Anti-hypertensive (Angiotensin converting enzyme-inhibitory) peptides released from milk proteins by proteolytic microorganisms and enzymes

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

Fatah BM Ahtesh
B.Eng. (Food Technology); M.Sc. and M.B.A in (Food and Agribusiness)

College of Health and Biomedicine

Victoria University, Melbourne
VIC, Australia

March 2016

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

I dedicate this PhD to my mother, sisters, brothers, father and my lovely family

ABSTRACT

This study was carried out to examine proteolytic activities of probiotic lactic acid bacteria (LAB) in different media and antihypertensive properties as influenced by, fermentation time, strain type and supplementation with or without an enzyme (Flavourzyme®). *Lactobacillus casei* (Lc210), *Bifidobacterium animalis* ssp12 (Bb12), *Lactobacillus delbrueckii subsp. bulgaricus* (Lb11842) and *Lactobacillus acidophilus* (La2410) were propagated in 12 % reconstituted skim milk (RSM) or 4 % whey protein concentrate (WPC) with or without supplementation (0.14 %) of Flavourzyme® for 12 h at 37°C. All the strains were able to grow in both media depending on the type of strains used and the time of fermentation. Moreover, all the strains showed higher proteolytic activity and produced more peptides with anti-hypersensitive properties when grown in RSM media for 12 h, compared with WPC. Combination with Flavourzyme® increased LAB growth, proteolytic and anti-hypersensitive activities. From the four strains used, Bb12 and La 2410 outperformed Lc210 and Lb11842. The highest angiotensin converting enzyme inhibitory (ACE-I) and proteolytic activities was shown by Bb12 combination with Flavourzyme®. Flavourzyme® led to increased bioactive peptides with ACE-I activity during fermentation at 37°C for 12 h.

The second phase was to determine the effects of Flavourzyme® on ACE-I peptides formed in skim milk and WPC during fermentation by *Lactobacillus helveticus* (Lh) strains, ASCC (881315, 881188, 880474 and 880953), based on proteolytic activity and the production of ACE-I peptides in different media, enzymatic supplementation and fermentation times. RSM (12 %) or WPC (4 %), with or without Flavourzyme® (0.14 % w/w), were fermented with *L. helveticus* strains separately at 37°C for 0, 4, 8 and 12 h. Proteolytic, *in vitro* ACE-I activities and growth were significantly affected ($P < 0.05$) by strains, media and enzyme supplementation. RSM supported higher growth, produced higher proteolysis and ACE-I, than WPC without enzyme supplementation. The strains Lh 881315 and Lh 881188 were able to increase ACE-I to 80 % after 8 h fermentation when combined with Flavourzyme® in RSM compared to the same strains without enzyme supplementation. Supplementation of media by Flavourzyme® was beneficial in increasing ACE-I peptides in both media. The best medium to release more ACE-I peptides was RSM with enzyme supplementation. The Lh 881315 with

Flavourzyme® outperformed all strains as indicated by highest proteolytic and ACE-I activities.

In addition to ascertain, the optimal proteolytic combination of microorganisms for the production of potent ACE-I peptides, resulted in further studies to determine the effects of dairy yeast *Kluyveromyces marxianus* LAF4, combined with probiotics (Lc210, Lb11842, La2410, Lh 881315, Lh 881188, Lh 880474 and Lh 880953), as a source of ACE-I properties. Consequently, this study examined the capacity of yeast strain with LAB to increase the hydrolysis of skim milk protein to obtain a fermented drink with high ACE-inhibition activity and bioactive peptides. Four different *Lactobacillus helveticus* strains and three selected probiotic LAB strains were combined with *Kluyveromyces Marxianus* LAF4 (*K. marxianus*) to ferment 12 % RSM at 37°C for 0, 4, 8 and 12 h and compared to RSM using the same strains without yeast, and using fermented skim milk with yeast as a control. The growth, pH value, proteolytic activity and ACE-I activity was examined at the different time of fermentation. Interestingly, the highest ACE-I activity were with separated strains compared to combination form, *K. marxianus* alone (60 %) or Lh181315 and Lh 880953 (70 % and 65 %) respectively at 37°C for 12 h ($P < 0.005$). Additionally, using *K. marxianus* in combination with LAB strains resulted in decreased milk protein hydrolyses (~30-55 %) at 12 h compared to the control due to alcohol production. The findings of this study have a number of important implications, such as the use of dairy yeast alone to produce a suitable functional dairy product containing ACE-I peptides instead of using a combination of yeast and LAB.

The third phase of this study was the identification of peptides from fermented skim milk protein hydrolysis by combination of Lh ASCC 8801315 and Flavourzyme®. This study presents the use of matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS/ MS) and Nano-liquid chromatography (Nano-LC/ MS/ MS) as a complement to reversed phase high-performance liquid chromatography (RP-HPLC) separation for the identification of ACE-I peptides from skim milk protein hydrolysate. As a preliminary step, RP-HPLC was used to isolate the different casein fractions from fermented skim milk. ACE-I activity of these fractions F1 (85.40 %) and F6 (90.31 %) with IC_{50} 0.01 mg mL^{-1} was performed using proteolytic strains of *L. helveticus* and Flavourzyme® using an agitation Bioreactor system. Nano-LC / MS / MS sequenced the peptides contained in the fractions. This procedure allowed the identification of 133

ACE-I peptides from α, β, and k-casein proteins with 99 % confidence from two fractions with most hypotensive effect were FFVAPFPGVFGK, GPVRGPFPIIV, and LHLPLPLL. These findings show the potential use of the *L. helveticus* strain to produce a functional fermented milk drink with a wide range of health benefits.

Based on the results in the third phase, milk casein hydrolysates containing peptides released during milk fermentation by the combination of Lh and Flavourzyme®, were isolated and used for *in vivo* animal studies. Milk peptides with ACE-I were extracted from a fermented skim milk with Lh 881315 and Flavourzyme®. ACE-I plays an important role in the regulation of hypertension: it catalyses the production of the vasoconstrictor peptide angiotensin-II and inactivates the vasodilator bradykinin. The fermentation processes showed higher proteolytic activity and the peptides released exhibited ACE-I properties. The effect of fermented low fat skim milk drink-based diets on the feed intake, weight and BP were investigated in spontaneously hypertensive rats (SHR). Fourteen-week-old male SHR were fed for ten weeks with either chow (NC), peptides added to chow (FC), or control skim milk powder added to chow (NFC). Food intake and body weights were measured daily and BP was measured weekly by tail-cuff plethysmography. BP decreased significantly ($P < 0.05$) from 6 to 10 weeks of FC groups (120 / 65 mm Hg) compared with the NC and NFC control groups, where BP increased significantly (220 /150 mmHg) ($P < 0.05$). The addition of fermented skim milk added to the chow did not change total energy intake in the FC group compared to the NFC group, yet the FC group weighed significantly less than both the NC and NFC groups by the end of the experiment. This implies, that the rats either had a change in metabolic energy or had impaired digestion and absorbance.

In the final phase of this research, the effects of processing and sensory characteristics of a fermented skim milk drink as functional milk product were examined. Using Lh 8801315 combined with Flavourzyme®, the efficiency of bioreactor increased cell viability and bioactive peptides with ACE-I properties during fermentation. The developed fermented skim milk containing bioactive peptides with improved sensory characteristics showed consumer acceptability. However increased acidity as well as bioactive peptides, led to increased bitterness of the fermented milk. The addition of 15 % sucrose and flavouring provided accepted positive changes in the fermented product.

Certificate

Professor Lily Stojanovska MSc. PhD
Centre for Chronic Disease
College of Health and Biomedicine
Victoria University
Melbourne, Australia

This is to certify that the thesis entitled "**ANTI-HYPERTENSIVE (ANGIOTENSIN CONVERTING ENZYME-INHIBITORY) PEPTIDES RELEASED FROM MILK PROTEINS BY PROTEOLYTIC MICRO-ORGANISMS AND ENZYMES**" submitted by Fatah Ahtesh in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bona fide research work carried out by him under my guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Professor Lily Stojanovska
Date: 21/03/2016

Declaration

I Fatah Basher Ahtesh declare that the PhD thesis entitled '*Angiotensin converting enzyme-inhibitory peptides released from milk proteins by proteolytic micro-organisms and enzymes'* 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 an award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Fatah BM. Ahtesh:

Date: / / 2016

Centre for Chronic Disease
College of Health and Biomedicine
Victoria University, Werribee campus
Victoria, Australia

Acknowledgements

This research was funded and supported by the Libyan government and Victoria University.

I would like to thank my principal supervisor, Professor Lily Stojanovska, College of Health and Biomedicine, Victoria University, for her guidance and valuable suggestions throughout the course of the study and preparation of the manuscripts. Her continuous diligence and patience over these years are genuinely appreciated.

I would also like to thank my co-supervisors, Associate Professor Michael Mathai and Dr. Vijay Mishra both from the College of Health and Biomedicine, Victoria University, for their contribution and valuable discussions during the studies.

I would also like to thank Dr. Osaana Donkor and Professor Vasso Apostolopoulos, College of Health and Biomedicine, Victoria University for their help, thesis editing and the friendly discussions we have had. In addition, I wish to thank Professor Todor Vasiljevic from the Advanced Food Systems Research Unit, College of Health and Biomedicine, and all the laboratory staff, especially the technical managers Mrs. Stacey Lloyd, and Mr. Joe Pelle for their technical help and patience. I appreciate the great friendship I have made during my research years at Victoria University.

Special thanks go to Anne Luxford, Animal Facilities Operations Supervisor, and Dr. Faten Zaibak (Faye) Manager, Animal Facilities and Biosafety as well as Western Health Sunshine Hospital staff and Monash University animal house facility staff for their help, training and cooperation.

I would like to express my profound gratitude to my great parents and family for their support.

List of Publication

Peer reviewed publications

1- Ahtesh, F., Stojanovska, L., Shah, N., & Mishra, V. K. (2016). Effect of Flavourzyme® on Angiotensin-Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by *Lactobacillus helveticus*. *Journal of food science*, 81(1), 43-57. doi:10.1111/1750-3841.13177.

2- Ahtesh F., Stojanovska L., Mathai M., Apostolopoulos V., & Mishra V. (2016). Proteolytic and angiotensin converting enzyme inhibitory activities of selected probiotic bacteria. *International journal of food science and technology*. doi: 10.1111/ijfs.13054[Ahead of print]

3- Ahtesh F., Apostolopoulos V., Shah, N. Mishra V., & Stojanovska L. (2016). Effects of *Kluyveromyces marxianus* LAF4 combined with probiotics as source of Angiotensin converting enzyme peptides. *Journal of Food Science*: Manuscript under review. ID JFDS-2015-2094.

4- Ahtesh F., Apostolopoulos V., Stojanovska L., & Mishra, V. K. (2016). Identification of bioactive peptides from fermented skim milk protein hydrolysis by combination of *Lactobacillus helveticus* ASCC 8801315 and Flavourzyme®. Submitted to *Journal of food Chemistry*. Manuscript under review.

5- Ahtesh F., Stojanovska L., Mishra V. K & Michael M. (2016). Dietary supplementation with milk-derived angiotensin- converting enzyme peptides decreases food intake, body weight and blood pressure in spontaneously hypertensive rats. Submitted to *Nutrition, metabolism and cardiovascular Disease*. Manuscript under review.

Oral Presentation

1- Fatah Ahtesh., Lily Stojanovska., Vijay Mishra., & Michael Mathai (2015). Antihypertensive peptides activity of fermented skim milk by combination of *Lactobacillus helveticus* and Flavourzyme® as beneficially affect blood pressure of spontaneously hypertension rats. Post Graduate Research Student Conference (14th August 2015). Victoria University Australia.

2- Fatah Ahtesh (2015). New Fermented Milk Drink lowers High Blood Pressure. 3 Minute Thesis presentation (3MT) Victoria University College of Health and Biomedicine Science Postgraduate Research Conference July 2015, Victoria University, Footscray, Australia

Poster Presentations

1- Ahtesh FB, Shah N & Mishra V (2012). Screening of probiotic proteolytic bacteria and proteases based on their proteolytic and angiotensin converting enzyme inhibitory activity. Poster presentation at the 45 Annual AIFST Convention, Adelaide, SA 15th – 17th July 2012

2- Fatah BM. Ahtesh, Lily Stojanovska, Vijay K. Mishra & Michael L. Mathai. (2015). Peptide Extract of Fermented Skim Milk formed by Combination of *Lactobacillus Helveticus* and Flavourzyme® reduce Blood Pressure of Spontaneously Hypertensive Rats. Poster presentation at AIFST Food Science summer school RMIT, 28-30 January 2015

Table of contents

List of Figures

List of tables

List of Abbreviations

AASI =Ambulatory arterial stiffness index

ACE = angiotensin-I converting enzyme

ANOVA = analysis of variance

APAF=Australian proteome analysis facility

Bb = *Bifidobacterium*

BSA = bovine serum albumin

BP = blood pressure

CE = Capillary electrophoresis

cfu = colony forming unit

CN = casein

°C = degree Celsius

CEP = cell-envelope protease

CVD = cardiovascular diseases

CID =Collision Induced Dissociation

DBP = Diastolic blood pressure

ECD = electrochemical detector

FAA= Free Amino Acids

FAO = Food and Agricultural Organization of the United Nations

FC = Fermented RSM containing peptides

FID = Flame ionization detector

g = gram

h = hour

HCl = hydrochloric acid

H_2SO_4 = sulphuric acid

HS = Head space

HDL = high-density lipoprotein

IC_{50} = half maximal inhibitory concentration

Ig = immunoglobulin

κ- CN = kappa-casein

kJ = kilojoules

Km= *Kluyveromyces marxianus*

L = litre

La = *Lactobacillus acidophilus*

LAB = lactic acid bacteria

Lb = *Lactobacillus delbrueckii* ssp. *bulgaricus*

Lh= *lactobacillus helveticus*

Loa C =lab-on-a-chip

M = Molar

M17 agar = agar for enumeration of *Streptococcus thermophilus*

MAP=Mean arterial blood pressure

min = minute

mL = millilitre

mm = millimetre

mM = millimolar

MRS = de Man Rogosa and Sharpe

mV = millivolts

MW = molecular weight

MPP = micro particulated protein

MRS = de Man Rogosa Sharpe

nA = nano-amps

NC = Normal rat feed chow control

NFC= Non-fermented RSM control

ng = nano-gram

nm = nano-meter

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

OPA = o-phthaldialdehyde

Pa = Pascal

pH = hydrogen ion concentration

RAS =renin-angiotensin system

RCA = reinforced clostridia agar

RP-HPLC = reversed-phase high performance liquid chromatography

rpm = revolution per minute

RSM = reconstituted skim milk

s = second

SBP = Systolic blood pressure

SHR = spontaneously hypertensive rats

sp. = species

ssp. = subspecies

St = *Streptococcus thermophilus*

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

TG = triglyceride

UV = ultraviolet

v/v = volume/volume

V = volts

VPR=volume pressure recording sensor

WHO = World Health Organization

WKS = weeks

WPC = whey protein concentrate

w/v = weight/volume

w/w = weight per weight

α- glu = α-glucosidase

α-La = alpha-lactalbumin

β-Lg = beta-lactoglobulin

μ = micro

μL = microlitre

μg = microgram

μ m = micrometre

IITCS=Tail Cuff Blood Pressure Systems

Chapter 1 Introduction

Developing and growing agricultural products, food industrialisation, and mechanisation have led to dramatic changes in lifestyle, particularly dietary pattern, which in turn have produced increased occurrence in chronic diseases such as cardiovascular, stroke, diabetes, hypertension and cancer. Indeed, obesity, hypertension and cardiovascular diseases have increased at an alarming rate worldwide in the last two decades, nearly 2.5-fold in Australia compared to the USA (Cameron et al., 2003; Roberfroid, 1999, 2000; World Health Organisation, 2002, 2003). Consequently, in recent years, people seeking healthier lifestyle prefer diets with low or fat free foods; this has led to the development of functional foods (Roberfroid, 1999a; Food and Agriculture Organization of the United Nations, & World Health Organization, 2002; Cameron et al., 2003; World Health Organization, 2003).

Functional foods are defined as foods 'that can beneficially affect one or more target functions in the body, beyond the adequate nutritional effect, in a way relevant to improved state of health and well-being and/or reduce the risk of disease' (Contor, 2001). Milk products, particularly fermented milk containing probiotics are most popular in this category of foods (Stanton et al., 2005). Probiotics are defined as 'live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host' (World Health Organisation, 2002). The benefits of utilising these organisms include maintenance of gut health, reduced allerginicity, increased bio-accessibility of lipids and proteins in foods, and lowering of blood pressure due to polyamines and bioactive peptides (Marteau et al., 1990; Santos, San Mauro, & Diaz, 2006; Tuohy et al., 2003). These bioactive peptides have the ability to reduce the risk of colorectal cancer, stimulate the immune response and reduce the risk of cancer, non-insulin dependent diabetes, obesity, cardiovascular disease and hypertension (Shah, 2007; Tuohy et al., 2003; Williams & Jackson, 2002). However, the health conscious consumer now requires additional health benefits from these products, which have opened new areas for research (Shah, 2007). Among these, peptides with blood-pressure- lowering effects have received considerable significance in being associated with the role of diet in prevention and treatment of disease (López-Fandiño, Otte, & van Camp, 2006). Blood pressure regulation is partially dependent on the rennin-angiotensin system (Silva & Malcata, 2005), in which the angiotensin-I converting enzyme (ACE) regulates the peripheral blood pressure and its inhibition can exert an anti-hypertensive effect (Gobbetti, Minervini, & Rizzello, 2004). Bioactive peptides are

defined as specific protein fragments that have positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). Upon oral administration, bioactive peptides may affect the major body systems, namely, the cardiovascular, digestive, immune and nervous systems, depending on their amino acid sequence (Erdmann, Cheung, & Schröder, 2008; FitzGerald et al., 2011; Yamamoto et al., 2010). These peptides are released through enzymatic breakdown of dairy proteins by digestive enzymes in the gastrointestinal tract (GIT) or extracellular proteinases formed by *lactobacilli* during their growth in milk (Seppo et al., 2003; van der Burg-Koorevaar & Schalk, 2010). The tri-peptides, Valyl-Prolyl-Proline (Val-Pro-Pro), and Isoleucyl-Prolyl-Proline (Ile-Pro- Pro) have been identified as antihypertensive agents, which inhibit the action of ACE (van der Burg-Koorevaar & Schalk, 2010). Most of the probiotic microorganisms are sensitive to food acidity and oxygen availability. Short-shelf-life fermented dairy products like yoghurt, are the most common functional foods on the market (Hekmat, Soltani, & Reid, 2009; Ozer et al., 2007; Stanton et al., 2003). During the fermentation process, probiotics produce a range of secondary metabolites, some of which have been associated with health promoting properties of which the notable ones are the B vitamins and bioactive peptides. The physiologically active peptides are produced from many food proteins during gastro-intestinal digestion and fermentation of food by lactic acid bacteria (LAB). The production of ACE-I peptides *in situ* in dairy products is the most appealing approach of generating these peptides. One of the most effective way to raise the number of these peptides is to ferment or co-ferment with highly proteolytic strains of LAB (Gobbetti et al., 2004), the challenge to this approach, however, lies in the selection of the appropriate strains or a combination of strains (Gobbetti et al., 2004; Meisel, 1998). ACE-inhibitory peptides produced in fermented milks using strains of proteolytic LAB (Nakamura et al., 1995a, 1995b; Seppo et al., 2002, 2003; Donkor et al., 2007) as well as the proteolytic system of LAB, have been well studied (Savijoki, Ingmer, & Varmanen, 2006; Van Beresteijn & Alting, 2002; Yamamoto, Akino, & Takano, 1994). Most of the ACE-inhibitory peptides have been created from α_{s1}-, α_{s2} and β- casein and β-lactoglobulin fractions of dairy products and only a few among the large number of peptides have been identified as anti-hypertensive under *in vitro* conditions and have proven to be clinically effective in animal and human studies (Korhonen and Pihlanto, 2006).

Therefore, controlled animal studies are needed to demonstrate the long-term physiological effects delivered by consuming such peptides. However, the market for probiotic-containing products shows a substantial increase in popularity recently, while scientific approaches to establishing the functional benefits of probiotic foods is still a challenge. Evidence from *in vitro* studies suggests beneficial effects; however, considerable progress has not yet been made in both effects on host health and mechanisms of action and whether viable microorganisms are necessary for health benefits, which require further clarification. Incorporation into other food commodities such as milk whey protein or yoghurt is promising and should be investigated (Hernández-Ledesma et al., 2014; Rijkers et al., 2011).

In the aforementioned study, low-fat fermented skim milk drink was formulated using a strain of Lh combined with Flavourzyme® and the influence of these on the physico-chemical and physiological properties were studied. Selection of probiotics and suitable media (RSM or WPC) were based on growth, proteolytic and ACE-I activity, along with the best combination with Flavourzyme®. β-casein, lacto globulin fractions of RSM or WPC are only a few among the large numbers of peptides identified as anti-hypertensive, which have proven to be clinically effective in animal and human studies (Korhonen and Pihlanto, 2006).

The specific aims of this project were:

1. To select suitable strains of *Lactobacillus casei* (Lc210), *Bifidobacterium animalis ssp12* (Bb12), *Lactobacillus delbrueckii subsp. bulgaricus* (Lb11842), *Lactobacillus acidophilus* (La2410), Lh strains, ASCC (881315, 881188, 880474 and 880953), *Kluyveromyces marxianus* (LAF4), and Flavourzyme® based on their proteolytic and ACE-inhibitory activities;

2. To select suitable media (RSM or WPC) based on bacterial growth;

3. To evaluate the extent of proteolysis and release of bioactive peptides by a combination of selected probiotic organisms during fermented low-fat skim milk drink production;

4. To identify and purify potential ACE-I peptides produced by the selected organisms during fermentation;

5. To develop a dairy product containing ACE-I peptides (with the best combination of probiotic bacteria and proteases), and perform organoleptic/sensory evaluation of fermented dairy drink;

6. To study the *in-vivo* antihypertensive effect of low-fat fermented skim milk drink on spontaneous hypertensive rats (SHR).

A review of the relevant literature forms Chapter 2. Chapter 3 focuses on the effects of media and probiotic strains in combination with or without Flavourzyme® on the production of bioactive peptides with ACE-I activity. The viability of (Lc210), (Bb12), (Lb11842), (La2410) and their proteolytic and ACE-I activities were assessed in RSM or WPC for different fermentation times (0, 4, 8 and 12 h) at 37°C. Chapter 4 examines ACE-I activity of peptides hydrolysed by Lh (881315, 881188, 880474 and 880953). Fermentations were terminated at different times (4, 8, and 12 h) at 37°C in RSM or WPC, and viability, proteolytic activity, bioactive peptides, and ACE-I activity were investigated. Chapter 5, on the other hand, investigates the influence of using *Kluyveromyces marxianus* LAF4 combined with probiotic strains to produce peptides with ACE-I properties. In Chapter 6 however, further investigation to produce bioactive peptides with ACE-I activity was carried out using a different enzyme namely, Flavourzyme®, in combination with Lh strains. The ACE-I activity of peptide fractions from fermented skim milk were also assessed. The *in vivo* testing of fermented skim milk containing peptides on SHR rats is discussed in Chapter 7.

Chapter 8 evaluates sensory characteristics of set-type fermented skim milk drink containing peptides with 95 % ACE-I activity. The overall conclusions and future directions of this project are summarised in Chapter 9, and finally, all relevant references are compiled in Chapter 10.

Chapter 2 Literature review

2.1 Background

Nutrition concepts today are moving away from prevention to the promotion of health and wellness, in keeping with consumer awareness of the link between diet and health. This trend has now created a demand for functional foods, or 'foods that contain some health-promoting component(s) beyond traditional nutrients' (Shah, 2001). The market for functional foods is large in the US, it was valued at US$21 billion (B) in 2006, with a 5 % annual growth forecast till 2011 (Parker, 2007) and increased to 25 % in 2014 (Leatherhead Food Research in 2014). According to the report by Leatherhead Food Research in 2014 (Figure 2.1), the global market for functional foods was worth an estimated united states dollar (USD)$ 43.27 B (Thomas, 2014). This represents an increase in value terms of 26.7 % compared with 2009 (Thomas, 2014). The market suffered during the global economic downturn, owing to consumers switching to cheaper groceries, whilst changes in regulations are also thought to have hindered growth (Thomas, 2014). In the European Union (EU) more pressure is being placed upon manufacturers of functional foods to provide robust scientific evidence backing up the health claims made by their products (Thomas, 2014). Other significant sectors in health promotion include digestive health and heart health-foods, worth USD $16 B and USD $13.75 B respectively in 2013 (Thomas, 2014). Leatherhead Food Research 2014 commented: 'The functional foods market has experienced fairly strong growth in certain parts of the world' (Thomas, 2014). For instance, more US consumers appear to be turning towards functional foods and drinks in order to address perceived nutritional shortfalls, away from dietary supplements. However, future growth is likely to be dependent upon the global economic situation (Thomas, 2014).

Foods can be modified to become 'functional' by enzymatic hydrolysis during gastrointestinal. This effectively releases bioactive peptides (specific protein fragments) from an inactive state in the protein molecule (Kitts & Weiler, 2003). The role of bioactive peptides in promoting wellness is acknowledged and attention is focused on its sources from milk proteins (FitzGerald & Meisel, 2003). Milk-derived bioactive peptides are regarded as highly prominent ingredients for health-promoting functional foods due to their physiological and physiochemical versatility (FitzGerald & Meisel, 2003). Depending on the amino acid sequence, these bioactive peptides may initiate a

number of different activities *in vivo*, e.g. antithrombotic and antihypertensive, immunomodulatory, antimicrobial and anti-oxidative (FitzGerald & Meisel, 2003).
To attain antihypertensive function *in vivo*, the ACE-I peptides have to be absorbed from the intestine in an active form, and reach the targeted organ. One of the challenges in oral ingestion is the stability of the ACE-I peptides (Fitgerald & Meisel, 2003). For these peptides to be effective, they need to pass from the intestine to the serum where they may be susceptible to brush border and intracellular peptidase activities, as well as be resistant to degradation by serum peptidases (Fitgerald & Meisel, 2003). In other words, they need to survive the degradation by gastrointestinal proteinases and peptidases, before being absorbed into the system (Fitgerald & Meisel, 2003).

Studies have shown that small peptides, such as di- and tri-peptides, are easily absorbed in the intestine (Hara et al., 1984). Most of the documented ACE-I peptides are short peptides with a proline residue at the carboxyl terminal end. Proline containing peptides are known to be resistant to degradation by digestive enzymes (Maxime, Marcotte, & Arcand, 2006). The literature reviewed thus indicates that further studies are required to better understand the blood-pressure-reducing mechanisms of milk peptides. Controlled animal studies are needed to demonstrate the long-term physiological effects delivered by consuming such peptides.

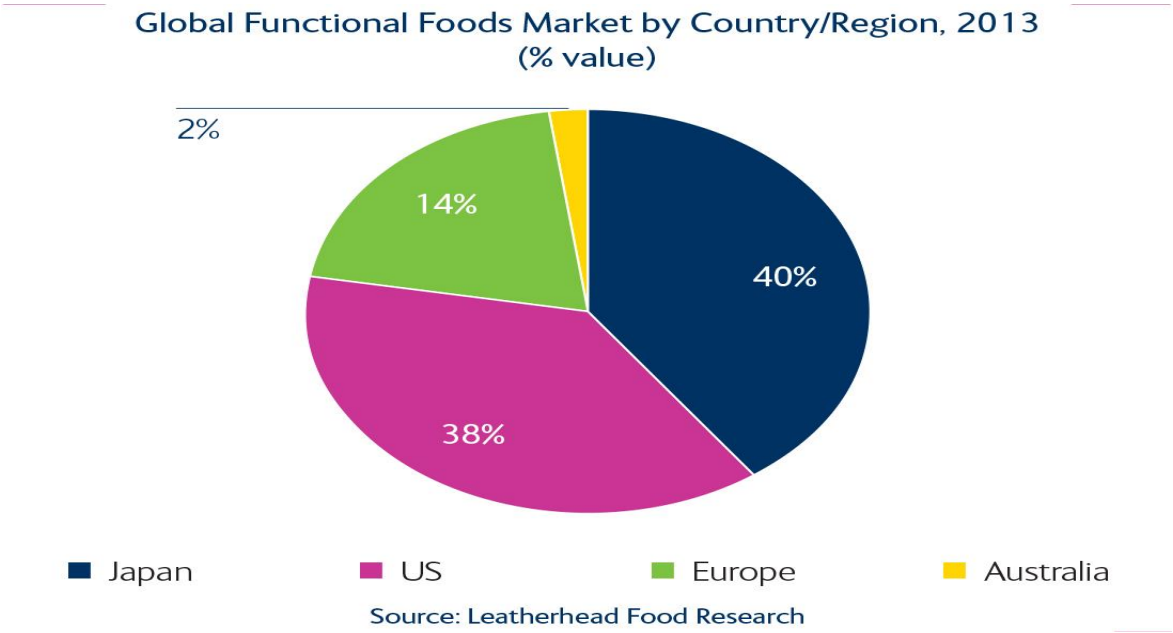


Figure 2.1 Global functional food market (Thomas, 2014).

2.2 Milk proteins

Milk is mainly an aqueous solution of lactose, inorganic and organic salts, and dispersed colloidal particles of milk proteins and larger emulsified lipid globules. Proteins and peptides, for example, metal-binding proteins, immunoglobulins, growth factors, enzymes, antibacterial agents and oligosaccharides present in milk, deliver important physiological and protective functions (FitzGerald & Meisel, 2003; Singh & Thompson, 2014). Dairy products play an important role in human nutrition. The average composition of main components of bovine milk is: 87.1 % water, 4.6 % lactose, 3.3 % milk proteins and 4 % milk fat (Walstra et al., 2005). Milk proteins are generally classified into two types namely caseins and whey proteins based on their solubility at pH 4.6 (Fox, 2003; Huppertz et al., 2006). Caseins are insoluble and consequently coagulated, with whey proteins (WPs) remaining soluble. Out of the total milk protein content, which is about 31 g/ L, caseins present about 80 % and the remaining 20 % are the whey proteins.

There are four types of caseins (αs1-, αs2-, β- and κ- casein) present in milk as large colloidal complexes or micelles composed of thousands of molecules with molecular mass ~108 Da. On the other hand, WPs most probably exist as monomers or as small quaternary structures. In comparison to caseins, which are extremely heat-stable and not coagulated when heated at 100ºC for 24 h or at 140ºC for up to 20 – 25 min (FitzGerald & Meisel, 2003), WPs are very heat sensitive. However, caseins are phosphorylated and the degree of phosphorylation varies among the individual caseins imparting to them molecular charges and thereby hydration, solubility, heat stability and metal binding, especially in this instance, Ca ions. As a result, high levels of calcium phosphate are available in milk in a soluble form. Additionally, under natural conditions and in a stable colloidal suspension of surrounding water-based liquid, the casein micelles are not aggregating as a result of an inter-micellar steric and electrostatic repulsion provided by the protruding polyelectrolyte region of κ-casein from the micellar surface. While whey proteins are not phosphorylated, they are richer in sulphur content (1.7 %) compared to caseins with ~ 0.8 % sulphur (FitzGerald & Meisel, 2003).

WPs are predominantly a mixture of β-lactoglobulin (β-Lg), α- lactalbumin (α-La), bovine serum albumin (BSA), immunoglobulins (Ig), protease peptones and other minor

proteins including lacto peroxidase, lysosome and lacto ferrin (Fitzsimons, Mulvihill, & Morris, 2007; Verheul & Roefs, 1998). There are different types of WP powders available on the market as concentrates, isolates and hydrolysates. The annual worldwide production of WP products is about 600,000 metric tons (Damodaran, Parkin, & Fennema, 2008). Whey protein concentration (WPC) contains up to ~ 85 % proteins, low levels of fat and cholesterol and typically a higher amount of bioactive compounds and lactose. However, the fat and lactose content present in WPC may exert detrimental effects on some functional properties and the overall protein quality. In comparison, whey protein isolate (WPI) contains more than 90 % of proteins with lower levels of fat, lactose and bioactive compounds (Morr & Ha, 1993). Therefore, they are relatively high quality protein powders with enhanced functionality (Morr & Ha, 1993). Whey protein hydrolysates are partially hydrolysed, pre-digested products enabling easy absorbance in the gut (FitzGerald & Meisel, 2003; Huppertz et al., 2005). The production of bioactive peptides in fermented milk has been widely studied, and the effectiveness of bioactive peptide depends on its amino acid sequence (FitzGerald & Murray, 2006; Pihlanto, Virtanen & Korhonen, 2010; Amigo & Recio, 2012; Chaves-López et al., 2012 and 2014; López-Expósito et al., 2013; Hernández-Ledesma et al., 2014; Singh & Thompson, 2014).

2.2.1 Milk products

There has been an increase in production of milk-based products, such as skim milk powder, yoghurt, fermented milk products and fermented WP products in the world (Hansen, 2002; Khan et al., 2013). Fermented milk products have been part of the diet in many countries such as Europe and the Middle East (Hansen, 2002; Khan et al., 2013), made by milk fermentation using yoghurt culture and/or Lactic acid bacteria (LAB). In yoghurt, lactose is converted into lactic acid by LAB, which gives a pleasant acidic flavour and the sweetness caused by the reduction of lactose (McKinley, 2005; Shah, 2007; Aslim et al., 2006).

2.2.2 Fermentation process, definition of fermented milks and yoghurt

The fermentation industry today is very much in a state of flux, with rapid changes in product spectrum, location and scale of processes occurring (McNeil & Harvey, 2008). This has been brought about by macroeconomic forces compelling the relocation of

large scale bioprocesses outside high labour cost regions, and the significant advances in the construction of advanced fermentation expression systems for making novel proteins and antibodies (McNeil & Harvey, 2008). Thus, fermentation skills and knowledge are now essential to driving forward systematic research into drug interactions, function of membrane proteins in health and disease, and are powering an unparalleled expansion in capability to combat serious diseases in the human population, including degenerative illnesses and cancers.

