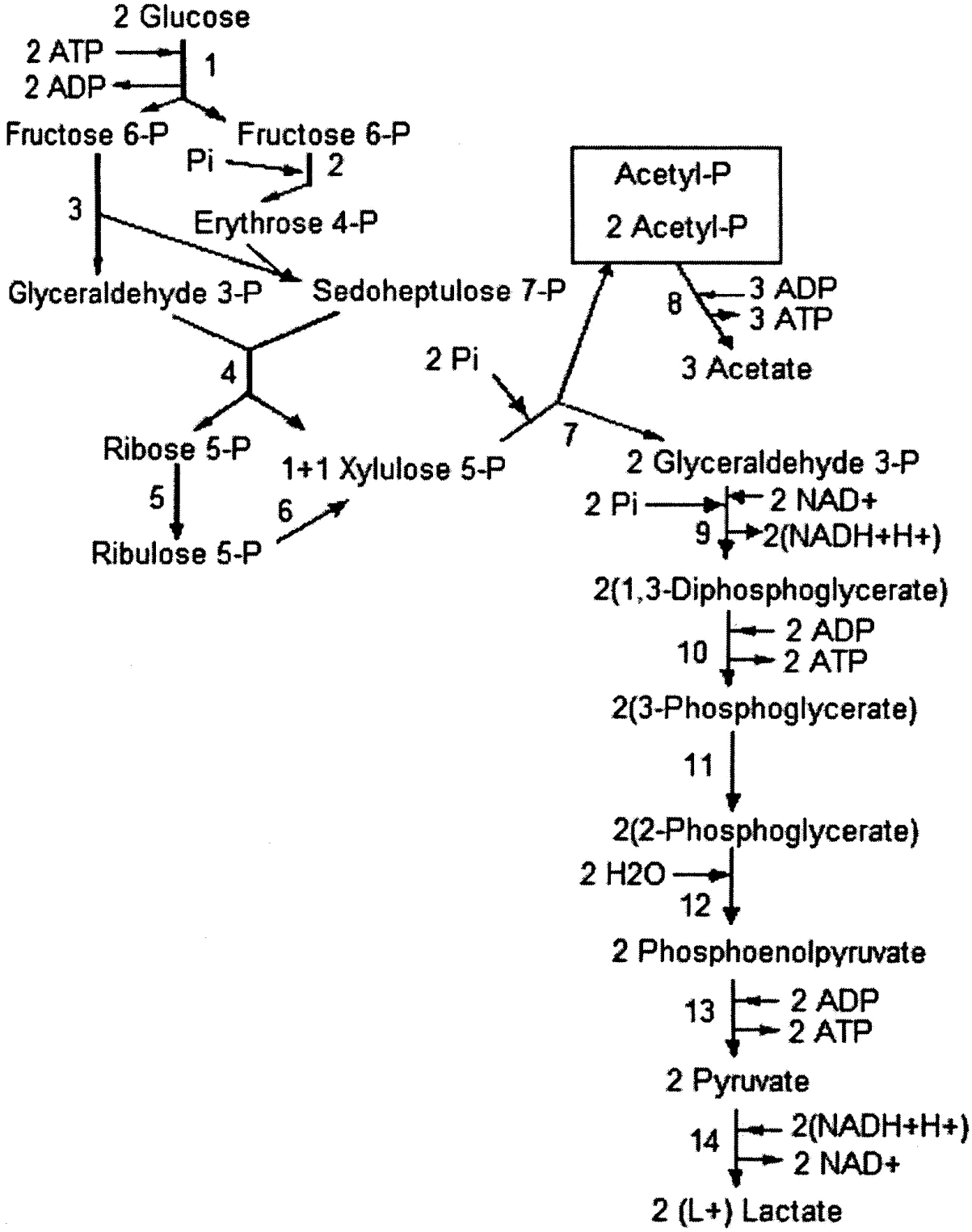


Figure 2.7. Metabolic pathway of *Bifidobacterium*. 1 = hexokinase and glucose-6-phosphate isomerase; 2 = fructose-6-phosphate phosphoketolase (F6PPK); 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphoketolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase; 11 = phosphoroclastic enzyme; 12 = formate dehydrogenase; 13 = alcohol dehydrogenase. Adapted from Ballongue (2004).

2.5.2 Proteolysis

Proteolysis is the most complex biochemical event which occurs during cheese ripening. Proteolysis in probiotic Cheddar cheeses is catalysed by proteinases and peptidases from several sources including indigenous enzyme from the milk, coagulant, starter, NSLAB and probiotic adjuncts. Because of the enzyme activity, the casein content decreases during ripening. Caseins account for 76 to 86% of the total milk proteins and are composed of α_{s1}-, α_{s2}-, β- and κ- caseins in a ratio of 3: 0.8: 3: 1 (Updhyay et al., 2004). The summary of the proteolysis which occurs in probiotic Cheddar cheeses during ripening is summarized in Figure 2.8.

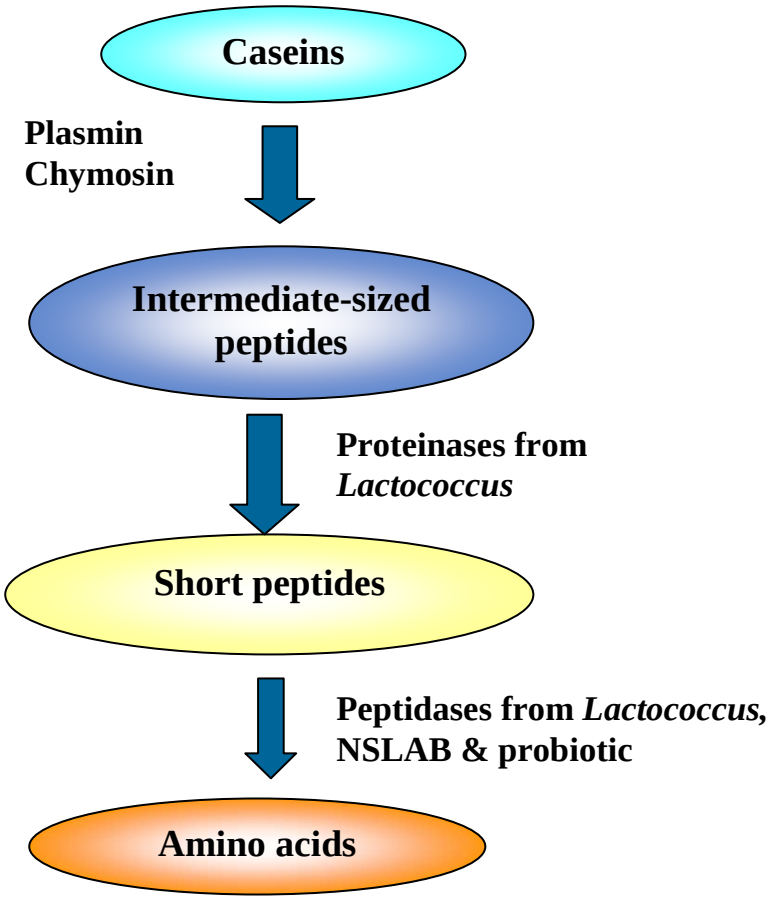


Figure 2.8. Proteolysis in probiotic Cheddar cheese during ripening.

Primary proteolysis of the cheese is caused by the coagulant and to a lesser extent by plasmin and perhaps somatic cell proteinases, which result in the formation of large-

(water-insoluble) and intermediate-sized (water-soluble) peptides. These intermediate-sized peptides are further hydrolysed by the coagulant and proteinases from starter, NSLAB and probiotic adjuncts. The production of small peptides and amino acids is caused by the action of the microbial proteinases and peptidases, respectively (Upadhyay et al., 2004).

Proteolysis contributes to the development of cheese texture via the hydrolysis of protein matrix of cheese, via the increase in pH through the production of NH_3 from amino acids catabolism and by increasing the water-binding capacity of the curd through the formation of new α– carboxylic and α– amino groups produced during hydrolysis of peptide bonds (Upadhyay et al., 2004). Proteolysis also contributes to flavour of the cheese through the liberation of short peptides and amino acids, some of which may have flavours and through the production of amino acids as precursor for a range of catabolic reactions which produce many important volatile compounds (McSweeney & Sousa, 2000). Following are the brief reviews of the role of indigenous enzyme from milk, coagulant, starter, NSLAB and probiotic to proteolysis of cheese during ripening.

2.5.2.1 Role of indigenous enzyme in milk on proteolysis during ripening

Milk contains a number of indigenous proteinases such as plasmin, which is produced from an inactive precursor, plasminogen. Plasmin is a heat stable trypsin-like serine proteinase with optimum activity at pH 7.5 and 37°C. Plasmin contributes more to proteolysis in Swiss-type cheese due to the fact that these cheeses are cooked at high temperature (~55 °C), at which most of the coagulant (chymosin) is inactivated, but on the other hand plasminogen is activated (Farkye & Fox, 1990). Plasmin also plays an important role in ripening of mould- or smear- ripened cheese varieties such as Camembert and Tilsit. Catabolism of lactic acid and deamination of amino acids with the production of NH_3 by the mould on the surface of the cheese result in an increase in the pH of the cheese to ~7.0. The increase in pH facilitates plasmin action, which contributes significantly to proteolysis of these cheeses (Upadhyay et al., 2004). Plasmin is most active on β-CN, hydrolysing it at three sites to produce γ-caseins and some proteose-peptones (Fox et al., 1994). Milk may also contain somatic cells, which contain lysosomes with a number of proteinases including cathepsins B, D, G, H, L and elastase (Keely & McSweeney, 2003). Most of these lysosomal enzymes in somatic cell can hydrolyse a wide variety of proteins including α_{s1}- and β-CN and the cleavage site has been studied by

several workers (Larsen et al., 1996; Considine et al., 2000, 2002, 2004). The presence of some of the lysosomal enzymes in milk, however, has not been confirmed yet.

2.5.2.2 Role of coagulant on proteolysis during ripening

Chymosin (EC.3.4.23.4) is the principal proteinase (88-94%) in the traditional calf rennet, the remainder being pepsin (EC 3.4.23.1). Chymosin is an aspartyl proteinase of gastric origin, secreted by young mammals. The principal role of chymosin in cheese making is to hydrolyse the Phe_{105} – Met_{106} bond of the micelle-stabilizing protein, κ-casein, as a result of which the colloidal stability of the micelles is destroyed, leading to gelation at temperature >20°C (refer to section 2.2.2.3). Most rennet added to milk during Cheddar cheese production is removed through the whey. Creamer et al. (1985) reported that ~6% of chymosin added to milk is retained in the curd, but the amount increased with decreasing pH at whey draining. Chymosin is weakly proteolytic (Upadhyay et al., 2004). During milk coagulation, α_{s1}-, α_{s2}- and β-CN are not hydrolysed, but may be hydrolysed in cheeses during ripening.

Chymosin cleaves β-CN at seven sites: Leu_{192}–Tyr_{193}, Ala_{189}–Phe_{190}, Leu_{165}–Ser_{166}, Gln_{167}–Ser_{168}, Leu_{163}–Ser_{164}, Leu_{139}–Leu_{150}, Leu_{127}–Thr_{128} in solution of 0.05 M sodium acetate buffer at pH 5.4 (Visser & Slangen, 1977). Chymosin cleaves α_{s1} at Phe_{23}-Phe_{24} and the small peptide α_{s1} (f1-23) is hydrolysed rapidly by starter proteinases. Chymosin was also known to cleave Leu_{11}-Pro_{12}, Phe_{28}-Pro_{29}, Phe_{32}-Gly_{35}, Leu_{40}-Ser_{41}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{144}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160}, Trp_{164}-Tyr_{165} and Phe_{179}-Ser_{180} at pH 5.2 in the presence of 5% NaCl, a similar condition in many cheese varieties. α_{s2}-CN is relatively resistant to proteolysis by chymosin. Cleavages sites of α_{s2}-CN by chymosin are restricted to hydrophobic region of the molecule, Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Tyr_{98}, Tyr_{98}-Leu_{99}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175} and Tyr_{179}-Leu_{180} (Upadhyay et al., 2004).

2.5.2.3 Role of starter, NSLAB and probiotic microorganisms on proteolysis

Starter bacteria contain cell envelope proteinases which contribute to ripening by hydrolysing intermediate size and short peptides from the caseins by the action of chymosin or plasmin. Starter bacteria also contain peptidases, which are responsible for

the hydrolysis of short peptides and the liberation of amino acids. The proteolytic system of *Lactococcus* is summarized in Figure 2.9.

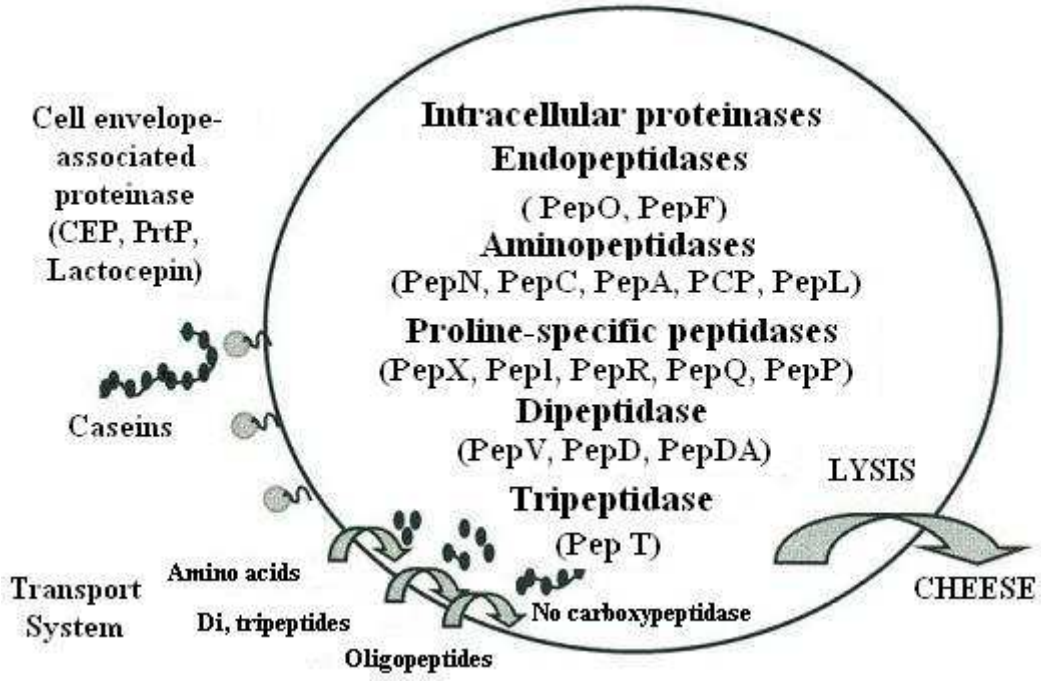


Figure 2.9. Proteolytic system of *Lactococcus* and other lactic acid bacteria. Adapted from McSweeney & Fox (2004).

Milk contains very low levels of small peptides and amino acids to support the growth of LAB. The proteolytic system of LAB enables them to grow in milk. Starter lactococci possess a cell envelope-associated proteinase (PrtP or lactocepin). PrtP contributes to the formation of small peptides in cheese, probably by hydrolyzing larger peptides produced by chymosin or plasmin. Cell envelope- associated proteinases with properties similar to the lactococcal lactocepins have also been isolated from a number of strains of *Lactobacillus* (Kunji et al., 1996).

Starter lactococci also possess intracellular peptidases, endopeptidases, exopeptidases (aminopeptidases, proline-specific peptidases), dipeptidases, tripeptidase and a number of oligopeptide, di-, tri- peptide and amino acid transport systems (Figure 2.9). The intracellular peptidases are responsible for the release of free amino acids especially after the cells have lysed. The proteolytic system of other LAB such as NSLAB and probiotic are generally similar (Kunji et al., 1996).

Addition of NSLAB as an adjunct to the normal starter to improve proteolysis, increase casein hydrolysis and enhance flavour development during Cheddar cheese

ripening has been studied by several researchers (Broome et al., 1990; Drake et al., 1996; Lynch et al., 1996; Muir et al., 1996). Probiotic bacteria also possess proteolytic system that may contribute to the release of small peptides and free amino acids in cheese (Shihata & Shah, 2000). Addition of probiotic *Lb. paracasei* or *Lb. acidophilus* in Cheddar cheese has been reported to increase proteolysis especially in the formation of low molecular mass peptides and free amino acids (Gardiner et al., 1998; Bergamini et al., 2006). In contrast to that, study conducted by Dinakar and Mistry (1994) found that proteolysis pattern was not affected by the addition of *Bifidobacterium* in Cheddar cheese (see Chapters 3 & 4 for the contribution of various probiotic microorganisms to proteolysis during ripening).

2.5.2.4 Measurement of proteolysis

Most nitrogenous compounds that contribute to cheese flavour are soluble in aqueous solvents. Quantification of nitrogen in water soluble extract (WSE) by using the macro-Kjeldahl method is commonly used as crude index of cheese ripening. Kuchroo and Fox (1982) compared various extraction procedures for Cheddar cheese and the procedure recommended for the preparation of WSE is grated cheese homogenized at 20^{o}C for 10 min with twice its weight of water; the slurry was held at 40^{o}C for 1 h, centrifuged and filtered. High performance liquid chromatography (HPLC) analysis of WSE has also been effectively used for the evaluation of cheese maturity. WSE extracted from 41 different commercial Cheddar cheeses with different ages showed chromatographic peaks with different relative areas of which varied with the age of the cheeses (McSweeney et al., 1993). WSE is also frequently used for the isolation of peptides and amino acids using RP-HPLC.

Trichloroacetic acid (TCA) is the protein precipitant that is commonly used to fractionate cheese nitrogen. The concentrations used varied from 2 to 12%. It was observed that rennet, and starter peptidases are responsible for the formation of some of the 12% TCA soluble nitrogen (McSweeney et al., 1993). Phosphotungstic acid (PTA) is a very discriminating protein precipitant. Free amino acids, except dibasic amino acids are soluble in 5% PTA, but peptides greater than about 600 Da are precipitated. Five percent PTA-SN has been widely used as an index of free amino acids in cheese. PTA-SN is produced primarily by the action of microbial peptidases. Both PTA-SN and TCA-SN

correlate significantly ($P < 0.001$) with the age and flavour intensity of Cheddar cheeses (Aston et al., 1983).

Electrophoresis has been applied widely to study primary proteolysis in cheese and is mostly related to the activities of residual coagulant and plasmin. α_{s1}–CN hydrolysed faster than β-CN in cheeses. α_{s1}–CN is the principal substrate for rennet during ripening and is converted to α_{s1}-I CN [α_{s1} (f1-23)] during the initial stage of ripening and other peptides at the later stage of ripening (see section 2.5.2.2). The level of residual α_{s1}–CN detected using gel electrophoresis is a good index of the level of general proteolysis at the early stage of ripening. β-CN is not extensively hydrolysed in bacterial ripened cheeses although in some varieties such as Emmental and Gouda, a considerable amount of γ-casein is formed via the action of plasmin, the activity of which appears to be dependent on the cooking temperature (see section 2.5.2.1).

2.6. Bioactive peptides

Bioactive peptides have been defined as 'peptides with hormone- or drug- like activity that eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses' (Fitzgerald & Murray, 2006). Milk protein is a rich source of biologically active peptides such as antihypertensive-, antithrombotic-, opioid-, immune-stimulating-, antimicrobial-, mineral carrying- and cholesterol lowering-peptides (Shah, 2000c). The activity of the peptides is based on the inherent amino acid composition and sequence. Bioactive peptides usually contain 3 – 20 amino acids per molecule. The peptides must both cross the intestinal epithelium and enter the blood circulation, or bind directly to specific epithelial cell-surface receptor sites in order to cause a biological response (Korhonen & Pihlanto-Leppälä, 2006). Most of these peptides are hidden in the inactive state in the original parent protein structure and may be released during food processing and storage. Bioactive peptides may be released through (a) hydrolysis by digestive enzymes such as trypsin and pepsin (FitzGerald et al., 2004; Korhonen & Pihlanto-Leppälä, 2006), (b) food processing (Van-Beresteijn et al., 1994) and (c) through hydrolysis by proteolytic microorganisms or through the action of proteolytic enzymes derived from the microorganisms (FitzGerald & Murray, 2006). This review will focus on antihypertensive peptides, the role of food processing and LAB in the liberation of these peptides, the occurrences of these peptides in cheeses and the identification methods for bioactive peptides.

2.6.1 Antihypertensive peptides

In the rennin-angiotensin system, Angiotensin-I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) plays a crucial role in the regulation of blood pressure and cardiovascular function (Figure 2.10).

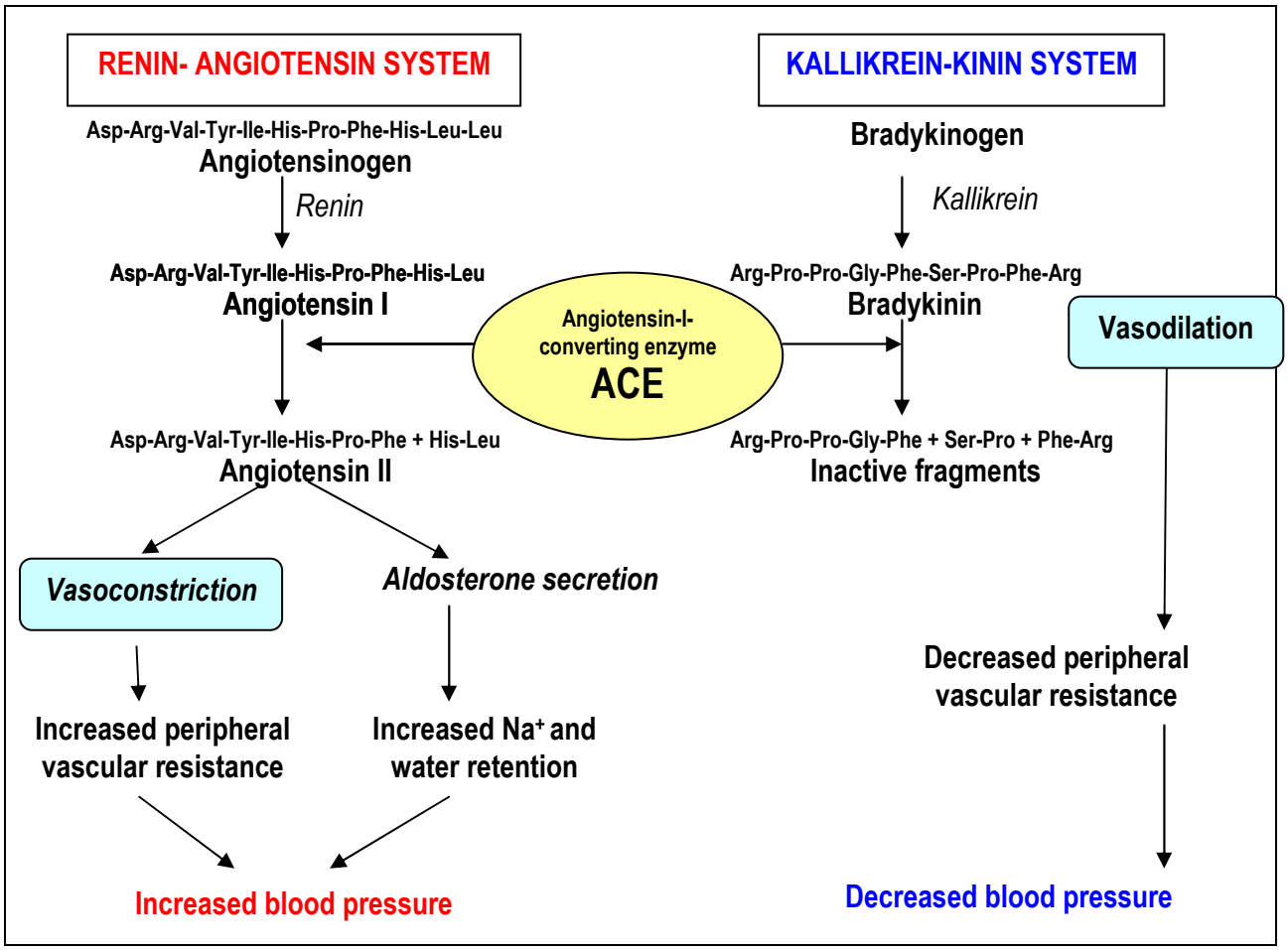


Figure 2.10. Regulation of blood pressure: role of angiontensin-I-converting enzyme in rennin- angiotensin system and Kallikrein-Kinin system. Adapted from Li et al. (2004).

ACE converts the inactive decapeptide angiotensin I by cleaving dipeptide from the C-terminus into angiotensin II, a potent vasoconstrictor. Angiotensin II is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure (Johnston, 1992). In the Kallikrein-Kinin system, ACE catalyses the degradation of bradykinin, a vasodilatory nonapeptide. ACE has also been shown to degrade ekephalins, neurotensin and substance P, which may interact with the cardiovascular system (Li et al., 2004). Inhibition of ACE is thus considered useful therapeutic approach in the treatment of hypertension.

ACE inhibition may also influence different regulatory systems involved in modulating blood pressure, immune defence, and nervous system activity (Meisel, 1998). The first reported competitive inhibitor of ACE is the naturally occurring peptides in snake venom (Ondetti et al., 1971). Many other ACE inhibitors have been discovered from enzymatic hydrolysis of bovine caseins, plant and other food proteins (Okamoto et al., 1995). ACE- inhibitory peptides have also been isolated from a variety of fermented dairy products including cheeses, fermented milk and yoghurt.

2.6.2 Role of food processing in the liberation of bioactive peptides

The structural and chemical changes that occur during the processing of food proteins may result in the release of bioactive peptides. In particular, heat and/or alkali treatment can generate additional inter- and intra-molecular covalent bonds that are resistant to hydrolysis. Such processing conditions also promote the racemic conversion of L-amino acids to D-isomers and consequently lead to the indigestible peptide bonds. The potential formation of indigestible peptide sequences during food processing may promote both formation and absorption of bioactive peptides that do not occur naturally in the precursor protein. For example, hydrolysed milk proteins used for hypoallergenic infant formulas for clinical application and as food ingredients consist exclusively of peptides including bioactive peptides (Van-Beresteijn et al., 1994). Cheese contains phosphopeptides as natural constituents and secondary proteolysis during cheese ripening leads to formation of various ACE- inhibitory peptides (Singh et al., 1997). Proteases from food itself, such as plasmin in milk, can hydrolyse proteins during food processing which may become precursor for formation of bioactive peptides.

2.6.3 Role of lactic acid bacteria in the liberation of bioactive peptides

Various oligopeptides and peptides are liberated during microbial fermentation of milk products. The proteolytic system of LAB has been explained in section 2.5.2. LAB can hydrolyse more than 40% of the peptide bonds of β-CN resulting in the formation of more than 100 different oligopeptides, which are in turn actively degraded by the complex peptidases system. The same pattern is reported for α_{s1}-CN (Kunji et al., 1996).

Consequently LAB could potentially generate a large variety of peptides including bioactive peptides.

Nakamura et al. (1995) reported that two peptides with amino acid residues of Val-Pro-Pro and Ile-Pro-Pro, isolated from sour milk fermented with *Lb. helveticus* and *Saccharomyces cerevisiae*, exhibited ACE inhibitory and antihypertensive activities. The concentrations of the peptides required to inhibit 50% of angiotensin-I-converting enzyme activity (IC_{50}) were 9 and 5 µmol L^{-1}, respectively. Several other ACE inhibitory peptides derived from β-CN including (f 6-14), (f 47-52) and (f 73-82) have been isolated from fermented milk with *Lb. delbrueckii* subsp. *bulgaricus* SS1 and *L. lactis* subsp. *cremoris* FT4 (Gobbetti et al., 2000). Ashar and Chand (2004) identified ACE-inhibitory peptides from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* or combination of *Streptococcus thermophilus* and *L. lactis* subsp. *lactis* with IC_{50} of 1.7 mg mL^{-1} and 1.4 mg mL^{-1}, respectively.

In addition to live microorganism, proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins. Yamamoto et al. (1994) reported that casein hydrolysed by the cell wall-associated proteinase from *Lb. helveticus* CP790 showed antihypertensive activity in spontaneous hypertensive rats (SHR). The peptides were derived from α_{s1}-CN including (f 24-31) and (f 170-199) and from β-CN including (f 168-175), (f 183-190), (f 113-127), (f 193-210), (f 70-97), (f 191-210) and (f 16-91). Among those peptides, (f 43-69) of the β-CN showed the highest ACE inhibitory activity with IC_{50} of 4 µmol L^{-1} (Yamamoto et al., 1994). Pan et al. (2004) hydrolysed skimmed milk with a cell-free extract of *Lb. helveticus* JCM1004 and purified the antihypertensive tripeptides Val-Pro-Pro and Ile-Pro-Pro, from the hydrolysate with IC_{50} of 9.12 ± 0.21 µmol L^{-1} and 5.15 ± 0.17 µmol L^{-1}, respectively. A significant ($P < 0.01$) decrease in systolic blood pressure in SHR was measured after a single gastric intubation of peptides, Val-Pro-Pro and Ile-Pro-Pro.

2.6.4 Bioactive peptides in cheeses

Ripened-type cheeses contain numerous peptides that originate mainly from casein released as a result of proteolysis during ripening. Various phosphopeptides have been found as natural constituent in Parmesan and Cheddar cheeses (Addeo et al., 1992; Singh et al., 1997). Smacchi and Gobbetti (1998) reported that peptides isolated from Italian cheeses (Crescenza, Gorgonzola, Mozzarella and Italico) were effective in reducing

activity of ACE. Some ACE-inhibitory peptides have also been isolated from other varieties such Parmesan cheese (Addeo et al., 1992), Gouda cheese (Meisel et al., 1997; Saito et al., 2000), Cheddar cheese (Ryahanen et al., 2001), Finish cheeses (Korhonen & Pihlanto-Leppala, 2001), Manchego cheese (Gomez-Ruiz et al., 2002) and Australian cheeses (Dionysius et al., 2000). Cheeses prepared by commercial enzymes (Neutrase® and Debitrase®) and *Lb. casei* enzymes (amino peptidase) were also reported to produce bioactive peptides (Haileselassie et al., 1999). Table 2.7 shows some examples of bioactive peptides that have been identified in various cheeses.

The appearance of these bioactive peptides is dependent on the ripening stage of the cheese. An α_{s1}-CN derived antihypertensive peptide isolated from Parmesan cheese at 6 mo of ripening could not be found after 15 mo (Addeo et al., 1992). Similarly the antihypertensive activity found in long-ripened Gouda cheese was half as much as that found in its medium-aged counterpart (Meisel et al., 1997). Following isolation from Gouda cheese, Saito et al. (2000) analysed the structure of the antihypertensive peptides and concluded that the strongest depressive effect on the systolic blood pressure (SBP) and the highest ACE-inhibitory capacity were associated with peptides found in 8 mo old water soluble extract of the cheese (maximum decrease in SBP was 24.7 ± 0.3 mm Hg after 6 h of gastric intubation at doses between 6.1 and 7.5 mg kg^{-1} body weight). Two peptides isolated from the water soluble extract were identified as α_{s1}-CN (f 1-9) and β-CN (f 60-68).

A fermented low-fat hard cheese produced with probiotic LAB was found to produce high amounts of ACE-inhibitory peptides derived from α_{s1}-CN during maturation (Ryahanen et al., 2001). Consistent with these findings, Gomez-Ruiz et al. (2002), who studied the ACE-inhibitory peptides in Manchego cheese, found the antihypertensive activity to decrease within the first 4 mo, was maximum at 8 mo of ripening and decreased again at 12 mo. The peptides emerged at the age of 3 mo and their level remained stable for 6 mo. These results suggest that the concentration of active peptides in cheeses increases with cheese maturation, but starts to stabilize or decline when proteolysis exceeds a certain level.

Table 2.7. Identified bioactive peptides from various cheeses

Product	Bioactive peptides	Bioactivity	References
Parmesan cheese	β-CN (f 8-16), (f 58-77) α_{s2}-CN (f 83-33)	Phosphopeptides ACE- inhibitory Precusor of β-casomorphins	Addeo et al. (1992)
Cheddar cheese	α_{s1}- and β-CN fragments	Phosphopeptides	Singh et al. (1997)
Gouda cheese	α_{s1}-CN (f 1-9), β-CN (f 60-68)	ACE- inhibitory	Meisel et al. (1997), Saito et al. (2000)
Enzyme modified cheese	β-CN (f 60-66)	Opioid activity ACE- inhibitory	Haileselassie et al. (1999)
Italian cheeses varieties: Mozarella, Crescenza, Italico, Gorgonzola	β-CN (f 58-72)	ACE- inhibitory	Smacchi and Gobbetti (1998)
Australian cheeses varieties: Cheddar, Edam, Swiss, Feta, Camembert, Blue vein	Active peptides not identified	ACE- inhibitory Immunomodulatory Opioid agonist	Dionysius et al. (2000)
Finnish cheeses varieties: Edam, Emmental, Turunmaa, Cheddar	Active peptides not identified	ACE- inhibitory	Korhonen and Pihlanto-Leppälä (2001)
Low fat Cheddar cheese	α_{s1}-CN (f 1-9), (f 1-7), (f 1-6)	ACE- inhibitory	Ryhanen et al. (2001)
Manchego cheese	Ovine α_{s1}-, α_{s2}- and β-CN fragments	ACE- inhibitory	Gomez-Ruiz et al. (2002)

In recent years, a few fermented dairy products with antihypertensive peptides have been launched in the Japanese, Finish and US markets (Table 2.8).

Table 2.8. Commercial dairy products with health claims based on bioactive peptides

Product	Bioactive peptides	Health claims	References
Ameal S[1] – sour milk	β- CN, κ- CN Val-Pro-Pro, Ile-Pro-Pro	Reduction of blood pressure	Hata et al. (1996), Takano (1998)
Evolus[2] – calcium enriched fermented milk drink	β- CN Val-Pro-Pro, Ile-Pro-Pro	Reduction of blood pressure	Seppo et al. (2002, 2003)
BioZate[3] – hydrolyzed whey protein isolate	β- lactoglobulin (f142-148)	Reduction of blood pressure	Klink (2002)
Festivo[4] – fermented low fat hard cheese	α_{s1}-CN (f1-9), (f 1-7), (f1-6)	No health claim yet	Ryhanen et al. (2001)

[1]Ameal S is produced by Calpis Food Industry Co Ltd, Japan; [2]Evolus is produced by Valio Oy, Finland; [3]BioZate is produced by Davisco Foods International Inc, USA; [4]Festivo is produced by MTT Agrifood Research, Finland.

The Japanese sour milk product "Ameal S"™ is made by inoculating skim milk with a starter containing *Lb. helveticus* and *S. cerevisiae*. The fermented drink is rich in the peptides Val-Pro-Pro and Ile-Pro-Pro which have shown to lower blood pressure in clinical trials with mild hypertensive humans with a maximum decrease in SBP of 14.1 ± 3.1 mm Hg after 8 wk (Hata et al., 1996). The product, "Evolus"™ also contains the ACE- inhibitory tripeptides Val-Pro-Pro and Ile-Pro-Pro. Evolus™ is the product of fermentation with *Lb. helveticus* LBK16H. This fermented milk drink has demonstrated hypertensive effects in SHR with a maximum decrease in SBP of 6.7 ± 3.0 mm Hg after 21 wk (Seppo et al., 2003).

A fermented low fat hard cheese "Festivo" was developed in Finland (Ryahanen et al., 2001) with probiotic LAB and was found to produce high amount of ACE-inhibitory peptides. A whey protein hydrolysate "BioZate", containing ACE-inhibitory peptide was recently developed by Davisco Foods International Inc. There was a significant decrease in both systolic and diastolic blood pressure of hypertensive men and women with a daily dose of 20 g after 6 wk (Klink, 2002).

The ACE inhibitory peptides derived from food products are not as potent as the drugs commonly used for hypertension treatment. Those products, however, hold a moderate bioactivity, and behave intrinsically and naturally as functional foods. They can be easily included in the daily diet and can represent a natural alternative to ACE inhibitor drugs.

2.6.5 Identification of peptides

Several methods are available for the sequencing and identification of peptides. The most popular methods that are currently used for the identification of bioactive peptides from dairy products are the N-terminal sequencing of the Edman degradation method (Singh et al., 1997; Saito et al., 2000; Ryahanen et al., 2001) and peptide sequencing using mass spectrophotometry (Gobbetti et al., 2000; Minervini et al., 2003).

2.6.5.1 Edman degradation

Automated Edman degradation chemistry consists of three steps, a) coupling of phenylisothiocyanate (PITC) with the alpha-amino group of the protein/peptide at pH 9-10 to form a phenylthiocarbamyl (PTC) group, b) cleavage by anhydrous acid trifluoroacetic acid (TFA) to generate an anilinothiazolinone (ATZ) amino acid and c) conversion of the ATZ derivative to the more stable phenylthiohydantoin (PTH) derivative. Finally, the PTH amino acids are analysed usually by HPLC (Figure 2.11).

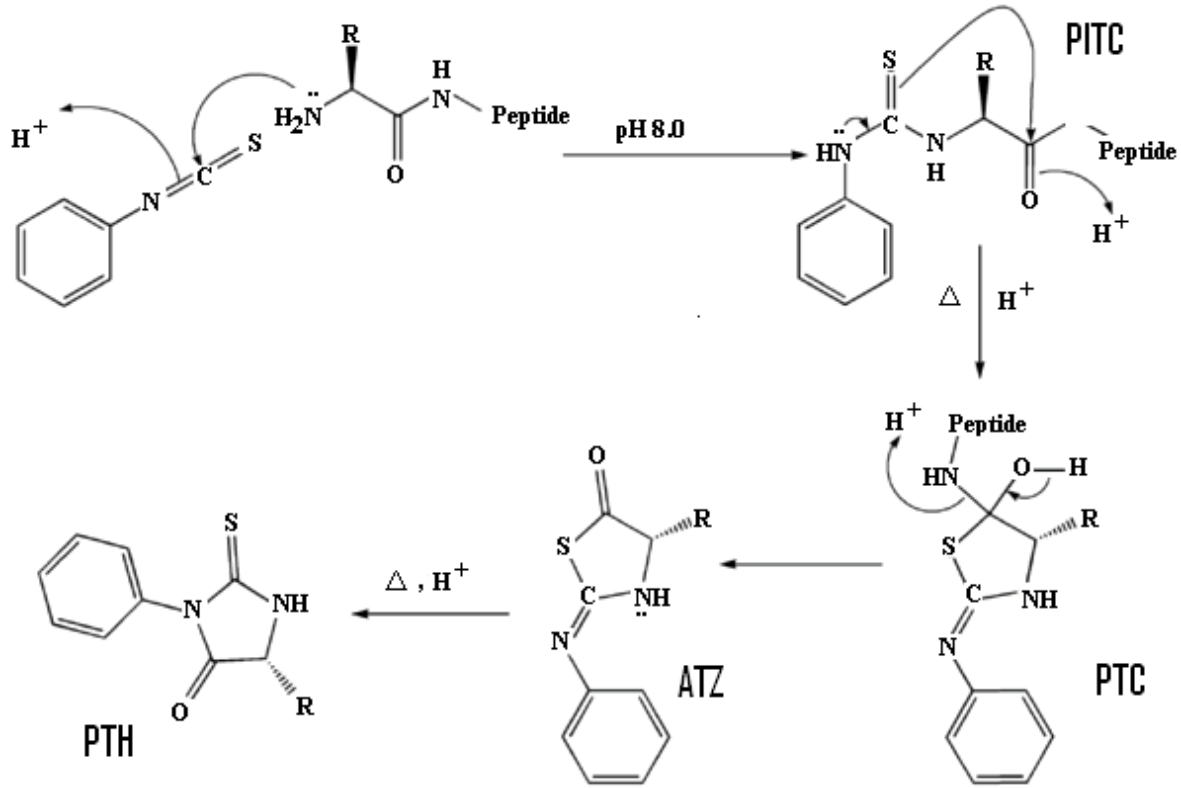


Figure 2.11. The Edman degradation reaction. PITC, phenylisothiocyanate; PTC, phenylthiocarbamoyl; ATZ, anilinothiazolinone; PTH, phenylthiohydantoin. Adapted from Yarwood (1989).

After one complete Edman reaction, the shortened peptide can be recycled to yield the PTH-derivative of the next residue and thus identifying the next amino acid (Yarwood, 1989) (see Appendix B for the example of RP-HPLC chromatogram for standard of PTH-amino acids). Some proteins may, however, undergo post-translational modifications that make the N-terminus unavailable for coupling to PITC (the 1st step in Edman chemistry). These proteins are effectively "blocked" and no sequencing data can be obtained from them in this state. A number of methods for removing N-terminal blocking groups have been reported without any success. A better approach for blocked proteins is to subject the sample to matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrophotometry analysis and/or chemically assisted fragmentation (CAF) chemistry to obtain sequence information.

2.6.5.2 Matrix assisted laser desorption ionization-time of flight

Purified peptides are incorporated into a matrix which helps the molecules ionize when hit by a laser. The resultant ions are accelerated by high voltage into a time of flight (TOF) tube. The velocity that these ions reach is dependant on their mass, with lighter ions acquiring a higher velocity than heavier ions. However, if the velocity of the ions is kept constant then the time that it takes for ions to hit a detector will be dependant only on their mass. The detector converts each of the times into a mass and produces a mass spectrum for each peptide, which is characteristic to that molecule. The mass spectrum can then be used to search a database to identify the peptide based on the molecular weight (see Chapter 6).

2.6.5.3 Chemical assisted fragmentation in combination with MALDI

Derivatization of tryptic peptides using an Ettan™ CAF MALDI sequencing kit in combination with MALDI-post source decay (PSD) is a fast, accurate and convenient way to obtain peptide sequencing data. CAF is based on solid-phase derivatization using a new class of water stable sulfonation agents; this strongly improves PSD analysis and simplifies the interpretation of acquired spectra.

CAF-MALDI chemistry is based on the introduction of a negatively charged group to the N-terminus of peptides generated by tryptic digestion. After derivatization, the

formation of a positively charged ion (net charge) introduces two protons into the peptide. One of these protons will primarily reside in the basic C-terminal side chain, while the other has a higher degree of freedom to resonate in the peptide backbone, assisting fragmentation. Only the y-ion fragments formed will retain a net positive charge and become separated in the reflectron, while N-terminal fragments are neutral and not detectable. With the generation of only y-ion series fragments, the mass between two peaks on the spectrum could be corresponded to the mass of individual amino acids (Flensburg et al., 2005) (see Chapter 6).

2.7. Acceleration of cheese ripening

The maturation of Cheddar cheese and the development of full flavour is a time consuming process. Any method which can accelerate the maturation and reduce storage time will provide substantial savings. Methods to improve maturation are based on increases in proteolysis resulting in increased flavour development. Research, however, has shown that accelerating proteolysis does not necessarily improve flavour development, suggesting that the production of amino acids is not the rate-limiting step in the development of cheese flavour. The principal role of proteolysis in the production of flavour compounds is the liberation of amino acids as precursors for a complex series of catabolic reactions that produce many important volatile flavour compounds. Accelerated maturation may involve the use of elevated temperature, exogenous enzymes, attenuated starters or addition of proteolytic strain of NSLAB (Azarnia et al., 2006).

2.7.1 Elevated ripening temperature

The biochemical reaction which generates flavour compounds or flavour precursors in cheese is accelerated by increasing the temperature at which the cheese is matured. Some studies have indicated that increasing ripening temperature from 6 to 15°C offers the producer a technologically simple method to achieve significant acceleration of cheese ripening (Aston et al., 1983; Law, 2001). After a maturation of six mo, cheeses ripened at 13°C scored 4.4 on a 0 to 8 scale for flavour intensity (corresponding to medium/mature cheese) while cheese stored at 6°C scored 3.2 (corresponding to mild Cheddar) (Law, 2001). The effect of higher ripening temperatures (8°C, 15°C, 17.5°C and 20°C) for

Cheddar cheeses stored for 32 wk was investigated by Aston et al. (1983). The levels of 12% TCA-SN and 5% PTA-SN increased with increasing temperature and duration of ripening. Cheeses stored at 20°C for 16, 24, 32 wk, however, were described as strongly off-flavoured, rancid, burnt or unclean. The maximum temperature at which cheeses could be stored for 32 wk without significant deterioration in quality was 15°C, at which cheeses showed levels of proteolysis after 12 wk similar to control cheeses stored at 8°C after 32 wk. In that study, the cheeses with higher level of proteolysis were less springy, softer and more fracturable.

An elevated ripening temperature may accelerate the loss of starter bacteria prior to complete lactose utilization, which may lead to off-flavours (Law, 2001). The growth of most NSLAB increased with higher ripening temperature. Fermentation of lactose by NSLAB produces some organic acid by-products such as formic acid and acetic acid (Fox et al., 1993). Excess of these compounds impairs the flavour balance of Cheddar cheeses. Appropriate ripening temperatures are required to maintain the balance growth of these bacteria to achieve the optimum cheese quality.

2.7.2 Exogenous enzymes and attenuated cultures in accelerated ripening

Since cheese ripening is essentially an enzymatic process, enzymes may be added to generate specific flavour in cheeses. The use of exogenous enzymes has the advantage of more specific action for accelerating flavour development compared to elevated temperatures. Addition of neutral proteinase from *Bacillus subtilis* (Neutrase), the aspartyl proteinase of *Aspergillus oryzae*, the alkaline protease of *Bacillus licheniformis* (Subtilisin) or the broad specificity proteinase from *Streptomyces griseus* (Pronase) to cheeses, significantly accelerate cheese ripening with enhanced cheese flavour, but with high level of bitterness (Wilkinson, 1993). Kheadr et al. (2000) recommended the use of an encapsulated bacterial proteinase or fungal proteinase to accelerate Cheddar cheese ripening without producing flavour or texture defects. The use of liposomes (artificial lipid membrane vesicles) as enzyme carriers also appeared to decrease the flavour defects that usually result from the addition of free enzymes. Cell free extracts (CFE) of *Lb. helveticus, Lb. bulgaricus, Lb. casei* or *L. lactis* have also been used to increase the level of small peptides and free amino acids in cheeses. Addition of Accelase (CFE from lactococci) to Neutrase-treated Cheddar cheese slurries was claimed to reduce bitterness due to the action of the starter peptidases to breakdown large peptides produced by Neutrase and/or chymosin to

smaller peptides and free amino acids (Wilkinson, 1993). The use of exogenous enzyme to accelerate ripening seems promising. Enzyme addition, however, is not permitted in all countries and the cost of enzymes such as proteinases, peptidases and lipases can be considerable, thus their use must be justified by a significant acceleration of flavour development.

Modified starter cultures such as (a) lysozyme-treated starters, (b) heat or freeze-shocked cells and (c) mutant starter cultures have been used to accelerate ripening. The objective of using modified/ attenuated starter is to increase the number of starter cells without detrimental effects on the acidification schedule during manufacture so that the cells contribute only to proteolysis during ripening (Wilkinson, 1993). Lysozyme and heat treatment cause early lysis of the cells which lead to early release of intracellular peptidases in cheese. Law (2001) indicated that the increase in the intracellular peptidases increased the concentration of free amino acids. However, no effect on the intensity of flavour over the control was noted. It was concluded that intracellular enzymes may not play a direct role in flavour development.

2.7.3 NSLAB in accelerated ripening

The addition of lactobacilli as an adjunct to the normal starter to increase casein hydrolysis and enhance flavour development during Cheddar cheese ripening has been studied by many researchers. Many lactobacilli added as adjuncts have been reported to increase the concentration of free amino acids (FAA) in Cheddar cheese and to enhance flavour development during ripening (Puchades et al., 1989; Lynch et al., 1996; Broome et al., 1990).

2.7.3.1 Influence of Lactobacillus helveticus *in cheese ripening*

Strains of *Lactobacillus helveticus* are homofermentative thermophilic LAB that are used in the manufacture of Swiss-type and long-ripened Italian cheeses. They are among the most nutritionally fastidious LAB, and require more exogenous amino acids than most other LAB (Kenny et al., 2003). At least 11 peptidases from *Lb. helveticus* have been characterised biochemically and/or genetically including the general aminopeptidases, PepC and PepN; proline-specific peptidases, PepX, PepI, PepQ and PepR; dipeptidases, PepD and

PepV; endopeptidases, PepE and PepO (Christensen et al., 1999), and more recently a tripeptidase PepT (Savijoki & Palva, 2000). In addition to the role that these peptidolytic activities play in growth they also contribute to overall proteolysis during cheese ripening through the release of amino acids (which are flavour precursors), decreasing bitterness by hydrolysing bitter peptides, changing the texture (by breakdown of the protein matrix) and increasing the pH and water binding capacity by the newly formed amino and carboxyl groups as peptide bonds are cleaved (Lawrence et al., 1987; Christensen et al., 2003).

Lb. helveticus may be added as adjunct in cheese manufacturing to improve flavour and accelerate ripening. *Lb. helveticus* DPC 4571 added as adjunct starter was previously shown to lyse during Cheddar cheese ripening releasing its intracellular enzymes, which led to an increase in proteolysis and improved cheese flavour (Kiernan et al., 2000). Autolysis is the spontaneous disintegration of bacterial cells which results in the liberation of the cytoplasmic content of the cell, including the intracellular enzymes. Autolytic starter bacteria strains release their intracellular enzymes at an early stage during ripening, thus have the ability to accelerate cheese ripening and enhance cheese flavour (Crow et al., 1995).

The proteolytic activity of *Lb. helveticus* is also related to the release of biologically-active peptides present in certain fermented milk. The major role of *Lb. helveticus* in the production of ACE-inhibitory peptides in fermented dairy products is evidenced in a number of studies, in which ACE-inhibitory activity has been determined in skimmed milk digested with cell-free extract of *Lb. helveticus* JCM1004 (Pan et al., 2004), bovine casein hydrolysed with an extracellular proteinase from *Lb. helveticus* CP790 (Yamamoto et al., 1994) and sodium caseinate hydrolyzed with a partially-purified proteinase of *Lb. helveticus* PR4 (Minervini et al., 2003). ACE-inhibitory activity has been determined also after fermentation of sodium caseinate by *Lb. helveticus* NCC 2765 (Robert et al., 2004) and in milk products fermented by *Lb. helveticus* CPN4 (Yamamoto et al., 1999), *Lb. helveticus* LKB-16 H (Seppo et al., 2002), *Lb. helveticus* CHCC637 and CHCC641 (Fuglsang et al., 2003), and a mixed-strain starter consisting of *Lb. helveticus* and *S. cerevisiae* (Nakamura et al., 1995).

3.0 Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei*, *Bifidobacterium* sp.*

3.1. Introduction

Probiotic bacteria are defined as 'living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition' (Ross et al., 2002). Foods containing probiotic bacteria are categorized as 'functional foods' and such products are gaining widespread popularity and acceptance throughout the developed world. A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhea, anticarcinogenic properties, reduction in blood cholesterol and improvement in immunity (Ballongue, 1993; Shah & Wu, 1999; Shah, 2000a,b). Daily consumption of high levels of probiotic bacteria, however, is required to confer health benefits. For dietary organisms to be beneficial in food systems, they should maintain viability in the food until the time of consumption and be present in significant numbers, at levels of at least 10^7 viable cells per gram or milliliter of product (Ishibashi & Shimamura, 1993). For this reason, changes in the numbers of viable bacteria during ripening period should be known.

Probiotic foods are currently restricted to fermented milk drinks and yoghurt, which have limited shelf life in contrast to hard cheeses including Cheddar. Incorporation of probiotic cultures in cheeses provides potential not only to improve health status and quality of products but also to increase the range of probiotic products. Cheeses have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to gastrointestinal tract as they tend to have higher pH, more solid consistency and relatively higher fat content. These offer protection to probiotic bacteria during storage and passage through the gastrointestinal tract. Cheeses also have higher buffering capacity than yoghurt (Stanton et al., 1998). Cheddar cheeses, however, have long ripening time hence development of probiotic Cheddar cheese requires a careful examination of the suitability of

* This chapter has been published. Ong, L., Henriksson, A., & Shah, N.P. (2006). Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei*, *Bifidobacterium* sp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal, 16*, 446 – 456.

particular strain(s) to maintain viability throughout the ripening and shelf life (Ross et al., 2002).

A number of studies have addressed development of probiotic cheeses using Cheddar cheese, goat cheese, Crescenza cheese, cottage cheese and fresh cheese (Ross et al., 2002). Dinakar and Mistry (1994) examined Cheddar cheese as a vehicle for incorporating bifidobacteria. The viability was retained for up to 24 wk at approximately 2 x 10^7 cfu g^{-1} and there were no adverse effects on cheese flavour, texture or appearance. Three *Bifidobacterium* species, *B. bifidum*, *B. longum* and *B. infantis*, were incorporated into Crescenza cheese (Gobbetti et al., 1997), which sustained cell counts of 10^8, 10^7 and 10^5 cfu g^{-1}, respectively for 14 days post manufacture. On the other hand, other studies have found low counts of probiotic bacteria particularly bifidobacteria during ripening (McBrearty et al., 2001). When bifidobacteria were used in combination with *Lactobacillus acidophilus* strain Ki as the starter in Gouda cheese manufacture (Gomes et al., 1998), there was also a significant effect on cheese flavour after 9 wk of ripening, possibly due to the production of acetic acid by bifidobacteria. These studies have demonstrated that Cheddar cheese has a great potential as a probiotic 'functional food'. However, it appears that the capabilities of probiotic strains to survive cheese manufacture and ripening vary from strain to strain.

In this study, six probiotic strains including *B. longum* 1941, *Lb. casei* 279, *Lb. acidophilus* 4962, *B. lactis* LAFTI®B94[†], *Lb. paracasei* LAFTI®L26[‡] and *Lb. acidophilus* LAFTI®L10 were examined as a potential candidate for incorporation in Cheddar cheeses. These strains have been selected based on their acid and bile tolerance, adhesion to intestinal cell line, anticarcinogenic properties, oxygen sensitivity and ability to modify gut microflora of human subjects (Lankaputhra & Shah, 1998; McIntosh et al., 1999; Crittenden et al., 2001).

The objectives of this study were thus to incorporate the selected probiotic strains into Cheddar cheese and examine the performance of these organisms in terms of their survival during manufacture and ripening and to examine the influence of these strains on product quality as assessed by the proteolytic patterns and the production of organic acid.

[†] *B. lactis* LAFTI®B94 has been reclassified as *B. animalis* subsp. *lactis* LAFTI®B94
[‡] *Lb. paracasei* LAFTI®L26 has been reclassified as *Lb. casei* LAFTI®L26

3.2. Materials and Methods

3.2.1. ***Starter and probiotic organisms***

Cheese starter culture, *Lactococcus lactis* subsp. *lactis* strain 227 and *Lactococcus lactis* subsp. *cremoris* strains 223 were obtained from Chr. Hansen (Bayswater, Vic, Australia) in freeze-dried form. The strains were activated by growing at least two times at 30^{o}C overnight in 12% (w/v) sterile reconstituted skim milk (RSM) containing 2% (w/v) glucose and 1.2% (w/v) yeast extract, prior to inoculation (2%, v/v) of the bulk culture in the same medium.

The probiotic strains, *Lb. acidophilus* 4962, *Lb. casei* 279 and *B. longum* 1941 were obtained from the Victoria University culture collection (Werribee, Vic, Australia), while *Lb. acidophilus* LAFTI®L10, *Lb. paracasei* LAFTI®L26 and *B. lactis* LAFTI® B94 were obtained from DSM Food Specialties Pty. Ltd. (Moorebank, NSW, Australia). *B. longum* 1941 (*B. longum* CSCC 1941) was originally obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) (Highett, Vic, Australia) while *Lb. acidophilus* 4962 and *Lb. casei* 279 were both originally obtained from the Australian Starter Culture Collection Center (ASCC) (Werribee, Vic, Australia). All *Lactobacillus* strains were subcultured (1%, v/v) at least two times at 37^{o}C overnight in 12 % (w/v) sterile RSM prior to use as a bulk culture (2%, v/v). Bifidobacteria were subcultured similarly using 1% inoculum in sterile RSM supplemented with 0.05% L-cysteine·hydrochloride (Sigma-Aldrich, St. Louis, MO, USA).

3.2.2. ***Cheddar cheese manufacture***

Cheddar cheeses were made with 10 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture using a pair of custom made cheese vats. Three batches of Cheddar cheeses were made. The first batch was a control batch with only starter lactococci (1.5% v/v) (Batch 1M), the second batch was produced with lactococci (1.5% v/v) and *Lb. acidophilus* 4962 (0.4% v/v), *Lb. casei* 279 (0.4% v/v), *B. longum* 1941 (0.4% v/v) (Batch 2M) and the third batch was produced with lactococci (1.5% v/v) and *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4% v/v), *B. lactis* LAFTI®B94 (0.4% v/v) (Batch 3M). Cheeses were made in three separate occasions.

Cheeses were manufactured according to the standard procedures (Kosikowski, 1997). Pasteurized whole milk was standardized with skim milk to give a casein to fat ratio

of 0.68. The milk was then tempered to 31^{o}C before inoculation with cheese starter culture and probiotic bacteria. The milk was left to ripen for 45 min before the addition of single strength chymosin (Chr. Hansen) at the rate of 0.2 mL per liter of milk. The milk coagulated after about 45 min and the resulted curd was cut with a cheese wire knife to 8 mm cubes. The curds were cooked to 38^{o}C by heating slowly at the rate of 1^{o}C rise per 5 min with agitation until the pH dropped to 6.1-6.2. The whey was drained and curds were cheddared at 38^{o}C and turned every 15 min until the pH dropped to 5.4-5.5. The curds were milled and salted (2% w/w) before putting into cheese moulds and pressed by placing a weight of 2.46 kg per 100 cm^{2} overnight.

The fresh cheese was removed from the mould, packed in oxygen barrier Cryovac® bags (Cryovac® Pty. Ltd., Fawkner, Vic, Australia) and heat sealed with a Multivac® vacuum packaging equipment (Multivac Sepp Haggenmüller, Wolfertschwenden, Germany) and ripened at 4^{o}C for 24 wk.

3.2.3. ***Cheese composition***

Grated cheese samples were analysed in duplicate for salt by Volhard method (AOAC 975.20, 1990), fat by Babcock method (AOAC 933.05, 1990), moisture by oven-drying at 102^{o}C (AOAC 926.08, 1990) and total protein by Kjeldahl method (AOAC 920.123, 1990) on a Kjeltec System II (with Digestion System 2000 and Distilling Unit 1002; Tecator, Höganäs, Sweden). The pH of cheese slurry prepared by blending 20 g of grated cheese with 12 mL of H_2O (Autralian Standard 2300.1.6, 1989) was measured with a pH meter (Model 8417, Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

3.2.4. ***Survival of bacteria in cheeses***

To assess the viability of the starter bacteria, probiotic organisms and non-starter lactic acid bacteria (NSLAB), samples of ripened milk, cooked curd, whey, cheddared curd and pressed curd (fresh cheese) were collected during production. Cheese samples were also collected at 4 weekly intervals during the 24 wk ripening period. The curd and Cheddar cheese samples (11g) were diluted in 99 mL of sterile 2% (w/v) tri-sodium citrate (Oxoid Ltd., West Heidelberg, Vic, Australia) at 40^{o}C. The sample was macerated in a stomacher 400 laboratory blender (Seward Medical, London, UK) for 4 min at high speed in stomacher bags to obtain slurry for the fist dilution and subsequent serial dilutions were

performed in 0.15% (w/v) peptone and water solution (Oxoid). The milk and whey sample were diluted directly in 0.15% peptone and water solution. Appropriate dilutions were pour-plated.

Starter lactococci were enumerated on M17 agar (Merck, South Granville, NSW, Australia) and incubated at 30^{o}C under aerobic condition for 72 h (Terzaghi & Sandine, 1975). Total lactobacilli during ripening were enumerated on LBS agar (Merck) and incubated at 30^{o}C in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, USA) with a Gas Generating Kit® (Oxoid) for 72 h.

Lb. acidophilus, *Lb. casei* and *Lb. paracasei* were enumerated as per Dave and Shah (1996) and Tharmaraj and Shah (2003). MRS-vancomycine (MRS-V) agar used for the selective enumeration of *Lb. casei* and *Lb. paracasei* was prepared by adding 2 mL of 0.5 mg mL^{-1} vancomycine (Sigma) solution to 1 L of molten MRS agar just before pouring to obtain 1 mg L^{-1} of final concentration. MRS-sorbitol (MRS-S) agar used for the selective enumeration of *Lb. acidophilus* was prepared by adding 10 mL of membrane filtered sterile 10% solutions (w/v) of sorbitol (Sigma) to 90 mL of molten MRS agar just before pouring. The plates were incubated anaerobically at 37^{o}C for 72 h.

Bifidobacterium was enumerated according to the method of Laroia and Martin (1991) and Tharmaraj and Shah (2003). A mixture of antibiotics, including 2 g of neomycin sulfate, 3 g of nalidixic acid, 60 g of lithium chloride, and 4 g of paromomycin sulfate (NNLP; Sigma), was prepared in 1 L of distilled water, filter- sterilized with 0.23 µm Millipore filters (Millipore Corp., Bedford, MA, USA) and stored at 4^{o}C until used. The mixture (5 mL) was added to 100 mL of MRS agar prior to plating. L-cysteine·hydrochloride (Sigma) was also added at the rate of 0.05% in order to decrease the redox potential of the medium. Plates were incubated anaerobically at 37^{o}C for 72 h.

3.2.5. ***Production of organic acids***

Production of lactic acid and acetic acid was determined using high performance liquid chromatography (HPLC) as per the method of Bruno et al. (2002). Grated cheese samples (5 g) were blended with 25 mL of 0.009 N sulfuric acid and 70 µL of 15.5 N nitric acid and homogenized with a Ultratorrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at 10,000 x g. After standing for 1 h in 50^{o}C water bath, the slurry was centrifuged for 20 min at 4000 x g at 4^{o}C. The soluble fraction (1.5 mL) located between the upper layer (fat) and the precipitate (casein) was further centrifuged (14,000 x g, 10 min) with a bench top centrifuge (Sorvall RT7, Newtown, CT, USA). The supernatant was

filtered using 0.23 µm millipore filters (Millipore) and approximately 1 mL aliquot from each sample was stored in HPLC vials at -20^{o}C until analysed. The HPLC system consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV/Vis tunable absorbance detector and a 730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable catridges H^+ (Bio-Rad Laboratories) maintained at 65^{o}C were used for the analysis. Sulfuric acid (0.009 N), filtered through a 0.45 mm membrane filter (Millipore) was used as a mobile phase at a flow rate of 0.6 mL min^{-1}. The detection device was the ultraviolet-visible detector set at 220 nm with running time of 15 min.

3.2.6. ***Assessment of proteolysis***

The water-soluble extracts (WSE) of the cheeses were prepared according to Kuchroo and Fox (1982). The nitrogen in the extract was estimated in duplicate by the Kjeldahl method. Non-protein nitrogen (TCA-SN) was estimated in 9 mL filtrate obtained after precipitation of the cheese homogenate with 12% TCA (Sigma-Aldrich). The extent of secondary proteolysis was assayed as the amount of nitrogen that was soluble in 5% PTA-SN (Sigma-Aldrich) extracts of 9 mL of the filtrate.

The proteolytic patterns of Cheddar cheeses were also analysed by SDS-PAGE using the stacking gel system by Laemmli (1970), as described by Shihata and Shah (2000). An aliquot of each cheese sample (0.050 g) was suspended in a mixture of 1 mL Tris (10 mM) (Sigma-Aldrich) – EDTA (1 mM) pH 8.0 buffer, 350 µL of 10% SDS (Sigma-Aldrich) and 50 µL of β-mercaptoethanol (Bio-Rad Laboratories Ltd., Watford, UK).

The samples were boiled at 5 min intervals until the cheese solids were completely dissolved. A 25 µL aliquot of stock solution was diluted with 100 µL of 2× treatment buffer (0.125 M Tris-chloride, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue (pH 6.8) (Sigma-Aldrich). Whole casein was dissolved in water at pH 10 and α-, β-, κ-caseins (Sigma-Aldrich) were dissolved in water at neutral pH to a concentration of 2 mg mL^{-1}. A 20 µL aliquot of this mixture was diluted with 40 µL of 2× treatment buffer.

A working volume (15 µL) of each sample and of each control (7.5 µL), the α-, β-, κ- and whole caseins were loaded into 12.5% running gels. A volume of broad range (20 µL), pre-stained SDS-PAGE standards (Bio-Rad) was used as a marker. SDS-PAGE gels

were run in a BIO-RAD Protean® II xi cell filled with tank buffer solution (0.025 M Tris, 0.192 M glycine, 0.1% SDS (pH 8.3), run by a power pac 300 run at 50 mA.

The gels were fixed in de-staining solution I (40% methanol, 7% acetic acid) for 30 min before staining with staining solution (0.025% Coomassie Brilliant blue, ICN Biochemicals Inc., Aurora, OH, USA), 40% methanol, 7% acetic acid) for 4 h. The gels were then de-stained in de-staining solution I for 1 h followed by de-staining in de-staining solution II (7% acetic acid, 5% methanol) until the background became clear.