The new dairy fermentation-derived medicines, including biopharmaceuticals, hold out the prospect of improved specificity of treatment and decreased side effects (McNeil & Harvey, 2008). It is truly a revolutionary period in clinical medicine as these new agents manufactured by fermentation routes enter the market (McNeil & Harvey, 2008). The new fermentation dairy products and therapeutic proteins are more complex and costly than previous products, but in essence, the need to focus upon the fermentation step is now clearer than ever. Basically, 'quality' of these products (the potency, efficacy, stability and immunogenicity) is determined by the upstream or fermentation stage. One of the fermentation processes is bioreactor. This process, in some form or another, has been in use for thousands of years, although up until the 1900s its use was limited to the production of potable alcohol (McNeil & Harvey, 2008). Since the 1940s onwards, that fermentation as it is known today, began to appear with the need to produce antibiotics during World War 2 (McNeil & Harvey, 2008). At this point, the need for process development to improve yields drove research. As it would not be practical to carry out this research on production scale equipment, small-scale bioreactors have become word (McNeil & Harvey, 2008). Bioreactors at this volume can be used for a number of purposes: monitoring and controlling pH by acid/base addition or CO_2/base addition, temperature regulation, sterile sampling capability and mixing such that the culture remains in suspension. All this should be achieved without damage to the organisms. Recently, the bioreactor processes have been developed for dairy fermentation products such as yoghurt (McNeil & Harvey, 2008).

Yoghurt is a coagulated milk product obtained by specific LAB fermentation, through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* as starter cultures in cow's milk. The microorganisms in the final product must be viable and abundant. On the other hand, yoghurt containing *Bifidobacterium* ssp. or

Lactobacillus acidophilus would be classified as 'fermented milk' (McKinley, 2005; Shah, 2007).

In some countries like the UK, Canada and the USA, the addition of other LAB to starter culture used to make yogurt is acceptable (Gilliland, 1991). All fermented milk products have something in common: they are all milk based, with an acidic pH resulting from the fermentation, contain a significant number of selected microorganisms that help preserve the milk and reduce the risk factors for diseases (Lahtinen et al., 2011). Fermentation processes lead to changes in the structure of milk proteins, resulting in the production of some amino acids and peptides from milk proteins (Gaudichon et al., 1994). One of the important processes prior to milk fermentation is heat treatment.

There are several benefits of using such a high level of heat treatment (pasteurisation), including:

1. Destruction of all pathogenic and most spoilage bacteria,

2. Inactivation of most enzymes, which may cause undesirable effects to the finished product,

3. Expulsion of toxic compounds and a decrease in the oxidation-reduction potential of the medium suitable for the growth of starter cultures by removal of oxygen,

4. Conversion of calcium into a soluble form leading to a decrease in time for milk coagulation.

2.2.3 Probiotics

The scientific understanding in the field of probiotic bacteria and the processes of bacterial fermentation are improving. The genera of bacteria and yeasts that are commonly used as probiotics are listed in (Table 2.1) (Amrane & Prigent, 1998b; Ramesh & Chandan, 2013). Many different types of fermented milk belong to or originate from a large diversity of microorganisms. The classification of probiotics includes different kinds of microorganisms (Lahtinen et al., 2011) listed below:

- Some Yeast: *Saccharomyces kefir* is part of the core of kefir, commonly fermented milk consumed in Eastern Europe. Some of *Kluyveromyces* (*Kluyveromyces marxianus*) is a species of yeast in the genus *Kluyveromyces marxianus* (*K. marxianus*) used

commercially to produce the lactase enzyme similar to that used by other fungi such as those in the genus (*Aspergillus*) in a Sudanese traditional fermented milk product (Rob). Rob is made from fermentation of cow, sheep and goat's milk. The bulk is made from cow's milk while a smaller proportion is prepared from either goat's or sheep's milk or a mixture of these two milks (Abdelgadir et al., 1998). Milk surplus is collected in a container, inoculated with a starter from the previous day and left to ferment overnight. The fermentation process usually starts in the evening when the animals return from grazing and the sour product is churned in the morning when the herd leaves for grazing. Freshly produced Rob has a pleasant taste with a pH of about 4.5. Other applications of *Kluyveromyces marxianus*, *Lactobacillus delbrueckii ssp. bulgaricus* and *Lactobacillus helveticus* are as starter cultures for sourdough bread making (Plessas et al., 2008). The use of mixed cultures led to higher total titratable acidities and lactic acid concentrations compared to traditionally made breads. Highest acidity (3.41 g lactic acid/kg of bread) and highest resistance to mould spoilage were observed when bread was made using 50% sourdough containing 1% *K. marxianus* and 4% *L. delbrueckii ssp. bulgaricus* (Plessas et al., 2008). The use of these cultures also improved the aroma of sourdough breads, as shown by sensory evaluations and as revealed by GC-MS analysis (Plessas et al., 2008).

- Specific strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) is involved in basically three groups of indigenous fermented products: non-alcoholic starchy foods, alcoholic beverages and fermented milk. These products are, to a great extent, made by spontaneous fermentation and consequently *S. cerevisiae* often coexists with other microorganisms, even though a microbiological succession usually takes place both between and within species. The function of *S. cerevisiae* is related to formation of alcohols and other aroma compounds, but stimulation of e.g. LAB, improvement of nutritional value, probiotic effects, and inhibition of undesired microorganisms and production of tissue-degrading enzymes may also be observed (Jespersen, 2003).

  Molds such as *Aspergillus* are proteases used in some cheese processes. Among proteases, aspartate proteases find application in industry for cheese making, as digestive aids, beer clarifiers, food protein modifiers, and de-bittering protein hydrolysate preparations (Rao et al., 1998). The cheese industry has a great demand for acid proteases (aspartate proteases). Aspartate proteases assist in clotting milk apart from playing a key role in flavor and texture development (Vioque et al. 2000).

Each genus is divided into numerous species. *Lactobacillus* is classified into 120 different species from *acetotolerans* to *zymae,* through more commonly encountered species like *acidophilus, casei, delbrueckii, helveticus, plantarum, reuteri*, and *rhamnosus*. A species can be again separated into sub species e.g., (*lactobacillus delbrueckii ssp. bulgaricus*).

Probiotics are a diverse community and can grow in different conditions. The optimal growth temperature (10°C; 2-30°C) for psychotropic cultures, medium temperature (25°C; 5-60°C) for mesophiles culture used mainly for cheese, and high temperature (40°C; 30-65°C) for thermophilic cultures, commonly used for fermented milks (Lahtinen et al., 2011).

During the fermentation process, LAB produces lactic acid and lowers the pH. The acidity is a self-limiting system that controls fermentation, as LAB is sensitive to high acidic pH. Therefore, the kinetics of exposure to acid may change the internal metabolism, and a longer exposure to acidic conditions will decrease the internal buffering capacity of LAB (Lahtinen et al., 2011).

Different cultures are used for different fermented milks, and different countries exhibit diversity by producing different milk varieties. For example, Kumis is made from mare's milk and some specific kefir grains in Russia. Dahi, a sweet yoghurt in India, is made from buffalo milk and is sometimes fermented in bamboo tubes with a mixture of LAB. In India, Lassi is made from milk blended with sugar, allowing some non-lactose-dependent bacteria to grow (Lahtinen et al., 2011).

Table 2.1 The genera of bacteria and yeasts that are commonly used as probiotics

Bifidobacterium	***Lactobacillus***	**Fungi**	**Others**
B. infantis	*L. salivarius*	*Saccharomyces*	
B. bifidum	*L. johnsonii*	*boulardii*	*Propionibacteriu*
B. acolescentis	*L. helveticus*	*Saccharomyces*	*freudenreichii*
B. thermophilum	*L. farciminis*	*cerevisiae*	*Enterococcus*
B. animalis	*L. acidophilus*		*faecium*
B. longum	*L. rhamnosus*		*Lactococcus*
B. breve	(GG)		*lactis*
B. lactis	*L. gasseri*		*Bacillus cereus*
	L. casei		*Bacillus clausii*
	L. paracasei		*Bacillus*
			oligonitrophilis
			Clostridium
			butyricum

(Penner, Fedorak, & Madsen, 2005)

2.3 Functional food products

2.3.1 Health and nutritional benefits of fermented milk products

Fermented milk products are known as cultured milk products that have been fermented with LAB such as *Lactobacillus* and *Streptococcus lactis*. Fermentation of milk increases the shelf-life of the product, in addition to improving the taste and digestibility. A variety of different strains of *Lactobacilli* have been used for a wide range of cultured dairy products with different flavours (Table 2.2). The efficacy of some probiotics against diarrhoea has been reported (Sampo & Lahtinen, 2011), and modern science is exploring different physiological targets of probiotics and follows on with the comparison with vitamins. There are various strains with different benefits involving different mechanisms that modulate multiple functions or pathways (Lahtinen et al., 2011). Studies reported for LAB strains in fermented milk products were responsible for health benefits for consumers (Shah, 2007; López-Expósito, Amigo, & Recio, 2012; Hernández-Ledesma et al., 2014). Health benefits of regular consumption

of milk products containing probiotics have been reported and they include: the development of intestinal microbial balance, improving symptoms of lactose intolerance, reduction of risk of colon cancer, protection against breast cancer, strengthening the immune system, lowering blood pressure and blood cholesterol levels, reduction in some forms of food allergies, and inhibiting the growth of pathogenic bacteria (Kawase et al., 2000; Alhaj et al., 2007; Fitzgerald et al., 2011; Marinik et al., 2013; Guo et al., 2015).

Table 2.2 Types of fermented milk products.

Dairy Products	Commercial names	Milk fat concentration	Typical shelf life at 4°C	Fermentation bacteria types	Description
Yogurt	yoghurt	0.5–4%	35–40 days	*Lactobacillus bulgaricus and Streptococcus thermophilus*	Thermophilic fermented milk cultured with *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Lactose-intolerant individuals may tolerate yoghurt better than other dairy products due to the conversion of lactose to the sugars glucose and galactose, and due to the fermentation of lactose to lactic acid carried out by the bacteria present in the yoghurt.
Kumis	kumiss, koumiss, kymy kymys,chigee	4%	10–14 days	*Lactobacilli* and yeasts	A carbonated fermented milk beverage traditionally made from horse milk.
Kefir	kephir, kewra, talai, mudu kekiya,	0-4%	10–14 days	Kefir grains, a mixture of bacteria and yeasts	A fermented beverage, originally from the Caucasus region, made with kefir grains; can be made

	milkkefir, búlgaros				with any sugary liquid, such as milk from mammals, soy milk, or fruit juices.
Acidophilus milk	acidophilus cultured milk	0.5-2 %	2 weeks	*Lactobacillus acidophilus*	Thermophilic fermented milk, often low fat (2 %, 1.5 %) or non-fat (0.5 %), cultured with *Lactobacillus acidophilus*
Cheese	Cheese	1-75 %	varies	a variety of bacteria and/or mold	Any number of solid fermented milk products.
Crème fraîche	creme fraiche	30-40 %	10 days	naturally occurring lactic acid bacteria in cream	Mesophilic fermented cream, originally from France; higher-fat variant of sour cream
Cultured buttermilk		1–2 %	10 days	*Lactococcus lactis*(*L. lactis*), *L. lactis* subsp. *cremoris, L. lactis subsp. lactis* biovar diacetylactis and *Leuconostoc mesenteroides* subsp. *cremoris*	Mesophilic fermented pasteurized milk
Cultured sour cream	sour cream	14-18 %	4 weeks	*L. lactis* subsp. *lactis*	Mesophilic fermented pasteurized cream with an acidity of at least

					0.5 %. Rennet extract may be added to make a thicker product. Lower fat variant of crème fraîche
Filmjölk	fil	0.1-4.5 %	10-14 days	*L. lactis. and Leuconostoc*	Mesophilic fermented milk, originally from Scandinavia
Viili	filbunke	0.1-3.5 %	14 days	*L. lactis* subsp. *cremoris, L. lactis subsp. lactis* biovar diacetylactis*, Leuconostoc mesenteroides* subsp. *cremoris* and *Geotrichum candidum*	Mesophilic fermented milk that may or may not contain fungus on the surface; originally from Sweden; a Finnish specialty

(Commission, 2012; Swedish, 2007; Virginie, Amilien, Hanne, & Vittersø, 2005)

2.3.2 Lactic acid bacteria (LAB)

Milk is a favourable media for bacterial growth (Martín et al., 2003; Galat et al., 2015; Li & Shah, 2015). This explains why raw milk is difficult to store, since the environment is rich in microbes that can contaminate milk and cause it to spoil (Lahtinen et al., 2011). Amongst those bacteria, some are detrimental to human health, called pathogens, or if humans became adapted to them, they are called cultures. Among these cultures, the LAB constitutes a group of gram-positive bacteria united by certain morphological, metabolic, and physiological characteristics (Lahtinen et al., 2011). LAB were used to refer to milk souring organisms which form the basis of the present classification of LAB (Lahtinen et al., 2011). LAB have traditionally been associated with food and animal feed fermentations, are generally considered beneficial microorganisms and some strains are considered as health-promoting (probiotic) bacteria (Lahtinen et al., 2011). LAB can use lactose as a source of energy and tolerate oxygen to survive transfers from pots to tanks (Lahtinen et al., 2011).

2.4 Lactic acid bacteria and blood pressure

2.4.1 Regulation of blood pressure

Increased blood pressure is one of the leading risk factors for cardiovascular disease. Hypertension is related to increased systolic blood pressure (SBP) and diastolic blood pressure (DBP): Up to 30 % of the world's adult population were hypertensive in 2000 (Kearney et al., 2005). Hypertension usually exists with other risk factors, including hypercholesterolemia, metabolic syndrome, and insulin resistance. Altogether, these conditions increase cardiovascular morbidity and mortality. Hypertension is an important public health challenge (Lahtinen et al., 2011). Long-term regulation of blood pressure is closely related to kidney function and body fluid volume homeostasis, while the short-term control of blood pressure has been attributed to the sympathetic nervous system (Wyss, 2001).

One of the key systems related to kidneys and body fluid volume is the renin-angiotensin system (RAS). The RAS consists of a cascade of enzymes and receptors, beginning from renin secreted by kidney juxtaglomerular cells and leading ultimately to the formation of angiotensin-II (Ang-II) and its binding to angiotensin-II type I receptors (AT1) (Figure 2.2) (Hong et al., 2008; Lemarié & Schiffrin, 2010). This leads

to arteriolar constriction, increase of blood pressure, salt and water retention via increased production of aldosterone (Lemarié & Schiffrin, 2010). The important aspects of the RAS in cardiovascular disease have been demonstrated by the clinical benefits of angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor blockers (Pripp & Ardö, 2007; Lemarié & Schiffrin, 2010). In molecular modelling, components of the RAS (especially ACE-I) have been used as potential targets of food derived antihypertensive compounds (Pripp & Ardö, 2007; Pripp et al., 2004). Blood vessels contribute to blood pressure regulation by controlling vascular resistance. Due to aging and increased blood pressure, arteries stiffen and gradually lose their ability to adjust to blood pressure changes (Ghiadoni et al., 2009). Although functional food products produced with LAB should not be considered as medications, they may be suitable for people with high blood pressure before pharmacological therapy is required and thereafter combined with medications.

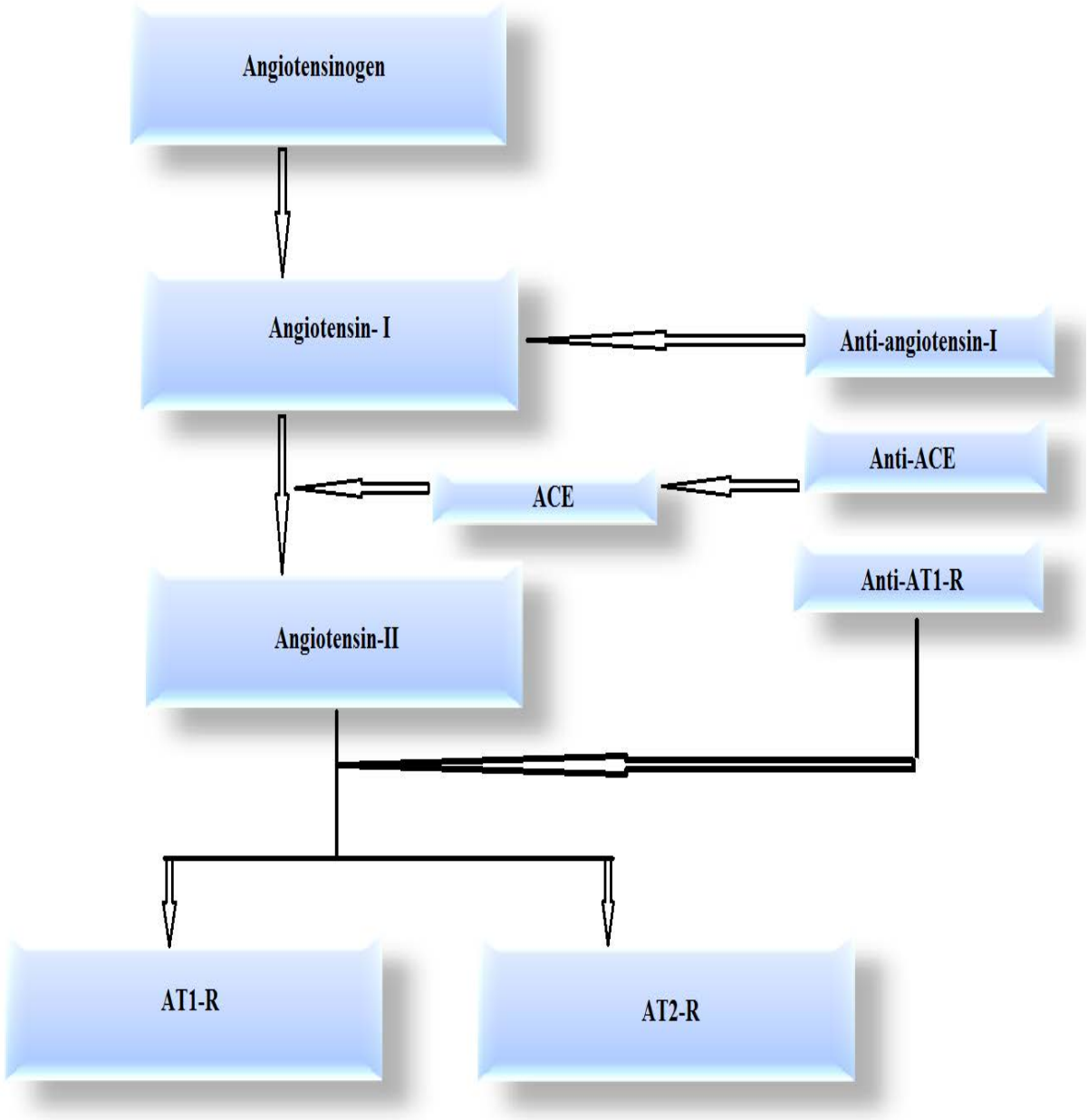


Figure 2.2 Targets to inhibit the renin-angiotensin system: (Hong et al., 2008)

2.4.2 Production of bioactive peptides

Peptides may be liberated from their parent proteins by enzymatic hydrolysis during gastrointestinal digestion, fermentation of milk with proteolytic starter cultures or hydrolysis by enzymes (Phelan et al., 2009). There are a number of methods, by which bioactive peptides with biological activity can be produced (Phelan et al., 2009). The

most common methods are heat, alkali, or acidic conditions that hydrolyse proteins, enzymatic hydrolysis of milk proteins and microbial activity of fermented milk products (Maruyama & Suzuki, 1982). Biologically active peptides are released by limited hydrolyses of well-known proteins. The most common method to produce bioactive peptides is through enzymatic digestion. For example, ACE-inhibitory peptides are most commonly produced by trypsin (Maruyama & Suzuki, 1982). However, other enzymes and various enzyme combinations of proteinases including alkalase, pepsin, pancreatin and enzymes from bacterial and fungal sources, have been used to produce bioactive peptides. Microbial enzymes have also been successfully used to produce ACE-inhibitory peptides from milk protein (Maeno et al., 1996). After hydrolysis of milk proteins, the peptides in hydrolysates are fractionated and enriched by means of various methods. Chemical measurements and analytical techniques are the critical components of the molecular understanding of the biological process where many bioactive peptides are involved. There are some methods, which have already been proven to be applicable for the identification and characterization of bioactive peptides derived from milk proteins. These methods are outlined in (Figure 2.3) (Schlimme & Meisel, 1995; Christensen et al., 1999).

LAB as *lactobacillus helveticus* is traditionally used in milk processing to produce cheese (Lahtinen et al., 2011). The release of amino acids by action of peptidases is an essential part of the LAB proteolytic system (Pederson et al., 1999). Tri-peptides, isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) have been generated from sour milk fermented with *L. helveticus CP790* and *Saccharomyces cerevisiae* (Nakamura et al., 1995). Several studies have reported that more than ten peptides have been defined as part of *L. helveticus* proteolytic system; (PepE, PepO, PepT, PepX, PepI, PepQ, PepR, PepD, PepV, PepC, PepN) (Christensen et al., 1999, Savijoki and Palva, 2000; Kenny et al., 2003; Stressler et al., 2013). Another study identified seven oligo endo peptidases and eight di- and tri–peptidases in *L. helveticus* strain CNRZ32 (Broadbent et al., 2011). The endo-peptidase PepO2 plays an important role to decrease the bitterness in cheese (Fernandez et al., 1994; Shao et al., 1997; Christensen et al., 2003; Chen et al., 2003; Kilpi et al., 2007). PepO2 specifically targets bonds containing amino acid proline (Dudley et al., 1996). A study on six amino peptidase activities in fermented milk using LAB described the mechanism of

regulation as dependant on a specific strain (Jensen and Ardö, 2010). *L. helveticus* peptidases also have higher proteolytic activity (Valence et al., 1998; Valence et al., 2000). It has been suggested that enzymes play an important role in hydrolysis of milk protein through the process of milk fermentation (Ueno et al., 2004). The whey fraction of yoghurt fermented with *L. helveticus* CPN4 has been found to contain dipeptide Tyr-Pro, which has shown significant antihypertensive effect in spontaneously hypertensive rats (SHR) (Yamamoto, Maeno, & Takano, 1999). Furthermore, proteolytic enzyme of LAB such as cell wall associated with serine protease, may be isolated, purified and used to produce bioactive peptides from casein (Minervini et al., 2003).

There are few studies which note the use of WPs as a source of bioactive peptides in fermentation with LAB (Yamamoto, Maeno, & Takano, 1999; Chatterton et al., 2006; Pihlanto et al., 2010; Tellez et al., 2011). *L. helveticus* strains (Lh) used to ferment WPs are able to hydrolyse α-lactalbumin to release bioactive peptides (Castro et al., 1996, Chatterton et al., 2006). Hydrolysates of WPs that release peptides with ACE-I activity have been investigated in several studies (Bayoumi & Griffiths, 2012; Illanés, 2011; Madureira et al., 2010; Pescuma et al., 2008; Pihlanto-Leppälä, 2000; Pihlanto et al., 2010; Saito, 2008; Wang et al., 2012; Welderufael, Gibson, & Jauregi, 2012). Table 2.3 shows a summary of various microorganisms and microbial enzyme strains that have been reported to produce bioactive peptides from milk proteins.

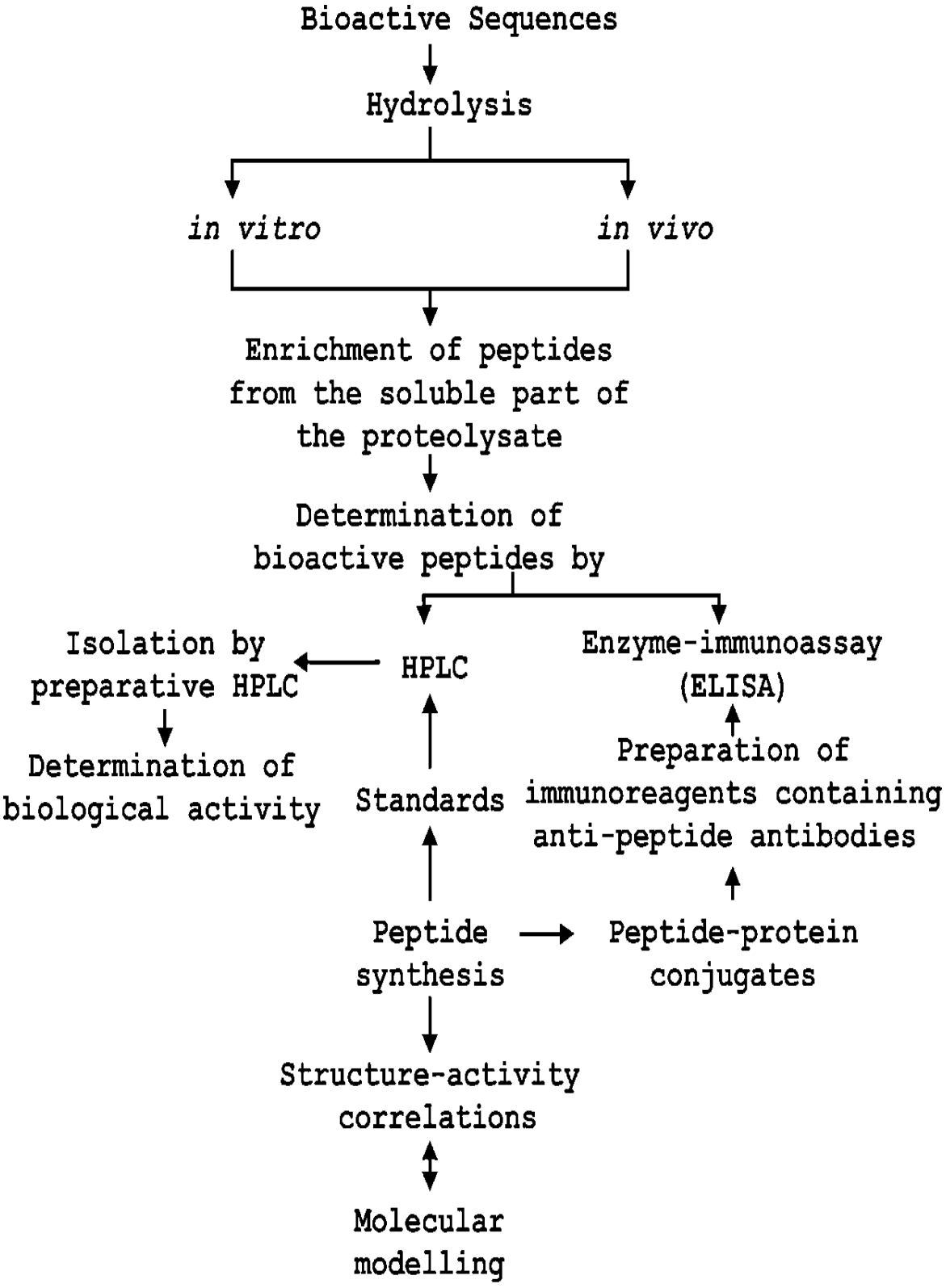


Figure 2.3 Outline of identification and characterisation of bioactive peptides derived from milk proteins (Schlimme & Meisel, 1995).

Table 2.3 Bioactive peptides released from milk proteins by various microorganisms and microbial enzymes.

Microorganisms	Precursor protein	Peptide sequence	Bioactivity	References
L. helveticus *S. cerevisiae*	β- CN * κ-CN	Ile-Pro-Pro, Val-Pro-Pro	ACE[#] inhibition , antihypertensive	Nakamura etal. (1995) Takano (1998)
L. helveticus JCM1004 cellfree extract	Skim milk hydrolysate	Ile-Pro-Pro, Val-Pro-Pro	ACE inhibition, antihypertensive	Pan et al. (2005)
Lactobacillus GG Enzymes + pepsin and trypsin	β- CN, αs1- CN	Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Met-Pro-Leu-Trp	ACE inhibition	Rokka et al. (1997)
L. helveticus CP790 proteinase	β- CN	Lys-Val-Leu-Pro-Val-Pro-Gln	ACE inhibition	Maeno et al. (1996)
L. helveticus CPN4	Whey proteins	Tyr-Pro	ACE inhibition	Yamamoto et al. (1999)
L. delbrueckii ssp. *bulgaricus* SS1, *Lactococcus lactic* ssp. cremoris FT4	β-cn, κ-CN	Many fragments	ACE inhibition	Gobbetti et al. (2000)
L. delbrueckii ssp.	β- CN	Ser-Lys-	ACE inhibition	Ashar and

bulgaricus		Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile		Chand (2004)
S. thermophilus + *L c. lactic* ssp. *lactic* biovar. diacetylactis	β- CN	Ser-Lys-Val-Tyr-Pro	ACE inhibition	Ashar and Chand (2004)
Lactococcus lactic	αs1-CN, αs2- CN, κ- CN	Many fragments	ACE inhibition	Minervini et al. (2003)
L. helveticus NCC 2765	β- CN	Tyr-Pro-Phe-Pro-Glu-Pro-Ile-Pro-Asn	Opioid	Meisel and Frister (1989)
Commercial products + digestion	αs1- CN	Thr-Thr-Met-Pro-Leu-Trp	ACE inhibition, immunomodulation	Maruyama et al. (1987)

CN * = casein; ACE# = Angiotensin-I converting enzyme

2.4.3 Dairy products as source of bioactive peptides

Dairy proteins possess physicochemical and biological properties of importance to human health. Specifically, dairy products contain nutrients that are needed for growth and development and are a rich source of proteins, lipids, minerals, vitamins and lactose. Studies during the last 15 years have shown that caseins and WPs are essential sources of bioactive peptides. Bioactive peptides have been defined as specific protein fragments, which have a positive effect on body functions and may ultimately influence health (Kitts & Weiler, 2003). Peptides generally contain two to 20 amino acid residues per molecule. Research has shown that peptides have immune-modulating, anti-hypertensive, antimicrobial and anti-oxidative activities. Specific peptides may have one or two different biological activities, and due to their physiological importance, milk-borne bioactive peptides are regarded as food

ingredients with health-promoting properties (Korhonen, 2004; Korhonen & Pihlanto, 2007). Therefore, milk derived peptides are potential candidates to be incorporated into food products and used to improve cardiovascular, skeletal and digestive functions or have an immune defence effect (Lahtinen et al., 2011). Therefore, it can be assumed that the number of bioactive peptides increases during the production of fermented milk products compared to raw milk and that the composition of the peptide fraction changes due to the proteolytic action of the employed microorganisms (Hayes et al., 2007). Some studies investigated bioactive peptides in traditional fermented milk products and identified angiotensin-converting enzyme inhibitory (ACE-I) peptide or antimicrobial peptides in fermented food products (Figure 2.4 and 2.5) (Gómez-Ruiz et al., 2002; Losito et al., 2006; Ebner et al., 2015). The major part of bioactive milk protein-derived peptides is ACE-inhibitors, influencing blood pressure by inhibiting the conversion of angiotensin-I to the vasoconstrictive angiotensin-II and the degradation of the vasodilator bradykinin to its inactive fragments (Hayes et al., 2007). The processes involved in the formation of bioactive peptides from milk proteins are shown in (Figure 2.4).

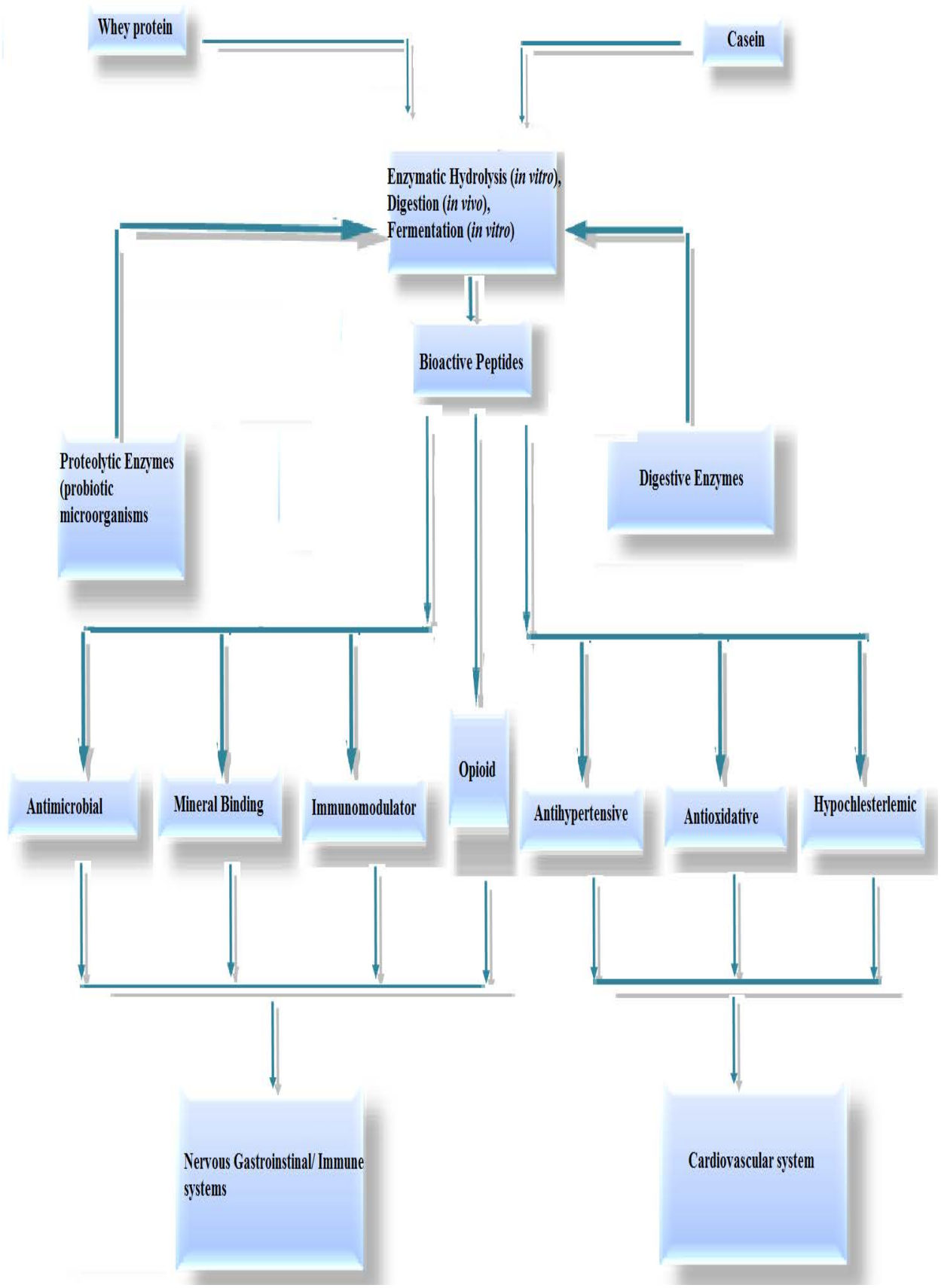


Figure 2.4 Formation of bioactive peptides from milk proteins.

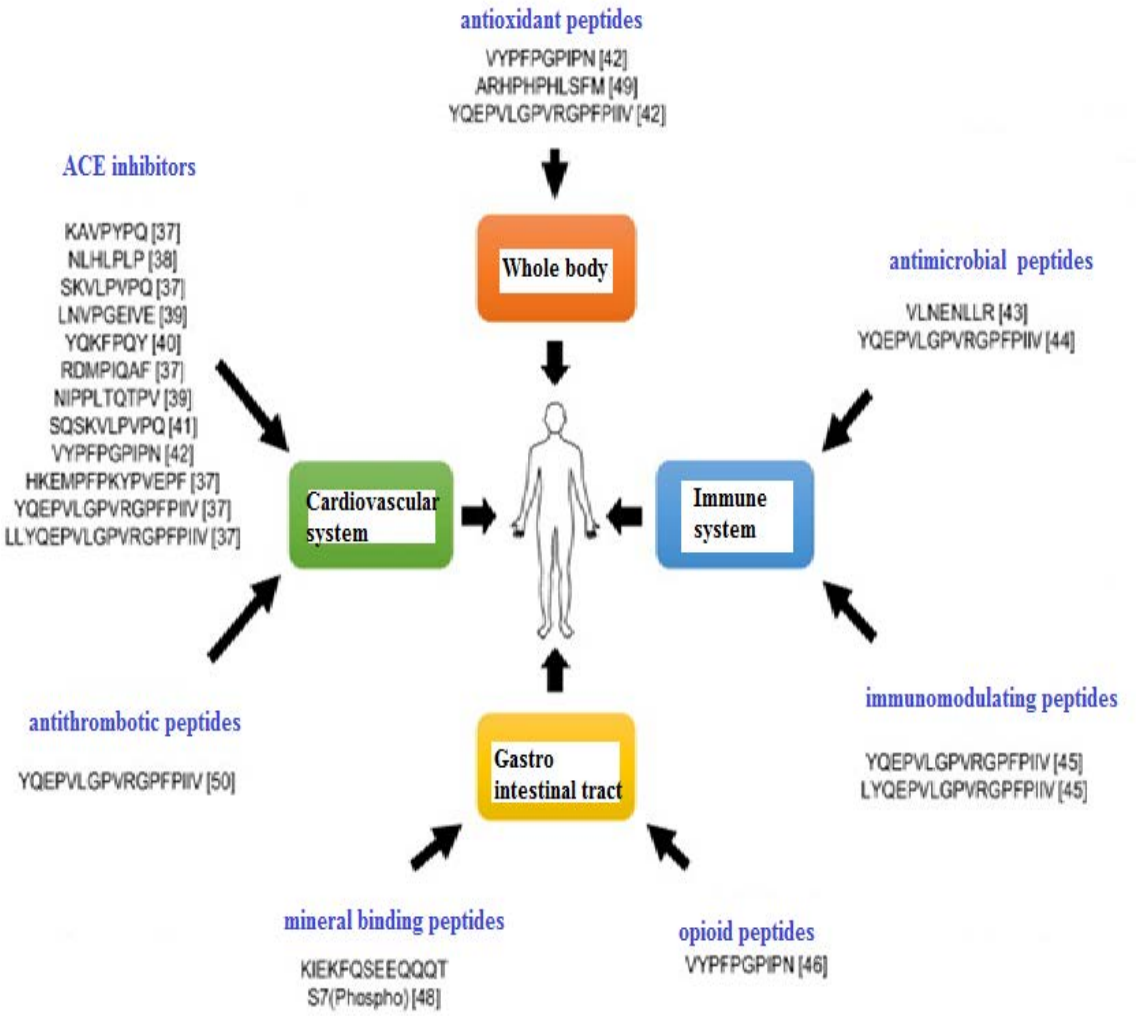


Figure 2.5 Bioactive peptides and their possible target sites in the human body. Peptide sequences are shown in single letter code (Ebner et al., 2015).

2.4.4 Physiological role of casein-based bioactive peptides

The predominant protein component in milk is casein, consisting of 80 % total milk protein (Table 2.4). Milk casein hydrolyses leads to the production of peptides, which have bioactive properties. There are four different types of caseins, namely αs1, αs2, β and κ-casein (Maubois & Léonil, 1989). A peptide derived from casein that has similar characteristics and pharmacological effect to morphine is known as casomorphin (Meisel & Schlimme, 1990). Similarly, other peptides released from caseins have been found to show immunomodulatory properties. Casein phosphor-peptides (CPPs) have been produced by gastrointestinal trypsin from αs1-, αs2- and β-caseins, and these

peptides have been shown to improve zinc and calcium absorbance (Hansen, Sandstrom, & Lonnerdal, 1996; Nagpal et al., 2011). In fact, peptides (Val-Pro-Pro or Ile-Pro-Pro) found in calpis sour milk and administered orally to spontaneous hypertensive rats show antihypertensive effects (Nakamura et al., 1995). Fermented milk containing peptides decreased systolic blood pressure post-administration. The antihypertensive effect of fermented milk containing two peptides Ile-Pro-Pro and Val-Pro-Pro was tested in hypertensive patients. As a result, systolic blood pressure decreased significantly at four and eight weeks after the beginning of ingestion (Nakamura et al., 1995). The physiological properties of casein-based bioactive peptides have been used in animal modelling systems however, they remain to be proven in humans.

Table 2.4 Concentration and biological functions of milk proteins.

Protein	**Concentration (g/l)**	**Function**
	Cow	
Total caseins	26.0	Ion carrier (Ca, PO_4, Fe, Zn, Cu) Precursors of bioactive peptides α-Casein
α-Casein	13.0	
β-Casein	9.3	
$_k$-Casein	3.3	
Total whey protein	6.3	
β-Lactoglobulin	3.2	
		β-Lactoglobulin 3´2 Retinol carrier, binding fatty acids, possible antioxidant
α –Lacto-albumin	1.2	Lactose- synthesis in mammary gland, Ca carrier, immunomodulation, anti-carcinogenic
Lacto-peroxidase	0.03	Antimicrobial

Lacto ferrin	0.1	Antimicrobial, anti-oxidative, immunomodulation, iron absorbance, anti-carcinogenic
Serum albumin	0.4	
Immunoglobulins (A, M, and G)	0.7	Immune protection
Glycomacro peptide	1.2	Antiviral, bifidogenic
Proteose-peptone	1.2	Not characterised
Miscellaneous	0.8	
Lysozyme	0.0004	Antimicrobial, synergistic effect with immunoglobulins and lacto-ferrin

(Yamauchi, 1992; Korhonen et al., 1998; Walstra & Jenness, 1984)

2.4.5 Physiological role of whey protein-based bioactive peptides

About 20 % of milk proteins are WPs, and these are not coagulated by acid (Brew, Castellino, Vanaman, & Hill, 1970). As a result, these proteins remain in solution as milk whey. Alfa-lactoalbumin (α-LA) is one of the main proteins in milk whey; Beta-Lactoglobulin consists of half the total protein in milk whey (Table 2.5). It contains 123 amino acids with a molecular weight of 14,175 K Da (Brew, Castellino, Vanaman, & Hill, 1970). Functional ingredients derived from milk including whey have a proven beneficial effect on human health (Park, 2009). WPs' bioactive peptides and their physiological effects have been less studied compared to bioactive peptides from caseins (Park, 2009). WPs and their derivatives provide important nutrients; immune system modulation; and bioactivities, including the inhibition of ACE activity, anti-carcinogenic activity, anti-microbial activity and hypocholesterolemic effects (Park, 2009). The enzymatic hydrolysis of WPs can offer a practical way to reduce its antigenic-protein fractions (Heyman, 1999). Enzymes from different origins may have variations in their hydrolytic capacity to break down WPs, and thereby may influence the physicochemical characteristics of the hydrolysates and their biological activities (FitzGerald & Meisel 2000; Bertrand - Harb et al. 2002). In addition, the hydrolysis of

WPs can yield a variety of new peptides, which may provide many physiological benefits for humans (Otte et al., 1997). WPs hydrolysates have been extensively prepared and used to nutritionally support human patients who have various physiological insufficiencies and abnormalities (Halken & Host, 1997). Therefore, they have potential use as health enhancing nutraceuticals for specific supplemental formulae for several chronic diseases. The various lacto-peroxidase, immunoglobulins, as well as the lacto ferrin, are known to protect the new born calf (Table 2.5) (McIntosh et al., 1995; McLeod A, 1996).

Table 2.5 Bioactive peptides derived from whey proteins.

Precursor protein	**Fragment**	**Peptide sequence**	**Name**	**Function**
α-Lactoalbumin	50-53	Tyr-Gly-Leu-Phe	α-Lactorphin	Opioid agonist ACE inhibition
α-Lactoglobulin	102-105 142-148 146-149	Tyr-Leu-Leu-Phe Ala-Leu-Pro-Met His-Ile-Arg His-Ile-Arg-Leu	β-Lactorphin	Non-opioid stimulatory effect on ileum ACE inhibition
			β-Lactotensin	Ileum contraction
Bovine serum albumin	399-404 208-216	Tyr-Gly-Phe-Gln-Asp-Ala Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg	Serorphin	Opioid

Lacto ferrin	17-42	Lys-Cys-Arg-Arg-Trp-Glu-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Pro-Ser-Ile-	Albutensin A	Ileum contraction ACE inhibition
		Thr-Cys-Val-Arg-Arg-Ala-Phe	Lacto ferrin	Antimicrobial

(Korhonen et al., 1998; Food Funct, 2011)

2.4.6 Bioactive components of milk and their physiological effects

The important attribute in metabolism of LAB is fermentation of carbohydrate with phosphorylation. LAB strains have the ability to ferment different types of carbohydrates. The major product of fermented milk is lactic acid. LAB can adapt to diverse conditions and change their metabolic activities and could lead to significantly different end products (Salminen, 1998; Salminen & von Wright, 2004). Lactose is used to produce lacto-oligosaccharides and lactulose which are used as probiotic bacteria growth promoters by enzymatic processes (Shah, 2000). Bioactive compounds of milk protein fractions have been extensively studied (Table 2.6), including other compounds which have physiological significance such as calcium. Calcium plays a role in blood pressure regulation. The possible protective role of calcium in avoidance of colon cancer has been researched at the Dutch Dairy Research Institute (Lapre, 1991; Medicine, 1997). Milk products play a role by providing calcium phosphate which binds to bile salts to prevent their toxic effect (Lapre, 1991; Medicine, 1997).

Fatty acids play a role as bioactive compounds in milk. For example, the role of conjugated linoleic acid in inhibition of cancer has been examined (Pariza, 1999). Fermented milk products such as kumis, yoghurt and kefir have a number of health benefits associated with their physiological effects. Recently, researchers have shown the beneficial physiological role of fermented milk products by probiotic organisms such as *Lactobacillus*, *Bifidobacteria* and *acidophilus* (Donkor et al., 2007; Lim, Lee, Park, Yoon, & Paik, 2011). The consumption of probiotic bacteria via fermented milk products has also been described to have a beneficial effect on the consumer by

restoring the balance in the intestinal micro-flora, which may have been lost due to antibiotic use or other conditions (McKinley, 2005; Donkor et al., 2007).

Table 2.6 Bioactive peptides released from bovine milk proteins.

Bioactive peptide	**Protein precursor**	**Bioactivity**
Casoxins	$_k$-Casein	Opioid antagonists
Casoplatelins	$_k$-Casein, transferrin	Antithrombotic
Casomorphins	α-, β-Casein	Opioid agonists
Casokinins	α-, β -Casein	Antihypertensive
a-Lactorphin	α -Lactoalbumin	Opioid agonist
β-Lactorphin	β -Lactoglobulin	Opioid agonist
Lacto ferroxins	Lacto ferrin	Opioid antagonists
Immuno peptides	α-, β -Casein	Immuno stimulants
Caseinophospho peptides	α-, β -Casein	Mineral carriers

(Meisel & Schlimme, 1990)

2.5 Antihypertensive peptides

The intervention and epidemiological studies mentioned above reported the consumption of low-fat dairy products is inversely related to the risk of hypertension (Lapre, 1991; Appel et al., 1997; Medicine, 1997; Toledo et al., 2009). Hypertension is

considered to be one of the risk factors for coronary heart diseases such as myocardial infarction and stroke (Eisele et al., 2013). According to the World Health Organisation (WHO), nearly one billion people around the world suffer from hypertension (Abu-Taraboush, Al-Dagal, & Al-Royli, 1998). Hypertension can be controlled by different types of drugs; the most commonly used are synthetic angiotensin converting enzyme (ACE) inhibitory drugs such as captopril and enalapril (Chaves-López et al., 2011; Griffiths & Tellez, 2013). However, these drugs have side effects; such as hypotension, increased potassium levels, reduced renal function, cough, angioedema, skin rashes, and fatal abnormalities (Nakamura et al., 1995; Sesoko S, 1985). There are strong possibilities of substituting synthetic ACE-I drugs with ACE-I peptides to control hypertension without the associated side effects (Mavromoustakos, 2004). These results have generated further studies on fermented milk with antihypertensive properties which have been related to milk proteins. Most of the clinical trial studies suggest that increased intake of protein is associated with lower blood pressure and attenuated blood pressure over time (Burke et al., 2001). Milk is rich in potassium and calcium and increased intake has been shown to lower blood pressure (Van Mierlo et al., 2006). In fact, the observed antihypertensive effect can often be attributed to specific peptides encrypted in the parent milk protein. The effects of antihypertensive peptides in regulating blood pressure have been studied by using spontaneous hypertensive rats (SHR) (Leclerc et al., 2002; Ono et al,. 1997; Wakai et al,. 2012; Wang et al., 2012).

In the regulation of blood pressure, ACE plays an important role. ACE-I peptides can be produced through either (a) hydrolysis by digestive enzymes, (b) hydrolysis by proteolytic microorganisms or (c) hydrolysis by proteolytic enzymes. A common way of producing ACE-I peptides in the gastrointestinal tract is by hydrolysis of digestive enzymes, namely trypsin and pepsin (Tauzin, Miclo, & Gaillard, 2002). ACE-I peptides can also be produced from milk proteins; through the hydrolysis of proteolytic microorganisms during fermentation of milk. The proteolytic system of LAB, such as *Lactococcus lactic*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* var. *bulgaricus*, have been well studied and characterised (Griffiths & Tellez, 2013). *Lactobacillus helveticus* strains have demonstrated high proteolytic capability by producing antihypertensive peptides through enzymes of cell-envelope proteinase (CEP) (Ono et al., 1997;Hébert et al., 1999;Wakai & Yamamoto, 2012; Wakai et al., 2013;

Boutrou et al., 2013). Other than using live microorganisms, proteolytic enzymes isolated from LAB have been successfully employed to release ACE-I peptides from food proteins (Boutrou et al., 2013; Griffiths & Tellez, 2013). Apart from LAB, Mizuno et al., (2004) suggested that *oryzae* protease might be a suitable enzyme to generate potent ACE-I peptides with an antihypertensive property. Other recent researchers have combined LAB and proteolytic enzymes (Flavourzyme®) to accelerate the production of bioactive peptides in milk and produced 32.8 mg /g bioactive peptides compared to 5.8 mg /g bioactive peptides from LAB fermentation alone (Eisele et al., 2013). ACE inhibition leads to a decrease in the level of vasoconstricting peptide, angiotensin-II, and a corresponding increase in the level of vasodilatory peptide, bradykinin, yielding an overall reduction in blood pressure (Griffiths & Tellez, 2013; Eisele et al., 2013). Clearly, reference peptides derived from caseins, tri-peptides Il-Pro-Pro and Val-Pro-Pro, are the most extensively studied. The hydrolysis of isoelectric casein with pepsin generates peptides corresponding to α_{s1}-casein *f* (90-94) (Arg-Tyr-Leu- Gly- Arg), α_{s1}-casein *f* (143-149) (Ala- Tyr- Phe- Tyr- Pro- Glu- Leu), and α_{s1}-casein *f* (89-95) (Tyr-Gln- Lys- Phe- Pro- Gln- Tyr), showing to exert antihypertensive effect after oral administration to SHR (del Mar Contreras et al., 2009). These peptides inhibited ACE, the pivotal enzyme in blood pressure regulation by IC_{50} value of (0.7, 6.6, and 20.1 μM) respectively (del Mar Contreras et al., 2009).

2.5.1 Casein derived tri-peptides

The effect of fermented milk containing peptides such as tri-peptides (Il-Pro-Pro and Val-Pro-Pro,) on blood pressure has been investigated, both in short and long term experimental studies. Different models of SHR and double transgenic rats harbouring human renin and angiotensinogen genes have been used (Lahtinen et al., 2011). The antihypertensive effect was first demonstrated by casein hydrolysate generated by purified proteinase from *L. helveticus* CP790, which are presented in (Table 2.7) (Yamamoto et al., 1994). Additionally, many *in-vitro* and *in-vivo* studies have been achieved to obtain more insight into the mechanisms of bioavailability of casein-derived tri-peptides. A single dose oral administration of casein hydrolysate or *L. helveticus* CP790 fermented milk led to decreased SBP of SHR by 21 or 35 mm Hg after 8 h of administration (Nakamura et al., 1995). Thereafter, ACE-I activity was found to be produced in sour milk during fermentation with *L. helveticus* and *S. cerevisiae*

(Nakamura et al., 1995). The fermented milk decreased SBP of SHR by 22 mm Hg after 6 h of oral administration (Nakamura et al., 1995). Tri-peptide, leucine-proline-proline (Leu-Pro-Pro) has been shown to inhibit ACE (Lehtinen et al., 2010). The amino acid sequences corresponding to Ile-Pro-Pro, Val-Pro-Pro and Leu- Pro- Pro were found in the primary structure of bovine β-casein (74-76 Ile-Pro-Pro.84-86 Val-Pro-Pro, 161-163 Leu-Pro- Pro) and k-casein (108-110 Ile- Pro- Pro) (Farrell et al., 2004). Long term studies have been mostly performed using young animals with normal blood pressure (Table 2.8). The development of hypertension has decreased significantly in rats receiving either pure Ile- Pro- Pro and Val- Pro- Pro in water or milk products fermented with *L. helveticus* (and / or *S. cerevisiae*) (Roy et al., 1999; Lehtinen et al., 2010; Domingues et al., 2010) (Tables 2.7 and 2.8).

Table 2.7 Milk-protein released peptides displaying hypertensive effects in SHR.

Milk protein	**Peptide fraction**	**Maximum decrease in systolic BP (mm Hg)**	**Reference**
αs_1 -Casein	f(1-9)	-9.3	Saito et al. (2000)
	f(23-24)	-34.0	Karaki et al. (1990)
	f(90-94)	-25.0	del Mar Contreras et
	f(104-	-13.0	al.(2009)
	109)	-20	Maeno et al. (1996)
	f(143-	-32.1	del Mar Contreras et
	149)	-14.0	al.(2009)
	f(146-		Yamamoto et al. (1999)
	147)		Karaki et al. (1990)
	f(194-		
	199)		
αs_2 -Casein	f(89-95)	-15.0	del Mar Contreras et
	f(189-	-5.0	al.(2009)
	192)	-3.0	Maeno et al. (1996)
	f(190-	-9.0	Maeno et al. (1996)

	197)		Maeno et al. (1996)
	f(198-202)		
β-Casein	f(59-61)	-21.0	Abubakar et al. (1998)
	f(59-64)	-22.0	Abubakar et al. (1998)
	f(60-68)	-7.0	Saito et al. (2000)
	f(74-76)	-28.3	Nakamura et al. (1995a)
	f(80-90)	-8.0	Abubakar et al. (1998)
	f(84-86)	-32.1	Nakamura et al. (1995a)
	f(140-143)	-2.0	Maeno et al. (1996)
		-32.2	Maeno et al. (1996)
	f(169-174)	-31.5	Maeno et al. (1996)
		-10.0	Karaki et al. (1990)
	f(169-175)		
	f(177-183)		
α-Lactalbumin	f(50-53)	-23.0	Mullally et al. (1996); Nurminen et al. (2000)
β-Lactoglobulin	f(58-61)	-20.0	Hernández-Ledesma et al.(2007)
	f(78-80)	-31.0	
	f(103-105)	-20.0	Abubakar et al. (1998) Hernández-Ledesma et al. (2007)
Bovine serum albumin	f(221-222)	-27.0	Abubakar et al. (1998)
β_2 -microglobulin	f(18-20)	-26.0	Abubakar et al., (1998)

Table 2.8 Experimental studies on the effects of tri-peptides Ile- Pro- Pro (IPP) and Val- Pro- Pro (VPP) on blood pressure.

Reference	Duration	Study Characteristics	Dose	Systolic Blood Pressure
Acute Experiments				
Yamamoto et al,.1994		Casein hydrolysate	15 mg / kg peptides	-22 mm Hg after 6 h
		L. helveticus CP790 fermented milk	15 mg / kg peptides	-35 mm Hg after 8 h
Nakamura et al,.1995		*L. helveticus* and *S. Cerevisae* fermented milk	0.3 mg / kg IPP,0.6 mg / kg VPP	-22 mm Hg after 6 h
Long Term Experiments				
Nakamura et al,.1996	16 wk	Diet containing 2.5 % lyophilized sour milk	Not specified	-19 mm Hg vs. control diet
Sipola et al,.2001	12wk	IPP and VPP in water	2.5-3.5 mg/ kg/d IPP+VPP	**-12** mm Hg vs. control
		L. helveticus fermented milk	2.5-3.5 mg/ kg/d IPP+VPP	**-17** mm Hg vs. control
Sipola et al,.2002	14wk	*L. helveticus* fermented milk	0.4 mg/ kg/ d IPP.0.6 mg / kg/ d VPP	**-21** mm Hg vs. control
		L. helveticus and *S. Cerevisae* fermented milk	0.2 mg/kg/ d IPP,0.3 mg/ kg/ d VPP	**-10** mm Hg vs. control
Jauhiainen et al,.2005	9wk	IPP and VPP in water	2.0 mg/kg/d IPP +VPP	**-8** mm Hg vs. control
		IPP ,VPP and minerals in water	1.7 mg/ kg /d IPP+VPP	**-13** mm Hg vs. control
		L. helveticus fermented milk	1.5 mg/ kg/ d IPP +VPP	**-17** mm Hg vs. control
	8wk	*L. helveticus* fermented milk	3.0-4.4 mg/ kg/ d IPP + VPP	-14 mm Hg vs. control

Jakala et al,.2009		Milk product produced by *L. helveticus* and proline-specific endo-protease	2.9-4.0 mg/ kg/ d IPP + VPP	-12 mm Hg vs. control
		Milk product produced by *L. helveticus* and proline-specific endo-protease and containing plant sterols	2.8-4.0 mg/ kg/ d IPP + VPP	**-7** mm Hg vs. control
Jakala et al,.2009	8wk	*L. helveticus* fermented milk	5.9-6.6 mg/ kg/ d IPP + VPP	-11 mm Hg vs. control
	(GK)	Milk product produced by *L. helveticus* and proline-specific endo-protease	4.6-5.1 mg/ kg/ d IPP + VPP	**-**12 mm Hg vs. control
		Milk product produced by *L. helveticus* and proline-specific endo-protease and containing plant sterols	4.8-5.3 mg/ kg/ d IPP + VPP	-10 mm Hg vs. control
Jakala et al,.2010	8wk	Tri-peptide powder in water (*L. helveticus* fermentation)	3.1-4.3 mg/ kg/ d IPP + VPP	-14 mm Hg vs. control
		Tri-peptide powder in water (*L. helveticus* and proline-specific endo-protease)	3.2-4.4 mg/ kg/ d IPP + VPP	-14 mm Hg vs. control
Jauhiainen et al.2010	3wk	IPP and VPP in water	10.9 mg/ kg/ d IPP + VPP	-3 mm Hg vs. control
	(dTGR)	*L. helveticus* fermented milk	5.4 mg/ kg/ d IPP + VPP	**-**19 mm Hg vs. control

Ehlers et al,.2011	6wk	Milk product produced by *L. helveticus* and proline-specific endo-protease and containing plant sterols	3.7-4.4 mg/ kg/ d IPP + VPP	-16 mm Hg vs. control

2.5.2 The mechanisms of peptide activity with antihypertensive properties

High blood pressure is regulated by renin-angiotensin and bradykinin (Meisel, 1998; Nagpal et al., 2011) (Figure 2.6). *In vitro*, several peptides of different lengths have been shown to inhibit ACE at micromolar concentrations (Lehtinen et al., 2010). Inhibition of ACE activity reduces angiotensin -II production and lowers blood pressure in hypertensive patients. The Bradykinin system involves ACE preventing degradation of the vasodilator, thus helping to control blood pressure. The two regulators of blood pressure; angiotensin and bradykinin are shown in (Figure 2.6). A study reported that hydrophobic tryptophan, phenyl alanine, proline and tyrosine were found to be most effective in lowering blood pressure (Meisel, 1998). ACE inhibitory peptides such as tri-peptides, Ile-pro-pro and Val-pro-pro from bovine casein were isolated from fermented milk by *Saccharomyces cerevisiae* and *L. helveticus* (Nakamura et al., 1995). Contreras et al. (2009) reported that tri-peptides released from fermented milk were found to reduce blood pressure. Similarly, Jauhiainen et al. (2010) observed that milk products containing bioactive tri-peptides had an antihypertensive effect in double transgenic rats. Several studies using SHR to determine hypertensive effect of fermented milk derived ACE-inhibitors have achieved promising results (Yamamoto et al.,1994; Yasunori Nakamura et al., 1995; Jauhiainen et al., 2010; Ehlers et al., 2011). Table 2.8 summarises the reduction in systolic BP reached in SHR using bioactive peptides hydrolysed from milk proteins. In clinical studies, different methods to evaluate endothelial function have been used. Ambulatory arterial stiffness index (AASI) can be calculated from 24-hour blood pressure recordings, and this has been shown to be an independent predictor of cardiovascular mortality (Dolan et al., 2006). Interestingly, a significant improvement in AASI was observed following a 10-week treatment with *L. helveticus* fermented milk (Jauhiainen et al., 2007).

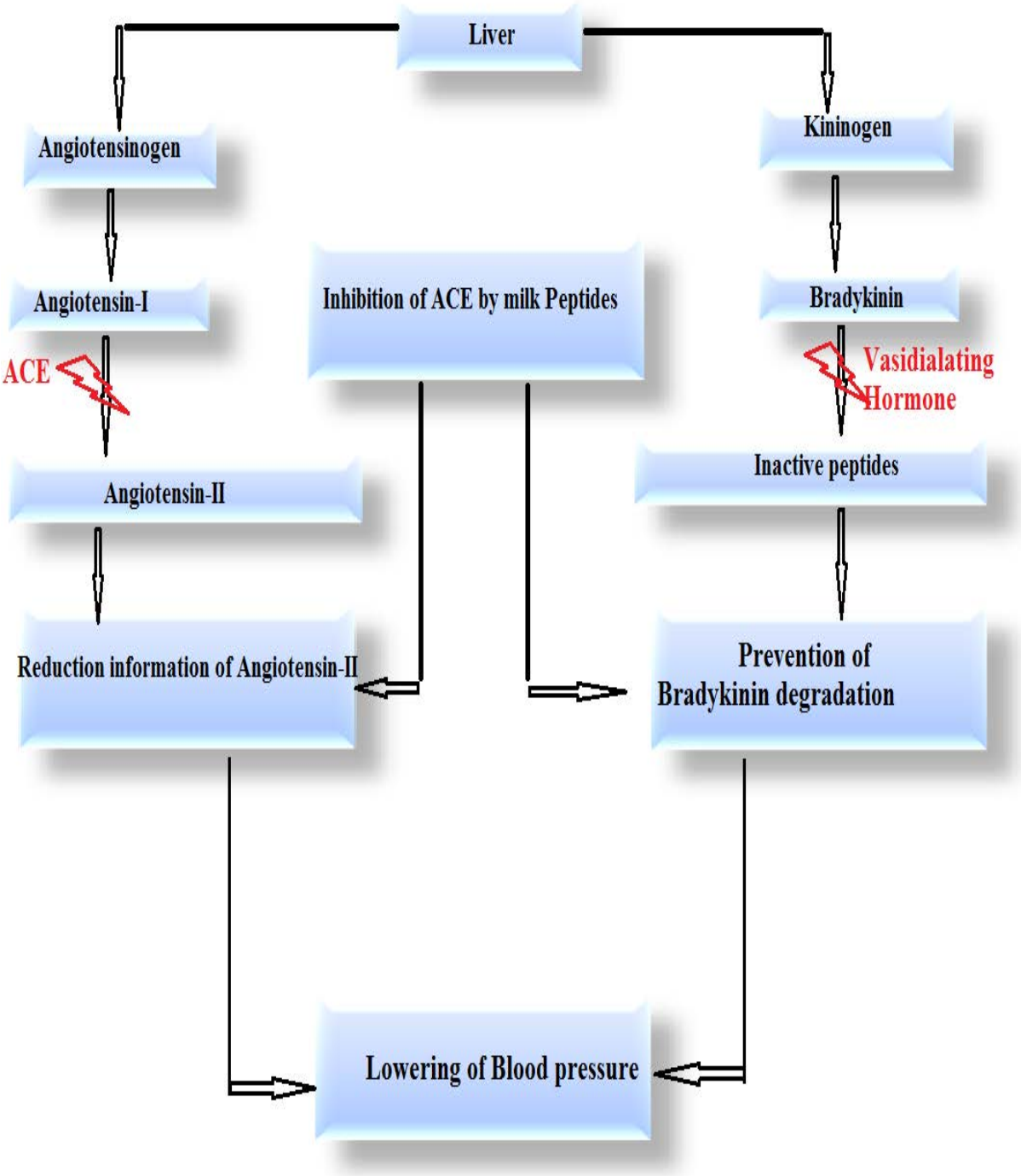


Figure 2.6 Regulation of blood pressure by angiotensin and bradykinin (Nagpal et al., 2011).

2.5.3 Angiotensin converting enzyme inhibitory peptides

Angiotensin-I converting enzyme (ACE-I) has been related to the renin-angiotensin system, which regulates peripheral blood pressure. Some peptides display antihypertensive activity. ACE-I activities were isolated from enzymatic hydrolysate of

bovine casein and their amino acid sequences are as follow: Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (CEI12), Phe-Phe-Val-Ala-Pro (CEI5), and Ala-Val-Pro-Tyr-Pro-Gln-Arg (CEIβ7) (Maruyama, et al., 1985). CEI5 is a penta-peptide derived from the hydrolysate of CEI12 with proline-specific endo-peptidase, and CEI β7 is a hepta-peptide derived from β-casein. These inhibitors potentiate bradykinin in the contraction of the uterus and the ileum of rats. The ileum was more sensitive to these inhibitors than the uterus (Maruyama, et al., 1985). ACE hydrolyses inactive angiotensin-I into the octa-peptide angiotensin-II (vasoconstrictor), which leads to increased blood pressure. ACE-I is also able to hydrolyse bradykinin (vasodilator) which is hypotensive. Peptides able to exhibit ACE-I activity are used as an antihypertensive drug (FitzGerald & Meisel, 2003; Tauzin et al., 2002). The main mechanical feature controlling this inhibitory reaction is the C-terminal tri-peptide sequence. It is proposed that these peptides may interact with subsides S_1, S_1' and S_2' at the active site of ACE (Brew, 2003) (Figure 2.7). It appears that ACE prefers substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions (Cheung et al., 1980). Generally, aliphatic, basic and aromatic residues are preferred in the penultimate positions while aromatic; proline and aliphatic residues are preferred in the ultimate positions. The positive charge of the β-amino group of Lys at the C-terminus has also been shown to contribute to the ACE-I potential of several peptides (Vermeirssen et al., 2003). Furthermore, structural studies [Nuclear magnetic resonance (NMR), molecular modelling], docking studies, design of mimetics and biological evaluation of angiotensin AT(1) receptor blockers have aided in new anti-hypertensive peptide inhibitors (Panagiotopoulos et al., 1996;Mavromoustakos et al., 2001; Hiroyuki et al., 2013).

ACE-inhibitory and antihypertensive peptides originating from milk usually contain up to ten amino acids (Yamamoto et al., 1993). The majority of milk-protein-derived ACE-inhibitors have moderate inhibitory potencies, usually within an IC_{50} range of 100-500 μ m /L, (Hayes et al., 2007). Thus, strain selection is one of the main factors that influence the release of ACE-inhibitors in dairy fermentations (Korhonen & Pihlanto, 2003; Takano, 2002). However, peptides with ACE-inhibitory activity may also be formed by *in vitro* hydrolysis of milk proteins using microbial and digestive enzymes (Otte et al., 2007; Paola Ortiz-Chaoa, 2009). It has been reported that fermented milk produced by mixing several types of microbes might contain a wider variety of functional substances

than milk cultured with a single strain (Kuwabara et al., 1995). Inclusion of probiotics to yoghurt has been shown to enhance *in vitro* ACE-inhibitory activity due to improved proteolytic hydrolyses (Donkor et al., 2007). The pH at the end of fermentation influences the ACE-inhibitory activity of fermented milk, which varies with the strain of LAB used (Nielsen et al., 2009). Additionally, LAB possesses a 'transport system' for amino acids, and di- tri- and oligo-peptides. As a result of this system, residual levels of peptides with bioactivity increases in fermented milks. The proteolytic systems of LAB species such as *L. casei, L. helveticus, L. delbrueckii* ssp. *bulgaricus, S. thermophilus, L. acidophilus* have been used to produce functional milk products containing antihypertensive peptides with ACE-inhibitory properties.