The gel images were recorded using a Fuji Film Intelligent Dark Box II with Fuji Film LAS-1000 Lite V1.3 software. The area and intensity of the bands were measured using Fuji Film Image Gauge V4.0 software (Fuji Photo Film Co. Ltd., Japan). Data from the analysis were expressed as the ratio of the area and the intensity of the band. The reduction in the intensity of bands during ripening with respect to the original intensity was expressed as percentage of hydrolysis.

3.2.7. ***Statistical analysis***

Data analysis was carried out with Minitab Statistical Package (Minitab Inc, State College, PA, USA). One way analysis of variance was used to study differences between means, with a significant level at $\alpha = 0.05$. All experiments were repeated three times and all analyses were carried out at least in duplicate. All data are presented as mean ± standard error of means.

3.3. Results and discussions

3.3.1. ***Cheese composition***

The composition of Cheddar cheeses with and without the addition of probiotic bacteria is summarized in Table 3.1. No significant differences ($P > 0.05$) were observed among the three types of cheeses for the moisture, salt, fat and protein content. The composition of the cheeses was within the suggested ranges of Cheddar cheeses with good quality and texture parameters (Fox, 1975, 1993). The result thus shows that addition of probiotic microorganisms into Cheddar cheese has no direct effect on cheese composition, which confirms the findings of Gardiner et al. (1998).

3.3.2. ***Survival of bacteria during manufacture of Cheddar cheese***

The survival and growth of the probiotic bacteria and lactococci were determined at different stages of the manufacture of Cheddar cheese as shown in Table 3.2. All probiotic microorganisms were found to survive the cheese manufacturing process. The probiotics were all inoculated at the rate of 4 mL of bulk culture of RSM per liter of pasteurized milk (0.4 % v/v). At this rate, the concentration of the probiotic in the inoculated-milk ranged between 6 to 7 $\log_{10}$ cfu g^{-1}. After inoculation, milk was left for ripening for 45 min at 31^{o}C. No significant differences ($P > 0.05$) were observed between the probiotic counts in milk before and after ripening. Counts of *Lb. casei* 279, *Lb. paracasei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 on cooked curd (after draining) and curd after cheddaring at 38^{o}C for 90 min increased significantly ($P < 0.05$) although by about half a log cycle. Results thus indicate that no or very slow growth occurred in all probiotics during the whole manufacturing process. Results were in agreement with those of Gomes et al. (1998) who observed no growth of *Bifidobacterium* sp. strain Bo and *Lb. acidophilus* strain Ki during the manufacture of Gouda cheese. Volume reduction due to the release of whey during draining has resulted in the increased in starter and probiotic counts by about one log cycle. Despite the loss of about 6 to 7 $\log_{10}$ cfu g^{-1} of probiotics in whey, the probiotic counts remained high in all cheeses (8 to 9 $\log_{10}$ cfu g^{-1}) at the end of the manufacturing stage. The significant differences ($P < 0.05$) observed between the lactococci counts in inoculated milk were attributed to the differences in the concentration of inocula of RSM used to propagate the culture. Unlike probiotic counts, significant differences ($P < 0.05$) were found between lactococci counts; in milk after inoculation and after ripening at 31^{o}C for 45 min; in cooked curd (after draining) and after cheddaring at 38^{o}C for 90 min. Lactococci counts increased consistently in all batches, which shows that they grew and were the primary acid producers throughout the cheese-making process.

Results from Table 3.2 thus show that when the probiotic bacteria were added as starter adjuncts together with starter lactococci, no alteration of cheese making procedure was necessary for incorporation of probiotic into Cheddar cheese. Compared to the experiments performed by Daigle et al. (1999) who used pre-fermented cream for the production of probiotic cheese with *B. infantis* and Dinakar and Mistry (1994) who added immobilized, freeze-dried *B. bifidium* at milling stage, our procedure offered much simplicity and may provide cost saving as only a relatively small inoculum (0.4% v/v) was

needed to achieve a high initial count (8 to 9 $\log_{10}$ cfu g^{-1}). This was possible as the probiotics were added as adjuncts and they were not required for acid production.

3.3.3. ***Survival of bacteria during ripening***

Survival and growth of lactococci, bifidobacteria and lactobacilli were determined during ripening period of 24 wk at 4°C (Figure 3.1). All probiotic bacteria added remained viable in Cheddar cheese during the 24 wk ripening period. Figure 3.1b shows viable counts of probiotic adjuncts that remained steady and decreased slightly but not significantly ($P > 0.05$) at the end of the ripening period reaching 8.3 $\log_{10}$ cfu g^{-1} for *Lb. acidophilus* 4962, 8.5 $\log_{10}$ cfu g^{-1} for *Lb. casei* 279 and 8.0 $\log_{10}$ cfu g^{-1} for *B. longum* 1941. Viable counts of probiotic adjunct of Batch 3M cheeses also showed a similar pattern, reaching 8.4 $\log_{10}$ cfu g^{-1} for *Lb. acidophilus* LAFTI®L10, 8.5 $\log_{10}$ cfu g^{-1} for *Lb. paracasei* LAFTI®L26 and 7.5 $\log_{10}$ cfu g^{-1} for *B. lactis* LAFTI®B94. In order to appreciate the beneficial effects of probiotic foods, it has been proposed that viable probiotic organisms should be present at levels of at least 7.0 $\log_{10}$ cfu per gram of a product (Ishibashi & Shimamura, 1993). All probiotic-containing cheeses developed in this study thus satisfied the criteria for a probiotic food product. Lactococci counts in all cheeses however decreased by one to two log cycles after 24 wk of ripening at 4°C probably due to unfavourable conditions in the cheese such as high salt in moisture, low pH, lack of fermentable carbohydrate and low ripening temperature.

During ripening period, a population of non-starter lactic acid bacteria (NSLAB) also proliferated in cheeses. NSLAB are chiefly composed of lactobacilli such as *Lb. plantarum*, *Lb. casei* and *Lb. brevis* (Gardiner et al., 1998). It is believed that NSLAB gain access to the cheesemilk during the manufacturing stage or they survive pasteurization in an attenuated state. Figure 3.1a shows the NSLAB counts of Batch 1M cheese on Lactobacillus Selective (LBS) agar, which remained relatively low for the first few weeks of ripening and increased to 7.6 $\log_{10}$ cfu g^{-1} at the end of ripening period of 24 wk at 4°C. The role of NSLAB in flavour development is not clear. A few studies indicated that NSLAB plays an important role in the development of cheese flavour (Broome et al., 1990; McSweeney et al., 1994), while others have indicated that NSLAB contribute only minimally to flavour development of Cheddar cheese (Peterson & Marshall, 1990). The effect of the level of NSLAB on cheese flavour will be investigated further in our study. The extent of growth of adventitious NSLAB in the control cheeses was typical for Cheddar cheese. Similar growth characteristics of adventitious NSLAB in Cheddar cheese produced

in open vats have been reported (Lynch et al., 1999). NSLAB counts were not performed on Batch 2M and Batch 3M cheeses because the probiotics added during the manufacturing of these cheeses were also able to grow on LBS agar.

3.3.4. ***Production of organic acids***

The metabolic activity of the microorganisms in cheese was monitored by estimating the metabolic products, lactic and acetic acids (Figure 3.2). Lactic acid production showed no discernible pattern probably because of utilization of lactic acid by NSLAB (Figure 3.2a). There was a slight increase in the concentration of lactic acid during the first month of ripening due to the use of residual lactose trapped in the curd. On the other hand, acetic acid concentration increased as the ripening time increased in all cheeses (Figure 3.2b). The highest final values found in the control cheese were 0.06% followed by 0.12% for both probiotic cheeses (Batches 2M and 3M). Concentration of acetic acid in probiotic cheeses was significantly higher ($P < 0.05$) compared to the control cheese, probably due to the addition of *Bifidobacterium* that were able to produce acetic acid during their metabolism. Bifidobacteria produce acetic acid and lactic acid from lactose via a fructose-6-phosphate shunt pathway (Dinakar & Mistry, 1994). The fermentation pathway results in 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose, therefore generating a theoretical molar ratio (acetic:lactic) of 3:2 (Scardovi & Trovatelli, 1965).

3.3.5. ***Assessment of proteolysis***

Assessment of proteolysis in the control and probiotic cheeses by determination of water soluble nitrogen (WSN), tricholoracetic acid soluble nitrogen (TCA-SN) and phospotungstic acid soluble nitrogen (TCA-SN) over 24 wk of ripening period at 4^{o}C is depicted in Figure 3.3. The ratio of WSN to total nitrogen (TN) in probiotic cheese increased progressively throughout the ripening period (Figure 3.3a). There were no significant differences ($P > 0.05$) between control and probiotic cheeses during the 24 wk ripening period. According to Fox et al. (1993), about 3-6% of the coagulant (chymosin) added to cheese milk is retained in the curd. Since WSN in the cheese is produced mainly by the primary proteolysis by the action of the coagulant (Visser, 1977), differences amongst these cheeses were not expected.

During the 24 wk of ripening period, the amount of TCA-SN also increased progressively (Figure 3.3b). Starter and non-starter bacterial proteinases are principally

responsible for the formation of TCA-SN (Fox et al., 1993). There were no significant differences ($P > 0.05$) in the level of TCA-SN between control and probiotic cheeses up to the 20 wk of ripening. At 20 wk of ripening period, more products of the primary proteolysis became available as substrates for the subsequent proteolysis by probiotic peptidases thus resulted in the increased levels of TCA-SN of probiotic cheeses. The level of TCA-SN shown in Figure 3.3b was also almost as high as that of WSN, which shows that once soluble peptides had been formed by rennet, bacterial peptidases hydrolyse them at a relatively rapid rate.

PTA-SN was used as an index of free amino acid in control and probiotic cheeses. PTA is a discriminating protein precipitant. Free amino acids, except di-basic amino acids are soluble in 5% PTA but peptides greater than 600 Da are precipitated (Fox et al., 1993). This method has been evaluated by Jarrett et al. (1982), who found a linear relationship between PTA-SN and total free amino acid extracted from Cheddar cheese. Figure 3.3c shows concentrations of total free amino acids that were similar ($P > 0.05$) in all cheeses until the 16 wk of ripening. Higher level of free amino acids developed thereafter in probiotic cheeses (Batch 2M and Batch 3M) indicated by the elevated ratio of PTA-SN to TN. This was probably due to the increased peptidase activity caused by the probiotic adjunct. The results also reflected the enhanced activity of intracellular peptidases, which became accessible upon limited lysis of starter lactococci. A similar trend in the changes of proteolytic pattern and the development of total free amino acid in probiotic cheeses was noted by Lynch et al. (1999), Gardiner et al. (1998) and McSweeney et al. (1994).

The extent of proteolysis of cheese during ripening was also monitored by SDS-PAGE. Three representative electrophoretic patterns of SDS-PAGE of Cheddar cheese during 24 wk ripening period at 4^{o}C are shown in Figure 3.4. Protein bands were identified based on molecular weight and also by comparison with standards of casein (CN), α_s- CN, β- CN and κ-CN. α_{s1}-CN and α_{s2}-CN could not be separated on 12% acrylamide gel because the molecular weights of these two fractions are very similar (Varnam & Sutherland, 1994). β-Lactoglobulin (MW 18, 300) and α-lactalbumin (MW 14, 000) were identified based on their molecular weight. The SDS-PAGE pattern was different between control (Batch 1M) and probiotic cheeses (Batch 2M and Batch 3M), but very little detectable differences were observed between the probiotic cheeses. Dinakar and Mistry (1994) found no significant differences between the electrophoresis patterns in cheese without probiotic bacteria and cheese supplemented with *B. bifidum* and similar results were observed by Corbo et al. (2001) who supplemented Canestro Pugliese hard cheese with *B. longum* and *B. bifidum*. This was possible because in both studies, only *Bifidobacterium* spp. were added.

Bifidobacterium strains are not highly proteolytic (Klaver et al., 1993; Shihata & Shah, 2000) and thus did not influence the proteolytic pattern of the probiotic cheese in those studies. A number of studies have shown that *Lb. acidophilus*, *Lb. casei* and *Lb. paracasei* are proteolytic and capable of releasing small peptides (proteinase of 145 kDa) and amino acids (X-prolyl-dipeptidyl-aminopeptidase) (Hickey et al., 1983; Gomes et al., 1998; Shihata & Shah, 2000) but their relative contribution in cheese has not yet been studied in detail.

As the ripening time increases, the concentrations of casein decrease and the concentration of lower MW breakdown products of the caseins increases. These products appear in SDS-PAGE in the area between β-casein and β-Lactoglobulin (Figure 3.4) (Basch et al., 1989; Brandsma et al., 1994).

In our study, hydrolysis of individual casein fractions was expressed as a percentage relative to concentration of casein at wk 1 (Figure 3.5). Most of the α_{s1}-CN hydrolysis occurred between 0 and 16 wk and the rate slowed down after 16 wk. At the end of the ripening period of 24 wk, 46.99%, 63.42% and 19.28% of the α_{s1}-CN was hydrolysed in Batch 2M, Batch 3M and the control cheese, respectively (Figure 3.5a). Hydrolysis of α_{s1}-CN was significantly faster ($P < 0.05$) in probiotic cheeses compared to the control cheese. Several important variables influence the rate of hydrolysis of this casein fraction, including salt to moisture ratio, milk quality, ripening temperature, bacterial count and species involved, residual coagulant in the cheese curd and the effects of different manufacturing procedures (Fox et al., 1993). Most of these variables were kept constant in the production and thus increases in the rate of proteolysis were mainly caused by the enhanced peptidase activity by the starter adjunct. The results were also reflected on the amount of PTA-SN that detected more product of proteolysis especially the free amino acids in probiotic cheeses as had been explained previously (Figure 3.3). The role of these amino acids in the production of certain aroma compounds in Cheddar cheese will be further investigated in our next study.

During ripening, β-CN does not undergo as much degradation as α_s-CN (Brandsma et al., 1994; Mistry & Kasperson, 1998). This shows that the bacterial peptidases hydrolyse α_{s1}-CN more readily than β-CN. Degradation of β –CN during cheese ripening occurs because of the action of chymosin and plasmin and small peptides formed from this hydrolysis cause bitterness in Cheddar cheese (Fox et al., 1993). Thomas and Mills (1981) reported that > 50% of β-CN is degraded after 4 wk with 4% salt in moisture (SM), but only 10% of β-CN is degraded with 8% SM. The salt percentages in this study were 4.56, 4.48, and 4.37% for Batch 1M, Batch 2M and Batch 3M, respectively. The percentage hydrolysis

of β-CN, however, was only 6.71, 9.23, and 12.75% for Batch 1M, Batch 2M and Batch 3M, respectively (Figure 3.5b).

Despite the significant difference observed between the cheeses regarding the percentage of hydrolysis of α_{s1}-CN and β-CN, it should be noted that proteolysis remained relatively low. These results could be due to the low temperature of ripening (4^{o}C) as compared to normal ripening temperature of Cheddar cheese (8-10^{o}C).

3.4. Conclusions

The results of the present study demonstrate that the probiotic organisms (*Lb. acidophilus* 4962, *Lb. acidophilus* LAFTI®L10, *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *B. longum* 1941 and *B. lactis* LAFTI®B94) survived the manufacturing process of Cheddar cheese at a high level without alteration of cheese making process. They were also able to maintain viability at > 7.5 $\log_{10}$ cfu g^{-1} at the end of ripening period of 24 wk at 4^{o}C. Addition of probiotic adjunct also did not alter the chemical composition (salt, fat, moisture and protein content), but acetic acid concentration was higher in probiotic cheeses. Although primary proteolysis was not affected by the addition of probiotic adjunct, the levels of secondary proteolysis indicated by the concentration of free amino acids was significantly higher. Electrophorectic patterns on SDS-PAGE show increase in percentage hydrolysis of α_{s1}-CN in probiotic cheese. These data thus suggested that Cheddar cheese is an effective vehicle for delivery of probiotic organisms. Our results also indicated that the addition of probiotic microorganisms in the production of Cheddar cheese may influence the proteolytic pattern.

Table 3.1. Composition of control and probiotic Cheddar cheeses[1]

Composition	Cheddar cheese		
	Control (Batch 1M)[2]	Mix probiotic (Batch 2M)[3]	Mix probiotic (Batch 3M)[4]
% Moisture	39.00 ± 0.78[a]	39.27 ± 0.81[a]	39.33 ± 0.46[a]
% Salt	1.78 ± 0.05[a]	1.76 ± 0.02[a]	1.72 ± 0.07[a]
% SDM[5]	2.91 ± 0.11[a]	2.90 ± 0.02[a]	2.83 ± 0.11[a]
% Fat	31.40 ± 0.85[a]	31.03 ± 0.26[a]	31.89 ± 0.93[a]
% FDM[6]	51.48 ± 0.83[a]	51.13 ± 1.11[a]	52.56 ± 1.20[a]
% Protein	27.46 ± 0.41[a]	27.70 ± 0.20[a]	28.07 ± 0.47[a]
pH	5.40 ± 0.03[a]	5.14 ± 0.03[b]	5.21 ± 0.04[b]

[ab] Means in rows with like superscripts do not differ ($P > 0.05$).
[1] Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate. Analyses were conducted during the first wk of Cheddar cheese ripening.
[2] Cheddar cheese produced with only cheese starter culture (1.5%, v/v).
[3] Probiotic cheese produced with cheese starter culture (1.5%, v/v), *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v).
[4] Probiotic cheese produced with cheese starter culture (1.5%, v/v), *Lb. acidophilus* LAFTI® L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI®B94 (0.4%, v/v).
[5] Salt in dry matter.
[6] Fat in dry matter.

Table 3.2. Survival (Log_{10} cfu g^{-1}) of cheese starter culture and probiotic adjunct during manufacturing of Cheddar cheese

Sample	Survival (Log_{10} cfu g^{-1})[1]								
	Control (Batch 1M)[2]	Mix probiotic (Batch 2M)[3]				Mix probiotic (Batch 3M)[4]			
	Starter	Starter	LA4962	LC279	BL1941	Starter	LAFTI®L10	LAFTI®L26	LAFTI®B94
Inoculated milk	7.38 ± 0.14^{aA}	7.54 ± 0.15^{abA}	6.77 ± 0.14^{cA}	6.77 ± 0.13^{cA}	6.32 ± 0.18^{cA}	7.94 ± 0.21^{bA}	6.60 ± 0.11^{cA}	6.77 ± 0.10^{cA}	6.36 ± 0.10^{cA}
Ripened milk	8.30 ± 0.02^{aB}	7.85 ± 0.18^{aB}	6.82 ± 0.19^{bA}	7.03 ± 0.17^{bA}	6.68 ± 0.31^{bA}	8.96 ± 0.31^{cB}	6.79 ± 0.09^{bAB}	7.16 ± 0.09^{abAB}	6.66 ± 0.080^{bAB}
Cooked curd	9.30 ± 0.31^{aC}	9.68 ± 0.55^{aC}	8.36 ± 0.45^{abB}	7.94 ± 0.53^{bB}	8.07 ± 0.08^{bB}	9.57 ± 0.35^{aBC}	7.85 ± 0.30^{bBC}	8.22 ± 0.05^{aBC}	7.56 ± 0.09^{bAB}
Whey	8.44 ± 0.09^{aB}	7.55 ± 0.10^{aA}	7.10 ± 0.25^{aA}	7.17 ± 0.23^{aA}	6.01 ± 0.19^{aA}	7.45 ± 0.18^{aA}	6.34 ± 0.17^{aA}	6.42 ± 0.17^{aA}	6.12 ± 0.13^{aA}
Cheddared curd	9.40 ± 0.34^{aC}	9.91 ± 0.26^{aC}	8.40 ± 0.15^{bB}	8.36 ± 0.13^{bBC}	8.37 ± 0.54^{bB}	9.66 ± 0.56^{aBC}	8.40 ± 0.09^{bC}	8.57 ± 0.03^{bC}	8.16 ± 0.13^{bB}
Pressed curd (Fresh cheese)	9.31 ± 0.23^{abcC}	9.70 ± 0.24^{beC}	8.66 ± 0.16^{cdB}	8.67 ± 0.17^{cdC}	8.63 ± 0.38^{cdB}	10.15 ± 0.40^{eC}	8.76 ± 0.07^{cdC}	8.74 ± 0.03^{cdC}	8.25 ± 0.06^{B}

[abcde] Means in rows with like superscripts do not differ ($P > 0.05$).
[ABC] Means in column with like superscripts do not differ ($P > 0.05$).
[1] Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.
[2] Cheeses with cheese starter culture only (1.5%, v/v).
[3] Cheeses with cheese starter culture (1.5%, v/v), *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v).
[4] Cheeses with cheese starter culture (1.5%, v/v), *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI®B94 (0.4%, v/v).

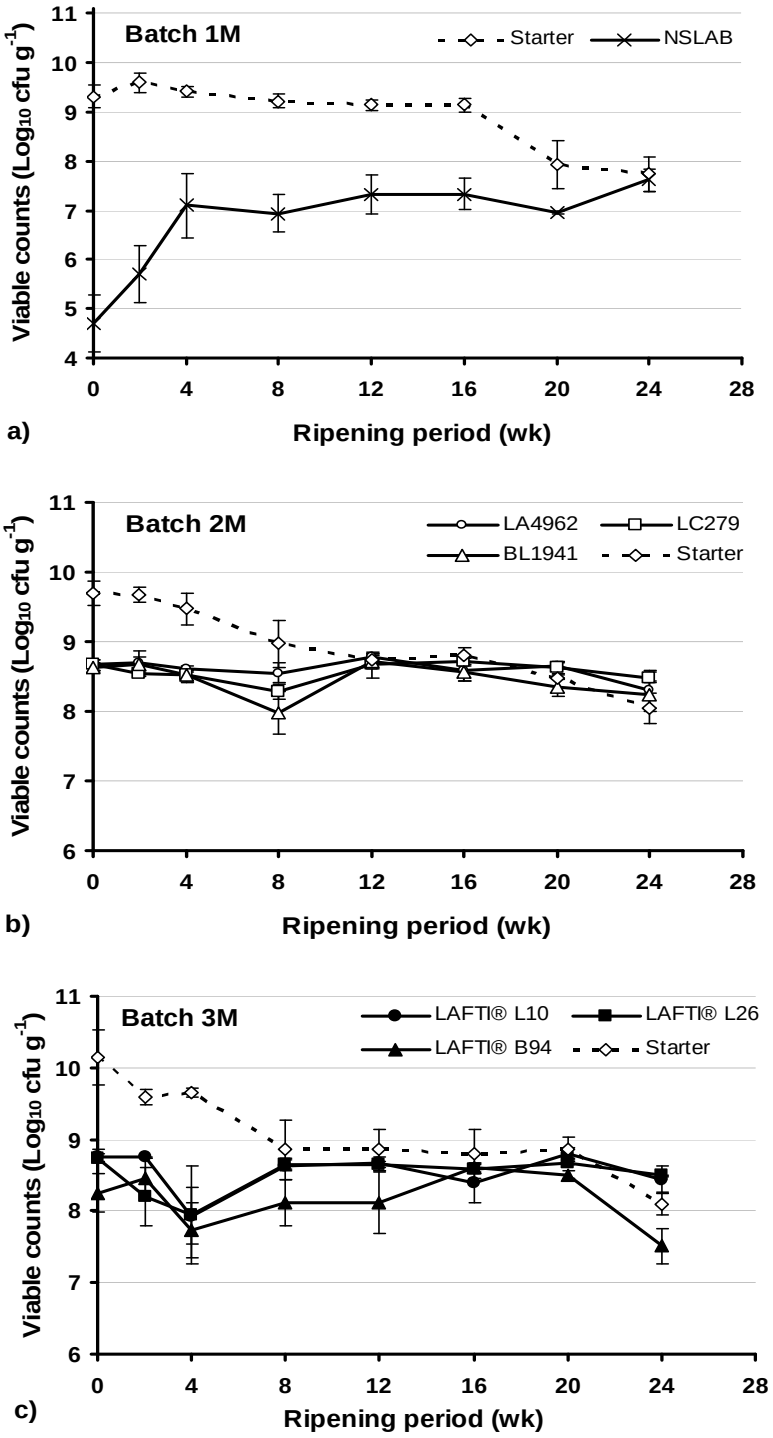


Figure 3.1. Survival of starter lactococci, probiotic adjunct and NSLAB in (a) control cheese with only starter culture (1.5% v/v) (Batch 1M), (b) probiotic cheese with starter culture (1.5% v/v), *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v) (Batch 2M), and (c) probiotic cheese with cheese starter culture (1.5%, v/v) and *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI®B94 (0.4%, v/v) (Batch 3M) during ripening at 4°C for 24 wk. Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.

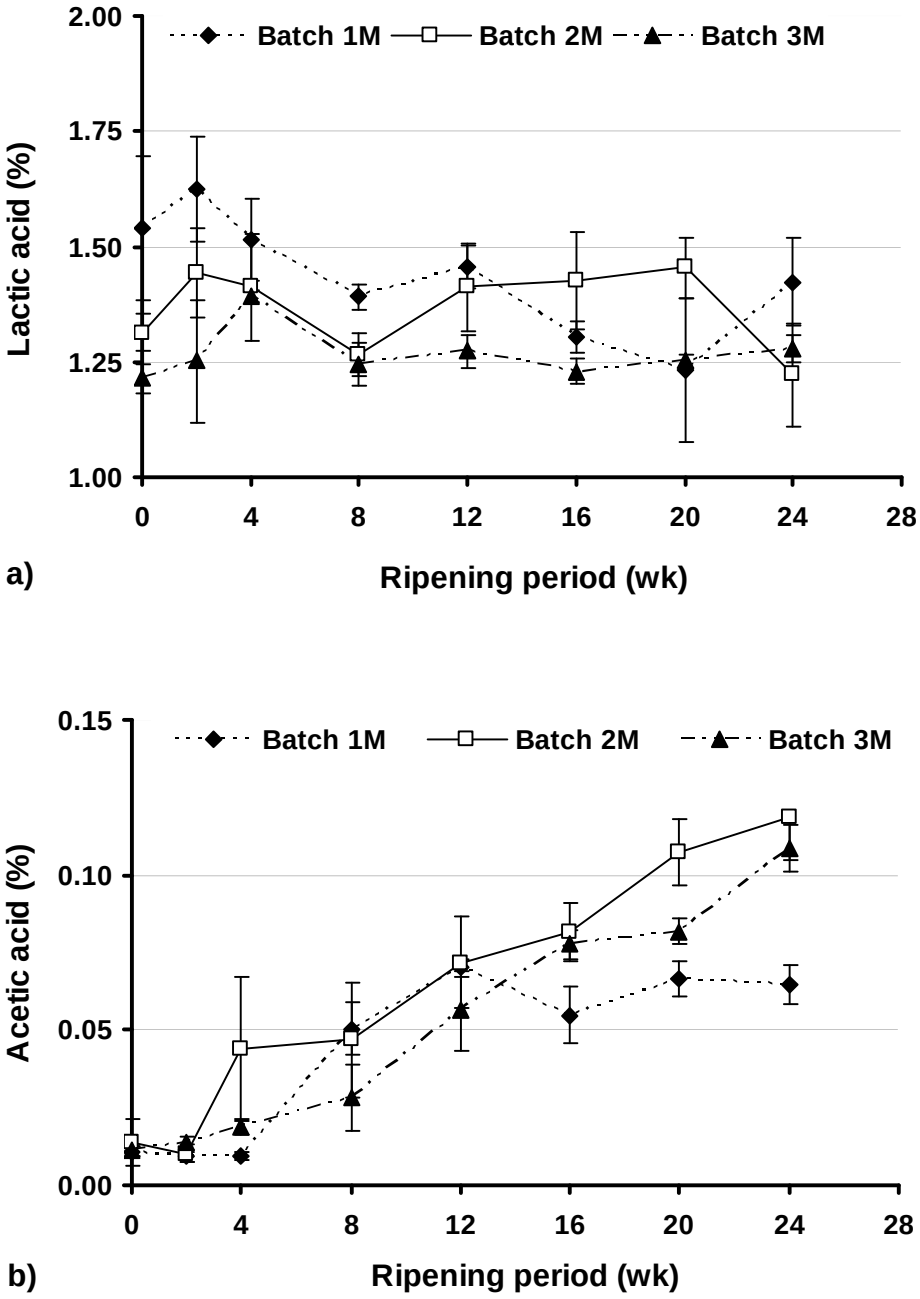


Figure 3.2. Concentration of (a) lactic acid and (b) acetic acid during ripening of Cheddar cheeses ripened at 4°C for 24 wk. Batch 1M = control cheese with only starter culture (1.5% v/v). Batch 2M = probiotic cheese with starter culture and *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v). Batch 3M = probiotic cheese with cheese starter culture (1.5%, v/v) and *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI®B94 (0.4%, v/v). Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.

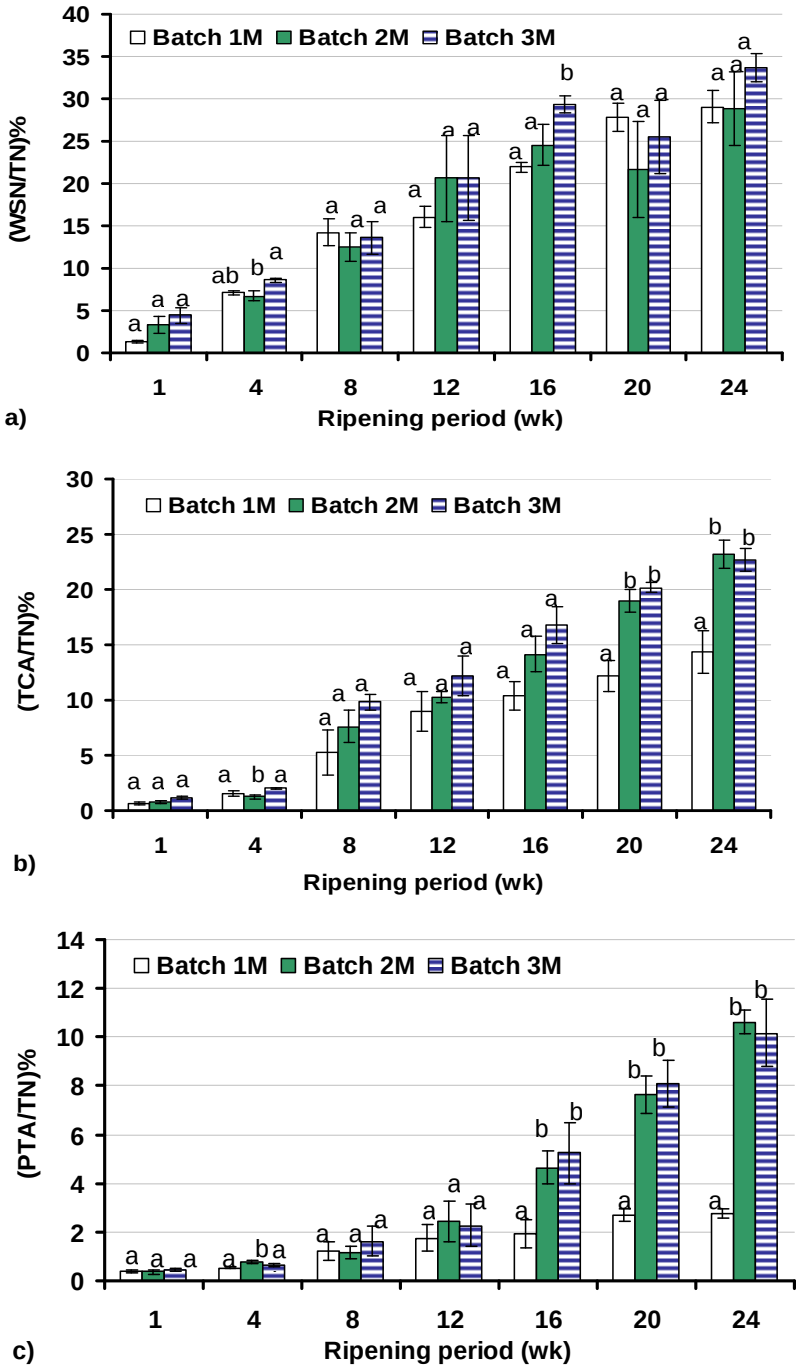


Figure 3.3. Concentration (expressed as percentage of total N) of (a) water-soluble nitrogen (WSN), (b) trichloroacetic acid-soluble nitrogen (TCA-SN), and (c) phosphotungstic acid-soluble nitrogen (PTA-SN) during ripening of Cheddar cheese at 4°C for 24 wk. Batch 1M = control cheese with only starter culture (1.5% v/v). Batch 2M = probiotic cheese with starter culture (1.5% v/v) and *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v). Batch 3M = probiotic cheese produced with cheese starter culture (1.5%, v/v) and *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI® B94 (0.4%, v/v). Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate. [abc] Means between treatments (Batch 1M, Batch 2M, Batch 3M) with like superscripts do not differ ($P > 0.05$).

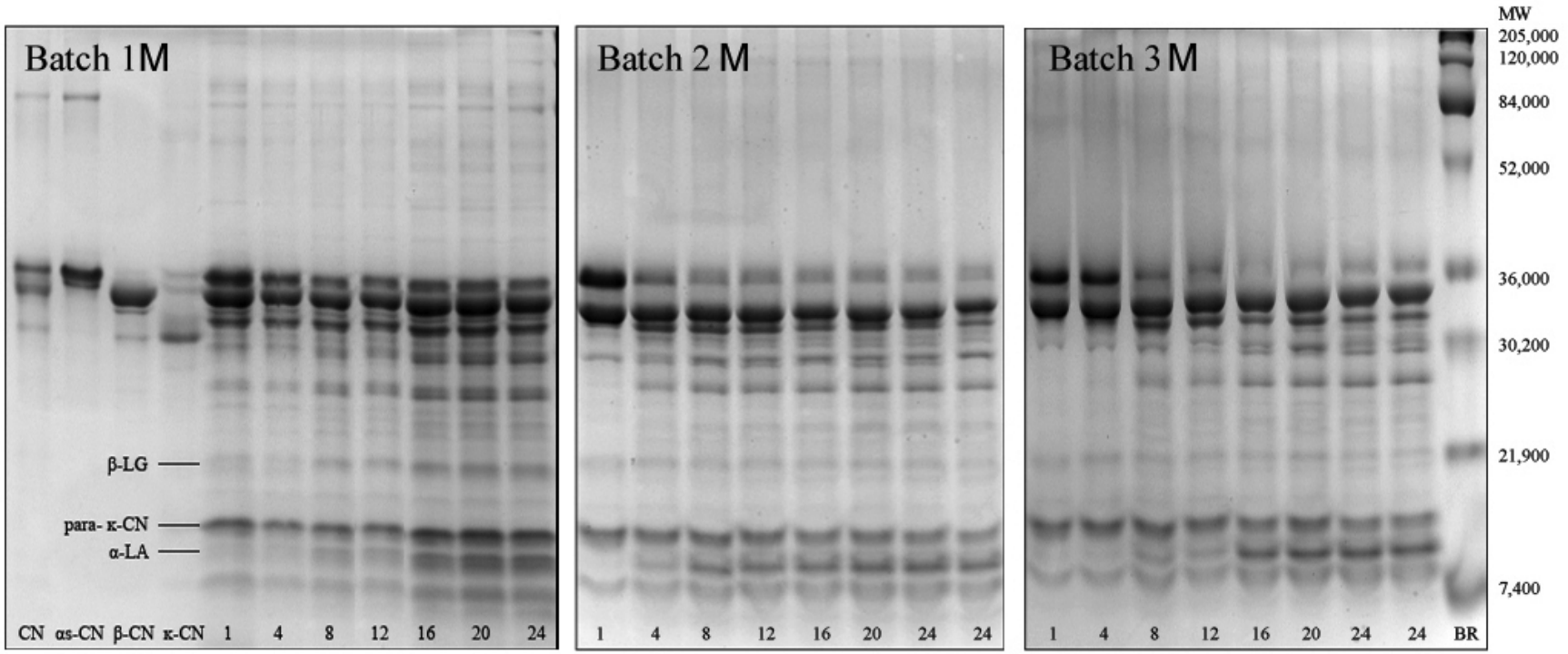


Figure. 3.4. Representative electrophoretic patterns of SDS-PAGE of Cheddar cheeses during ripening period (wk 1, 4, 8, 12, 20, 24). Batch 1M = control cheese with only starter culture (1.5% v/v). Batch 2M = probiotic cheese with starter culture (1.5%, v/v) and *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v). Batch 3M = probiotic cheese with cheese starter culture (1.5%, v/v) and *Lb. acidophilus* LAFTI® L10 (0.4% v/v), *Lb. paracasei* LAFTI® L26 (0.4%, v/v) and *B. lactis* LAFTI® B94 (0.4%, v/v). CN = casein. BR = broad range prestained molecular weight (MW) standards containing: myosin (MW 205,000), β-galactosidase (MW 120,000), bovine serum albumin (MW 84,000), ovalbumin (MW 52,000), carbonic anhydrase (MW 36,000), soybean trypsin inhibitor (MW 30,200), lysozyme (MW 21,900) and aprotinin (MW 7,400). LA = lactalbumin. LG = lactoglobullin.

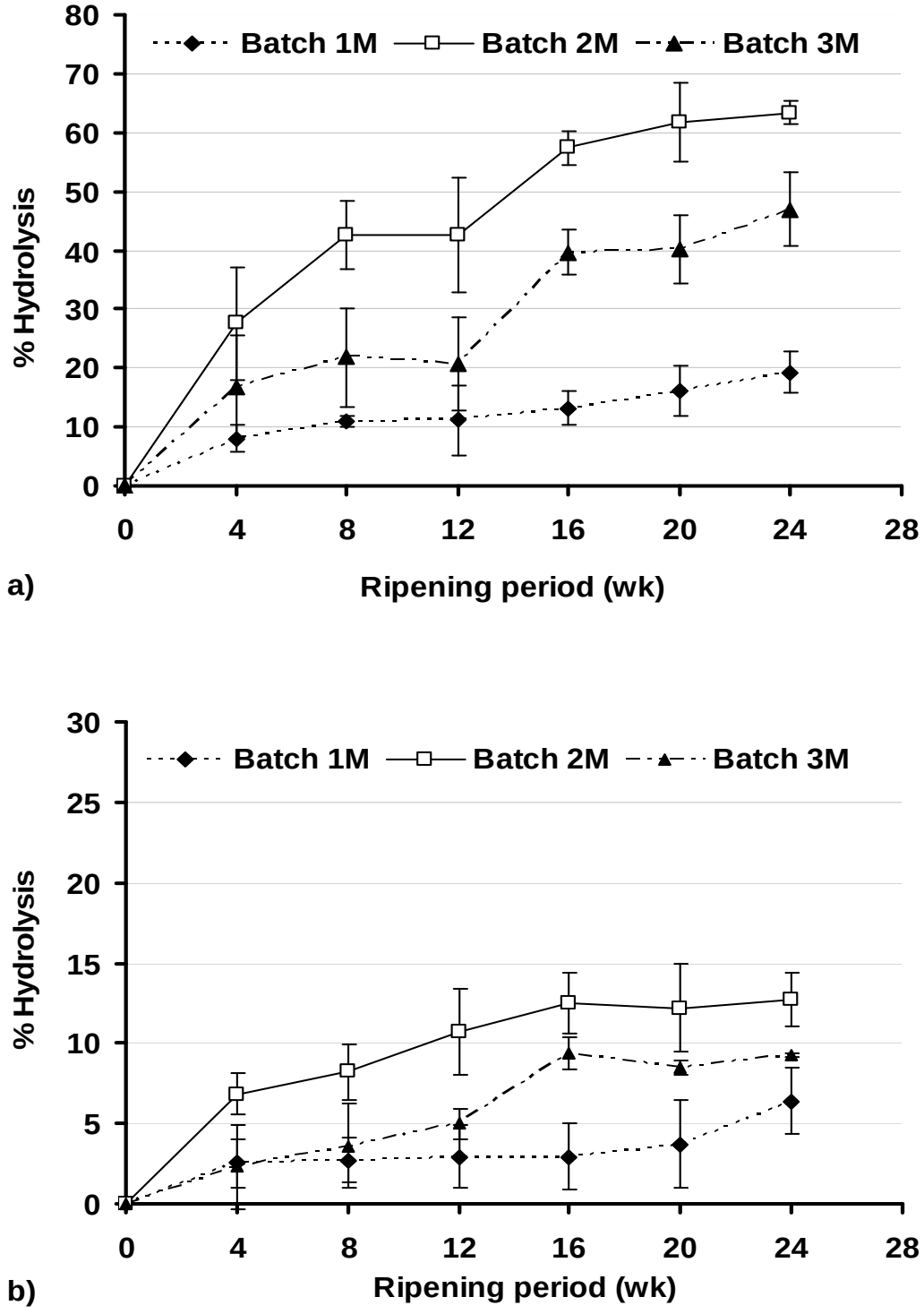


Figure 3.5. Protein hydrolysis (a) α_{s1}-CN and (b) β-CN during ripening of Cheddar cheeses. Batch 1M = control cheese with only starter culture (1.5% v/v). Batch 2M = probiotic cheese with starter culture (1.5% v/v) and *Lb. acidophilus* 4962 (0.4%, v/v), *Lb. casei* 279 (0.4%, v/v) and *B. longum* 1941 (0.4%, v/v). Batch 3M = probiotic cheese with cheese starter culture (1.5%, v/v) and *Lb. acidophilus* LAFTI®L10 (0.4% v/v), *Lb. paracasei* LAFTI®L26 (0.4%, v/v) and *B. lactis* LAFTI®B94 (0.4%, v/v). Results are expressed as mean ± standard error of means of at least 3 sets of data.

4.0 Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* sp.*

4.1. Introduction

The application of probiotic bacteria in food products is increasing due to potential health benefits associated with the consumption of these bacteria. A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhea, anticarcinogenic properties, reduction in blood cholesterol and improvement in immunity (Ballongue, 1993; Shah & Wu, 1999; Shah, 2000a,b). For dietary organisms to be beneficial in food systems, they should maintain viability in the food until the time of consumption and be present in significant numbers, at levels of at least 10^7 viable cells per gram or milliliter of a product (Ishibashi & Shimamura, 1993). For this reason, changes in the numbers of viable bacteria during storage period should be known.

Yoghurt and fermented milk have received most attention as carriers of probiotic bacteria, but foods such as Cheddar cheese (Dinakar & Mistry, 1994; Gardiner et al., 1998), Gouda cheese (Gomes et al., 1995), cottage cheese (Blanchette et al., 1996), Crescenza cheese (Gobbetti et al., 1997), frozen yoghurts (Laroia & Martin, 1991) and ice-cream (Hekmat & McMahon, 1992) have also been studied as carriers of probiotic microorganisms.

Cheeses have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to gastrointestinal tract as they tend to have higher pH, more solid consistency and relatively higher fat content. These offer protection to probiotic bacteria during storage and passage through the gastrointestinal tract. Cheeses also have higher buffering capacity than yoghurt (Stanton et al., 1998). Cheddar cheeses, however, have long ripening time hence development of probiotic Cheddar cheese requires

* This chapter has been published. Ong, L., Henriksson, A., & Shah, N.P. (2007a). Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* and *Bifidobacterium* sp. *International Dairy Journal*, *17*, 67 - 78.

a careful examination of the suitability of particular strain(s) to maintain viability throughout the ripening and shelf life (Ross et al., 2002).

A series of chemical and biochemical reactions occur during Cheddar cheese ripening including glycolysis, lipolysis and most importantly proteolysis (Fox et al., 1993). Proteolysis plays a critical role in determining the typical sensory characteristics and represents a significant indicator of quality, as shown for Cheddar cheese (Fox & McSweeney, 1996). Proteolysis is caused by enzymes contained in milk (plasmin) and rennet (pepsin and chymosin) or released by microorganisms. The activities of these enzymes reduce the concentration of casein (α_{s1}, α_{s2}, β and κ caseins) and lead to the formation of large and intermediate size peptides. These peptides may be further hydrolysed by proteolytic enzymes, originating from the microflora (starter bacteria, non-starter lactic acid bacteria and probiotic adjunct) of the cheese, into small peptides and free amino acids (FAA), which are important for the development of Cheddar flavour (Cliffe et al., 1993; Lynch et al., 1999).

Six probiotic strains (*Lactobacillus acidophilus* 4962, *Lb. casei* 279, *B. longum* 1941, *Lb. acidophilus* LAFTI®L10, *Lb. paracasei* LAFTI®L26[**] and *B. lactis* LAFTI®B94[††]) were examined in this study as a potential candidate for incorporation in Cheddar cheeses. Previously, these strains were used together as a mix culture into Cheddar cheese and all six probiotics were able to maintain their viability at high level > 7.5 $\log_{10}$ cfu g^{-1} at the end of 24 wk of ripening at 4°C (Chapter 3.0). Although there was no direct influence of addition of probiotic organisms on the composition (protein, fat, moisture and salt content) of Cheddar cheese, acetic acid concentration was found to be higher in probiotic cheeses and proteolytic pattern of probiotic cheeses was also significantly different ($P < 0.05$) when compared with the control cheese. The probiotic strain that was responsible for the differences in proteolytic pattern and organic acid profile, however, was not identified. The influence of these individual probiotic organisms to the changes in quality of Cheddar cheese has not yet been fully elucidated. The objective of the study was to investigate the performance of individual probiotic strains of *Lb. acidophilus, Lb. casei*, *Lb. paracasei* and *Bifidobacterium* sp. in Cheddar cheeses in terms of their ability to survive during ripening, their influence on the proteolytic pattern and the production of organic acid.

[**] *Lb. paracasei* LAFTI®L26 has been reclassified as *Lb. casei* LAFTI®L26
[††] *B. lactis* LAFTI®B94 has been reclassified as *B. animalis* subsp. *lactis* LAFTI®B94

4.2. Materials and methods

4.2.1. ***Starter and probiotic organisms***

Cheese starter culture, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and probiotic organisms *B. longum* 1941, *Lb. casei* 279, *Lb. acidophilus* 4962, *B. lactis* LAFTI®B94, *Lb. paracasei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 were obtained from the culture collections mentioned in section 3.2.1. The organisms were activated and grown under the conditions described in section 3.2.1.

4.2.2. ***Cheddar cheese manufacture***

Cheddar cheeses were made with 10 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture using a pair of custom made cheese vats. Seven batches of Cheddar cheeses were made including a control and six different probiotic cheeses as shown in Table 4.1. The complete set (7 batches) was produced randomly in 4 days with the same batch of pasteurised milk and at least 2 replications were produced the following weeks.

Cheeses were manufactured according to the standard procedures of Kosikowski (1977) as described previously in section 3.2.2. All cheeses were packed in oxygen barrier Cryovac® bags (Cryovac® Pty. Ltd., Fawkner, Vic, Australia) and heat-sealed with a Multivac® vacuum packaging equipment (Multivac Sepp Haggenmüller, Wolfertscgwenden, Germany) and ripened at 4^{o}C for 24 wk.

4.2.3. ***Cheese composition***

The composition of the cheeses including the salt, fat, moisture, protein contents and pH of the cheeses were determined according to the procedure described in section 3.2.3.

4.2.4. ***Survival of bacteria in cheeses***

Viability of the starter bacteria, probiotic organisms and non-starter lactic acid bacteria (NSLAB) were assessed during production and during ripening at 4^{o}C for 24 wk. Samples of ripened milk, cooked curd, whey, cheddared curd and pressed curd (fresh cheese) were collected during production. Cheese samples were also collected at 4 wk intervals during the 24 wk ripening period. Samples for enumeration were prepared as

described in section 3.2.4. Starter lactococci were enumerated on M17 agar (Merck, South Granville, NSW, Australia) and incubated at 30°C under aerobic condition for 72 h (Terzaghi & Sandine, 1975). Total lactobacilli during ripening were enumerated on LBS agar (Merck) and incubated at 30°C in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, USA) with a Gas Generating Kit® (Oxoid) for 72 h. The probiotic adjuncts were enumerated using various selective media as described in section 3.2.4 and incubated anaerobically at 37°C for 72 h. Since starter lactococci and *Bifidobacterium* sp. do not grow on Lactobacillus Selective agar (LBS), the NSLAB counts of control cheese (Batch 1), BL 1941 cheese (Batch 2) and *B. lactis* LAFTI®B94 cheese (Batch 3) were obtained from LBS agar. On the other hand, the NSLAB counts of cheeses with *Lb. casei* 279 (Batch 4), *Lb. paracasei* LAFTI®L26 (Batch 5), *Lb. acidophilus* 4962 (Batch 6) and *Lb. acidophilus* LAFTI®L10 (Batch 7) were obtained by subtracting the count of probiotic organisms on the selective agar from the count of total lactobacilli on LBS agar.

4.2.5. ***Production of organic acids***

The concentration of lactic acid and acetic acid was determined using high performance liquid chromatography (HPLC) as described in section 3.2.5.

4.2.6. ***Proteolytic activity of starter and probiotic organisms in reconstituted skim milk***

All bacterial strains were grown overnight at 37°C in MRS broth (Merck). To minimize carryover of free amino acids during inoculation, 10 mL of cells were washed and resuspended to the original volume with 0.32 mM sodium phosphate, pH 7.2. Cells were inoculated (2%) into RSM (12% w/v) and incubated at their optimum temperatures (37°C) for 18 h. A control, which was the uninoculated RSM, was also incubated at 37°C for 18 h. A 2.5 mL sample of each incubated RSM was then mixed with 10 mL of 0.75 M trichloroacetic acid (TCA) and 1 mL of water to 5 mL of sample to give a final concentration of 0.47 M (7.7%) TCA. The samples were filtered using a Whatman number 4A filter paper after 10 min incubation at room temperature (~22°C) before assayed. The *o*-phthaldialdehyde (OPA) method (Church et al., 1983) described by Shihata and Shah (2000) was used to determine the concentration of free amino groups in the filtrate. Triplicate aliquots from each TCA filtrate were analysed using a Pharmacia LKP Novaspek II Spectophotometer (Pharmacia, Biotech, Uppsala, Sweden).

4.2.7. ***Assessment of proteolysis***

The water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) of the cheeses were determined as per the method described in section 3.2.6.

The proteolytic patterns of Cheddar cheeses were also analysed by SDS-PAGE using the stacking gel system as described in section 3.2.6. The gels were fixed in de-staining solution I (40% methanol, 7% acetic acid) for 30 min before staining with staining solution (0.025% Coomassie Brilliant blue (ICN Biochemicals Inc., Aurora, OH, USA), 40% methanol, 7% acetic acid) for 4 h. The gels were then de-stained in de-staining solution I for 1 h followed by de-staining in de-staining solution II (7% acetic acid, 5% methanol) until the background became clear. The gel images were recorded using a Fuji Film Intelligent Dark Box II with Fuji Film LAS-1000 Lite V1.3 software. The area and intensity of the bands were measured using Fuji Film Image Gauge V4.0 software (Fuji Photo Film Co. Ltd., Japan). Data from the analysis were expressed as the ratio of the area and the intensity of the band. The reduction in the intensity of bands during ripening with respect to the original intensity was expressed as percentage of hydrolysis.

4.2.8. ***Statistical analysis***

Data analysis was carried out with Minitab Statistical Package (Minitab Inc, State College, PA, USA). One-way analysis of variance was used to establish differences between means, with a significant level at $\alpha = 0.05$. A total of 21 batches of cheeses were produced from the seven variations and all analyses were carried out at least in duplicate. All data are presented as mean ± standard error of means.

4.3. Results and discussions

4.3.1. Cheese composition

The composition of control and probiotic Cheddar cheeses is summarized in Table 4.2. The moisture content of the cheeses in this study was higher than the typical moisture of 36 to 39% for normal Cheddar (Kosikowski, 1977). There was, however, no significant difference ($P > 0.05$) in the moisture content of control and probiotic cheeses. The salt content and the percentage of salt in dry matter varied between cheeses. The differences reflect the difficulties in controlling the cheese-making parameters in laboratory scale production (e.g. it was hard to achieve constant pressure during pressing without proper cheese pressing equipment and this also affected the amount of salt released with the whey during pressing). All the other compositional values (fat, fat in dry matter, pH and protein content) for the different type of cheeses were not significantly different ($P > 0.05$). The result thus confirms the findings of Gardiner et al. (1998) and Ong et al. (2006) that the addition of probiotic microorganisms into Cheddar cheese has no direct effect on cheese composition.

4.3.2. Survival of bacteria during manufacture of Cheddar cheeses

The probiotic microorganisms were initially inoculated at the rate of 1.2 % v/v. At this rate, the concentration of the probiotic in the inoculated-milk ranged between 7 to 8 $\log_{10}$ cfu g^{-1} (Table 4.3). The starter culture count increased by about half a log cycle during milk ripening at 31^{o}C for 45 min in almost all cheeses. On the other hand, the counts of probiotic organisms during milk ripening remained relatively constant. Volume reduction during whey draining followed by cooking resulted in increase in the counts of starter bacteria by more than one log cycle, whereas the counts of probiotic only increased by half to one log cycle. The counts of starter organisms continued to increase after cheddaring at 38^{o}C for about 90 min with only slight increase in the counts of probiotic organisms. Overall, the lactococci counts increased consistently in all batches, suggesting that they were the primary acid producers throughout the cheese-making process. Their performances were also not affected by the addition of any of the probiotic adjuncts. On the other hand, most of the probiotic organisms only grew slowly during the whole manufacturing process because of the insufficient amounts of amino acids and low molecular weight peptides in milk to sustain their growth (Fox et al., 1993). About 5.5 to 7.5 $\log_{10}$ cfu g^{-1} of probiotic

bacteria were lost during draining (Table 4.3). All probiotic microorganisms were found to survive the cheese manufacturing process and were present at a high level of 8 to 9 $\log_{10}$ cfu g^{-1} at the end of the manufacturing process (Table 4.3).

4.3.3. ***Survival of bacteria during ripening***

All probiotic bacteria added were able to maintain their viability in Cheddar cheeses during the ripening period of 24 wk at 4^{o}C (Figure 4.1). The survival patterns of *B. longum* 1941, *B. lactis* LAFTI®B94, *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10 in Cheddar cheeses were similar. At the beginning of the ripening period, all six probiotic organisms declined by about half a log cycle. After 4 wk, their count remained relatively constant, which indicated the ability of each of the probiotic strains to adapt quickly to the cheese environment. At the end of the ripening period, *Lb. acidophilus* strains (LA 4962 and LAFTI®L10) showed the highest decline as compared to *B. longum* 1941, *B. lactis* LAFTI® B94, *Lb. casei* 279 and *Lb. paracasei* LAFTI®L26. All probiotic organisms, however, were able to maintain their viability at the level of > 8.0 $\log_{10}$ cfu g^{-1}.

On the other hand, lactococci counts in all cheeses decreased by one to two log cycles after 24 wk of ripening at 4^{o}C probably due to unfavorable conditions in the cheese such as high salt in moisture, low pH, lack of fermentable carbohydrate and low ripening temperature. Despite the variation in the type of probiotic adjunct added, the decline in counts of starter lactococci in different batches of the cheeses was not significantly different ($P < 0.05$). This indicates that the probiotic adjunct did not affect the performance of starter lactococci during ripening. A similar trend was also observed in our previous study (Chapter 3, section 4.3.3).

A population of non-starter lactic acid bacteria (NSLAB) also proliferated in cheeses during ripening. NSLAB are chiefly composed of lactobacilli such as *Lb. plantarum*, *Lb. casei* and *Lb. brevis* (Gardiner et al., 1998). As shown in Figure 4.1, NSLAB counts remained relatively low for the first few weeks of ripening and increased to about 7 $\log_{10}$ cfu g^{-1} at the end of ripening period of 24 wk at 4^{o}C (Batches 1 – 3). Similar growth characteristics of adventitious NSLAB in Cheddar cheese produced in open vats have been reported (Lynch et al., 1999). NSLAB counts could not be presented for the first 8 wk of ripening in cheeses with *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10 (Batches 4 – 7). These probiotic organisms were able to grow on LBS agar used for the enumeration of NSLAB. Counts of NSLAB in cheeses with

the addition of these probiotic bacteria were obtained by subtracting the count of probiotic bacteria on selective media from the total lactobacilli count on LBS agar. The subtraction method, however, did not work for the first 8 wk of ripening because the levels of NSLAB were more than one log lower than the counts of probiotic organisms. At the end of the ripening period, the counts of NSLAB in control and all probiotic cheeses were not significantly different, which shows that addition of the probiotic bacteria did not influence the growth of the indigenous microflora of the Cheddar cheeses.

4.3.4. ***Production of organic acid***

The metabolic activity of the microorganisms in the cheeses was monitored by estimating the metabolic products, lactic and acetic acids. Lactic acid concentration increased slightly during the first few weeks of ripening due to the used of residual lactose trapped in the curd. At the end of ripening, the concentration of lactic acid was not significantly different between cheeses ($P > 0.05$). No other discernible pattern was observed in the concentration of lactic acid. Acetic acid concentration, however, increased as the ripening time increased in all cheeses including the control (Figure 4.2). At the end of ripening, the concentration of acetic acid in different probiotic cheeses varied significantly ($P < 0.05$).

Acetic acid concentration in cheeses with *Lb. acidophilus* strains and control cheese was not significantly different ($P > 0.05$). At the end of 24 wk, the concentration of acetic acid was 0.06% for both cheeses with *Lb. acidophilus* 4962 (Batch 6) and for control cheese (Batch 1) and 0.05% for cheese with LAFTI®L10 (Batch 7). Results show that *Lb. acidophilus* strains used in this study were not able to produce acetic acid as their metabolic end products. The presence of a small amount of acetic acid in both control and *Lb. acidophilus* cheeses was due to the ability of NSLAB and starter bacteria to produce acetate from lactose or citrate or from amino acids (Fox & McSweeney, 1996). On the other hand, cheeses with *Lb. casei* and *Lb. paracasei* had considerable concentrations of acetic acid at the end of ripening, reaching 0.07% for cheese with *Lb. casei* 279 (Batch 4) and 0.10% for cheeses with *Lb. paracasei* LAFTI®L26 (Batch 5). Desai et al (2004) reported that some strains of *Lb. casei* were able to produce acetic acid as their metabolic end products.

Acetic acid concentrations in cheese with *Bifidobacterium* sp. were also significantly higher ($P < 0.05$). At the end of 24 wk of ripening *B. longum* 1941 cheese (Batch 2) had 0.13% of acetic acid followed by 0.10% for cheese with LAFTI®B94 (Batch 3). *Bifidobacterium* produces acetic acid and lactic acid from lactose via a fructose-6-phosphate

shunt pathway (Bruno et al., 2002). The fermentation pathway results in 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose, therefore generating a theoretical molar ratio (acetic:lactic) of 3:2 (Scardovi & Trovatelli, 1965). Dinakar and Mistry (1994) incorporated *B. bifidum* into Cheddar cheese and found that this organism was able to remain viable in Cheddar cheese up to the end of 24 wk but the strain was not metabolically active and did not produce acetic acid. This was expected since the *Bifidobacterium* used in that study was immobilized and microencapsulated prior to addition at milling stage. The cells were not exposed to the cheese environment, but enveloped with κ-carragenan, thus would have restricted the used of lactose by *Bifidobacterium* to produce acetic acid.

4.3.5. ***Proteolytic activity of starter and probiotic organisms in reconstituted skim milk***

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. Figure 4.3 represents the proteolytic activities in RSM of probiotic bacteria *B. longum* 1941, *B. lactis* LAFTI®B94, *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10. The proteolytic activity of these bacterial strains is expressed as the amount of free amino groups measured as a difference in absorbance values at 340 nm, after substraction of values for the control RSM. As shown in Figure 4.3, *Lb. casei* 279 showed the highest proteolytic activity releasing the highest amount of free amino groups followed by *Lb. acidophilus* 4962, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* LAFTI® L10 and *Bifidobacterium* sp.

4.3.6. ***Assessment of proteolysis in Cheddar cheeses***

Assessment of proteolysis in the control and probiotic cheeses by determination of WSN, TCA-SN and PTA-SN over the 24 wk of ripening period at 4^{o}C is depicted in Figure 4.4. The ratio of WSN to total nitrogen (TN) in all cheeses increased progressively, but the increase was more intense after 16 wk of the initial ripening period (Figure 4.4a). During the first 12 wk of ripening, there were no apparent differences among all cheeses in the level of WSN. Fox (1993) explained that about 3-6% of the coagulant (Chymosin) added to cheese milk is retained in the curd. Differences in the level of WSN at the early stage of ripening period (0-12 wk) among these cheeses were not expected because most of the water-soluble peptides were the products of primary proteolysis produced by the action of the coagulant and plasmin (Visser, 1977). After 20 wk, higher WSN was detected in *Lb.*

casei 279 cheese (Batch 4) and *Lb. paracasei* LAFTI®L26 cheese (Batch 5), because both strains have considerably higher proteolytic activity (Figure 4.3) and thus higher casein hydrolytic ability, which contributed to the release of more water soluble peptides. At the end of the ripening period, levels of WSN for all probiotic cheeses were higher and significantly different ($P < 0.05$) from the control cheese due to the presence of more proteolytic enzymes with the addition of probiotic cheeses.

The amount of TCA-SN also increased progressively and was more intense after 8 wk (Figure 4.4b). Starter and non-starter bacterial proteinases are principally responsible for the formation of TCA-SN (Fox et al., 1993). Cheeses made with *B. longum* 1941 (Batch 2), *Lb. acidophilus* 4962 (Batch 6) and *Lb. casei* 279 (Batch 4) in general had the highest level of TCA-SN (Figure 4.4b), which shows that when soluble peptides had been formed by rennet and starter culture, the peptidases and proteinases from these probiotic adjuncts were able to hydrolyse them effectively thus releasing more intermediate and smaller size peptides. This was more apparent after 12 wk especially as more products of the primary proteolysis were available as substrates for the subsequent proteolysis by the probiotic organisms.

Higher concentration of free amino acids was observed in probiotic cheeses (Figure 4.4c). A similar trend in the development of total free amino acid in probiotic cheeses was noted in our previous study (Chapter 3, Figure 3.3c) and also by Gardiner et al. (1998). It is interesting to note that although both *B. longum* 1941 and *B. lactis* LAFTI® B94 were weakly proteolytic strains (Figure 4.3), both cheeses with the addition of those strains had elevated concentration of free amino acids at the end of the ripening period (Figure 4.4c). It thus suggests that the peptidases from *Bifidobacterium* sp. were able to hydrolyze the product of the primary proteolysis to mostly smaller peptides and amino acids that were soluble in PTA. Shihata and Shah (2000) found that although *Bifidobacterium* sp. possesses very little proteolytic activity, they had high levels of intracellular aminopeptidases activity. Minagawa et al (1985) described the exopeptidase system of several *Bifidobacterium* strains and demonstrated the presence of aminopeptidase, iminopeptidase and carboxypeptidase activities.

The concentration of free amino acids in *Lb. acidophilus* LAFTI®L10 cheese (Batch 7), *Lb. casei* 279 cheese (Batch 4) and *Lb. acidophilus* 4962 cheese (Batch 6) was not significantly different and the FAA levels were almost as high as those of *Bifidobacterium* sp. cheeses (Batches 2 & 3). *Lb. paracasei* LAFTI®L26 cheese (Batch 5) on the other hand, had the lowest level of free amino acids when compared to the other probiotic cheeses

which shows that although *Lb. paracasei* LAFTI®L26 was highly proteolytic (Figure 4.3), its proteolytic enzymes produced mainly large to medium size peptides during ripening.

Proteolysis pattern of the cheeses during ripening was also monitored by SDS-PAGE. Electrophoretic pattern of cheeses with *B. longum* 1941 was similar with that with *B. lactis* LAFTI®B94; *Lb. casei* 279 was similar with *Lb. paracasei* LAFTI®L26 and *Lb. acidophilus* 4962 was similar with *Lb. acidophilus* LAFTI®L10. The electrophoretic patterns of the control and probiotic cheeses were presented in Figure 4.5. Protein bands were identified based on molecular weight and also by comparison with standards of casein (CN), α_s-CN, β-CN and κ-CN. α_{s1}-CN and α_{s2}-CN could not be separated on 12% acrylamide gel because the molecular weights of these two fractions are very similar (Varnam & Sutherland, 1994). β-Lactoglobulin (MW 18, 300) and α-lactalbumin (MW 14, 000) were identified based on their molecular weight. In general, the electrophoretic patterns of Cheddar cheese during ripening at 4^{o}C for 24 wk show that as the ripening time increases, the concentrations of α_s-CN, β-CN decreased and the concentration of lower molecular weight breakdown products of the caseins increased. These products, which appear in SDS-PAGE in the area between β-casein and β-lactogolobulin (Figure 4.5) (Basch et al., 1989; Brandsma et al., 1994), serve as substrates for microbial proteinases and peptidases, which lead to the formation of smaller peptides and amino acids. The rate of hydrolysis of α_s-CN and β-CN was, however, different between batches.