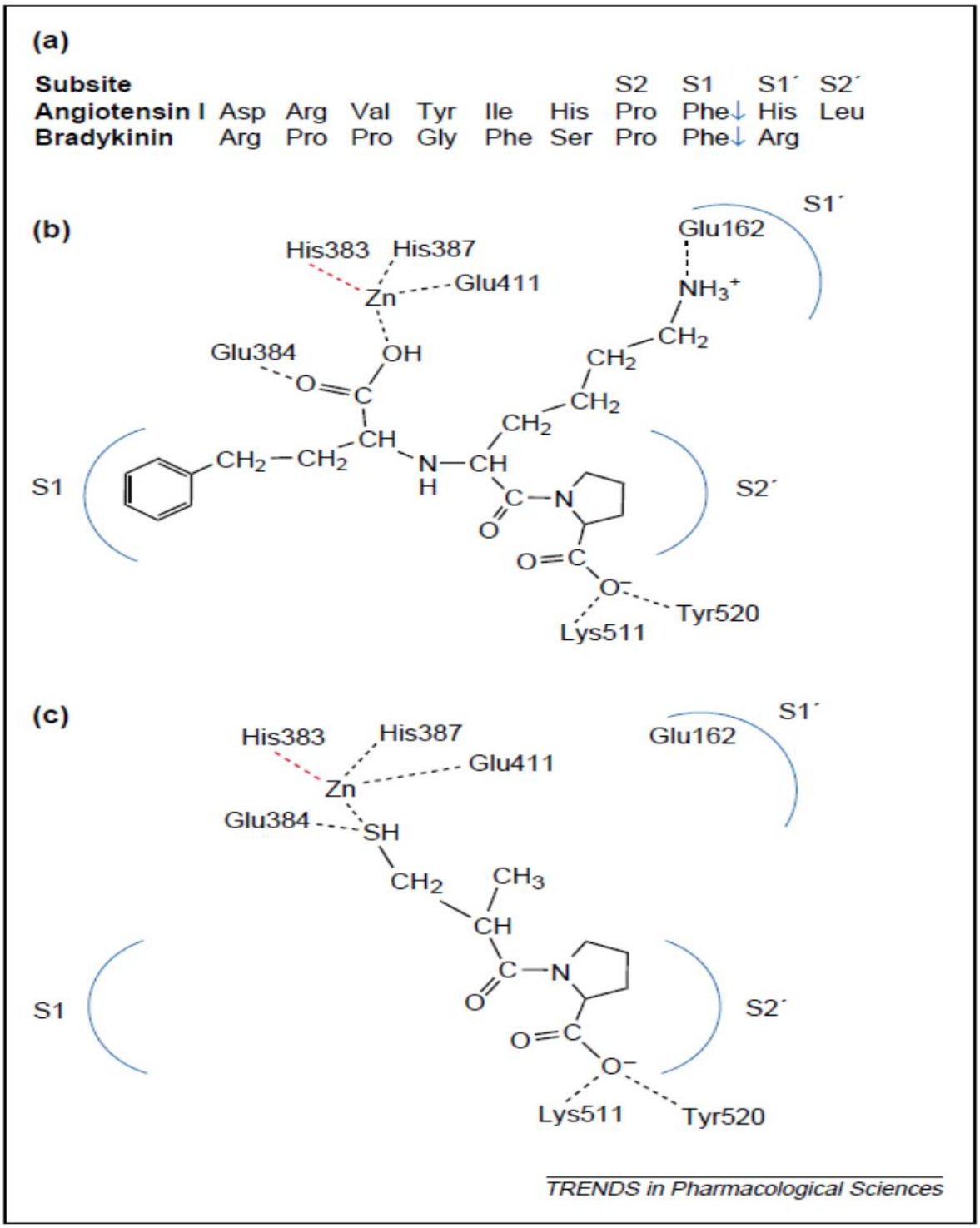


Figure 2.7 Molecular recognition of substrates by testis-specific angiotensin-converting enzyme (ACE) (a) the sites of cleavage by ACE in the substrates angiotensin I and bradykinin are shown. ACE is a di-peptidyl carboxy-peptidase and catalyses the cleavage of the bond between substrate residues that occupy the S1 and S10 sites (Brew, 2003).

2.6 Sensory evaluation of food products

Sensory evaluation is defined as a scientific judgment of food quality using senses, such as smell, taste, touch and sight. These sensory attributes have been developed by existing techniques. The developed procedures promote economic interest and establish the worth or acceptance of a product. Sensory evaluation is divided into two categories, namely: objective and subjective testing. Objective testing employs the use of laboratory instruments with no involvement of the senses, whereas subjective testing involves panellists. Both tests are essential in sensory evaluation (Meilgaard et al., 2006).

After food production and before reaching the marketplaces, new food products have to go through many tests to accurately judge how well people will accept them (Lawless, 2010). The companies have to evaluate the new product by particular food gastronomy and have to respond to some potential questions including: (i) Will people like the product? (ii) Will they buy the product? What price? (iii) How can the product be successfully marketed to people? And (iv) Will they prefer the product over others? Useful information can be obtained by posing specific questions to panels about age, sex, religion, geographic nationality, location, employment and education (Bopp, 1997). Food sensory evaluation has been used as a useful tool for new product development by assisting in product matching and improvement. Evaluation of a product could be required to determine the effects an experiment has on it. Lastly, marketing and quality control are additional application of sensory testing (Stone et al., 2012).

2.6.1 Sensory attributes

Attributes of food items are typically perceived in the following order:

- Appearance
- Odour/aroma/fragrance
- Consistency and texture
- Flavour (aromatics, chemical feeling, taste)

➢ Appearance

The appearance of the product and/ or the package is often the only attribute that is used to base a decision on to purchase or consume a product. Sensory analyses must pay

meticulous attention to every aspect of the appearance of test samples (Meilgaard et al., 2006) and must often attempt to obliterate or mask many unattractive test samples with coloured lights, opaque containers, etc.

➢ Odour/aroma/fragrance

The odour of a product is detected when its aroma volatiles enter the nasal passage and they are detected by the olfactory system. Odour is discussed when the volatiles are sniffed through the nose. Aroma is the odour of a food product and fragrance is the odour of a perfume or cosmetic, while aromatics are the volatiles detected by the olfactory system from a substance in the mouth (Meilgaard et al., 2006).

➢ Consistency/texture

The third set of attributes to be considered are those perceived by sensors in the mouth other than taste and chemical feeling. Texture is also perceived by the skin and muscles of the body, other than those in the mouth when evaluating personal care and home care products (Meilgaard et al., 2006).

➢ Flavour

Flavour, as an attribute of food, beverages and seasonings, has been defined as the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts (Amerine et al., 2013). However, for the purposes of practical sensory analyses, the term is restricted to the impressions perceived via chemical senses from a product in the mouth. This is defined in this manner as flavour and includes (Meilgaard et al., 2006):

- The aromatics, i.e. olfactory perception caused by volatile substances released from a product in the mouth via the posterior nares
- The tastes, i.e. gustatory perceptions (salty, sweet, sour, bitter) caused by soluble substances in the mouth
- The chemical feeling factors that stimulate nerve ends in the soft membranes of the buccal and nasal cavities (astringency, spice heat, cooling, bite, metallic flavour).

2.6.2 Hedonic scale

The subjective assessment of a food product uses the hedonic scale method (Figure 2.8). It measures the level of the liking of a product. This test relies on consumers' ability to rate their feelings of like or dislike. Hedonic testing is commonly used with experienced panel members as well as untrained panel members (Poste, 1991). When using hedonic scale testing, food samples are offered in succession and the subject is told to elect how much the panellist 'likes' or 'dislikes' the product and to mark the scales accordingly. The hedonic scale is anchored verbally with nine different categories ranging from 'like extremely' to 'dislike extremely' (Figure 2.8). Several different methods of the scale have been used with success; however, the differences in the scale form is likely to cause marked changes in the distribution of reactions and statistical factors such as variances and means (ASTM, 1968). The test attitudes and characteristics of the subjects and expectation have reflective effects on the results. Consequently, the investigator must be cautious about making implications on the basis of evaluation of average ratings obtained in different experiments (Stone et al., 2012). There is no question that for some products a subset of the population of consumers may alter the ordering; however, the usefulness of the benchmark is not lost. This degree of stability is especially important for companies that seek to develop a database for their own products as well as to have a means for rapid assessment of formulation changes. In addition to these scaling techniques, there is another scale such as, semantic differential, appropriateness measures and summative scales. These scales are used primarily by market research to measure consumer behaviour as it impacts on product image, social issues and attitudes. They impact on sensory evaluation when results are used to direct product formulation efforts or when results are compared with those from a sensory test (Stone et al., 2012).

Name----------

Product code----------

Date----------

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

____Like extremely

____Like very much

____Like moderately

____Like slightly

____Neither like or dislike

____Dislike slightly

____Dislike moderately

____Dislike very much

____Dislike extremely

Comments:

Figure 2.8 An example of nine point hedonic scale; Like extremely-9; Dislike extremely-1.

2.6.3 The other sensory experiments

There are different sensory experiments designated by the following definitions besides the hedonic scale used in sensory evaluation of food products:

Preference or acceptance tests: Determine representative population preferences and require numerous panellists.

Duo-Trip test: In this test, three samples are offered to the taster. One is labelled "R"(reference) and the other two are coded. One coded sample is identical with "R" and the other is different.

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

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

Paired comparison test: In the paired comparison test there are two coded samples that represent the standard or control. Experimental treatments are presented to the panellist who is asked to indicate which sample has the greater or lesser degree of intensity of a specified characteristic, such as sweetness and hardness. If more than two treatments are being considered, each treatment is compared with every other in the series.

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

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

Scoring: Coded samples are evaluated by the panellist who records his/her reactions on a descriptive graduated scale. These scores are given numerical values by the panellist who analyses the results.

Flavour-profile method: It consists of a small laboratory panel of six or eight testers trained in the method of measuring the flavour profile of food products. Descriptive words and numbers, with identifiable meaning to each panel member, are used to express the comparative strength of each note on the scale.

Dilution tests: Dilution tests include the determination of the identification threshold for the material under study (Poste, 1991; Stone et al., 2012).

Chapter 3 Proteolytic and angiotensin converting enzyme inhibitory activities of selected probiotic bacteria

This chapter has been accepted for publication. Fatah Ahtesh, Lily Stojanovska, Michael Mathia, Vasso Apostolopoulos and Vijay Mishra (2015). Proteolytic and angiotensin converting enzyme inhibitory activities of selected probiotic bacteria. *International journal of Food Science and Technology*: IJFST-2015-19010. In print (Appendex. I)

3.1 Introduction

The most extensively studied microorganisms are lactic acid bacteria (LAB), *Streptococcus*, *Lactococcus, Lactobacillus*, and *Bifidobacterium* (Castro et al., 1996; Christensen et al., 1999; Ziadi et al., 2010). LAB, which includes probiotic organisms, are fastidious in nature, demanding several essential growth factors (Donkor et al., 2007). The proteolytic systems of LAB have been studied widely and the enzymes involved have been isolated and characterised (Shihata & Shah, 2000). However, *Bifidobacterium* strains are not as proteolytic as other LAB, which explains why *Bifidobacterium* grow slowly in milk and may require supplementation from external sources (Dave & Shah, 1998; Gomes et al., 1998). Milk products, such as skim milk, although they are rich growth media, contain low concentrations of free amino acids and peptides to efficiently support growth of LAB (Shihata & Shah, 2000). Therefore, through proteolytic activity of LAB, bioactive peptides and amino acids are released from parent proteins in milk to support growth (Gobbetti et al., 2000).

There are two methods of releasing milk peptides namely, by milk fermentation with LAB and by enzymatic hydrolysis of proteins. The cell wall of LAB is able to hydrolyse caseins into peptides by extracellular proteinases and intracellular peptidases (Korhonen & Pihlanto, 2006; Otte et al., 2007). Some of these peptides are classified as having angiotensin converting enzyme inhibition (ACE-I) activity (Yamamoto et al., 1994). Angiotensin-I-converting enzyme (ACE) plays a role in the regulation of blood pressure by catalysing the production of vasoconstrictor Angiotensin-II and inactivating the vasodilator and bradykinin (Doolittle, 1983; Brown and Vaughan, 1998). Milk products with ACE-I peptides are fermented between 6-48 h, at optimal temperatures for the strains used, reaching pH 4 - 5 in most products (Salminen & von Wright, 2004; Muguerza et al., 2006). Some ACE-I peptides may be intermediate products of hydrolysis which, upon further fermentation, are degraded into inactive peptides. Other ACE-I peptides may stop or end the protein hydrolysis, e.g., many di- and tri-peptides, which would be formed upon longer fermentation (He et al., 2013; Boutrou et al., 2013). Furthermore, the effect of pH on ACE-I activity of fermented milk increases with reducing pH until pH 3.5 is reached (Nakamura et al., 1995; Nakamura et al., 1995; Donkor et al., 2006). These peptides generally exist as an inactive form in milk proteins and, following enzymatic hydrolysis, active peptides are released. Furthermore, enzymatic hydrolysates possess a number of physiological properties such as

antioxidant and ACE-I activity (Rajapakse et al., 2005; Unal & Akalin, 2012). Protease such as Flavourzyme®, also known as a proteolytic enzyme aids in digestion of different kinds of proteins (Chen et al., 2007). It is able to break down bonds by a process of protein hydrolysis converting it into smaller chains of amino acids (Roy et al., 2000; Korhonen & Pihlanto, 2003). Proteins have a complex folded structure requiring these types of enzymes to disassemble the molecule in very specific ways (Tsai et al., 2008; Ahn et al., 2012).

Since the activity of ACE-I and antihypertensive peptides could be affected by the method of production, there is a need to evaluate the efficiency of proteases in releasing ACE-I peptides from milk proteins. Therefore, probiotic strains *Lactobacillus casei* (Lc210), *Bifidobacterium animalis spp12* (Bb12), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb11842), *and Lactobacillus acidophilus* (La2410) were screened separately, or in combination with the commercial proteolytic enzyme protease (Flavourzyme®) for production of bioactive peptides with ACE-I activity in short fermentation time using two different media in 12 % reconstituted skim milk (RSM) or 4 % whey protein concentrates (WPC) by measuring the bacterial growth, proteolysis and ACE-I activity.

3.2 Material and Methods

3.2.1 Substrates and chemicals

A number of substrates and chemicals namely O-phthaldialdehyde (OPA), Hippuryl–histidyl–leucine (HHL), trichloroacetic acid (TCA), bacteriological medium, bacteriological agar, trifluoroacetic acid (TFA), HCl, ACE enzyme were purchased from Sigma Chemical Company (Sigma Aldrich, NSW Australia). De Man Rogosa and Sharpe (MRS) were purchased from Oxoid, Ltd., VIC Australia. Flavourzyme® 1000 L (Protease enzymes .EC 3.4.11.1), an amino peptidase with an activity of [1000 Leucine Amino-peptidase unit (LAPU g^{-1})] was purchased from Novozymes Australia, NSW Australia. Skim milk powder (SM) was purchased from Murray Goulburn Co-operative Co. Ltd, VIC Australia and whey protein concentrate (WPC-35) powder was purchased from United Milk Tasmania Ltd., TAS Australia. Acetonitrile and reinforced clostridia agar (RCA) were purchased from Merck, Darmstadt, Germany and bacteriological peptone from Oxoid, Ltd., VIC Australia. Peptone solution was obtained from Merck

Pty. Ltd., VIC Australia. Stuart colony was from Scientific Counter UK. Anaerobic kit was obtained from (Oxoid™, AnaeroGen Australia). Advantech #231 filter paper was from Advantech Australia, NSW Australia. Four strains of *Lactobacillus casei* (Lc210), *Bifidobacterium animalis* ssp12 (Bb12), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb11842), and *Lactobacillus acidophilus* (La2410) were obtained from Dairy Innovation Australia Ltd, Werribee, VIC Australia. UV-VIS spectrophotometer was from LKB NOVASPEC II Pharmacia, LKB Bio- Chrom UK. Centrifuge was from Beckman Coulter, Avanti J-265xPI. Freeze-dried was purchased from Freeze-drier model FD-300; Air vac Engineering Pty. Ltd., VIC Australia. Column (C18) was purchased from Phenomenex, NSW Australia. Membrane filter was purchased from Schleicher & Schuell GmbH, Dassel Germany. Reversed phase (RP) - HPLC was from Varian Analytical Instruments, CA USA.

3.2.2 Experimental design, media preparation and fermentation

Table 3.1 shows 16 different fermented samples of RSM and WPC and Flavourzyme® as control media were prepared using 12 % RSM, skim milk powder (SMP) composition are (52 % lactose, 37 % protein, 8.6 % ash and 1.2 % fat) or 4 % WPC powder (47.5 % lactose, 35 % protein, 9 % ash, 2.5 % fat) separately. All media powder (SM or WPC) were reconstituted using sterilized water. Reconstituted media were heat treated in water path (20-30) min at 90°C. This optimal temperature and time for sterilization to avoid milk protein denaturation, cooled to 40°C and each medium was inoculated with 1 % v/v of *Lactobacillus casei* (Lc210), *Bifidobacterium animalis ssp12* (Bb12), *Lactobacillus delbrueckii subsp. bulgaricus* (Lb11842), and *Lactobacillus acidophilus* (La2410) separately and in combination with or without 0.14 % (w/w) Flavourzyme®. Fermentation was carried out for 12 h at 37°C and samples were collected at 0, 4, 8 and 12 h and stored at -20°C for further analysis.

Table 3.1 Experimental design and the coding used in the study to analyse and measure the pH, growth, proteolytic activity and % of ACE-inhibitory activities during (0, 4, 8 and 12 h) fermentation of probiotic strains in12 % RSM or 4 % WPC and with or without combination of Flavourzyme®.

Media used	**Code**	**Cultures used without combination**	**Code**	**Combination of cultures with Flavourzyme® (1 % v/v each)**
RSM	L.b	*L.bulgaricus*	L.b	*L.bulgaricus* + Flavourzyme®
	L.A	*L. acidophilus*	L.A	*L. acidophilus*+ Flavourzyme®
	L.C	*L. casei*	L.C	*L. casei* + Flavourzyme®
	Bb	*L. Bifidobacterium*	Bb	*L. Bifidobacterium* +Flavourzyme®
	control			Flavourzyme®
WPC	L.b	*L.bulgaricus*	L.b	*L.bulgaricus* + Flavourzyme®
	L.A	*L. acidophilus*	L.A	*L. acidophilus*+ Flavourzyme®
	L.C	*L. casei*	L.C	*L. casei* + Flavourzyme®
	Bb	*L. Bifidobacterium*	Bb	*L. Bifidobacterium* +Flavourzyme®
	control			Flavourzyme®

3.2.3 Bacterial Counts

Four probiotic strains, Lc210, Bb12, Lb11842 and La2410, separately or combined with Flavourzyme® were added to 12 % RSM or 4 % WPC media. Bacterial growth was measured by pour-plate method. Appropriate serial dilutions were made using 0.1 % peptone solution and the strains were incubated at 37°C for 48 h using anaerobic jars with anaerobic kit. The colony enumeration system used was the Stuart colony. The growth of LAB strains was examined every 4 h up to 12 h during the fermentation process at 37°C. Plates showing 25 to 250 colonies were counted and expressed as colony forming units per mL (cfu mL^{-1}) of sample.

3.2.4 Preparation of crude water-soluble peptide extract

The crude water-soluble peptide extract was prepared from 250 mL fermented sample by centrifugation (J2-HS rotor, Beckman Instruments Inc., Palo Alto, CA USA) at 15,000 x g for 30 min at 4°C. The supernatant was filtered through a 0.45 µm membrane filter (Schleicher & Schuell GmbH, Dassel Germany) and freeze-dried using Dynavac freeze drier (Dynavac Eng. Pty. Ltd., Melbourne Australia). The freeze-dried samples were stored at -80°C for further analysis.

3.2.5 Determination of proteolytic activity

Proteolytic activity of Lc210, Bb12, Lb11842 and La 2410 was determined using the O-pathalaldehyde (OPA) method as previously described (Church et al., 1983). Briefly, 3 mL of sample was mixed with equal volume of 1 % trichloroacetic acid followed by filtration using Advantech #231 filter paper. Filtrate 150 µL was mixed with 3 mL of OPA reagent and allowed to react at room temperature for 2 min. The OPA reagent was prepared by adding 25 mL of 100 mM di-sodium tetra-borate, 2.5 mL of 20 % (w / w) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL methanol and 100 µl of β-mercaptoethanol in 50 mL total volume of the reagent. Absorbance of the samples was measured at 340 nm using UV-VIS spectrophotometer (LKB NOVASPEC II Pharmacia, LKB Bio- Chrom UK). The relative absorbance between the control and sample was used as an indication of proteolysis.

3.2.6 Determination of ACE inhibitor activity

ACE-I activity was determined according to the previously described method (Donkor et al., 2007). Briefly, 10 mL of fermented media WPC or RSM was centrifuged at 4000 x g at 4°C for 30 min and the supernatant was freeze-dried for 72 h (Freeze-drier model FD-300; Air vac Engineering Pty. Ltd., VIC Australia). The freeze-dried powder (40 mg) was dissolved in 2 mL Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride. ACE enzyme and Hippuryl-L-histidyl-L-leucine (HHL) (Sigma, St. Louis, MO USA) were prepared in Tris buffer. Fifty µL of 3.0 mM HHL, 50 µL of 1.25 MU ACE enzyme (from rabbit lung), and 50 µL of experimental samples were placed in a glass tube and incubated for 1 h at 37°C ensuring mixing for the first 30 min. Glacial acetic acid (150 µL) was added to stop the reaction. The reaction mixture was stored at -20°C before further analysis of released hippuric acid (HA) by HPLC. An external

standard curve of hippuric acid was prepared to quantify the resultant hippuric acid in fermented samples. An aliquot (20 µL) of the mixture was injected into Gemini® C18 110 Å (100 mm x 4.6 mm, 5 µm) (Phenomenex, Pty Ltd., NSW Australia) using Varian HPLC equipped with an auto sampler. The separation was conducted at room temperature (~22°C) at a mobile phase flow rate of 0.6 mL min^{-1}. The mobile phase consisting of 12.5 % (v/v) acetonitrile (Merck Pty. Ltd., VIC Australia) in MilliQ-water, and pH was adjusted to 3.0 using glacial acetic acid. Ultraviolet-visible detector was set at 228 nm. The % ACE-I was calculated as follows:

$$ACEI\ \% = \frac{HA\ (control) - HA\ (sample)}{HA\ (control)} \times 100$$

Where ACE-I = angiotensin converting enzyme inhibition and HA = Hippuric acid.

3.2.7 RP-HPLC analysis of water-soluble peptides extract

The water soluble peptides of fermented RSM samples were profiled by a reversed phase (RP) - HPLC using the method as previously described (Donkor et al. 2007) with some modifications. Briefly, 50 mL of dissolved freeze-dried samples of fermented RSM or WPC for 12 h (with or without supplementation of Flavourzyme®) were centrifuged at 15,000 x g for 30 min at 4°C (Beckman Instruments Inc. Palo Alto, CA USA) respectively. The supernatant was filtered through a 0.45 µm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) and 20 µL of the sample was injected into a C_{-18} monomeric column (5 µm, 300 Å, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA). The peptides were eluted by a linear gradient from 100 – 0 % solvent A (0.1 % TFA in deionised water) and solvent B (0.1 % TFA in 90 % acetonitrile/water v/v) over 70 min. Peaks were detected using a Varian UV/vis detector set at 214 nm. Separations were carried out at room temperature (~22°C) at a flow rate of 1.0 mL min^{1}.

3.2.8 Statistical analysis

All results are expressed as mean values of 3 replicates with standard deviation. One way ANOVA was performed to investigate the differences in the treatments (bacterial strains, growth media, presence or absence of Flavourzyme® and fermentation time). Fisher`s (least significant difference; LSD) test was used to investigate significant

differences among the means. All statistical analyses were carried out using SAS Version 9.0 software (SAS Institute Inc., Cary, NC, USA).

3.3 Results and Discussion

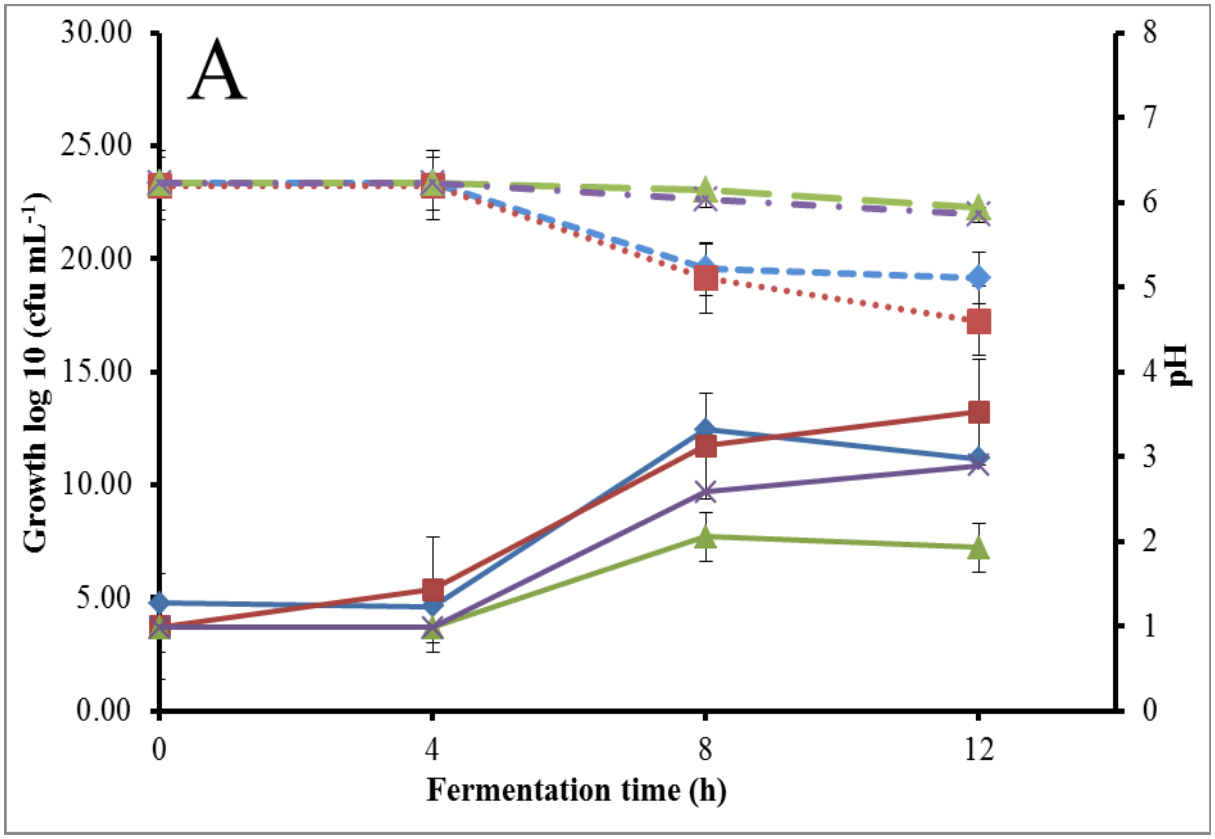
3.3.1 Preferential growth of selected LAB strains in RSM media with Flavourzyme® compared to WPC

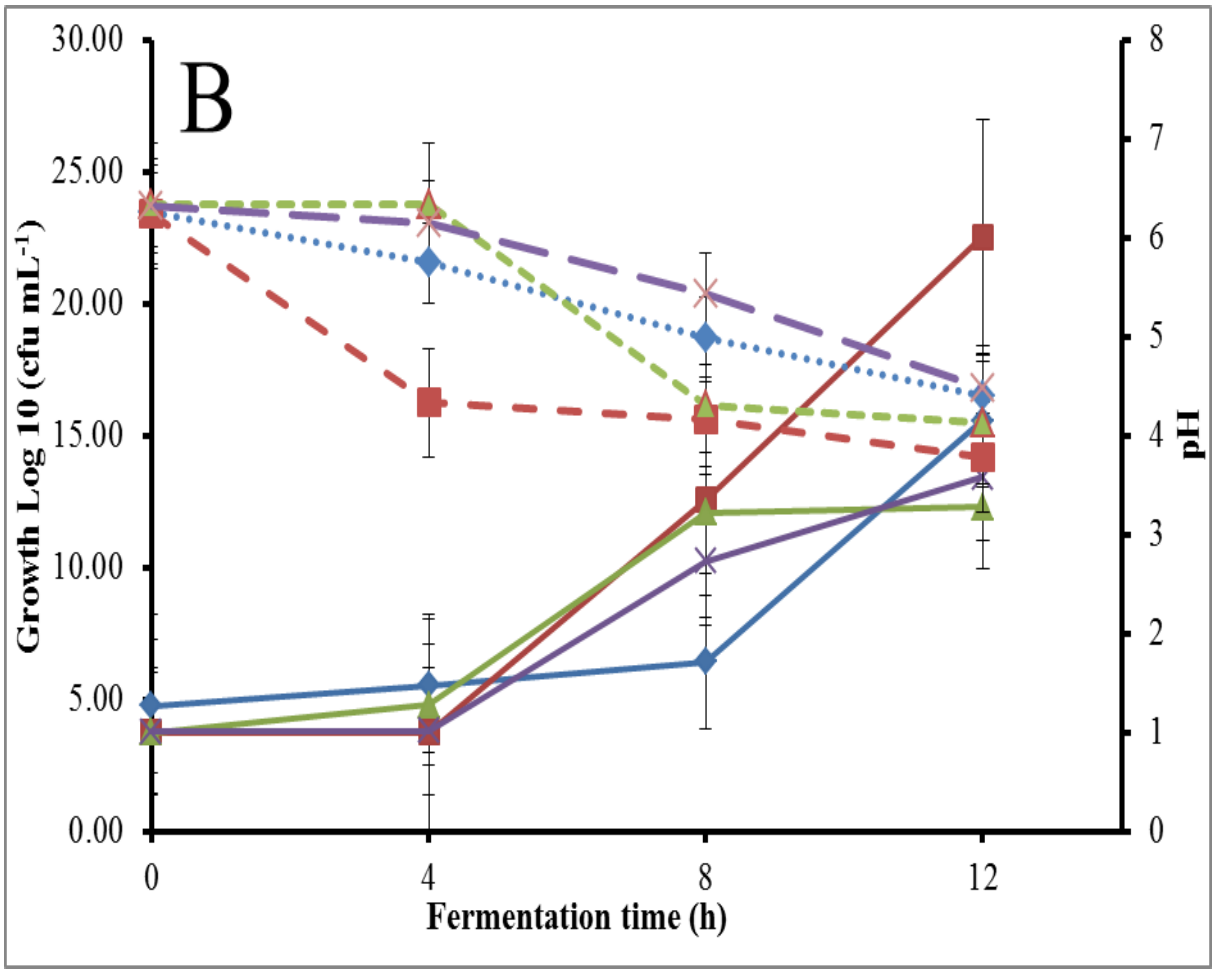
Bacterial growth and pH value in RSM and WPC with and without Flavourzyme® at 37°C for 0, 4, 8 and 12 h are shown in (Figure 3.1). All strains grew differently in the media with varying pH. In general, higher bacterial growth logarithm was presented in RSM media compared with WPC media and was dependent on bacteria type and fermentation time ($P < 0.05$), (Figure 3.1). This may be attributed to the superior nutrient profile of RSM compared to WPC or due to heat treatments which leads to WPC protein denaturation (Dissanayake et al., 2012; Ramos et al., 2012; Dissanayake et al., 2013). The growth logarithm of the Bb12 was 12 cfu mL^{-1} without Flavourzyme® in RSM at 12 h and pH 4.6 (Figure 3.1A). The highest growth logarithm was 25 cfu mL^{-1} of *Bifidobacterium animalis* ssp12 (Bb12) with Flavourzyme® in RSM at 12 h and pH 3.6 (Figure 3.1B). Moreover, (Bb12) growth logarithm was weak in WPC without Flavourzyme® (5 cfu mL^{-1}) in the same fermentation time at pH 5.5 (Figure 3.1C). However, in WPC with Flavourzyme® the growth logarithm of Bb12 was 20 cfu mL^{-1} at pH 4 (Figure 3.1D). Interestingly, the growth logarithm of *Lactobacillus casei* (Lc 210) was sharply increased between 8-12 h in RSM with Flavourzyme® (Figure 3.1B). Whilst the growth logarithm was stable in the same media without Flavourzyme® (Figure 3.1A), and in WPC with or without Flavourzyme® (Figure 3.1C and D), the optimal growth characteristics was with strain *Bifidobacterium animalis* ssp12 (Bb12), and the most effective media was RSM supplemented with Flavourzyme®.

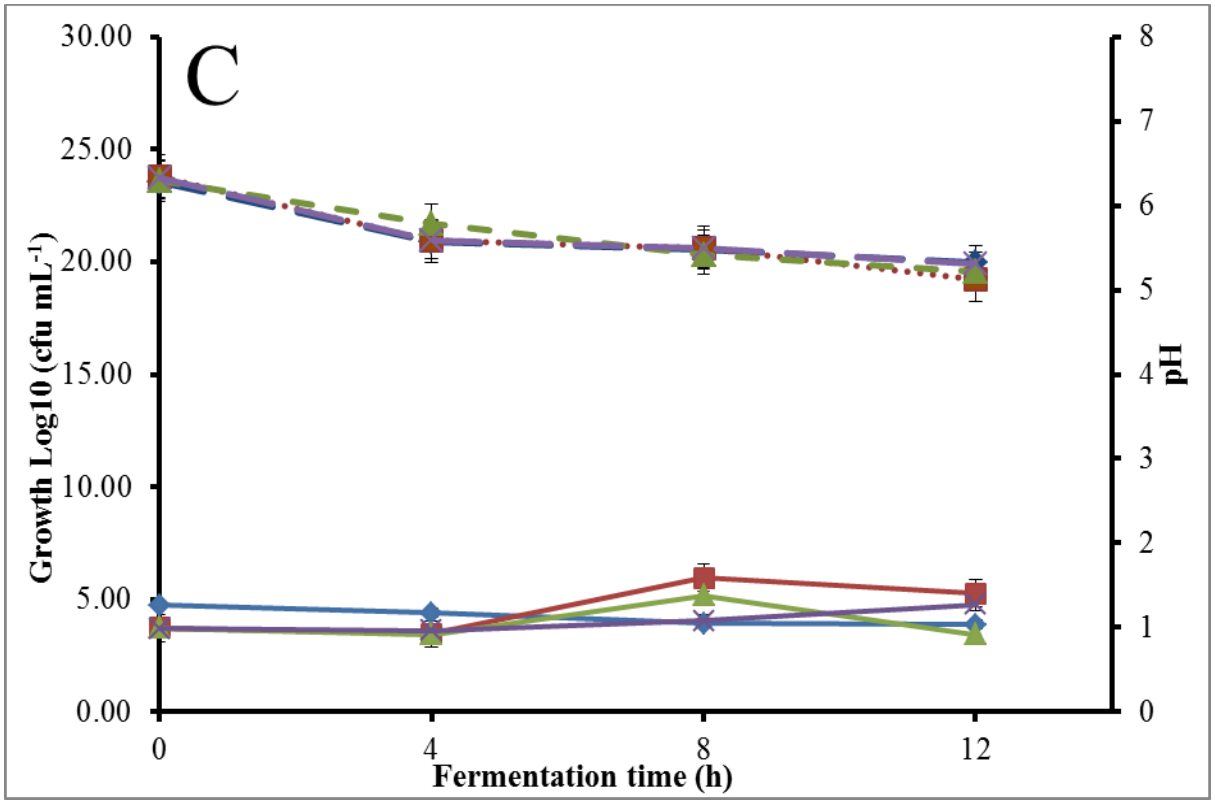
Analysis of variance showed that bacterial growth was significantly ($P < 0.05$) affected by media supplementation with Flavourzyme®, fermentation time and strain type (Kilpi et al., 2007; Leclerc et al., 2002). Combination with Flavourzyme® increased the bacterial growth in both media types ($P < 0.05$); however, the growth and pH was higher in RSM media compared to WPC media (Figure 3.1). This implies that Flavourzyme® facilitates bacterial growth through its proteolytic action resulting in an increased amount of free amino acids. It has been reported that heat treatment at low pH

of WPC causes denaturation of WPC proteins (Dissanayake et al., 2013), in addition to having negative effects on milk proteins and peptides (Davies et al., 1998). Bb12 and Lc210 showed higher growth (~14 cfu mL^{-1}) at pH 4 and (~7 cfu mL^{-1}) at pH 4.9 respectively in 12 % RSM with Flavourzyme® supplementation compared to Lb11842 (12 cfu mL^{-1}) at pH 4.2 and La 2410 (10 cfu mL^{-1}) and pH 5.2 at 8 h (Figure 3.1), suggesting that LAB strains prefer substrate by enzymes. However, at 12 h incubation, Bb12 in 12 % RSM with Flavourzyme® showed significantly ($P < 0.05$) higher growth than Lc210, Lb11842 and La 2410 at pH 3.9 (Figure 3.1B). On the other hand, Bb12 showed low growth after 8 h fermentation (~12.5 cfu mL^{-1}) at pH 4.6 in 12 % RSM without Flavourzyme® supplementation (Figure 3.1A). The highest ($P < 0.05$) growth in the 4 % WPC media with Flavourzyme® supplementation was also shown by Bb12 after 12 h (~20 cfu mL^{-1}) at pH 4 but not in the first 8 h of fermentation (Figure 3.1D). Due to Flavourzyme® has the ability to hydrolyse milk proteins to free amino acids for all the media types resulted to an increase in bacterial growth compared with the media without Flavourzyme®.

Hence, supplementation of Flavourzyme® effects the growth. In general, Bb12 and Lc210 strains were always more numerous than Lb11842 and La2410 during any given period for both media and were higher in RSM than WPC, most likely due to differences in protein type and protein structure of both media, variation amongst strains and utilization of limiting nutrients (Ramchandran et al., 2008; Dissanayake et al., 2013). It is likely that the growth of *L. delbrueckii ssp. bulgaricus* was lower due to low pH level (Tharmaraj & Shah, 2003). Similar growth characteristics were noted using LAB strains under the same media and same conditions (Ramchandran et al., 2010).


A
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Growth log 10 (cfu mL-1)
8
7
6
5
4
3
2
1
0
pH
0
4
8
12
Fermentation time (h)


B
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Growth Log 10 (cfu mL-1)
8
7
6
5
4
3
2
1
0
pH
0
4
8
12
Fermentation time (h)



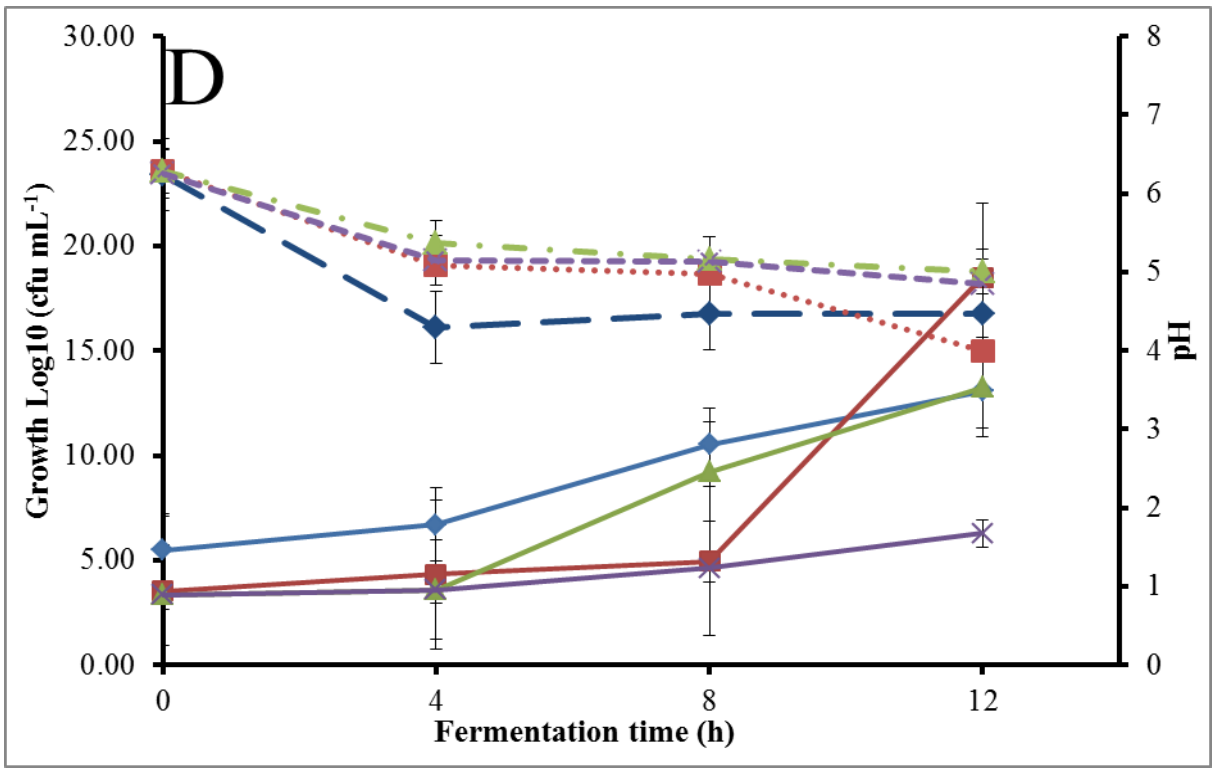


Figure 3.1 Growth (log 10 cfu ml^{-1}) and pH of (—◆—) *L. casei* (Lc210), (—■—) *Bifidobacterium animalis ssp12* (Bb12), (—▲—) *L. delbrueckii* subsp. *bulgaricus* (Lb11842), (—✳—) *L. acidophilus* (La 2410) at 37°C for 12 hours (Error bars represent standard error of the mean) in RSM (**A**), RSM with Flavourzyme® (**B**), WPC (**C**) and WPC with Flavourzyme® (**D**).

3.3.2 Proteolytic activity is higher in RSM media supplemented with Flavourzyme®

Proteolytic activities of the four bacterial strains, Lc210, Bb12, Lb11842 and La2410 were measured to determine the free amino acids generated after 0, 4, 8 and 12 h of fermentation at 37°C in RSM or WPC and are shown in (Figure 3.2 (Line)). All organisms showed an increase in proteolysis with time (Figure 3.2). In general, supplementation with Flavourzyme® led to significantly increased processing of hydrolysis during 12 h fermentation time in both media. The proteolytic activity of strain (Bb12) was sharply increased, starting with initial fermentation at 4-8 h followed by no significant changes at 12 h fermentation in RSM supplemented with Flavourzyme®, while proteolytic activity of La2410 and Lc210 were significantly increased during 12 h in the same media (Figure 3.2B). On the other hand, hydrolyses of the same strains were weak in RSM or WPC without supplementation (Figure 3.2A, C), even though strain Bb12 demonstrated high proteolytic activity in the same medium without supplementation (Figure 3.2A). Interestingly, strain La2410 showed the highest proteolytic activity in WPC supplemented with Flavourzyme® compared to the other strains in same media (Figure 3.2D). Highest proteolytic activity was observed in RSM media for all four strains compared to WPC media ($P < 0.05$). RSM supplemented with Flavourzyme® showed the highest proteolytic activity which increased with time, this correlated to a similar trend in the growth pattern (Figure 3.2).

At 4 h the proteolytic activities of Lc 210, Bb12, Lb11842 and La2410 ranged between 35-65 % higher in RSM with Flavourzyme® compared to RSM without Flavourzyme® (Figure 3.2). Flavourzyme® has the ability to hydrolyse RSM's caseins (Tsai et al., 2008) more rapidly than whey proteins in WPC. This suggests that the addition of Flavourzyme® reduces the fermentation time required to achieve high proteolytic activity. As evidenced, protease enzymes hydrolyse large proteins to intermediate peptides (Tsai et al., 2008; Barbana & Boye, 2010), which in turn facilitated the activities of four strains (Figure 3.2). Similarly, the proteolytic activity and ACE-inhibition of LAB strains grown in 12 % RSM without Flavourzyme® supplementation with *Bifidobacterium* showed very high proteolytic capability compared to *L. delbrueckii ssp. bulgaricus* 1368, *L. casei* 15286, and *L. acidophilus* 4461 (Ramchandran et al., 2008 & 2010). The ability of *Bifidobacterium* to utilize almost all types of substrates could explain their high proteolytic capability (Ramchandran & Shah, 2008). However, *delbrueckii* subsp. *bulgaricus* has a poor ability to grow in milk

media due to its weak proteolytic activity. In general, proteolytic activities were significantly different ($P < 0.05$) between strains at any given fermentation time (Dave et al., 1998; Ramchandran et al., 2010). Similarly, the type of media used, as well as with or without Flavourzyme® supplementation, showed significant differences (Figure 3.2C, 3.2D), with the highest proteolytic activity in WPC media and Flavourzyme® observed with La 2410 at 12 h fermentation time and not at 8 h (Figure 3.2D). Bb12 and La2410 showed significantly ($P < 0.05$) higher proteolytic activity in both 12 % RSM and 4 % WPC with Flavourzyme® compared to *L. casei and L. delbrueckii* subsp. *bulgaricus*. These patterns of proteolysis correspond with the growth patterns of these organisms. Several reports have shown wide variations in the proteolytic abilities of different LAB strains (Oberg et al., 1991; Ramchandran & Shah, 2008). The highest proteolytic activities in RSM media with Flavourzyme® supplemented at 12 h was shown by strain Bb12 (Figure 3.2B). Flavourzyme® supplementation in WPC did not have the same effect as in RSM, especially at a lower fermentation time of 4 h and 8 h (Figure 3.2), which may be due to the effect of heat treatment and pH on the protein of WPC (Gauthier & Pouliot, 2003; Dissanayake et al., 2012). Flavourzyme® has the ability to hydrolyse peptides to free amino acids (Tsai et al., 2008) for all the media types due to an increase in bacterial growth (Figure 3.1, 3.2). Similarly, increasing proteolytic activity of probiotic organisms in RSM during fermentation process results in better survival (Donkor et al., 2006).

3.3.3 ACE-inhibition is influenced by strain type, media, fermentation time and Flavourzyme®

The percentage of ACE-I activity of fermented RSM or WPC with Lc 210, Bb12, Lb11842 and La 2410 strains with or without Flavourzyme® supplementation at 37°C for (0-12 h) fermentation period is shown in (Figure 3.2 (bars)). Bacterial strain, media type, supplementation with or without Flavourzyme® and fermentation time demonstrated significant ($P < 0.05$) effects on ACE-I activity. Fermented RSM by LAB facilited with Flavourzyme® showed the strongest inhibitory activity on ACE-I (Tsai et al., 2008). Flavourzyme® alone was used for fermentation of RSM or WPC as a control (Figure 3.2B, 3.2D). At 0 h fermentation time, ACE-I showed no activity for all strains (Figure 3.2). Interestingly, strain La 2410 showed higher ACE-I activity in WPC or WPC with Flavourzyme® compared to Lc210, Bb12, Lb 11842, while this strain had

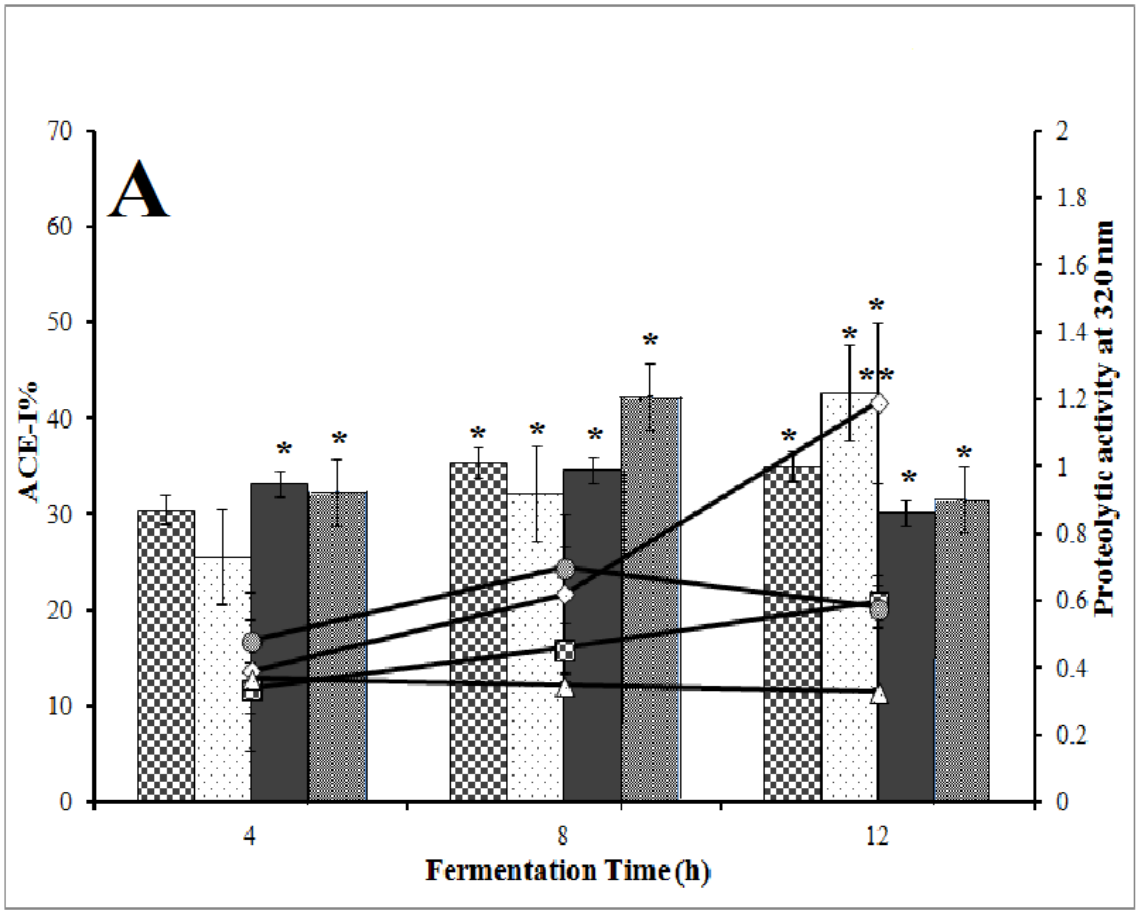
lesser ACE-I activity in RSM compared to the same strains used (Figure. 3.2C, D). Strain Bb12 (55 %) in RSM facilitated with Flavourzyme® at 12 h showed the highest ACE-I activity (Figure 3.2B (bars)). As the same time, the highest ACE-I activity at 8 h of fermentation was strain Lb11842 (50 %) in the same media compared to Flavourzyme® as control (25 %) at the same fermentation time. However, the same strain activity reached 35 % at 12 h in WPC Flavourzyme®-facilitated (Figure. 3.2D (bars)). The highest ACE-I activity in WPC Flavourzyme®-facilitated and without Flavourzyme®-facilitated was strain La 2410 (42 %), (Figure 3.2C, 2D (bars)). The best media to increase ACE-I activity was RSM with Flavourzyme® by strain Bb12 at 12 h, this correlated to a similar trend in the Proteolytic activity and growth pattern.

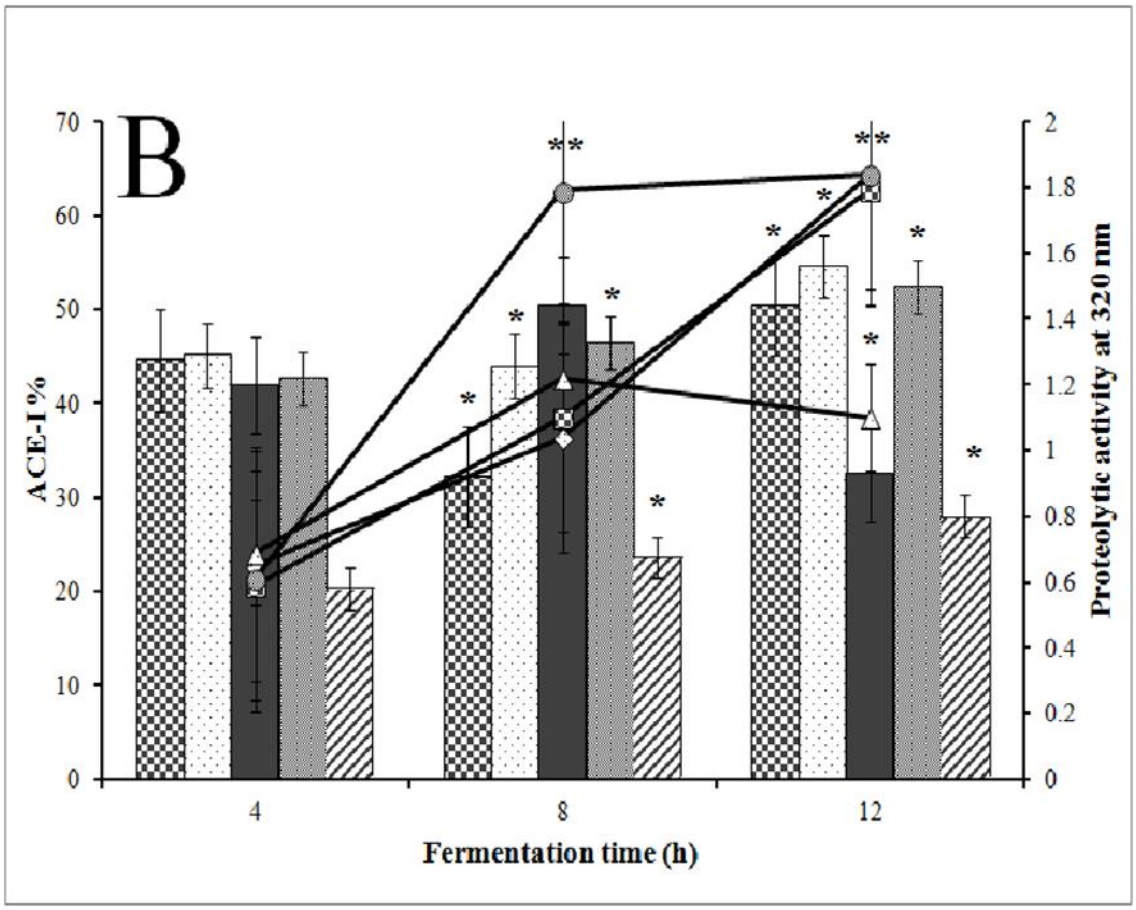
Flavourzyme® plays a role in increasing the production of bioactive peptides by hydrolysing proteins present in the media, resulting in the production of large and intermediate peptides. These become available to LAB strains, which utilize them as a source of essential and growth-stimulating amino acids (Juillard et al., 1998). These bacterial cells possess cell-envelope-located proteinases, which are able to degrade caseins/ oligo-peptides into peptides, allowing the internalization of the released peptides and intracellular peptidases and further hydrolysing them into smaller peptides and amino acids (Figure 3.3) (Juillard et al., 1998; Kunji et al., 1998). The differences observed in the two media (RSM and WPC) herein may be attributed to differences in the type of proteins present (Pan & Guo, 2010).

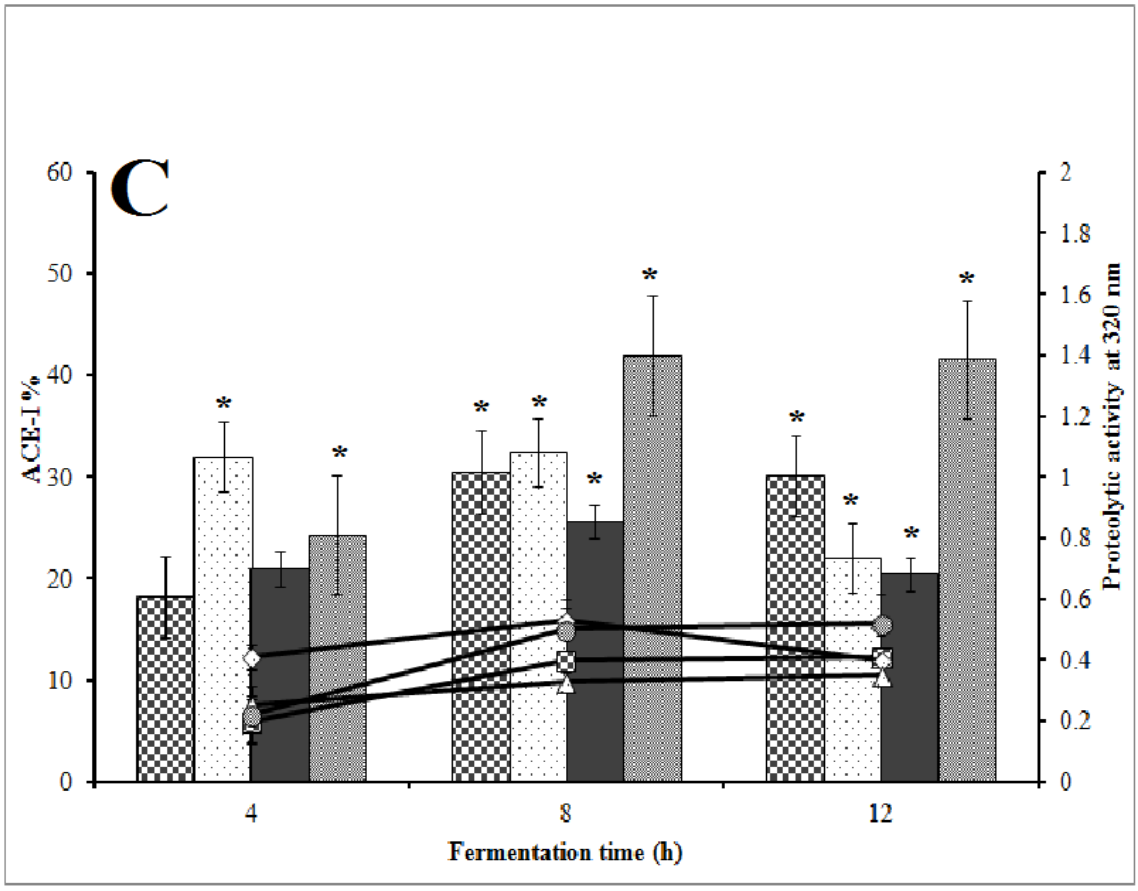
Previous studies have noted a similar trend with LAB fermentation in 12 % RSM showing ACE-I activity (Fitzgerald & Murray, 2006; Donkor et al., 2007). In both RSM and WPC media, supplementation with Flavourzyme® leads to increased ACE-I activity for all four bacterial strains with time of fermentation ($P < 0.05$). Bb 12 and La2410 had the highest ACE-I activity in both media types supplemented with Flavourzyme® compared to Lb 11842 and Lc 210 during 0- 12 h fermentation at 37°C (Figure 3.2).

ACE-I activity increased as the fermentation period increased from 4 h to 12 h for all strains due to increased protein hydrolyses. This likely resulted in high peptide production with ACE-I activity. However, the effect was more pronounced at 8 h fermentation as ACE-I activity increased from 30 to ≥ 52 % in RSM supplemented with Flavourzyme® compared to 12 h (55 %) (Figure 3.2A, B). Whilst the highest ACE-

I activity in WPC with Flavourzyme® was stable between 8 h and 12 h (39 %) (Figure 3.2D), this implies that Flavourzyme® supplementation can be used to achieve the production of ACE-I activity peptides in a shorter time of fermentation at 8 h instead of 12 h. Generally, strains showing a greater percentage of ACE-I activity also indicates higher proteolysis, except in the case of *L. acidophilus*. In fact, oligo-peptides that cannot be transported into the cell usually remain in the media to exhibit bioactivity (Meisel and Bockelmann., 1999).




B
ACE-I %
Proteolytic activity at 320 nm
Fermentation time (h)
0
10
20
30
40
50
60
70
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
4
8
12


C
ACE-I %
Proteolytic activity at 320 nm
Fermentation time (h)
0
10
20
30
40
50
60
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
4
8
12

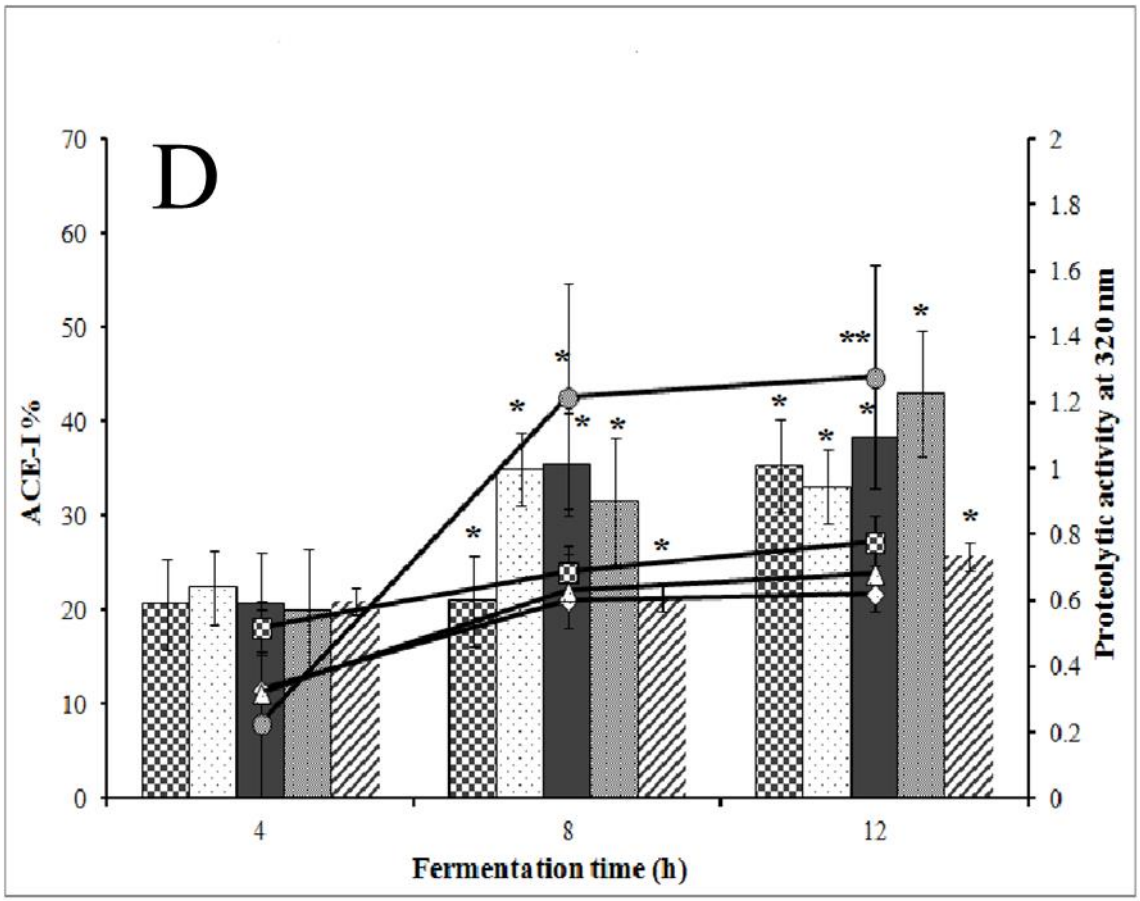


Figure 3.2 % ACE-inhibitory activities (bars) and Proteolytic activity (line) of 12% RSM (A) and 4% WPC (C) fermented by selected LAB.*L. casei* ( )(Lc210), *Bifidobacterium animalis* ssp12( )(Bb12), *L. delbrueckii* subsp. *bulgaricus* ( )(Lb11842), *L. acidophilus*( )(La2410) , Flavourzyme® as control and combination of LAB with Flavourzyme® ( )(F+) (B and D) at 37°C up to 12 h .

3.3.4 RP-HPLC analysis of water-soluble peptides extract suggested that LAB's selected strains with Flavourzyme® is most optimal

The profiles of water-soluble peptides extract of 12 h fermented WPC or RSM hydrolysis by Lc210, Bb12, Lb11842 and La2410 strains combined with Flavourzyme® is shown in (Figure 3.3). The RP-HPLC elution profile of the hydrolysates was based on the hydrophobicity group of peptides (He et al., 2013). Both media hydrolyzed proteins providing peptides in the retention time range of 10-85 min in WPC with Flavourzyme® (Figure 3.3A), and, 10-60 min in RSM by strains Bb 12, Lb11842, La2410, and Lc210, respectively (Figure 3.3B). The effect of Flavourzyme® in both media and in particular RSM with Flavourzyme® was the most effective media to increase milk protein

hydrolysis as peptide peaks are shown in (Figure 3.3A, B). Strain Bb 12 had the largest number of peptide peaks between retention time (0-40) and (50-75), and this correlated to a similar trend in the ACE-I activity. Combination with Flavourzyme® generally aids strains in RSM media to increase proteolysis as evidenced by the presence of increased peptide's peaks (Figure 3.3B), compared to the same strains growing in WPC media (Figure 3.3A). On the other hand, based on the number of peaks, Bb12 and La2410 with Flavourzyme® were the best in terms of production of peptides in RSM. However, the supplementation was more beneficial to all strains in RSM compared with the same strains in WPC (Figure 3.3). Bb12 with Flavourzyme® in RSM was the most optimal in terms of providing an increased number of peaks. This corroborated our observation of high ACE-I activity (Figure 3.2).

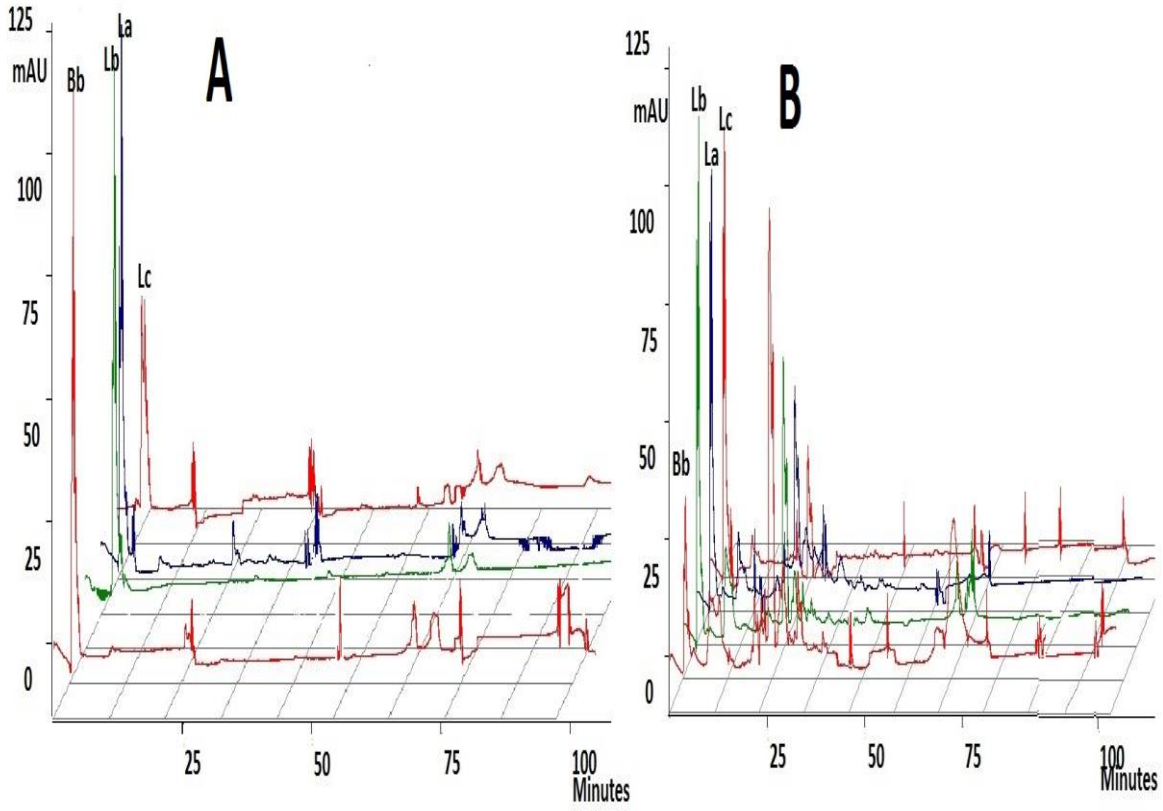


Figure 3.3 RP-HPLC peptides profile of water soluble extracts obtained from fermented skim milk with *L. casei* (Lc210), *Bifidobacterium animalis ssp12* (Bb12), *L. delbrueckii* subsp. *bulgaricus* (Lb11842), *L. acidophilus* (La2410) at 37°C for 12 h, combined with Flavourzyme® in WPC (A) compared to a combination with Flavourzyme® in RSM (B) .