The α_s-CN was hydrolysed faster in almost all probiotic cheeses as shown by the disappearance of the α_s- CN band at the early stage of ripening period (Figure 4.5). This was especially very obvious for cheeses made with *Lb. casei* 279 and *Lb. paracasei* LAFTI®L26. On the other hand, α_s- CN band for cheeses made with *Bifidobacterium* sp. was hydrolysed almost at the same rate as control cheeses. This was expected because *Bifidobacterium* strains used in this study were not highly proteolytic (Figure 4.3). Our findings are in agreement with those of Dinakar and Mistry (1994), who found no significant differences between the electrophoresis patterns of cheeses without probiotic bacteria and those supplemented with *B. bifidum*. Similar results were also observed by Corbo et al. (2001) who supplemented Canestro Pugliese hard cheese with *B. longum* and *B. bifidum*.

The progress of hydrolysis of the individual casein fractions is depicted in Figure 4.6. Percentage hydrolysis of casein fraction was expressed as relative to concentration of casein at wk 1. Most of the α_s-CN hydrolysis occurred at the early stage of ripening, between 0 and 16 wk and the hydrolysis slowed down after 16 wk. The percentages of α_s-CN hydrolysed at the end of 24 wk were 69.5%, 57.6%, 42.9%, 40.0%, 27.9% and 27.8%

for *Lb. paracasei* LAFTI®L26, *Lb. casei* 279, *Lb. acidophilus* 4962, *Lb. acidophilus* LAFTI®L10, *B. longum* 1941 and *B. lactis* LAFTI®B94 cheeses, respectively. The most important contributor to the continuous casein matrix of a Cheddar cheese is α_{s1}-CN (Lawrence et al., 1987), and hydrolysis of this casein is believed to be responsible for the softening of Cheddar cheese texture (Grappin et al., 1985).

During ripening, β-CN does not undergo as much degradation as α_s-CN (Brandsma et al., 1994; Mistry & Kasperson, 1998). This shows that the bacterial peptidases were able to hydrolyse α_s-CN faster than β-CN. Addition of probiotic adjuncts increased the percent hydrolysis of β–CN slightly (~ 5%) but significantly ($P < 0.05$), except for cheese with *Lb. paracasei* LAFTI®L26 (Batch 5), which shows that the proteolytic enzymes from *Lb. paracasei* LAFTI®L26 had very low preference for β–CN.

4.4. Conclusions

All probiotic adjuncts survived the cheese making process at a high level without alteration of cheese making process. At the end of 24 wk ripening at 4°C, these organisms maintained the levels of > 8.0 $\log_{10}$ cfu g^{-1}. Acetic acid concentration was higher in cheeses with *B. longum* 1941, *B. lactis* LAFTI® B94, *Lb. casei* 279 and *Lb. paracasei* LAFTI®L26. No significant difference in the level of acetic acid was found amongst cheeses with *Lb. acidophilus* sp. and control cheeses. *Lb. casei* 279 and *Lb. paracasei* LAFTI®L26 had higher casein hydrolytic activity which was reflected on the release of more water-soluble peptides and higher percentage hydrolysis of α_s-CN. Concentration of free amino acids on cheese with *Lb. paracasei* LAFTI®L26 was not higher than other probiotic cheeses, showing that the bacterial peptidases in the cheese produced more intermediate size peptides rather than smaller peptides and amino acids. Although *Bifidobacterium* sp. was found to be weakly proteolytic, both cheeses with these strains had the highest concentration of free amino acids at the end of the ripening period. Once the products of primary proteolysis were available, the proteolytic enzyme of *Bifidobacterium* sp. was able to hydrolyze them very rapidly to produce smaller peptides and amino acids. These data thus suggest that the six probiotic strains studied can be applied successfully in Cheddar cheeses. Addition of these probiotic adjuncts also resulted in the increased hydrolysis of α_{s1}-CN and thus in the release of small molecular weight peptides.

Table 4.1. Production scheme of probiotic Cheddar cheeses

Cheddar cheese[1]	Probiotic adjunct (1.2% v/v)
Batch 1	None
Batch 2	*B. longum* 1941
Batch 3	*B. lactis* LAFTI®B94
Batch 4	*L. casei* 279
Batch 5	*L. paracasei* LAFTI®L26
Batch 6	*L. acidophilus* 4962
Batch 7	*L. acidophilus* LAFTI®L10

[1] All Cheddar cheeses were produced with cheese starter culture (1.5% v/v) of mix *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*.
[2] 1.2 % v/v = 12 mL of bulk culture of RSM added to every liter of pasteurized milk.

Table 4.2. Composition of control and probiotic Cheddar cheeses[1]

Composition	Cheddar cheese[2]						
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7
% Moisture	39.00 ± 0.78[a]	40.60 ± 0.78[a]	43.30 ± 0.38[a]	39.80 ± 0.38[a]	39.00 ± 0.99[a]	43.3 ± 1.1[a]	40.60 ± 1.35[a]
% Salt	1.78 ± 0.05[a]	1.85 ± 0.04[a]	1.90 ± 0.04[a]	1.71 ± 0.02[b]	1.70 ± 0.06[b]	1.95 ± 0.05[a]	1.95 ± 0.07[a]
% SDM[3]	2.91 ± 0.11[a]	3.68 ± 0.07[b]	3.28 ± 0.06[c]	3.31 ± 0.03[c]	3.28 ± 0.08[c]	3.11 ± 0.08[ac]	3.35 ± 0.11[bc]
% Fat	31.40 ± 0.85[a]	33.10 ± 0.64[a]	30.45 ± 0.59[a]	33.25 ± 0.32[a]	32.50 ± 0.83[a]	31.00 ± 0.79[a]	30.64 ± 1.02[a]
% FDM[4]	51.48 ± 0.83[a]	55.39 ± 1.07[a]	54.40 ± 1.05[a]	52.54 ± 0.51[a]	51.58 ± 1.31[a]	55.72 ± 1.42[a]	53.70 ± 1.79[a]
% Protein	27.46 ± 0.41[a]	25.54 ± 0.49[a]	28.41 ± 0.55[a]	25.80 ± 0.25[a]	26.88 ± 0.68[a]	25.51 ± 0.65[a]	26.74 ± 0.89[a]
pH	5.40 ± 0.03[a]	5.20 ± 0.10[a]	5.26 ± 0.10[a]	5.25 ± 0.05[a]	5.34 ± 0.14 [a]	5.15 ± 0.13[a]	5.22 ± 0.17[a]

[ab] Means in rows with like superscripts do not differ ($P > 0.05$).

[1] Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate. Analyses were conducted during the first wk of Cheddar cheese ripening.

[2] Batch codes are as detailed in Table 4.1.

[3] Salt in dry matter.

[4] Fat in dry matter.

Table 4.3. Viability (Log_{10} cfu g^{-1}) of cheese starter culture and probiotic adjunct during manufacturing of Cheddar cheese

Sample[2]		Viability (Log_{10} cfu g^{-1})[1]					
		Inoculated milk	**Ripened milk**	**Cooked curd**	**Whey**	**Cheddared curd**	**Pressed curd (Fresh cheese)**
Batch 1	Starter	7.37 ± 0.14^{aA}	7.90 ± 0.02^{aA}	9.20 ± 0.31^{bA}	7.39 ± 0.09^{aA}	9.49 ± 0.34^{bA}	9.31 ± 0.23^{bA}
Batch 2	Starter lactococci BL 1941	7.84 ± 0.20^{abA} 7.18 ± 0.18^{abA}	8.00 ± 0.40^{abA} 7.27 ± 0.18^{abA}	9.08 ± 0.60^{acA} 8.28 ± 0.12^{bcA}	7.20 ± 0.36^{bA} 7.27 ± 0.18^{aA}	9.59 ± 0.24^{cA} 8.63 ± 0.57^{cA}	9.80 ± 0.04^{cA} 8.71 ± 0.37^{cA}
Batch 3	Starter lactococci LAFTI®B94	7.43 ± 0.11^{aA} 7.27 ± 0.37^{aA}	7.78 ± 0.20^{abA} 7.22 ± 0.24^{aA}	8.90 ± 0.29^{bcA} 7.98 ± 0.12^{aA}	7.18 ± 0.48^{aA} 5.54 ± 0.08^{bA}	9.20 ± 0.30^{cA} 8.30 ± 0.27^{aA}	9.84 ± 0.09^{cA} 8.60 ± 0.57^{aA}
Batch 4	Starter lactococci LC 279	7.11 ± 0.18^{aA} 8.01 ± 0.12^{abA}	7.78 ± 0.52^{abA} 8.23 ± 0.42^{abA}	9.08 ± 0.14^{bcA} 8.80 ± 0.29^{aA}	7.11 ± 0.18^{aA} 7.45 ± 0.11^{bA}	9.43 ± 0.48^{cA} 8.82 ± 0.22^{aA}	9.71 ± 0.03^{cA} 8.84 ± 0.22^{aA}
Batch 5	Starter lactococci LAFTI®L26	7.52 ± 0.38^{aA} 7.85 ± 0.52^{aA}	8.05 ± 0.26^{abA} 7.95 ± 0.26^{aA}	9.54 ± 0.48^{bA} 8.61 ± 0.57^{aA}	7.15 ± 0.23^{aA} 7.43 ± 0.11^{aA}	9.90 ± 0.50^{bA} 8.90 ± 0.59^{aA}	9.58 ± 0.17^{bA} 8.65 ± 0.44^{aA}
Batch 6	Starter lactococci LA 4962	7.10 ± 0.47^{aA} 7.01 ± 0.46^{aA}	7.39 ± 0.11^{aA} 7.34 ± 0.37^{abA}	8.96 ± 0.45^{bA} 8.12 ± 0.41^{abA}	7.41 ± 0.37^{aA} 7.53 ± 0.19^{abA}	9.00 ± 0.23^{bA} 8.43 ± 0.43^{abA}	9.42 ± 0.11^{bA} 8.73 ± 0.13^{bA}
Batch 7	Starter lactococci LAFTI®L10	7.94 ± 0.26^{abA} 7.35 ± 0.24^{abA}	8.36 ± 0.45^{abA} 7.48 ± 0.38^{abA}	9.37 ± 0.23^{abA} 8.15 ± 0.21^{bA}	7.69 ± 0.12^{aA} 6.27 ± 0.32^{aA}	9.55 ± 0.64^{bA} 8.59 ± 0.13^{bA}	9.60 ± 0.08^{bA} 8.60 ± 0.57^{bA}

[abcd] Means in rows with like superscripts do not differ ($P > 0.05$).
[AB] Means in column with like superscripts do not differ ($P > 0.05$).
[1] Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.
[2] Batch codes are as detailed in Table 4.1.

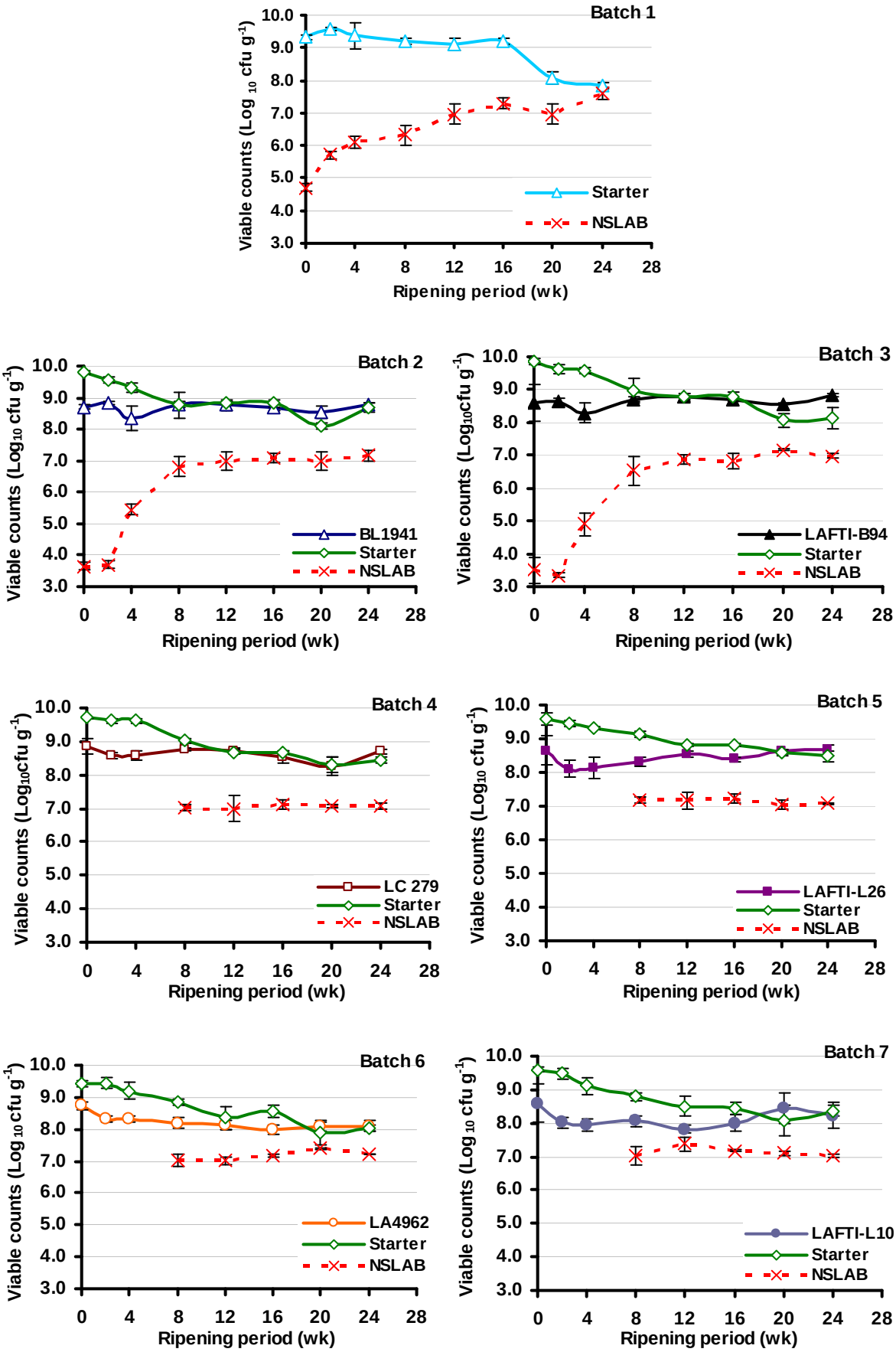


Figure 4.1. Survival of starter lactococci, probiotic adjuncts and NSLAB in Cheddar cheese during ripening period of 24 wk at 4°C. Batch codes are as detailed in Table 4.1. Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.

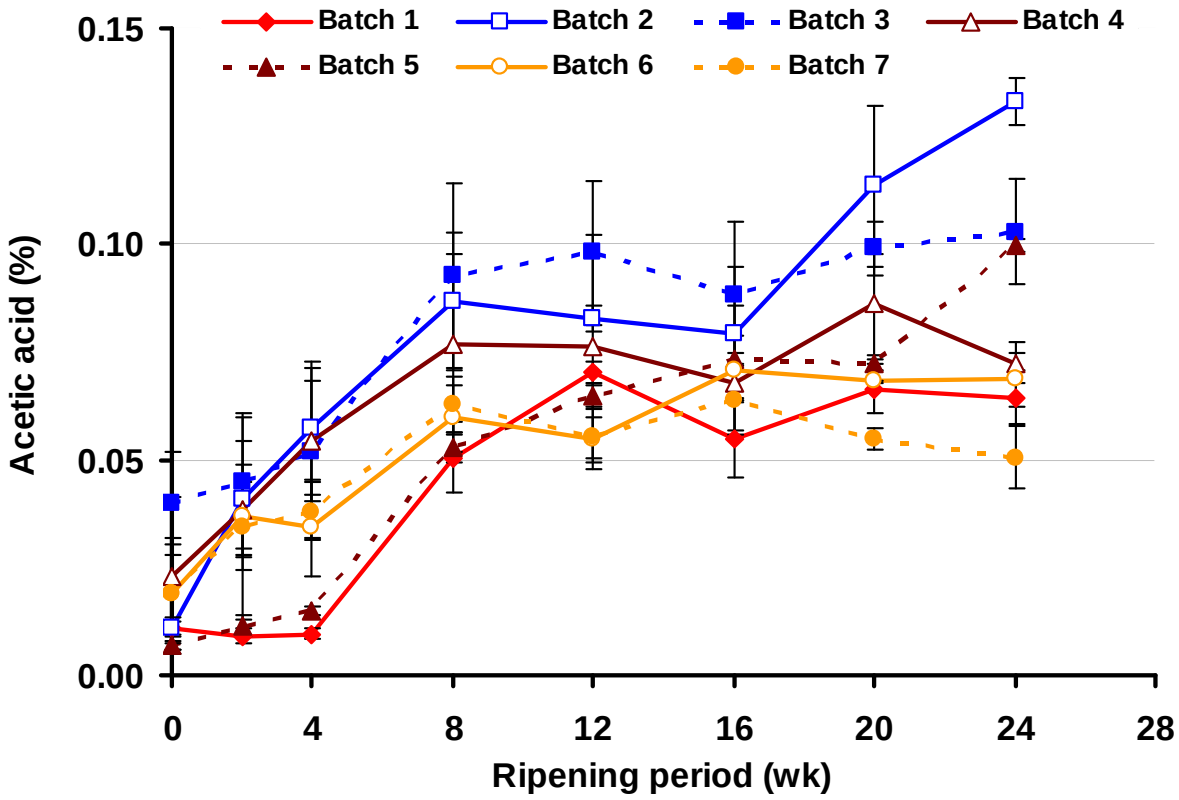


Figure 4.2. Acetic acid concentration of Cheddar cheeses during ripening at 4°C for 24 wk. Batch codes are as detailed in Table 4.1. Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate.

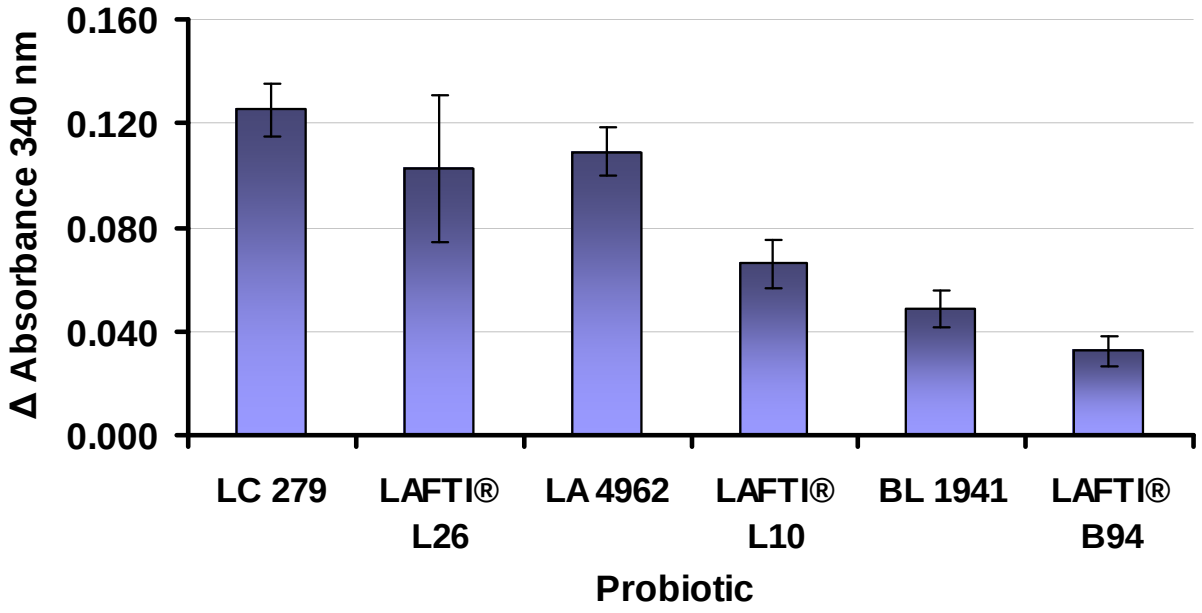


Figure 4.3. Indication of proteolytic activity of probiotic bacteria *B. longum* 1941, *B. lactis* LAFTI®B94, *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10 after incubation in reconstituted skim milk (RSM) for 18 h. Data represent differences in absorbance values after subtracting the value for the control RSM.

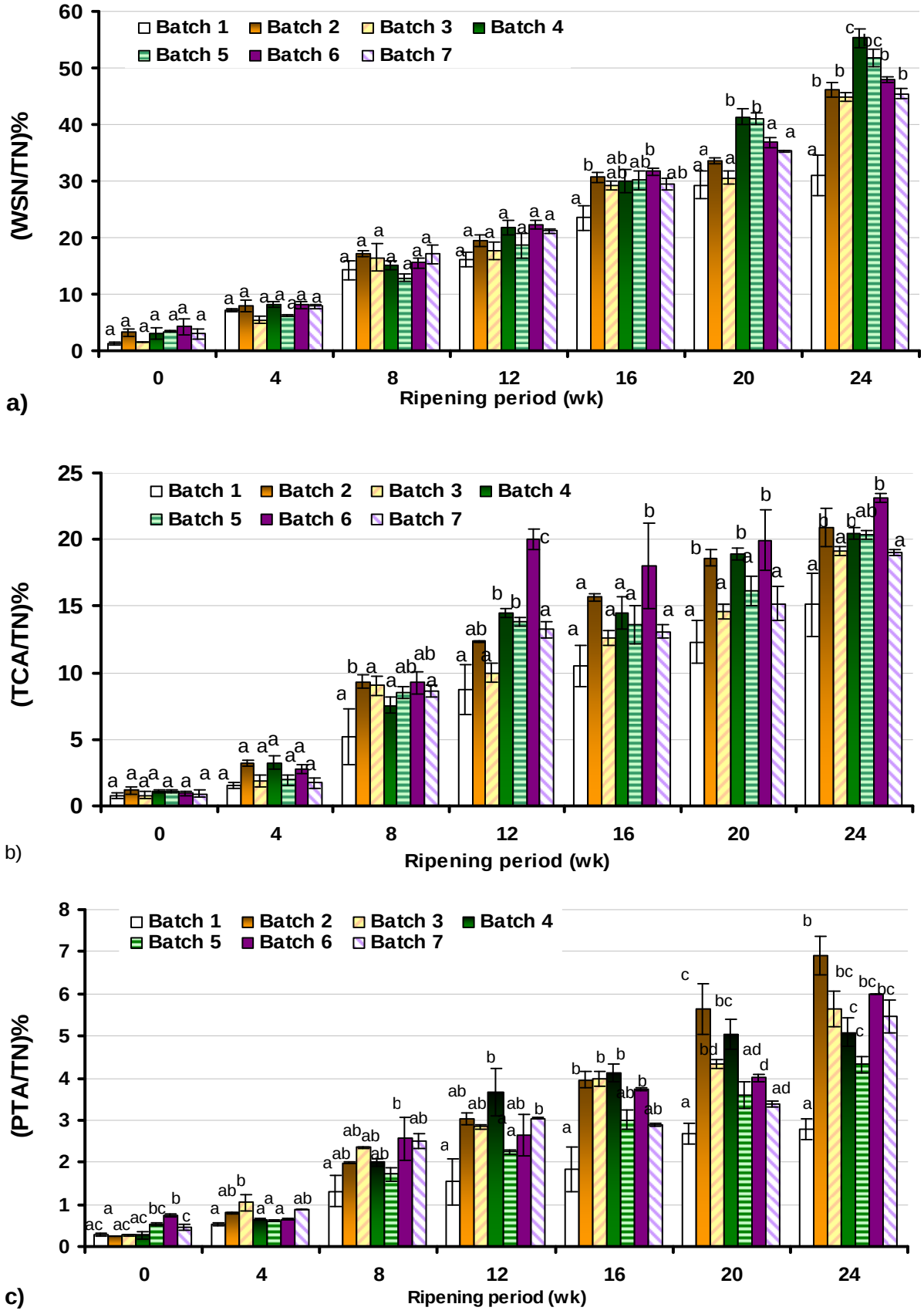


Figure 4.4. Concentration (expressed as percentage of total N) of (a) water-soluble nitrogen (WSN), (b) trichloroacetic acid-soluble nitrogen (TCA-SN), (c) phosphotungstic acid-soluble nitrogen (PTA-SN) of Cheddar cheeses during ripening at 4°C for 24 wk. Batch codes are as detailed in Table 4.1. Results are expressed as mean ± standard error of means; n = 3 sets of data analysed in duplicate. [abc] Means between treatments (Batches) with like superscripts do not differ ($P > 0.05$).

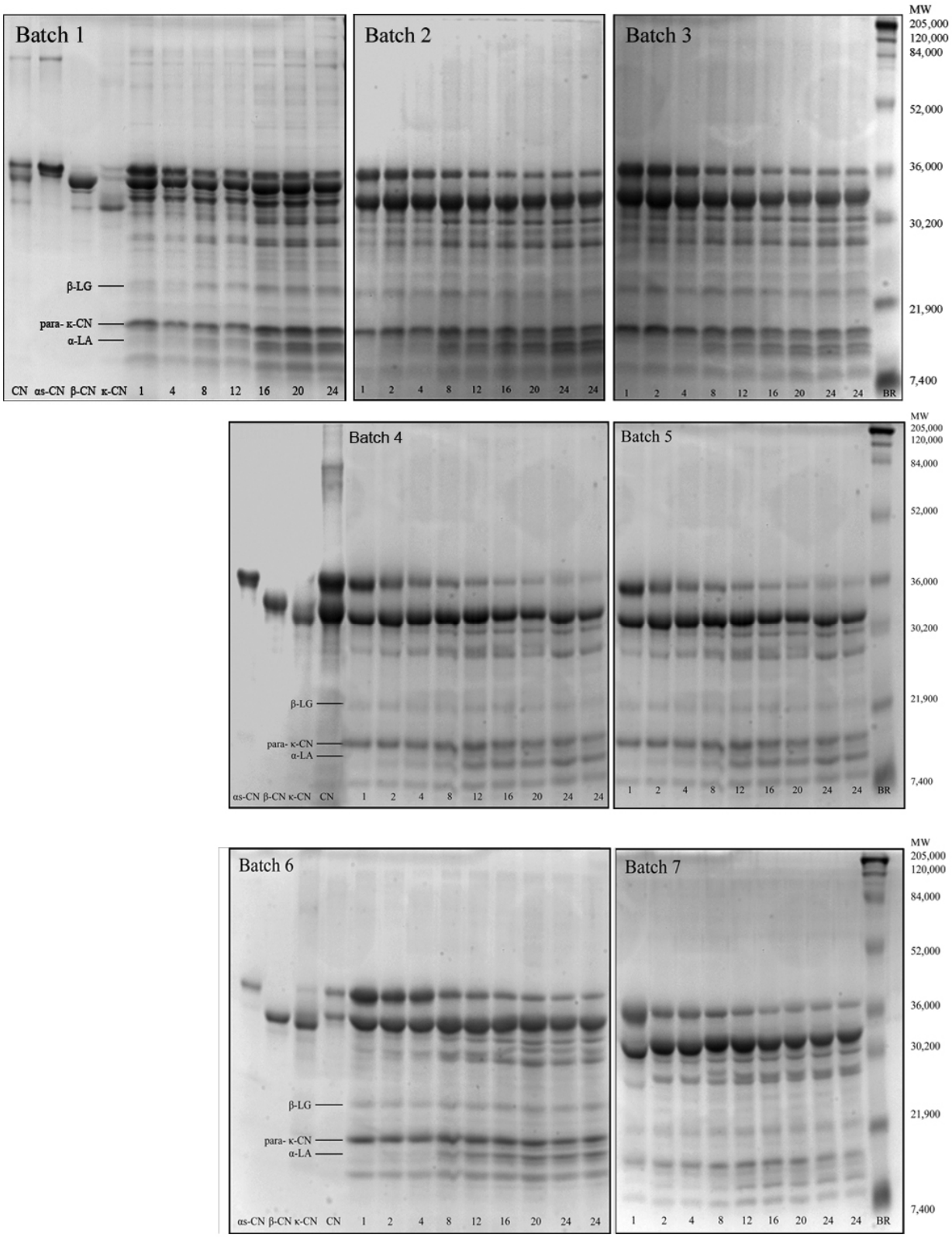


Figure 4.5. Electrophoretic patterns of SDS-PAGE of Cheddar cheese during ripening period (wk 1, 2, 4, 8, 12, 16, 20, 24, 24). Batch codes are as detailed in Table 4.1. CN = casein. BR = broad range prestained molecular weight (MW) standards containing: myosin (MW 205,000), β-galactosidase (MW 120,000), bovine serum albumin (MW 84,000), ovalbumin (MW 52,000), carbonic anhydrase (MW 36,000), soybean trypsin inhibitor (MW 30,200), lysozyme (MW 21,900) and aprotinin (MW 7,400). LA = lactalbumin. LG = lactoglobullin.

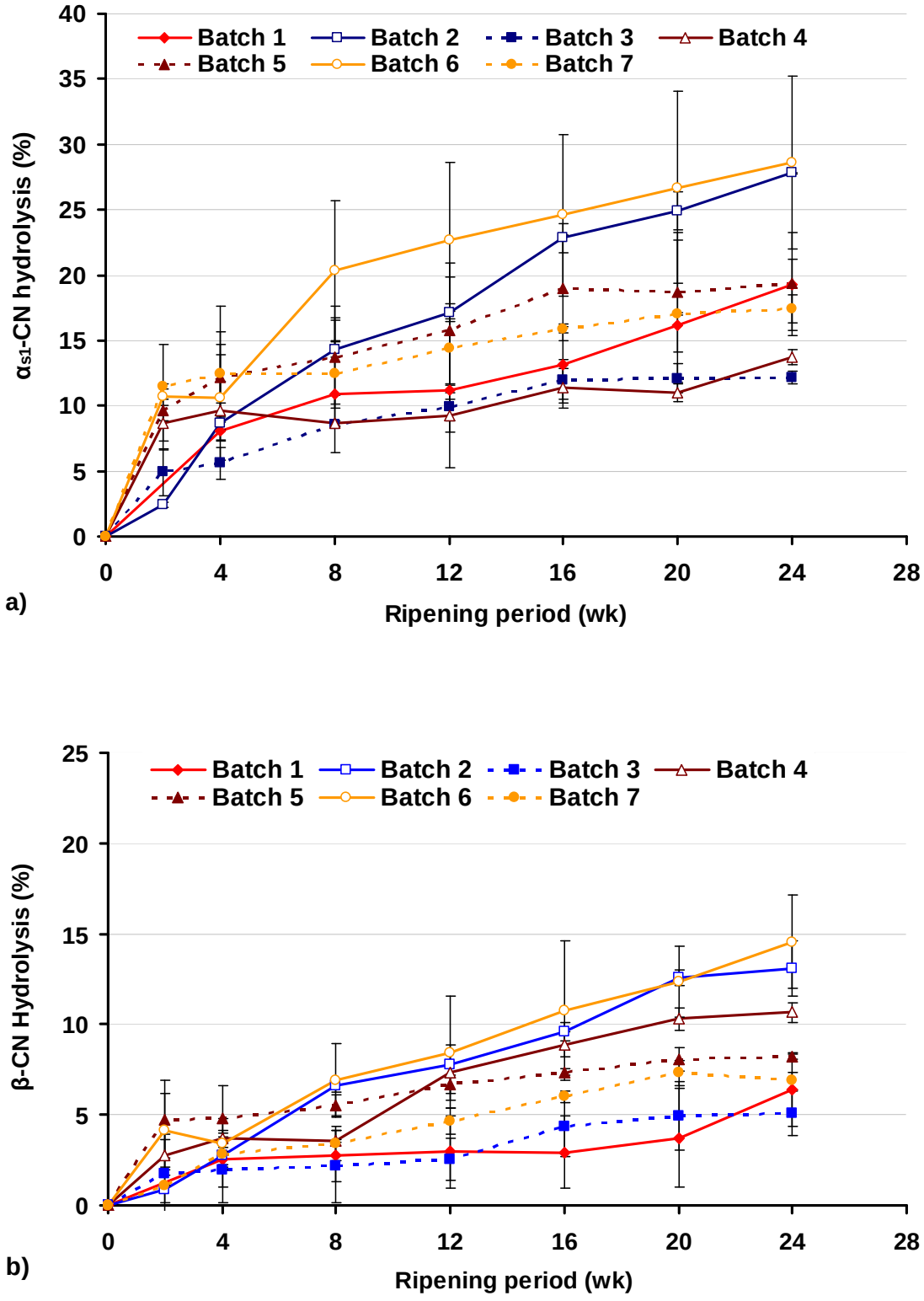


Figure 4.6. Protein hydrolysis (%) of (a) α_{s1}-CN and (b) β-CN during ripening of Cheddar cheese. Batch codes are as detailed in Table 4.1. Results are expressed as mean ± standard error of means of at least 3 sets of data.

5.0 Sensory evaluation of Cheddar cheeses produced with *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* sp.*

5.1. Introduction

The ripening of cheese involves three primary biochemical processes namely glycolysis, lipolysis, and proteolysis. Proteolysis plays a critical role in determining the typical sensory characteristics and represents a significant indicator of quality, as shown for Cheddar cheese (Fox & McSweeney, 1996). Proteolysis is caused by enzymes contained in milk (plasmin, cathepsin), rennet (pepsin and chymosin) and microbial enzymes released by starter bacteria and non-starter lactic acid bacteria (NSLAB). A gradual decomposition of caseins occurs due to the combined action of the various proteolytic enzymes. Product of proteolysis including peptides and amino acids has been shown to be important for the development of Cheddar flavour (Thomas & Mills, 1981; Cliffe et al., 1993; Lynch et al., 1999).

Addition of lactobacilli in cheeses has been associated with an increased proteolysis and intensification of flavour (McSweeney et al., 1994; Drake et al., 1996; Lane & Fox 1996; Lynch et al., 1999). Proteolytic enzymes produced by certain probiotic adjuncts were also found to degrade bitter peptides (Koka & Weimer, 2000). Broadbent et al. (2002) showed that lactococcal proteinases and rennet are responsible for the formation of bitter peptides from caseins in Cheddar cheese. Although bitter taste is considered a normal component of cheddar flavour, excessive bitterness may limit consumer acceptance of cheeses. Identification of an adjunct culture that produces a premium quality of Cheddar cheese will thus be useful to the industry.

Glycolysis also influences the flavour of Cheddar cheese. It involves the conversion of lactose to lactic acid mainly by starter bacteria, reducing the pH of typical Cheddar to 5.1 – 5.4. During ripening, residual lactose (0.8-1.5%) is metabolized to predominantly L-lactate. Oxidation of lactate by starter bacteria, NSLAB or probiotic adjunct in general

*A version of this chapter has been published. Ong, L., Henriksson, A., & Shah, N.P. (2007b). Chemical analysis and sensory evaluation of Cheddar cheeses produced with *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* sp. *International Dairy Journal*, *17*, 937 - 945.

produces 1 mole of acetate and 1 mole of CO_2 and consumes 1 mole of O_2 per mole of lactate utilized (Fox et al., 1993). Acetate may also be produced by starter bacteria or probiotic adjunct such as *Lactobacillus* and *Bifidobacterium* from lactose or citrate or amino acid and is usually present at fairly high concentrations in Cheddar cheeses (Fox & McSweeney, 1996). Acetate is considered to contribute to cheese flavour, although high concentrations may cause off-flavours. When *Bifidobacterium lactis* was used in combination with *Lactobacillus acidophilus* strain Ki as the starter in Gouda cheese manufacture (Gomes et al., 1995), there was a significant effect on cheese flavour after 9 wk of ripening, possibly due to the production of acetic acid by the *Bifidobacterium*. Production of acetic acid in cheeses especially with the addition of probiotic adjunct thus requires a careful examination.

In our previous study (Chapters 3.0 & 4.0), six probiotic organisms (*B. longum* 1941, *Lb. casei* 279, *Lb acidophilus* 4962, *B. lactis* LAFTI®B94[*], *Lb. paracasei* LAFTI®L26[†] and *Lb. acidophilus* LAFTI®L10) were used for the development of probiotic Cheddar cheeses. These organisms have been selected based on their acid and bile tolerance, adhesion to intestinal cell line, anticarcinogenic properties, oxygen sensitivity and ability to modify gut microflora of human subjects (Lankaputhra & Shah, 1998; McIntosh et al., 1999; Crittenden et al., 2001). These strains were shown to maintain their viability at high level of > 7.0 $\log_{10}$ cfu g^{-1} at the end of ripening period of 24 wk at 4°C. Addition of probiotic adjuncts also changed the proteolytic pattern and the organic acid profiles of the cheeses. The objective of this study was to investigate the influence of *B. longum* 1941, *Lb. casei* 279, *Lb acidophilus* 4962, *B. lactis* LAFTI®B94, *Lb. paracasei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 added individually and in combination, on sensory properties of cheddar cheeses after ripening for 9 months at 4°C as affected by acetic acid production and proteolysis.

[*] *B. lactis* LAFTI®B94 has been reclassified as *B. animalis* subsp. *lactis* LAFTI®B94
[†] *Lb. paracasei* LAFTI®L26 has been reclassified as *Lb. casei* LAFTI®L26

5.2. Materials and Methods

5.2.1. ***Cheddar cheese samples***

Cheddar cheeses were made with 10 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) using a pair of custom made cheese vats. Three batches of Cheddar cheeses including a control cheese with only starter lactococci (Batch 1M), and probiotic cheeses produced with starter lactococci and mixture of *Lactobacillus acidophilus* 4962, *Lb. casei* 279 and *Bifidobacterium longum* 1941 (Batch 2M) or *Lb. acidophilus* LAFTI®L10, *Lb. paracasei* LAFTI®L26 and *B. lactis* LAFTI®B94 (Batch 3M) as described in Chapter 3.0 (section 3.2.2) were evaluated in a series of sensory evaluation. Seven batches of Cheddar cheeses including a control cheese with starter lactococci only (Batch 1) and six probiotic cheeses with starter lactococci and probiotic adjuncts (Batches 1-7) as described in Chapter 4.0 (Table 4.1) were also evaluated.

The cheeses were manufactured in triplicates according to the standard procedures of Kosikowski (1977) as described in sections 3.2.2 and 4.2.2 and ripened for 9 months at 4^{o}C. The chemical analyses of the cheeses during the first 24 wk of ripening is reported in Chapters 3.0 and 4.0. There was a delay in obtaining ethic approval of the protocol for sensory evaluation. The ethics committee take longer than it was anticipated. Hence sensory evaluation was carried out on the 9 months sample. The results of the chemical analysis after prolonged ripening (at 9 months) and their correlation with the sensory results are reported in this Chapter.

5.2.2. ***Sensory evaluation of cheeses***

Staff and students of Victoria University were recruited as the sensory panelists for a series of triangle tests (n = 36), acceptance rating test (n = 30) and evaluation for specific attributes (n = 30). All panelists signed a Victoria University human subject's consent form (Appendix A.1). The panelists were familiar with basic sensory evaluation techniques and prior to sensory evaluation they participated in briefing sessions. Panelists had access to deionized water and unsalted soda crackers to help cleanse their palates.

For sensory evaluation, cheese samples were removed from the refrigerator and cut into pieces (about 1.5 x 1.5 x 1.5 cm in size) and placed on white plates coded with a random 3-digit numbers one hour prior to evaluation at room temperature (25^{o}C). Cheese

cubes from the three replications of the same batch were mixed randomly so that all replications from the same batch were presented equal number of times.

For the triangle test (Appendix A.6), panelists were asked to choose the odd cheese in a series of eight triangle tests conducted in two days (4 combinations each day). The probiotic cheeses were presented against the control cheese to find out whether there were any significant differences between the control and the probiotic cheeses. The cheeses were presented as AAB, ABA, BAA, BBA, BAB and ABB, where 'A' was probiotic cheese and 'B' was control cheese and each combination was presented an equal number of times. Significant differences were determined using the method of Roessler et al. (1978).

For acceptance rating test (Appendix A.5), panelists were instructed to evaluate their perception of the overall liking of the cheeses on a 10-point intensity scale (1 = dislike extremely, 10 = like extremely). Prior to tasting, panelists completed a questionnaire on frequency of cheese consumption (<1 once per wk, 2-3 times per wk, 4-5 times per wk, or > 5 times per wk) and cheese preference (mild, medium or sharp matured cheese) (Appendix A.4). Panelists evaluated all cheeses in two days (five at each session), with a control cheese included as a reference in each session. The manner in which the treatment combinations were divided between the sessions and the order in which the cheeses were presented was randomized to minimize the carryover effects (Muir & Hunter, 1991). Panelists were instructed to cleanse their palates before proceeding to the next sample.

Panelists evaluated specific attributes, which included Cheddary flavour, bitterness, sour-acid, vinegary, creamy, hardness and crumbliness using a 10-point intensity scale (Appendix A.7). Panelists also evaluated all cheeses in two sessions (five at each session including a control at each session), as described previously. A wide range of descriptive vocabulary (attributes) for Cheddar flavour has been defined by Delahunty and Murray (1997) and Murray and Delahunty (2000). Drake et al. (1996) selected six specific attributes (bitter, sour-acid, oaky/nutty, creamy, firmness and crumbliness for the sensory evaluation of Cheddar cheeses made with the addition of adjunct lactobacilli. In our study similar terms were used with some modifications. Vinegary attribute was added to the attribute lists to determine the correlation between acetic acid concentrations obtained from instrumental analysis and human sensory perception of vinegary taste of the cheeses. Panelists received 3 main sessions of training (3 days) prior to sensory evaluation. In the first session panelists were trained for their ability to detect sour-acid taste, bitterness and vinegary taste of different concentration of lactic acid, caffeine and acetic acid in water. Detection threshold was determined using a series of duo-trio tests (Appendix A.2). In the second session, panelists were trained to recognize the oaky/nutty flavour of Cheddar cheese by using three

different type of commercial cheese ("mild", "tasty" and "vintage" cheeses). The attribute oaky/nutty, which corresponded to the flavour characteristic of premium quality Cheddar cheese (Bodyfelt et al., 1988), was changed to "Cheddary", which was defined as the general flavours of Cheddar cheese (Hulin-Bertaud et al., 2000). Panelists were instructed to rate the Cheddary intensity using 1 to 10 scale (10 = highest intensity, 1 = lowest intensity) of three different types of commercial Cheddar cheeses ("mild" aged 3 months, "tasty" aged 9 months and "vintage" aged > 12 months). In the third session, panelists were trained for their ability to detect sour-acid taste, bitterness and vinegary taste of different concentration of lactic acid, caffeine and acetic acid in a cream cheese. Detection threshold was determined using a series of duo-trio tests. Panelists were also trained for their ability to rank products with different concentration of lactic acid, acetic acid and caffeine from lowest intensity to highest intensity in water and in cream cheese (Appendix A.3). Repeated testing was performed until panelists were competent to rank different intensity of lactic acid, acetic acid and caffeine both in water and in cream cheese.

5.2.3. ***Cheese composition***

The composition of the cheeses including the salt, fat, moisture, protein contents and pH of the cheeses after ripening at 4^{o}C for 9 months were determined according to the procedure described in section 3.2.3.

5.2.4. ***Acetic acid concentration***

The concentrations of acetic acid after ripening at 4^{o}C for 9 months were determined using high performance liquid chromatography (HPLC) as described in section 3.2.5.

5.2.5. ***Assessment of proteolysis***

The water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) of the cheeses were determined as per the method described in section 3.2.6. The proteolytic patterns of Cheddar cheeses were also analyzed by assessing the percentage hydrolysis of α_s-casein and β-casein using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE) as described in section 3.2.6.

5.2.6. ***Statistical analysis (data treatment)***

Data analysis was carried out with Minitab statistical package (Minitab Inc, State College, PA, USA). One-way analysis of variance was used to find out differences between means, with a significant level at $\alpha = 0.05$. When significant differences were found among treatments, means were compared using Tukey's test. The significance in the differences of data obtained from the triangle test was determined using the expanded statistical tables of Roessler et al. (1978). Simple linear correlation analysis was used to determine a relationship between mean scores of sensory attributes and that of chemical analysis.

5.3. Results and discussions

The results of the sensory analysis (n = 36) based on a triangle test to differentiate between control and probiotic Cheddar cheese are shown in Table 5.1. Batches 2 to 8 were presented against control cheese Batch 1. Batches 2M and 3M were presented against control cheese Batch 1M. There was a significant difference ($P < 0.05$) between probiotic cheeses made using *B. longum* 1941, *B. lactis* LAFTI®B94, *Lb. casei* 279, *Lb. paracasei* LAFTI®L26, *Lb. acidophilus* LAFTI®L10 and the control cheese. Only the cheese made using *Lb. acidophilus* 4962 was similar to the control cheese ($P > 0.05$). Triangle test results also show that the control cheese Batch 1M (Chapter 3.0) and control cheese Batch 1 (Chapter 4.0) were not significantly different (4 no of correct judgements; n = 36; $P < 0.05$). Batches 1 and 1M were produced using similar methods (section 3.2.2) and had only 2 wk differences in ripening age. The same amount of cheeses from Batch 1M and Batch 1 were thus mixed and used as the control cheese for acceptance and specific attributes scorring test.

Addition of *Lactobacillus* adjuncts has been reported previously to improve Cheddar cheese flavour (Broome et al., 1990; McSweeney et al., 1994), but in other case they were responsible for flavour defects (Lee et al., 1990). Acceptability of the control and probiotic cheeses in our study is shown in Table 5.2. There was no significant difference ($P < 0.05$) between acceptance scores of control cheeses presented at sensory sessions day 1 and day 2. Acceptance scores of other treatments presented at day 1 and day 2 could then be compared. The acceptance scores of cheeses with the addition of probiotic adjuncts were not significantly different ($P > 0.05$) to the control except for cheeses made with the addition of *Lb. casei* (Batch 4) and mixture of ABC culture (*Lb. acidophilus* 4962, *B. longum* 1941, *Lb. casei* 279) (Batch 2M), which received the lowest acceptance scores. The results thus show

that Cheddar cheeses made with probiotic adjuncts *B. longum* 1941, *B. lactis* LAFTI®B94, *Lb. paracasei* LAFTI®L26 or *Lb. acidophilus* LAFTI®L10 received acceptance scores comparable to that of control cheese. Results from the questionnaire form completed by the panelists showed that the type of cheese preferred, sex, age and frequency of cheese consumption did not affect the acceptance scores ($P > 0.05$). About 80% of panellists who participated for the acceptance rating consumed cheese at 1-2 times per wk or more, 70% were female, 73% were between the ages of 18 and 35 years and 53% preferred mild cheeses.

Scores for specific attributes of the Cheddar cheeses are shown in Table 5.3. Out of the seven attributes assessed, creamy, sour-acid, vinegary, bitterness and hardness of the cheeses were significantly different among various cheeses ($P < 0.05$). Cheeses with the addition of probiotic adjuncts received higher scores for bitterness, sour-acid taste and vinegary taste when compared to those without probiotic. Bitterness scores in cheese with *Lb. casei* 279 (Batch 4) and *Lb. paracasei* LAFTI®L26 (Batch 5) were significantly higher ($P < 0.05$) than those for the control cheese (Batch 1). Table 5.4 shows that there was a negative and significant correlation between the bitterness scores and the acceptability of the cheeses ($r = -0.809$, $P= 0.008$). The resulting bitter off-flavours have probably masked the liberated aroma component in the cheeses and may explain the reason for lower acceptance scores obtained. These results supported an earlier observation of El Soda et al. (2000), who reported that addition of lactobacilli enhanced bitterness when incorporated into Cheddar cheese. In that study, the enhanced bitterness was related to the complex peptidases system of the lactobacilli used. Table 5.4 also shows that the acceptance scores were influenced by the sour-acidic scores ($r = -0.858$, $P = 0.003$). Higher levels of bitterness and development of more intense sour-acid flavour in Cheddar cheeses containing adjunct cultures of Lactobacilli have been reported by Lee et al. (1990) and Lynch et al. (1999). Lynch et al. (1999), however found that the bitterness scores were only higher at the early stage of ripening (up to 6 months); thereafter, the bitterness scores for the cheeses with adjunct lactobacilli were similar to those without adjunct lactobacilli. Both bitterness and the sour-acid taste of the cheeses needed to be controlled in order to produce probiotic cheeses with acceptable quality. Although there was a significant correlation between Cheddary and acceptances scores, ANOVA showed that the Cheddary scores were not significantly different ($P > 0.05$) among various cheeses (Table 5.3).

5.3.1. *Relationship between compositional variables of Cheddar cheeses and their sensory characteristic*

The results of the composition (percentage moisture, fat, protein, salt and pH), degree of proteolysis (percentage of WSN/TN, TCA-SN/TN, PTA-SN/TN, hydrolysis of α_{s1}-casein and β-casein) and acetic acid concentration of Cheddar cheeses after ripening at 4^{o}C for 9 months are shown in Table 5.5. Simple linear correlation between the mean scores of the specific attributes and the chemical analysis is shown in Table 5.6. No significant differences ($P > 0.05$) were observed among the experimental cheeses for the moisture, salt, fat and protein content, which confirmed our previous findings that addition of probiotic microorganisms has no direct effect on cheese composition (Chapter 3.0, section 3.3.1 & Chapter 4.0, section 4.3.1). The relationship between the main compositional variables (protein, fat, salt, moisture and pH) of the cheeses and their sensory characteristics were investigated (Table 5.6). There were no significant correlations between the composition variables (protein, salt and moisture content) of the cheeses and sensory attributes scores. The fat content of the cheeses tended to show positive but not significant correlation with the creamy scores of the cheeses (r = 0.627, $P = 0.071$). There were, however, negative and significant correlation between the fat content and mean scores for hardness (r = -0.731, $P = 0.025$). The pH of probiotic cheeses was in general lower than that of control cheese, but this did not influence the sour-acid scores of the cheeses. It is interesting to note, however, that vinegary scores were negatively and significantly correlated to the pH of the cheeses (r = -0.830, $P = 0.006$) (Table 5.6).

5.3.2. *Relationship between acetic acid concentration and sensory attributes of Cheddar cheeses*

Concentrations of acetic acid in cheese with *Bifidobacterium* sp. (Batch 2 & 3), *Lb. casei* (Batch 4) and *Lb. paracasei* (Batch 5) after ripening at 4^{o}C for 9 months were significantly higher ($P < 0.05$) as compared to the control cheese (Table 5.5). *Lb. casei* is known to produce acetic acid (Shihata & Shah, 2000). On the other hand, *Bifidobacterium* sp. produced acetic acid and lactic acid from lactose via a fructose-6-phosphate shunt pathway (Dinakar & Mistry, 1994) as described in Chapter 3.0 section 3.3.4.

The level of acetic acid in the Cheddar cheeses was assessed during sensory evaluation as the level of vinegary taste as shown in Table 5.3. During the course of training, panelists' average threshold of acetic acid was determined using a series of duo-

trio tests. It was found that their acetic acid threshold in water was less than 0.006% (v/v). The vinegary taste, however, was more difficult to be detected in the presence of other components such as fat, protein, acid and salt. When different concentrations of acetic acid were mixed into a product such as cream cheese, the detection threshold increased more than ten fold to 0.06% - 0.100% (v/w). The detection thresholds both in water and in cream cheese, however, were much lower than the concentration of acetic acid in experimental Cheddar cheeses (Table 5.5).

Production and accumulation of acetic acid in the probiotic cheeses were reflected in the sensory scores. Although all cheeses with adjuncts probiotic organisms received higher vinegary attribute scores (Table 5.3), there was no significant correlation between vinegary and acceptability scores (Table 5.4). Similarly, there was no significant correlation between the mean scores of vinegary attribute and acetic acid concentration of the cheeses ($r = 0.271$, $P = 0.481$; Table 5.6). The result indicated that, although panelists were able to detect the presence of acetic acid in the probiotic Cheddar cheeses, they were unable to detect the differences in the concentration of acetic acid between different batches of cheeses. Results also indicated that the vinegary flavour of the cheeses in this study was still within the acceptable range.

5.3.3. *Relationship between proteolytic pattern of Cheddar cheeses and their sensory characteristics*

Higher levels of proteolysis were detected in probiotic cheeses after 9 months of ripening at 4^{o}C as indicated by the release of more water-, TCA- and PTA-soluble peptides in the probiotic cheeses (Table 5.5). The soluble nitrogenous compounds provided by casein proteolysis contribute directly to cheese flavour and texture (Fox et al., 1993). These nitrogen fractions generally increased as cheeses aged, corresponding to the continued breakdown of casein and large peptides into small peptides and amino acids by the action of starter culture enzymes and residual rennet (Lau et al., 1991). The levels of proteolysis was particularly high in cheeses with the addition of *Lb. casei* 279 (Batch 4) and *Lb. paracasei* LAFTI®L26 (Batch 5), because both strains have considerably higher proteolytic activity (Chapter 4.0, section 4.2.5). At the end of the 9 months ripening period at 4^{o}C, percentage hydrolysis of α_{s1}- CN in all probiotic cheeses except *B. longum* 1941 (Batch 3) cheese was significantly higher ($P < 0.05$) than control cheeses.

The increased proteolysis in probiotic cheeses showed that the cheeses ripened faster than controls. The results were expected to influence the Cheddary scores of the

cheeses, but Table 5.3 shows that the Cheddary scores were not significantly different between batches. The scores of commercial cheeses used during the training session included 3.11 ± 0.54, 5.60 ± 0.41 and 8.90 ± 0.62 for "mild", "tasty" and "vintage" cheeses, respectively. The average scores of Cheddary attributes as shown in Table 5.3 indicated that the experimental cheeses were comparable to the commercial "tasty" cheese. The findings also show that the probiotic adjuncts used in this study did not increase the level of perceived maturity. The control cheese, despite having lower WSN, TCA-SN and PTA-SN had a Cheddary score comparable to the probiotic cheeses (Table 5.3).

Previously, excessive proteolysis was associated with the increase in bitterness (Lee et al., 1990). Table 5.6 shows that there was a positive and significant correlation between the scores of bitterness and the level of WSN ($r = 0.755$, $P = 0.046$). According to Lemieux and Simard (1992), bitterness develops when small to medium-size hydrophobic peptides produced by the coagulant and microbial enzymes accumulate to the levels that exceed desirable taste thresholds. Residue 193-209 of the C-terminal region of β-casein and residue 1-9 of the C-terminal region of α_{s1}-casein have been associated with bitterness in cheese (Lemieux & Simard, 1992). Reverse phase - HPLC chromatograms of the Cheddar cheeses in our study indicated the presence of β-casein (f 193-209) and α_{s1}-casein (f 1-9) (Chapter 6.0, section 6.3.4). Broadbent et al. (2002) performed regression analysis of bitter flavour scores from the trained sensory panel and concentration of β-casein (f 193-209) and α_{s1}-casein (f 1-9) and found that these peptides had positive correlation with bitterness. Hydrolysis of these peptides is associated with decreased bitterness in Cheddar cheese. The total area of the peak of these peptides during ripening is discussed in Chapter 6.0 section 6.3.5.

A level of salt in moisture (S/M) > 4.5% is necessary to prevent the development of bitterness in cheese (Mistry & Kasperson, 1998). These ratios reflect the amount of free water available for microbial growth. A lower level of S/M and high water activities allow excessive bacterial growth, promote excessive proteolysis and lipolysis and in turn lead to defective body, texture (open, soft, greasy), flavour (unclean, bitter) and consumer unacceptability (Beresford & Williams, 2004).

The moisture content of the experimental cheeses was slightly higher than average moisture content of commercial cheeses. The higher moisture content resulted in the lowering of the percentage of S/M. Although there was no significant difference ($P > 0.05$) between the salt content of the cheeses, some cheeses such as those with *Lb. casei* 279 (Batch 4) and *Lb. paracasei* LAFTI®L26 (Batch 5), had only about 4.3% S/M. The

percentage of S/M would probably be one of the factors that contribute to the increase in bitterness.

The most important contributor to the continuous casein matrix of a Cheddar cheese is α_{s1}-CN (Lawrence et al., 1987), and hydrolysis of this casein is believed to be responsible for the softening of Cheddar cheese texture (Grappin et al., 1985). Cheese with the addition of *B. longum* 1941 (Batch 2), *Lb. casei* 279 (Batch 4) and *Lb. paracasei* LAFTI®L26 (Batch 5) received significantly lower hardness scores than the control (Table 5.3). The scores however did not influence the acceptability of the cheeses (Table 5.4) and no correlation was observed between the degree of proteolysis (percentage of WSN/TN, TCA-SN/TN, PTA-SN/TN) and panelists' scores for hardness (Table 5.6).

5.4. Conclusions

Sensory panelists perceived all probiotic cheeses except that with *Lb. acidophilus* 4962 to be significantly different than the control cheese without probiotic. Acceptability of probiotic cheese with *Lb. casei* 279 was significantly lower than that of the control cheese with bitterness and sour-acid taste as the major defects. Concentration of acetic acid in cheeses with *Bifidobacterium* sp., *Lb. casei* 279 and *Lb. paracasei* LAFTI®L26 was significantly higher than the control cheese. Vinegary scores, however, did not significantly influence the acceptability of the cheeses and panelists were not able to detect the various concentrations of acetic acid between the different batches of cheese. Concentrations of soluble nitrogen and hydrolysis of casein were higher in probiotic cheeses, but increased proteolysis did not significantly influence the Cheddary attribute scores of the cheeses. There was, however, a positive and significant correlation between the scores of bitterness and the level of water-soluble nitrogen. Although there was no significant correlation between the composition variables (protein, salt and moisture content) and scores of sensory attributes, cheeses with the lowest percentage of S/M received the highest bitterness scores. Some strains used in the study have potential for use in the production of probiotic Cheddar cheese with acceptable quality.

Table 5.1. Differences between control and probiotic cheeses as perceived by triangle test (n = 36) after 9 months of ripening at 4°C

Cheddar cheese[1]	No. of correct judgments	P[2]
Batch 2	22	< 0.05
Batch 3	20	< 0.05
Batch 4	26	< 0.05
Batch 5	18	< 0.05
Batch 6	17	> 0.05
Batch 7	23	< 0.05
Batch 2M	25	< 0.05
Batch 3M	20	< 0.05

[1]Batch codes used are as detailed in section 5.2.1. Probiotic cheeses, Batches 2 to 8 were presented against control cheese Batch 1. Batches 2M and 3M were presented against control cheese Batch 1M.
[2]Probiotic cheeses are significantly different with control cheese if $P < 0.05$ (95% confidence level). Significance of triangle tests was determined using the expanded statistical tables of Roessler et al. (1978).

Table 5.2. Mean overall acceptance of control and probiotic Cheddar cheeses after 9 months of ripening at 4°C (n = 30)

Cheddar cheese[1]	Mean overall acceptance
Control cheese	6.35 ± 0.47[a]
Batch 2	6.33 ± 0.42[a]
Batch 3	4.93 ± 0.45[ab]
Batch 4	3.59 ± 0.38[c]
Batch 5	5.03 ± 0.38[ab]
Batch 6	5.60 ± 0.40[ab]
Batch 7	5.10 ± 0.35[ab]
Batch 2M	4.33 ± 0.40[bc]
Batch 3M	5.40 ± 0.29[ab]

[1] Batch codes used are as detailed in section 5.2.1.

[abc] Means in a column followed by the same letter are not significantly different ($P > 0.05$). Results are expressed as mean of scores ± standard error of means. 1 = dislike extremely, 10 = like extremely.

Table 5.3. Scores obtained from judging specific attributes of control and probiotic Cheddar cheeses after 9 months of ripening at 4°C (n = 30)

Sensory attributes	Cheddar cheeses[1]									ANOVA[2]	
	Control cheese	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 2M	Batch 3M	F	P
Flavour											
Cheddary	5.94 ± 0.34^{a}	5.93 ± 0.35^{a}	5.40 ± 0.33^{a}	5.13 ± 0.42^{a}	5.57 ± 0.38^{a}	5.40 ± 0.43^{a}	5.40 ± 0.44^{a}	5.72 ± 0.45^{a}	5.57 ± 0.32^{a}	0.57	0.82
Creamy	5.26 ± 0.38^{a}	6.20 ± 0.36^{ab}	5.73 ± 0.37^{ab}	6.07 ± 0.35^{ab}	6.57 ± 0.32^{b}	6.00 ± 0.39^{ab}	5.10 ± 0.31^{ab}	5.00 ± 0.41^{ab}	5.53 ± 0.31^{ab}	2.34	**0.02**
Sour-acidic	3.23 ± 0.42^{ab}	3.70 ± 0.45^{ab}	4.93 ± 0.46^{ab}	5.33 ± 0.53^{b}	4.03 ± 0.47^{ab}	4.03 ± 0.49^{ab}	4.80 ± 0.36^{ab}	4.30 ± 0.42^{ab}	3.87 ± 0.41^{ab}	2.32	**0.02**
Vinegary	2.44 ± 0.39^{a}	3.47 ± 0.50^{ab}	3.67 ± 0.53^{ab}	4.23 ± 0.52^{b}	3.40 ± 0.51^{ab}	3.63 ± 0.46^{ab}	4.20 ± 0.53^{b}	5.00 ± 0.41^{b}	4.17 ± 0.41^{ab}	3.06	**0.00**
Bitter	3.84 ± 0.63^{a}	3.73 ± 0.47^{a}	5.57 ± 0.54^{ab}	6.83 ± 0.50^{b}	6.37 ± 0.42^{bc}	4.27 ± 0.53^{ab}	4.67 ± 0.38^{ab}	4.83 ± 0.51^{ab}	4.53 ± 0.51^{ac}	4.95	**0.00**
Texture											
Hardness	5.52 ± 0.49^{a}	4.27 ± 0.47^{b}	4.97 ± 0.41^{ab}	4.40 ± 0.44^{bc}	3.77 ± 0.41^{b}	5.80 ± 0.46^{ac}	5.67 ± 0.37^{abc}	5..27 ± 0.53^{abc}	5.57 ± 0.39^{abc}	4.20	**0.00**
Crumbliness	5.24 ± 0.60^{a}	5.03 ± 0.58^{a}	5.03 ± 0.51^{a}	4.43 ± 0.58^{a}	4.47 ± 0.56^{a}	5.30 ± 0.53^{a}	5.57 ± 0.44^{a}	6.33 ± 0.53^{a}	5.97 ± 0.62^{a}	1.11	0.35

[1] Batch codes used are as detailed in section 5.2.1.
[2] One way analysis of variance of means. ^{abc} Means in a row followed by the same letter are not significantly different ($P > 0.05$). Results are expressed as mean of scores ± standard error of means. (1 = low intensity, 10 = high intensity).

Table 5.4. Correlation between mean scores of sensory attributes and the mean scores of cheeses acceptability

Sensory Attributes	Correlation between mean scores of sensory attributes (r/ *P*)[1]						
	Acceptance	Cheddary	Creamy	Sour-acidic	Vinegary	Bitter	Hardness
Cheddary	**0.715** **0.030**						
Creamy	0.136 0.728	-0.069 0.860					
Sour-acidic	**-0.858** **0.003**	-0.547 0.128	-0.181 0.640				
Vinegary	-0.603 0.086	-0.504 0.166	-0.554 0.122	0.592 0.093			
Bitter	**-0.809** **0.008**	**-0.673** **0.047**	0.361 0.339	**0.704** **0.034**	0.124 0.750		
Hardness	0.119 0.761	-0.089 0.820	**-0.757** **0.018**	-0.221 0.568	0.360 0.341	-0.524 0.148	
Crumbliness	0.046 0.907	0.189 0.626	**-0.853** **0.003**	-0.010 0.980	0.605 0.084	-0.531 0.141	**0.718** **0.029**

[1]Correlation between mean scores of sensory attributes was expressed as r. There was significant correlation between sensory attributes if $P < 0.05$ (95% confidence level).