3.4 Conclusions

The proteolytic and ACE-I activities of *Lactobacillus casei* (Lc 210), *Bifidobacterium animalis ssp12* (Bb12), *Lactobacillus delbrueckii subsp. bulgaricus* (Lb11842), *Lactobacillus acidophilus* (La2410) were higher in RSM media compared with WPC media. *Bifidobacterium animalis ssp12* (Bb12) and *Lactobacillus acidophilus* (La 2410) demonstrated higher ACE-inhibitory and proteolytic activities compared to *Lactobacillus delbrueckii subsp. bulgaricus* (Lb11842) and *Lactobacillus casei* (Lc 210). However, the combination of Flavourzyme® and *Bifidobacterium animalis ssp12* (Bb12) have the highest proteolytic and ACE-I activities in RSM. The supplementation of media with Flavourzyme® increased proteolysis and thus increased ACE-I activities of all four bacterial strains. Moreover, Flavourzyme® supplementation of media could be used to reduce fermentation time from 12 h to 8 h.

Chapter 4 Effect of Flavourzyme® on Angiotensin Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by *Lactobacillus helveticus*

This chapter has been published. Ahtesh, F., Stojanovska, L., Shah, N., & Mishra, V. K. (2016). Effect of Flavourzyme® on Angiotensin-Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by *Lactobacillus helveticus*. *Journal of food science*, 81(1), M135-M143. (Appendex. I)

4.1 Introduction

Hypertension is considered a risk factor for coronary heart disease such as myocardial infarction and stroke (FitzGerald et al., 2004). According to the World Health Organisation, nearly one billion people worldwide suffer from hypertension (World Health Organisation, 2013). Angiotensin converting enzyme catalyses conversion of Angiotensin-I to Angiotensin-II (a vasoconstrictor), which contributes to hypertension and heart failure. Hypertension is usually controlled by a number of medications, the most common being a synthetic angiotensin converting enzyme inhibitory (ACE-I) drug such as captopril and enalapril (Hansson et al. 1999; Turner and Hooper 2002). ACE-I drugs decrease active angiotensin-II production from inactive angiotensin-I (Erdos, 1975; FitzGerald et al., 2004). Angiotensin-II receptor antagonists are agents used to modify the renin-angiotensin-aldosterone system through blocking angiotensin receptors, resulting in a decrease in blood pressure (Miura, Karnik, & Saku, 2011). However, long term use of synthetic ACE-I drugs may result in side effects such as cough, skin rash or development of impaired renal function (Sesoko & Kaneko, 1985; Coulter and Edwards 1987; Morgan, Anderson, & MacInnis, 2001; Acharya et al., 2003).

Peptides such as Val-Pro-Pro and Ile-Pro-Pro derived from milk proteins have been identified to have similar beneficial effects of ACE-I action opening the possibilities of replacing or complementing synthetic drugs (FitzGerald & Meisel, 2000; Pan et al. 2005; Tsai et al., 2008; Nielsen et al., 2009; Yamaguchi et al., 2009; Pihlanto, A., Virtanen, T., & Korhonen, H., 2010; Phelan and Kerins, 2011). Lactic acid bacteria (LAB) used to produce fermented dairy products (i.e. yoghurt, fermented milk, cheeses) have been shown to produce peptides with varied but significant ACE-I activities during fermentation as reported in several studies (Korhonen, 2009; Phelan & Kerins, 2011; Korhonen & Pihlanto 2003; Korhonen & Pihlanto, 2006; Korhonen & Pihlanto, 2007; Hernández-Ledesma et al., 2011). The use of specific LAB or proteases for producing ACE-I peptides from various milk media (yoghurt, cheese, sour milk) have been reported (van der Ven et al., 2002; Donkor et al., 2005; Pan et al., 2005; Kilpi et al., 2007; Meena et al., 2008; Tsai et al., 2008; Korhonen, 2009; Hamme et al., 2009; Ramchandran & Shah 2010; Ramchandran & Shah, 2011; Tellez et al., 2011; Chaves-López et al., 2012; García-Tejedor et al., 2013). Bioactive peptides which have ACE-I

activity have been derived from hydrolysis of proteins using skim milk and whey protein concentrate (Madureira et al., 2010; Donkor et al., 2007). Such peptides have clinically documented effects in the reduction of hypertension in humans (Aihara et al., 2005; Agyei et al., 2015). The production of these bioactive peptides thorough fermentation depends on several factors, such as growth media, fermentation time, temperature, pH and the type of LAB and strain used (Ramesh et al., 2012).

Lactobacillus helveticus (*L. helveticus*) is homo fermentative thermophilic LAB that possesses strong proteolytic activity and is used in the production of cheese and fermented milk beverages (Griffiths & Tellez., 2013). Due to its high proteolytic activity, *L. helveticus* is more effective compared to other LAB such *as L. delbrueckii sp. bulgaricus* and *L. acidophilus* in the production of ACE-I peptides (Korhonen & Pihlanto, 2006). Several studies have reported the use of *L. helveticus* for production of ACE-I peptides using milk media (Maeno et al., 1996; Leclerc et al., 2002; Kilpi et al,. 2007; Nielsen et al., 2009; Sun et al., 2009; Pan and Guo 2010a; Otte et al., 2011; Singh et al., 2011; Lim et al., 2011; Unal and Akalin, 2012; Griffiths & Tellez, 2013). The effect of temperature, fermentation time and initial pH of fermented milk by *L. helveticus* has been reported for sour milk production (Pan, & Guo, 2010). Proteinases have been used extensively in the production of ACE-I peptides from dairy proteins (Pihlanto-Leppala, 2000). Pan and others (2005) used cell-free enzyme extract from *L. helveticus* consisting of proteinase, amino peptidase and x-prolyl-dipeptidyl amino peptidase to produce Val-Pro-Pro and Ile-Pro-Pro with potent ACE-I activities. Tsai and others (2008) reported a tenfold increase in the production of ACE-I peptides when milk was fermented by *Streptococcus thermophiles* and *Lactobacillus bulgaricus* in the presence of a proteinase. Since there are no published reports on the production of ACE-I peptides from milk employing proteases and *helveticus,* the objective of this study was to assess and compare *L. helveticus* strains for production of ACE-I peptides using two milk media (12 % reconstituted skim milk (RSM) and 4 % whey protein concentrate (WPC) with or without protease (Flavourzyme®) supplementation by measuring the bacterial growth, proteolytic activity and *in vitro* ACE-I activity. Therefore, the present study was performed to evaluate the hypothesis that a combination of Flavourzyme® and *L. helveticus* significantly increases ACE-I percentage in milk media.

4.2 Material and Methods

4.2.1 Experimental design and bacteria propagation

Bacteria and enzymes

L. helveticus strains ASCC 881315, 881188, 880474 and 880953 were obtained from Dairy Innovation Australia Ltd, Werribee, VIC, Australia and stored in a 40 % glycerol de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Ltd., West Heidelberg, VIC, Australia) at −80°C. For activation, an aliquot (100 µL) of each strain was individually transferred into the MRS broth and incubated at 37°C for 24 hours (h). Weekly subculturing of bacteria into the MRS broth was performed to maintain the bacterial activity. Prior to each experiment, bacteria were subcultured three times and fermented for 12 h in 12 % RSM or 4 % WPC. Flavourzyme® 1000 L (EC 3.4.11.1), an amino peptidase with an activity of 1000 Leucine Amino-peptidase (LAPU g-1), was obtained from Novozymes Australia., NSW Australia. Table 4.1 shows the types of experimental media, strains and enzyme combinations used in the study.

Table 4.1 Experimental design to analyse and measure the pH, growth, proteolytic activity and % of ACE-inhibitory activities during (0, 4, 8 and 12 h) fermentation of *L. helveticus* strains in 12 % RSM or 4 % WPC and with or without combination of Flavourzyme®.

Media used		*L. helveticus* strains used without combination	Combination of *L. helveticus* strains (1 % v/v each with Flavourzyme® 0.14%)
RSM		881315	881315+Flavourzyme®
		881188	881188+Flavourzyme®
		880474	880474+Flavourzyme®
		880953	880953+Flavourzyme®
	control	Flavourzyme®	
WPC		881315	881315+Flavourzyme®
		881188	881188+Flavourzyme®
		880474	880474+Flavourzyme®
		880953	880953+Flavourzyme®
	control	Flavourzyme®	

4.2.2 Media preparation

Fermentation media, preparation and procedure

Skim milk powder (52 % lactose, 37 % protein, 8.6 % ash and 1.2 % fat) and WPC (47.5 % lactose, 35 % protein, 9 % ash and 2.5 % fat) were obtained from Murray Goulburn Co-operative Co. Ltd., VIC and United Milk Tasmania Ltd., TAS Australia, respectively. RSM (12 %) and WPC (4 %) were prepared by dissolving appropriate quantities of skim milk powder and WPC in distilled water. Both media (RSM and WPC) were heated to 90°C for 20 minutes (min), cooled to room temperature and inoculated with 1% of *L. helveticus* strains with or without 0.14 % (w/w) Flavourzyme®1000 L. Fermentation was conducted at 37°C and samples collected at 4 h, 8 h and 12 h and stored at -20°C for analysis of bacterial growth, proteolytic and ACE-I activities and peptide profiling by Reverse Phase – High Performance Liquid Chromatography (RP-HPLC).

4.2.3 Measurement of bacterial growth

Growth was assessed every 4 h up to 12 h during fermentation in 12 % RSM or 4% WPC as described in the procedure in section 3.2.3.

4.2.4 Determination of proteolytic activity

Proteolytic activity during fermentation was determined according to the procedure described in section 3.2.5.

4.2.5 Determination of ACE-Inhibitory activity

ACE inhibitory activity was measured according to the procedure described in section 3.2.6.

4.2.6 RP-HPLC analysis of water-soluble peptides extract

Water-soluble peptides extract was analysed according to the procedure described in section 3.2.7.

4.2.7 Statistical analysis

All results were expressed as mean values of three replicates with standard deviation. One-way ANOVA was performed to investigate the significant differences in the

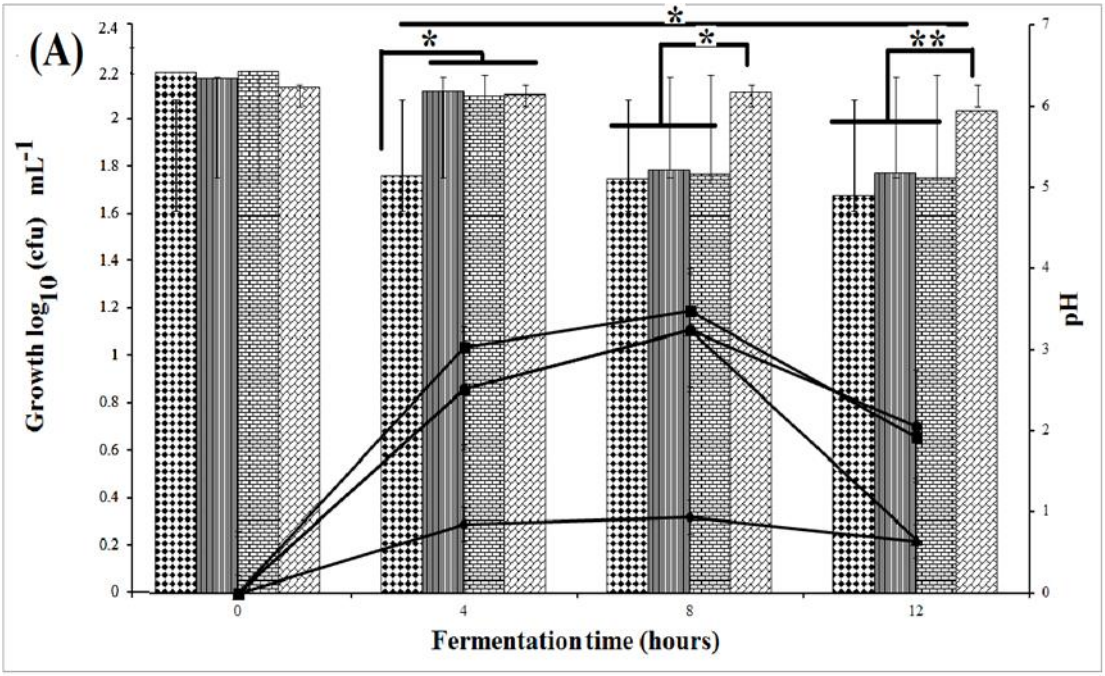
treatments (strains, growth media, presence or absence of Flavourzyme® and fermentation time). The level of significance was tested at 5% level ($P < 0.05$). Fisher`s (least significant difference; LSD) test was used to investigate significant differences among the treatment means. Correlation analysis was carried out between variables for the same bacteria strain, growth media and presence or absence of Flavourzyme®. The degree of correlation between these variables was expressed as Pearson coefficient (r) and corresponding *P* values. All statistical analyses were carried out using SAS V9.0 software (SAS Inc., Cary, NC, USA).

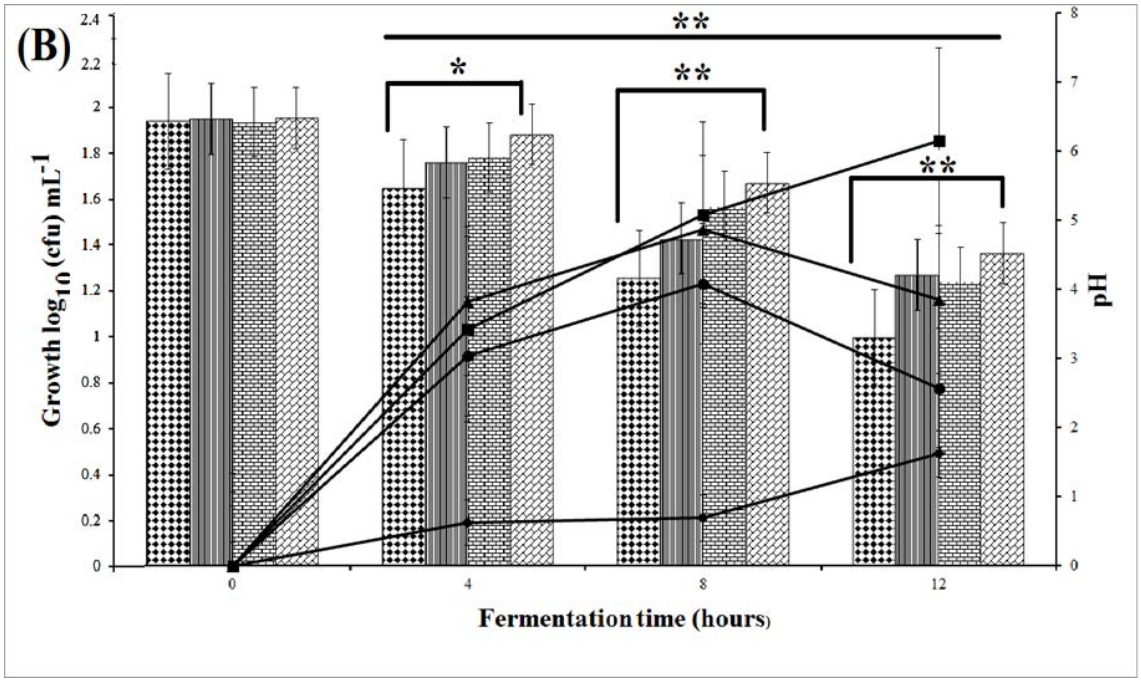
4.3 Results and Discussion

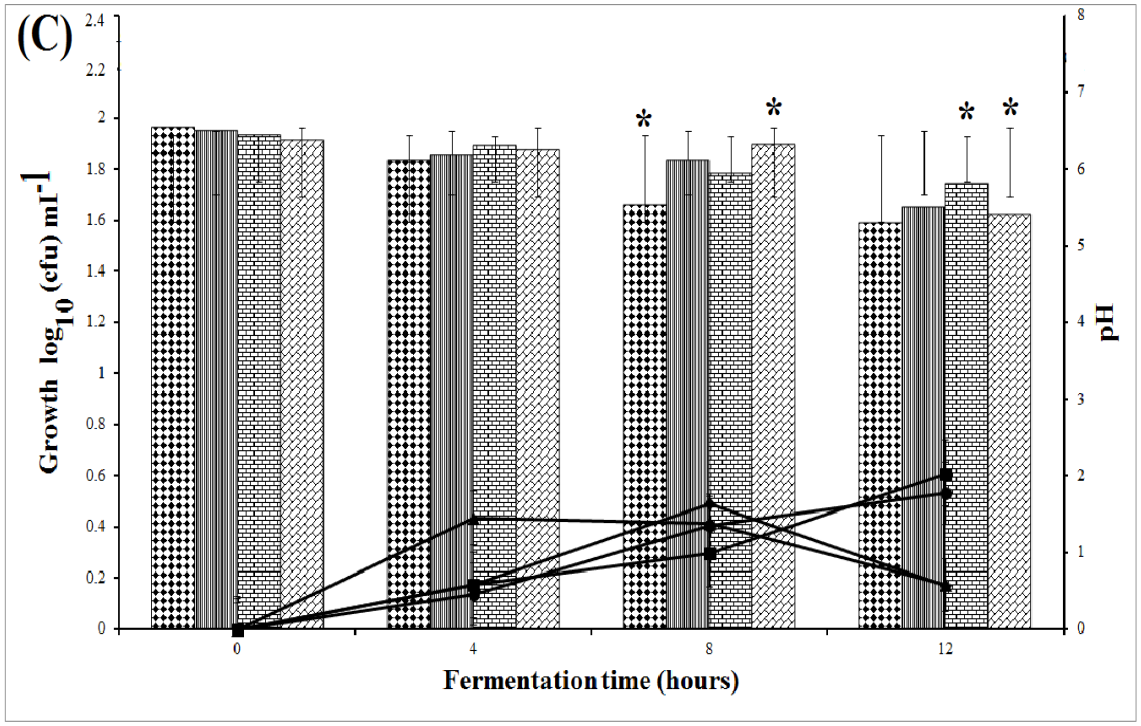
4.3.1 Preferential growth of *L. helveticus* in RSM media with Flavourzyme® compared to WPC

Figure 4.1 shows the microbial growth and the pH of media during fermentation with *L. helveticus* strains. All strains were able to grow in both media (Figure 4.1). Analysis of variance showed that the bacterial growth was significantly ($P < 0.05$) affected by media, media supplementation with protease (Flavourzyme®), fermentation time and strain type. Higher growth was significantly noted ($P < 0.05$) in RSM compared to WPC. This may be attributed to a superior nutrient profile of RSM compared to WPC (Kilpi et al., 2007; Leclerc et al., 2002) and higher specificity to caseins than whey proteins. Flavourzyme® led to increased growth in both media owing to higher proteolysis releasing more peptides and amino acids required for bacterial growth (Kenny et al., 2003). While *L. helveticus* 881315 showed the least growth (0.6 cfu mL^{-1}) at pH 4 , *L. helveticus* 881188 (2.0 cfu mL^{-1}) at pH 4.2 showed the highest growth compared to other strains in RSM containing Flavourzyme® at 12 h for the entire duration of fermentation. *L. helveticus* strains 880474 (1.5 cfu mL^{-1}) and pH 5 at 8 h and 880953 (1.2 cfu mL^{-1}) and pH 5.5 at 8 h, also showed increased growth compared to 881315 in RSM. It appears that Flavourzyme® supplementation prolonged the log phase in 881188, whereas 880474 and 880953 strains went into a decline phase after 8 h (1.2 cfu mL^{-1}) and pH 4 and (0.6 cfu mL^{-1}) and pH 4.6. *L. helveticus* 881188 showed the highest growth (1.3 cfu mL^{-1}) and pH 4.2 at 8 h and decreased into (1.0 cfu mL^{-1}) and pH 4.9 at 12 h of fermentation in WPC with Flavourzyme®. In general, WPC showed a weak growth for all strains without the combination of Flavourzyme® compared to the same strains in combination with Flavourzyme® (Figure 4.1). However, growth for all

strains in WPC with Flavourzyme® increased significantly at 8 h and declined after 8 h of fermentation at pH 3.4, possibly due to low pH and heat treatment of WPC thereby reducing available nutrients for growth as previously reported (Zisu and Shah 2003; Dissanayake et al., 2013). Furthermore, accumulation of lactic acid in the media may have contributed to a decrease in bacterial growth observed after 8 h. Similar growth characteristics are known for most LAB as reported by Leroy and de Vuyst (2001) for *L. sakei* CTC 494. The differences observed in bacterial growth in the two media are related to the different nature of proteins present (Leroy & de Vuyst, 2001). Caseins in general are more susceptible to hydrolysis by *L. helveticus* enzymes than whey proteins (Griffith & Tellez, 2013).




(B)
Growth log10 (cfu) mL-1
pH
Fermentation time (hours)
*
**
**
**


(C)
Growth log10 (cfu) mL-1
pH
Fermentation time (hours)
*
*
*
*

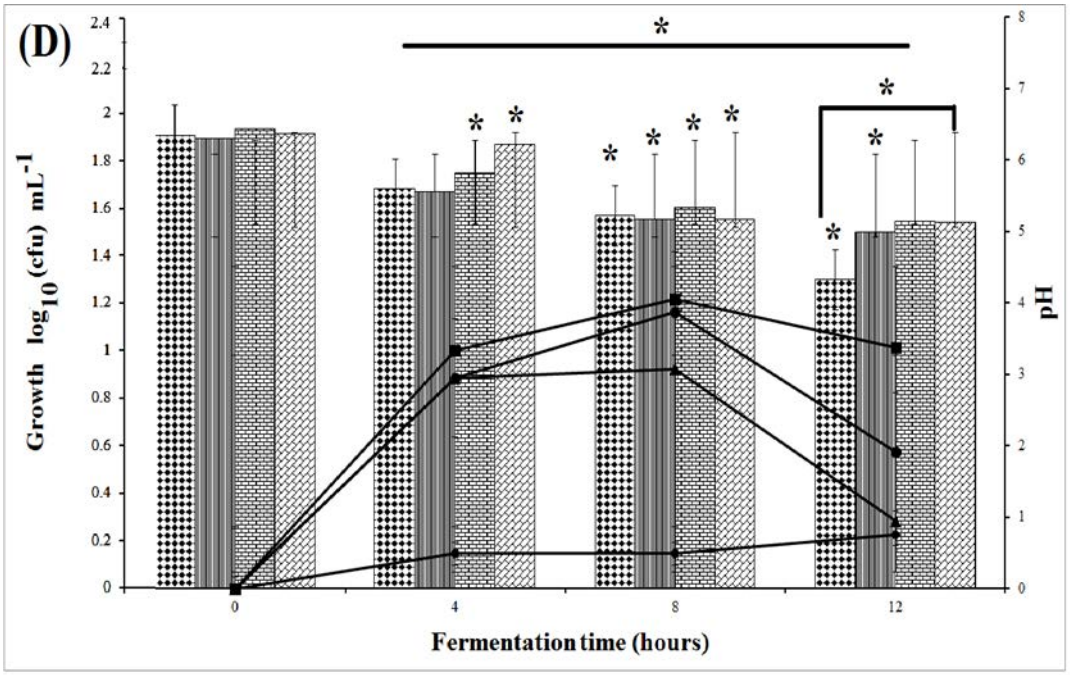


Figure 4.1 Growth (line) of *L. helveticus* strains and pH (bars) in (A) RSM, (B) RSM with Flavourzyme®, (C) WPC and (D) WPC with Flavourzyme® fermented at 37°C for 12 h, (881315), (881188), (880474) and (880953). The vertical lines depict standard deviation and lines above signify differences at (*) $P < 0.05$ and (**) $P < 0.01$.

4.3.2 Proteolytic activity is higher in RSM media with Flavourzyme®

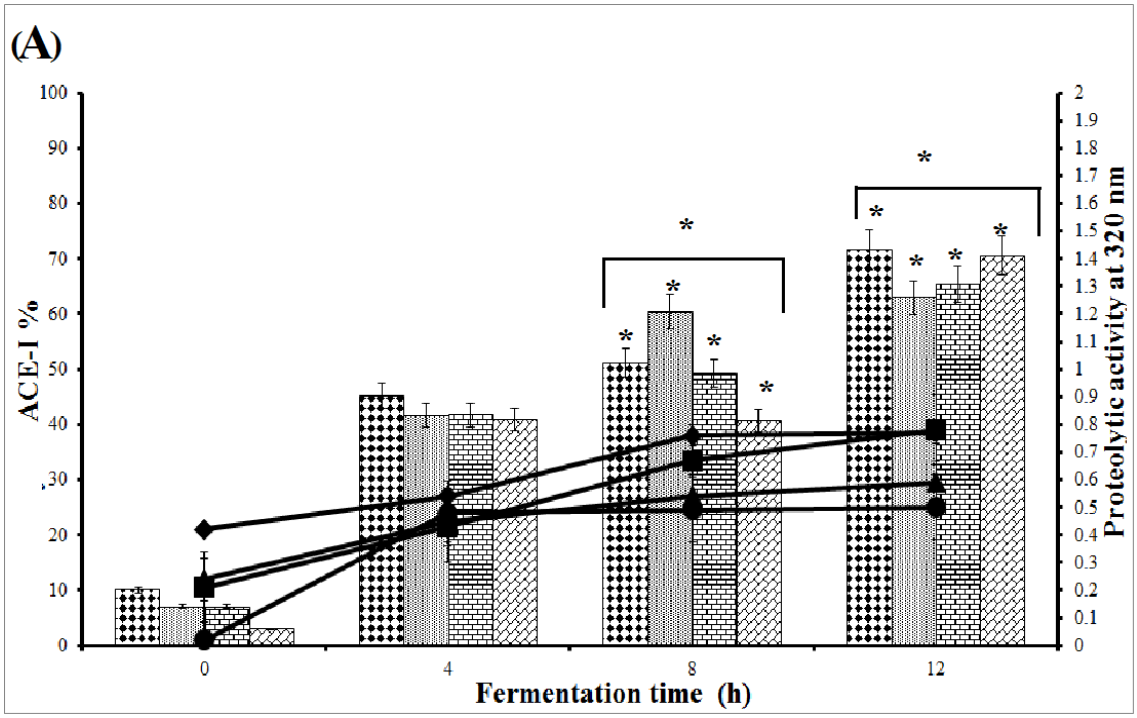
An increase in the amount of free amino acids (NH_3) groups as released by hydrolysis of milk proteins were quantified by the OPA method by measuring the change in the absorbance at 320 nm and are presented in Figure 4.2. Irrespective of *L. helveticus* strains grown in RSM or WPC with or without Flavourzyme® supplementation, the proteolysis continued at 37°C for 12 h (Figure 4.2). Higher proteolysis was noted in fermented RSM than WPC, with or without supplementation with Flavourzyme®, indicating that all strains preferred RSM proteins as substrate over WPC. The proteolysis remained significantly lower (≤ 0.5) in WPC compared to RSM (> 0.78), indicating that proteins of RSM were the preferred substrate by proteolytic enzymes of *L. helveticus* strains investigated. This correlated with a similar trend noted in the growth pattern (Figure 4.1). The order of proteolytic activity of *L. helveticus* strains in RSM was 881315 > 881188 > 880474 > 880953. Supplementation of RSM with Flavourzyme® increased the proteolytic activity of all strains significantly (< 0.05),

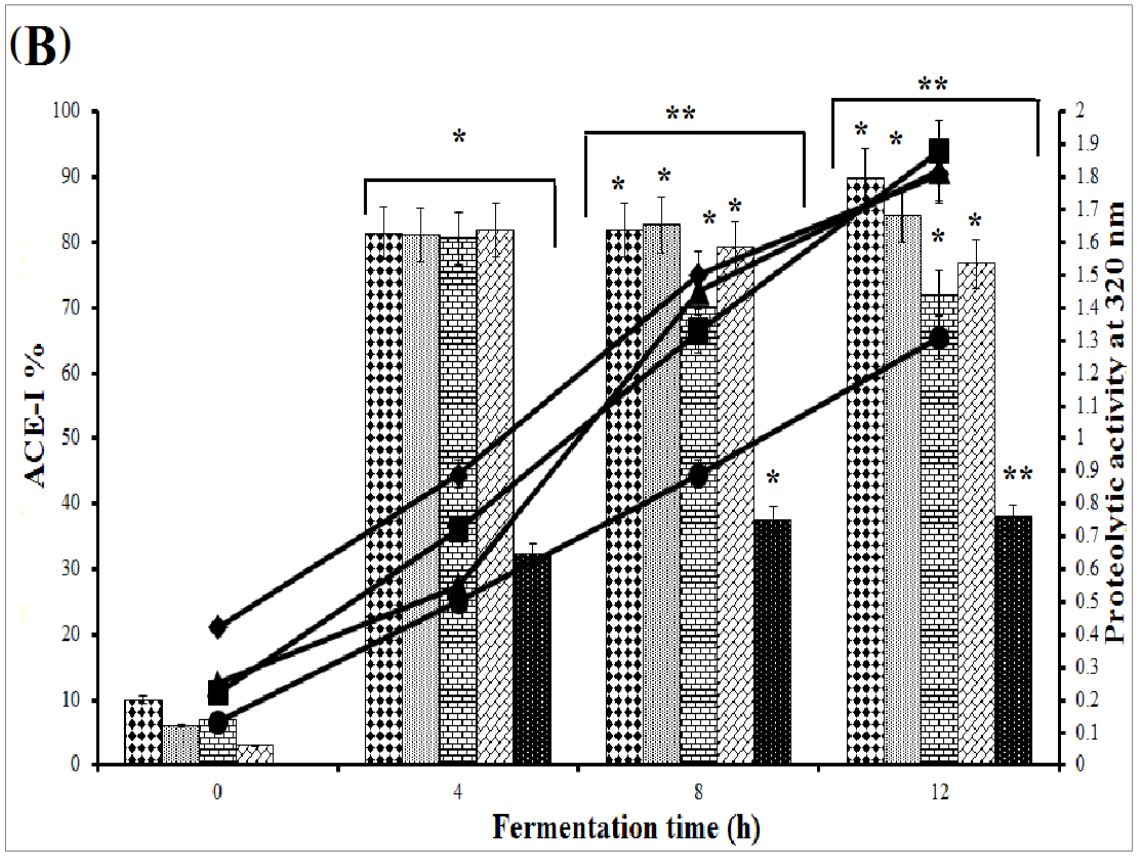
reaching a maximum absorbance > 1.8 in 12 h by *L. helveticus* 881315, 880474 and 881188 (Figure 4.2). Interestingly, the proteolytic activity of strain 881315 was high during 12 h (Figure 4.2) despite poor growth in both media (Figure 4.1). The activity in RSM with Flavourzyme® was approximately higher by 45-60 % than that without Flavourzyme® even after 4 h of fermentation and was sustained over the 12 h duration of fermentation. However, except for *L. helveticus* 880953, the response to Flavourzyme® in increasing proteolysis was similar after 8 h of fermentation. Flavourzyme® appears to have hydrolysed large proteins present in RSM to intermediate peptides, which were used by *L. helveticus* to produce small peptides and free amino acids (Leclerc et al., 2002). Co-fermentation of RSM with Flavourzyme® supplementation with *L. helveticus* strains reduced the time required for a given degree of proteolysis. These results suggest that proteolysis was enhanced in the higher protein containing media supplemented with Flavourzyme® and that casein was a better substrate compared to whey proteins for all *L. helveticus* strains (Griffith & Tellez, 2013). In addition, the amount of free NH3 groups in the media continued to increase over 12 h except for media without Flavourzyme® for which the amount did not increase as much after 8 h (Figure 4.2). Matar et al., (1996) have noted differences in the proteolytic activities between strains.