Table 5.5. Influence of probiotic microorganisms on composition and proteolytic profiles of Cheddar cheeses after 9 months of ripening at 4°C

Cheddar cheeses[1]	Composition and proteolytic profiles[2]										
	Moisture %	Salt %	Fat %	Protein %	pH	Acetic acid %	WSN[3] %	TCA-SN[3] %	PTA-SN[3] %	α_{s1} – CN[4] %	β – CN[4] %
Control cheese	38.50 ± 0.65^{a}	1.88 ± 0.06^{a}	31.60 ± 0.65^{a}	27.56 ± 0.81^{a}	5.19 ± 0.09^{a}	0.15 ± 0.01^{a}	35.78 ± 1.04^{a}	16.83 ± 0.50^{a}	4.49 ± 0.42^{a}	27.07 ± 3.31^{a}	12.86 ± 0.65^{a}
Batch 2	40.10 ± 0.71^{a}	1.85 ± 0.04^{a}	32.90 ± 0.68^{a}	26.55 ± 0.95^{a}	5.15 ± 0.18^{a}	0.30 ± 0.05^{b}	49.21 ± 2.08^{bc}	25.82 ± 0.98^{b}	9.24 ± 0.50^{b}	31.55 ± 1.96^{a}	17.12 ± 2.63^{a}
Batch 3	40.85 ± 1.15^{a}	1.91 ± 0.03^{a}	31.15 ± 0.90^{a}	28.51 ± 0.88^{a}	5.14 ± 0.12^{a}	0.26 ± 0.01^{b}	49.40 ± 1.80^{bc}	21.18 ± 1.80^{ab}	7.12 ± 0.31^{bc}	28.08 ± 1.47^{a}	12.78 ± 1.33^{a}
Batch 4	39.20 ± 0.88^{a}	1.72 ± 0.04^{a}	32.35 ± 0.42^{a}	26.80 ± 0.65^{a}	5.11 ± 0.08^{a}	0.25 ± 0.01^{ab}	58.47 ± 2.25^{b}	23.79 ± 1.55^{b}	7.91 ± 0.55^{bc}	62.94 ± 1.20^{b}	16.05 ± 0.25^{b}
Batch 5	38.75 ± 0.98^{a}	1.71 ± 0.06^{a}	32.70 ± 0.89^{a}	26.85 ± 0.74^{a}	5.12 ± 0.14^{a}	0.25 ± 0.01^{ab}	55.93 ± 1.95^{bc}	19.45 ± 1.50^{ab}	6.80 ± 0.15^{ac}	75.38 ± 4.20^{b}	13.79 ± 0.22^{a}
Batch 6	41.20 ± 0.90^{a}	1.85 ± 0.05^{a}	31.50 ± 0.56^{a}	26.30 ± 0.86^{a}	5.09 ± 0.13^{a}	0.20 ± 0.01^{ab}	52.98 ± 3.04^{bc}	24.71 ± 1.89^{b}	8.11 ± 0.32^{bc}	45.45 ± 4.30^{c}	15.23 ± 1.16^{a}
Batch 7	39.70 ± 1.20^{a}	1.95 ± 0.07^{a}	30.75 ± 0.85^{a}	26.55 ± 0.74^{a}	5.14 ± 0.14^{a}	0.18 ± 0.01^{ab}	53.05 ± 3.10^{bc}	20.98 ± 1.42^{ab}	6.61 ± 0.45^{ac}	47.17 ± 1.22^{c}	12.24 ± 2.29^{a}
Batch 2M	38.62 ± 1.10^{a}	1.76 ± 0.04^{a}	31.20 ± 0.76^{a}	27.41 ± 0.80^{a}	5.07 ± 0.11^{a}	0.23 ± 0.01^{ab}	47.15 ± 0.61^{c}	22.66 ± 0.90^{ab}	7.99 ± 0.57^{bc}	65.08 ± 3.00^{b}	14.30 ± 3.18^{a}
Batch 3M	38.85 ± 0.89^{a}	1.85 ± 0.06^{a}	31.96 ± 0.84^{a}	27.67 ± 0.77^{a}	5.11 ± 0.08^{a}	0.23 ± 0.01^{ab}	45.32 ± 3.01^{ac}	22.17 ± 0.95^{ab}	8.13 ± 0.45^{bc}	51.50 ± 1.77^{c}	13.07 ± 1.03^{a}

[1] Batch codes used are as detailed in section 5.2.1.
[2] Results are expressed as mean ± standard error of means; n = 3 sets of data analyzed in duplicate. ^{abc} Means in column with like superscripts do not differ ($P > 0.05$).
[3] The concentration of soluble nitrogen is presented as percentage over total nitrogen. WSN = water soluble nitrogen, TCA-SN = trichloroacetic acid soluble nitrogen, PTA-SN = phosphotungstic acid soluble nitrogen.
[4] Percentage hydrolysis of casein.

Table 5.6. Correlation between results of chemical analysis and mean scores of sensory attributes

Sensory Attributes	Correlation between results of chemical analysis and mean scores of sensory attributes (r/ *P*)[1]										
	Moisture	Salt	Fat	Protein	pH	Acetic acid	WSN[2]	TCA-SN[2]	PTA-SN[2]	α_s – CN[2]	β – CN[2]
Flavour											
Cheddary	-0.391	-0.116	0.030	0.160	0.328	-0.138	**-0.807**	-0.335	-0.296	-0.414	-0.014
	0.298	0.776	0.939	0.682	0.388	0.722	**0.009**	0.379	0.439	0.268	0.972
Creamy	0.111	-0.185	0.627	-0.475	0.247	0.446	0.425	0.142	**-0.757**	0.116	0.530
	0.776	0.633	0.071	0.196	0.521	0.229	0.254	0.716	**0.018**	0.766	0.142
Sour-acidic	-0.100	-0.389	0.120	0.335	-0.139	0.388	-0.515	0.121	0.218	-0.368	-0.003
	0.798	0.301	0.759	0.378	0.721	0.302	0.156	0.756	0.574	0.342	0.995
Vinegary	0.086	0.055	-0.137	0.055	**-0.830**	0.271	0.376	0.579	0.618	0.369	0.124
	0.825	0.888	0.724	0.888	**0.006**	0.481	0.318	0.102	0.076	0.328	0.750
Bitter	-0.124	-0.469	0.295	0.044	0.241	0.240	**0.755**	-0.110	-0.033	0.627	-0.003
	0.751	0.202	0.441	0.910	0.531	0.534	**0.046**	0.777	0.932	0.071	0.994
Texture											
Hardness	0.340	0.533	**-0.731**	0.221	-0.453	-0.594	-0.329	0.035	-0.078	-0.291	-0.425
	0.374	0.140	**0.025**	0.568	0.221	0.091	0.388	0.930	0.843	0.448	0.255
Crumbliness	-0.060	0.155	-0.529	0.418	-0.611	-0.192	-0.385	0.128	0.201	-0.022	-0.293
	0.878	0.690	0.143	0.263	0.081	0.621	0.306	0.742	0.605	0.955	0.444
Acceptance	0.119	0.355	-0.072	-0.094	0.189	-0.136	-0.564	-0.100	-0.128	-0.648	-0.012
	0.761	0.348	0.854	0.809	0.627	0.728	0.114	0.798	0.742	0.059	0.976

[1]Correlation between mean scores of sensory attributes and chemical analysis was expressed as r. There is significant correlation between sensory attributes scores and results of chemical analysis if $P < 0.05$ (95% confidence level).

[2] WSN = water soluble nitrogen, TCA-SN = trichloroacetic acid soluble nitrogen, PTA-SN = phosphotungstic acid soluble nitrogen, CN = casein.

6.0 Isolation and purification of angiotensin converting enzyme-inhibitory peptides from probiotic Cheddar cheeses*

6.1. Introduction

Milk protein is a rich source of biologically active peptides such as antihypertensive, antithrombotic, opioid, immunostimulating, antimicrobial, mineral carrying and cholesterol-lowering peptides (Shah, 2000c). Most of these peptides are hidden in the inactive state in the original parent protein structure and may be released by proteolysis. Protease such as plasmin in milk can hydrolyze milk proteins during cheese ripening. Proteolytic enzymes from bacterial cultures may be responsible for the breakdown of protein into peptides and amino acids. Intracellular peptidases of lactic acid bacteria may also contribute to further degradation after cell lysis.

Angiotensin-I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) increases blood pressure by converting angiotensin-I to angiotensin-II, a potent vasoconstrictor and by degrading bradykinin, a vasodilatory peptide (Johnston, 1992). ACE inhibition results in an antihypertensive effect and may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity (Meisel, 1998). The first reported competitive inhibitor of ACE is the naturally occurring peptides in snake venom (Ondetti et al., 1971). Many other ACE inhibitors have been discovered from enzymatic hydrolysis of bovine caseins (CNs), plant and other food proteins (Okamoto et al., 1995).

Various studies have been reported on ACE-inhibitory peptides found in fermented milk products (Nakamura et al., 1995; Takano, 1998; Seppo et al., 2003). Nakamura et al. (1995) reported that two peptides with amino acid residues of Val-Pro-Pro and Ile-Pro-Pro, isolated from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, exhibited ACE inhibitory and antihypertensive activities. The concentrations of the peptides required to inhibit 50% of angiotensin-I-converting enzyme activity (IC_{50}) were 9 and 5 μmol L^{-1}, respectively. Several other ACE inhibitory peptides derived from β-CN including (f 6-14), (f 47-52) and (f 73-82) have been isolated from fermented milk with

*A version of this chapter has been published. Ong, L., Henriksson, A., & Shah, N.P. (2007c). Angiotensin converting enzyme-inhibitory activity of Cheddar cheeses made with the addition of probiotic *Lactobacillus casei* sp. *Lait, 87,* 149-165.

Lb. delbrueckii subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4 (Gobbetti et al., 2000). Peptides derived from α_{s1}-CN including (f 24-31) and (f 170-199) and those from β-CN such as (f 168-175), (f 183-190), (f 113-127), (f 193-210), (f 70-97), (f 191-210) and (f 16-91) by *Lb. helveticus* CP790 proteinase have shown to exhibit ACE inhibitory activities after oral administration to spontaneously hypertensive rats. Among those peptides, (f 43-69) of the β-CN showed the highest ACE inhibitory activity with IC_{50} of 4 μmol L^{-1} (Yamamoto et al., 1994).

Ripened-type cheeses contain numerous peptides that originate mainly from casein released as a result of proteolysis during ripening. Some ACE-inhibitory peptides have been isolated from several Italian Cheeses (Crescenza, Gorgonzola, Mozzarella and Italico) (Smacchi & Gobbetti, 1998) and other cheeses (Camembert, Edam, Gouda, Cheddar, Roquefort, Emmentaler and Parmesan) (Okamoto et al., 1995; Meisel et al., 1997; Ryahanen et al., 2001). The appearance of these bioactive peptides is influenced by proteolysis, but only to a certain degree. An α_{s1}-CN derived antihypertensive peptide isolated from Parmesan cheese at 6 month of ripening could not be found after 15 month (Addeo et al., 1992). Similarly the antihypertensive activity found in long-ripened Gouda cheese was half as much as that found in its medium-aged counterpart (Meisel et al., 1997). Consistent with these findings, Gomez-Ruiz et al. (2002) who studied the ACE-inhibitory peptides in Manchego cheese found the antihypertensive activity to decrease within the first 4 month, was maximum at 8 month of ripening and decreased again at 12 month. Following isolation from Gouda cheese, Saito et al. (2000) analyzed the structure of the antihypertensive peptides and concluded that the strongest depressive effect on the systolic blood pressure and the highest ACE-inhibitory capacity was associated with peptides found in 8 month old cheese. A fermented low-fat hard cheese produced with probiotic lactic acid bacteria was found to produce high amounts of ACE-inhibitory peptides derived from α_{s1}-CN during maturation (Ryahanen et al., 2001). The peptides emerged at the age of 3 month and their level remained stable for 6 month.

Chapters 3.0 & 4.0 reports the used of probiotic organisms including *Lb. casei* 279 or *Lb. casei* LAFTI®L26 as adjunct in the development of probiotic Cheddar cheeses. The probiotic organisms were able to maintain a high level of viability of > 7.0 $\log_{10}$ cfu·g^{-1} at the end of ripening period of 6 month at 4ºC with minimal effect on the cheese composition. Addition of the probiotic adjuncts also changed the proteolytic pattern and more peptides were released into probiotic cheeses. The objective of the present report was to study the influence of probiotic adjuncts on the ACE-inhibitory activity of the cheeses during ripening and to isolate, purify and identify the ACE-inhibitory peptides.

6.2. Materials and Methods

6.2.1. ***Starter and probiotic organisms***

Cheese starter culture, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and probiotic organisms *B. longum* 1941, *Lb. casei* 279, *Lb. acidophilus* 4962, *B. lactis* LAFTI®B94, *Lb. paracasei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 were obtained from the culture collections mentioned in section 3.2.1. The organisms were activated and grown under the conditions described in section 3.2.1.

6.2.2. ***Cheddar cheeses and preparation of water soluble extract (WSE)***

Cheddar cheeses were made with 10 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) using a pair of custom made cheese vats. Three batches of Cheddar cheeses including a control cheese with only starter lactococci (Batch 1M), and probiotic cheeses made with starter lactococci and mixture of *Lb. acidophilus* 4962, *Lb. casei* 279 and *B. longum* 1941 (Batch 2M) or *Lb. acidophilus* LAFTI®L10, *Lb. paracasei* LAFTI®L26 and *B. lactis* LAFTI®B94 (Batch 3M) as described in Chapter 3.0 (section 3.2.2) and seven batches of Cheddar cheeses including a control cheese with starter lactococci only (Batch 1) and six probiotic cheeses with starter lactococci and probiotic adjuncts (Batches 1-7) as described in Chapter 4.0 (Table 4.1) were analyzed for the ACE-inhibitory activity.

The cheeses were manufactured in triplicates according to the standard procedures of Kosikowski (1977) as described in sections 3.2.2 and 4.2.2 and ripened for 9 months at 4°C. Cheese samples were collected at wk 1, 6, 12, 24 and 36. To obtain the WSE, the cheese (10 g) was homogenized in 10 mL of water with an ultraturrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at 10,000 x *g* for 2 min. The slurry was centrifuged (Sorvall, Newtown, CT, USA) for 20 min at 4000 x *g* and 4°C. The soluble fraction located between the upper layer (fat) and the precipitate (casein) was filtered through Whatman No. 41 filter paper. The extracts were further centrifuged at 4000 x *g* for 20 min at 4°C and filtered through glass wool to obtain the clear supernatant (WSE). The extracts were then concentrated by freeze drying (Dynavac FD300; Airvac Engineering Pty. Ltd., Rowville, Australia) at 20°C and –100 kPa for 72 hour. The freeze-dried WSE was then stored in vacuum at 20°C for analysis with reverse phase – high performance liquid chromatography (RP-HPLC) and for determination of ACE-inhibitory activity.

6.2.3. ***Reverse-phase HPLC of cheese WSE***

An aliquot of freeze-dried WSE of 40 mg was dissolved in 1 mL of solvent A (10% of acetonitrile; Merck, South Granville, NSW, Australia) containing 0.05% trifluoroacetic acid (TFA) solution (Sigma-Aldrich, St. Louis, MO, USA), centrifuged (14,000 x *g*, 10 min) using a bench top centrifuge (Sorvall RT7, Newtown, CT, USA) and filtered through a 0.45 µm filter (Millipore Corp., Bedford, MA, USA). RP-HPLC was performed using Varian HPLC (Mulgrave, Vic, Australia) consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength ultraviolet-visible tunable absorbance detector and a 730 data module. A sample size of 50 µL was injected into the reverse-phase column (C18, 250 mm x 4.6 mm, 5 µm, Grace Vdac, Hesperia, CA, USA) with a guard column and disposable cartridge (10 mm, 12 µm, Grace Vydac). The separation was conducted at room temperature (~22^{o}C) at a flow rate of 0.75 mL min^{-1}. The eluent B was 60% acetonitrile containing 0.05% TFA. A linear gradient was applied from 0 to 80% eluent B over 100 min. The detection device was the ultraviolet-visible detector set at 215 nm.

6.2.4. ***ACE-inhibitory activity of cheese WSE***

The ACE-inhibitory activity of the freeze-dried WSE was measured using the spectrophotometric assay of Cushman and Cheung (1971) as modified by Donkor et al. (2005). The method is based on the liberation of hippuric acid from hippuryl-histidyl-leucine (HHL, Sigma) catalysed by ACE. Each assay mixture contained 200 µL HHL solution (3.8 mmol^{-1} HHL, 100 mmol L^{-1} sodium borate buffer, 300 mmol L^{-1} NaCl, pH 8.3), 2 mU ACE (from rabbit lung; Sigma) and 35 µL sample solution (15 mg freeze-dried WSE in 1 mL of distilled water). After 30 min of incubation at 37ºC, the hippuric acid was extracted with 1.7 mL ethyl acetate. The mixture was centrifuged and 1.5 mL of the organic phase (ethyl acetate) was transferred to a fresh test tube and evaporated to dryness on a water bath for 15 min at 100ºC. The residue containing hippuric acid was dissolved in 1 mL deionised water and the solution was measured using Cary IE UV visible spectrophotometer (Varian) at 228 nm against deionised water as a blank. The percent inhibition was calculated as follows: ACE-inhibition (%) = [1- (A-C)/(B-D)] x 100, where A is the absorbance with ACE, HHL and ACE-inhibitory sample, B is the absorbance with ACE and HHL without ACE-inhibitory sample, C is the absorbance with HHL and ACE-inhibitory

sample without ACE and D is the absorbance with HHL without ACE and ACE-inhibitory sample. The inhibition was also expressed as IC_{50} (the concentration of an ACE inhibitor needed to inhibit 50% of ACE activity). All inhibition values reported are the means of two determinations of three replicates (n = 6).

6.2.5. ***Protein assays***

The protein content in the cheese WSE and in the purified fractions was determined by the Folin-Lowry method (Lowry et al., 1951). A sample (100 µL) was mixed with an alkaline-copper reagent (3 mL) and folin phenol reagent (0.3 mL; two-fold dilution with distilled water of folin-phenol; Sigma). After the solution was allowed to stand for 30 min, the absorbance was measured at 650 nm using a Pharmacia LKP Novaspek II Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). All determinations were carried out in duplicate.

6.2.6. ***Isolation of ACE–inhibitory peptides***

For peptide separation, the WSE from 24 wk old cheese was used. A sample size of 100 µL (80 mg freeze-dried WSE in 1 mL of solvent A) was injected into a reverse-phase column and forty fractions from each batch of cheeses were collected using a fraction collector (Pro Star 704; Varian) on a 2-min collection time basis. This step was repeated 10 times and the 40 fractions from the various chromatographic runs were pooled and concentrated using a vacuum evaporator (SpeedVac SC110 concentrator, Savant Instruments Inc., Farmingdale, NY, USA) to a final volume of ~1 mL and filtered through a low protein binding 0.2 µm filters (Millipore Corp., Bedford, MA, US). An aliquot of the concentrated fractions (50 µL) was used to determine the ACE-inhibitory activity. The fractions with the highest ACE-inhibitory activity were subjected to further separation and purification by RP-HPLC. The chromatographic conditions used were as described in section 6.2.3.

6.2.7. ***Identification of peptides***

Purified peptides were sequenced by an automated Edman degradation method using a protein sequencer (490 Procise, Perkin Elmer Co. Ltd., Applied Biosystem Division, Foster City, CA, USA) with a PTH-C18 column (2.1 x 220 mm; Perkin Elmer Co. Ltd.). Chemicals used in the 140C microgradient delivery system included two types of

solvent (Perkin Elmer Co. Ltd.): A3 (3.5% vol/vol aqueous tetrahydrofuran) and B (acetonitrile and isopropanol).

The molecular mass of purified peptides was analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics Inc., Billerica, MA, USA). The matrix used for the analysis was α-cyano-4-hydroxycinnamix acid (Aldrich Chemical Co., Milwaukee, WI, USA). A saturated solution of matrix was prepared in acetonitrile/water (1:1) containing 0.25% (vol/vol) TFA. The crystal matrix-analyte was ionized by a 337 nm nitrogen laser pulse and accelerated under 25000 V before passing through the time-of-flight mass spectrophotometer. The instrument was set in the positive linear mode.

Peptide sequences were also obtained by using chemically assisted fragmentation (CAF) in conjunction with MALDI-TOF-MS. Ettan™ CAF MALDI sequencing kit (Amersham Biosciences, Uppsala, Sweden) was used to derivatize purified peptides. Peptides were bound to a prepacked reversed-phase C18 matrix in pipette tips (ZipTip™ pipette tips, Millipore Corp.). Acquisition of spectra in reflectron and post source decay (PSD) modes was performed using an Ettan MALDI-TOF-MS. For each analysis, the selected mass of the peptide from the reflectron spectrum was subjected to PSD mode. Mass differences between the fragment ions were calculated to determine the peptide sequence.

6.2.8. ***Statistical analysis***

Data analysis was carried out with Minitab statistical package (Minitab Inc., State College, PA, USA). One-way analysis of variance was used to find out differences between means of ACE-inhibition, IC_{50} and total area of peaks with a significant level at $\alpha = 0.05$.

6.3. Results

6.3.1. ***ACE-inhibitory activity of cheese WSE***

The ACE-inhibitory activity of WSE of cheese samples is shown in Figure 6.1. ACE-inhibitory activity of the cheeses increased significantly ($P < 0.05$) especially during the first 12 wk of ripening. The inhibitory activity continued to increase in all cheeses for 24 wk and stabilized after that period. The ACE-inhibition in cheeses with *Lb. casei* 279 (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5) at 24 wk were higher than that of the control

cheeses (Batch 1). The differences were, however, not statistically significant ($P > 0.05$). The IC_{50} (concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity) of the cheeses during ripening at 4°C for 36 wk is shown in Figure 6.2. The IC_{50} (concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity) was the lowest after 24 wk of ripening in the probiotic cheeses (Batches 2-7, 2M & 3M; 0.20 - 0.29 mg mL^{-1}) compared to 36 wk for cheeses without any probiotic (Batches 1 & 1M; 0.28 – 0.31 mg mL^{-1}). The IC_{50} of the probiotic cheeses at 24 wk of ripening was significantly lower ($P < 0.05$) than that of the control cheese. The IC_{50} among the cheeses at 36 wk of ripening was, however, not significantly different ($P < 0.05$). The result shows that cheeses with the addition of probiotic had higher ACE-inhibitory activity during the first 24 wk of ripening at 4°C. There was no significant difference ($P > 0.05$) between the percentage inhibition or the IC_{50} of the cheeses between the ripening period of 24 wk and 36 wk. The isolation of ACE-inhibitory peptides from the cheeses was thus performed on 24 wk old cheeses.

Previously we have reported that cheese with the addition of *Lb. casei* 279 or *Lb. casei* LAFTI®L26 had increased proteolysis with higher concentration of water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) as compared to the control or other probiotic cheeses (Chapter 3.0, section 3.3.5 & Chapter 4.0, section 4.3.6). The ACE-inhibitory activity of cheeses with *Lb. casei* sp. (Batches 4 & 5) as compared to the control cheese (Batch 1) was thus further investigated.

6.3.2. ***Reverse-phase HPLC of cheese WSE***

The RP-HPLC peptide profiles of control Cheddar cheeses (Batch 1) and probiotic Cheddar cheeses with *Lb. casei* 279 (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5) at wk 1, 12, 24 and 36 are shown in Figure 6.3. More peptides were released into the cheeses as the ripening period increased. The number of peaks and total area increased significantly ($P < 0.05$) during the first 12 wk, continued to increase slowly but insignificantly ($P > 0.05$) until 24 wk and remained constant after that period in all cheeses (Table 6.1). The results show that the rate of proteolysis in the cheeses was more extensive at the early stage of ripening. Although cheeses with *Lb. casei* sp. had higher level of proteolysis than the control cheese, the peptide profiles of the three different batches of cheeses, however, were very similar (Figure 6.1). There are no significant differences ($P > 0.05$) in total area or the number of peaks between the cheeses (Table 6.1).

6.3.3. ***Isolation of ACE-inhibitory peptides***

Forty fractions from each batch of the cheeses (Batches 1, 4 & 5) were collected by RP-HPLC connected to a fraction collector on a 2-min collection time basis. The ACE-inhibitory activities of the 40 fractions collected from control cheese (Batch 1), probiotic cheese with *Lb. casei* 279 (Batch 4) and probiotic cheese with *Lb. casei* LAFTI®L26 (Batch 5) are shown in Figures 6.4. Several fractions distributed throughout the acetonitrile gradient consistently showed high ACE-inhibition index especially in the control cheese. The ACE-inhibition and IC_{50} of fraction 9 were 87% (IC_{50}, 0.20 mg·mL^{-1}), 60% (IC_{50}, 0.18 mg·mL^{-1}) and 64% (IC_{50}, 0.25 mg·mL^{-1}) in Batches 1, 4 and 5, respectively. Fraction 15 consistently showed a high ACE-inhibition and low IC_{50} value, especially in probiotic cheeses. The ACE-inhibition and IC_{50} of fraction 15 were 42% (IC_{50}, 0.16 mg·mL^{-1}), 79% ($IC_{50,}$ 0.12 mg·mL^{-1}) and 69% (IC_{50}, 0.14 mg·mL^{-1}) in Batches 1, 4 and 5, respectively. Fraction 27 showed a consistently high ACE-inhibition and low IC_{50} value in all three batches of cheeses. The ACE-inhibitions and IC_{50} of fraction 27 were 54% (IC_{50}, 0.12 mg·mL^{-1}), 61% ($IC_{50,}$ 0.09 mg·mL^{-1}) and 63% (IC_{50}, 0.11 mg·mL^{-1}) in Batches 1, 4 and 5, respectively. Fraction 28 was collected from a higher concentration of acetonitrile gradient. The ACE-inhibition and IC_{50} of fraction 28 were 57% (IC_{50}, 0.14 mg·mL^{-1}), 50% ($IC_{50,}$ 0.17 mg·mL^{-1}) and 46% (IC_{50}, 0.14 mg·mL^{-1}) for Batches 1, 4 and 5, respectively.

Fractions 9, 15, 27 and 28 obtained from the three different batches of the cheeses were re-injected into RP-HPLC and the profiles of the fractions were found to be very similar. Only fractions obtained from *Lb. casei* LAFTI®L26 (Batch 5) were subsequently purified by further RP-HPLC with different gradient or isocratic run to obtain pure peptides for identification purpose.

6.3.4. ***Identification of peptides***

N-terminal sequencing of the peptides was obtained by using an automated Edman degradation technique on a protein sequencer and the molecular weights of the peptides were determined by MALDI-TOF-MS. The details of the fractions are shown in Table 6.2. Most of the fractions collected contained a mixture of several peptides. The first five residues of fraction 9 obtained from the N-terminal sequencing were Arg-Pro-Lys-His-Pro. Following sequence interpretation and molecular weight determination, the peptides in fraction 9 were identified as α_{s1}-CN (f 1-9), α_{s1}-CN (f 1-7) and α_{s1}-CN (f 1-6) (Table 6.2).

The first five residues obtained from the N-terminal sequencing of fraction 15 were Asp and Glu in the first, Lys and Val in the second, Ile in the third, His and Glu in the fourth, and Pro in the fifth residue. The mass spectra of fraction 15 only corresponded to sequence Asp-Lys-Ile-His-Pro-Phe with molecular molecular weight of 755.4 $g \cdot mol^{-1}$. Thus only one peptide derived from β-CN (f 47-52) was identified from fraction 15 (Table 6.2). The first five residues of fraction 27 obtained from the N-terminal sequencing were Phe and Lys, Val and Lys, Ala and Tyr, Pro and Lys, Phe and Val in the first, second, third, fourth and fifth residues, respectively. The sequence interpretation and the mass spectrums (1053.3 and 1132.4 $g \cdot mol^{-1}$) showed that fraction 27 had peptides derived from α_{s1}-CN (f 102-110) and α_{s1}-CN (f 24-32) (Table 6.1). Sequences and molecular weight of fraction 28 corresponded to peptide derived from β-CN (f 193-209). Sample of chromatograms obtained during the N-terminal sequencing using Edman degradation method are shown in Appendix B.

The identification method was also confirmed by using chemically assisted fragmentation (CAF) chemistry followed by MALDI-TOF-MS determination (Figure 6.5). Purified peaks were derivatized using the Ettan™ CAF-MALDI sequencing kit. CAF-MALDI chemistry is based on the introduction of a negatively charged group to the N-terminus of peptides generated by tryptic digestion. Only y-ions, which retain a net positive charge, are separated and detected while N-terminal fragments are neutral and not detectable. With the generation of only y-ion series fragments, the mass between two peaks on the spectrum could be corresponded to the mass of individual amino acids. The first 9 residues obtained by using CAF-MALDI of fraction 28 were identified as Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val (Figure 6.5). Thus the peptide was confirmed as β-CN (f 193-209).

6.3.5. ***Formation of peptides during ripening***

After 24 wk of ripening, control cheese had more of the fraction 9 as compared to the probiotic cheeses. Figure 6.6 shows that the total area of fraction 9 was higher in control cheese (Batch 1) after 24 wk, which possibly explained the high ACE-inhibitory index in fraction 9 of control cheese (Figure 6.4). The total area of fraction 9 reduced after 24 wk in all cheeses (Figure 6.6). Further ripening, possibly degraded these peptides to smaller peptides which may have changed their bioactivity. The peak area of fraction 27 increased significantly ($P < 0.05$) as the ripening time increased to 24 wk in all cheeses (Batches 1, 4 & 5) and decreased significantly ($P < 0.05$) in cheese with *Lb. casei* 279 (Batch 4) and remained relatively constant in cheese with *Lb. casei* LAFTI®L26 (Batch 5) after 24 wk of ripening (Figure 6.6). Total area of fraction 27 in control cheese continued to increase even

after 24 wk of ripening (Figure 6.6). The peak area of fraction 28 increased during ripening especially during the first 3 month in Batches 1, 4 and 5, but decreased after further ripening (Figure 6.6).

6.4. Discussion

It has been reported that proteinases of lactic acid bacteria can hydrolyze more than 40% of the peptide bonds of β-CN resulting in the formation of more than 100 different oligopeptides, which are in turn actively degraded by the complex peptidase system. The same pattern is reported for α_{s1}-CN. Consequently lactic acid bacteria could potentially generate a large variety of peptides including bioactive peptides (Kunji et al., 1996). Rokka et al. (1997) have reported the release of a variety of bioactive peptides by enzymatic proteolysis of UHT milk fermented with probiotic *Lb. casei* ssp. *rhamnosus* strain. The type of dairy product, the technology adapted, and the selection of strain based on the specificity of proteolysis are factors that influence the proteolytic activation of bioactive peptides. ACE-inhibitory peptides have been found in several types of cheeses, which differ with respect to the type of starter and the ripening condition used (Gouldsworthty et al., 1996; Haileselassie et al., 1998; Saito, 2000; Ryahanen, 2001).

In our study, probiotic organism was added as an adjunct in Cheddar cheeses production and the ACE-inhibitory activity was monitored during a ripening period of 36 wk at 4ºC. The rate of increase of the ACE-inhibitory activity during ripening was very similar to the rate of proteolysis of the cheeses reported previously (Chapter 4.0, section 4.2.6). Proteolysis was reported to be more extensive in probiotic cheeses after ripening for 24 wk at 4ºC. The IC_{50} of the probiotic cheeses at 24 wk of ripening was significantly lower ($P < 0.05$) than the control cheese (Batch 1). The data also show that ACE-inhibitory activity was higher in cheeses received a higher degree of proteolysis. The IC_{50} among the cheeses at 36 wk of ripening was, however, not significantly different ($P < 0.05$). ACE-inhibition is thus dependent on the extent of proteolysis but only to a certain extent. Our results support the previous findings of Addeo et al. (1992), Gomez-Ruiz et al. (2002), Ryahanen et al. (2001), and Saito et al. (2000).

ACE is predominantly an ectoenzyme with two catalytic sites, one on each lobe of the extracellular portion. Structure activity correlations among different peptides inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tri-peptide sequence of the substrate (Maruyama et al., 1987). ACE appears to prefer substrates or inhibitors that contain mainly hydrophobic (aromatic or branched side chains) amino acid

residues at the three C-terminal positions. The structure-activity relationship of ACE-inhibitory peptides has not yet been established and different antihypertensive sequences have been derived from a large number of food proteins. Likewise, most of the potential ACE-inhibitory peptides identified in this study have different structure and sequences.

In cheeses, bovine α_{s1}-CN can be rapidly hydrolyzed by chymosin at Phe^{23}-Phe^{24} to yield α_{s1}-CN (f 1-23) and α_{s1}-CN (f 24-199) (Minkiewicz et al., 2000). α_{s1}-CN (f 1-23) is hydrolyzed rapidly in cheese by lactococcal cell envelope proteinases to several small peptides resulting in the formation and accumulation of peptides α_{s1}-CN (f 1-9) and α_{s1}-CN (f 1-13) in Cheddar during ripening (Singh et al., 1997). In the present study, three peptides from the same N-terminal region of α_{s1}-CN (f 1-23) were isolated, which corresponded to α_{s1}-CN (f 1-9) (f 1-7) and α_{s1}-CN (f 1-6) (Table 6.2). The IC_{50} of the peptides was not determined. These peptides were previously isolated from a fermented low-fat hard cheese produced with probiotic bacteria and have shown to have ACE-inhibitory properties (Ryahanen et al., 2001). Peptide α_{s1}-CN (f 1-9) isolated from Gouda cheese gave very low IC_{50} value of 13.4 $\mu mol \cdot L^{-1}$ and antihypertensive effect of -9.3 ± 4.8 mm Hg on spontaneously hypertensive rats (SHR) (Saito et al., 2000). The peptide derived from α_{s1}-CN (f 1-6) had been isolated from sheep sodium caseinate hydrolysate and was shown to have ACE-inhibitory activity with an IC_{50} of 30.1 $\mu g \cdot mL^{-1}$ (Minkiewicz et al., 2000), probably due to the presence of hydrophobic Pro-Ile residues at the C-terminal end. Synthesized peptide α_{s1}-CN (f 1-6) treated with trypsin and chymotrypsin was also resistant to hydrolysis (Minkiewicz et al., 2000).

The hexapeptide Asp-Lys-Ile-His-Pro-Phe that originates from β-CN (f 47-52) was also isolated in our study (Table 6.2). It is interesting to note that this peptide had the first N-terminal amino acid and the last four C-terminal amino acids in common with the octapeptide angiotensin-II generated by ACE hydrolysis of the decapeptide angiotensin-I. Commercial drugs used in antihypertension therapy are based on compounds which may compete for the receptor sites of the vasoconstrictor angiotensin-II due to their partial homology with the product of ACE activity (angiotensin-II). Angiotensin-II receptor antagonists (such as losartan) competitively block angiotensin–II-induced vascular contraction (Jagadesh, 1998). A similar peptide was found in milk fermented by *L. lactis* subsp. *cremoris* FT4 (Gobbetti et al., 2000). In that study, this peptide was chemically synthesized and the ACE-inhibitory activity was confirmed (IC_{50} = 193.9 $mg \cdot L^{-1}$). The chemically synthesized peptide was also resistant to hydrolysis by trypsin and chymotrypsin (Gobbetti et al., 2000). Biochemical properties and cleavage site of proteinases and peptidases of *L. lactis* strain have been studied in detail (Juillard et al., 1995). The cleavage

sites of the peptide bonds residue 46-47 and 52-53 of β-CN are hydrolyzed by all of the lactococal proteinases studied. The ACE inhibitory index of fraction 15 was higher in probiotic cheeses (Figure 6.4). The proteolytic enzymes from the probiotic adjuncts could possibly hydrolyze residue 46-47 and 52-53 of β-CN resulting in the liberation of more of this peptide in probiotic cheeses.

The fraction 27 contained a mixture of two peptides derived from α_{s1}-CN (f 102-110) and α_{s1}-CN (f 24-32) (Table 6.2). The peptide Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu derived from α_{s1}-CN (f 102-110) had within its sequence the hexapeptide Tyr-Lys-Val-Pro-Gln-Leu of α_{s1}-CN (f 104-109), which has been proven to have in-vitro ACE-inhibitory activity (IC_{50} = 22 $\mu mol \cdot L^{-1}$) (Maeno et al., 1996). This peptide, however, did not show a major antihypertensive effect after oral administration to spontaneously hypertensive rats (Maeno et al., 1996). A similar peptide derived from α_{s1}-CN (f 101-107) of ovine cheese was isolated from Manchego cheese and had a potent ACE-inhibitory activity with an IC_{50} of 77.1 $\mu mol \cdot L^{-1}$ (Gomez-Ruiz et al., 2002). This peptide has a high homology with the peptide α_{s1}-CN (f 102-110) found in our study. The synthesized peptide of fragment α_{s1}-CN (f 101-107) was, however, not resistant to hydrolysis with a pancreatic extract and the ACE-inhibitory activity decreased after digestion (Gomez-Ruiz et al., 2004).

The peptide corresponding to α_{s1}-CN (f 24-32) probably originated from α_{s1}-CN (f 24-199) via hydrolysis by chymosin (cleavage sites Phe^{23}-Phe^{24} and Phe^{32}-Gly^{33}). Peptides from within this sequence, α_{s1}-CN (f 24-27), (f 25-27), (f 27-30) have been produced from bovine casein of enzymatic hydrolysis and have shown bradykinin-potentiating activity on the uteri and ilea of rats (Maruyama et al., 1987; FitzGerald & Meisel, 2000). The IC_{50} of some of the above peptides varied largely from 2 to > 1000 $\mu mol \cdot L^{-1}$.

According to Fox et al. (1993), about 3-6% of the coagulant (chymosin) added to cheese milk is retained in the curd. After cheese processing, peptide corresponding to α_{s1}-CN (f 24-32) was possibly released as the action of the remaining coagulant to a certain extent. However, the decrease in the area of the peak corresponding to α_{s1}-CN (f 24-32) in probiotic cheeses (Batches 4 and 5) indicated that this peptide was hydrolyzed, possibly by the proteolytic enzyme produced by the probiotic adjunct. Carboxypeptidase activity in lactobacilli (Atlan et al., 1993) and in nonstarter lactic acid bacteria (NSLAB) in Feta cheese (Michaelidou et al., 1998) has been reported to hydrolyze α_{s1}-CN (f 24-32) to α_{s1}-CN (f 24-30) and smaller fragments, which further supported the possibility that the proteolytic enzymes from the adjunct probiotic were responsible for the decrease in total area of fraction 27. Peptides derived from β-CN (f 193-209) was isolated from the fractions eluted with a higher concentration of acetonitrile gradient (Table 6.1). Leu^{190}-Tyr^{191} and Leu^{192}-

Tyr^{193} were known chymosin cleavage sites in solution (Exterkate et al., 1997). Cell-wall-associated proteinases also appear to cleave Leu^{192}-Tyr^{193} in solution (Fox et al., 1994). Both peptides were thus most probably the product of hydrolysis by chymosin and cell-wall-associated proteinases from starter lactococci and/ or from probiotic adjunct. Peptides derived from β-CN (f 193-209) has previously been isolated from casein by extracellular proteinase from *Lb. helveticus* CP790 and have shown to have ACE-inhibitory activity with IC_{50} of 101 $\mu g \cdot mL^{-1}$ (Yamamoto et al., 1994). Other studies also reported the various bioactivities of these peptides such as immunomodulating properties (Coste et al., 1992) and antimicrobial activity (Sandre et al., 2001). Peptide β-CN (f 193-209) and α_{s1}-CN (f 1-9) have been reported to cause bitterness in cheese (Broadbent et al., 2002). Sensory evaluation of Cheddar cheeses made with *Lb. casei* 279 showed bitterness as the major defect (Chapter 5.0, section 5.3).

6.5. Conclusion

Addition of probiotic organisms has potential to increase the ACE-inhibitory activity of the cheeses during ripening. This may suggest that the proteolytic enzyme of the probiotic organisms could possibly play a role in increasing the production of ACE-inhibitory peptides in Cheddar cheeses. Some of the peptides isolated were not resistant to further proteolysis and bioactivity may have been enhanced only after subsequent proteolysis. These peptides may become potential substrates for the production of other ACE-inhibitory peptides by the proteolytic enzyme of the probiotic organism. Further study on the individual ACE-inhibitory peptides isolated in this study is needed to better understand the correlation between the proteolytic enzyme produced by the probiotic organisms on the generation of these peptides and other ACE-inhibitory peptides generated by subsequent proteolysis of these peptides.

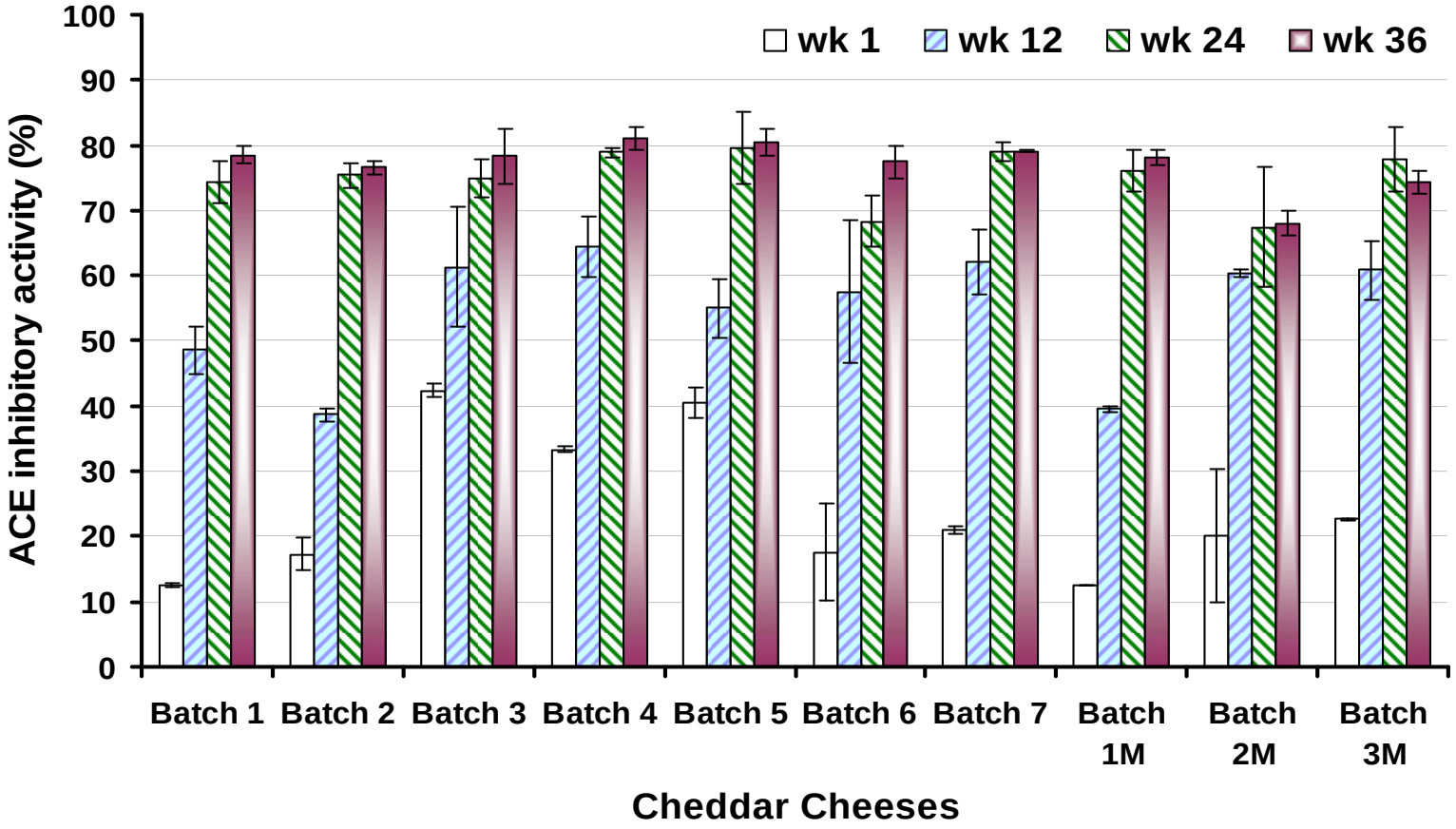


Figure 6.1. ACE-inhibitory activity of water-soluble extract (WSE) of control and probiotic Cheddar cheeses at wk 1, 12, 24 and 36. Results were expressed as mean ± SE (n = 6). Batch codes are as detailed in section 6.2.2.

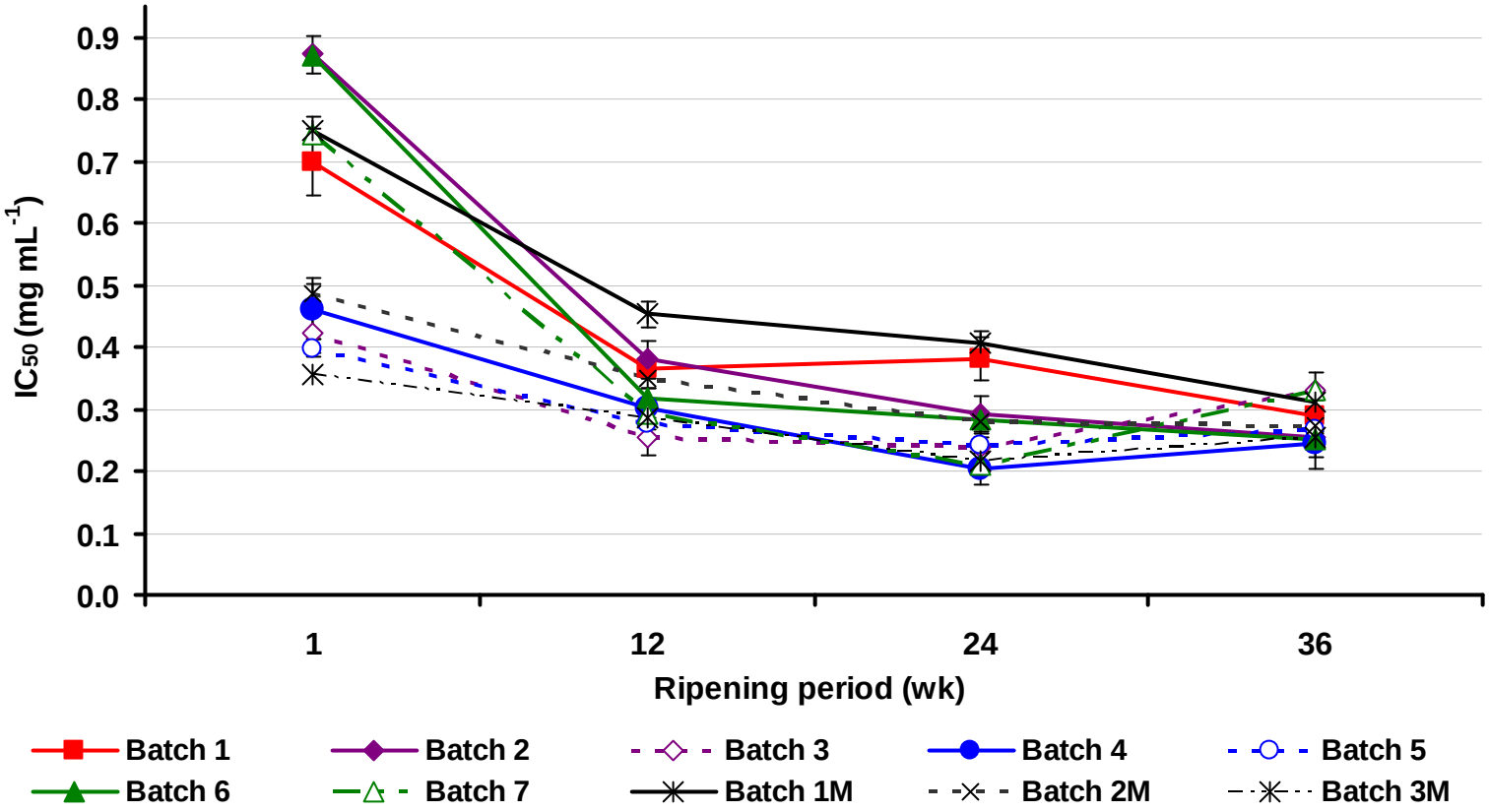


Figure 6.2. The ACE-inhibitory activity presented as IC_{50} of water-soluble extract (WSE) of control and probiotic Cheddar cheeses at wk 1, 12, 24 and 36. IC_{50} is concentration of ACE inhibitor needed to inhibit 50% of ACE activity. Results were expressed as mean ± SE (n = 6). Batch codes are as detailed in section 6.2.2.

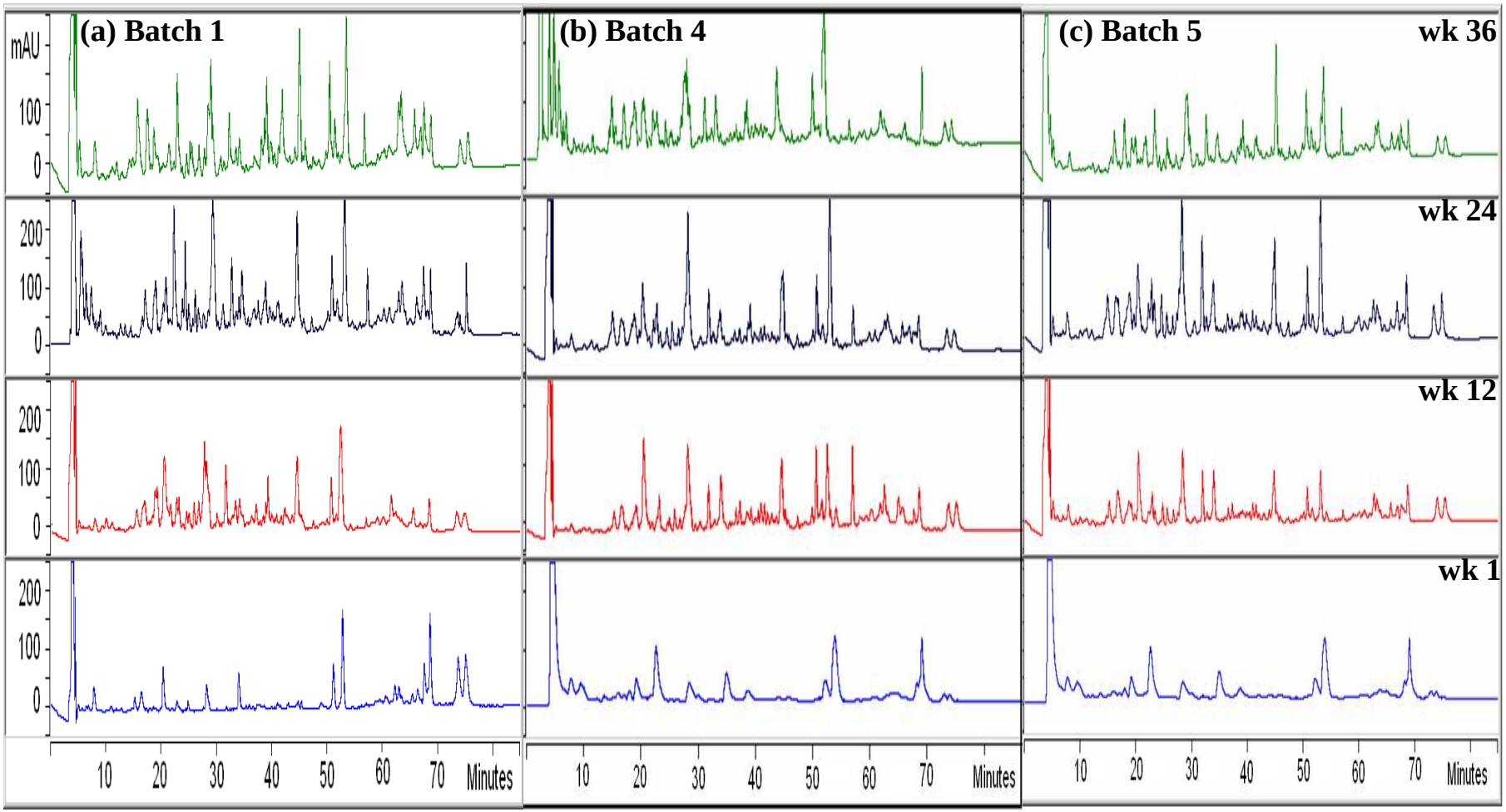


Figure 6.3. Peptide profiles of water-soluble extract (WSE) of (a) control Cheddar cheese (Batch 1), (b) probiotic Cheddar cheeses with *Lb. casei* 279 (Batch 4) and (c) probiotic Cheddar cheese with *Lb. casei* LAFTI®L26 (Batch 5) at wk 1, 12, 24 and 36. Eluent A was 10% acetonitrile containing 0.05% TFA solution. Eluent B was 60% acetonitrile containing 0.05% TFA. Gradient: 0 to 100 min, 0 to 80% eluent B; 100 to 105 min, 80 to 100% eluent B; 105 to 110 min, 100 to 0% eluent B. Detection was at 215 nm.

Table 6.1. Total area and number of peaks of peptide profiles obtained from water-soluble extract of control cheese (Batch 1) and probiotic Cheddar cheeses produced with *Lb. casei* 279 (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5) adjunct

Ripening period (wk)	Batch 1	Batch 4	Batch 5
No. of peaks[1]			
1	27 ± 3^{aA}	33 ± 2^{aA}	29 ± 6^{aA}
12	58 ± 4^{abB}	57 ± 3^{aB}	64 ± 5^{bB}
24	66 ± 5^{aBC}	66 ± 4^{aBC}	71 ± 4^{aBC}
36	68 ± 2^{aC}	67 ± 4^{aC}	72 ± 3^{aC}
Total area[1] (x 10^8)			
1	0.819 ± 0.05^{aA}	0.923 ± 0.06^{aA}	0.885 ± 0.09^{aA}
12	1.95 ± 0.08^{aB}	2.03 ± 0.07^{aB}	2.04 ± 0.09^{aB}
24	2.51 ± 0.09^{aC}	2.39 ± 0.12^{aC}	2.52 ± 0.11^{aC}
36	2.43 ± 0.11^{aC}	2.58 ± 0.09^{aC}	2.45 ± 0.15^{aC}

[1] Results were expressed as mean ± SE (n = 6).
[ab] Means in rows with like superscripts do not differ ($P > 0.05$).
[ABC] Means in column with like superscripts do not differ ($P > 0.05$).

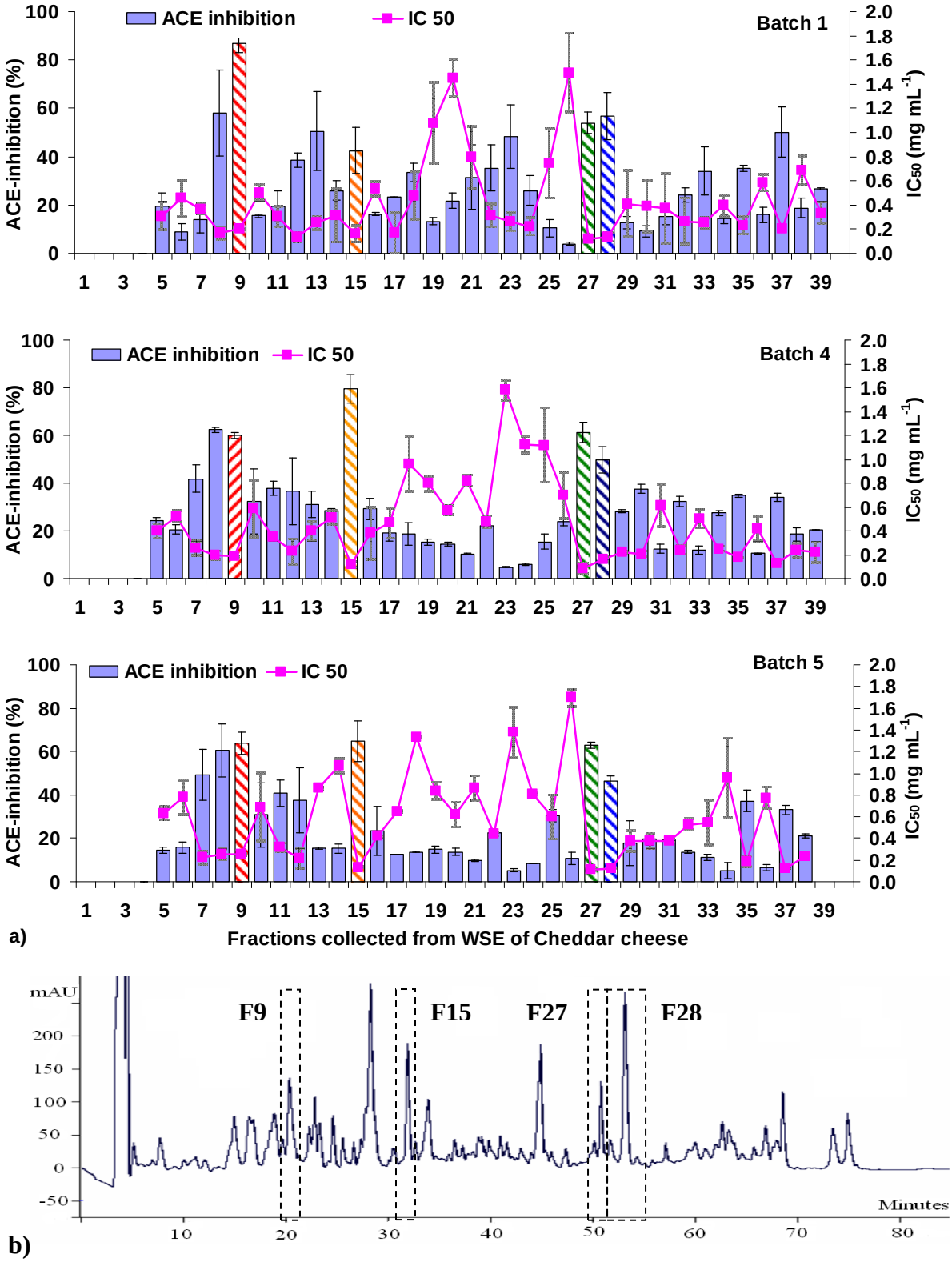
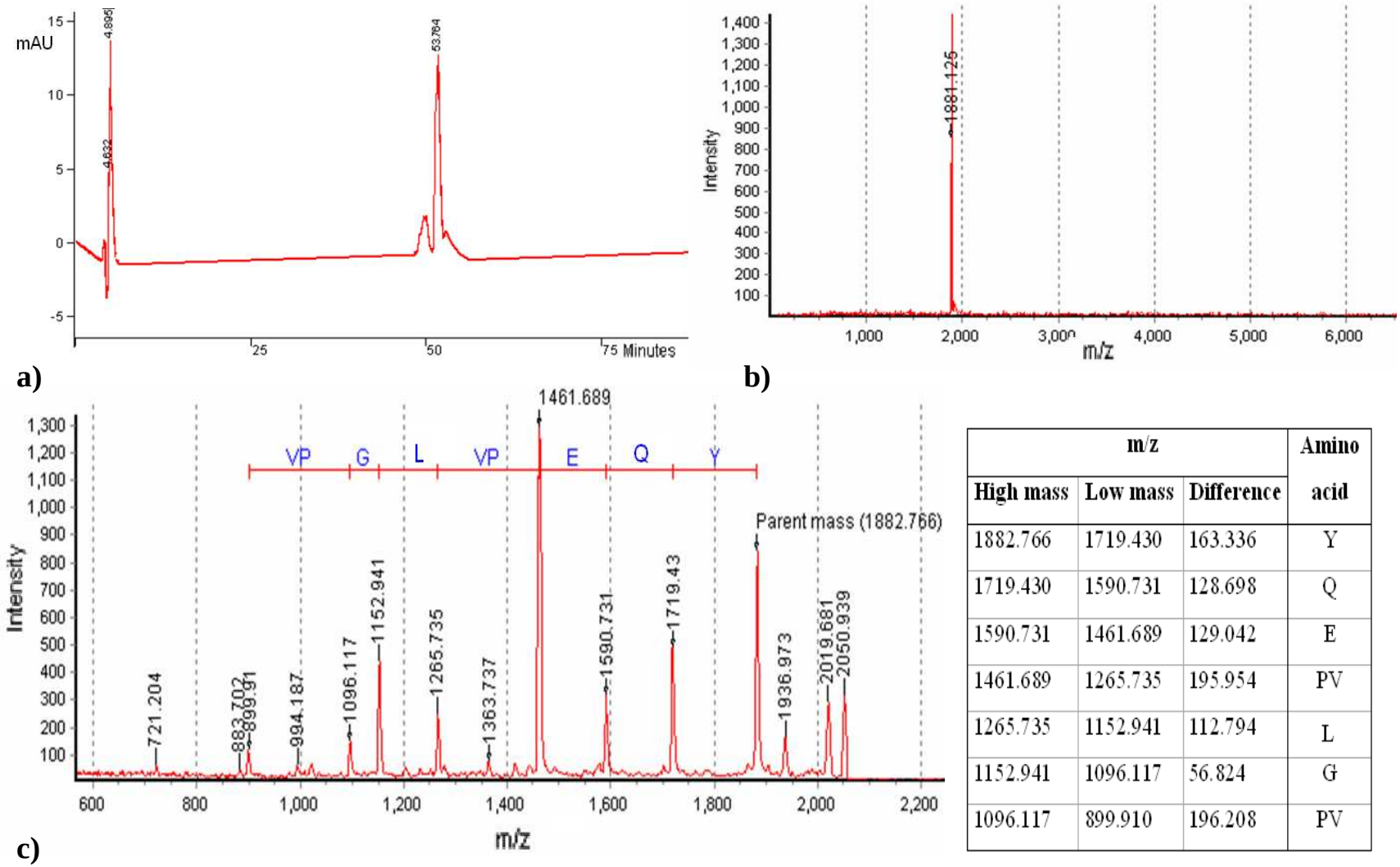


Figure 6.4. (a) The ACE-inhibitory activity (%) of 40 fractions collected from water-soluble extract (WSE) of control Cheddar cheese (Batch 1), probiotic Cheddar cheeses with *Lb. casei 279* (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5). IC_{50} is concentration of ACE inhibitor needed to inhibit 50% of ACE activity. Results were expressed as mean ± SE (n = 6). Fractions without results mean that no inhibitory activity was obtained. (b) RP-HPLC profiles of fractions with the highest ACE-inhibitory activity. Fractions 9, 15, 27 and 28 were further collected and re-injected into RP-HPLC for further purification (RP-HPLC condition as described in section 6.2.3 & section 6.2.6).



m/z			Amino acid
High mass	**Low mass**	**Difference**	
1882.766	1719.430	163.336	Y
1719.430	1590.731	128.698	Q
1590.731	1461.689	129.042	E
1461.689	1265.735	195.954	PV
1265.735	1152.941	112.794	L
1152.941	1096.117	56.824	G
1096.117	899.910	196.208	PV

Figure 6.5. (a) RP-HPLC profiles of fractions 28 of Cheddar cheeses produced with the addition of probiotic *Lb. casei* LAFTI® L26 (condition as described in section 6.2.3) (b) Molecular weight of purified peptide from fraction 28 obtained with MALDI-TOF-MS (condition as described in section 6.2.7) (c) Chemically assisted fragmentation (CAF) of purified peptide from fraction 28. The first nine amino acids of the N-terminal was identified as YQEPVLGPV (CAF condition as described in section 6.2.7). Following sequence interpretation and molecular weight determination, the peptide was identified as β-CN (f 193-209).

Table 6.2. Identified peptides in the fractions obtained from water-soluble extract of Cheddar cheese produced with *Lb. casei* LAFTI®L26

Fraction	Sequences	Origin	Experimental molecular mass ($g \cdot mol^{-1}$)	Theoretical molecular mass ($g \cdot mol^{-1}$)
9	Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln (RPKHPIKHQ)	α_{s1}-CN (f 1-9)	1140.7	1140.4
9	Arg-Pro-Lys-His-Pro-Ile-Lys (RPKHPIK)	α_{s1}-CN (f 1-7)	877.0	875.1
9	Arg-Pro-Lys-His-Pro-Ile (RPKHPI)	α_{s1}-CN (f 1-6)	745.4	746.9
15	Asp-Lys-Ile-His-Pro-Phe (DKIHPF)	β-CN (f 47-52)	755.4	755.5
27	Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe (FVAPFPEVF)	α_{s1}-CN (f 24-32)	1053.3	1052.2
27	Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu (KKYKVPQLE)	α_{s1}-CN (f 102-110)	1132.4	1134.4
28	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (YQEPVLGPVRGPFPIIV)	β-CN (f 193-209)	1881.1	1881.3

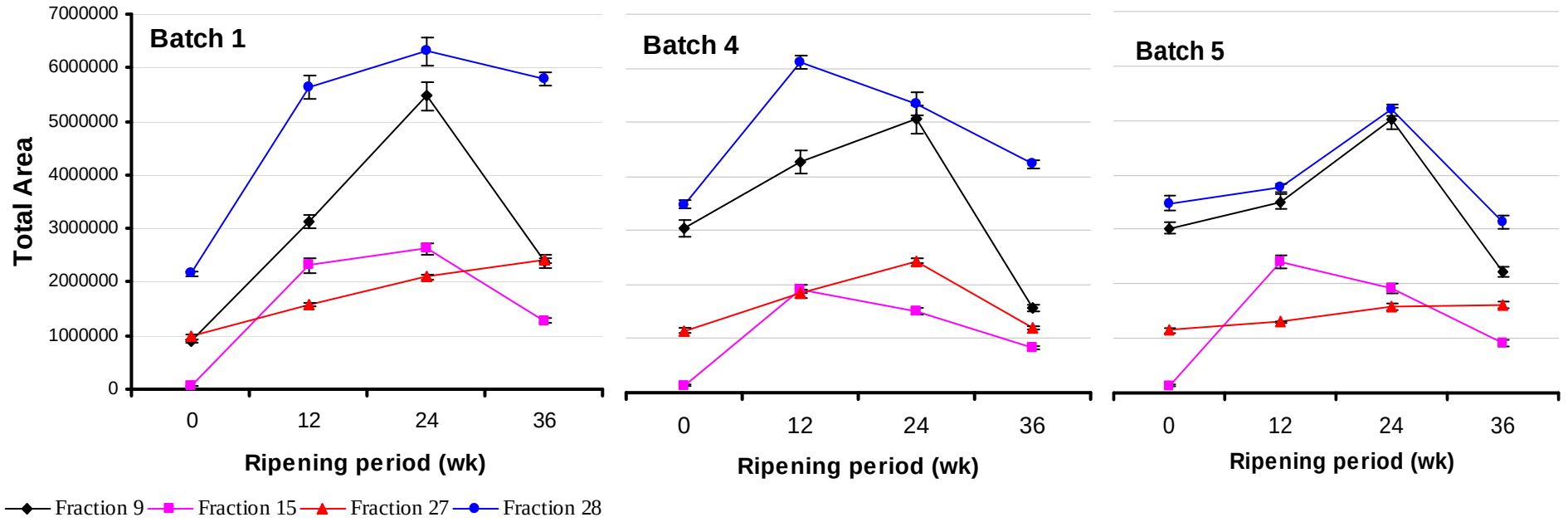


Figure 6.6. Total area of ACE-inhibitory fractions obtained from water soluble extract of control Cheddar cheese (Batch 1) and probiotic cheeses made with *Lb. casei* 279 (Batch 4) or *Lb. casei* LAFTI®L26 (Batch 5) during ripening at 4ºC for 36 wk. Total area is the area of the peak (ACE-inhibitory fractions highlighted in Figure 6.3) obtained from HPLC-RP. Results were expressed as total area ± standard error (n = 6).

7.0 Influence of ripening temperatures on proteolytic pattern, sensory evaluation and angiotensin converting enzyme- inhibitory activity of probiotic Cheddar cheeses*

7.1. Introduction

A higher ripening temperature has been reported to accelerate cheese ripening, thus promoting faster flavour development, reducing storage time, and providing savings to the producer (Law, 2001). An elevated ripening temperature, however, may accelerate the loss of starter bacteria prior to complete lactose utilization, which may lead to off-flavours (Law, 2001).

Production of organic acid may occur in cheeses as a result of bacterial metabolism and breakdown of milk proteins, fat, lactose and citrate during manufacture and storage. They play an important role in the flavour development of cheeses. The organic acid content also reflects the type of fermentation and indicates deviations of the expected course of maturation that may lead to defects (Careri et al., 1996; DeLiano et al., 1996). Several studies correlated the age of cheeses with the level of organic acid (Bevilacqua & Califano, 1992; Careri et al., 1996; Lues & Bekker, 2002). An increase in ripening temperature was reported to increase the metabolic rates of cheese microflora including non-starter lactic acid bacteria (NSLAB) during ripening (Lues & Bekker, 2002). Fermentation of lactose by NSLAB produces some organic acid by-products such as formic acid and acetic acid (Fox et al., 1993). Excess of these compounds impairs the flavour balance of Cheddar cheeses.

*Versions of this chapter have been submitted to *International Dairy Journal* and *LWT Food Science and Technology*.

Ong, L., Henriksson, A., & Shah, N.P. (2007d). Probiotic Cheddar cheese Part I. Influence of ripening temperatures on survival of probiotic microorganisms, cheese composition and organic acid profiles. *International Dairy Journal* (revised manuscript submitted in April 2007).

Ong, L., Henriksson, A., & Shah, N.P. (2007e). Probiotic Cheddar cheese Part II. Influence of ripening temperatures on proteolysis and sensory characteristic of Cheddar cheeses. *International Dairy Journal* (revised manuscript submitted in April 2007).

Ong, L., Henriksson, A., & Shah, N.P. (2008a). Influence of ripening temperatures and probiotic adjuncts on the *in vitro* angiotensin converting enzyme-inhibitory activity of Cheddar cheeses. *LWT Food Science and Technology* (doi:10.1016/j.lwt.2007.11.026).

Appropriate ripening temperatures are required to maintain the balance growth of these bacteria to achieve the optimum cheese quality (Kleter, 1977; Puchades et al., 1989).

A high ripening temperature has also been reported to increase the proteolytic activity of the cheese microflora (Folkertsma et al., 1996). Starter lactococci contributes to ripening by releasing proteinases and peptidases for hydrolysing intermediate-sized peptides produced by the action of chymosin (rennet) and plasmin (indigenous enzymes in milk) to smaller peptides and free amino acids. The counts of starter lactococci decline during ripening due to unfavourable conditions in cheeses such as high salt in moisture, low pH and lack of fermentable carbohydrates (Ong et al., 2006). The proteolytic enzymes of the starter are also released into the cheese matrix when the cells lyse after death (Beresford & Williams, 2004). The proteinases and peptidases of NSLAB have been found to contribute to ripening (Williams & Banks, 1997). Probiotic bacteria also possess proteolytic system that may contribute to the release of small peptides and free amino acids in cheeses (Shihata & Shah, 2000). Addition of probiotic *Lactobacillus paracasei* in Cheddar cheeses has been reported to increase the level of free amino acid in cheeses (Gardiner et al., 1998). Excessive proteolysis may, however, form peptides and free amino acids that cause off-flavours such as bitterness (Broadbent et al., 2001). Although bitter taste is considered a normal component of the Cheddar flavour, excessive bitterness may limit consumer acceptance of cheeses (Lemieux & Simard, 1992). Appropriate ripening temperatures would thus be necessary to control the excessive proteolysis in cheeses.

In addition to the role of the proteolytic enzymes to overall proteolysis, they may also contribute to a release of biologically-active peptides such as angiotensin converting enzyme (ACE)-inhibitory peptides. The role of ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) in the regulation of blood pressure and cardiovascular function are discussed in section 2.6.1. ACE inhibition leads to a decrease in the level of the vasoconstrictory peptide, therefore may result in an antihypertensive effect and may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity (Meisel, 1998). ACE-inhibitory peptides have been isolated from various cheese varieties such as Italian, Parmesan, Gouda, Cheddar, Finnish and Manchego (Addeo et al., 1992; Smacchi & Gobbetti, 1998; Saito et al., 2000; Ryahanen et al., 2001; Korhonen & Pihlanto-Leppälä, 2001; Gomez-Ruiz et al., 2002). Only a few studies have investigated the release of ACE-inhibitory peptides in probiotic cheeses.

Studies on the ACE-inhibitory activity of cheeses during ripening show that inhibitory activity increased as proteolysis progressed (section 6.1). However, the bioactivity decreased when proteolysis exceeded at a certain level. To date, no studies have

investigated the influence of ripening temperatures on the ACE-inhibitory activity of Cheddar cheeses during ripening.

Six probiotic organisms including *Bifidobacterium longum* 1941, *Lactobacillus casei* 279, *Lb. acidophilus* 4962, *B. animalis* subsp. *lactis* LAFTI®B94 (B94), *Lb. casei* LAFTI®L26 (L26) and *Lb. acidophilus* LAFTI®L10 (L10) were selected based on the criteria described in section 2.1.3. The objective of the study was to examine the influence of ripening temperatures of 4 and 8°C on a) the survival of the probiotic bacteria, compositional changes in cheeses and the organic acid profiles; b) the proteolytic pattern and sensory properties of Cheddar cheeses and; c) the ACE-inhibitory activity of Cheddar cheeses during a ripening period of 24 wk.

7.2. Materials and methods

7.2.1. ***Starter and probiotic organisms***

Cheese starter culture, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and probiotic organisms *B. longum* 1941, *Lb. casei* 279, *Lb. acidophilus* 4962, *B. animalis* subsp. *lactis* LAFTI®B94, *Lb. casei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 were obtained from the culture collections mentioned in section 3.2.1. The organisms were activated and grown under the conditions described in section 3.2.1.

7.2.2. ***Cheddar cheese making***

Cheddar cheeses were made with 10 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture using a pair of custom made cheese vats. Seven batches of Cheddar cheeses were made including a control and six different probiotic cheeses as shown in Table 7.1. The complete set (7 batches) was produced randomly in 4 days with the same batch of pasteurised milk and at least 2 replications were produced the following weeks.

Cheeses were manufactured according to the standard procedures of Kosikowski (1977) as described previously in section 3.2.2. The adjunct cultures were added at the same time as the starter culture. All cheeses were packed in oxygen barrier Cryovac® bags (Cryovac® Pty. Ltd., Fawkner, Vic, Australia) and heat-sealed with a Multivac® vacuum packaging equipment (Multivac Sepp Haggenmüller, Wolfertscgwenden, Germany). Each

batch of the cheese was assigned in two equal portions to two ripening temperatures (4 and 8°C) and ripened for 24 wk.

7.2.3. ***Cheese composition***

The composition of the cheeses including the salt, fat, moisture, protein contents and pH of the cheeses were determined according to the methods described in section 3.2.3. Cheese composition was analyzed at day 1 (before ripening) and at the end of ripening (wk 24).

7.2.4. ***Survival of bacteria in cheeses***

Viability of the starter bacteria, probiotic organisms and NSLAB was assessed during production and ripening period of 24 wk at 4 and 8ºC. Samples of ripened milk, cooked curd, whey, cheddared curd and pressed curd (fresh cheese) were collected during production. During ripening, cheese samples were collected at day 1, wk 6, wk 12 and wk 24. Samples for enumeration were prepared as described in section 3.2.4. Starter lactococci, probiotic microorganisms and NSLAB were enumerated using various selective media as described in section 4.2.4.

7.2.5. ***Production of organic acids***

The concentration of lactic, acetic, citric, butyric, formic, propionic and succinic acid was determined using high performance liquid chromatography (HPLC) as described in section 3.2.5.

7.2.6. ***Assessment of proteolysis***

Proteolysis of the control and probiotic cheeses was analyzed at day 1 and wk 6, 12 and 24 during ripening at 4 and 8°C. The water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) of the cheeses were determined as per the method described in section 3.2.6. The proteolytic patterns of Cheddar cheeses were also analysed by SDS-PAGE using the stacking gel system as described in section 3.2.6. Data from SDS-PAGE analysis were expressed as the

ratio of the area to the intensity of the band. The reduction in the intensity of bands during ripening with respect to the original intensity was expressed as percentage hydrolysis.

7.2.7. ***Sensory evaluation of cheeses***

Staff and students of Victoria University (n = 15) were recruited as sensory panelists for a series of scoring test for specific attributes. The panelists were familiar with basic sensory evaluation techniques for Cheddar cheeses and were further trained for their ability to detect cheddary flavour, sour-acid taste, bitterness and vinegary taste as per the method described in section 5.2.2. Prior to sensory evaluation they also participated in briefing sessions. All panelists signed a Victoria university human subject's consent form (Appendix A.1).

Sensory evaluation was conducted for the cheeses after ripening for 6, 12 and 24 wk. Cheese samples were removed from the refrigerator and cut into pieces (about 1.5 x 1.5 x 1.5 cm in size) and placed on white plates coded with a random 3-digit numbers one hour prior to evaluation at room temperature (25^{o}C). A total of 14 variations (Table 7.1) of cheeses were presented to the panelists in randomly order over two days (7 cheeses per day). The sensory evaluation was repeated the following week where replicates of the cheeses were presented. Panelists had access to deionized water and unsalted water crackers to help cleanse their palates.

Prior to tasting, panelists completed a questionnaire on frequency of cheese consumption (<1 once per wk, 1-2 times per wk, 2-3 times per wk, 4-5 times per wk, or >5 times per wk) and cheese preference (mild, medium or sharp matured cheese). Panelists evaluated specific flavour attributes, which included the cheddary intensity, bitterness, sour-acid and vinegary using a 10-point intensity scale (1 = low intensity, 10 = high intensity). Panelists also evaluated texture attributes (hardness and crumbliness) using a 10-point scale (hardness, 1 = extremely soft, 10 = extremely hard; crumbliness, 1 = firm, 10 = extremely crumbly) as described previously in section 5.2.2.

7.2.8. ***Determination of ACE-inhibitory activity***

Cheese water soluble extracts (WSE) were prepared as per the method described in section 6.2.2. The extracts were concentrated by freeze drying (Dynavac FD300; Airvac Engineering Pty. Ltd., Rowville, Australia) at –20°C and –100 kPa for 72 h. The freeze-

dried WSE was then stored in vacuum at -20^{o}C for peptide analysis with reverse phase-high performance liquid chromatography (RP-HPLC) and for determination of ACE-inhibitory activity.

The ACE-inhibitory activity of the freeze-dried WSE was measured using the spectrophotometric assay of Chusman and Cheung (1971) as described in section 6.2.4. The ACE-inhibitory activity was also expressed as IC_{50} (the concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity). The IC_{50} was determined as per the method described in section 6.2.4 and 6.2.5 with modification. The IC_{50} was determined using graphical extrapolation by plotting ACE-inhibition as a function of different protein concentrations. To create a graphical correlation between ACE-inhibition and protein concentration, each sample was adjusted to at least three concentration levels. The protein content of the samples was determined using the Folin-Lowry method (Lowry, Resebrough, Farr, & Randall, 1951). The IC_{50} was then determined from the linear regression as the protein concentration in the sample required to inhibit 50% of the ACE activity.

7.2.9. ***Peptide profiles of cheese WSE***

A 40 mg aliquot of freeze-dried WSE was dissolved in 1 mL of solvent A containing 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich), centrifuged (14,000 x *g*, 10 min) using a bench top centrifuge (Sorvall RT7, Newtown, CT, USA) and filtered through a 0.45 µm filter (Millipore Corp., Bedford, MA, USA). The condition of RP-HPLC was as described in section 6.2.3 with modification. Eluent A in section 6.2.3 with 10% acetonitrile (Merck, South Granville, NSW, Australia) and 0.05% TFA was changed to 0.1% TFA without acetonitrile. Eluent B was 60% acetonitrile containing 0.05% TFA. A linear gradient was applied from 0 to 80% eluent B over 100 min. The detection device was an ultraviolet-visible detector set at 215 nm.

7.2.10. ***Isolation and purification of ACE-inhibitory peptides***

A sample size of 100 µL (80 mg freeze-dried WSE in 1 mL of solvent A) was injected into a reverse-phase column and 17 fractions were collected. This step was repeated 10 times and the 17 fractions from the various chromatographic runs were pooled and concentrated using a vacuum evaporator (SpeedVac SC110 concentrator, Savant Instruments Inc., Farmingdale, NY, USA) to a final volume of ~1 mL and filtered through a low protein binding 0.2 µm filters (Millipore Corp.). An aliquot of the concentrated

fractions (35 µL) was used in the determination of the ACE-inhibitory activity. The fractions with the highest ACE-inhibitory activity were subjected to further separation and purification by RP-HPLC. The chromatographic conditions used were as described in section 7.2.9.

7.2.11. ***Identification of peptides***

The molecular mass of purified peptides was analysed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics Inc., Billerica, MA, USA) under the condition described in section 6.2.7.

Peptide sequences were obtained by using chemically assisted fragmentation (CAF) in conjunction with MALDI-TOF-MS (Conrotto & Hellman, 2005). Ettan^{TM} CAF MALDI sequencing kit (Amersham Biosciences, Uppsala, Sweden) was used to derivatize purified peptides. Peptides were bound to a prepacked reversed-phase C18 matrix in pipette tips (ZipTip™ pipette tips; Millipore Corp.). Acquisition of spectra in reflectron and post source decay (PSD) modes was performed using an Ettan MALDI-TOF-MS. For each analysis, the selected mass of the peptide from the reflectron spectrum was subjected to PSD mode. Mass differences between the fragment ions were calculated to determine the peptide sequence.

Purified peptides were also identified by an automated Edman degradation method (Yarwood, 1989) using a protein sequencer (490 Procise, Perkin Elmer Co. Ltd., Applied Biosystem Division, Foster City, CA, USA) with a PTH-C18 column (2.1 x 220 mm; Perkin Elmer Co. Ltd.) as described in section 6.2.7.

7.2.12. ***Statistical analysis***

Analysis of variance was carried out to analyze viable counts of probiotic and starter lactococci during production. When significant differences were found among treatments, means were compared using Tukey's test (P = 0.05). The composition of cheeses, viable counts, concentration of organic acids, concentration of soluble nitrogen, hydrolysis of casein, sensory evaluation scores and the ACE-inhibitory activity of the cheeses during ripening at 4 and 8^{o}C for 24 wk were analyzed as a split plot in time design using General Linear Model procedure of the SAS system (SAS Inst. Inc., Cary, NC, USA). Multiple comparisons of means were achieved using pdiff option (t – test). The main plot factors were probiotics and replications (blocks) with ripening temperature as the split-plot factor. This design was additionally split with ripening time as additional split-plot factor.

The statistical model (split-plot linear model) used was:

$$Y_{ijkl} = \mu + P_i + B_j + \delta_{ij} + T_k + (P*T)_{ik} + (P*B)_{jk} + \gamma_{ijk} + t_l + (t*P)_{il} + (t*B)_{jl} + (t*T)_{kl} + (P*T*t)_{ikl} + \varepsilon_{ijkl}$$

where Y_{ijk} = response for probiotic i (i = 1, 2, 3, 4, 5, 6 or 7), block j (j = 1, 2 or 3), temperature k (k = 1, 2) and time l (l = 1, 2, 3, 4); μ = constant (population mean); P_i, B_j, T_k, t_l, $(P*T)_{ik}$, $(P*B)_{ik}$, $(t*P)_{il}$, $(t*B)_{jl}$, $(t*T)_{kl}$, $(P*T*t)_{ikl}$ = the effects of the probiotic i, block j, temperature k, time l and interaction of probiotic x temperature, probiotic x block, time x probiotic, time x block, time x temperature, probiotic x temperature x time, respectively; and δ_{ij}, γ_{ijk}, ε_{ijkl} = the main plot and two subplots random components, respectively. All significant differences were at least $P < 0.05$. A total of 21 batches of cheeses were made from the seven variations (Table 7.1). All results presented are means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM).

7.3. Results and discussions

7.3.1. ***Cheese composition during ripening***

The composition of control and probiotic Cheddar cheeses after ripening at 4 and 8ºC for 24 wk is summarized in Table 7.2. The percentages of moisture, salt, salt in moisture phase (SM), fat and protein of the fresh cheeses (before ripening) among batches were not significantly different ($P > 0.05$). The result confirms with our previous findings that addition of probiotic microorganisms did not affect the main compositional variables of Cheddar cheeses (Chapter 3.0, section 3.2.1 & Chapter 4.0, section 4.2.1). At the end of ripening period of 24 wk, the percentage salt, fat and protein remained relatively constant ($P > 0.05$). The moisture content, however, reduced significantly in all cheeses ripened at 8ºC ($P < 0.05$) (Table 7.2; Table 7.5). With the reduction in the moisture content, percentages of fat and protein in the cheeses increased slightly but insignificantly after 24 wk.

The salt in cheese influences cheese ripening through its effect on water activity. The salt concentration influences microbial growth, various enzyme activities and proteolysis of cheeses. Lower SM has been correlated with higher microbial growth, increased acid production, increased proteolysis and increased bitterness (Mistry & Kasperson, 1998). The percentages of salt and SM in this study were not significantly different among batches even after ripening for 24 wk ($P > 0.05$) (Table 7.2). Any changes

in the viability of the probiotic and starter organisms, organic acid profiles and proteolysis of the cheeses were thus not due to the concentration of salt in the cheeses.

The pH of the cheeses at day 1 was similar among the treatments ($P > 0.05$). The pH of the cheeses reduced significantly after 24 wk of ripening at 4 and 8°C ($P < 0.05$) (Figure 7.1; Table 7.5). According to Fox et al. (1993), about 98% of lactose is removed through the whey as lactose or lactate during draining. Acid production during ripening was the result of residual lactose fermentation by starter and probiotic microorganisms. There were significant effects of the type of probiotic microorganisms used, ripening temperatures and their interactions on the pH of the cheeses ($P < 0.05$) (Table 7.5). The pH of the cheeses with *B. longum* 1941 (Batch 2), *B. animalis* B94 (Batch 3), *Lb. casei* 279 (Batch 4) or *Lb. acidophilus* L10 (Batch 7) ripened at 8°C was significantly lower ($P < 0.05$) than that ripened at 4ºC after 24 wk (Figure 7.1). The pH of the cheese made with *Lb. casei* 279 (Batch 4) was less than 4.6 and this value was much lower than that of commercial Cheddar cheeses. At higher ripening temperature probiotic microorganisms and starter lactococci had higher metabolic activity, which probably contributed to the increased acidification. The decrease in pH can also be attributed to increased organic acid production.

The drop in pH after 24 wk was probably one of the factors that caused the significant reduction in moisture content. Small portions of whey were observed in the packages of the cheeses stored at 8°C after 24 wk probably due to the induced protein to protein bonds in the casein matrix as the pH decreased that led to syneresis. VanVliet and Walstra (1994) made a similar observation of an increased rate of syneresis in skim milk gels as the pH decreased.

7.3.2. ***Survival of bacteria during manufacture of Cheddar cheese***

Average counts of starter lactococci and probiotic in the inoculated-milk were 7.5 $\log_{10}$ cfu g^{-1} and 7.0 $\log_{10}$ cfu g^{-1}, respectively (Table 7.3). There were no significant differences ($P < 0.05$) in the counts of starter culture and those of probiotic organisms in the milk among batches indicating that all cheeses were made with similar concentration of starter culture and probiotic bacteria. During milk ripening (31°C, 45 min) the counts of starter culture increased by about half a log cycle in all cheeses. The counts of probiotic organisms after milk ripening, however, remained relatively constant. Cooking increased the temperature of the cheese curds to 38°C. Increase in temperature accelerated metabolism of bacteria enclosed within the curd. The counts of starter bacteria in the cooked curds increased by more than one log cycle, whereas those of probiotic organisms only increased

by half to one log cycle. The counts of starter organisms continued to increase during cheddaring (38°C, 90 min), while there was only a slight increase in the counts of probiotic organisms. The significant increase in the starter lactococci counts during production shows that starter lactococci were the primary acid producers throughout the cheese-making process. Their performance was also not affected by the addition of any of the probiotic adjuncts. There was about 8 $\log_{10}$ cfu g^{-1} of the probiotic bacteria in the whey. The count of probiotic in cheeses remained at the level of 8 to 9 $\log_{10}$ cfu g^{-1} at the end of the manufacturing process.

7.3.3. ***Survival of bacteria during ripening***

The counts of starter lactococci decreased by about two log cycles after 24 wk of ripening at 4 and 8ºC (Table 7.4), probably due to the combination of low pH, high NaCl and lack of fermentable carbohydrate. Despite the variation in the type of added probiotic adjunct, the counts of starter lactococci in different batches of the cheeses were not significantly different ($P > 0.05$). The counts of starter lactococci were, however, affected by the ripening temperature ($P < 0.05$) (Table 7.5). Lactococci counts of cheeses produced with *B. animalis* B94 (Batch 3), *Lb. casei* L26 (Batch 5) or *Lb. acidophilus* 4962 (Batch 6) ripened at 8ºC were significantly lower than those ripened at 4ºC after 24 wk ($P < 0.05$).

The probiotic counts of cheeses with different probiotic microorganisms were not significantly different throughout the ripening period ($P > 0.05$) (Table 7.4) (Table 7.5). Ripening temperature also did not affect the counts of probiotic in all cheeses ($P > 0.05$). The results show that cheeses can become an effective carrier for probiotic to consumer. Cheeses also have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to consumer. Cheeses have higher pH, a more solid consistency, a higher fat content and a higher buffering capacity than yoghurt, which would offer more protection to probiotic organisms in the gastrointestinal tract. Addition of 5 g cheese to 10 mL of gastric juice increased the pH from 2.00 to 4.74, whereas 5 mL of yoghurt increased the pH to only 3.65 (Gardiner et al., 1998).

Another group of microorganisms present in the cheeses was the NSLAB (see review in section 2.4.1). The contribution of NSLAB to ripening only increases with longer ripening and higher temperatures. NSLAB use residual lactose, sugars from the glycomacropeptide of casein and glycoproteins of the milk-fat globule membrane, autolysis and proteolytic products formed during ripening (Peterson & Marshall, 1990). The effects on flavour and texture can be positive, negative or neutral, depending on the type of strains

that predominate and their associated ripening properties. The initial level of NSLAB was 3.10 ± 0.32 $\log_{10}$ cfu g^{-1} and increased to 5.39 ± 0.05 $\log_{10}$ cfu g^{-1} and 6.09 ± 0.41 $\log_{10}$ cfu g^{-1} in 24 wk for the control cheeses (Batch 1) ripened at 4 and 8^{o}C, respectively. The initial counts of NSLAB in cheeses with the addition of *Lb. casei* 279 (Batch 4), *Lb. casei* L26 (Batch 5), *Lb. acidophilus* 4962 (Batch 6) and *Lb. acidophilus* L10 (Batch 7) could not be obtained from LBS agar used for the enumeration of NSLAB because the probiotic out-numbered their growth in those media at the beginning of the ripening period. At 24 wk, the counts of NSLAB in these cheeses (Batches 4-7) were obtained by subtracting the count of probiotic organisms in selective media from the count of total lactobacilli on LBS agar. The counts of NSLAB in probiotic cheeses (Batches 2-7) were about 7.5 – 8.5 $\log_{10}$ cfu g^{-1} at 24 wk (Table 7.4) and there was no significant difference in counts of NSLAB at 4 or 8^{o}C after 24 wk ($P > 0.05$) (Table 7.5). Law (2001) found that NSLAB increased more rapidly at 13^{o}C than at 6^{o}C. Cromie et al. (1987) reported that NSLAB counts increased more rapidly in cheese stored at higher temperatures (15-20^{o}C) as compared to control cheese (8^{o}C). The difference in ripening temperatures (4 & 8^{o}C) used in this study was not large enough to give any statistical significance to the growth of NSLAB. The level of NSLAB in our cheeses was comparable to commercially produced Cheddar cheeses (Peterson & Marshall, 1990).

7.3.4. ***Production of organic acid***

The effect of different probiotic organisms and ripening time and temperature on the organic acid contents of the control and probiotic cheeses is presented in Figures 7.2-7.5. The main organic acids in probiotic Cheddar cheeses throughout ripening were lactic, acetic, citric and butyric acids. Our results are consistent with the findings of Bouzas et al. (1993) and Lues and Bekker (2002). An increase in concentration during ripening was observed in all organic acid analyzed except for citric acid. Lactic acid was present in much greater abundance than acetic, citric, butyric, formic, propionic and succinic acid. *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* metabolize lactose to L(+) lactic acid; the glucose moiety is metabolized via the Embden-Meyerhof (EM) pathway while galactose is metabolized via the tagatose pathway. *Lactobacillus* sp. on the other hand, converts galactose via the Leloir pathway to glucose-6-P, which is then metabolized to DL-lactate via the EM pathway (Fox et al., 1993). The lactic acid content increased significantly in cheeses stored at 4 and 8^{o}C after 24 wk ($P < 0.05$) (Figure 7.2). There were significant effects of the type of probiotic microorganisms used, ripening time, ripening temperatures

and their interactions on the concentration of lactic acid in the cheeses ($P < 0.05$) (Table 7.5). Probiotic cheeses had higher concentration of lactic acid especially in those with *Lb. casei* 279 (Batch 4) or *Lb. casei* L26 (Batch 5) ripened at 8^{o}C (Figure 7.2).

Acetic acid can be produced from citrate, lactose and amino acids (Aston & Dulley, 1982). Acetic acid was detected in all cheeses at levels which increased with time ($P < 0.05$) (Figure 7.3). There were significant effects of the type of probiotic microorganisms used, ripening time, ripening temperatures and their interactions on the concentration of acetic acid in the cheeses ($P < 0.05$) (Table 7.5). Concentration of acetic acid in cheeses ripened at 8^{o}C was significantly higher than those stored at 4^{o}C ($P < 0.05$). Concentration of acetic acid in cheeses made with *Bifidobacterium* sp. (Batches 2 and 3) and *Lb. casei* (Batches 4 and 5) were significantly higher ($P < 0.05$) than that of the control cheese (Batch 1). Desai et al. (2004) reported that some strains of *Lb. casei* were able to produce acetic acid as their metabolic end products. *Bifidobacterium* produces acetic and lactic acids from lactose via a fructose-6-phosphate shunt pathway (Chapter 2.0, section 2.5.1). The fermentation pathway results in 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose, therefore generating a theoretical molar ratio (acetic:lactic) of 3:2 (Scardovi & Trovatelli, 1965). This increase could also be due to the higher amount of free amino acids in cheeses produced with the addition of probiotic adjuncts, which might have served as precursors for the formation of acetic acid. Molimard and Spinnler (1996) confirmed that acetic acid can be produced from the degradation (oxidative deamination or decarboxylation) of amino acids (alanine and serine).

Citrate in milk is metabolized by lactic acid bacteria into flavour components, such as acetic acid, acetaldehyde and diacetyl (Hugenholz, 1993). The percentage of citric acid decreased significantly after ripening for 24 wk at both 4 and 8^{o}C ($P < 0.05$) (Figure 7.4). Our finding is in agreement with the result reported for citric acid in Cheddar (Lues & Botha, 1998). Thomas (1987) reported that citrate in Cheddar cheese decreased slowly to almost zero at 6 mo, presumably as a result of metabolism by lactobacilli which became the major component of the NSLAB flora. Ripening at 8^{o}C as compared to 4^{o}C, however, did not significantly affect the disappearance rate of citric acid in the cheeses ($P > 0.05$) (Table 7.5). The disappearance rate of citric acid between cheeses stored at 12^{o}C was significantly higher than those stored at lower temperature in the study performed by Bouzas et al. (1993). The ripening temperatures used in that study was, however, much higher than that used in our study. There was no significant difference in the concentration of citric acid between cheeses with different probiotic adjuncts ($P > 0.05$).

The concentration of butyric acid in the cheeses after ripening for 24 wk at 4 and 8^{o}C is shown in Figure 7.5. The most important low-molecular-weight fatty acid formed by lipase from milk, starter and NSLAB that contributes to the aroma and flavour of Cheddar cheese is butyric acid (Bhowmik & Marth, 1990). Butyric acid can be produced by the lipolytic activity of cheese microflora on milk fat. The butyric acid content of the probiotic cheeses was not significantly different to the control cheese after 24 wk ($P > 0.05$). Ripening temperatures, however, have a significant effect on the concentration of butyric acid in the cheeses ($P < 0.05$) (Table 8.5). Some *Lactobacillus* strains were reported to have lipolytic activity, which increased the rate of fat hydrolysis (Kondyli et al., 2002). Butyrate production might also be accounted by other mechanisms. Intracellular lipases excreted during the death of starter bacteria might be responsible for the increased concentration of butyric acid. Aston and Dulley (1982) reported that free fatty acids were produced in Cheddar cheese slurries manufactured from milk whose fat had been replaced by vegetable lipids. These lipids contain no fatty acids of chain length less than C_{12} thus eliminating lipolysis as a direct source. The free fatty acids such as butyric acid might also be produced via the deamination of amino acids liberated through proteolysis in the cheeses.

A small amount of propionic acid and succinic acid was also detected in the cheeses (concentration too little to quantify). Propionic and succinic acids were only detected after 12 and 24 wk of ripening. Propionic and succinic acids production in the cheeses was probably due to non-starter contamination. Strains of *Lactobacillus* including *Lb. casei* produced propionic and succinic acids (Ocando et al., 1993).

7.3.5. ***Assessment of proteolysis***

7.3.5.1. Concentration of soluble nitrogen (SN)

Water soluble nitrogen, TCA-SN and PTA-SN are commonly used proteolytic indices of cheese ripening (Fox et al., 1993). The ratio of WSN, TCA-SN and PTA-SN to the total nitrogen (TN) of Cheddar cheeses during ripening at 4 and 8^{o}C for 24 wk is presented in Figure 7.6. Water soluble fraction of Cheddar cheeses contains numerous small and medium sized peptides, free amino acids and their degradation products. The level of WSN increased significantly in all cheeses after ripening for 24 wk ($P < 0.05$) (Figure 7.6a). About 14% of the TN in fresh cheese (before ripening, day 1) was extracted as WSN, which increased to about 58 and 62% after 24 wk of ripening at 4 and 8^{o}C, respectively. Statistical analysis of the data (Table 7.6) shows that the level of WSN was not affected by ripening

temperatures, probiotic microorganisms and their interaction ($P > 0.05$). The result shows that addition of probiotic did not influence the proteolysis of the cheeses at this level. This was expected because WSN is primarily the product of proteolysis from residual rennet or by proteinases present in the curd such as plasmin or cell envelope proteases from the starter microorganisms (Sousa et al., 2001). The ripening temperature also did not significantly affect the level of WSN between cheeses stored at 4 and 8^{o}C ($P > 0.05$). Folkertsma et al. (1996) found that Cheddar cheeses made from pasteurised milk and ripened at 12 or 16^{o}C developed higher levels of WSN than those ripened at 8^{o}C. The ripening temperature used in that study, however, was much higher than that used in our study.

Trichloroacetic acid-soluble nitrogen increased progressively in all cheeses during ripening (Figure 7.6b) at a rate dependant on the ripening temperature used (Table 7.6). Cheeses ripened at 8^{o}C had higher TCA-SN than those ripened at 4^{o}C after 24 wk of ripening. About 5% of the TN was soluble in 12% TCA in fresh cheeses and increased to about 13 and 16% after 24 wk at 4 and 8^{o}C, respectively (Figure 7.6b). TCA-SN of cheeses made with *Lb. casei* 279, *Lb. casei* L26, *Lb. acidophilus* 4962 or *Lb. acidophilus* L10 (Batches 4, 5, 6 & 7) ripened at 8^{o}C was significantly higher than that stored 4^{o}C after 24 wk of ripening ($P < 0.05$). Fox, et al. (1995) reported that rennet is responsible for the production of some of the 12% TCA-SN and the level of this fraction is higher in cheeses acidified by starter than in chemically acidified cheeses indicating that bacterial proteinases and peptidases are also responsible for the formation of some of the 12% TCA-SN. TCA-SN of cheeses made with *B. animalis* B94, *Lb. casei* 279, *Lb. casei* L26 or *Lb. acidophilus* L10 (Batches 3, 4, 5 & 7) was higher than that of the control cheeses after 24 wk of ripening at 4 and 8^{o}C. There was, however, no significant effect ($P > 0.05$) of the type probiotic microorganisms used on the concentration of TCA-SN (Figure 7.6b; Table 7.6). Further ripening would probably contribute to the statistical significant of the effect of probiotic microorganisms to the concentration of TCA-SN.

The level of PTA-SN has been widely used as an index of free amino acids (FAA) in cheeses (Fox et al., 1993). PTA-SN is produced primarily by the action of microbial peptidases. Peptides greater than 600 Da are precipitated in 5% PTA. PTA-SN increased with time in all cheeses and was higher in probiotic cheeses (Batches 2-7), especially in those ripened at higher temperature (Batches 2B-7B; Figure 7.6c). There were significant ($P < 0.05$) effects of the type of probiotic microorganisms used, maturation time, maturation temperatures and their interactions on the concentration of PTA-SN (Figure 7.6c; Table 7.6). About 1.5% of the TN was soluble in 5% PTA in fresh cheeses and increased to about 2.9% and 4.4% after 24 wk of ripening at 4 and 8^{o}C, respectively. PTA-SN of cheeses made

with *B. animalis* B94, *Lb. casei* 279 or *Lb. casei* L26 (Batches 3, 4 & 5) was significantly higher than that of the control cheese (Batch 1) after 24 wk of ripening at 4 and 8°C ($P < 0.05$). The results suggested that probiotic microorganisms contribute to the release of small peptides and free amino acids in Cheddar cheeses. Similarly, other studies reported that probiotic adjunct could contribute to the increased level of PTA-SN (Gardiner et al., 1998). Ripening temperature of 8°C accelerated the proteolytic activity of probiotic microorganisms in the cheeses, especially in those with the addition of *Lb. casei* sp. (Batches 5B & 6B).

7.3.5.2. SDS-PAGE of cheese samples during ripening

SDS-PAGE of the control cheeses (Batches 1A, B) and probiotic cheeses with *B. animalis* B94 (Batches 3A, B), *Lb. casei* L26 (Batches 5A, B) or *Lb. acidophilus* L10 (Batches 7A, B) during the ripening period of 24 wk at 4 and 8°C are shown in Figure 7.7. SDS-PAGE of Batches 3A, B are similar to Batches 2A, B; Batches 5A, B are similar to Batches 4A, B; and Batches 7A, B are similar to Batches 6A, B. Thus only SDS-PAGE diagram of Batches 1A, B; Batches 3A, B; and Batches 5A, B is presented. Various protein fractions as separated by electrophoresis are labeled as bands 1 to 10 in Figure 7.7. Protein bands were identified based on the molecular weight (MW). The intensities of both α_{s1}-CN (band 1) and β-CN (band 2) bands decreased throughout the ripening period (Figure 7.7). As the concentration of α_{s1}-CN and β-CN decreased, the concentration of intermediate size or low molecular weight breakdown products of the casein increased. These products appeared in SDS-PAGE in the area between β-CN (band 2) and β-lactoglobulin (band 8). The α_{s1}- CN was hydrolyzed faster than β-CN in all cheeses as shown by the disappearance of the α_{s1}- CN band even at the early stage of ripening period (Figure 7.7). This was especially obvious for cheeses made with the addition of *Lb. casei* sp. (Figure 7.7; Batches 5A, B) or *Lb. acidophilus* sp. (Figure 7.7; Batches 7A, B).

The difference in electrophoretic diagrams of control cheeses stored at 4°C and 8°C was obvious. As the intensity of α_{s1}-CN (band 1) and β-CN (band 2) bands decreased, band 6 started to appear after 24 wk in the control cheeses ripened at 8°C (Batch 1B), but not in those ripened at 4°C (Batch 1A). Band 6 appeared as early as 12 wk in cheeses with the addition of *Lb. casei* sp. and 24 wk in all other probiotic cheeses ripened at 4 and 8°C. The disappearance of bands 3, 4 and 8 was also faster in probiotic cheeses than the control, especially when the cheeses were stored at 8°C (Figure 7.7). The disappearance of these

bands (3, 4, 8) probably led to the formation of smaller peptides (bands 9 & 10) or other peptides smaller than 7400 Da that could not be detected in SDS-PAGE gel used.

Percentage hydrolysis of α_{s1}-CN and β-CN in the control cheese (Batch 1) and probiotic cheeses (Batches 2-7) after 24 wk of ripening at 4 and 8^{o}C is presented in Figure 7.8. Percentage hydrolysis of α_{s1}-CN and β-CN increased significantly with time ($P < 0.05$) (Table 7.6). Hydrolysis of α_{s1}-CN in the cheeses was more extensive at the early stage of ripening (during the first 12 wk) (Figures 7.8a & 7.8b). Statistical analysis of the data shows that there were significant effects of the type of probiotic microorganisms used, maturation time, maturation temperatures and their interactions on the degree of hydrolysis of α_{s1}-CN ($P < 0.05$) (Table 7.6). Cheeses made with *Lb. casei* sp. (Batches 4 & 5) had the highest degree of α_{s1}-CN hydrolysis followed by cheeses made with *Lb. acidophilus* sp. (Batches 6 & 7) or *Bifidobacterium* sp. (Batches 2 & 3) and control cheese (Batch 1). This was expected because *Lb. casei* sp. had higher proteolytic activity than other probiotic microorganisms used in this study. The results from OPA-based spectrophotometric assay showed that *Lb. casei* sp. released the highest amount of free amino groups followed by *Lb. acidophilus* or *Bifidobacterium* sp. after 18 h incubation in RSM at 37^{o}C (section 4.2.5). Percentage hydrolysis of α_{s1}-CN in cheeses made with *Lb. casei* sp. ripened at 8^{o}C was significantly higher than those ripened at 4^{o}C ($P < 0.05$). About 75% of the α_{s1}-CN was hydrolysed in cheeses with *Lb. casei* sp. (Batches 4B & 5B) after ripening for 24 wk at 8^{o}C compared with about 65% in those ripened at 4^{o}C (Batches 4A & 5A).

Only about 30% of the β-CN was hydrolysed in all cheeses (Batches 1-7) as compared to more than 50% of the α_{s1}-CN, which shows that bacterial proteinases and peptidases have preference over α_{s1}-CN than β-CN. According to Fox et al. (1993), β-CN is more resistant to proteolysis than α_{s1}-CN. There were significant effects of the type of probiotic microorganisms used, maturation time, maturation temperatures and their interaction on the degree of hydrolysis of β-CN ($P < 0.05$) (Table 7.6). About 30% and 38% of the β-CN were hydrolysed after ripening for 24 wk at 4 and 8^{o}C, respectively. Percentage hydrolysis of β-CN in control cheese (Batch 1) and cheeses made with *B. longum* 1941 (Batch 2), *B. animalis* B94 (Batch 3) or *Lb. acidophilus* 4962 (Batch 6) ripened at 8^{o}C was significantly higher than that ripened at 4^{o}C.

7.3.6. ***Sensory evaluation for specific flavour and texture attributes of Cheddar cheeses***

Acceptability of the control and probiotic cheeses stored at 4 and 8^{o}C after ripening for 6, 12 and 24 wk is shown in Table 7.7. Acceptability of the cheeses increased as the ripening time increased ($P < 0.05$). Acceptability of cheeses with probiotic microorganisms was comparable to that of the control cheese ($P > 0.05$) after 24 wk of ripening at 4^{o}C. Acceptability scores of cheeses with *B. animalis* subsp. *lactis* (Batch 3B) or *Lb. casei* 279 (Batch 4B) were significantly lower than that of the control cheese (Batch 1B) ($P < 0.05$) after 24 wk of ripening at 8^{o}C possibly due to the higher concentration of acetic acid in cheeses with *B. animalis* subsp. *lactis* or *Lb. casei* 279. The acceptability of cheeses ripened at 8^{o}C was not significantly different to those ripened at 4^{o}C ($P > 0.05$) except for the cheeses with *Lb. casei* L26 (Batch 5). The acceptability of cheeses with *Lb. casei* L26 ripened at 8^{o}C (Batch 5A) was significantly higher than that ripened at 4^{o}C (Batch 5A) after 24 wk of ripening.

A wide range of descriptive vocabulary (attributes) for Cheddar flavour has been defined by Delahunty and Murray (1997) and Murray and Delahunty (2000) as discussed in Chapter 5.0, section 5.2.2. Drake et al. (1996) selected the specific attributes including bitter, sour-acid, oaky/nutty, firmness and crumbliness for the sensory evaluation of Cheddar cheeses made with the addition of adjunct *Lactobacillus*. In the present study, similar terms were used. Vinegary attribute was added to the attribute list to find out the correlation between acetic acid concentrations obtained from instrumental analysis and human sensory perception of vinegary taste of the cheeses. The attribute oaky/nutty, which corresponded to the flavour characteristic of premium quality Cheddar cheese (Bodyfelt et al., 1988), was changed to “cheddary”, which was defined as the general flavour of Cheddar cheese (Hulin-Bertaud et al., 2000).

Scores for flavour attributes (cheddary, bitterness, sour-acid and vinegary) of the cheeses ripened at 4 and 8^{o}C at 6, 12 and 24 wk are shown in Figure 7.9. Scores for cheddary increased as the ripening period increased in all cheeses (Figure 7.9a). At the early stages of ripening (6 & 12 wk), the cheddary scores between different batches of the cheeses were not significantly different ($P > 0.05$). After 24 wk of ripening, scores of cheddary for cheeses ripened at 8^{o}C were higher than those ripened at 4^{o}C. There was a significant time x temperature effect on the cheddary scores of the cheeses (Table 7.9). During ripening, more peptides and free amino acids were released into the serum of the cheeses with ripening time. Hydrolysis of caseins by rennet, plasmin and starter bacteria in the initial stages of maturation released a considerable quantity of peptides of large or

intermediate size. These peptides probably became the substrates for peptidases of the starter, NSLAB and probiotic bacteria, which gave rise to smaller peptides and free amino acids that contribute directly to the flavour or by acting as precursors of aromatic substances such as amines, acids, thiols, thioesters (Fox et al., 1993).

The scores for cheddary flavour for commercial cheeses used during the training session was 4.01 ± 0.49, 5.80 ± 0.43 and 8.80 ± 0.47 for "mild", "tasty" and "vintage" cheeses, respectively. The average scores of cheddary flavour for experimental cheeses ripened at 4^{o}C indicated that panelists perceived the cheeses as "mild" cheese and those ripened at 8^{o}C to be comparable to commercial "tasty" cheese in terms of their maturity. Data collected from the panelists showed that the preference for type of cheese, age and frequency of cheese consumption did not affect the sensory scores ($P > 0.05$). About 73% of sensory panelists consumed cheeses 1-2 times per wk, 87% were between the ages of 18 and 35 years and 50% preferred mild cheeses.

The correlation between the compositional analysis, level of organic acid and proteolysis to the sensory scores of the cheeses is presented in Table 7.8. There was a positive and significant correlation between the proteolysis indices including WSN, TCA-SN, PTA-SN, percentage hydrolysis of α_{s1}-CN and β-CN and the scores of cheddary flavour (Table 7.8). The results confirm that the products of proteolysis are important for flavour development of Cheddar cheeses (Aston & Creamer, 1986). Increase in ripening temperature, increased the proteolysis and thus the Cheddary scores of the cheeses. There was also a strong and positive correlation between the level of organic acids such as lactic, acetic and butyric acids to the scores of cheddary flavour (Table 7.8). Production of organic acids may occur in cheeses as a result of normal bacterial metabolism and breakdown of milk proteins, fat, lactose and citrate during manufacture and storage. These acids and breakdown products play an important role in the flavour of cheeses.

Bitterness in cheese results from the accumulation of hydrophobic short peptides, which can originate from α_{s1}-CN or β-CN (Broadbent et al., 2002). El-Soda et al. (2000) reported that lactobacilli extracts enhanced bitterness when incorporated into Cheddar cheese curd due to the release of the complex peptidase system of the *Lactobacillus*. It was expected that the increase in proteolysis with the addition of adjunct probiotic would probably increase the scores of bitterness for the cheeses. There was, however, no significant correlation between the level of proteolysis and the scores of bitterness for the cheeses (Table 7.8). The bitterness scores between cheeses with and without adjunct probiotic at both 4 and 8^{o}C were also not significantly different ($P > 0.05$). It is interesting to see that scores for bitterness reduced after 12 wk of ripening and increased again after 24

wk in most of the cheeses (Figure 7.9b). Residue 193-209 of the C-terminal region of β-casein and residue 1-9 of the C-terminal region of α_{s1}-casein have been associated with bitterness in cheeses (Lemieux & Simard, 1992). These peptides were found in 6 wk cheeses and were possibly formed by the action of chymosin (rennet). Hydrolysis of these peptides is associated with decreased bitterness in Cheddar cheeses. In our study, β-CN (193-209) and α_{s1}-CN (1-9) were isolated in all cheeses using reverse-phase HPLC and the concentration varied as the ripening time increased (Figure 7.15). These peptides were possibly broken down at the early stage of ripening resulting in lower bitterness scores after 12 wk. As the ripening time increased, other short peptides and amino acids were released into the cheeses and would possibly contribute to further increase in bitterness.

Scores of sour-acid in the probiotic cheeses were higher than that of the control cheese. Scores of sour-acid were higher in cheeses ripened at 8^{o}C as compared to 4^{o}C. Increase in ripening temperature from 4 to 8^{o}C, however, did not significantly affect (P > 0.05) the scores of sour-acid in the control cheeses and those with *Lb. acidophilus* (Batches 1, 6 & 7). Cheeses with the addition of *Bifidobacterium* sp. and *Lb. casei* sp. ripened at 8^{o}C for 24 wk (Batches 2B, 3B, 4B & 5B) had the highest scores of sour-acid (Figure 7.9c). Cheeses ripened at 8^{o}C also received higher vinegary scores especially in those with *Bifidobacterium* sp. or *Lb. casei* 279 (Batches 2B, 3B, 4B) (P < 0.05) (Figure 7.9d). The scores for sour-acidic and vinegary were mainly influenced by the concentration of lactic and acetic acids of the cheeses (Table 7.8).

Scores for texture attributes (hardness and crumbliness) of the cheeses stored at 4 and 8^{o}C after 6, 12 and 24 wk are shown in Figure 7.10. The most important contributor to the continuous casein matrix of a Cheddar cheese is α_{s1}-CN (Lawrence et al., 1987), and hydrolysis of this casein is believed to be responsible for the softening of Cheddar cheese texture (Grappin et al., 1985). Hardness in the cheeses decreased slightly as the ripening time increased, but not significantly (P > 0.05). Scores for crumbliness of the cheeses showed no discernable pattern. Statistical analysis of the data showed that the mean scores for hardness and crumbliness of the cheeses were not affected by the ripening temperatures and probiotic adjuncts (P > 0.05) (Table 7.9). There was a significant negative correlation between the level of WSN, TCA-SN, PTA-SN, percentage hydrolysis of α_{s1}-CN and β-CN and hardness scores showing that increased proteolysis with time was responsible for the softening of the cheeses.

*7.3.7. **ACE-inhibitory activity of control and probiotic cheeses during ripening***

Release of bioactive peptides as a result of the proteolytic activity of lactic acid bacteria (LAB) has become the subject of intense research due to the beneficial effects of using food-grade microorganisms to enrich foods with bioactive peptides (Gobbetti et al., 2002). Proteolytic system of LAB is very complex. Starter lactococci possess a cell envelope-associated proteinase (PrtP or lactocepin) that contributes to the formation of small peptides (probably by hydrolyzing larger peptides produced by chymosin or plasmin) and a multitude of intracellular peptidases that are responsible for the hydrolysis of short peptides and the liberation of amino acids in cheeses. The proteolytic system of probiotic bacteria has also been studied in detail by Shihata and Shah (2000). Cell envelope-associated proteinases with properties similar to the lactococcal lactocepins have been isolated from a number of strains of *Lactobacillus* (Kunji et al., 1996). Consequently these microorganisms could potentially generate a large variety of peptides including bioactive peptides.

The ACE-inhibitory activity of WSE of control (Batch 1) and probiotic cheeses (Batches 2-7) during ripening at 4 and 8^{o}C for 24 wk is shown in Figure 7.11a. The ACE-inhibitory activity of the cheeses increased significantly ($P < 0.05$) during ripening, especially during the first 6 wk and reached a maximum at 24 wk. Ripening temperatures had a significant effect ($P < 0.05$) on the percentage of ACE-inhibition of the cheeses during ripening (Table 7.11). Probiotic microorganisms had no significant effect ($P > 0.05$) on the percentage of ACE-inhibition of the cheeses at the early stage of ripening. Table 7.11, however, shows that there was a significant interaction ($P < 0.05$) of probiotic microorganisms with time, indicating the contribution of probiotic microorganisms to an increased level of ACE-inhibition of the cheeses as the ripening progressed. Percentage ACE-inhibition of cheeses made with the addition of *Lb. casei* 279 (Batch 4), *Lb. casei* L26 (Batch 5) or *Lb. acidophilus* L10 (Batch 7) was significantly higher ($P < 0.05$) than that of the control cheese (Batch 1) after 24 wk of ripening at both 4 and 8^{o}C.

The ACE-inhibitory activity of the cheeses is also expressed as IC_{50} (concentration of the sample required to inhibit 50% of the ACE activity). Figure 7.11b shows the IC_{50} value of the cheeses when the percentage ACE-inhibition was maximum during ripening (at 24 wk). IC_{50} of cheeses made with the addition of probiotic microorganims (Batches 2-7) were significantly lower ($P < 0.05$) than that of the control cheese (Batch 1) at 24 wk. IC_{50} of cheeses ripened at 8^{o}C was not significantly different to those ripened at 4^{o}C at 24 wk ($P > 0.05$). The lowest value of the IC_{50} and therefore the highest ACE-inhibitory activity

corresponded to the cheese made with the addition of *Lb. acidophilus* L10 (Batch 7, Figure 7.11b).

The higher percentage of ACE-inhibition in cheeses ripened at 8°C as compared to those ripened at 4°C was probably due to the accumulation of more ACE-inhibitory peptides in the 8°C cheeses as a result of the increased proteolysis. Previously we have reported that cheeses ripened at 8°C had higher level of proteolysis than those ripened at 4°C as indicated by the increase in TCA-SN, PTA-SN and percentage hydrolysis of α_{s1}-casein in the cheeses (section 7.3.5). It is interesting to note that the IC_{50} of the cheeses ripened at 4 and 8°C were, however, not significantly different ($P > 0.05$). The results indicated that most of the active peptides present in the cheeses ripened at 4°C were relatively similar to those ripened at 8°C.

Peptide profiles of control and probiotic cheeses obtained using RP-HPLC after 24 wk of ripening at 4 and 8°C are shown in Figure 7.12. Although cheeses made with probiotic bacteria had higher ACE-inhibitory activity, Figure 7.12 shows that the peptides profiles of the cheeses were very similar. Isolation and purification of ACE-inhibitory peptides were performed using only WSE of cheeses with the highest ACE-inhibitory activity.

7.3.8. Isolation of ACE-inhibitory peptides from cheese made with Lb. acidophilus *L10 adjunct*

Water soluble extract of cheese with the highest ACE-inhibitory activity (Batch 7, ripening temperature 8°C) was injected into RP-HPLC column in order to fractionate the peptides. Seventeen fractions were collected manually as shown in Figure 7.13a. The ACE-inhibitory activity of the 17 fractions was shown in Figures 7.13b. Several fractions distributed throughout the acetonitrile gradient exhibited high level of ACE-inhibition and low IC_{50} values, especially fractions 1, 4, 15 and 16. Percentage ACE-inhibition and IC_{50} of fractions 1, 4, 15 and 16 were 54% (IC_{50}, 0.17 mg mL^{-1}), 66% (IC_{50}, 0.22 mg mL^{-1}), 81% (IC_{50}, 0.19 mg mL^{-1}) and 73% (IC_{50}, 0.20 mg mL^{-1}), respectively. These fractions were purified using RP-HPLC followed by identification of peptides.

7.3.9. Identification of ACE-inhibitory peptides

Details of peptides identified from fractions with high ACE-inhibitory activity are shown in Table 7.10. Peptide from fraction 1 was identified using chemically assisted fragmentation (CAF) chemistry and MALDI-TOF. CAF-MALDI chemistry is based on the

introduction of a negatively charged group to the N-terminus of peptides generated by tryptic digestion. In this method, only y-ions, which retain a net positive charge, are separated and detected while N-terminal fragments are neutral and not detectable. With the generation of only y-ion series fragments, the mass between two peaks on the spectrum could be corresponded to the mass of individual amino acid (Conrotto & Hellman, 2005). An example of CAF-fragmentation mass spectrum used in the identification of peptides is shown in Figure 7.14. Molecular weight of the purified peak determined using MALDI-TOF-MS was m/z 854.67 (Figure 7.14a). The first five residues obtained using CAF-MALDI of fraction 1 were identified as Ala-Arg-His-Pro-His (Figure 7.14b). Following the sequence interpretation and molecular weight determination, this peptide was identified as Ala-Arg-His-Pro-His-Pro-His originating from κ-casein (CN) (f 96-102).

Tryptic digestion using Ettan^{TM} – CAF MALDI sequencing kit did not work on peptides in fraction 4. Trypsin predominantly cleaves proteins at the carboxyl side of the amino acid lysine and arginine, except when either is followed by proline. Fraction 4 was thus sequenced using the automated Edman degradation method (Yarwood, 1989). Fraction 4 contained a mixture of several peptides. The first five residues of fraction 4 obtained using the Edman N-terminal sequencing were Arg-Pro-Lys-His-Pro. Peptides from fraction 4 were thus identified as α_{s1}-CN (f 1-9), α_{s1}-CN (f 1-7) and α_{s1}-CN (f 1-6) with theoretical molecular weights of 1140.4, 875.1 and 746.9, respectively. Peptides from fractions 15 and 16 were both identified in a similar manner using the Edman N-terminal sequencing and CAF-MALDI sequencing methods. Peptides in fractions 15 and 16 were identified as α_{s1}-CN (f 24-32) and β-CN (f 193-209), respectively (Table 7.10). Most of these peptides have been identified in probiotic cheese with *Lb. casei* L26 (Chapter 6, Table 6.2).

7.3.10. ***Influence of probiotic organisms and ripening temperature on the formation of ACE-inhibitory peptides during ripening***

The total area of fractions 1, 4, 15 and 16 during ripening at 4 and 8^{o}C for 24 wk in control (Batch 1) and probiotic cheeses (Batches 2-7) is shown in Figure 7.15. The effects of probiotic microorganisms, ripening temperatures and their interaction on the formation of fractions 1, 4, 15 and 16 are shown in Table 7.11. All fractions (1, 4, 15 & 16) were detected even at the early stage of ripening, but their relative abundance shown by the total area of the peaks changed during the 24 wk of ripening. The total area of fraction 1, that was identified as κ-CN (f 96-102), increased as the ripening time increased ($P < 0.05$). The increase in the total area of fraction 1 during ripening was significantly affected ($P < 0.05$)

by the probiotic microorganisms and ripening temperatures (Table 7.11). Total areas of fraction 1 in probiotic cheeses (Batch 2-7) were significantly higher ($P < 0.05$) than those of the control cheeses (Batch 1) at both 4 and 8^{o}C (Figure 7.15a). After 24 wk of ripening, cheese made with the addition of *Lb. acidophilus* L10 (Batch 7) ripened at 8^{o}C had the highest total area of fraction 1 as compared to that of other cheeses (Figure 7.15a).

Total area of fraction 4, which contained a mixture of three peptides α_{s1}-CN (f 1-9), α_{s1}-CN (f 1-7) and α_{s1}-CN (f 1-6), decreased during the 24 wk of ripening at 4^{o}C. The total area of fraction 4 also decreased during the first 12 wk of ripening at 8^{o}C, but increased as the ripening progressed to 24 wk (Figure 7.15b). Changes in the total area of fraction 1 with time, was significantly affected by the probiotic microorganisms and ripening temperatures ($P < 0.05$) (Table 7.11).

Fractions 15 and 16 were isolated at a higher acetonitrile gradient. Total area of the peak of fraction 15 (α_{s1}-CN (f 24-32)) increased during the early stage of ripening (up to 12 and 6 wk in cheeses ripened at 4 and 8^{o}C, respectively) and decreased as the ripening progressed to 24 wk. Higher amount of fraction 15 was formed in cheeses ripened at 4^{o}C as compared to that ripened at 8^{o}C during the early stage of ripening. At the end of ripening period of 24 wk at 4 and 8^{o}C, control cheese (Batch 1) had the highest amount of fraction 15 as compared to other cheeses (Batches 2-7). Fraction 16 was detected in fresh cheeses before ripening. This peptide (β-CN (f 193-209)) accumulated in the cheeses especially during the first 12 wk of ripening and the amount remained constant after that period (Figure 7.15d).

7.3.11. ***Profiles of the isolated ACE-inhibitory peptides***

Caseins (α_{s1}-, α_{s2}-, β- and κ-CN) have been the major precursor for the generation of bioactive peptides. In this study, various peptides from α_{s1}-, β- and κ-CN were identified (Table 7.10). The ACE-inhibitory peptides isolated in this study showed various peptide sequences of varying lengths. The structure activity relationship for food-derived ACE inhibitors has not been well established. However, some general features have been reported (Meisel et al., 1997; FitzGerald et al., 2004). It appears that binding to ACE is strongly influenced by the C-terminal tripeptide sequence. Many substrates and competitive inhibitors of ACE contain hydrophobic amino acid in this region. The presence of the positive charge lysine and arginine as the C-terminal residue was also suggested to contribute to the ACE-inhibitory potency. Longer chain inhibitors may also contribute to the ACE-inhibitory potency (FitzGerald et al., 2004).

The peptide Ala-Arg-His-Pro-His-Pro-His corresponding to κ-CN (f 96-102) was identified in fraction 1 (Table 7.10). Reports on ACE-inhibitory peptides derived from hydrolysis of κ-CN are rare and correspond to very short sequences such as κ-CN (f 38-39), (f 25-34), and (f 24-26) (Gobbetti et al., 2002). Most of the bioactive peptides from κ-CN have antithrombotic activity. Most recently, a peptide from within this sequence, κ-CN (f 96-100) was isolated from probiotic yoghurt and was found to have *in vitro* ACE-inhibitory activity with an IC_{50} of 9.64 ± 3.67 μg mL^{-1} (Donkor et al., 2007). Lactocepins from a number of *Lactococcus* strains were found to be able to cleave Met^{95}-Ala^{96} and His^{102}-Leu^{103} of κ-CN (Fox et al., 1994). Peptide κ-CN (f 96-102) was detected at the early stage of ripening and accumulated in the cheeses throughout the ripening period. It is interesting to note that total area of the peak that corresponded to this peptide was significantly higher in probiotic cheeses indicating the possible contribution of proteolytic enzymes from probiotic bacteria in the production of peptide κ-CN (f 96-102) in cheese during ripening.

In the cheese system, α_{s1}-CN was hydrolyzed by chymosin at Phe^{23}-Phe^{24} to yield α_{s1}-CN (f 1-23) and α_{s1}-CN (f 24-199) (Minkiewicz et al., 2000). Lactococcal cell envelope proteinases and endopeptidases from probiotic bacteria are able to rapidly hydrolyze α_{s1}-CN (f 1-23) to several small peptides resulting in the formation and accumulation of peptides α_{s1}-CN (f 1-9) and α_{s1}-CN (f 1-13) in Cheddar cheese during ripening (Singh et al., 1997; Janer et al., 2005). Peptide α_{s1}-CN (f 1-9) isolated from Gouda cheese gave very low IC_{50} value of 13.4 μg mL^{-1} and antihypertensive effect of -9.3 ± 4.8 mm Hg on spontaneously hypertensive rats (SHR) (Saito et al., 2000). Peptide α_{s1}-CN (f 1-6) isolated from sheep sodium caseinate hydrolysate exhibited a potent ACE-inhibitory activity (IC_{50} of 30.1 μg mL^{-1}) and was resistant to hydrolysis by trypsin and chymotrypsin (Minervini et al., 2003). In the present study, three peptides from the same N-terminal region of α_{s1}-CN (f 1-23) were isolated, which corresponded to α_{s1}-CN (f 1-9) (f 1-7) and α_{s1}-CN (f 1-6) (Table 7.10). The total area of the peaks in fraction 4 only increased at the later stage of ripening (Figure 7.15b) probably due to the action of intracellular peptidases that were released in the cheeses after starter or probiotic cell lyses. Similar peptides were isolated from "Festivo" cheese, produced with a commercial starter mixture of *Lactococcus, Leuconostoc, Propionibacterium, Lactobacillus* sp. as well as probiotic strains of *Lb. acidophilus* and *Bifidobacterium* sp. (Ryahanen et al., 2001). Maximum ACE-inhibitory activity of "Festivo" cheese was found after 13 wk of ripening.

The peptide corresponding to α_{s1}-CN (f 24-32) probably originates from α_{s1}-CN (f 24-199) via hydrolysis by chymosin (cleavage sites Phe^{23}-Phe^{24} and Phe^{32}-Gly^{33}). This peptide has a high homology with the 12 residue peptide (C12) α_{s1}-CN (f 23-34) isolated

from a tryptic hydrolysate of casein (FitzGerald et al., 2004). Townsend et al. (2004) reported that peptide C12 had a potent ACE-inhibitory effect in hypertensive human. A C12 containing hydrolysate was orally administered (100-200 mg kg^{-1} C12 with 877 or 1754 mg alginic acid) on a daily basis to 10 hypertensive human volunteers over a 7 day cycle (5 of the possible treatments) resulting in a blood pressure reduction of 9 mm Hg. In the present study, peptide α_{s1}-CN (f 24-32) was detected in fresh cheese and accumulated during the early stage of ripening (12 wk) at 4 and 8^{o}C. The total area of fraction 15, however, decreased as the proteolysis progressed. Proteolytic enzymes from the probiotic adjunct were probably responsible for the decrease in total area of fraction 15. Carboxypeptidase activity in lactobacilli and in nonstarter lactic acid bacteria (NSLAB) in Feta cheese were reported to hydrolyze α_{s1}-CN (f 24-32) to α_{s1}-CN (f 24-30) and smaller fragments during ripening (Michaelidou et al., 1998). Peptides from within this sequence, α_{s1}-CN (f 24-27), (f 25-27), (f 27-30) produced from bovine casein of enzymatic hydrolysis, however, also showed bradykinin-potentiating activity on the uteri and ilea of rats (Maruyama et al., 1987; FitzGerald & Meisel, 2000).

A peptide derived from β-CN (f 193-209) was identified in fraction 16 (Table 7.10). Leu^{190}-Tyr^{191} and Leu^{192}-Tyr^{193} were known chymosin cleavage sites in solution (Exterkate, et al, 1997). Cell-wall-associated proteinases also appear to cleave Leu^{192}-Tyr^{193} in a solution (Fox et al., 1994). This peptide was present in the cheeses at the early stage of ripening, accumulated to its maximum after 12 wk and remained relatively constant until the end of the ripening period of 24 wk at both 4 and 8^{o}C. Peptides β-CN (f 193-209) has previously been isolated from casein by extracellular proteinase from *Lb. helveticus* CP790 and have shown to exhibit ACE-inhibitory activity with IC_{50} of 101 μg mL^{-1} (Yamamoto et al., 1994). In several cheese varieties, peptide β-CN (f 193-209) was associated with bitter taste defects (Singh et al., 2003).