4.3.3 ACE-Inhibitory activity is influenced by strain type, media and Flavourzyme® combination

The amount and type of peptides produced during hydrolysis is known to influence ACE-I activity. An *in vitro* assay was used to measure this activity following the method of Donkor et al., (2007). The ACE-I activity of *L. helveticus* strains (881315, 881188, 880474 and 880953) in RSM or WPC with or without Flavourzyme® at 37°C for 12 h are presented in (Figure 4.2). Flavourzyme® alone was used as a control. ACE-I activity differed significantly between strains. *L. helveticus* 881315 and 881188 showed higher ACE-I activity compared to other strains in RSM (Figure 4.2). Flavourzyme® enhanced the production of ACE-I peptides as previously reported for other LAB (Tsai et al., 2008). ACE-I activity for all strains in both media increased significantly during the fermentation period ($P < 0.05$). However, differences existed between strains and media used when compared at the same time of fermentation. Media type, strains, supplementation of Flavourzyme® and fermentation time had significant ($P < 0.05$)

effects on ACE-I activity. As with proteolytic activity, ACE-I activity also increased as fermentation time increased for all strains. Supplementation of RSM with Flavourzyme® significantly ($P < 0.05$) increased ACE-I activity of *L. helveticus* strains. Except for *L. helveticus* 880474, ACE-I increased from 40-60 % to ≥ 85 % in RSM with Flavourzyme® supplementation after 8 h of fermentation. The inhibition increased during fermentation when *L. helveticus* 881315 and 881188 were used from 10- 89.8 % and from 5-85 % in RSM with supplementation, respectively (Figure 4.2B). While the same strains in WPC with Flavourzyme® were present, the ACE-I increased from 10- 65 % and 5-60 % during 12 h, respectively (Figure 4.2D). Since both of these strains demonstrated high proteolysis, co-fermentation with enzyme appeared to have produced higher amounts of ACE-I peptides as evident in increased number of peaks (Figure 4.3). The inhibitory activity remained high at 12 h for all strains except *L. helveticus* 880474, which showed a significant drop in ACE-I after 4 h of fermentation. There was no significant difference ($P < 0.05$) in ACE-I between hydrolysates produced from WPC with or without Flavourzyme® at 4 h fermentation. Thereafter, ACE-I increased differentially among the strains and a maximum of 89.8 % observed for *L. helveticus* 881315 with Flavourzyme® in RSM at 12 h. However, the growth of the same strain was weak for the entire period of 12 h, it not only produced ACE-I peptides but also accumulated them without further conversion to free amino acids required for growth. This indicates that peptidase enzymes are not very active in strain 881315 as similar observation was previously reported for peptidase deficient mutant of *L. helveticus* CNRZ32 (Kilpi et al., 2007). Data also suggest a delayed effect of the addition of Flavourzyme® in WPC. The increase in ACE-I due to the addition of Flavourzyme® was significantly ($P < 0.05$) higher in RSM compared to WPC (Banks, Law, Leaver, & Horne, 1995; Patel & Creamer, 2008) and since ACE-I almost doubled in the first 8 h of fermentation, Flavourzyme® supplementation can be used to reduce the time of hydrolysis required for the production of ACE-I peptides. The differences observed between RSM and WPC may be attributed to differences in the type of proteins present and therefore the variety of peptides present in the hydrolysates (Matar & Goulet, 1996; Pan & Guo, 2010). Preference to casein by proteinases has been well documented (Matar & Goulet, 1996; Cheison et al., 2007; Lim et al. 2011; Griffiths & Tellez, 2013). Strains 881315 and 881188 appear to be the best of the four tested strains in providing maximum ACE-I (60-70 %). Flavourzyme® supplementation increased ACE-I of both media to 80-88 %.


(A)
ACE-I %
Proteolytic activity at 320 nm
Fermentation time (h)
0
4
8
12


(B)
ACE-I %
Proteolytic activity at 320 nm
Fermentation time (h)
0
4
8
12

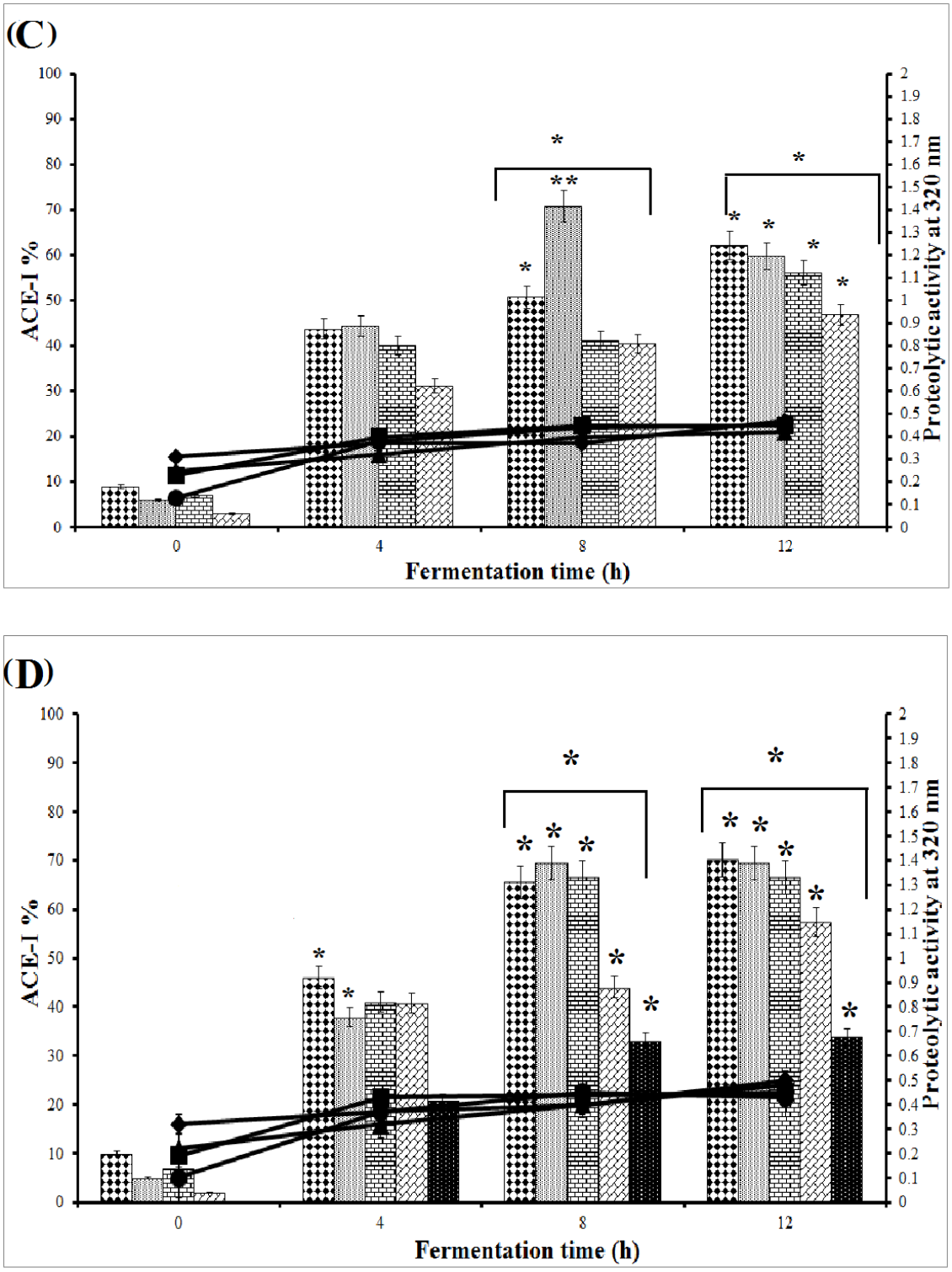


Figure 4.2 Proteolytic activity (line) and ACE-I % (bars) of *L. helveticus* strains (—◆— 881315), (—■— 881188), (—▲— 880474), (—●— 880953) and (—◆— Flavourzyme®) as control at 37°C for 12 h in (**A**) RSM, (**B**) RSM with Flavourzyme®, (**C**) WPC and (**D**) WPC with Flavourzyme® . The vertical lines depict standard deviation and lines above signify differences at (*) $P < 0.05$ and (**) $P < 0.01$.

4.3.4 RP-HPLC analysis of water-soluble peptide extracts

The peptide profiles of water-soluble extracts of 12 h fermented skim milk, with or without supplementation of Flavourzyme® by the two best performer strains, *L. helveticus* 881315 and 881188 showing high proteolytic and ACE-I activities, are shown in (Figure 4.3). The RP- HPLC elution profile of the hydrolysates is mainly based on the hydrophobicity of the peptides. In the control unfermented RSM, only one peak appeared at 10 min (not shown). The chromatograms (Figures 4.3A, 4.3C) show that 881315 and 881188 strains without enzyme supplementation hydrolysed skim milk proteins into peptides that showed the retention time range of 10-40 min and 10-45 min, respectively. Supplement with Flavourzyme® (Figure 4.3B and 4.3D) generally increased proteolysis as evident by the presence of more peptides appearing in the range of 10-65 min (881315) and 10-45 min (881188). Since peptide profile extended beyond 45 min for extracts from hydrolysates achieved by co-fermentation by strain 881315 combined with Flavourzyme®, this combination was optimal in terms of providing peptides that are more hydrophilic and in higher amounts that the other three treatments. This trend is also followed by high ACE-I activity (Figure 4.2). However, the effect of supplementation was more beneficial to strain 881315 than that to 881188.

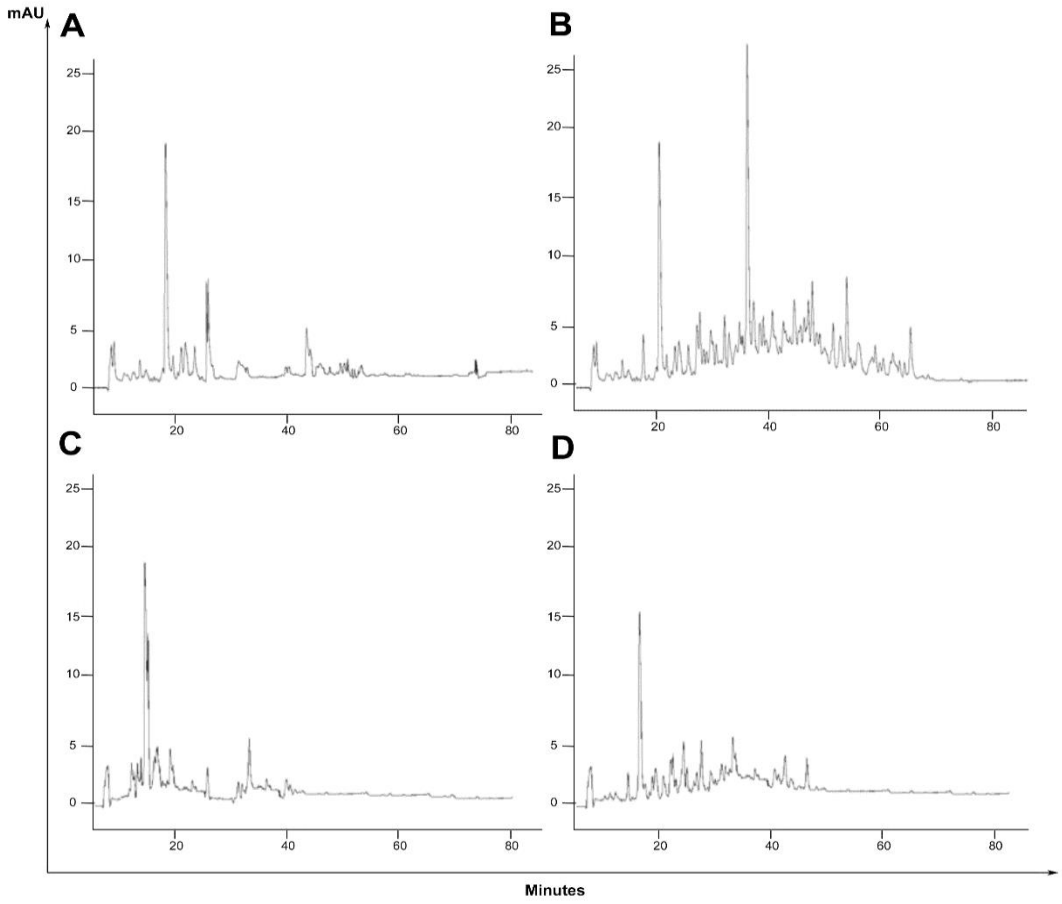


Figure 4.3 RP-HPLC peptide profile of water soluble extracts obtained from skim milk fermented by *L. helveticus* strains 881315(**A**), 881315 with Flavourzyme® (**B**), 881188 (**C**) and 881188 with Flavourzyme® (**D**) after 12 h fermentation at 37 °C.

4.3.5 Correlation between proteolytic activity, ACE-Inhibition and bacterial growth

The correlation between proteolytic activity and anti-hypertensive properties expressed as ACE-I and bacterial growth expressed as (cfu) for the same bacterial strain are presented in (Tables 4.2 and 4.3) for RSM and WPC respectively. A significant correlation in growth with all measurements for each strain in RSM was evident except *L. helveticus* 880953, which did not grow well in both media ($P < 0.05$). This correlated to proteolytic and ACE-I activity of strains (Table 4.2). This suggests that Flavourzyme® enhanced the proteolytic and ACE-I activities of *L. helveticus* in RSM. ACE-I activity positively and strongly correlated with proteolytic activity for each strain, both with or without Flavourzyme® ($P < 0.05$) (Table 4.2), implying that

increased proteolytic activity increased the production of ACE-I peptides. Moreover, ACE-I activity showed a strong correlation with bacterial growth in RSM with or without Flavourzyme®, for all strains except *L. helveticus* 880953.

Table 4.2 Pearson coefficient correlations (r), proteolytic activity (OPA), ACE-inhibitory activity (ACE) and bacterial growth (CFU) for strain, *L. helveticus* 881315, *L. helveticus* 881188, *L. helveticus* 880474 and *L. helveticus* 880953 grown in 12 % RSM at 37°C for 12 h with or without Flavourzyme® combination.

***L. helveticus* strains**	**Variables**	**Without Flavourzyme®**			**With Flavourzyme®**		
		OPA	ACE	CFU	OPA	ACE	CFU
881315	OPA	1.000	0.988**	0.918**	1.000	0.979**	0.946**
	ACE		1.000	0.945**		1.000	0.864**
	CFU			1.000			1.000
881188	OPA	1.000	0.991*	0.874*	1.000	0.966*	0.852*
	ACE		1.000	0.882*		1.000	0.694**
	CFU			1.000			1.000
880474	OPA	1.000	0.962**	0.805*	1.000	0.978*	0.835*
	ACE		1.000	0.825*		1.000	0.928*
	CFU			1.000			1.000
880953	OPA	1.000	0.989*	0.690**	1.000	0.891*	0.863*
	ACE		1.000	0.588**		1.000	0.597*
	CFU			1.000			1.000

*$P < 0.05$, ** $P < 0.01$.

Table 4.3 Pearson coefficient correlations (r), proteolytic activity (OPA), ACE-inhibitory activity (ACE) and bacterial growth (CFU) of, *L. helveticus* 881315, *L. helveticus* 881188, *L. helveticus* 880474 and *L. helveticus* 880953 grown in 4 % WPC at 37°C for 12 h with or without Flavourzyme® combination.

***L. helveticus* strains**	**Variables**	**Without Flavourzyme®**			**With Flavourzyme®**		
		OPA	ACE	CFU	OPA	ACE	CFU
881315	OPA	1.000	0.615*	0.713*	1.000	0.530*	0.580*
	ACE		1.000	0.927**		1.000	0.949**
	CFU			1.000			1.000
881188	OPA	1.000	0.946*	0.866*	1.000	0.626*	0.562*
	ACE		1.000	0.947*		1.000	0.978**
	CFU			1.000			1.000
880474	OPA	1.000	0.615**	0.666*	1.000	0.867*	0.907*
	ACE		1.000	0.894*		1.000	0.971*
	CFU			1.000			1.000
880953	OPA	1.000	0.716**	0.567*	1.000	0.686*	0.608*
	ACE		1.000	0.827*		1.000	0.634**
	CFU			1.000			1.000

*P<0.05,**P<0.01.

4.4 Conclusions

Production of ACE-I peptides by *L. helveticus* varied between the strains due to the differences in proteolytic activity. Casein-rich RSM supported higher growth, higher proteolytic activity and produced higher ACE-I activities by all *L. helveticus* strains. Therefore, RSM is superior to WPC as a medium for production of ACE-I peptides irrespective of supplementation with protease, which generally increased hydrolysis of proteins to produce more ACE-I peptides. Beneficial effects of protease supplementation were more pronounced in the first 8 h of fermentation and also sustained thereafter. However, *L. helveticus* 881315 showed the lowest growth. The highest ACE-I activity was observed in 12 % RSM supplemented with Flavourzyme® and up to 12 h fermentation by *L. helveticus* 881315 and 881188 at 37°C, respectively. These conditions will aid in the production of a functional fermented drink with high ACE-I activity.

Chapter 5 Effects of *Kluyveromyces marxianus* LAF4, combined with probiotic strains as a source of angiotensin converting-enzyme peptides

This chapter has been submitted for publication. Ahtesh F., Apostolopoulos V., Vijay M., Stojanovska L., (2016). Effects of *Kluyveromyces marxianus* LAF4 combined with probiotics as source of Angiotensin converting enzyme peptides. *Dairy science journal.* (Appendix. I).

5.1 Introduction

Probiotic microorganisms are defined as 'live microorganisms that when administered in adequate amounts confer health benefits to the host' (Sanders, 2008; World Health Organisation, 2013). Probiotic foods are 'food products that contain a living probiotic organism in adequate concentration, so that after their ingestion, the postulated effect is obtained and is beyond that of usual nutrient suppliers' (Saxelin et al., 2003). Milk is a rich growth medium that contains proteins and essential factors that are capable of supporting growth of LAB. ACE-I peptides activity has been extensively studied in the last few years (Hong et al., 2008; Fragasso et al., 2012; García-Tejedor et al., 2013). ACE-I peptides such as, antihypertensive peptides, decrease the level of vasoconstricting peptide, Angiotensin-II and therefore reduce blood pressure (Martinez-Maqueda et al., 2012; Arbia et al., 2013). 2012; García-Tejedor et al., 2013).

Bioactive peptides, derived from milk proteins, have been found to have similar ACE-I activity and may be used to decrease hypertension with no known side effects (Pfeffer et al., 2003; Tsai et al., 2008; Hernández-Ledesma et al., 2011; Wang et al., 2012; Unal and Akalin, 2012). The side effects of ACE-I drugs include coughing, skin rashes and impaired renal function (Sesoko S, 1985; Turner and Hooper, 2002; Fragasso et al., 2012). The beneficial alternatives of ACE-I drugs are peptides which are isolated from fermented milk products using probiotics (Hartmann and Meisel, 2007; Hernández-Ledesma et al., 2011; Ahtesh et al., 2016a). Probiotics consist of either yeast, in particular *Saccharomyces* or *Kluyveromyces marxianus* (*K. marxianus*) (Penner, Fedorak, & Madsen, 2005).

For thousands of years yeasts have been used to produce a wide range of fermented traditional foods and beverages (Chaves-López et al., 2011b; Roostita and Fleet, 1996). Yeast fermentation of milk involves hydrolysis of lactose and galactose, assimilation of lactate, lipolytic and proteolytic activity (Roostita & Fleet, 1996). Yeasts are considered fundamental in the production of some fermented milk products, such as Kumis and Kefir. Kumis is traditional fermented cow milk produced and consumed in South West Colombia (Chaves-López et al., 2011). Recently, skim milk fermented with yeast and LAB exhibited particular metabolic profiles, which possess great variability in ACE-I-inhibitory properties, contributing to the ACE-inhibitory activity of Colombian Kumis (Chaves-López et al., 2011). Natural fermented milk beverages are manufactured in many countries and yeasts are included in the production process of some of them (Graham, 2006). In different Colombian

Kumis, several yeast species have been found, and most of them have been generally described in indigenous Asian or African fermented milk products, contributing considerably to the development of the final flavours of the products (Graham, 2006; Kebede et al., 2007). In Asian fermented milks, the most prevalent species is *Kluyveromyces marxianus* (*K. marxianus*) often associated with *Saccharomyces spp* (Jespersen, 2003). It has been reported that yeasts isolated from dairy products have proteolytic characteristics (Jakobsen and Narvhus, 1996).

K. marxianus is dairy yeast that may have a promising and viable application in obtaining bioactive peptides from lactoglobulin by fermenting whey protein (Belem et al., 1999). The use of *K. marxianus* in milk fermentation is also a good source for producing oligo-nucleotides (flavour enhancers in food products); oligosaccharides (a prebiotic that stimulates the growth of *Bifidobacterium* sp. in animal and human intestines) and oligo peptides (immunostimulatory) (Belem and Lee, 1998; García-Tejedor et al., 2013; Chaves-López et al., 2012). *K. marxianus* has been reported as a promising candidate for the generation of antihypertensive peptides from whey proteins α -lactalbumin and β -lactoglobulin, (Belem et al., 1999) or in combination with *Lactobacillus rhamnosus* during 168 h of milk fermentation (Hamme et al., 2009). Yeast species such as *K. marxianus*, *Saccharomyces cerevisiae* and *Candida parapsilosis* have been documented to produce peptides with ACE-I activity (García-Tejedor et al., 2013; Gonzalez-Gonzalez et al., 2011; Chaves-López et al., 2012; Hamme et al., 2009; Kebede et al., 2007). Recently, *K. marxianus* isolated from Colombian Kumis was able to produce fermented milk with ACE-I activity (Chaves-López et al., 2012). However, the *in vivo* antihypertensive effect of casein and whey-derived bioactive peptides generated by yeast strains has not yet been demonstrated.

Herein, the efficacy of dairy yeast to generate milk protein-derived peptides with ACE-I activity was investigated. For this purpose, different strains of *Lactobacillus; L. casei* (Lc210), *L. delbrueckii subsp. bulgaricus* (Lb11842), *L. acidophilus* (La2410), and four different strains of *L. helveticus* (Lh) ASCC-881315, 881188, 880474 and 880953 combined separately with *K. marxianus* LAF4 were screened for their ability to grow in 12 % RSM to produce hydrolysates containing ACE-I peptides. The effect of fermentation time, media, probiotic strain and the presence or absence of yeast was evaluated.

5.2 Material and Methods

5.2.1 Experimental design and culture propagation

Fifteen different fermented treatments in 12 % RSM treatments with probiotic bacteria *L. casei* (Lc210), *L. delbrueckii ssp. bulgaricus* (Lb11842), *L. acidophilus* (La2410), *L. helveticus*; Lh 881315, Lh 881188, Lh 880474 and Lh 880953 were tested and the experiments were triplicated (Table 5.1) the treatments were obtained from Dairy Innovation Australia Ltd. The selected probiotic bacteria were stored in de Man Rogosa and Sharpe (MRS) broth containing 40 % glycerol (Oxoid, Ltd., West Heidelberg, Victoria, Australia) at −80 °C. *K. marxianus* LAF4/ 10U were obtained from Chr. Hansen Pty. Ltd, France. For activation 100 µL aliquot of each strain was individually transferred into sterile 10 mL MRS broth and incubated at 37 °C for 24 hours (h) prior to each experiment. The microorganisms were cultured three times and incubated for 12 h in 12 % RSM at 37 °C.

Table 5.1 The experimental design and codes used in the study to analyse and measure the pH, growth, proteolytic activity and % of ACE-inhibitory activities during (0, 4 , 8 and 12 h) fermentation of probiotic strains in 12 % RSM with or without *Kluyveromyces marxianus LAF4***.**

Media used	**Code**	**Cultures used without combination**	**Code**	**Combination of cultures with Kluyveromyces (1 % v/v each)**
RSM	Lb	*L.bulgaricus*	Lb	*L.bulgaricus* + *Kluyveromyces*
	La	*L. acidophilus*	La	*L. acidophilus*+ *Kluyveromyces*
	LC	*L. casei*	LC	*L. casei* + *Kluyveromyces*
	Control			*Kluyveromyces*
	Lh	L h 881315	Lh	L h 881315+ *Kluyveromyces*
	Lh	L h 881188	Lh	L h 881188+ *Kluyveromyces*
	Lh	L h 880474	Lh	L h 880474+ *Kluyveromyces*
	Lh	L h 880953	Lh	L h 880953 + *Kluyveromyces*

5.2.2 Media Preparation

Media were prepared using RSM (52 % lactose, 37 % protein, 8.6 % ash and 1.2 % fat). All media were sterilised by heat treatment at 90 °C for 20 min, cooled to 40 °C. Each media was inoculated with 1 % v/v of *L. casei* (Lc210), *L. delbrueckii ssp. bulgaricus* (Lb11842), *L. acidophilus* (La2410), *L. helveticus* ASCC*;* Lh 881315*,* Lh 881188, Lh 880474 and Lh 880953 separately and in combination with or without *K. marxianus* and a fermented sample using *K. marxianus* alone as control (Table 5.1). Fermentation was carried out for 12 h and samples were collected at 0, 4, 8 and 12 h at 37 °C and immediately analysed and after that stored at -20 °C for further analysis.

5.2.3 Measurement of bacterial and yeast growth

Growth was assessed every 4 h up to 12 h during fermentation in 12 % RSM as described in the procedure in section 3.2.3.

5.2.4 Determination of proteolytic activity

Proteolytic activity during fermentation was determined according to the procedure described in section 3.2.5.

5.2.5 Determination of ACE inhibitor activity

ACE inhibitory activity was measured according to the procedure described in section 3.2.6.

5.2.6 Preparation of water-soluble peptides extract

Water-soluble peptides extract was analysed according to the procedure described in section 3.2.7.

5.3 Statistical analysis

All results were expressed as mean values of 3 replicates with standard deviation. ANOVA was performed to investigate the significant differences in the treatments: bacteria strains with yeast, growth, and fermentation time using Minitab software. The level of significance was tested at $P < 0.05$. Fisher`s (least significant difference; LSD) test was used to investigate significant differences among the treatment means.

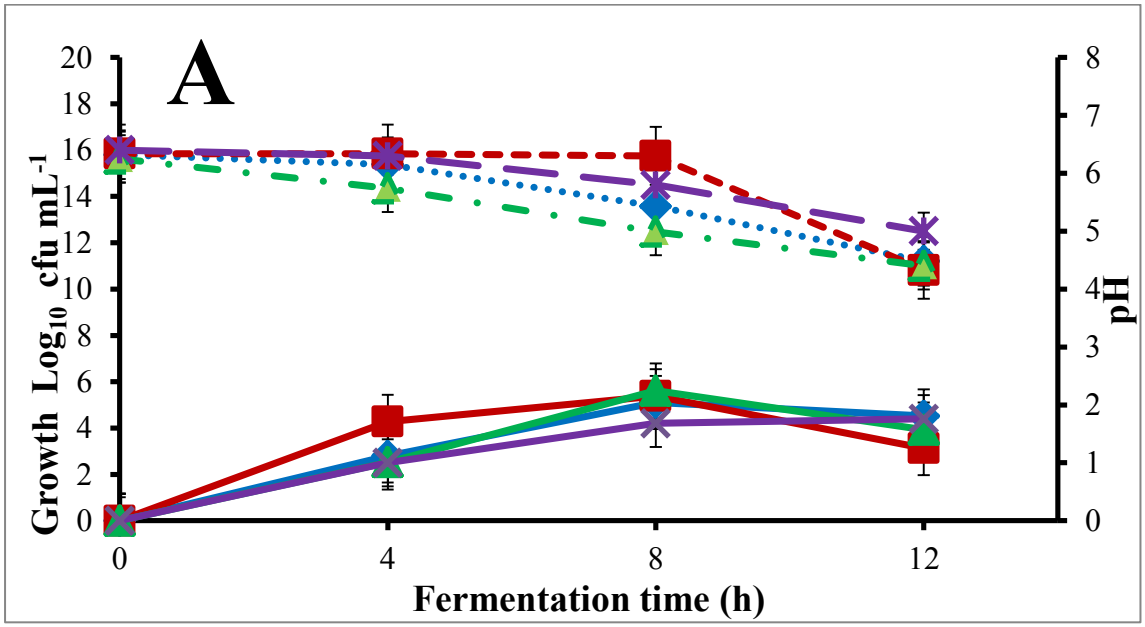
5.4 Results and Discussion

5.4.1 Enumeration of organisms

Preferential growth of selected LAB strains in RSM media without combination of *Kluyveromyces marxianus* LAF4

The bacterial growth and pH measurements are shown in (Figure 5.1, 5.2). The pH was measured using a pH meter (model 8417, Hanna Instruments, Singapore). In the first 4 h of fermentation, log cfu counts of Lh 881315, Lh 881188, and Lh 880474 strains in samples with yeast cells were approximately 2.5 to 5.4 log cfu mL^{-1}. Log cfu counts increased significantly from (5.4 - 6.47 log cfu mL^{-1}) ($P > 0.05$) at 4 h and 8 h. PH was (2.9-5) after which Lh strains showed gradual growth decrease at 12 h. This was most likely due to alcohol production by yeast (Figure 5.1 A, B) compared to the LAB strains alone in RSM in the same conditions (Figure 5.2 A, B). Whereas, regarding *L. acidophilus* (La), *L. delbrueckii subsp. bulgaricus* (Lb) or *L. Casei* (Lc), the growth decreased from (2.5-4.9) log cfu mL^{-1} between 4 and 12 h, pH (4-5.2). Bacterial growth significantly ($P < 0.05$) affected fermentation time, supplemented with *K. marxianus* and strain type (Leclerc et al., 2002; Kilpi et al., 2007). *L. acidophilus* (La), *L. delbrueckii subsp. bulgaricus* (Lb), or *L. Casei* (Lc), the growth increased from 2.5 to 6 log cfu mL^{-1} between 4 and 8 h. Similarly, growth decreased from 6 to 5.5 log cfu mL^{-1} between 8 and 12 h fermentation, due to alcohol production (Belem & Lee, 1999) (Figure 5.1, 5.2), compared to 4.6 log cfu mL^{-1} for *K. marxianus* as control at pH 3.5 (Figure 5.1A, B). In general, the lowest pH value obtained for Lh strains (881315, 881188, and 880474) with *K. marxianus* on average after 4 h fermentation ranged between 3.2 to 3.5 compared with *K. marxianus* as control pH 5.1 (Belem & Lee, 1999; Yadav et al., 2014). The growth of probiotic strains, Lh 881315, Lh 880474 and La, Lb and Lc were slightly decreased between 8 h and 12 h, whilst the growth was stable between 8 h and 12 h for Lh 881188. This suggests an imbalance of glycolytic metabolism over oxidative metabolism and growth limitation during fermentation, due possibly to lactose consumption and ethanol production by yeast (Belem & Lee, 1999; Yadav et al., 2014). However, a previous study (chapter 3 and 4) used the same strains to ferment RSM separately reported that *L. helveticus* 881188 showed the highest growth compared to other strains in RSM whilst, *L. helveticus* 881315 showed the least growth at pH 3.4, possibly due to low pH and heat treatment reducing available nutrients for growth (Ahtesh et al., 2016b). There was no significant ($P > 0.05$) difference in growth between yeast and strain combination in the initial fermentation in RSM. In general, *K. marxianus* LAF4 decreased log counts of approximately (~12 to ~2.8 log cfu mL^{-1}) between 4 h and 12 h was observed

(Figure 5.1, 5.2). Similar counts have been reported using LAB and yeasts (*Saccharomyces. sp* and *Candida. sp)* to hydrolyse milk protein (Isono, Shingu, & Shimizu, 1994; Kebede et al., 2007). In comparison, a study demonstrated that yeasts and LAB strains counts ranged from 6.0 to 8.0 log cfu mL^{-1} after two days of milk fermentation (Mathara et al., 2004). Overall, the highest growth observed in RSM fermented using LAB strains with yeast was Lh 881188 and with *K. marxianus* at 8 h compared to *K. marxianus* as control (Figure 5.1B). Similarly, a study by Mathara et al. (2004) reported bacterial counts in milk fermented with yeast and bacteria as 5.8 log cfu mL^{-1} for *Enterococcus* and 4.24 to 7.44 log cfu $mL,^{-1}$ for yeast at pH 4.5. However, Chaves-López et al., (2012) reported substantial increased cell counts for *K. marxianus* KL26A during 72 h fermentation. The yeast counts recorded in this study were similar to those reported previously (Isono et al., 1994; Mathara et al., 2004), which indicated *Saccharomyces. sp* and *Candida. sp* growth in Tanzanian and Kenyan traditional fermented milks ranged from 6.0 to 8.0 log cfu mL^{-1} and 4.3 to 7.4 log cfu mL^{-1} respectively. The highest log coliform counts in our study increased gradually from 4.5 log cfu mL^{-1} to a maximum of 12 cfu mL^{-1} for Lh 881188 after 8 h of fermentation at 37°C (Figure 5.2). This corresponded to a final pH range of 3.1 at 12 h fermentation, which was dependent on strain type as well as yeast supplementation (Figure 5.1). Conversely, Roostita et al., (1996) reported that *K. marxianus* showed strong utilisation of lactose and weak metabolism of citrate, protein and fat resulting in the production of ethanol with strong growth responses.