7.4. Conclusions

The percentages salt, fat and protein remained relatively constant after 24 wk of ripening of cheeses. The moisture content and the pH of the cheeses reduced significantly in all cheeses ripened at 8ºC. Starter lactococci counts in cheeses produced with *B. animalis* B94, *Lb. casei* L26 or *Lb. acidophilus* 4962 ripened at 8ºC were significantly lower than those ripened at 4ºC after 24 wk. Probiotic microorganisms remained viable at the end of 24 wk and their viability was not affected by the ripening temperatures. Probiotic cheeses had higher concentration of lactic acid especially in those with *Lb. casei* 279 or *Lb. casei* L26 ripened at 8^{o}C. The acetic acid concentration in cheeses made with *Bifidobacterium* sp. and *Lb. casei* sp. was significantly higher than that of the control cheese at 4 and 8^{o}C. Ripening temperature of 8^{o}C as compared to 4^{o}C increased the level of proteolysis indicated by the increased TCA-SN, PTA-SN and percentage hydrolysis of α_{s1}-CN and β-CN in the cheeses. There were positive and significant correlations between the levels of WSN, TCA-SN, PTA-SN, lactic acid, acetic acid, butyric acid, percentage hydrolysis of α_{s1}-CN, β-CN and the scores of cheddary flavour. Scores of sour-acid and vinegary were significantly higher in cheeses with the addition of *Bifidobacterium* sp. and *Lb. casei* 279 ripened at 8^{o}C as compared to 4^{o}C. Both sour-acid and vinegary scores were positively and significantly correlated to the level of lactic and acetic acids. ACE-inhibitory activity of probiotic cheeses with *Lb. casei* 279, *Lb. casei* LAFTI® L26 and *Lb. acidophilus* LAFTI® L10 was significantly higher than that of the control cheese after 24 wk of ripening at 4 and 8^{o}C. The IC_{50} of the cheeses, however, was not significantly affected by the increasing ripening temperature from 4 to 8^{o}C. Cheeses made with the addition of *Lb. acidophilus* LAFTI®L10 ripened at 8^{o}C had the highest ACE-inhibitory activity. Ripening temperature of 4 and 8^{o}C can be applied successfully for the ripening of probiotic Cheddar cheeses.

Table 7.1. Control and probiotic Cheddar cheeses[1]

Probiotic adjunct (1.2% v/v)[2]	Ripening temperature	
	4°C	8°C
None (control)	Batch 1A	Batch 1B
B. longum 1941	2A	2B
B. animalis subsp. l*actis* LAFTI®B94	3A	3B
Lb. casei 279	4A	4B
Lb. casei LAFTI®L26	5A	5B
Lb. acidophilus 4962	6A	6B
Lb. acidophilus LAFTI®L10	7A	7B

[1] Cheeses were produced in triplicate with cheese starter culture (1.5% v/v) of mix *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Each batch of cheese was divided into two equal portion and stored at 4°C (A) and 8°C (B).

[2] 1.2 % v/v = 12 mL of bulk culture of RSM added to every liter of pasteurized milk.

Table 7.2. Composition of control and probiotic Cheddar cheeses ripened at 4 and 8ºC after 24 wk

Composition (%)	Ripening period	Ripening temperature (ºC)	Cheddar cheese[1]							SEM[2]
			Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	
Moisture	day 1	N/A	40.52^{A}	42.27^{A}	42.14^{A}	42.92^{A}	43.14^{A}	42.08^{A}	42.65^{A}	0.99
	wk 24	4	39.09^{AB}	39.78^{B}	39.30^{B}	39.28^{B}	40.62^{AB}	39.17^{B}	39.27^{B}	
	wk 24	8	37.94^{B}	38.31^{B}	38.22^{B}	39.62^{B}	39.95^{B}	39.25^{B}	39.82^{B}	
Salt	day 1	N/A	2.05^{A}	2.07^{A}	2.06^{A}	2.06^{A}	2.17^{A}	2.01^{A}	1.91^{A}	0.11
	wk 24	4	2.08^{A}	1.97^{A}	2.00^{A}	2.04^{A}	1.97^{A}	1.90^{A}	1.88^{A}	
	wk 24	8	1.94^{A}	1.97^{A}	1.96^{A}	2.02^{A}	2.13^{A}	1.97^{A}	1.89^{A}	
Salt in moisture	day 1	N/A	5.11^{A}	4.94^{A}	4.91^{A}	4.91^{A}	5.10^{A}	4.83^{A}	4.52^{A}	0.36
	wk 24	4	5.34^{A}	4.96^{A}	5.10^{A}	5.20^{A}	4.85^{A}	4.85^{A}	4.81^{A}	
	wk 24	8	5.10^{A}	5.13^{A}	5.12^{A}	5.35^{A}	5.35^{A}	5.01^{A}	4.74^{A}	
Fat	day 1	N/A	32.17^{A}	31.71^{A}	32.60^{A}	32.36^{A}	32.51^{A}	33.33^{A}	31.82^{A}	0.89
	wk 24	4	33.28^{A}	32.87^{A}	33.50^{A}	33.13^{A}	33.23^{A}	33.79^{A}	33.16^{A}	
	wk 24	8	33.37^{A}	32.95^{A}	33.90^{A}	33.50^{A}	34.20^{A}	34.13^{A}	33.13^{A}	
Protein	day 1	N/A	26.99^{A}	27.38^{A}	27.45^{A}	27.64^{A}	27.71^{A}	28.15^{A}	27.42^{A}	1.52
	wk 24	4	29.22^{A}	27.34^{A}	28.66^{A}	27.47^{A}	28.21^{A}	28.29^{A}	27.68^{A}	
	wk 24	8	26.89^{A}	28.05^{A}	27.53^{A}	28.45^{A}	28.32^{A}	28.72^{A}	28.31^{A}	

[1]Batch codes used are as in Table 7.1. Results are presented as means of a least 6 observations ($n \geq 6$).
[2]Pooled standard error of means
[AB]Means in column of the same composition with different superscript are significantly different ($P < 0.05$).

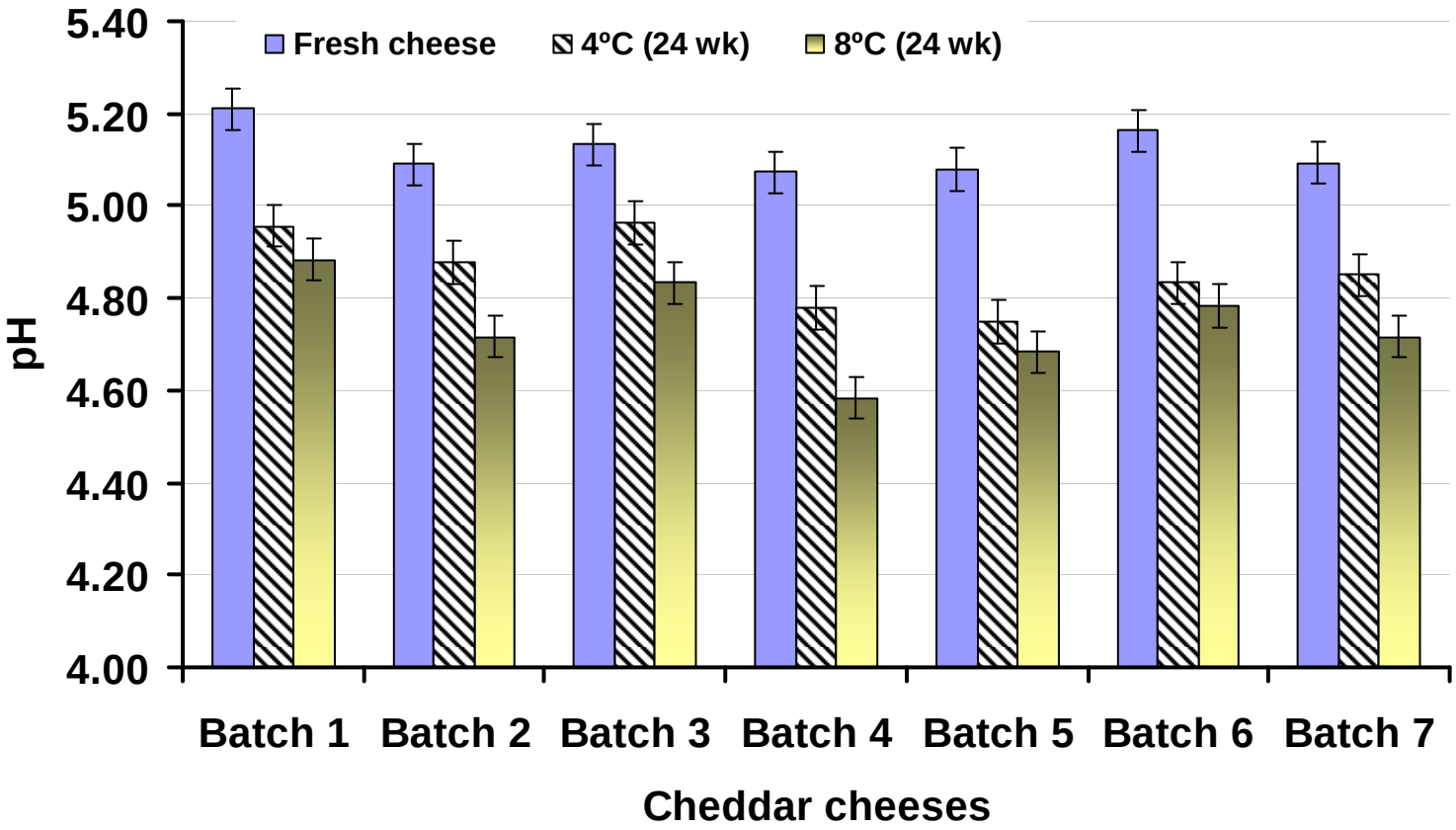


Figure 7.1. pH of control and probiotic Cheddar cheeses after ripening at 4 and 8ºC for 24 wk. Results are expressed as means of a least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.05). [abc], pH of fresh cheeses (day 1) and cheeses ripened for 24 wk at 4 and 8ºC with different superscripts are significantly different ($P < 0.05$).

Table 7.3. Viable counts (Log_{10} cfu g^{-1}) of cheese starter lactococci and probiotic microorganisms during manufacturing of Cheddar cheeses

Sample[1]		Viable counts (Log_{10} cfu g^{-1})					
		Inoculated milk	**Ripened milk**	**Cooked curd**	**Whey**	**Cheddared curd**	**Pressed curd (Fresh cheese)**
Batch 1	Starter	7.39 ± 0.05^{aA}	7.80 ± 0.03^{aA}	8.77 ± 0.04^{bA}	8.16 ± 0.07^{bA}	9.60 ± 0.07^{bA}	9.10 ± 0.63^{bA}
Batch 2	Starter	7.86 ± 0.02^{aA}	8.35 ± 0.15^{bB}	8.84 ± 0.08^{bcA}	8.25 ± 0.03^{abA}	9.68 ± 0.06^{cA}	9.73 ± 0.12^{cA}
	BL 1941	6.94 ± 0.10^{aA}	7.02 ± 0.07^{aA}	7.78 ± 0.15^{bA}	7.64 ± 0.15^{abA}	8.11 ± 0.16^{bA}	8.29 ± 0.29^{bA}
Batch 3	Starter	7.40 ± 0.20^{aA}	8.23 ± 0.13^{bB}	8.96 ± 0.03^{bcAB}	8.24 ± 0.01^{bA}	9.63 ± 0.06^{cA}	9.57 ± 0.12^{cA}
	LAFTI® B94	7.32 ± 0.03^{aA}	7.29 ± 0.04^{aA}	8.07 ± 0.13^{bA}	7.55 ± 0.02^{abA}	8.28 ± 0.05^{bA}	8.38 ± 0.12^{bA}
Batch 4	Starter	7.64 ± 0.07^{aA}	7.86 ± 0.04^{aA}	8.90 ± 0.05^{bcA}	8.38 ± 0.06^{bA}	9.59 ± 0.05^{cA}	9.59 ± 0.11^{cA}
	LC 279	7.26 ± 0.09^{aA}	7.36 ± 0.04^{aA}	8.18 ± 0.09^{bA}	7.87 ± 0.08^{abA}	7.99 ± 0.11^{bA}	8.01 ± 0.17^{bA}
Batch 5	Starter	7.83 ± 0.09^{aA}	8.25 ± 0.12^{abB}	9.13 ± 0.04^{cBC}	8.39 ± 0.14^{bA}	9.64 ± 0.03^{cA}	9.62 ± 0.05^{cA}
	LAFTI® L26	7.29 ± 0.09^{aA}	7.25 ± 0.04^{aA}	7.80 ± 0.08^{abA}	7.77 ± 0.04^{abA}	8.12 ± 0.05^{bA}	8.14 ± 0.10^{bA}
Batch 6	Starter	7.65 ± 0.10^{aA}	7.78 ± 0.06^{aA}	9.11 ± 0.02^{cBC}	8.33 ± 0.17^{bA}	9.65 ± 0.06^{dA}	9.74 ± 0.02^{dA}
	LA 4962	6.98 ± 0.05^{aA}	7.14 ± 0.15^{aA}	7.78 ± 0.10^{bcA}	7.66 ± 0.05^{bcA}	8.20 ± 0.13^{cB}	8.34 ± 0.15^{cA}
Batch 7	Starter	7.81 ± 0.08^{aA}	8.11 ± 0.08^{aB}	9.22 ± 0.02^{cC}	8.41 ± 0.12^{bA}	9.71 ± 0.05^{cA}	9.73 ± 0.02^{cA}
	LAFTI® L10	7.12 ± 0.16^{aA}	7.06 ± 0.09^{aA}	7.71 ± 0.09^{bA}	7.65 ± 0.05^{bA}	8.15 ± 0.21^{bA}	8.19 ± 0.33^{bA}

[abc] Means in rows with different superscripts are significantly different ($P < 0.05$).
[ABC] Means in column among counts of starter lactococci and among counts of probiotic with different superscripts are significantly different ($P < 0.05$).
[1] Batch codes used are as in Table 7.1. Results are expressed as mean ± standard error of means ($n \geq 6$).

Table 7.4. Viable counts (Log_{10} cfu g^{-1}) of starter lactococci, probiotic microorganisms and NSLAB during ripening period of 24 wk at 4 and 8°C in Cheddar cheeses

Ripening Period	Ripening Temperature (°C)	Viable counts (Log_{10} cfu g^{-1})							SEM[2]
		Batch 1[1]	Batch 2[1]	Batch 3[1]	Batch 4[1]	Batch 5[1]	Batch 6[1]	Batch 7[1]	
Starter lactococci									
day 1	-	9.10^{aAB}	9.72^{aA}	9.57^{aA}	9.59^{aA}	9.62^{aA}	9.74^{aA}	9.73^{aA}	0.26
wk 6	4	9.48^{aB}	9.43^{aA}	9.42^{aA}	9.38^{aA}	9.54^{aA}	9.4^{aAB}	9.60^{aA}	
wk 6	8	8.5^{aAC}	8.68^{aB}	8.50^{aB}	8.48^{aB}	8.73^{aB}	8.54^{aC}	8.49^{aB}	
wk 12	4	8.67^{aAC}	8.22^{aBC}	8.71^{aB}	8.05^{aB}	7.99^{aB}	9.05^{aBC}	7.95^{aBC}	
wk 12	8	8.10^{aCD}	7.61^{aCD}	7.46^{aC}	7.40^{aC}	8.05^{aBC}	7.08^{aD}	7.17^{aD}	
wk 24	4	7.93^{aCD}	7.69^{aCD}	8.64^{aB}	7.81^{aBC}	8.37^{aB}	8.65^{aC}	7.70^{aCD}	
wk 24	8	7.41^{aD}	7.15^{aD}	7.53^{aC}	7.11^{aC}	7.23^{aC}	6.81^{aD}	7.24^{aD}	
Probiotic microorganisms									
day 1	-	-	8.29^{aA}	8.38^{aA}	8.01^{aAB}	8.14^{aA}	8.34^{aA}	8.19^{aA}	0.28
wk 6	4	-	8.31^{aA}	8.58^{aA}	7.97^{aAB}	8.28^{aA}	8.58^{aA}	8.31^{aA}	
wk 6	8	-	8.38^{aA}	8.81^{aA}	8.23^{aA}	8.54^{aA}	8.24^{aA}	8.27^{aA}	
wk 12	4	-	8.10^{aA}	8.26^{aA}	7.84^{aAB}	8.22^{aA}	8.46^{aA}	7.62^{aA}	
wk 12	8	-	8.11^{aA}	8.35^{aA}	8.59^{aA}	8.46^{aA}	7.99^{aA}	7.67^{aA}	
wk 24	4	-	8.16^{aA}	8.09^{aA}	7.52^{aB}	8.49^{aA}	8.45^{aA}	7.76^{aA}	
wk 24	8	-	8.29^{aA}	8.48^{aA}	7.87^{aAB}	8.26^{aA}	8.03^{aA}	7.92^{aA}	
Non starter lactic acid bacteria									
wk 24	4	5.39^{aA}	7.43^{bA}	7.44^{bA}	7.44^{bA}	8.23^{bA}	7.52^{bA}	7.61^{bA}	0.41
wk 24	8	6.09^{aA}	7.94^{bA}	8.38^{bA}	8.49^{bA}	8.44^{bA}	8.29^{bA}	8.09^{bA}	

[1]Probiotic microorganisms added are as detailed in Table 7.1. Results are presented as means of a least 6 observations ($n \geq 6$).
[2] Pooled standard error of means
[ab] Means in rows with different superscripts are significantly different ($P < 0.05$).
[A,B,C,D]Means in column of the same organism with different superscript are significantly different ($P < 0.05$).

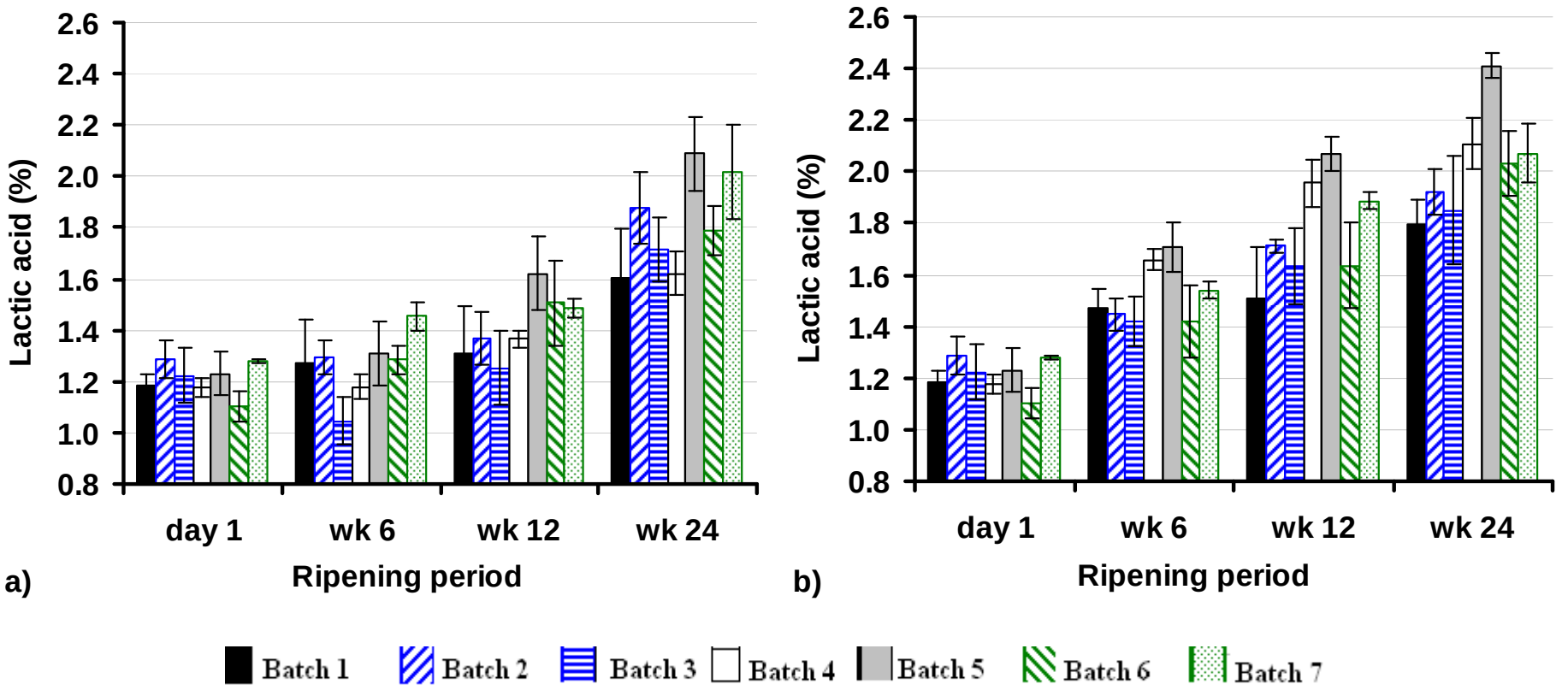


Figure 7.2. Concentration of lactic acid (%) in control and probiotic Cheddar cheeses during ripening at (a) 4°C and (b) 8°C for 24 wk. Results are expressed as means of a least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.10). Batch codes used are as in Table 7.1.

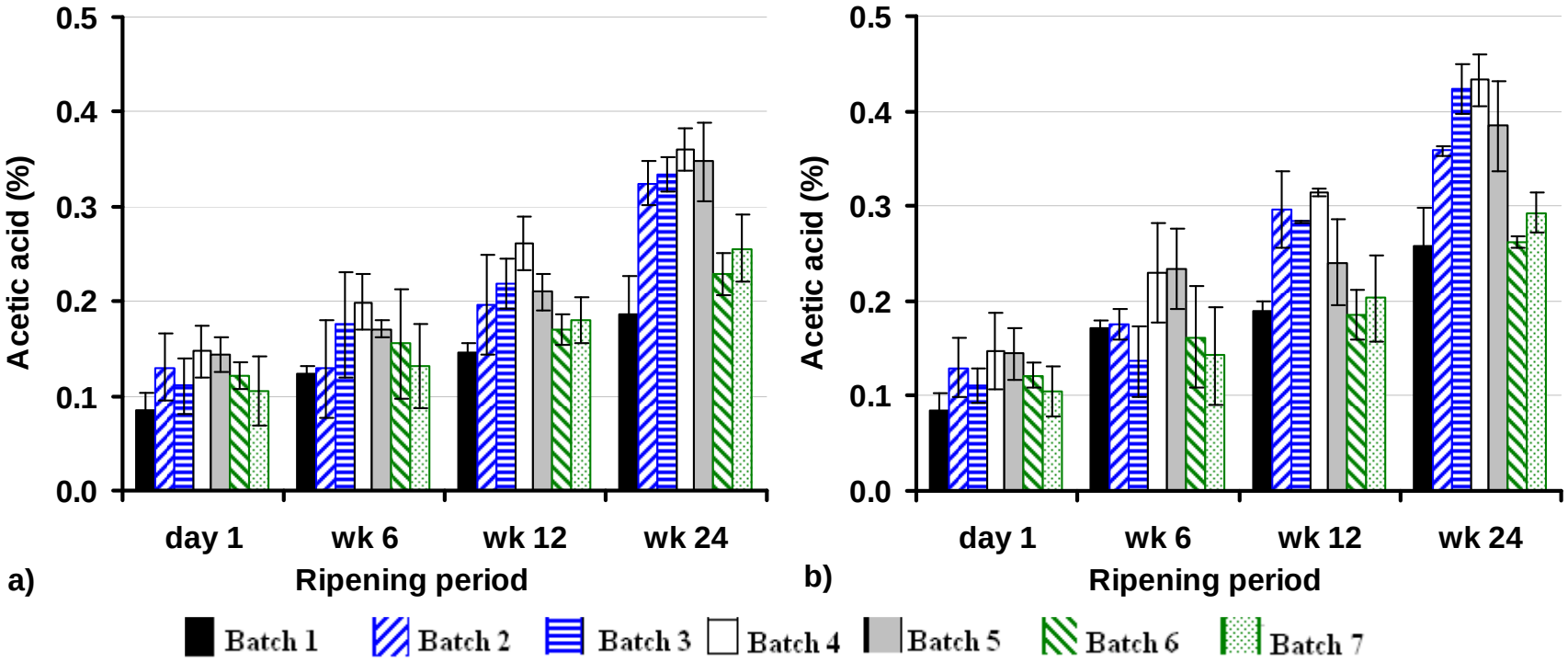


Figure 7.3. Concentration of acetic acid (%) in control and probiotic Cheddar cheeses during ripening at (a) 4°C and (b) 8°C for 24 wk. Results are expressed as means of a least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.04). Batch codes used are as in Table 7.1.

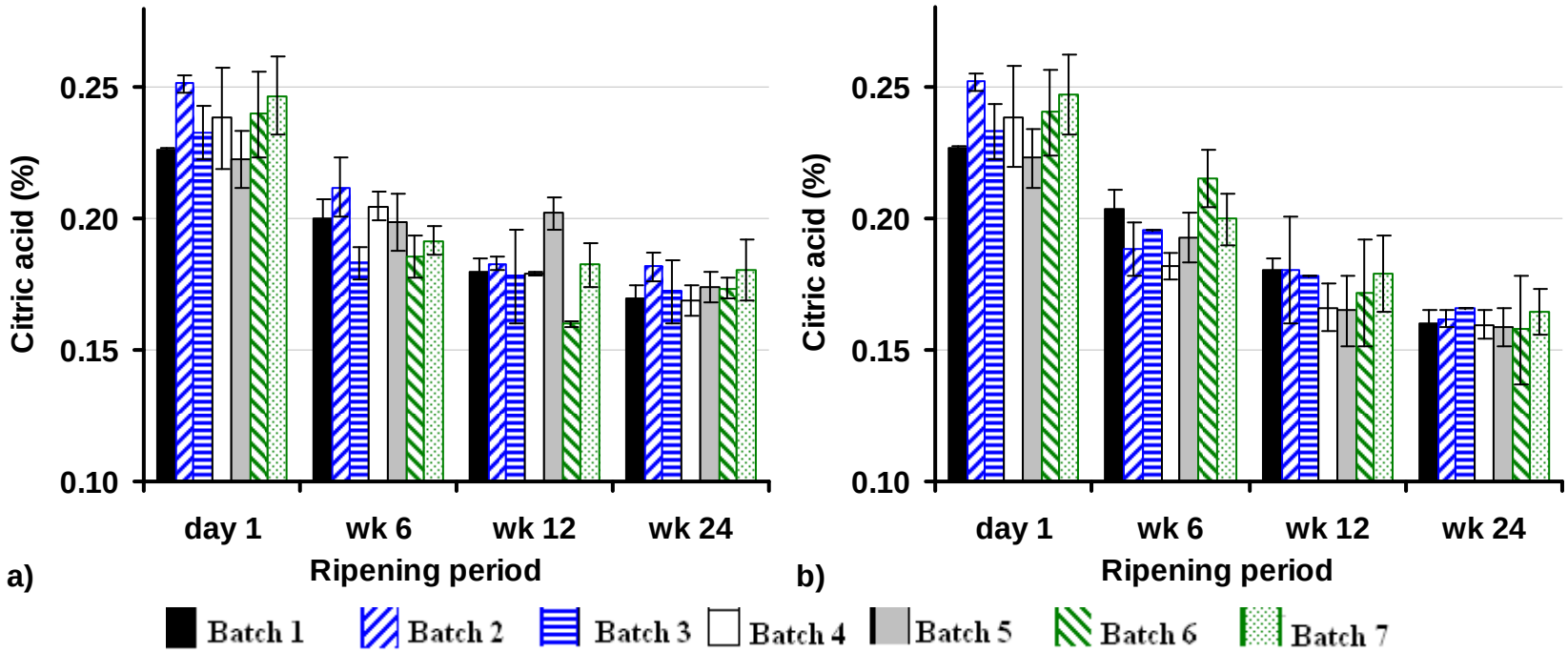


Figure 7.4. Concentration of citric acid (%) in control and probiotic Cheddar cheeses during ripening at (a) 4°C and (b) 8°C for 24 wk. Results are expressed as means of a least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.02). Batch codes used are as in Table 7.1.

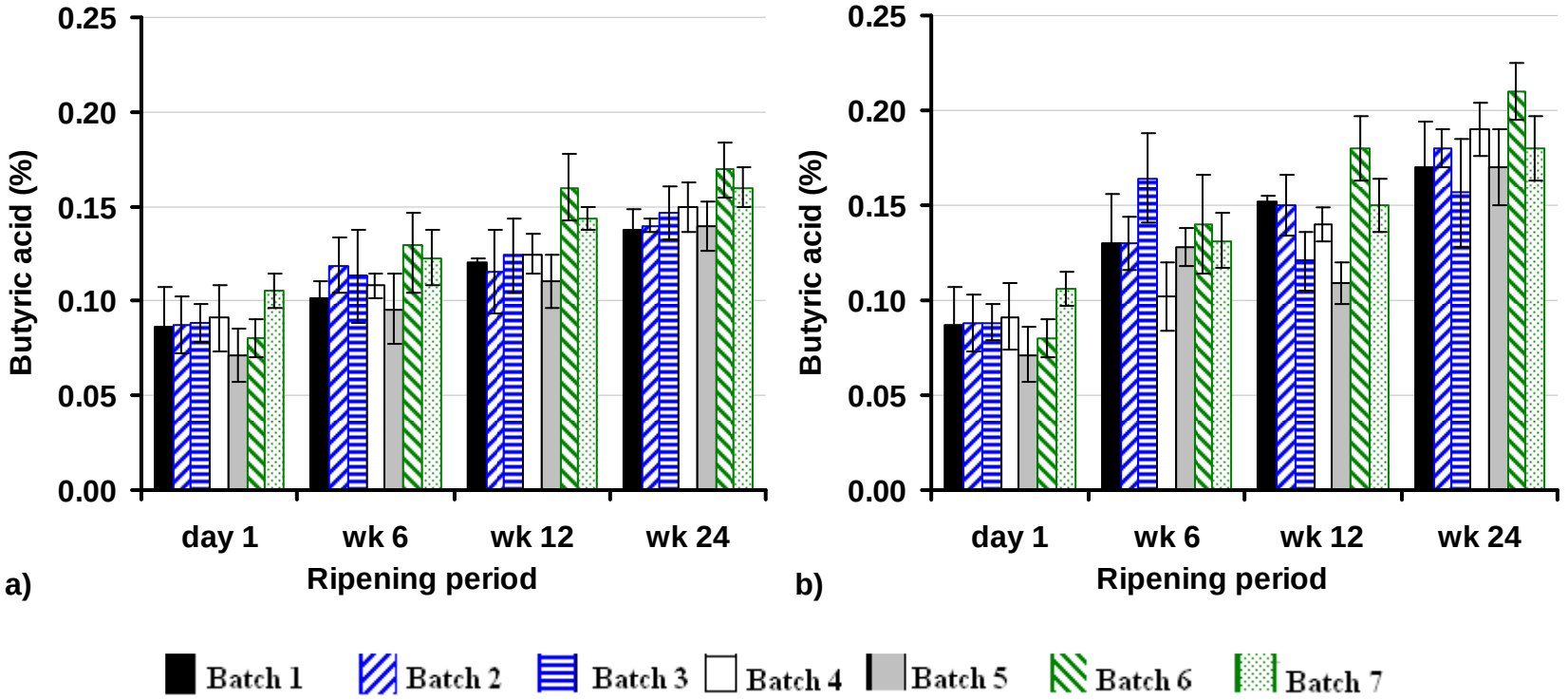


Figure 7.5. Concentration of butyric acid (%) in control and probiotic Cheddar cheeses during ripening at (a) 4°C and (b) 8ºC for 24 wk. Results are expressed as means of a least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.05). Batch codes used are as in Table 7.1.

Table 7.5. Effect of probiotic microorganisms, ripening temperatures, ripening time and interaction between probiotic microoganisms, ripening time and temperatures on composition of cheeses, bacterial counts and organic acid concentrations during ripening

Variables & interaction	Probabilities (*P*)[1]								
	pH	Moisture	Lactococci	Probiotic	NSLAB	Lactic acid	Acetic acid	Citric acid	Butyric acid
Probiotic microorganisms (P)	**<0.001**	0.604	0.827	0.224	0.728	**0.045**	**<0.001**	0.236	0.060
Block (B)	0.458	0.352	0.724	0.815	0.654	0.435	0.538	0.894	0.354
Ripening temperature (T)	**0.001**	**0.013**	**0.001**	0.545	0.544	**0.002**	**<0.001**	0.183	**0.021**
P* T	0.9103	0.973	**0.027**	0.111	0.115	**0.031**	**<0.001**	0.831	0.622
Ripening time (t)[2]	**<0.001**	**<0.001**	**<0.001**	**0.011**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t * P	**<0.001**	0.667	0.466	0.346	0.266	**<0.001**	**<0.001**	0.962	0.846
t * T	**0.002**	0.804	**<0.001**	0.954	0.812	**0.001**	**<0.001**	0.727	0.323
P * T * t	**<0.001**	0.973	0.494	0.996	0.894	**<0.001**	**<0.001**	0.999	0.997

[1] Data were analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were probiotics (P) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

[2] Repeated measures over ripening time were performed at day 1, wk 6, wk 12, and wk 24 (except for pH and moisture, which was analysed at day 1 and wk 24).

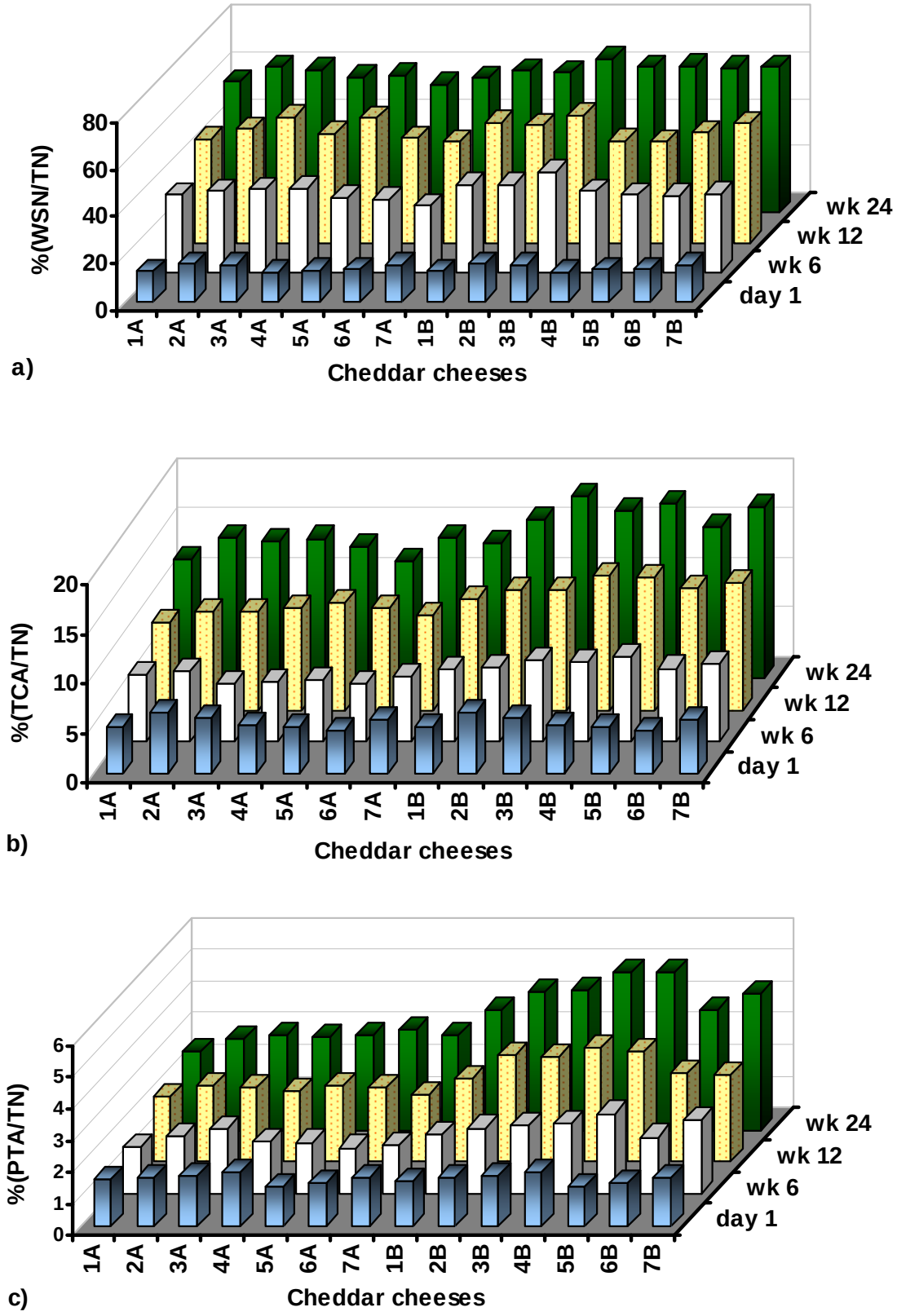


Figure 7.6. Concentration (expressed as percentage of total nitrogen) of A) water-soluble nitrogen (WSN) (Pooled standard error of mean, SEM = 2.94), B) trichloroacetic acid-soluble nitrogen (TCA-SN) (SEM = 0.80), C) phosphotungstic acid-soluble nitrogen (PTA-SN) (SEM = 0.20) during ripening of Cheddar cheeses at 4 and 8^{o}C for 24 wk. Batch codes are as Table 7.1. Results are expressed as mean of at least 6 observations ($n \geq 6$).

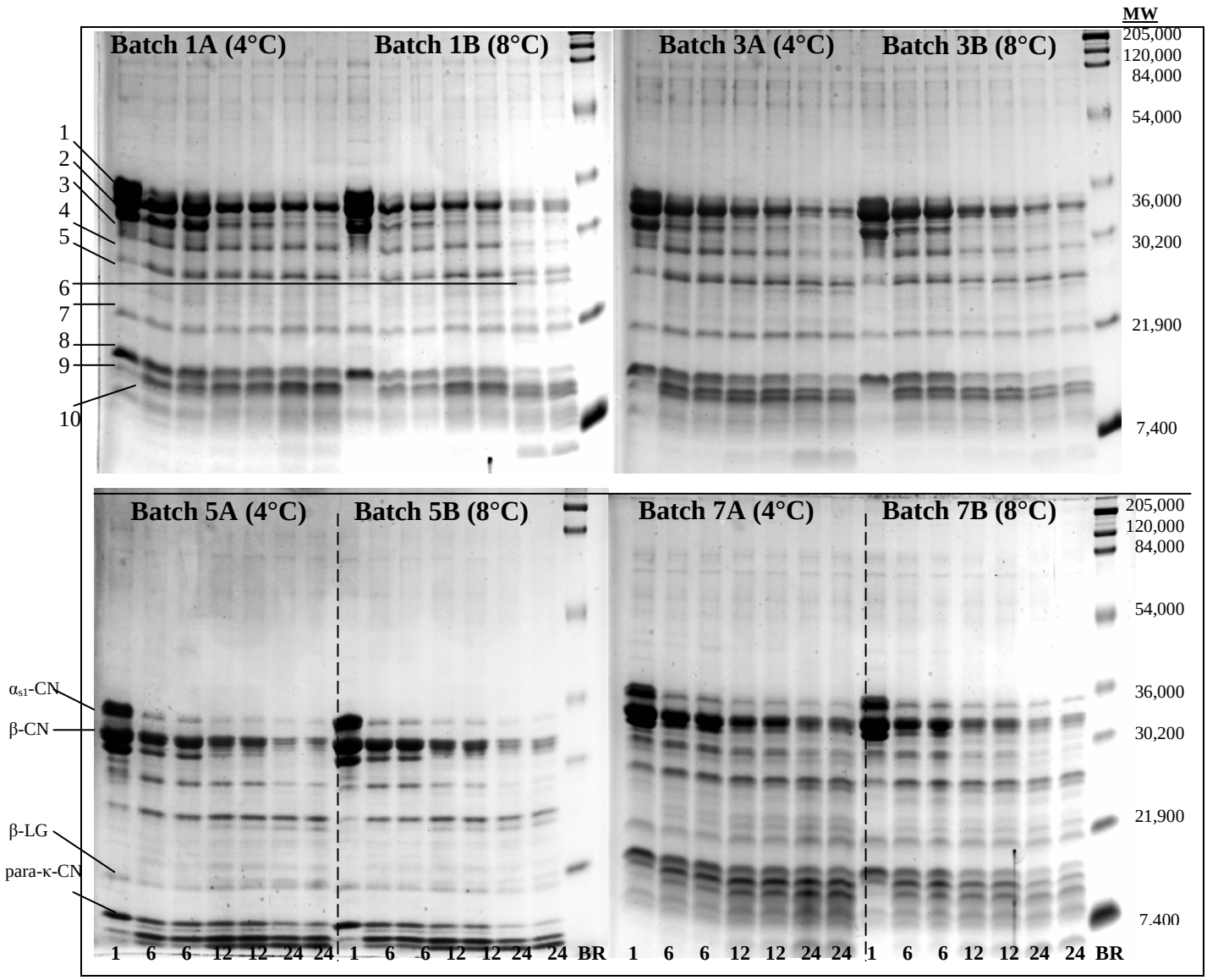


Figure 7.7. Electrophoretic patterns of SDS-PAGE of Cheddar cheeses during ripening (day 1, wk 6, 6, 12, 12, 24 and 24) at 4 and 8°C. Batch codes are as in Table 7.1. Separated protein fractions are labeled as bands 1 to 10. CN = casein, LG = lactoglobullin, BR = broad range prestained molecular weight (MW) standards containing: myosin (MW 205,000), β-galactosidase (MW 120,000), bovine serum albumin (MW 84,000), ovalbumin (MW 52,000), carbonic anhydrase (MW 36,000), soybean trypsin inhibitor (MW 30,200), lysozyme (MW 21,900) and aprotinin (MW 7,400).

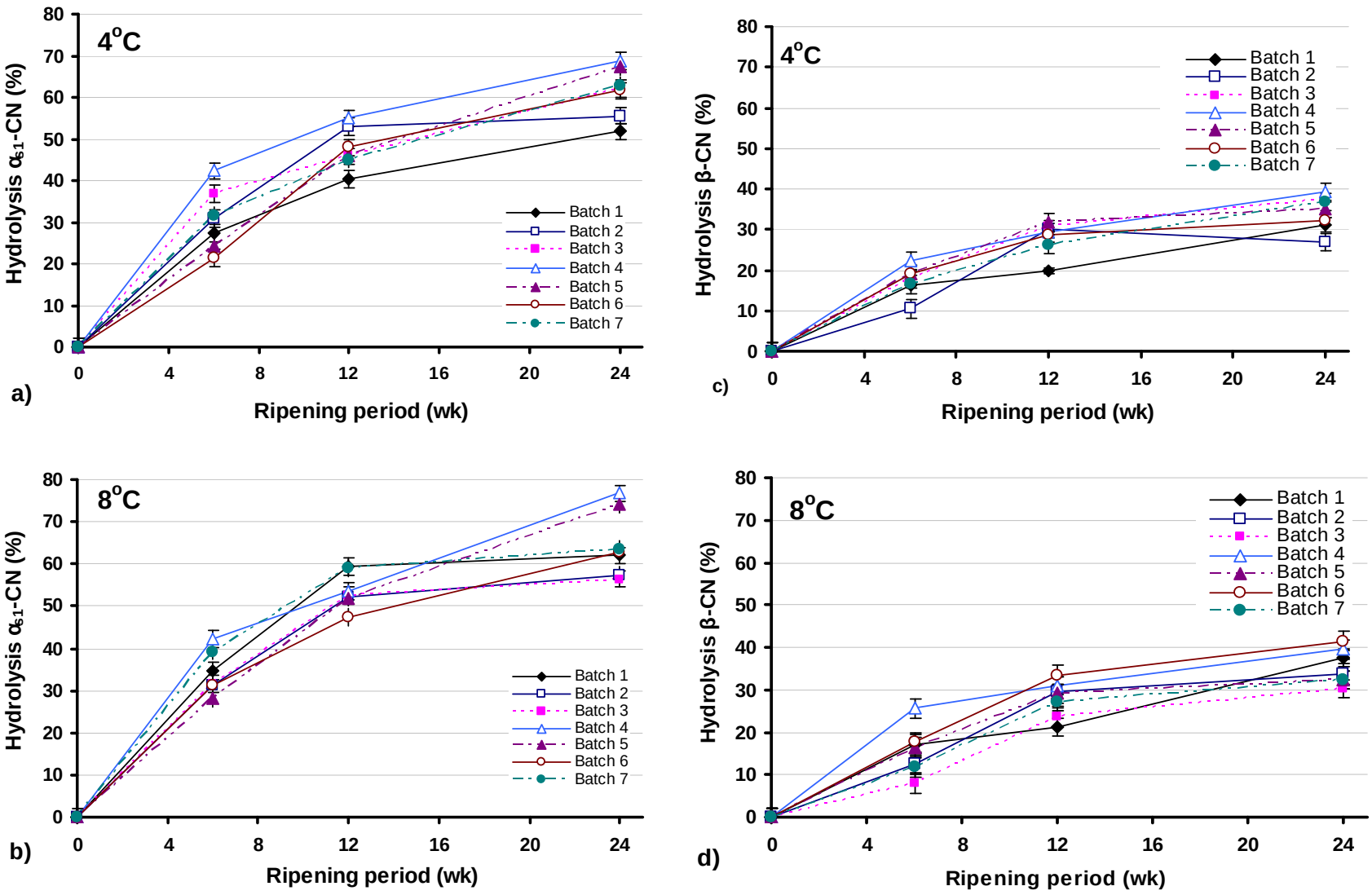


Figure 7.8. Percentage hydrolysis of α_{s1}-CN (A, B); β-CN (C, D) during ripening of Cheddar cheese at 4 and 8°C for 24 wk. Batch codes are as Table 7.1. Results are expressed as mean of at least 6 observations ($n \geq 6$). Error bars are pooled standard error of means (SEM = 2.02 and 2.20 for percentage hydrolysis of α_{s1}-CN and β-CN, respectively).

Table 7.6. Effect of probiotic microorganisms, ripening temperatures, ripening time and interaction between probiotic microoganisms, ripening time and temperatures on proteolysis of Cheddar cheeses

Variables & interaction	Probabilities (*P*)[1]				
	WSN[2]	TCA-SN[2]	PTA-SN[2]	α_{s1} – CN[2]	β – CN[2]
Probiotic microorganisms (P)	0.412	0.114	**0.027**	**<0.001**	**<0.001**
Block (B)	0.745	0.588	0.316	**0.002**	0.118
Ripening temperature (T)	0.064	**<0.001**	**<0.001**	**<0.001**	**<0.001**
P* T	0.713	0.578	**0.003**	**<0.001**	**<0.001**
Ripening time (t)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t * P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t * T	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
P * T * t	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

[1] Data were analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were probiotics (P) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

[2] WSN = water soluble nitrogen, TCA-SN = trichloroacetic acid soluble nitrogen, PTA-SN = phosphotungstic acid soluble nitrogen, α_{s1} – CN = percentage hydrolysis of α_{s1} – casein, β – CN = percentage hydrolysis of β – CN.

Table 7.7. Means overall acceptance of control and probiotic Cheddar cheeses during ripening at 4 and 8°C for 24 wk

Ripening period (wk)	Ripening temperature (°C)	Acceptance scores[1]						
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7
6	4	3.55^{Aa}	3.41^{Aa}	3.08^{Ac}	2.54^{Ab}	3.23^{Aac}	2.91^{Abc}	3.32^{Aac}
6	8	3.84^{Aa}	3.29^{Aac}	3.05^{Aac}	2.49^{Ab}	3.25^{Aac}	3.02^{Abc}	3.33^{Aac}
12	4	5.78^{Ba}	5.64^{Ba}	5.67^{Ba}	5.75^{Ba}	5.46^{Bac}	5.17^{Bbc}	5.61^{Bac}
12	8	5.72^{Bab}	6.65^{Bb}	6.30^{Bab}	5.64^{Ba}	5.65^{Ba}	5.57^{Ba}	5.82^{Bab}
24	4	6.40^{Ca}	5.92^{Ba}	5.84^{Ba}	5.60^{Ba}	5.53^{Ba}	5.98^{BCa}	6.24^{BCa}
24	8	6.97^{Ca}	6.42^{Ba}	5.81^{Bb}	5.54^{Bb}	6.50^{Ca}	6.44^{Ca}	6.92^{Ca}

^{ABC}Means in column with different superscript are significantly different ($P<0.05$).

^{abc} Means in row with different superscript are significantly different ($P<0.05$).

[1]Panelists (n = 15) tested 3 replicates of all 14 variations (mean scores were the average of 45 sensory scores). Standard error of means = 0.43. Batch codes are as Table 7.1.

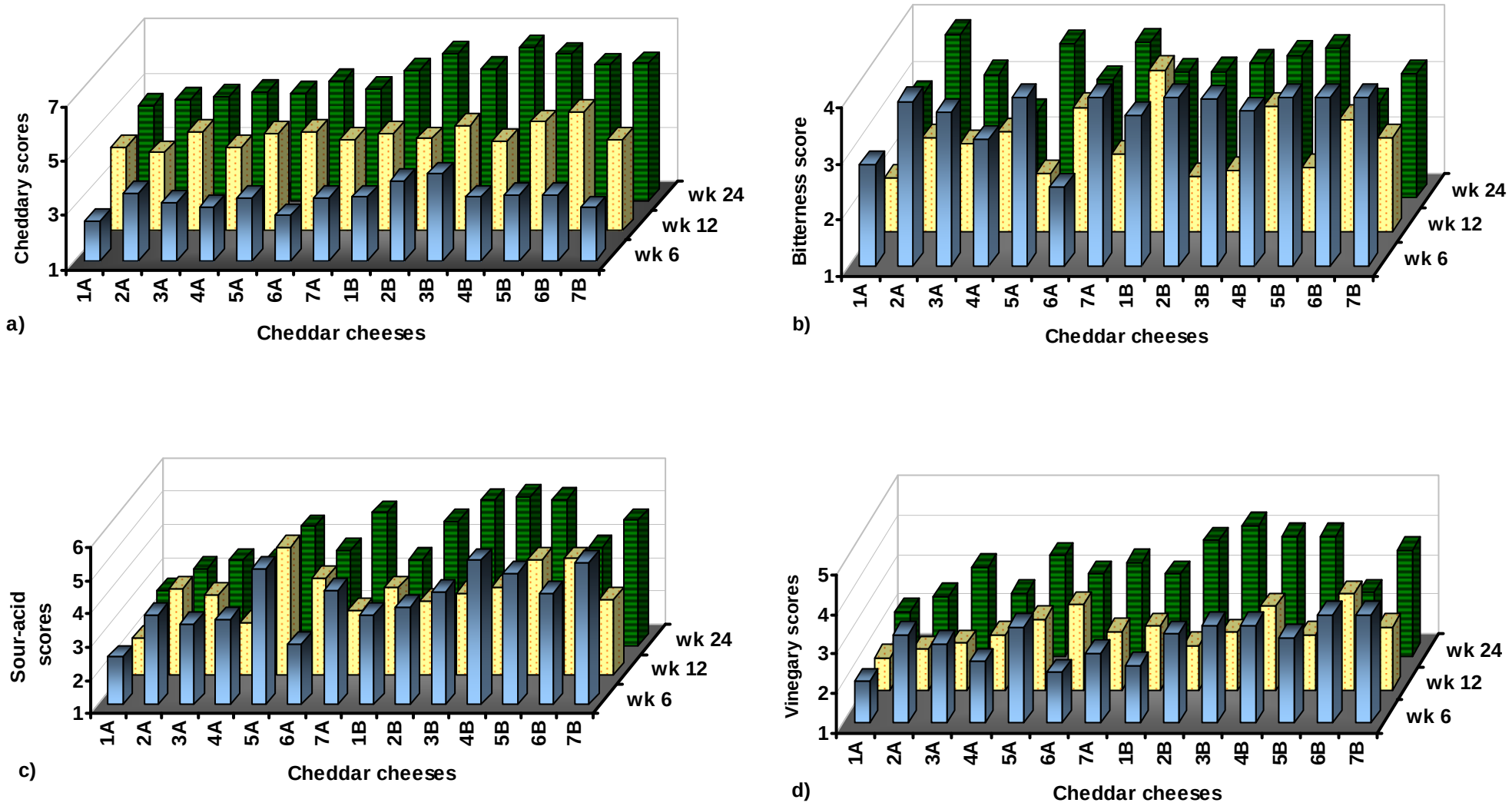


Figure 7.9. Mean of flavour scores a) Cheddary, b) Bitterness, c) Sour-acid, d) Vinegary of control and probiotic Cheddar cheeses during ripening (wk 6, wk 12 and wk 24) at 4 and 8^{o}C (0 = low intensity, 10 = high intensity). Panelists (n = 15) tested 3 replicates of all 14 variations (mean scores were the average of 45 scores, SEM = 0.331, 0.449, 0.212 and 0.231 for cheddary, bitterness, sour-acid and vinegary scores, respectively). Batch codes are as Table 7.1.

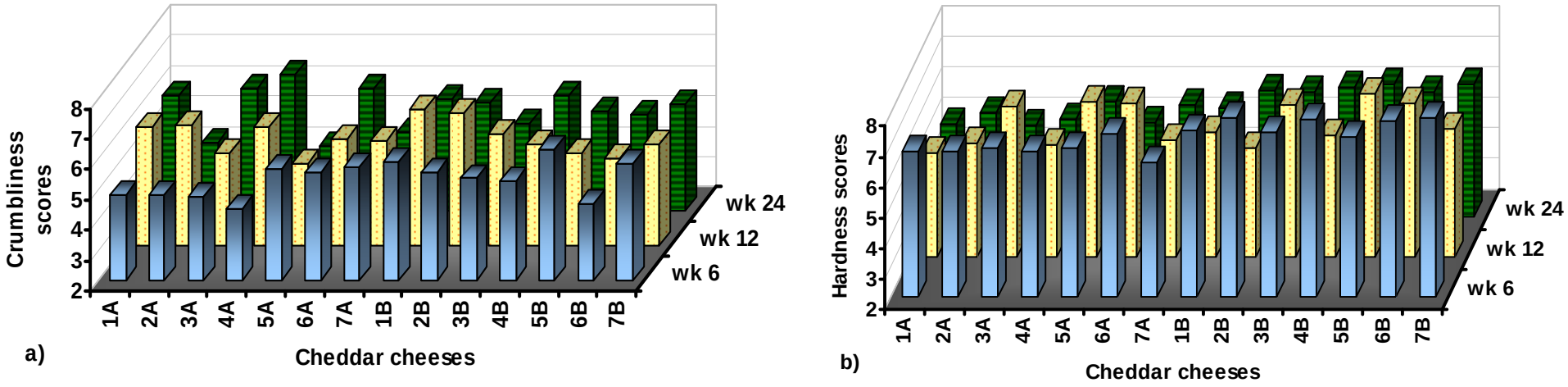


Figure 7.10. Mean of texture scores a) Hardness (0 = extremely soft, 10 = extremely hard), b) Crumbliness (0 = firm, 10 = extremely crumbly) of control and probiotic Cheddar cheeses during ripening (wk 6, wk 12 and wk 24) at 4 and 8^{o}C. Sensory panelists (n = 15) tested 3 replicates of all 14 variations (mean scores were the average of 45 readings, SEM = 0.612 and 0.415 for hardness and crumbliness scores, respectively). Batch codes are as Table 7.1.

Table 7.8. Correlation of results of chemical analysis and sensory attributes scores

Sensory Attributes	**Correlation of results of chemical analysis and sensory attributes scores (r/ *P*)[1]**													
	Moisture	**Salt**	**Fat**	**Protein**	**pH**	**Lactic acid**	**Acetic acid**	**Citric acid**	**Butyric acid**	**WSN[2]**	**TCA-SN[2]**	**PTA-SN[2]**	**α_{s1} – CN[2]**	**β – CN[2]**
Flavour Cheddary	**-0.753**	-0.291	**0.688**	0.303	**-0.736**	**0.738**	**0.774**	**-0.753**	**0.558**	**0.860**	**0.917**	**0.902**	**0.839**	**0.770**
	<0.001	0.062	**<0.001**	0.051	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Sour-acidic	0.161	0.054	0.082	-0.175	**-0.450**	**0.327**	**0.491**	-0.225	0.036	0.083	0.298	**0.421**	0.166	0.105
	0.308	0.734	0.606	0.267	**0.003**	**0.034**	**0.001**	0.151	0.823	0.600	0.055	**0.005**	0.292	0.506
Vinegary	0.064	-0.003	0.152	-0.099	**-0.367**	**0.415**	**0.453**	-0.257	0.239	0.197	**0.372**	**0.474**	0.217	0.095
	0.688	0.987	0.337	0.534	**0.017**	**0.006**	**0.003**	0.100	0.128	0.211	**0.015**	**0.002**	0.168	0.501
Bitter	**0.508**	0.156	**-0.361**	**-0.436**	0.130	-0.083	-0.007	0.249	0.102	-0.286	-0.206	-0.130	-0.242	**-0.419**
	0.001	0.323	**0.019**	**0.004**	0.413	0.585	0.964	0.111	0.520	0.066	0.190	0.411	0.122	**0.006**
Texture Hardness	**0.648**	**0.328**	-0.085	**-0.390**	0.284	-0.292	**-0.457**	**0.348**	**-0.341**	**-0.631**	**-0.351**	**-0.509**	**-0.631**	**-0.613**
	<0.001	**0.034**	0.591	**0.011**	0.068	0.061	**0.002**	**0.024**	**0.027**	**<0.001**	**0.023**	**0.001**	**<0.001**	**<0.001**
Crumbliness	-0.140	0.028	-0.085	0.130	-0.135	-0.061	0.020	-0.056	0.166	-0.002	0.027	0.079	0.078	0.006
	0.376	0.859	0.591	0.413	0.394	0.702	0.902	0.726	0.295	0.992	0.863	0.620	0.624	0.969
Acceptance	**-0.899**	**-0.423**	**0.611**	**0.448**	**-0.615**	**0.574**	**0.619**	**-0.617**	**0.499**	**0.828**	**0.831**	**0.714**	**0.812**	**0.780**
	<0.001	**0.005**	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

[1] Correlation between mean scores of sensory attributes and chemical analysis was expressed as r. Significant level was expressed as *P*.

[2] WSN = water soluble nitrogen, TCA-SN = trichloroacetic acid soluble nitrogen, PTA-SN = phosphotungstic acid soluble nitrogen, α_{s1} – CN = percentage hydrolysis of α_{s1} – casein, β – CN = percentage hydrolysis of β – CN.

Table 7.9. Effect of probiotic microorganisms, ripening temperatures, ripening time and interaction between probiotic microoganisms, ripening time and temperatures on sensory scores of Cheddar cheeses

Variables & interaction	**Probabilities (*P*)[1]**						
	Acceptance	Cheddary	Bitterness	Sour-acid	Vinegary	Crumbliness	Hardness
Probiotic microorganisms (P)	**0.006**	0.257	0.837	**0.008**	**0.021**	0.438	0.689
Block (B)	0.321	0.236	0.128	0.212	0.124	0.128	0.355
Ripening temperature (T)	**0.008**	**<0.001**	0.258	**<0.001**	**<0.001**	0.521	0.354
P* T	0.953	0.613	0.080	0.512	0.613	0.124	0.341
Ripening time (t)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.114	0.063
t * P	0.646	0.067	**0.030**	**<0.001**	**<0.001**	0.287	0.214
t * T	0.311	**0.001**	0.318	**<0.001**	**<0.001**	0.384	0.628
P * T * t	0.994	0.868	0.605	**<0.001**	**<0.001**	0.969	0.899

[1]Data were analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were probiotics (P) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

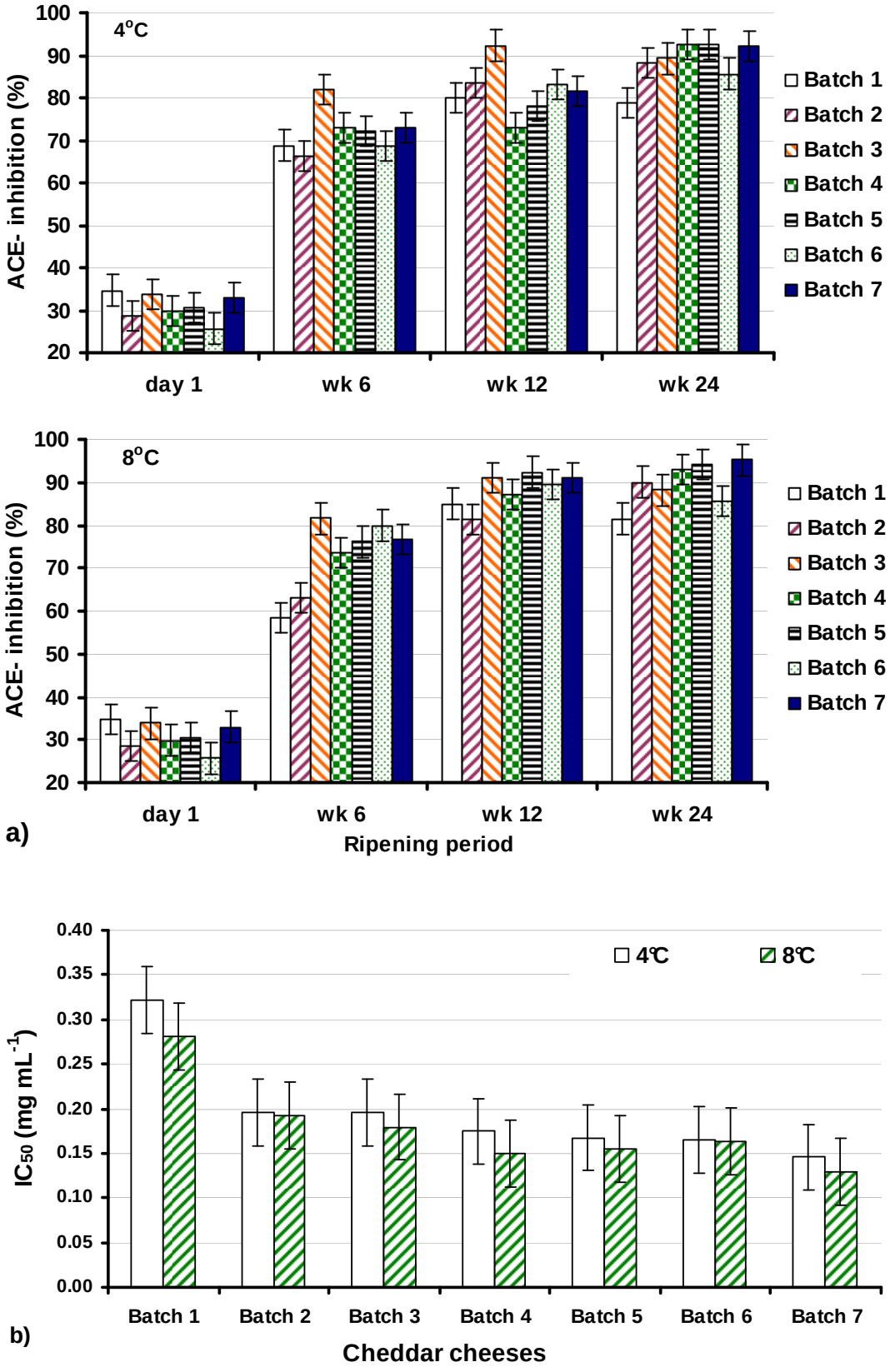


Figure 7.11. Angiotensin converting enzyme (ACE)-inhibitory activity of control and probiotic Cheddar cheeses presented as: (a) Percentage ACE-inhibition of water-soluble extract (WSE) of cheeses during ripening at 4 and 8°C for 24 wk and (b) IC_{50} (concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity) of WSE of cheeses after ripening at 4 and 8°C for 24 wk. Batch codes are as Table 1. Error bar is pooled standard error of mean (SEM = 3.56 and 0.04 for ACE-inhibition index and IC_{50}, respectively).

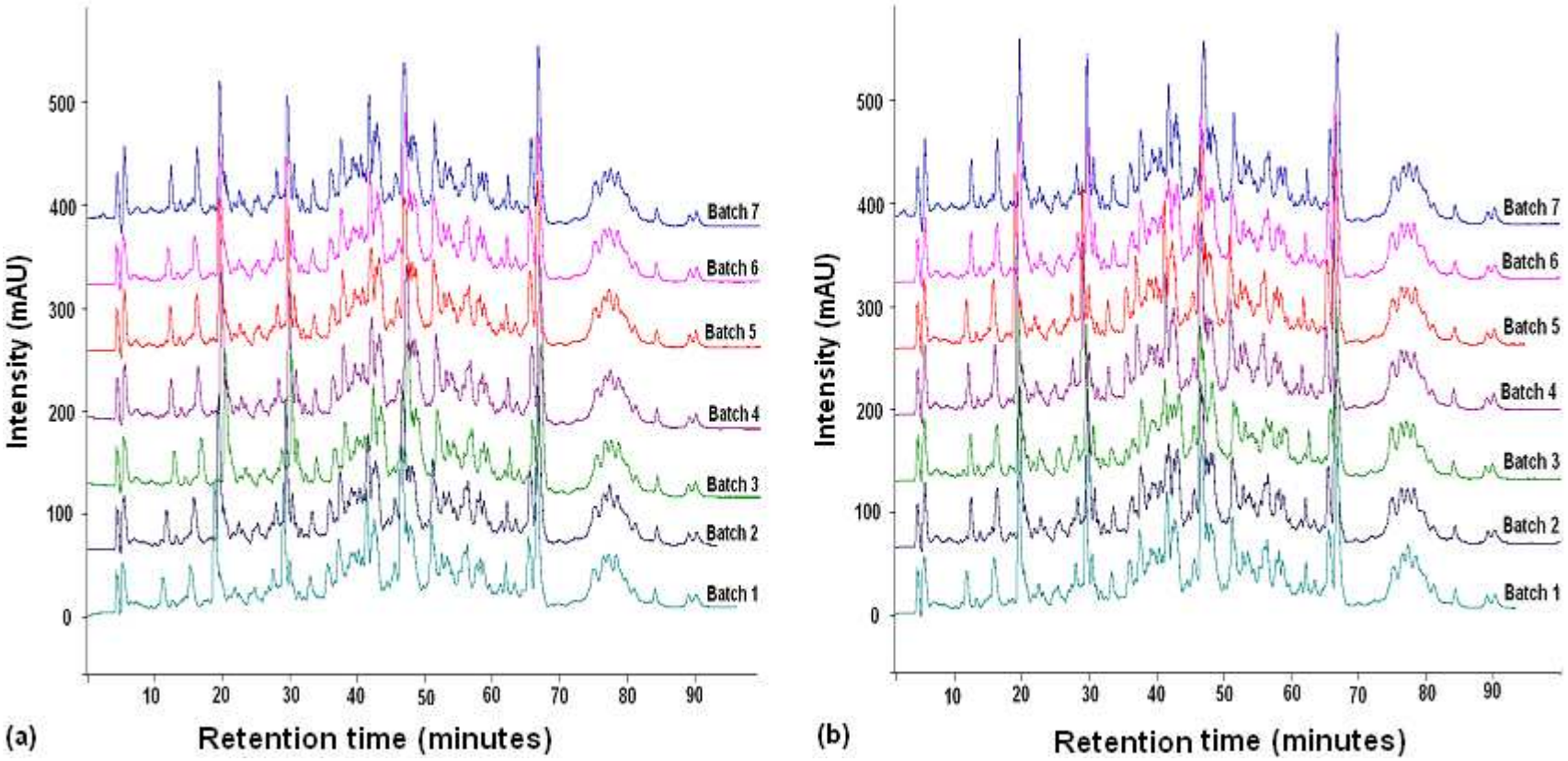


Figure 7.12. Peptide profiles of control and probiotic Cheddar cheeses after ripening at (a) 4°C and (b) 8°C for 24 wk. RP-HPLC conditions as described in section 7.2.9.

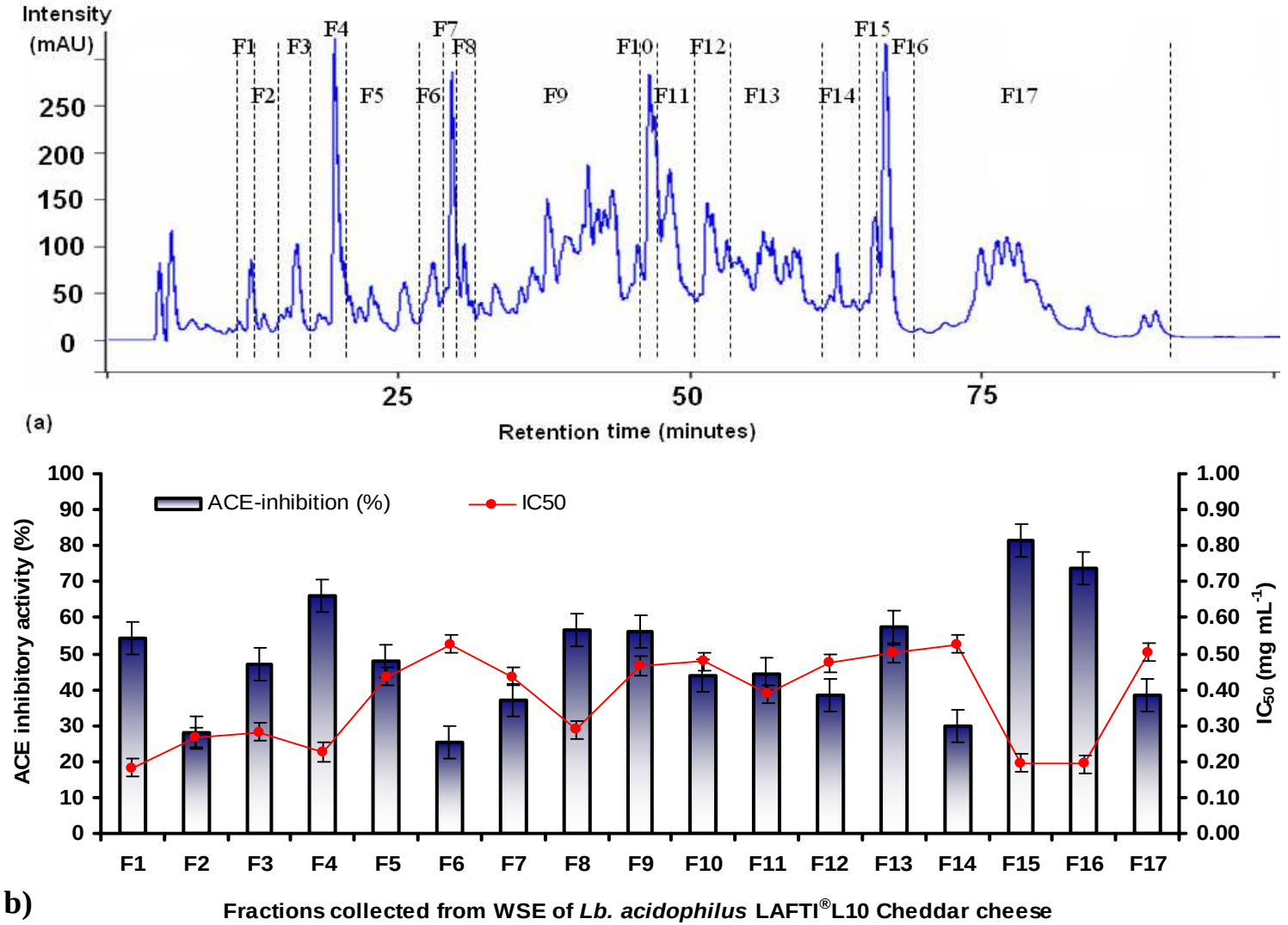


Figure 7.13. (a) Fractionation by RP-HPLC of WSE obtained from cheese made with *Lb. acidophilus* LAFTI® L10 after ripening at 8°C for 24 wk (RP-HPLC conditions as described in section 7.2.9 & section 7.2.10). Collected fractions are termed with F followed by a number. (b) Angiotensin converting enzyme-inhibitory activity presented as percentage ACE-inhibition and IC_{50} (concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity) of the collected fractions from RP-HPLC system. Error bar is pooled standard error of mean (SEM = 4.51 and 0.03 for ACE-inhibition index and IC_{50}, respectively).

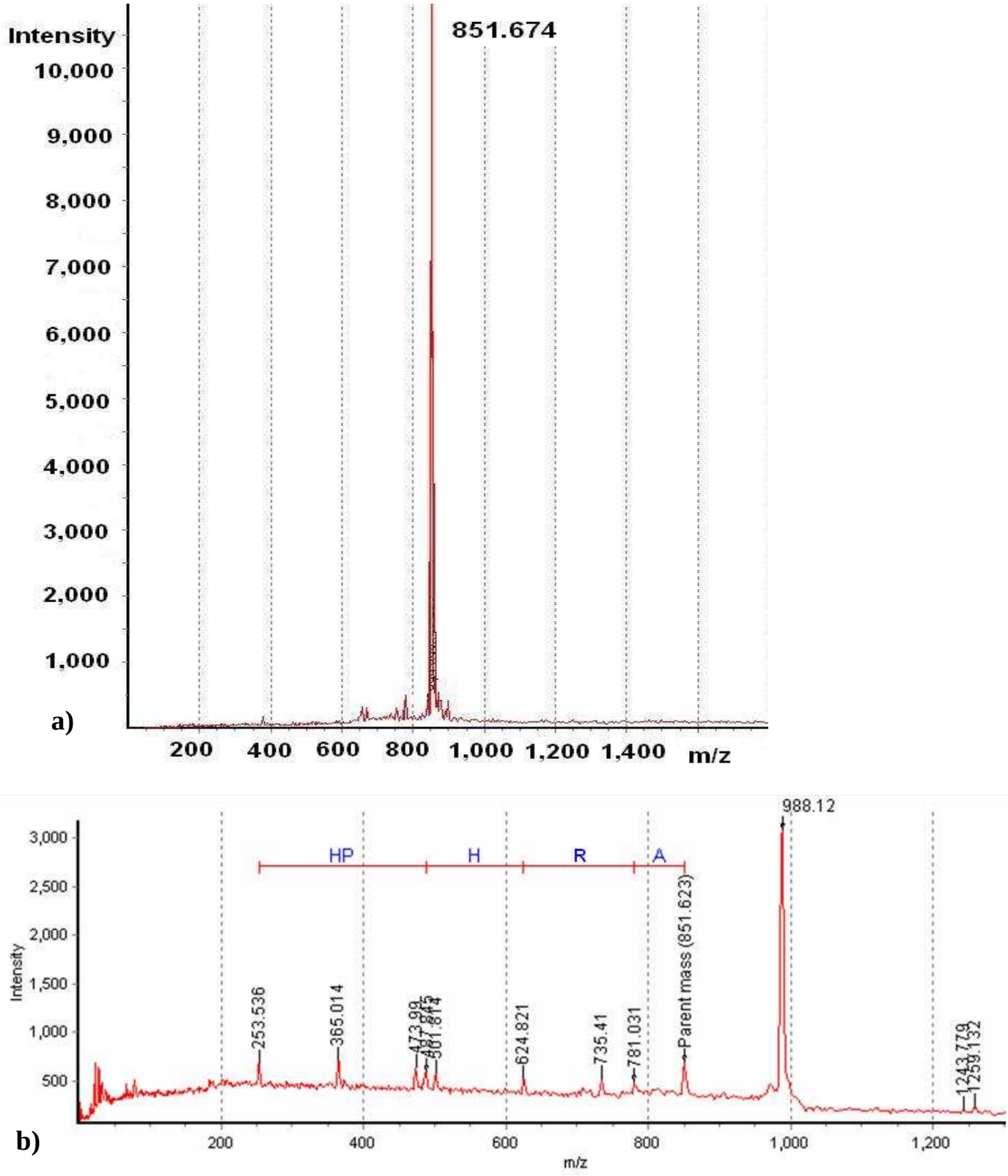


Figure 7.14. (a) Molecular weight of purified peptide from fraction 1 obtained using MALDI-TOF-MS (condition as described in section 7.2.11). (b) Chemically assisted fragmentation (CAF) of purified peptide from fraction 1. The first five amino acids of the N-terminal were identified as ARHPH (CAF condition as described in section 7.2.11). Following sequence interpretation and molecular weight determination, the peptide was identified as ARHPHPH.

Table 7.10. Identification of ACE-inhibitory peptides collected from WSE of cheese made with the addition of *Lb. acidophilus* LAFTI® L10 after ripening at 8°C for 24 wk

Fractions	Sequences	Origin	Experimental molecular mass (Dalton)	Theoretical molecular mass (Dalton)
F1	Ala-Arg-His-Pro-His-Pro-His (ARHPHPH)	κ-CN (f 96-102)	851.7	850.9
F4	Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln (RPKHPIKHQ)	α_{s1}-CN (f 1-9)	1140.5	1140.4
F4	Arg-Pro-Lys-His-Pro-Ile-Lys (RPKHPIK)	α_{s1}-CN (f 1-7)	877.0	875.1
F4	Arg-Pro-Lys-His-Pro-Ile (RPKHPI)	α_{s1}-CN (f 1-6)	745.4	746.9
F15	Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe (FVAPFPEVF)	α_{s1}-CN (f 24-32)	1053.3	1052.2
F16	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (YQEPVLGPVRGPFPIIV)	β-CN (f 193-209)	1881.2	1881.3

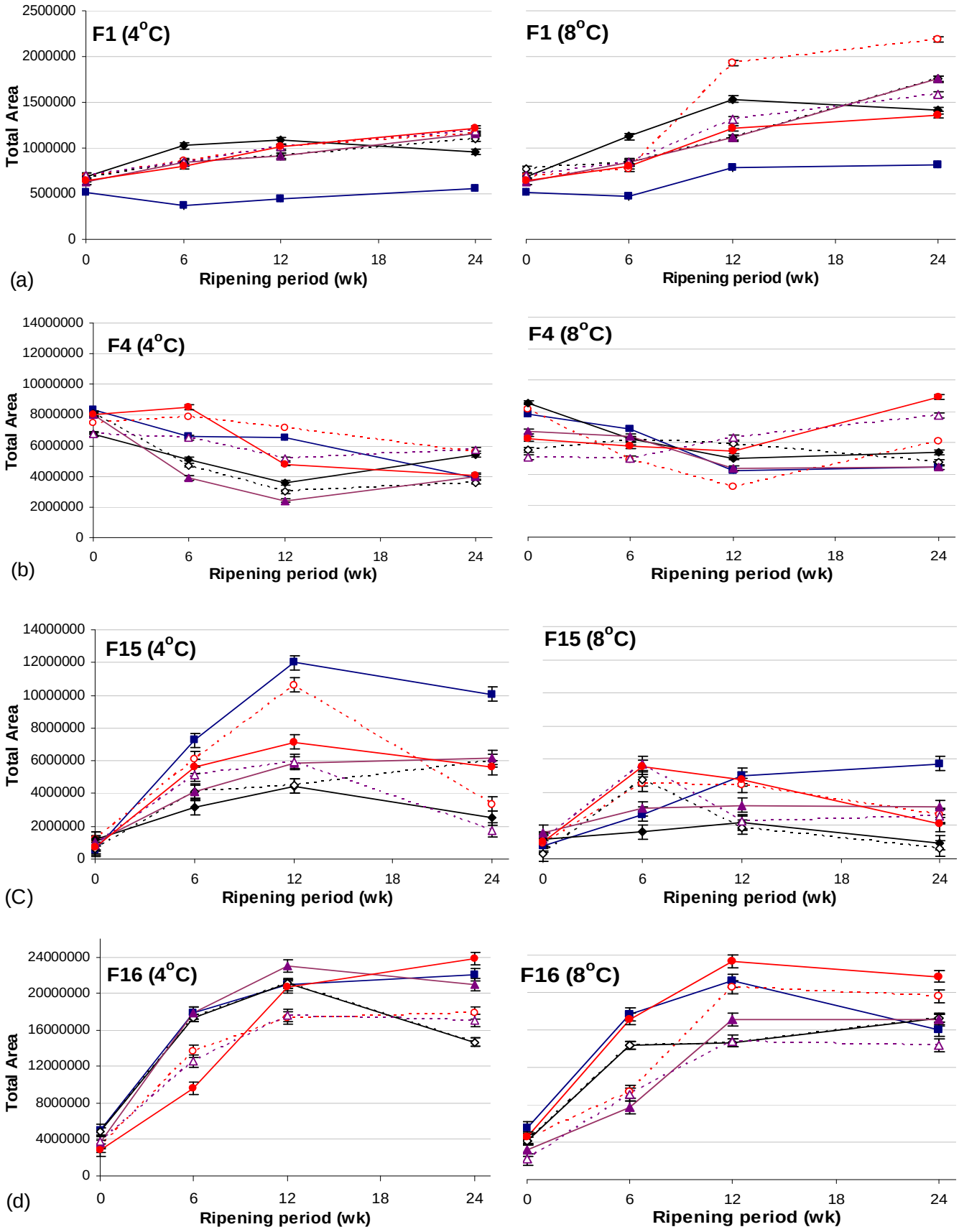


Figure 7.15. Total area of fractions with ACE-inhibitory activity collected from WSE of control (Batch 1, —■—), and probiotic Cheddar cheeses (Batch 2, —♦—; Batch 3, --◊--, Batch 4, —▲—; Batch 5, --Δ--, Batch 6, —●—; Batch 7, --○--) during ripening at 4 and 8°C for 24 wk. (a) fraction 1, F1; (b) fraction 4, F4; (c) fraction 15, F15 and (d) fraction 16, F16. Error bar is pooled standard error of mean (SEM = 33927, 242855, 139766, 432704 and 671931 for F1, F4, F15 and F16, respectively). Batch codes are as Table 7.1.

Table 7.11. Effect of probiotic microorganisms, ripening temperatures, ripening time and their interaction on ACE-inhibitory activity of Cheddar cheeses and total area of ACE-inhibitory peptides during ripening at 4 and 8°C for 24 wk

Variables & interaction	**Probabilities (*P*)[1]**				
	ACE-inhibition	**Fraction 1[2]**	**Fraction 4[2]**	**Fraction 15[2]**	**Fraction 16[2]**
Probiotic microorganisms (P)	0.093	**<0.001**	0.802	0.188	0.118
Block (B)	0.131	0.061	0.993	0.994	0.578
Ripening temperature (T)	**0.012**	**0.001**	0.221	**<0.001**	**0.052**
P* T	0.139	0.001	0.475	**0.001**	**0.020**
Ripening time (t)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t * P	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t * T	0.079	**<0.001**	**<0.001**	**<0.001**	**<0.001**
P * T * t	0.789	**<0.001**	**<0.001**	**0.005**	**<0.001**

[1]Data were analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were probiotics (P) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

[2]Total area of ACE-inhibitory fractions of control and probiotic cheeses obtained using RP-HPLC during ripening at 4 and 8°C for 24 wk.

8.0 Influence of probiotic *Lactobacillus acidophilus* and *Lb. helveticus* on proteolysis, organic acid profiles and ACE-inhibitory activity of Cheddar cheeses ripened at 4, 8 and 12°C*

8.1. Introduction

Growing public awareness of diet related health issues has fuelled the demand for probiotic foods. Health benefits for consuming food products containing live probiotic bacteria are discussed in Chapter 2.0, section 2.1.4. Cheddar cheese has been found to be an excellent carrier for probiotic bacteria to the consumer (Dinakar & Mistry, 1994; Daigle et al., 1999). Results from Chapter 7.0 also showed that cheeses made with *Lactobacillus acidophilus* LAFTI®L10 had good potential for the development of Cheddar cheeses with bioactive properties. It is also possible that the beneficial health effects of cheese can be increased based on the peptides released as a result of proteolysis during ripening. Cheese made with *Lb. acidophilus* LAFTI®L10, however, was not the cheese with the highest level of proteolysis.

A few studies have reported that increasing ripening temperature from 6 to 15°C offers a technologically simple method to increase proteolysis, thus accelerate cheese ripening (Law, 2001). The biochemical reaction which generates flavour compounds or flavour precursors in cheese is also accelerated by increasing the ripening temperature. An elevated ripening temperature may, however, accelerate the loss of starter bacteria prior to completing lactose utilization (Fox et al., 1993). The growth of most non-starter lactic acid bacteria (NSLAB) increases with higher ripening temperature and fermentation of lactose by NSLAB produces organic acid by-products such as formic, acetic, butyric and propionic acids (Fox et al., 1993). Excess of these compounds impairs the flavour balance of Cheddar cheeses. Folkertsma et al., (1996) reported that the texture of cheeses deteriorated after prolonged ripening at 16°C. An appropriate ripening temperature is thus required to maintain a balance growth of NSLAB to achieve an optimum cheese quality.

*A version of this chapter has been submitted to *Journal of Food Science.* Ong, L. & Shah, N.P. (2008b). Influence of probiotic *Lactobacillus acidophilus* and *Lb. helveticus* on proteolysis, organic acid profiles and ACE-inhibitory activity of Cheddar cheeses ripened at 4, 8 and 12°C. *Journal of Food Science* (doi:10.1111/j.1750-3841.2008.00689.x).

The use of lactobacilli as an adjunct to the normal starter to increase proteolysis and enhance flavour development during cheese ripening has also been studied by several researchers (Broome et al., 1990; Lynch et al., 1996). A highly proteolytic strain of *Lb. helveticus* added as adjunct in Cheddar cheese was shown to lyse during ripening releasing its intracellular enzymes, which led to an increase in proteolysis and improved cheese flavour (Kiernan et al., 2000). At least 11 peptidases from *Lb. helveticus* have been characterised biochemically and/or genetically including the general aminopeptidases (PepC and PepN); proline-specific peptidases (PepX, PepI, PepQ and PepR); dipeptidases (PepD and PepV); endopeptidases (PepE and PepO) and tripeptidase (PepT) (Christensen et al., 1999; Savijoki & Palva, 2000). In addition to the role of these enzymes to overall proteolysis during cheese ripening, they may also contribute to a release of biologically-active peptides such as angiotensin converting enzyme (ACE)-inhibitory peptides.

The role of ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) in the regulation of blood pressure and cardiovascular function are discussed in section 2.6.1. Inhibition of ACE is considered a useful therapeutic approach in treatment of hypertension. The first reported competitive inhibitor of ACE is the naturally occurring peptides in snake venom (Ondetti et al., 1971). ACE-inhibitory peptides have been isolated from milk fermented with *Lb. helveticus* CPN4 (Yamamoto et al., 1999), *Lb. helveticus* LKB-16 H (Seppo et al., 2002), *Lb. helveticus* CHCC637 and CHCC641 (Fuglsang et al., 2003), and a mixed-strain starter consisting of *Lb. helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995).

Lb. helveticus H100 and probiotic *Lb. acidophilus* L10 used in this study were selected based on the proteolytic activity, proven health effects, superior stability and ability to survive through gastro-intestinal tract (McIntosh et al., 1999; Pidcock et al., 2002; Gunaranjan et al., 2007). To date, very few studies have evaluated the influence of ripening temperatures on the survival of probiotic bacteria in Cheddar cheeses. Most of the studies pertaining to production of the ACE-inhibitory peptides were performed in fermented milk or yoghurt with proteolytic strain of lactic acid bacteria (LAB) mainly *Lb. helveticus.* Only a few studies have investigated the release of ACE-inhibitory peptides in cheeses made with the addition of probiotic bacteria. The objectives of this study were to improve the proteolysis and the ACE-inhibitory activity of probiotic cheeses made with *Lb. acidophilus* LAFTI®L10 adjunct and to study the influence of *Lb. helveticus* and ripening at 4, 8 and 12^{o}C on composition of cheeses, organic acid profiles, proteolysis and ACE-inhibitory activity of Cheddar cheeses.

8.2. Materials and Methods

8.2.1. ***Cheese starter culture,*** **Lactobacillus** ***adjunct and probiotic organism***

A cheese starter culture consisting of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* was obtained from DSM Food Specialties Pty. Ltd. (Moorebank, NSW, Australia) in freeze-dried form. The probiotic *Lb. acidophilus* LAFTI®L10 and *Lb. helveticus* H100 were obtained from DSM in frozen form. *Lb. helveticus* H1188, H474, H1315 were obtained from Australian Starter Culture Collection Center (ASCC) (Werribee, Australia). The starter culture was activated by growing two times at 30°C overnight in 12% (w/v) sterile reconstituted skim milk (RSM), whereas the probiotic *Lb. acidophilus* L10 and *Lb. helveticus* H100, H1188, H474 and H1315 were activated by growing two times at 37°C overnight in 12% (w/v) RSM.

8.2.2. ***Cheddar cheese making***

Cheddar cheeses were made with 20 L pasteurized milk and 1.5% (v/v) inoculum of the mixed strain starter culture using a pair of custom made cheese vats. Three batches of Cheddar cheeses were made. The first batch was a control made with starter lactococci (1.5% v/v) only (control), the second batch was made with starter lactococci (1.5% v/v) and *Lb. acidophilus* L10 (1.2% v/v) (L10), while the third batch was made with starter lactococci (1.5% v/v), *Lb. acidophilus* L10 (1.2% v/v) and *Lb. helveticus* H100 (1.2% v/v) (H100). Each batch of cheeses was made in triplicate and a total of 9 batches of cheeses were made. All 9 batches of cheeses were made randomly in 5 days with the same lot of pasteurized milk.

Cheeses were made according to the procedure as described in section 3.2.2. Fresh cheese was removed from the mould, packed in oxygen barrier Cryovac® bags (Cryovac® Pty. Ltd., Fawkner, Vic, Australia) and heat sealed with a Multivac® vacuum packaging equipment (Multivac Sepp Haggenmüller, Wolfertscgwenden, Germany). Each batch of the cheeses was divided into three equal portions and assigned to three ripening temperatures (4, 8 & 12°C) and ripened for 24 wk.

8.2.3. ***Cheese composition***

The composition of the cheeses including the salt, fat, moisture, protein contents and pH of the cheeses were determined according to the methods described in section 3.2.3.

8.2.4. ***Viability of bacteria in cheeses***

Counts of the starter bacteria, probiotic organism and *Lb. helveticus* adjunct were enumerated in ripened milk, cooked curd, whey, cheddared curd, pressed curd (fresh cheese) and in cheese samples at day 1 (before ripening), and at wk 6, wk 12 and wk 24. The curd and Cheddar cheese samples (11g) were diluted in 99 mL of sterile 2% (w/v) tri-sodium citrate (Oxoid Ltd., West Heidelberg, Vic, Australia) at 40^{o}C. Each sample was macerated in a stomacher 400 laboratory blender (Seward Medical, London, UK) for 4 min at high speed in stomacher bags to obtain slurry for the first dilution and subsequent serial dilutions were performed in 0.15% (w/v) peptone and water solution (Oxoid). The milk and whey samples were diluted directly in 0.15% peptone and water solution. Appropriate dilutions were pour-plated.

Starter lactococci were enumerated using M17 agar (Merck, South Granville, NSW, Australia) incubated at 30^{o}C under aerobic condition for 72 h (Terzaghi & Sandine, 1975). NSLAB was enumerated using LBS agar (Merck) incubated at 30^{o}C for 72 h in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, USA) with a gas generating kit® (Oxoid).

Lb. acidophilus L10 was enumerated using reinforced clostridial–clindamycine agar (RCA-clindamycine). RCA (Oxoid) was prepared according to the manufacturer instructions. To prepare RCA-clindamycine agar, 100µL of the 0.05% (w/v) filter-sterillised clindamycine (Sigma-Aldrich, St. Louis, MO, USA) stock solution was added to molten RCA agar (pH 5.5) along with 2 mL of 0.2% (w/v) bromocresol green (Sigma-Aldrich) stock solution just before pouring. *Lb. helveticus* H100 was enumerated using MRS agar (pH 5.4) incubated at 45°C for 3 days.

8.2.5. ***Organic acid analysis***

Concentrations of lactic, acetic, citric, propionic and butyric acids in cheeses during ripening were determined using high performance liquid chromatography (HPLC) as described in section 3.2.5.

8.2.6. ***Proteolytic activity of adjunct bacteria in reconstituted skim milk***

All organisms were grown overnight at 37^{o}C in MRS broth (Merck). To minimize carryover of free amino acids during inoculation, 10 mL of cells were washed and resuspended to the original volume with 0.32 mM sodium phosphate, pH 7.2. Cells were inoculated (2%) into RSM (12% w/v) and incubated at their optimum temperatures (37^{o}C) for 18 h. The proteolytic activity of the organism was determined using the *o*-phthaldialdehyde (OPA) method as described in section 4.2.6.

8.2.7. ***Assessment of proteolysis***

Proteolysis of the cheeses was assessed by estimating the amount of soluble nitrogen in water, 12% trichloroacetic acid (TCA; Sigma-Aldrich) and 5% phosphotungstic acid (PTA; Sigma-Aldrich) using the Kjeldahl method as previously described in section 3.2.6.

8.2.8. ***Sensory evaluation of cheeses***

Staff and students of Victoria University were recruited as sensory panelists for a series of acceptance rating test (n = 15) and scoring test for specific attributes (n = 15). The panelists were familiar with basic sensory evaluation techniques for Cheddar cheeses and were further trained for their ability to detect cheddary flavour, sour-acid taste, bitterness and vinegary taste as previously described in section 5.2.2. Prior to sensory evaluation they also participated in briefing sessions. All panelists signed a Victoria University human subject's consent form (Appendix A.1).

Sensory evaluation was conducted for the cheeses after ripening for 6, 12 and 24 wk. A total of 27 cheeses (9 variations and 2 replicates) were presented to the panelists in randomly order over a period of five days. Cheese cubes from the three replications of the same batch were mixed randomly so that all replications from the same batch were presented equal number of times. One hour prior to sensory evaluation, cheese samples were removed from the refrigerator and cut into pieces (about 1.5 x 1.5 x 1.5 cm in size) and placed on white plates coded with a random 3-digit numbers. Panellists had access to deionized water and unsalted water crackers to help cleanse their palates.

For acceptance rating test, panellists were instructed to evaluate their perception of the overall acceptance of the cheeses on a 10-point intensity scale (1 = dislike extremely, 10

= like extremely). Prior to tasting, panellists completed a questionnaire on frequency of cheese consumption (<1 once per wk, 2-3 times per wk, 4-5 times per wk, or >5 times per wk) and cheese preference (mild, medium or sharp matured cheese). Panellists also evaluated specific flavour and texture attributes, which included sour-acid, vinegary, bitterness, creaminess, cheddary intensity, hardness and crumbliness using a 10-point intensity scale as per the method described in section 5.2.2.

8.2.9. ***Determination of ACE-inhibitory activity of cheeses water-soluble extract (WSE)***

Cheese WSE was prepared as per the method described in section 6.2.2. The extracts were concentrated by freeze drying (Dynavac FD300; Airvac Engineering Pty. Ltd., Rowville, Australia) at –20°C and –100 kPa for 72 h. The freeze-dried WSE was then stored in vacuum at -20^{o}C for determination of ACE-inhibitory activity.

The ACE-inhibitory activity of the freeze-dried WSE was measured using the spectrophotometric assay of Cushman and Cheung (1971) as described in section 6.2.4. The ACE-inhibitory activity of the cheeses was also expressed as IC_{50} (the concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity). The IC_{50} was determined as per the method described in section 6.2.4 and 6.2.5 with modification. The IC_{50} was determined using graphical extrapolation by plotting ACE-inhibition as a function of different protein concentrations. To create a graphical correlation between ACE-inhibition and protein concentration, each sample was adjusted to at least three levels of concentrations by standard volume dilution. The protein content of the samples was determined using the Folin-Lowry method (Lowry et al., 1951). The IC_{50} was then determined from the linear regression as the protein concentration in the sample required to inhibit 50% of the ACE activity.

8.2.10. ***Statistical analysis***

One-way analysis of variance was used to establish differences between means of data obtained from OPA-analysis (Minitab Inc, State College, PA, USA). Data obtained during ripening was analyzed as a split plot in time design using General Linear Model procedure of the SAS system (SAS Inst. Inc., Cary, NC, USA). Multiple comparisons of means were achieved using pdiff option (t – test). The main plot factors were variation in adjunct bacteria (Control, cheeses made with starter culture only; L10, cheeses made with starter culture and *Lb. acidophilus* L10 adjunct; H100, cheeses made with starter culture,

Lb. acidophilus L10 and *Lb. helveticus* H100 adjuncts) and replications (blocks) with ripening temperatures (4, 8 & 12^{o}C) as the split-plot factor. This design was additionally split with ripening time (day 1, wk 6, wk 12 & wk 24) as additional split-plot factor. All significant differences were at least $P < 0.05$. All results presented are means of at least 6 observations ($n \geq 6$). Simple linear correlation analysis was used to determine a relationship between mean scores of sensory attributes and that of chemical analysis.

8.3. Results and discussions

8.3.1. ***Cheese composition***

The composition of control, L10 and H100 cheeses made with or without the addition of *Lb. helveticus* H100 and/or *Lb. acidophilus* L10 ripened at 4, 8 and 12°C after 24 wk is summarized in Table 8.1. At the end of ripening period of 24 wk, the percentage of salt, fat and protein remained relatively constant in all cheeses stored at 4, 8 and 12°C ($P > 0.05$). The moisture content, however, reduced significantly after 24 wk ($P < 0.05$). The changes in moisture content after ripening was significantly affected by the interactions of time x temperature and time x adjunct bacteria (Table 8.2). Moisture content of control and L10 cheeses ripened at 12°C was significantly lower ($P < 0.05$) than that ripened at 4°C after 24 wk. The pH of the cheeses also reduced significantly after 24 wk of ripening at 4, 8 and 12^{o}C; the extent of the decrease depended on factors such as adjunct bacteria, temperature, time and interactions between the factors ($P < 0.05$) (Table 8.2). The cheese curd normally contains about 0.8 to 1.5% lactose at the end of manufacture (Fox et al., 1993). Acid production during ripening is normally due to the residual lactose fermentation by the cheese microflora, mainly the starter bacteria (Singh, et al., 2003). The decrease in pH during ripening could also be attributed to the increase in organic acid concentration as will be discussed below. The pH of the control, L10 and H100 cheeses was not significantly different before ripening (day 1) ($P > 0.05$). After 24 wk of ripening at 4, 8 and 12°C, the pH of L10 and H100 cheeses was significantly lower than that of the control cheese ($P < 0.05$). This finding is in agreement with that reported by Madkor et al. (2000) that adjunct lactobacilli also metabolized residual lactose in the cheese curd and produced acid.

8.3.2. ***Viability of bacteria in cheeses***

The survival and growth of starter lactococci, probiotic bacteria, NSLAB and *Lb. helveticus* adjunct were determined during different stages of Cheddar cheese-making. Cheese-milk for different batches of the cheeses (control, L10 and H100) were inoculated with the same concentration of starter lactococci ($P > 0.05$). There were about 10^7 cfu g^{-1} of starter lactococci in the milk at the beginning of cheese-making process, which increased significantly ($P < 0.05$) during milk-ripening, cooking and cheddaring reaching about 10^9 cfu g^{-1} at the end of the process (Figure 8.1). There was about 10^7 cfu g^{-1} of *Lb. acidophilus* L10 in the milk at the beginning of cheese-making process, which increased by about one log at the end of the process (Figure 8.1). The counts of starter lactococci among control, L10 and H100 cheeses during cheese-making were, however, not significantly different ($P > 0.05$). Also, addition of *Lb. helveticus* to the probiotic cheeses (H100) did not affect the count of the probiotic bacteria during cheese-making ($P > 0.05$).

Viability of starter lactococci and NSLAB in Cheddar cheeses during ripening for 24 wk at 4, 8 and 12°C are shown in Figure 8.2. The counts of starter lactococci decreased significantly as the ripening period increased ($P < 0.05$; Figure 8.2a), probably due to the unfavorable conditions in cheeses such as low water activity, low redox potential, low pH, high salt and lack of fermentable carbohydrate. The count of starter lactococci during ripening was significantly affected by factors such as adjunct bacteria, temperature and time and interactions between the factors ($P < 0.05$) (Table 8.2). The lower count of starter lactococci in L10 and H100 cheeses as compared to that of the control was possibly due to the low pH of cheeses made with adjunct bacteria as mentioned previously.

Non starter lactic acid bacteria are not deliberately added as a part of the starter culture but are contaminants which gain access to the cheese during cheese-making stage or survive milk-pasteurization in an attenuated state (Beresford & William, 2004). NSLAB are chiefly composed of lactobacilli such as *Lb. plantarum*, *Lb. casei* and *Lb. brevis* (Gardiner et al., 1998) and can impact on cheese quality in both beneficial and detrimental ways. The presence of NSLAB in commercial cheeses is associated with the development of more intense Cheddar flavour in a shorter time (Puchades et al., 1989). In our study, the count of NSLAB was expressed as the count of total lactobacilli, which included the contaminants, *Lb. helveticus* adjuncts and/or probiotic bacteria.

The count of NSLAB during ripening was significantly affected by factors such as adjunct bacteria, time and interactions between time x adjunct bacteria, time x temperature, time x adjunct bacteria x temperature ($P < 0.05$) (Table 8.2). Although no lactobacilli were

added (Figure 8.1), there was 2.1 $\log_{10}$ cfu g^{-1} NSLAB in control cheese at day 1 (Figure 8.2b). The count of NSLAB increased to 7.5 $\log_{10}$ cfu g^{-1} in control cheeses ripened at 12°C and only to about 5.5 log cfu g^{-1} in control cheeses ripened at 4 and 8°C after 24 wk. The level of NSLAB in our study is in agreement with that reported by Peterson and Marshall (1990) for commercially produced Cheddar cheese. Cromie et al. (1987) and Folkertsma et al. (1996) also reported that elevation of ripening temperature from 7 to 12^{o}C resulted in a more rapid development of NSLAB in cheeses.

Viability of probiotic bacteria and *Lb. helveticus* H100 adjunct in Cheddar cheeses during ripening for 24 wk at 4, 8 and 12°C is shown in Figure 8.3. The count of the probiotic bacteria during ripening was significantly affected by factors such as adjunct bacteria, temperature, time and interactions between the factors ($P < 0.05$) (Table 8.3). *Lb. acidophilus* L10 survived better at lower ripening temperature (Figure 8.3a). The count of the probiotic bacteria remained at $>10^7$ cfu g^{-1} in L10 cheeses ripened at 4 and 8^{o}C after 24 wk. There was about one log reduction in count of probiotic bacteria in L10 cheeses after 24 wk of ripening at 12°C. Probiotic bacteria count of H100 cheeses was also significantly lower than that of L10 cheeses ($P < 0.05$). There was a possible competition for growth factors such as nutrients between the *Lb. helveticus* and the probiotic bacteria in the H100 cheeses during ripening. The count of probiotic bacteria was $<10^6$ cfu g^{-1} in the cheese with the addition of *Lb. hevelticus* H100 after 24 wk of ripening at 12°C. The count of *Lb. helveticus* in H100 cheeses during ripening at 4, 8 and 12°C is depicted in Figure 8.3b. Count of *Lb. helveticus* adjunct in H100 cheeses ripened at 12°C was lower than that ripened at 4 and 8°C. The lower counts of adjunct bacteria in cheeses ripened at 12°C were also reflected in the NSLAB (total lactobacilli) counts of L10 and H100 cheeses (Figure 2b).

8.3.3. ***Concentration of organic acids***

Glycolysis is an essential biochemical event during the production of cheeses. It involves the conversion of lactose to sugar constituents or organic acid by LAB. About 98% of the lactose in milk is lost in whey during cheese-making and a relatively small amount of lactose remains in the cheese curd (Fox et al., 1993). This residual lactose can be fermented by starter and NSLAB leading to the production of organic acids. The metabolic activity of the microorganisms in the cheeses was monitored by estimating the metabolic products including lactic, acetic, citric, propionic and butyric acids during ripening at 4, 8 and 12°C for 24 wk (Figure 8.4). An increase in concentration during ripening was observed in all

organic acids analysed except for citric acid. The concentration of organic acids measured in this study (lactic, acetic, citric and propionic acids, except for butyric acid) was significantly ($P < 0.05$) affected by factors such as adjunct bacteria, temperature, time and interactions between time and the factors ($P < 0.05$) (Table 8.2). Significant interaction between adjunct bacteria x temperature was also observed for the concentration of citric and propionic acids ($P < 0.05$) (Table 8.2).

Lactic acid was present in much greater abundance than other organic acids. Formation of lactic acid is essential for flavour development and keeping quality (Upreti et al., 2006). Concentration of lactic acid increased significantly as the ripening time increased in all cheeses ($P < 0.05$) (Figure 8.4a). The largest increase in concentration of lactic acid was during the first 6 wk of ripening. *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* metabolize lactose to L(+) lactic acid; the glucose moiety is metabolized via the Embden-Meyerhof (EM) pathway while galactose is metabolized via the tagatose pathway. Upreti et al. (2006) made a similar observation that the largest decrease in lactose content and thus the formation of lactic acid occurred at day 1 of ripening and that starter bacteria had minimal ability to metabolize residual lactose in the cheeses after day 1. St-Gelais et al. (1991) also observed an increase in lactic acid content from day 1 to 2 mo of ripening at 4°C; thereafter, lactic acid content did not change until the end of the ripening period of 6 mo. This finding illustrates the possibility of hetero-fermentation during cheese ripening that will occur in order for starter bacteria to survive and for NSLAB to grow. Figure 8.4a shows that the concentration of lactic acid during ripening in cheeses ripened at 8 and 12°C was significantly higher than that at 4°C ($P < 0.05$). Cheeses with lactobacilli adjuncts also had significantly higher concentration of lactic acid than the control cheese ($P < 0.05$). *Lactobacillus* sp. converts galactose via the Leloir pathway to glucose-6-P, which is then metabolized to DL-lactate via the EM pathway (Fox et al., 1993).

Acetic acid was detected in all cheeses at levels which increased with time (Figure 8.4b). McGregor and White (1990) and Bouzas et al. (1993) also reported acetic acid to increase steadily over the maturation period. Acetic acid can be produced from citrate, lactose and amino acids (Aston & Dulley, 1982). Figure 8.4b shows that concentration of acetic acid was significantly higher ($P < 0.05$) in cheeses with the addition of *Lactobacillus* sp (L10 and H100 cheeses). This increase could be due to the higher amount of free amino acids in cheeses produced with the addition of *Lactobacillus* adjuncts, as discussed later (Figure 8.5), which might have served as precursors for the formation of acetic acid. Molimard and Spinnler (1996) confirmed that acetic acid can be produced from the degradation (oxidative deamination or decarboxylation) of amino acids (alanine and serine).

Concentration of acetic acid in L10 cheeses was not significantly different to that of H100 cheeses after 24 wk ripening at 4, 8 and 12^{o}C ($P > 0.05$).

The concentration of citric acid decreased as the ripening time increased ($P < 0.05$) (Figure 8.4c). Addition of *Lactobacillus* sp. and higher ripening temperatures also increased the rate of hydrolysis of citric acid (Figure 8.4c). Citric acid was not detected in L10 and H100 cheeses after 24 wk of ripening at 12°C. Thomas (1987) reported that citrate in the Cheddar cheeses was found to decrease slowly to almost zero at 6 mo, presumably as a result of metabolism by lactobacilli, which became the major component of the NSLAB flora. Citrate is involved in the Krebs or citric acid cycle, where it acts both as a substrate and a product. Citrate can be used as a substrate by citrate-fermenting strains of LAB to produce pyruvic acid, carbon dioxide, and acetic acid (Bouzas et al., 1993). The later reference also reported that the disappearance rate of citric acid in cheeses ripened at 12°C was significantly higher ($P < 0.05$) than that ripened at a lower temperature.

Propionic acid was only detected in the cheeses at the later stages of ripening in particular after 12 wk (Figure 8.4d). Propionic acid was not detected in 24 wk old control cheeses ripened at 4°C. Traces of propionic acid, however, were detected in control cheeses ripened at 8 and 12°C. Cheeses with the addition of *Lactobacillus* sp. (L10 and H100 cheeses) had higher concentration of propionic acid than the control cheeses, especially in those ripened at 8 and 12°C. The *Lb. helveticus* adjunct used in this study appeared to release propionic acid as a part of their metabolic products. The concentration of propionic acid in H100 cheeses was significantly higher than that of the control and L10 cheeses ($P < 0.05$). deLiano et al. (1996) reported an increase in propionic acid concentration in 8 mo pickled white cheese at the end of ripening and explained that the increase might be due to the action of esterases and lipases of the starter lactococci and NSLAB. Some strains of *Lactobacillus* were reported to produce propionic acid (Ocando et al., 1993). Proteolysis of side chains of amino acids from casein, and non-specific esterase activities have also been suggested to be the sources of propionic acid during maturation of cheeses (Bouzas et al., 1993; deLiano et al., 1996). In our study, only a small amount of butyric acid (< 0.05%) was detected in the cheeses during ripening. No particular trend was observed in the concentration of butyric acids, although other study has reported a gradual increase in butyric acid over a 6 mo ripening period, attributed to the action of esterases and lipases of starter lactococci and NSLAB (deLiano et al., 1996).

*8.3.4. **Assessment of proteolysis***

The OPA-based spectrophotometric assay detects the released α-amino groups, which result from the proteolysis of milk proteins, thus giving a direct measurement of proteolytic activity. The proteolytic activities in RSM of *Lb. helveticus* strains (H100, H 1188, H474, H1315) and probiotic bacteria *B. longum* 1941, *B. animalis* LAFTI®B94, *Lb. casei* 279, *Lb. casei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10 is presented in Figure 8.5. The proteolytic activity of these bacterial strains is expressed as the amount of free amino groups measured as a difference in absorbance values at 340 nm, after substraction of values for the control RSM. As shown in Figure 8.5, all *Lb. helveticus* sp. had significantly higher ($P < 0.05$) proteolytic activity as compared to the probiotic microorganisms, releasing higher amount of free amino groups. Among the *Lb. helveticus* strains, only commercial strain *Lb. helveticus* H100 was used in the cheese-making. The proteolytic activities of other *Lb. heveticus* strains 1188, H474 and H1315 are presented just for comparison purpose. *Lb. helveticus* H100 had considerably high proteolytic activity when compared to other *Lb. helveticus* strain analysed (Figure 8.5).

Proteolysis in the cheeses during ripening was assessed by analysing the proteolytic indices including water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN). Proteolysis contributes to the development of cheese texture via the hydrolysis of protein matrix of cheese, via the increase in pH through the production of NH_3 from amino acids catabolism and by increasing the water-binding capacity of the curd through the formation of new α–carboxylic and α–amino groups produced during hydrolysis of peptide bonds (Upadhyay, et al., 2004). Proteolysis also contributes to flavour of the cheese through the liberation of short peptides and amino acids, some of which may have flavours, and through the production of amino acids as a precursor for a range of catabolic reactions which produce many important volatile compounds (McSweeney & Sousa, 2000). Intensified proteolysis generally leads to an accelerated ripening of the product, which is desired as long as no effect on the storage quality is encountered.

The ratios of WSN, TCA-SN and PTA-SN to total nitrogen (TN) of Cheddar cheeses during ripening at 4, 8 and 12°C for 24 wk are presented in Figure 8.6. The level of WSN in the cheeses increased with time (Figure 8.6a) and was significantly affected by interaction of time x adjunct bacteria, time x temperature and time x adjunct bacteria x temperature (Table 8.2). The increase was the largest during the first 6 wk of ripening. WSN is primarily the product of proteolysis from residual rennet or from proteinases

present in the curd such as plasmin or cell envelope proteases from the cheese microflora (Sousa et al., 2001). WSN in L10 and H100 cheeses after 24 wk of ripening at 4, 8 and 12°C was significantly higher than that of the control cheeses (Figure 8.6a) ($P < 0.05$). This result indicates the contribution of proteolytic enzyme released by the probiotic *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts in increasing the proteolysis at this level. Increased in ripening temperature also improved the proteolysis of the cheeses. The WSN of L10 cheeses ripened at 12°C after 24 wk of ripening was significantly higher than those ripened at 4 or 8°C ($P < 0.05$). The pH of L10 and H100 cheeses ripened at higher ripening temperature (8 and 12^{o}C) was significantly lower than that at 4^{o}C. The difference in pH may also contribute to the changes in primary proteolysis, since proteinase such as plasmin is not active when pH is below 5.2.

Concentration of TCA-SN of control, L10 and H100 cheeses during ripening was significantly affected by factors such as adjunct bacteria, temperature, time and interactions between time x adjunct bacteria, time x temperature and time x adjunct bacteria x temperature (Table 8.2). The concentration of TCA-SN increased progressively as the ripening time increased ($P < 0.05$) (Figure 8.6b). Fox et al. (1993) reported that rennet and bacterial proteinases and peptidases are responsible for the formation of some of the 12% TCA-SN. Level of TCA-SN during ripening was significantly higher in L10 and H100 cheeses than that of the control cheeses ($P < 0.05$). This increase indicated the contribution of adjunct bacterial proteinases and peptidases activity. Increased in ripening temperature also increased the TCA-SN of the cheeses during ripening. Concentration of TCA-SN between L10 and H100 cheeses during ripening was, however, not significantly different ($P > 0.05$).

The level of PTA-SN has been widely used as an index of free amino acids (FAA) in cheeses (Fox et al., 1993). PTA-SN is produced primarily by the action of microbial peptidases. Concentration of PTA-SN of control, L10 and H100 cheeses during ripening was significantly affected by factors such as adjunct bacteria, temperature, time and interactions between the factors (Table 8.2). The concentration of PTA-SN increased with time and with higher ripening temperatures (Figure 8.6c). The concentration of PTA-SN in cheeses with the addition of *Lb. helveticus* H100 and/or probiotic *Lb. acidophilus* L10 was also significantly higher ($P < 0.05$) than that of the control cheeses.

The high levels of soluble nitrogen in the L10 and H100 cheeses reflect the contribution of the adjunct bacteria in promoting more proteolytic activity in cheeses. Addition of *Lb. helveticus* in the probiotic cheese (H100 cheese) did not significantly improve the level of PTA-SN during ripening as compared to that of L10 cheeses ($P >$

0.05). Kiernan et al. (2002) reported that a decline in *Lb. helveticus* during ripening of Cheddar cheeses increased the level of intracellular enzymes in the cheeses that led to an increase in proteolysis. The decline in counts of *Lb. helveticus* in our study (Figure 8.3b) was less than that reported by Kiernan et al. (2002), which possibly explains as to why there was no increase in proteolysis of cheeses with the addition of *Lb. helveticus* adjunct. The use of *Lb. helveticus* strain with strong autolytic activity rather than strong proteolytic activity would possibly be better for increasing proteolysis of cheeses. In addition to that, as the pH of H100 cheese reach about 4.6, casein breakdown would stop as casein is not soluble at this pH (Fox et al., 1993). The low pH, further explain why proteolysis slow down at the later stage of ripening when the pH of the cheese reach about 4.6 (Figure 8.6a).

8.3.5. ***Sensory evaluation of cheeses with* Lactobacillus *sp. adjunct***

Acceptability of the Cheddar cheeses stored at 4, 8 and 12^{o} C after ripening for 6, 12 and 24 wk is shown in Table 8.3. Despite the improvement in the secondary proteolysis of cheeses with the addition of *Lactobacillus* sp., the acceptability of the cheeses (L10 and H100) was not significantly higher than the control cheese ($P > 0.05$). There were significant effects of the type of adjunct microorganisms used, ripening time, ripening temperatures and their interactions on the acceptance scores of the cheeses ($P < 0.05$) (Table 8.4). At the early stage of ripening (12 wk), cheeses with the addition of *Lactobacillus* sp. received a significantly lower acceptance scores than the control ($P < 0.05$). The acceptability of the cheeses with *Lactobacillus* sp., however, improved and was comparable to the control cheese after prolonged ripening (24 wk). The acceptability at the early stage of ripening was probably influenced by the lower pH (Table 8.1) and higher concentration of lactic acid (Figure 8.4a) of the probiotic cheeses as compared to the control cheeses. As the ripening time increased, there was an increase in the production of peptides and free amino acids in probiotic cheeses (Figure 8.6). Free amino acids are precursor for flavour development. The release of other flavour components probably masked the acidity and improved the flavour of the cheeses with *Lactobacillus* sp. at 24 wk (L10 and H100 cheeses).

Scores for specific attributes (sour-acid, vinegary, bitterness, creaminess, cheddary, hardness and crumbliness) of the cheeses ripened at 4, 8 and 12^{o}C at 6, 12 and 24 wk are shown in Figure 8.7. Scores for cheddary increased as the ripening period increased in all cheeses (Figure 8.7). There were significant effects of the type of adjunct microorganisms

used, ripening temperatures and ripening time on the cheddary scores of the cheeses ($P < 0.05$) (Table 8.4). The increased in ripening temperatures improved the scores of cheddary. Improvement in the proteolysis and the increase concentration of organic acid of the probiotic cheeses was also reflected on the significantly higher ($P < 0.05$) cheddary scores of these cheeses as compared to the control cheese. As mentioned previously, the counts of starter lactococci decreased faster in L10 and H100 cheeses. Early studies showed that Cheddar cheese had good flavour when the starter used exhibited poor survival in cheese (Kenny et al., 2006). Autolysis of starter bacteria in the cheeses allows the releases of their intracellular enzymes during ripening and may thus accelerate cheese ripening and improve the cheddary flavour. Addition of *Lb. helveticus* H100 to the probiotic cheeses, however, did not improve the cheddary scores of the probiotic cheese any further (Figure 8.7).

Sensory panels scored the sour-acid attributes of L10 and H100 cheeses higher, especially at the early stage of ripening (Figure 8.7). The scores of vinegary were rated very low in all cheeses (Figure 8.7). There were significant effects of the type of adjunct microorganisms used, ripening time, ripening temperatures and their interactions on the scores for sour-acid and vinegary ($P < 0.05$) (Table 8.4). The scores of sour-acid and vinegary were higher in L10 and H100 cheeses as compared to the control cheeses (Figure 8.7).

Accumulation of hydrophobic short peptides, originating from α_{s1}-CN or β-CN may cause bitterness in cheeses. Scores of bitterness at the early stage of ripening were not significantly different ($P > 0.05$) among control cheese, L10 and H100 cheeses (Figure 8.7). It is interesting to note that bitterness in the cheeses increased up till 12 wk and decreased after that period in the probiotic cheeses especially in the cheeses that received higher degree of proteolysis or ripened at higher temperatures (Figure 8.7). Proteolytic enzymes from probiotic *Lb. acidophilus* LAFTI®L10 and *Lb. helveticus* H100 could probably degrade the bitter peptides and the proteolytic activity of these microorganisms were optimized when the cheeses were ripened at 8 and 12°C as compared to 4°C. Scores for creaminess decreased as the ripening time and temperature increased (Figure 8.7). Creaminess or milky taste is one of the characteristic of young cheese. During ripening, the liberation of the flavour components and the increased concentration of organic acid reduced the creaminess of the cheeses. This was especially apparent in 24 wk L10 and H100 cheeses.

Scores for hardness at the beginning of the ripening period were higher in the cheeses with the addition of *Lactobacillus* sp. as compared to the control cheese (Figure 8.7). The hardness of the cheeses with adjunct *Lactobacillus,* however, decreased

significantly after 24 wk possibly due to the increased proteolysis ($P < 0.05$). Crumbliness of the cheese decreased significantly with time ($P < 0.05$) and cheeses with adjunct *Lactobacillus* sp. were also regarded as less crumbly (Figure 8.7).

8.3.6. ***ACE-inhibitory activity of cheeses WSE***

The increase in proteolysis in cheeses with adjuncts bacteria (L10 and H100 cheeses) indicated that more peptides were released into the cheeses as compared to the control cheeses. Some peptides released during ripening of probiotic Cheddar cheeses were potential ACE-inhibitory peptides (Chapter 6.0, section 6.4 & Chapter 7.0, section 7.3.11). The ACE-inhibitory activity of control, L10 and H100 cheeses during ripening at 4, 8 and 12°C is depicted in Figure 8.8 and Table 8.2 shows the percentage of ACE-inhibition as affected by factors such as adjunct bacteria, temperature, time and interaction among the factors ($P < 0.05$).

The ACE-inhibitory activity of WSE of the cheeses analyzed at 6 wk intervals increased with time and was maximum at 24 wk in control and L10 cheeses (Figure 8.8a). Probiotic cheese with added *Lb. helveticus* (H100 cheese), on the other hand, had the highest ACE-inhibitory activity at 12 wk. The percentage of ACE-inhibition of H100 cheeses at 24 wk was significantly lower than at 12 wk ($P < 0.05$). As shown in Figure 8.8a, ACE-inhibition of L10 cheeses was also significantly higher than that of the control cheeses after 24 wk of ripening at 4, 8 and 12°C ($P < 0.05$). Increased in ripening temperature from 4 to 8 and 12°C also further improved the ACE-inhibition of L10 cheeses. ACE-inhibitory activity of the cheeses is also expressed as IC_{50} (concentration of the sample required to inhibit 50% of the ACE activity) (Figure 8.8b). As shown in Table 8.2, the IC_{50} of control, L10 and H100 cheeses was significantly affected by adjunct bacteria, temperature, time and interaction between adjunct bacteria x temperature, time x adjunct bacteria, time x adjunct bacteria x temperature ($P < 0.05$). The interaction between time and temperature did not significantly affect the IC_{50} of the cheeses ($P > 0.05$). IC_{50} among L10 cheeses ripened at 4, 8 and 12°C was not significantly different ($P > 0.05$). It was possible that most of the active peptides in the cheeses ripened at different ripening temperatures were similar, but varied in concentration.

8.4. Conclusions

Probiotic *Lb. acidophilus* L10 survived better in cheeses ripened at 4 and 8°C as compared to that at 12°C. Their counts remained at 10^7-10^8 cfu g^{-1} after 24 wk of ripening at 4 and 8°C. The count of probiotic *Lb. acidophilus* L10 declined by about 2 logs in the presence of *Lb. helveticus* H100 after 24 wk of ripening at 12°C. Cheeses with *Lb. helveticus* H100 and/or *Lb. acidophilus* L10 adjuncts had higher concentration of lactic, acetic and propionic acid than the control cheese. Increased in ripening temperature improved the proteolysis of the control, L10 and H100 cheeses. Cheeses with added *Lb. helveticus* H100 adjunct and/or *Lb. acidophilus* L10 had higher levels of WSN, TCA-SN and PTA-SN as compared to the control cheeses. L10 cheeses also had higher ACE-inhibitory activity than the control cheeses. The ACE-inhibition increased in cheeses ripened at higher temperatures. The IC_{50} of the cheeses ripened at 4, 8 and 12°C, however, was not significantly different. The use of *Lb. helveticus* did not further improve the proteolysis and the ACE-inhibitory activity of the probiotic cheeses. Our results indicated that addition of probiotic *Lb. acidophilus* L10 into Cheddar cheeses ripened at 4, 8 and 12°C could have potential to improve the quality of cheeses and health status of the product through increased ACE-inhibitory activity.

Table 8.1. Composition of Cheddar cheeses during ripening at 4, 8 and 12°C for 24 wk

Composition	Ripening temperature (^oC)	Ripening period	Cheddar cheeses			SEM
			Control	L10	H100	
Moisture (%)	N/A	day 1	42.88^{aA}	43.58^{aA}	42.36^{aA}	0.38
	4	wk 24	40.45^{aB}	41.32^{aB}	40.42^{aB}	
	8	wk 24	39.88^{aBC}	40.47^{aC}	40.11^{aB}	
	12	wk 24	39.51^{aC}	39.48^{aD}	39.61^{aB}	
Salt (%)	N/A	day 1	1.97^{aA}	2.17^{aA}	1.97^{aA}	0.09
	4	wk 24	2.03^{aA}	1.95^{aA}	2.03^{aA}	
	8	wk 24	1.97^{aA}	1.97^{aA}	1.93^{aA}	
	12	wk 24	1.98^{aA}	2.05^{aA}	2.13^{aA}	
SM (%)	N/A	day 1	4.59^{aA}	4.97^{bAB}	4.65^{abA}	0.16
	4	wk 24	5.03^{aB}	4.71^{aA}	5.04^{aAB}	
	8	wk 24	4.93^{aB}	4.86^{aA}	4.72^{aA}	
	12	wk 24	4.98^{aB}	5.19^{abB}	5.38^{bB}	
Fat (%)	N/A	day 1	31.78^{aA}	32.14^{aA}	32.11^{aA}	0.33
	4	wk 24	32.74^{aA}	32.55^{aA}	32.97^{aA}	
	8	wk 24	32.80^{aA}	33.01^{aA}	32.46^{aA}	
	12	wk 24	33.23^{aA}	32.38^{aA}	33.22^{aA}	
Protein (%)	N/A	day 1	28.25^{aA}	28.32^{aA}	28.60^{aA}	0.35
	4	wk 24	28.44^{aA}	28.23^{aA}	29.01^{aA}	
	8	wk 24	27.81^{aA}	28.32^{aA}	28.34^{aA}	
	12	wk 24	28.44^{aA}	28.17^{aA}	28.87^{aA}	
pH	N/A	day 1	5.14^{aA}	5.11^{aA}	5.08^{aA}	0.03
	4	wk 24	4.91^{aB}	4.73^{bB}	4.75^{bB}	
	8	wk 24	4.89^{aB}	4.60^{bB}	4.58^{bC}	
	12	wk 24	4.80^{aB}	4.64^{bB}	4.62^{bBC}	

^{AB}Means in column of the same composition with different superscript are significantly different (P<0.05).
^{ab} Means in row with different superscript are significantly different (P<0.05).
SEM, pooled standard error of mean. Results are presented as mean of at least 6 observations ($n \geq 6$).
SM, salt in moisture. N/A, not applicable.
Control, cheese made with starter culture only; L10, cheese made with starter culture and *Lb. acidophilus* L10 adjunct; H100, cheese made with starter culture, *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts.

Table 8.2. Probabilities (*P*) of changes in means of composition variables (pH and moisture), counts of microorganisms (probiotic, lactococci and NSLAB), concentration of organic acids (lactic, acetic, citric, propionic and butyric), proteolysis level (WSN, TCA-SN and PTA-SN) and ACE-inhibitory activity (percentage of ACE-inhibition and IC_{50}) of control, L10 and H100 cheeses during ripening at 4, 8, and 12°C for 24 wk

Factors and interactions	Probabilities (*P*)[a]														
	Composition		Counts of microorganisms			Organic acid concentration					Proteolysis			ACE-I activity	
	pH	Moisture	NSLAB[c]	Lactococci	Probiotic	Lactic	Acetic	Citric	Propionic	Butyric	WSN[c]	TCA-SN[c]	PTA-SN[c]	ACE-I[c]	IC_{50}[c]
Main plot															
Block (B)	0.1977	0.4589	0.1208	0.3189	0.6686	0.7087	0.9124	0.4758	0.7243	0.3523	0.1782	0.3924	0.9305	**0.0141**	0.2860
Adjuncts (A)[b]	**<.0001**	0.4040	**<.0001**	**<.0001**	0.0537	**0.0003**	**0.0007**	**0.0001**	**<.0001**	**0.0286**	**0.0394**	**0.0070**	**0.0005**	**0.0059**	**0.0202**
Sub-plot															
Temperature (T)[b]	**0.0009**	0.3354	0.3162	**<.0001**	**0.0004**	**0.0002**	**0.0017**	**<.0001**	**<.0001**	**0.0196**	**<.0001**	**<.0001**	**<.0001**	**0.0108**	**0.0244**
B*T	0.5405	0.5798	0.1671	0.7458	0.3600	0.4358	0.4782	0.0906	0.5883	0.6285	0.7518	0.4822	0.8866	0.2420	0.5134
A*T	**0.0315**	0.8069	**<.0001**	**0.0702**	**0.0222**	0.3890	0.4361	**<.0001**	**<.0001**	0.8803	0.1434	0.9410	**0.0390**	**0.0031**	**0.0035**
Sub-plot															
Time (t)[b]	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0012**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
B*t	**<.0001**	**<.0001**	**<.0001**	0.2162	**0.0027**	**0.0112**	**0.0145**	**<.0001**	0.9890	0.9449	0.0700	0.0272	0.1643	**<.0001**	**0.0331**
A*t	**<.0001**	**0.0260**	**<.0001**	**<.0001**	**<0.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	0.3926	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
T*t	**0.0020**	**0.0432**	**<.0001**	**<.0001**	**<0.0001**	**<.0001**	**0.0003**	**<.0001**	**<.0001**	0.8327	**<.0001**	**<.0001**	**<.0001**	**0.0248**	0.3843
A*T*t	**0.0003**	0.3956	**<.0001**	**<.0001**	**0.0004**	**0.0365**	**0.0092**	**<.0001**	**<.0001**	0.9760	**0.0013**	**<.0001**	**<.0001**	**<.0001**	**0.0031**

[a]Data was analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were adjuncts (A) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

[b]Variations in adjuncts include: Control, cheese made without adjunct and with starter culture only; L10, cheese made with starter culture and *Lb. acidophilus* L10 adjunct; H100, cheese made with starter culture, *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts. Variations in temperature include: ripening at 4, 8 and 12°C. Repeated measures over ripening time were performed at day 1, wk 6, wk 12, and wk 24 (except for pH and moisture, which was analysed at day 1 and wk 24).

[c]NSLAB = non starter lactic acid bacteria, WSN = water soluble nitrogen, TCA-SN = trichloroacetic acid soluble nitrogen, PTA-SN = phosphotungstic acid soluble nitrogen, ACE-I = Angiotensin converting enzyme-inhibition (%), IC_{50} = concentration of ACE peptides needed to inhibit 50 % of ACE activity.

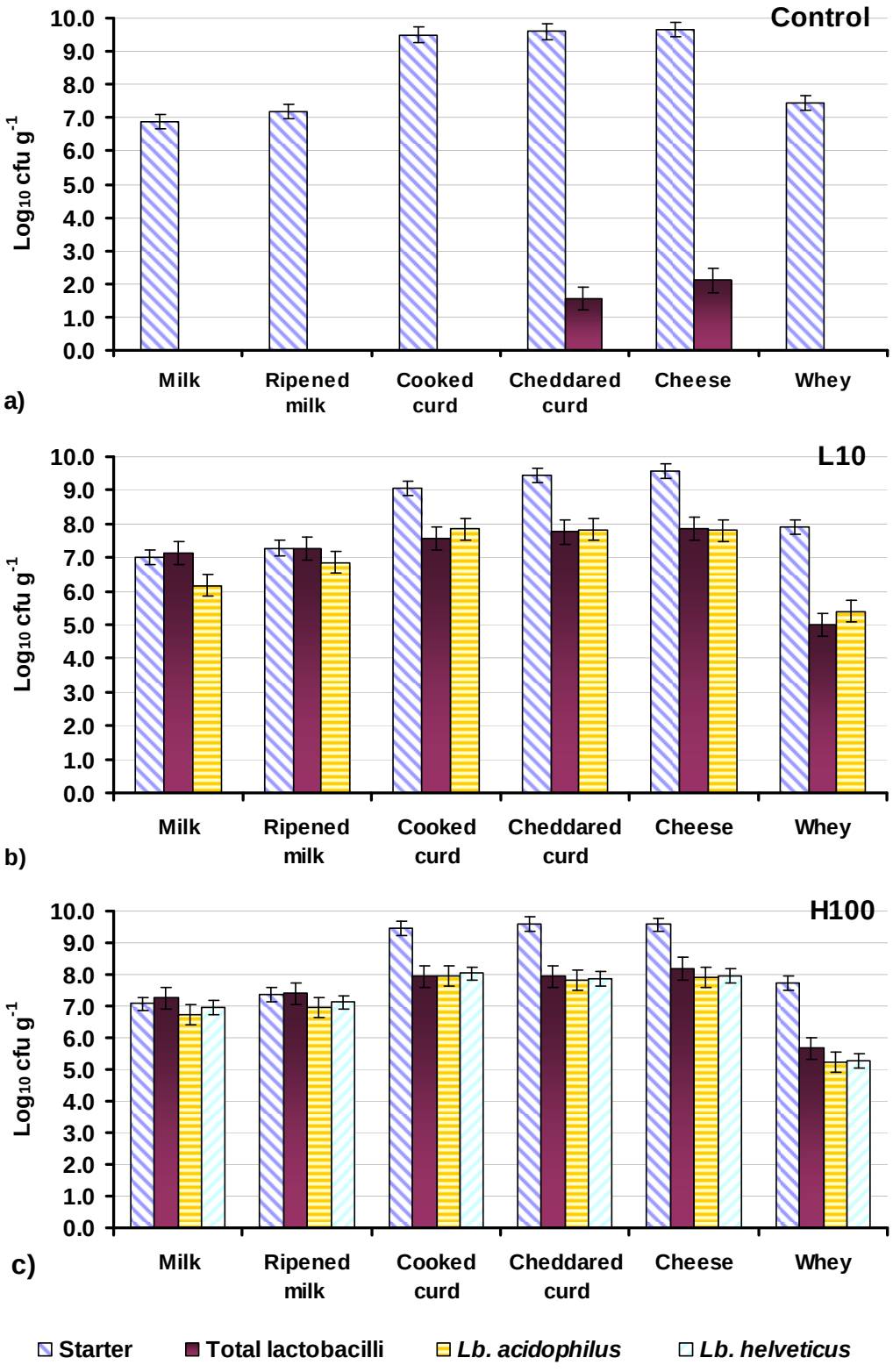


Figure 8.1. Counts of starter lactococci, total lactobacilli, *Lb. acidophilus* L10 and *Lb. helveticus* H100 in control Cheddar cheeses without probiotic (Control); probiotic cheeses with *Lb. acidophilus* L10 adjunct (L10); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (H100) during cheese making. Results are presented as means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.22, 0.35, 0.32 and 0.22 for counts of starter lactococci, total lactobacilli, *Lb. acidophilus* L10 and *Lb. helveticus*, respectively).

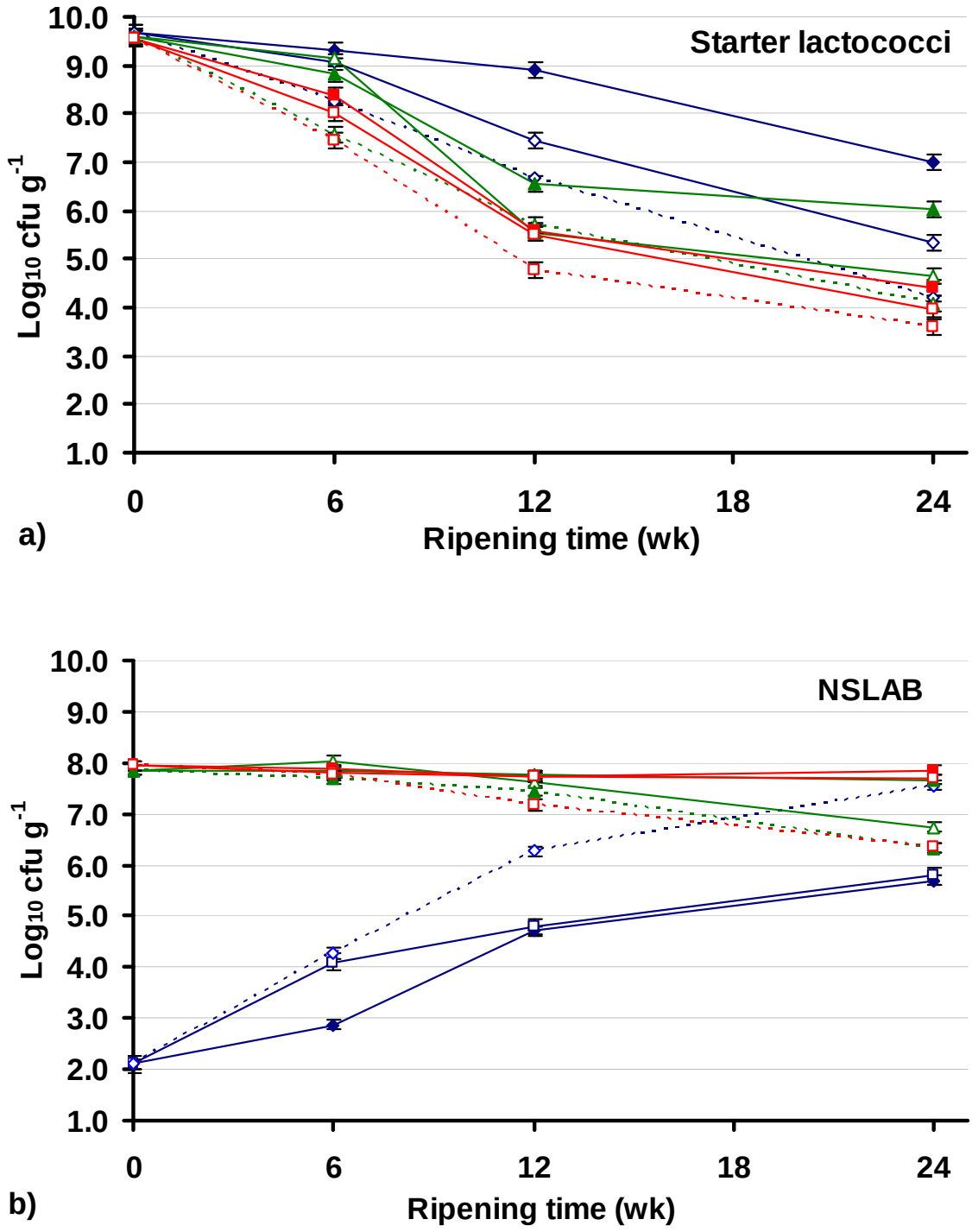


Figure 8.2. Viability of (a) starter lactococci and (b) NSLAB (total lactobacilli) in control Cheddar cheese without probiotic (—♦— Control 4°C; —◊—, Control 8°C; --◊--, Control 12°C); probiotic cheeses with *Lb. acidophilus* L10 adjunct (—▲— L10 4°C; —Δ— L10 8°C; --Δ--, L10 12°C); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (—■—, H100 4°C; —□—, H100 8°C; --□--, H100 12°C) during ripening at 4, 8 and 12°C for 24 wk. Results are presented as means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.16 and 0.10 for counts of starter lactococci and NSLAB, respectively).

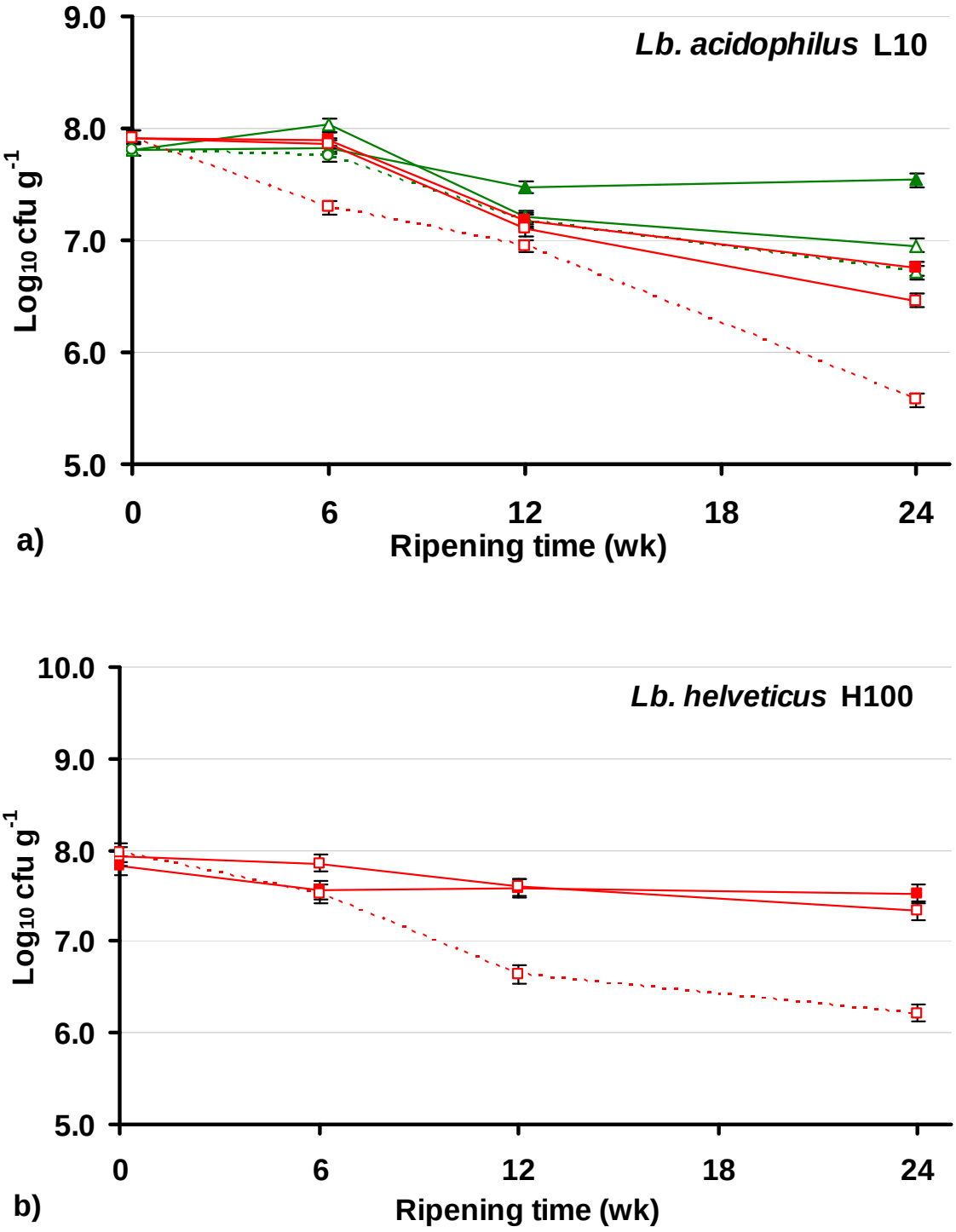


Figure 8.3. Viability of (a) probiotic *Lb. acidophilus* in L10 cheeses and H100 cheeses; (b) *Lb. helveticus* in H100 cheeses during ripening at 4, 8 and 12°C for 24 wk (—▲— L10 4°C; —Δ— L10 8°C; --Δ--, L10 12°C; —■—, H100 4°C; —□—, H100 8°C; --□--, H100 12°C). Results are presented as means of at least 6 observations (n ≥ 6). Error bar is pooled standard error of means (SEM = 0.06 and 0.15 for counts of probiotic bacteria and *Lb. helveticus* adjunct, respectively).

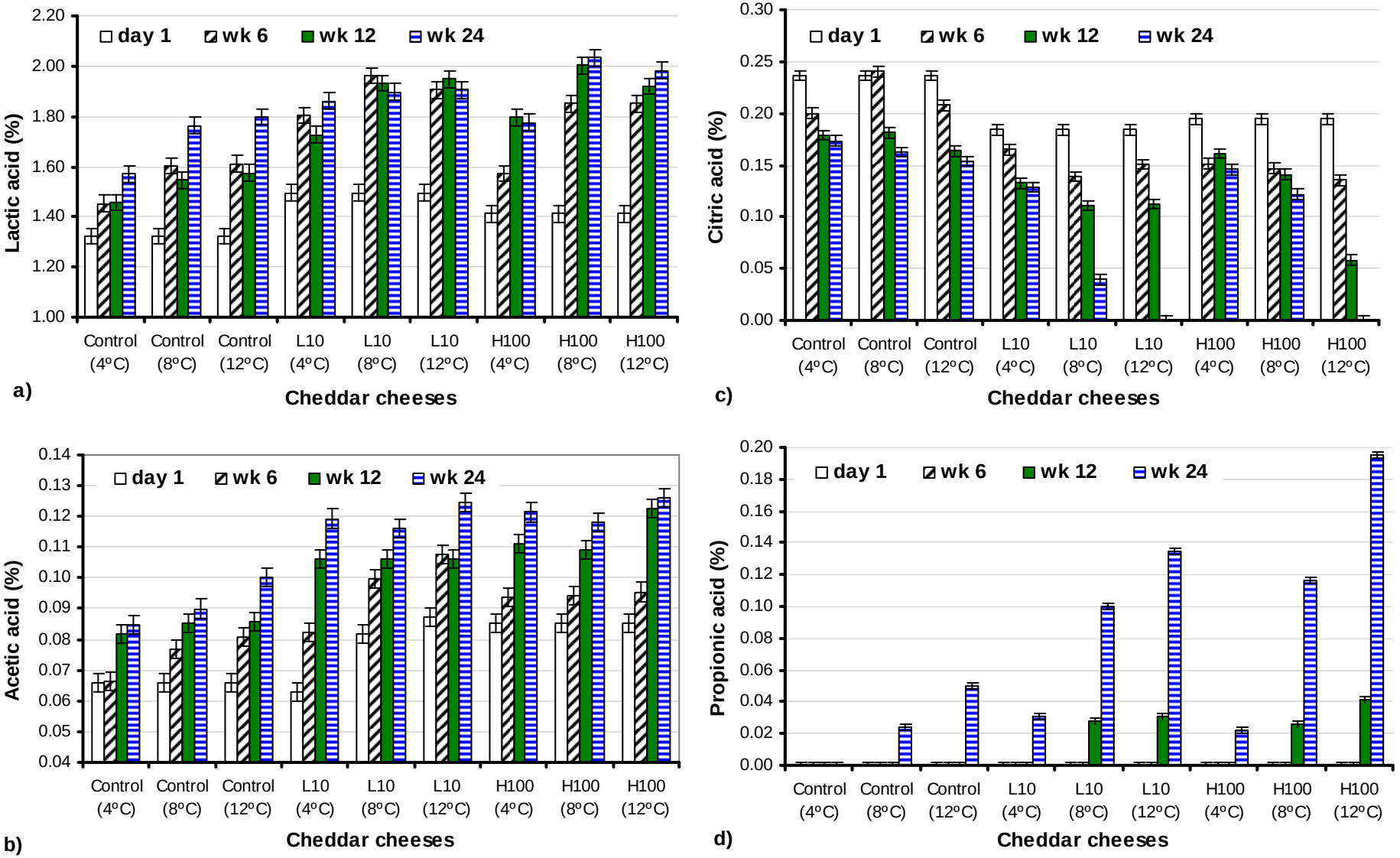


Figure 8.4. Concentration (%) of (a) lactic acid, (b) acetic acid, (c) citric acid, and (d) propionic acid in control Cheddar cheese without probiotic (Control); probiotic cheeses with *Lb. acidophilus* L10 adjunct (L10); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (H100) during ripening at 4, 8 and 12°C for 24 wk. Results are presented as means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 0.032, 0.003, 0.005 and 0.001 for concentration of lactic, acetic, citric and propionic acids, respectively).

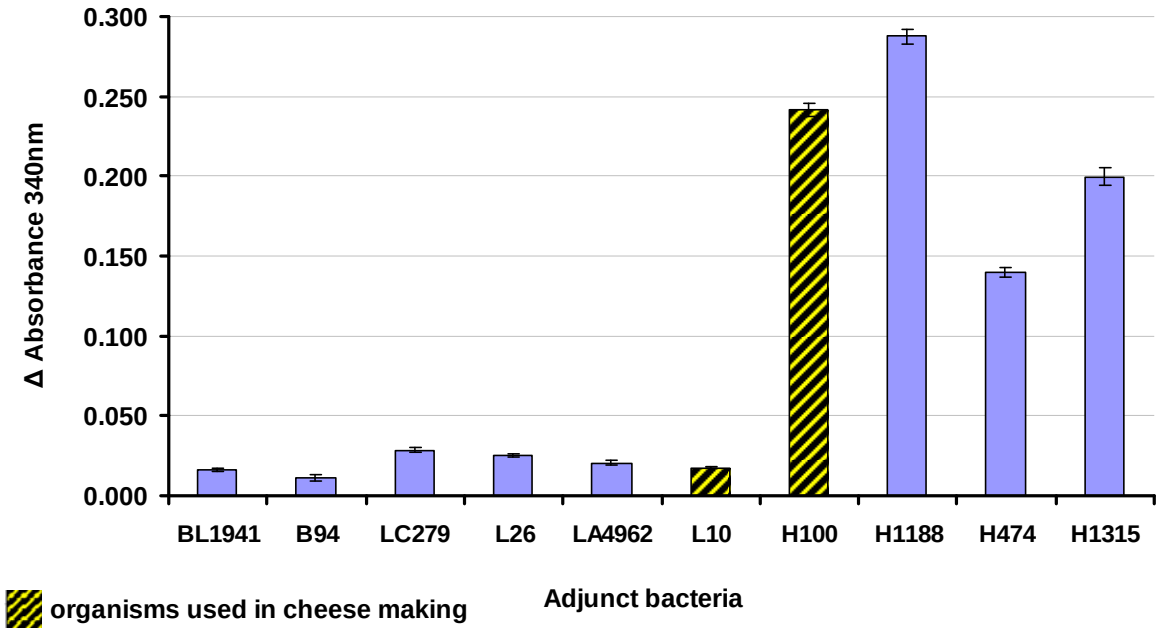


Figure 8.5. Indication of proteolytic activity of *Lb. helveticus* sp. (H100, H1188, H474, H1315) and probiotic bacteria *B. longum* 1941, *B. animalis* LAFTI®B94, *Lb. casei 279*, *Lb. casei* LAFTI®L26, *Lb. acidophilus* 4962 and *Lb. acidophilus* LAFTI®L10 after incubation in reconstituted skim milk (RSM) for 18 h. Data represent differences in absorbance values after subtracting the value for the control RSM. Results are presented as means of at least 6 observations ($n \geq 6$) ± standard error of means.

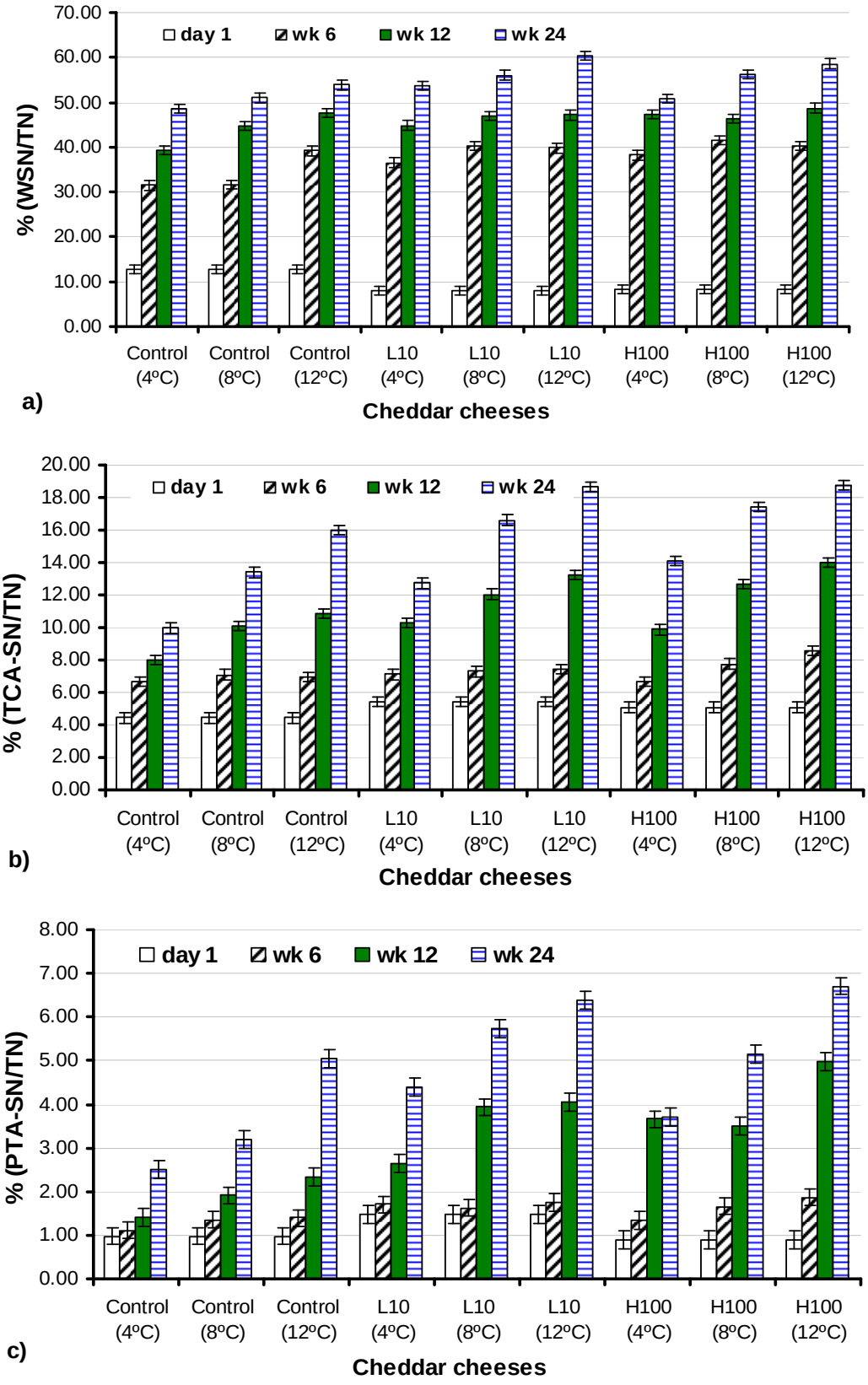


Figure 8.6. Concentration (expressed as percentage of total nitrogen) of (a) water-soluble nitrogen (WSN), (b) trichloroacetic acid-soluble nitrogen (TCA-SN), and (c) phosphotungstic acid-soluble nitrogen (PTA-SN) in control Cheddar cheese without probiotic (Control); probiotic cheeses with *Lb. acidophilus* L10 adjunct (L10); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (H100) during ripening at 4, 8 and 12°C for 24 wk. Results are presented as means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 1.02, 0.21 and 0.10 for concentration of WSN, TCA-SN and PTA-SN, respectively).

Table 8.3. Means overall acceptance of control and probiotic Cheddar cheeses during ripening at 4, 8 and 12°C for 24 wk

Ripening temperature (^{o}C)	Ripening period (wk)	Overall acceptance scores[1]		
		Control[2]	L10[2]	H100[2]
4	6	5.55^{aA}	5.42^{aAC}	5.07^{aAC}
8	6	5.90^{aA}	4.33^{bBC}	4.55^{bA}
12	6	6.19^{aA}	3.84^{bB}	4.44^{bA}
4	12	5.58^{aA}	4.28^{bBC}	4.27^{bAB}
8	12	5.30^{aA}	4.12^{bBC}	3.40^{bB}
12	12	6.04^{aA}	3.56^{bB}	3.47^{bB}
4	24	6.44^{aA}	4.97^{bC}	5.95^{aC}
8	24	6.84^{aA}	6.31^{aA}	6.14^{aC}
12	24	5.78^{abA}	6.22^{bA}	4.97^{aAC}

[AB] Means in column with different superscript are significantly different ($P<0.05$).
[ab] Means in row with different superscript are significantly different ($P<0.05$).
[1] Results are presented as mean of at least 6 observations ($n \geq 6$). Standard error of means = 0.41.
[2] Control, cheese made with starter culture only; L10, cheese made with starter culture and *Lb. acidophilus* L10 adjunct; H100, cheese made with starter culture, *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts.

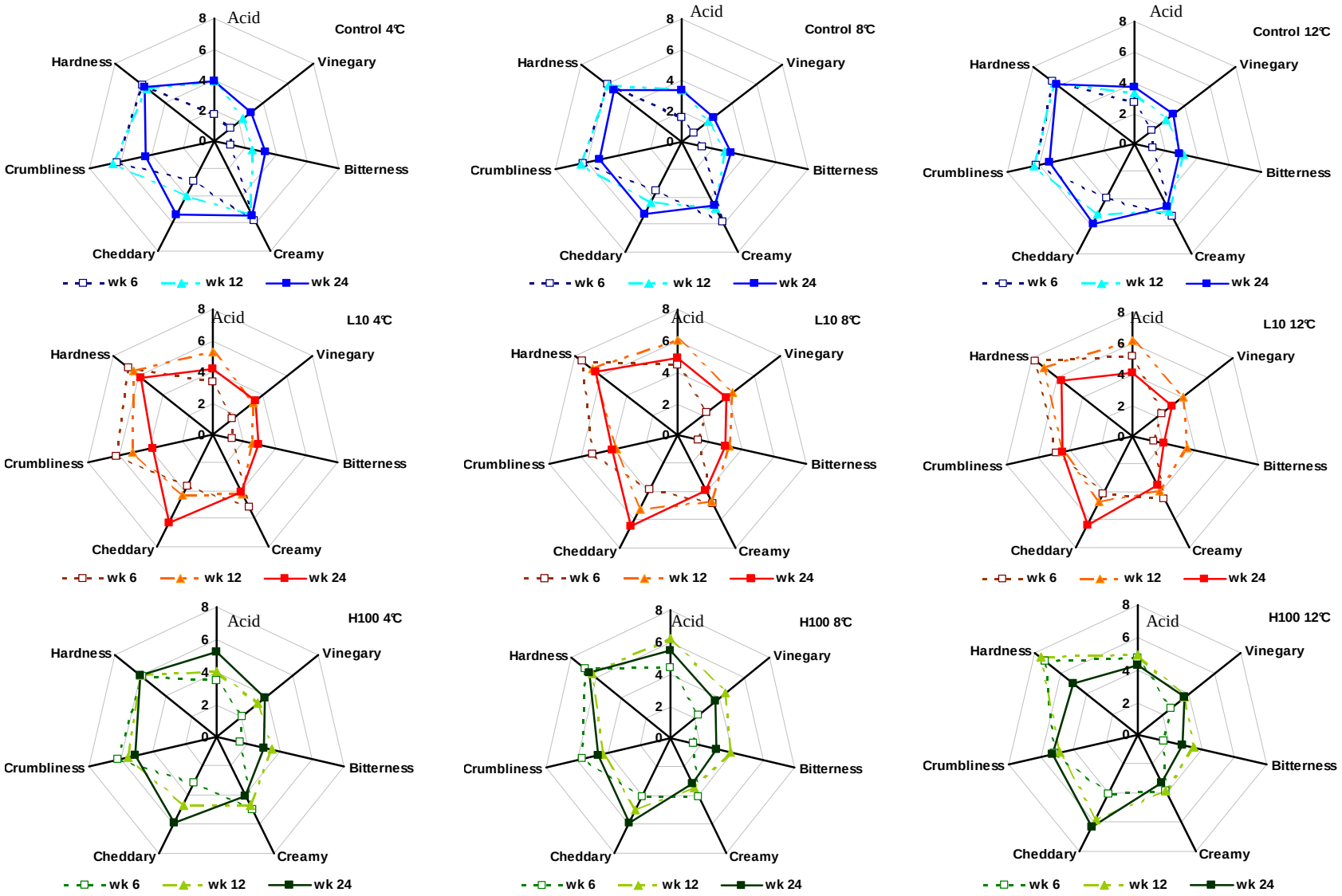


Figure 8.7. Mean of sensory scores of control Cheddar cheeses without probiotic (Control); probiotic cheeses with *Lb. acidophilus* L10 adjunct (L10); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (H100) during ripening at 4, 8 and 12°C for 24 wk. Results are means of at least 45 scores ($n \geq 45$). Standard error of mean = 0.35, 0.39, 0.41, 0.25, 0.45, 0.55 and 0.38 for acid, vinegary, bitterness, creamy, cheddary, crumbliness and hardness, respectively).

Table 8.4. Probabilities (*P*) of changes in means of sensory evaluation scores in control, L10 and H100 cheeses during ripening at 4, 8 and 12°C for 24 wk

Factors and interactions	Probabilities (*P*)[1]							
	Acceptance	Acid	Vinegary	Bitterness	Creamy	Cheddary	Crumbliness	Hardness
Main plot								
Block (B)	**0.0295**	0.2403	0.7231	**0.0152**	**0.0374**	0.3454	0.6187	0.5670
Adjuncts (A)[2]	**<.0001**	**0.0005**	**0.0063**	**0.0002**	**0.0004**	**0.0373**	**0.0004**	**0.0018**
Sub-plot								
Temperature (T)[2]	**0.0005**	**0.0008**	**0.0005**	**0.0243**	**0.0001**	**0.0012**	0.0546	**0.0002**
B*T	**<.0001**	0.1952	**0.0103**	**0.0412**	0.6374	0.9841	0.8776	**0.0153**
A*T	**0.0068**	**0.0008**	**0.0002**	0.7002	**0.0153**	0.3206	0.0980	**0.0470**
Sub-plot								
Time (t)[2]	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
B*t	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
A*t	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
T*t	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
A*T*t	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**

[1]Data was analyzed as a split plot design using general linear model procedure of SAS system. The main plot factors were adjuncts (A) and replications (blocks) with ripening temperature (T) as the split-plot factor. This design was additionally split with ripening time (t) as additional split-plot factor. Statistical significance was at least $P < 0.05$.

[2]Variations in adjuncts include: Control, cheese made without adjunct and with starter culture only; L10, cheese made with starter culture and *Lb. acidophilus* L10 adjunct; H100, cheese made with starter culture, *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts. Variations in temperature include: ripening at 4, 8 and 12°C. Repeated measures over ripening time were performed at wk 6, wk 12, and wk 24.

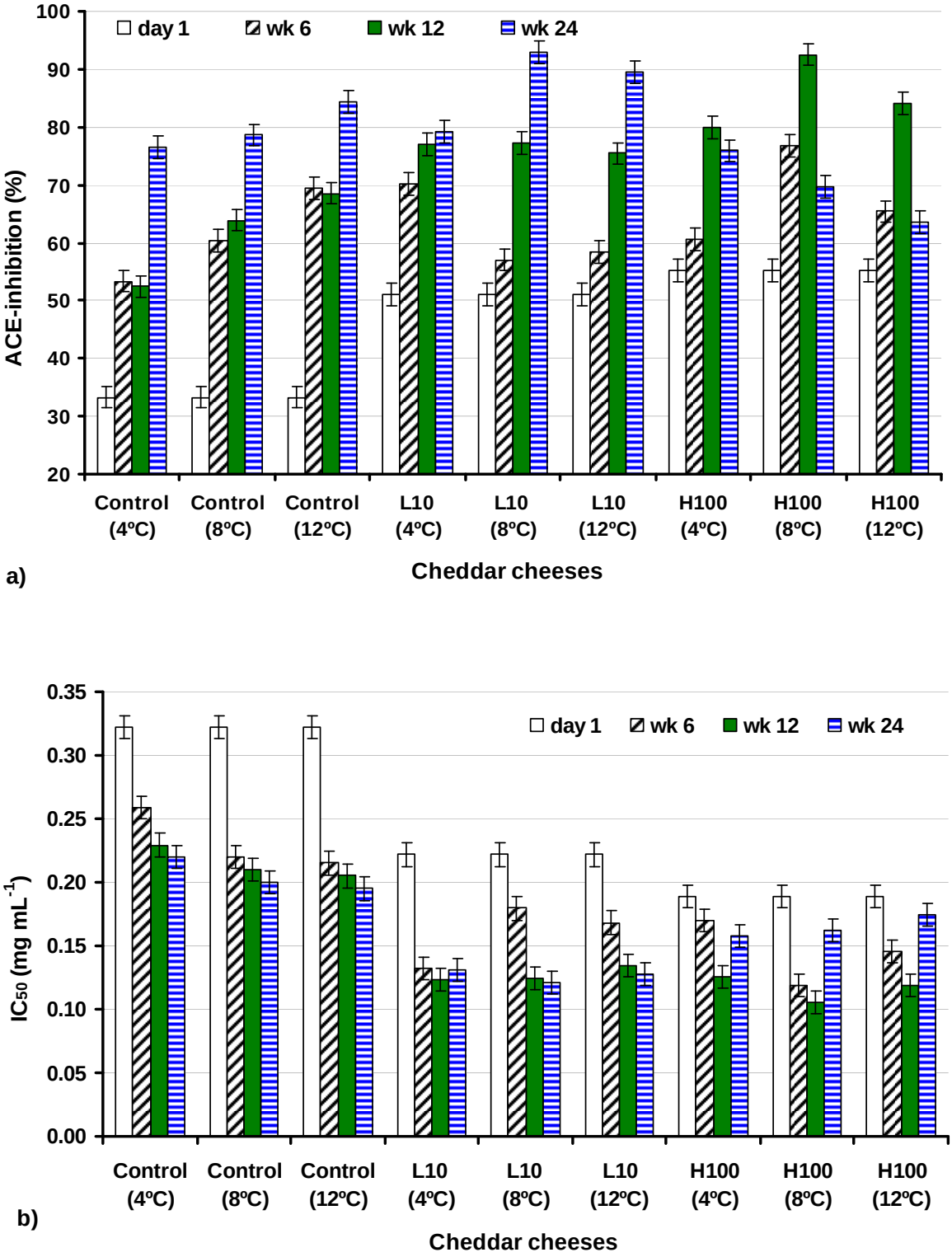


Figure 8.8. Angiotensin converting enzyme (ACE)-inhibitory activity presented as (a) percentage of ACE-inhibition and, (b) IC_{50} (concentration of ACE inhibitor needed to inhibit 50% of ACE activity) of control Cheddar cheese without probiotic (Control); probiotic cheeses with *Lb. acidophilus* L10 adjunct (L10); and probiotic cheeses with *Lb. acidophilus* L10 and *Lb. helveticus* H100 adjuncts (H100) during ripening at 4, 8 and 12°C for 24 wk. Results are presented as means of at least 6 observations ($n \geq 6$). Error bar is pooled standard error of means (SEM = 1.93 and 0.01 for percentage of ACE-inhibition and IC_{50} values, respectively).

9.0 Summary of Results

This study shows that probiotic bacteria can be added as adjunct together with the starter lactococci during cheese-making. The probiotic adjuncts survived at high level at the end of cheese-making process (8.0 - 9.0 $\log_{10}$ cfu g^{-1}). They were also able to maintain viability of > 7.5 $\log_{10}$ cfu g^{-1} at the end of ripening at 4^{o}C for 24 wk. Acetic acid concentration in probiotic cheeses with *B. longum* 1941, *B. animalis* subsp. *lactis* LAFTI®B94, *Lb. casei* 279 or *Lb. casei* LAFTI®L26 was higher when compared to the other cheeses. Each probiotic organisms influenced the proteolytic pattern of Cheddar cheese in different ways. Cheeses made with the addition of *Lb. casei* 279 and *Lb. casei* LAFTI®L26 showed higher level of α_{s1}-CN and β-CN hydrolysis when compared to the other cheeses. Probiotic combination of *Lb. acidophilus* 4962, *B. longum* 1941 and *Lb. casei* 279 or *Lb. acidophilus* LAFTI®L10, *B. animalis* subsp. *lactis* LAFTI®B94 and *Lb. casei* can also be used in the probiotic Cheddar cheese development. When used in combination, these probiotic organisms did not show any level of antagonism and each probiotic survived at the level of > 7.5 $\log_{10}$ cfu g^{-1} at the end of ripening.

Sensory evaluation of the cheeses showed that probiotic cheeses except those with *Lb. acidophilus* 4962 were found to be significantly different from the control cheeses made without any probiotic organism. The acceptability scores of the cheeses were, however, not significantly different among the cheeses except for that with *Lb. casei* 279 with bitterness and sour-acid taste as the major defects. Improvement in proteolysis also resulted in the release of more peptides in the probiotic cheeses including those with bioactive properties. The concentration of ACE-inhibitory peptides needed to inhibit 50% of ACE activity was the lowest after 24 wk of ripening in the probiotic cheeses (0.20 - 0.29 mg mL^{-1}) compared to 36 wk for cheeses without any probiotic (0.28 – 0.31 mg mL^{-1}). Various ACE-inhibitory peptides corresponding to the α_{s1}-casein [(f 1-6), (f 1-7), (f 1-9), (f 24-32) and (f 102-110)] and β-casein [(f 47-52) and (f 193-209)] were isolated from WSE of cheeses made with *Lb. casei* LAFTI®L26.

Both 4 and 8^{o}C can be used for ripening of probiotic Cheddar cheeses. Ripening at 8^{o}C as compared to 4^{o}C increased the level of organic acids and the proteolysis of the cheeses. The increase in ripening temperature improved the ACE-inhibition index of the cheeses. The IC_{50} of cheeses ripened at 4°C was, however, not significantly different to that

ripened at 8°C. The lowest value of the IC_{50} (0.13 mg mL^{-1}) and therefore the highest ACE-inhibitory activity corresponded to the cheese with the addition of *Lb. acidophilus* LAFTI®L10 ripened at 8°C. ACE-inhibitory peptides including κ-CN (f 96-102), α_{s1}-CN (f 1-9), α_{s1}-CN (f 1-7), α_{s1}-CN (f 1-6), α_{s1}-CN (f 24-32) and β-CN (f 193-209) were identified from WSE of cheese with *Lb. acidophilus* LAFTI®L10. Most of the ACE-inhibitory peptides accumulated during ripening, and as proteolysis proceeded, some of the peptides were hydrolyzed into smaller peptides.

Ripening temperature of 12°C further improved the proteolysis and the ACE-inhibitory activity of probiotic cheeses made with *Lb. acidophilus* LAFTI®L10 adjunct. The counts of *Lb. acidophilus* LAFTI®L10 adjunct in the cheeses remained at $>10^6$ cfu g^{-1} after 24 wk of ripening at 12°C. Concentrations of lactic, acetic and propionic acids of cheeses made with *Lb. acidophilus* LAFTI®L10 were significantly higher than those of the control cheeses after 24 wk of ripening at 12°C. Proteolysis of the cheeses was improved as the ripening temperature increased. Increased in ripening temperature from 4°C to 8 and 12°C aslo increased the percentage of ACE-inhibition. The IC_{50} value among cheeses ripened at 4, 8 and 12°C, however, was not significantly different. Elevated ripening temperature can be used to improve proteolysis and ACE-inhibitory activity of probiotic Cheddar cheese. Addition of *Lb. helveticus* H100, however, did not further improve the proteolysis and the ACE-inhibitory activity of the probiotic cheeses. This study shows that some probiotic organisms used in this study can be added successfully in Cheddar cheeses with acceptable organic acid, proteolysis and sensory profiles. In addition of to their well known probiotic effects, addition of probiotic *Lb. casei* 279, *Lb. casei* LAFTI®L26 and *Lb. acidophilus* LAFTI®L10 may play an important role in improving the ACE-inhibitory activity of Cheddar cheeses.

10.0 Future Research Directions

This study demonstrated that Cheddar cheeses can be a potential carrier for probiotic organisms to the consumer. The ripening periods used in this study are up to 36 wk. Some commercial 'tasty' and 'vintage' Cheddar cheeses, however, are ripened for longer than 36 wk. The effect of the probiotic organisms on the quality of the Cheddar cheeses during prolonged ripening may be useful to study, particularly to get an insight into the potential flavour developments or defects at a later stage of ripening.

The potential health benefits of milk protein-derived peptides have been a subject of growing commercial interest in the context of health promoting functional foods. A few potential ACE-inhibitory peptides were isolated and identified in this study. Many scientific, technological and regulatory issues, however, must be resolved before these peptides can be optimally exploited for human nutrition and health. The physiological function of these peptides needs to be established *in vivo* through clinical trials with animal and human studies. There is also a need to scale-up the production of bioactive peptides in cheeses. This study demonstrated that microbial fermentation with the help of lactic acid bacteria and probiotic organisms provides a natural technology applicable for the production of bioactive peptides. The commercial production of specific peptide sequences can also be achieved by the use of recombined enzyme technology or specific production strains (genetic engineered microorganisms) or the use of specific peptidases isolated from suitable microorganisms.

The ACE-inhibitory peptides isolated in this study show various peptide sequences of varying lengths and structures. The length and structure of the peptides may influence the adsorption of these peptides in the gastro-intestinal tract. The ability of the peptides to with-stand further hydrolysis by gastric juices needs to be addressed. More detailed studies are also required for a better understanding of the blood pressure reducing mechanisms of the peptides. Molecular studies such as proteomics are needed to assess the mechanisms by which these peptides exert their activity.

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12.0 Appendices

Appendix A - Sensory evaluation forms

A.1. Consent form for research project involving human subject

CONSENT FORM

I,_____of (address) _____certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the experiment entitled:

"Influence of Probiotic Bacteria on Proteolytic Pattern and Sensory Attributes of Cheddar Cheese"

being conducted at Victoria University, by Prof Nagendra Shah and his student Lydia Ong.

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

(Name of researcher): Lydia Ong **on (date)**_____

(Signature of researcher):_____

and that I have had the opportunity to have any questions and answered. I also understand that I can withdraw from this experiment anytime and that this withdrawal will not jeopardise me in any way. I have also been informed that the information I provide will be kept confidential

I freely consent to participation involving the use on me of these procedures.

- Triangle tests Yes☐ No☐
- Cheese grading/scoring Yes☐ No☐
- Duo-trio and intensity ranking test Yes☐ No☐
- To be contacted if needed in the future Yes☐ No☐

If yes, your telephone number:_____and/or e-mail add:_____

Signature:_____Date:_____

Any queries about your participation in this project may be directed to the researcher (Professor Nagendra Shah, Phone: (03) 9919-8289). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MCMC, Melbourne, 8001 (telephone no: 03-9688 4710).

A.2. Form for duo-trio test for determination of flavour threshold

Duo-trio test

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product : cream cheese
Name :
Date :

Instructions:
On your tray you have a marked of control sample (R) and 6 sets of three coded samples. One sample is identical with R and the other is different. Which of the coded sample is different with R? Use the water crackers and water to wash your palate after tasting each sample.

	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
Sample 1	863	445	101	995	818	643
Sample 2	134	216	247	837	389	451
Odd sample						

Thank you very much for your participation

A.3. Form for intensity ranking test for products with various concentration of lactic acid, acetic acid and caffeine

Intensity ranking test

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product : Cream cheese with different concentration of lactic acid
Name :
Date :

Instructions:
You have been given 6 three coded samples. Please rank the sample from the least intensity to the highest intensity. Use the water crackers and water to wash your palate after tasting each sample.

Least intensity ---------- Highest intensity					

Thank you very much for your participation

A.4. Panelist questionnaire

Panelist Questionnaire

Name:

Please put a tick next to your answer for the following questions:

Sex : Male_____ Female _____

Age: < 18 years _____
18- 25 years _____
25- 35 years _____
35- 55 years _____

Frequency of cheese consumption:
≤1 per week _____
1-2 per week _____
2-3 per week _____
3-4 per week _____
> 5 per week _____

Preferred type of cheese:
Mild_____ Tasty (medium)_____ Matured _____

A.5. Form for acceptance rating test

Acceptability of Cheddar cheeses

Product : Cheddar cheese
Name :
Date :

Instructions:
You have been given 5 three coded samples. Please score the Cheddar cheese samples on a 1 to 10 scale (1 = dislike extremely, 10 = like extremely). Use the water crackers and water to wash your palate after tasting each sample.

Attributes	123	861	754	678	459
Acceptability					

Thank you very much for your participation

A.6. Form for triangle test

Triangle Test

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product : Cheddar cheese
Name :
Date :

Instructions:
You have been given 4 sets of three coded samples. In each set, two samples are taken from the same batch and the other from another batch. Within each set, circle the code number of the **ODD** sample. Please taste samples in order from left to right. Use the water crackers and water to wash your palate after tasting each sample.

Set	Codes		
1.	733	409	651
2.	767	377	420
3.	283	612	229
4.	865	134	962

A.7. Scoring for specific attributes form

Scoring for specific attributes

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product : Cheddar Cheese
Name :
Date :

Instruction:

You have been given 5 three coded samples. Please score the Cheddar cheese samples on a 1 to 10 scale for all parameters listed:

1.Cheddary	1 = none (very mild)	10 = high intensity (mature)
2.Creamy / milky	1 = not creamy	10 = very creamy
3.Sour- acid	1 = not acidic	10 = very acidic
4.Vinegary	1 = not detected	10 = high intensity
5.Bitterness	1 = not bitter	10 = very bitter
6.Hardness	1 = soft	10 = hard
7.Crumbliness	1 = crumbly (do not hold together)	10 = firm, stick together

Please score one sample at a time. Use the water crackers and water to wash your palate after tasting each sample.

SAMPLES

Attributes	123	861	754	678	459
1.Cheddary					
2.Creamy / milky					
3.Sour-acid					
4.Vinegary					
5.Bitterness					
6.Hardness					
7.Crumbliness					

Cheddary: general flavours of Cheddar cheese.
Creamy/ milky: flavour associated with fresh milk, creamy product, condensed milk.
Sour-acid: sour, taste sensation of lactic or citric acid.
Vinegary: flavour associated with vinegar.
Bitterness: chemical-like, aspirin, taste sensation of caffeine.

Thank you very much for your participation

Appendix B – Peptide sequencing using automated Edman degradation method

Automated Edman degradation chemistry consists of three steps as described in section 2.6.5.1. During the Edman degradation, phenylisothiocyanate (PITC) reacted with the amino acid residue at the amino terminus under basic conditions to form a phenylthiocarbamyl derivative (PTC-protein). Trifluoroacetic acid then cleaved off the first amino acid as its anilinothialinone derivative (ATZ-amino acid) and leaves the new amino terminus for the next degradation cycle. The ATZ amino acid was then removed by extraction with ethyl acetate and converted to a phenylthiohydantoin derivative (PTH-amino acid). The PTH-amino acid was transferred to a reversed-phase C-18 column (PTH-C18 colum, 2.1 x 220 mm; Perkin Elmer Co. Ltd.) for detection at 270 nm. A standard mixture of 19 PTH-amino acids (Applied Biosystems, Foster City, CA, USA) was also injected onto the column for separation (usually as the first cycle of the sequencing run) (Figure 12.1).

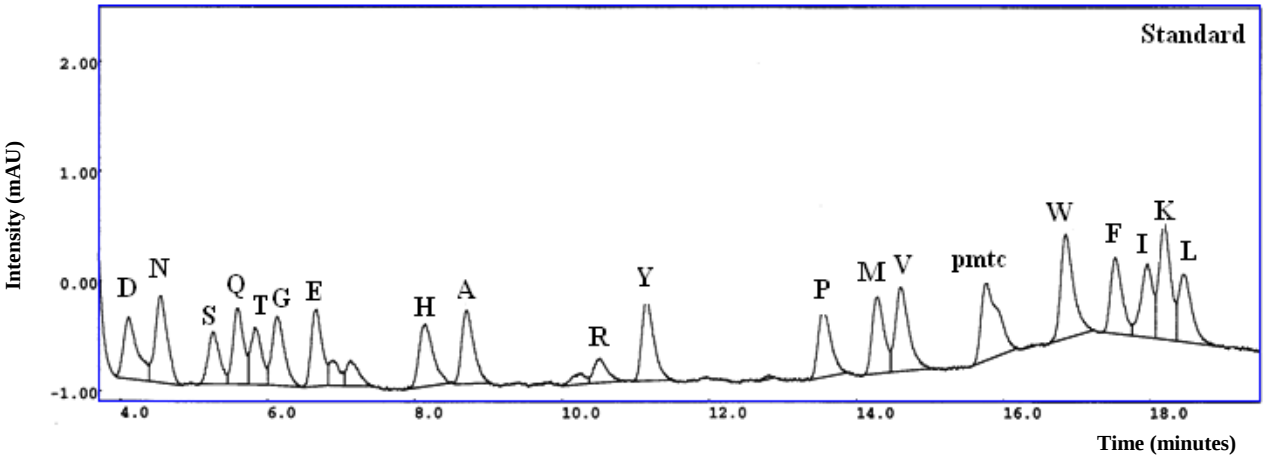

Figure 12.1. Standard of phenylthiohydantoin derivative (PTH) amino acids (5 pmol)

The chromatogram (Figure 12.1) provides standard retention times of the amino acids for comparison with each Edman degradation cycle chromatogram. Example of the chromatograms obtained during the N-terminal sequencing of peptides α_{s1}-CN (f 1-6), β-CN (f 47-52) and β-CN (f 193-209) are shown in Figures 12.2, 12.3 and 12.4a, b. To determine the amino acid present at a particular residue number, the chromatogram from the residues of interest was compared with the chromatogram from the previous residue by overlaying one on top of the other. From this, the amino acid for the particular residue could be determined. This process was repeated sequentially to provide the N-terminal sequence of the peptide.

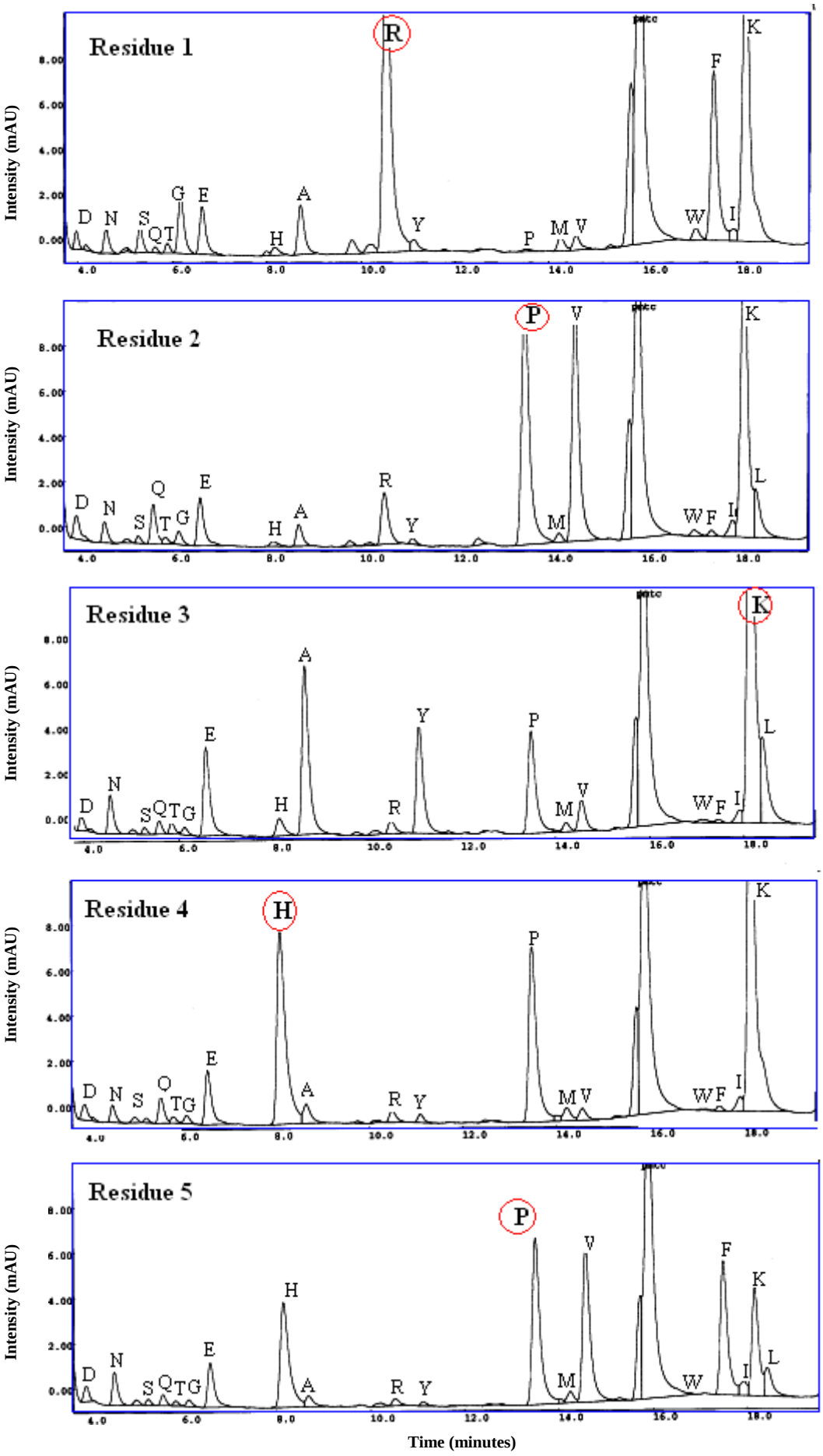

Figure 12.2. Residues 1 to 5 of peptide derived from α_{s1}-CN (f 1-9)

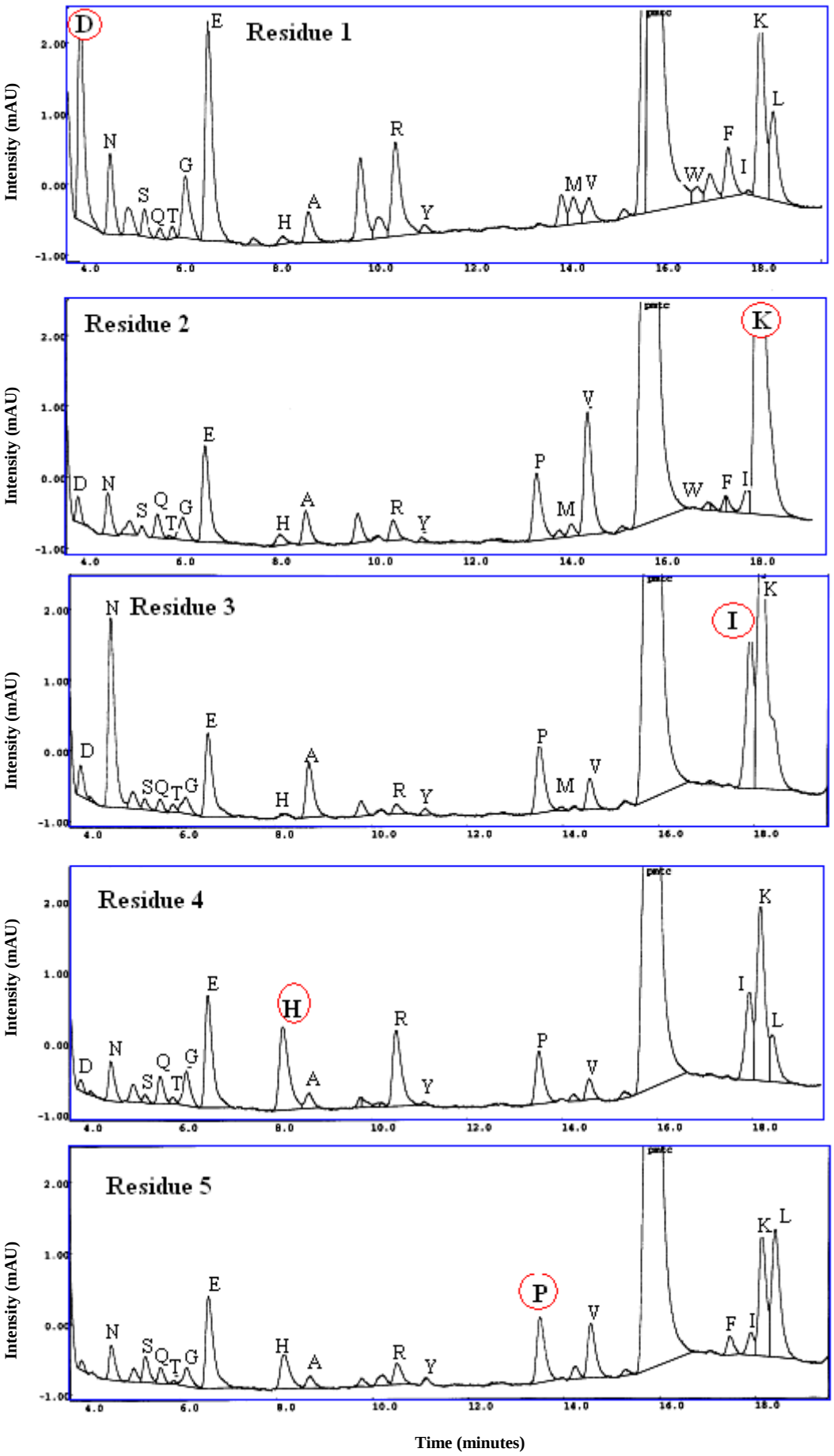

Figure 12.3. Residues 1 to 5 of peptide derived from β-CN (f 47-52)

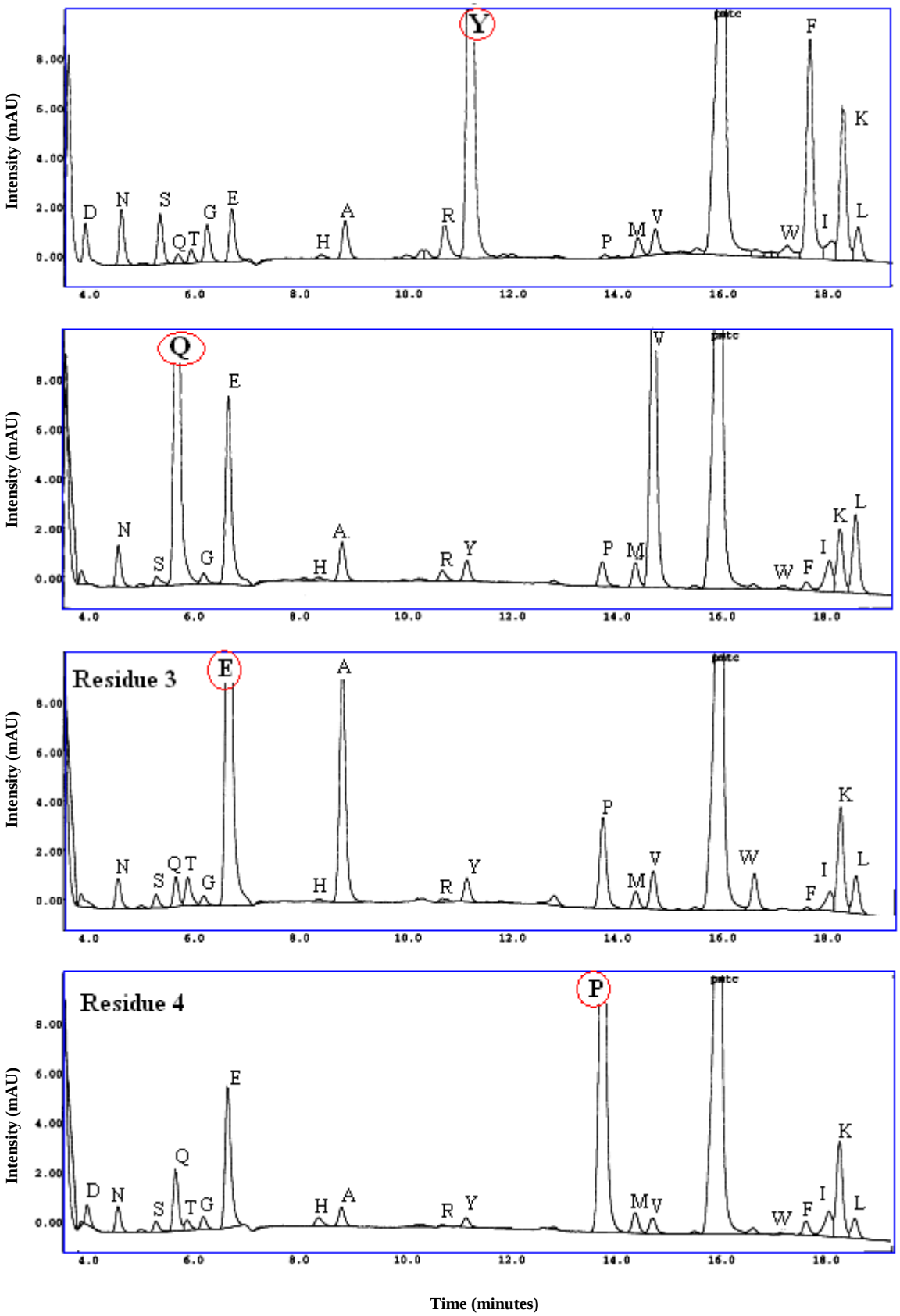

Figure 12.4a. Residues 1, 2, 3 and 4 of peptide derived from β-CN (f 193-209)

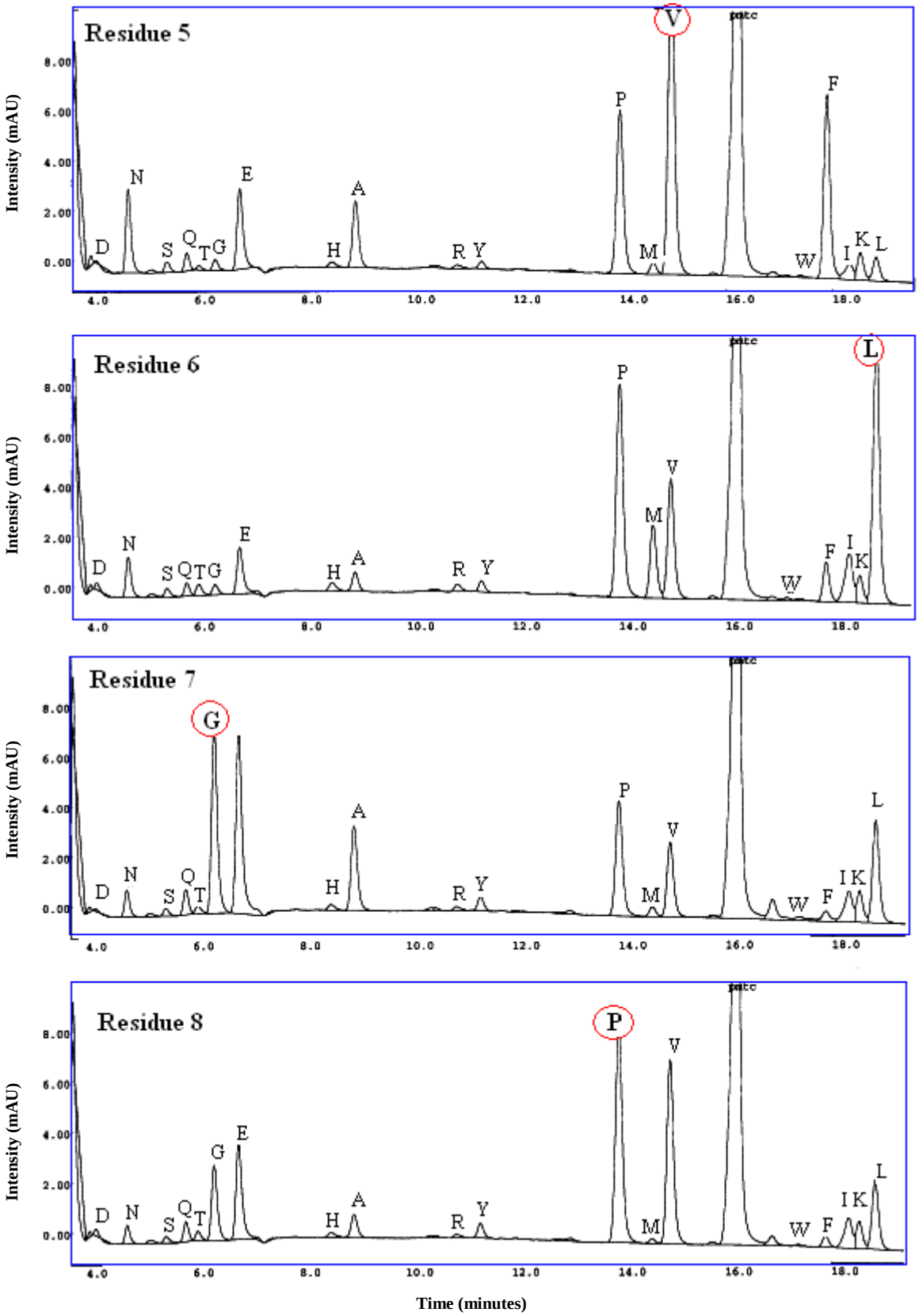

Figure 12.4b. Residues 5, 6, 7 and 8 of peptide derived from β-CN (f 193-209)