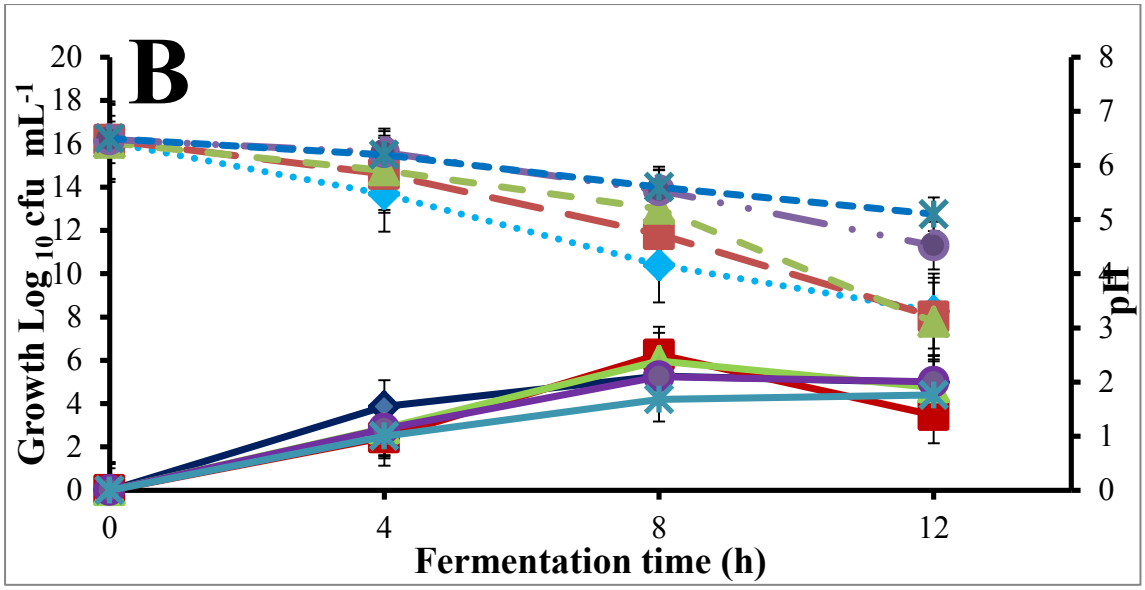
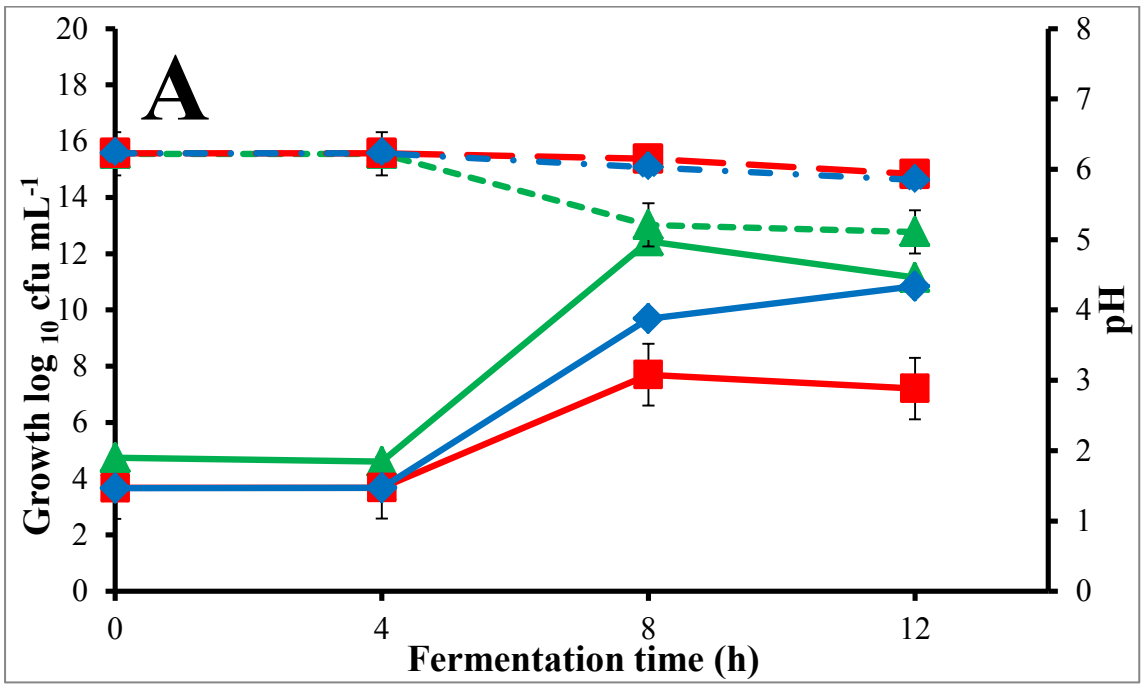


Figure 5.1 The growth (log_{10} cfu mL^{-1}) (lines-right)) and pH (dotted line-lift) characteristics. (**A**) Combination of *Kluyveromyces marxianus* with each of *L. acidophilus (—◆—), L. delbrueckii subsp. bulgaricus (—■—)* or *L. Casei (—▲—*), *and Kluyveromyces marxianus (—✱—) as control*. (**B**) Combination of *Kluyveromyces marxianus with L. helveticus* strains Lh 881315(—◆—), Lh 881188(—■—), Lh 880474(—▲—) and Lh 880953(—●—), separately in 12 % RSM at 37 °C for 12 h, (results were expressed as mean values of 3 replicates with standard deviation).



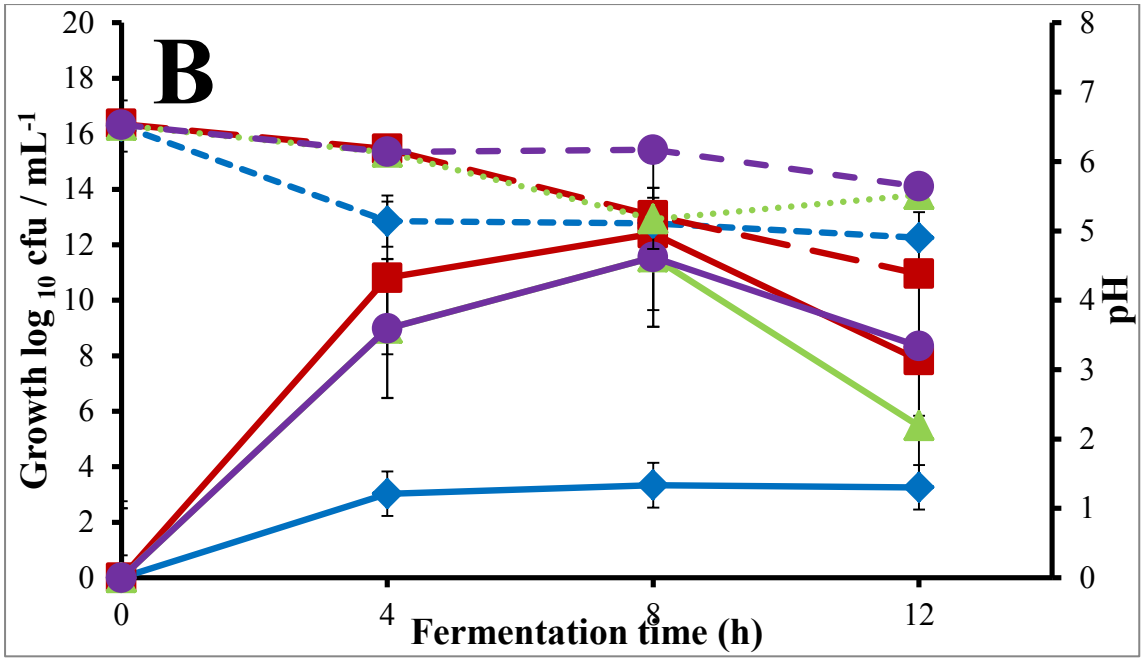


Figure 5.2 The growth (log_{10} cfu mL^{-1}) (lines-right)), and pH (dotted line-lift) characteristics of (**A**) *L. acidophilus* ( ) *L. delbrueckii subsp. bulgaricus* ( ), or *L. Casei* ( ) and (**B**) *L. helveticus strains* Lh 881315( ), Lh 881188( ), Lh 880474( ) and h 880953( ), separately in 12 % RSM at 37°C for 12 h. (results were expressed as mean values of 3 replicates with standard deviation).

5.4.2 Proteolytic activity of *Kluyveromyces* is higher in fermented RSM media

The effectiveness of proteolytic activity of *K. marxianus* in combination with LAB strains in RSM compared to strains alone without combination were assessed for the ability to hydrolyse skim milk protein. Most of the combinations with strains and yeast had the ability to hydrolyse milk proteins into amino acids compared with fermented RSM by yeast alone (Figure 5.3 A, B). O-pathalaldehyde (OPA) index of yeast and Lh 881315 was higher than Lh (881188, 880474 and 880953) strains (Figure 5.3), compared to strains alone in RSM at 37°C for 12 h (Figure 5.4). It has been reported that the proteolytic activity of *K. marxianus* is higher than *Saccharomyces cerevisiae*, evidenced by increased amino acid content in milk (Roostita & Fleet, 1996). Similarly, yeasts from Kumis showed proteolytic activity although at lower levels (Chaves-López et al., 2012). The greatest increase in proteolytic activity was detected in samples fermented in combinations of Lh 881315 with *K. marxianus*, Lh 881188 with *K. marxianus*, compared to *K. marxianus* as control. Whilst, results reported by Roostita

& Fleet (1996) and Chaves-López et al., (2012) reported that *K. marxianus* had greater proteolytic activity than *S. cerevisiae* as evidenced by the increase of the amino acids content in milk. Proteolytic activities were also detected in different fermentation times. However, the level of activity was dependent on strain type. Those results indicated that *Kluyveromyces marxianus* LAF4 has high proteolytic activity.

5.4.3 ACE-inhibition activity of fermented skim milk

Results presented in (Figure 5.3, 5.4) show ACE-I activity of LAB and *K. marxianus* used as controls and in combination of *K. marxianus* with LAB in fermented skim milk at 37°C for 12 h. The ACE-I increased at varying activities in all samples up to 8 h fermentation after which the activity was almost stable for all combinations, compared with *K. marxianus* alone in which ACE-I activity increased up to 12 h ($P < 0.05$) (Figure 5.4 A). The highest ACE-I activity reached was sample fermented with *K. marxianus* alone which yielded 60 % ACE-I at 12 h, compared with the samples fermented with combination forms. However, ACE-I activities between strains in combination with *K. marxianus* showed varying differences ($P < 0.05$) (Figure 5.3) whereas, *L. Casei* with *K. marxianus* showed the lowest ACE-I activity (15 %) (Figure 5.3 A) compared to the same strain without combination (50 %) (Figure 5.4 B). A study by Hamme et al., (2009) noted that ACE-I activities of different yeast strains, *Candida lusitaniae* KL4, *P. kudriavzevii* KL52 and *G. geotrichum* KL20A in milk whey, were high (60 % - 72 %) after 52 h of fermentation at 37°C. These results with long fermentation time are possibly due to production of ethanol by *K. marxianus* lactose during the fermentation period (Roostita & Fleet, 1996). In the conditions tested, yeasts efficiently degraded casein since almost complete hydrolysis of the protein was observed (Jakobsen & Narvhus, 1996; Amrane & Prigent, 1998; Chaves-López et al., 2012; García-Tejedor et al., 2013). The agreements with experiments by García-Tejedor et al. (2013) in a study using different strains of yeast including *K. marxianus* to ferment milk separately for seven days, the highest ACE-I provoked by casein-derived peptides was 55 %, corresponding to hydrolysates generated by *K. lactis* Kl3 and *K. marxianus* K2. However, in this study the ACE-I activity of *K. marxianus* as control was 60 % at 12 h of fermentation compared to combination with strains (45 %) for strain Lh 881315 and 880953. In a recent study using the same strains, Lh 881315 and 880953 separately, to ferment 12 % RSM demonstrated that the highest ACE-I activity was 75 % in the same conditions (Ahtesh et al., 2016b). Hence, supplementation of *K. marxianus* affects the ACE-I activity which correlated to growth

activity of strands. As demonstrated by this study on the production kinetics of ACE-I peptides, the fermentation of milk by yeasts and or combination of yeast with LAB strains are prone to a dynamic system where peptides are constantly released; some of them are subsequently hydrolysed and most likely utilized for cell growth, while others accumulate during fermentation. It is well known that the production of high quality fermented dairy products depends on the proteolytic system of strains used, since peptides and the amino acids formed have a direct impact on flavour or act as flavour precursors (Williams and Banks, 1997).

Overall, these results clearly show increased ACE-I activity in the samples after fermentation depending on the strain type and fermentation time. In addition, this current research study shows that there is an increase of ACE-I activity in the samples fermented by yeast of *K. marxianus* separate more than using a combination with LAB strains. In fact, Chaves-López, et al. (2012) reported that peptide profiles show characteristic differences among strains. In fact, samples fermented with *K. marxianus* and LAB led to a decreased number of peaks between 8 and 12 h fermentation due to increased alcohol production by *K. marxianus* yeast during the fermentation period and that results affect of bacteria growth (Roostita & Fleet, 1996).

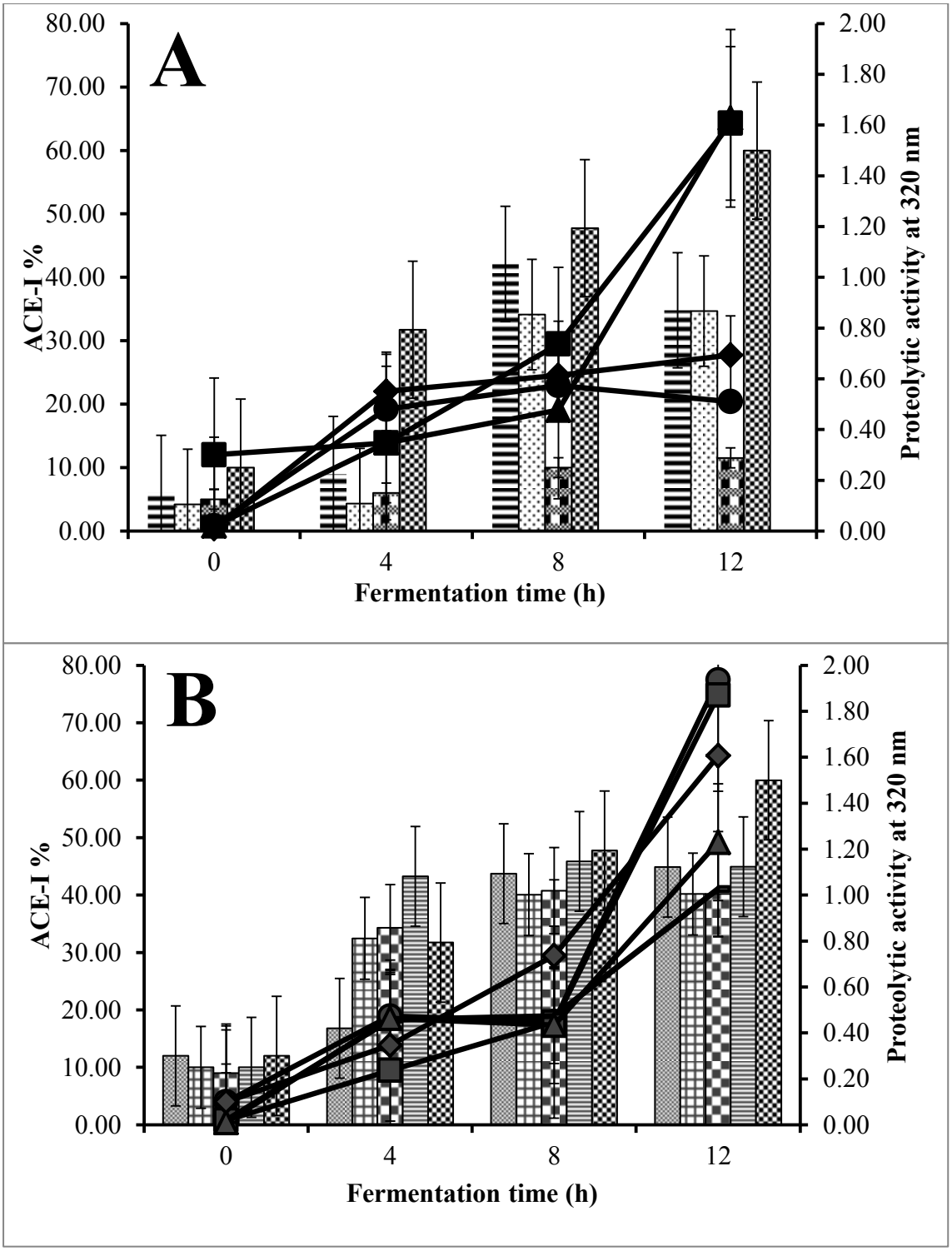


Figure 5.3 The absorbance of proteolytic activity at 320nm (lines-right)) and ACE-I % (bars-lift) of 12 % RSM fermented by a combination of (**A**) *Kluyveromyces marxianus* ( ) with LAB strains; *L. acidophilus* ( ), *L. delbrueckii subsp. bulgaricus* ( ), *L. Casei* ( ) (**B**) and Combination of *Kluyveromyces marxianus* ( ) with, *L. helveticus* strains; Lh 881315 ( ), Lh 881188 ( ), Lh 880474 ( ) and Lh 880953 ( )at 37°C for 12 h fermentation. (results were expressed as mean values of 3 replicates with standard deviation).

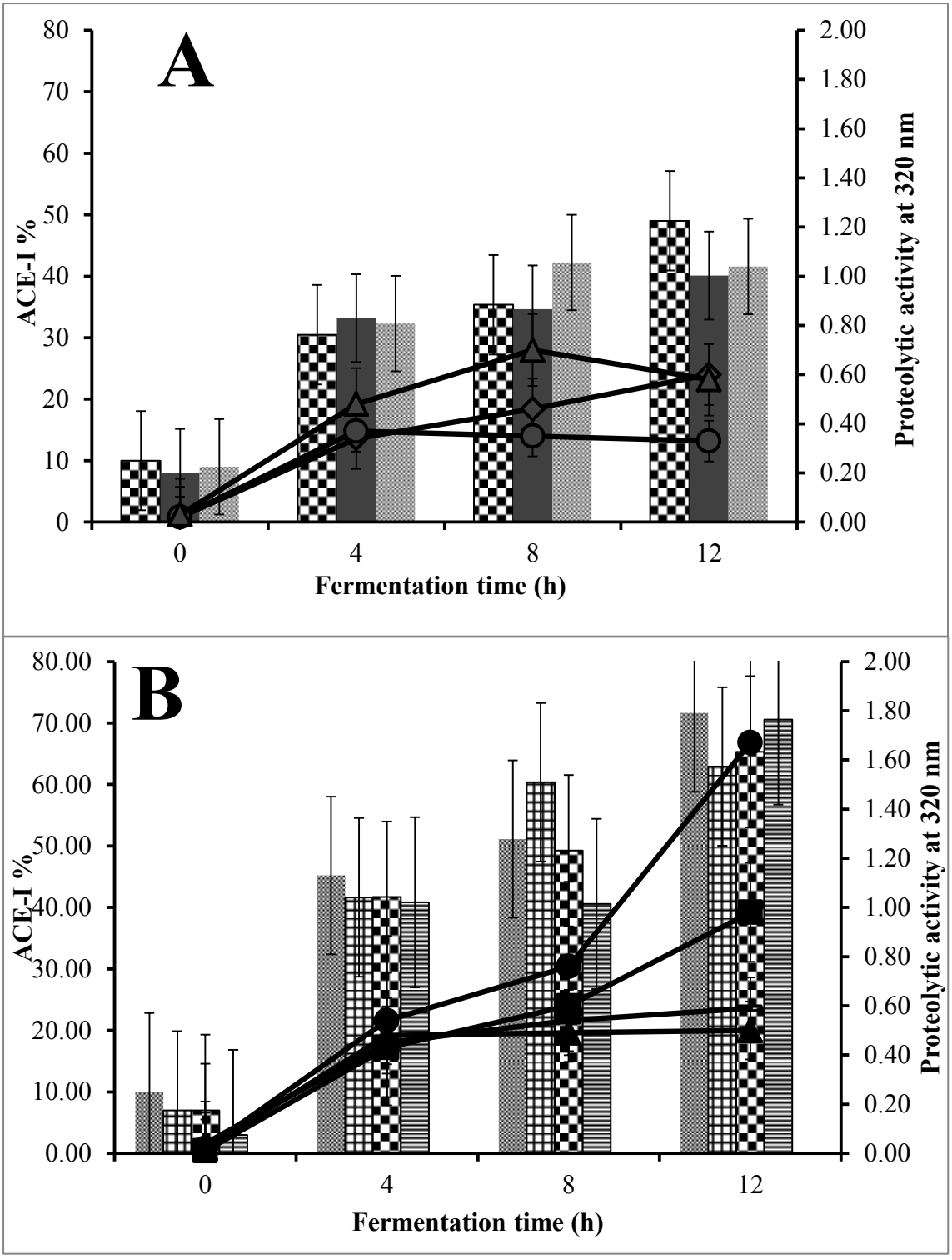


Figure 5.4 The absorbance of proteolytic activity at 320 nm (lines-right)) and ACE-I % (bars-lift) of 12 % RSM fermented by (**A**) LAB strains; *L. acidophilus* ( ), *L. delbrueckii subsp. bulgaricus* ( ), *L. Casei* ( ) at 37°C for 12 h fermentation. (**B**) LAB strains; *L. helveticus* strains; Lh 881315 ( ), Lh 881188 ( ), Lh 880474 (and, Lh 880953 ( ), at 37°C for 12 h fermentation. (results were expressed as mean values of 3 replicates with standard deviation).

5.4.4 RP-HPLC analysis of water-soluble peptide extracts

Reverse phase-HPLC was used to show the degree of proteolysis in fermented skim milk using a combination of LAB with *K. marxianus*, *K. marxianus* alone as control (1) and untreated skim milk as control (2) as presented in Figure 5.5 and Figure 5.6. A higher number of peptides were released when *K. marxianus* alone fermented in RSM for 12 h compared with *K. marxianus* combined with LAB strains and untreated RSM as control (Figure 5.5 and 5.6). In general, peptide peaks were appearing between 5 to 37 mins (Figure 5.5 and 5.6, line B). Whilst in line (F), peptide peaks were appearing between 5-45 mins due to milk caseins incomplete hydrolysis (Figure 5.6, lines D and F). However, peaks were observed between retention times 5 to 37 and the number of peaks were more when samples were fermented by *K. marxianus* alone as control (Figure 5.5 and 5.6, line B) compared to the untreated skim milk and to the combination forms which have less number of peaks. Chaves-López et al., (2012) reported that peptide profiles show characteristic differences among strains. In fact, samples fermented with *K. marxianus* and LAB led to a decreased number of peaks between 8 and 12 h fermentation due to increased alcohol production by *K. marxianus* yeast during the fermentation period (Figure 5.4 and 5.5) and that results correlated to bacterial growth (Roostita & Fleet, 1996).

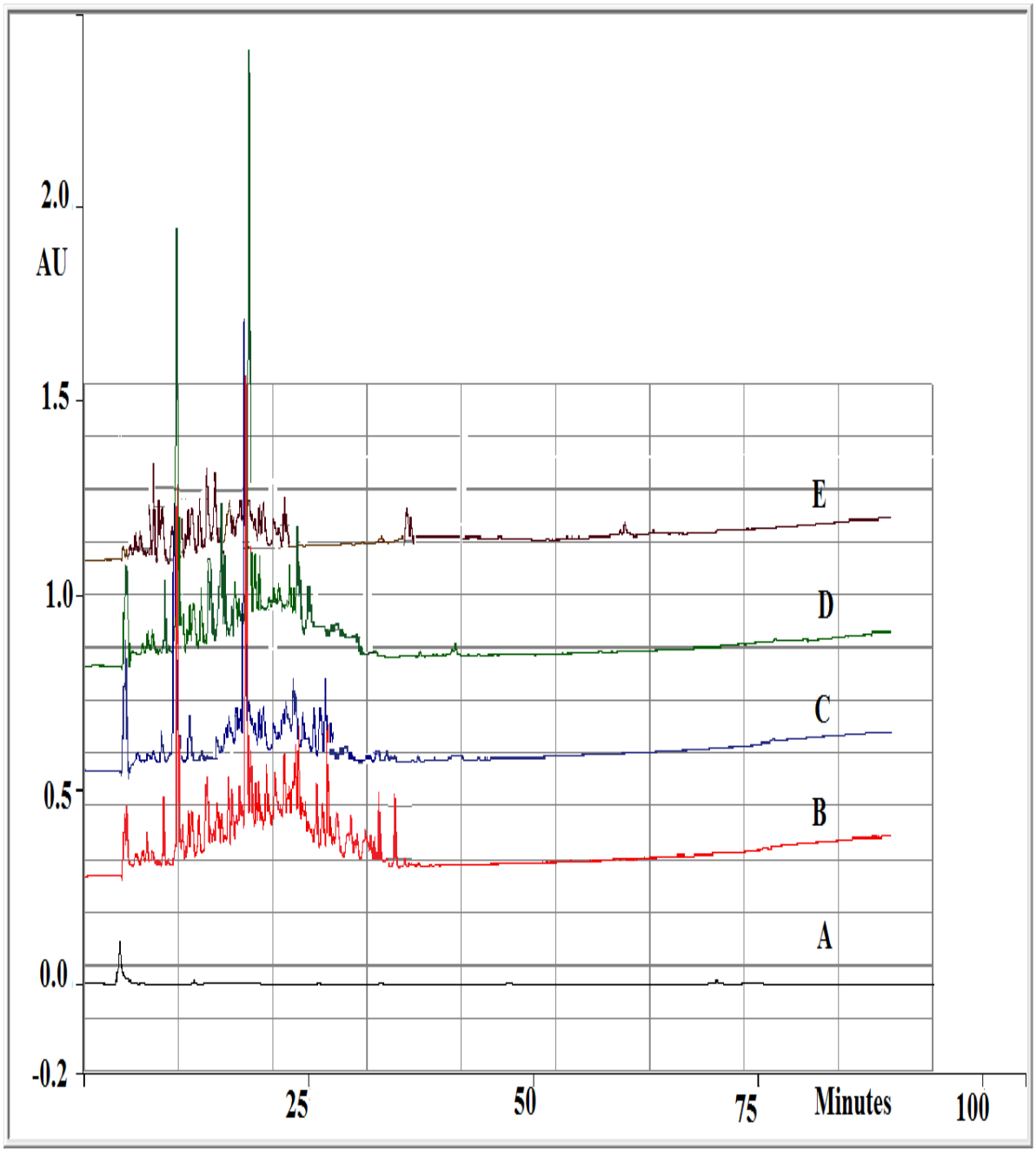


Figure 5.5 RP-HPLC peptide profile of water soluble extracts obtained from fermented skim milk with combination of LAB strains and *Kluyveromyces Marxianus;* (**A**) untreated RSM as control, (**B**) fermented skim milk by *K. marxianus* only, (**C**) *K. marxianus* and *Lactobacillus Casei*, (**D**) *K. marxianus* and *Lactobacillus delbrueckii subsp. bulgaricus* and (**E**) *K. marxianus* with *Lactobacillus acidophilus* during 12 h fermentation at 37° C.

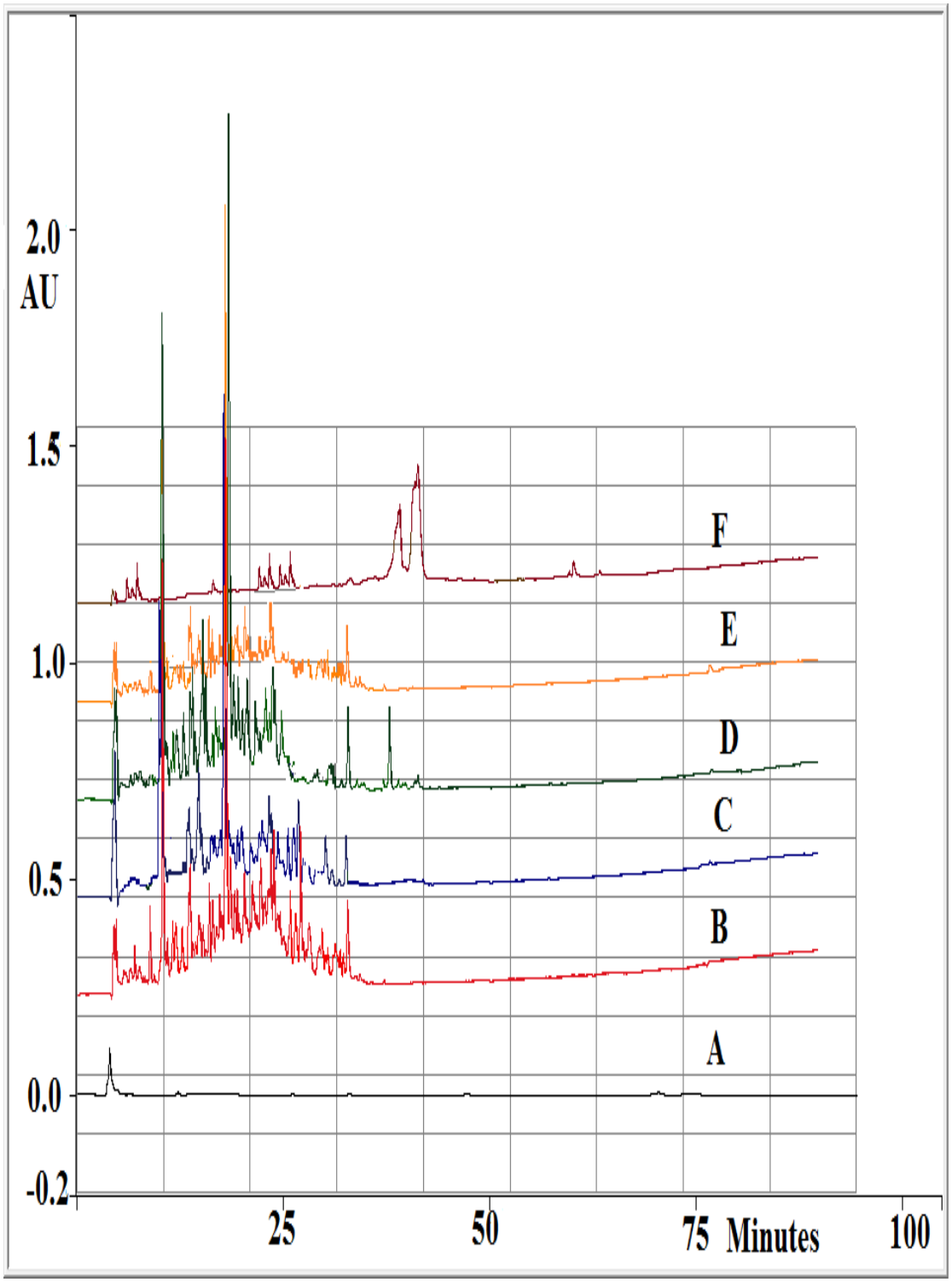


Figure 5.6 RP-HPLC peptides profile of water soluble extracts obtained from fermented skim milk made with combination of *L. helveticus* strains and *Kluyveromyces marxianus;* (**A**) untreated RSM as control (**B**), fermented skim milk by *K. marxianus* yeast only, (**C**) *K. marxianus* yeast and, Lh 880953, (**D**) *K. marxianus* yeast and Lh 881315, (**E**) *K. marxianus*. Yeast and Lh 880474, and (**F**) *K. marxianus* yeast with Lh 881188, during 12 h fermentation at 37°C.

5.5 Conclusion

The *K. marxianus* produced > 60 % ACE-I in 12 % RSM and there appears to be no significant advantage of co-culturing it with LAB for the conditions investigated. Since, the combination of *K. marxianus* with LAB led to reduced bacterial count, the production of ACE-I peptides was adversely impacted. Further research is needed to discover the understanding of the mechanism of inhibition and adaptation for the design of suitable combination for ACE-I peptides production strategies. Also, new yeast strains with superior biotechnological capabilities need to be further evaluated.

Chapter 6 Identification and purification of peptides from skim milk protein hydrolyses by combination of *L. helveticus* 8801315 and Flavourzyme®.

This chapter has been submitted for publication. Ahtesh F., Apostolopoulos V., Vijay M., Stojanovska L., (2016). Identification and purification of peptides from skim milk protein hydrolyses by combination of *L. helveticus* 8801315 and Flavourzyme®. *Journal of Food Chemistry.* (Appendix. I).

6.1 Introduction

In addition to providing a source of nutrients and energy, fermented dairy products are also a source of bio-functional peptides that may impart improved health benefits when ingested (FitzGerald & Murray, 2006). Hypertension (high blood pressure) affects 1/4 adults worldwide (He et al., 2013; Otte et al., 2007), with 1/3 in the western population (Kearney et al., 2005). Angiotensin converting enzyme-inhibitory (ACE-I) peptides released from milk proteins have been used to treat hypertension in spontaneous hypertensive rats (SHR) (Fernández-Musoles et al., 2013; Ramchandran & Shah, 2011; Seppo et al., 2002; Wang et al., 2012; Yoshii et al., 2001). Several ACE-I peptides have been isolated from enzymatic hydrolysis of milk proteins (Hernández-Ledesma et al., 2004; Hernández-Ledesma et al., 2011; Hernández-Ledesma et al., 2014; Tauzin et al., 2002). Based on their extensive proteolytic systems, they are perfectly adapted to grow in milk (Degraeve & Martial-Gros, 2003). Several identified peptide sequences have been released from different milk proteins after fermentation and are able to inhibit ACE activity (Eisele et al., 2013; Jauregi & Welderufael, 2010; Pihlanto-Leppälä, 2000). In particular, regarding fermented skim milk, the authors previously reported that *Lactobacillus helveticus* (*L. helveticus*) strains combined with Flavourzyme® lead to increased ACE-I activity of fermented skim milk (Ahtesh et al., 2016b). In addition, skim milk fermented with *L. helveticus* and *Saccharomyces*, displayed a systolic blood pressure (SBP) decrease in mildly hypertensive human volunteers ranging from 4.6 to 14.1 mmHg (Fitzgerald et al., 2006). These human hypertensive effects have, in part, been attributed to the release of potent casein-derived tri-peptide inhibitors of ACE during fermentation. Tri-peptides such as, Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP) have positive effects on human and rat blood pressure (Jauhiainen et al., 2005; Lehtinen et al., 2010; López Expósito & Recio, 2006; Narva et al., 2004; Jauhiainen et al., 2010). ACE-inhibition has two biological activities: deca-peptide angiotensin-1, (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and nano-peptide bradykinin, (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) activities (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Two peptides, Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and Hip-Phe-Arg have similar binding affinity tendencies as bradykinin and Angiotensin-II, showing substrate specificity of ACE-I activity (Cheung et al., 1980). Several studies have used different processes for the production of bioactive peptides with ACE-I activity based on enzymatic hydrolysis of milk protein such as fermentation by bioreactor (Otagiri et al., 1985; Welderufael, Gibson, & Jauregi, 2012; Wu, Aluko, & Nakai, 2006). ACE-I

activity was correlated with a high degree of hydrolysis (Gonzalez-Gonzalez et al., 2011). For instance, different strains of *L. helveticus* have the ability to express proline-peptidases leading to the release of peptides IPP and VPP during milk fermentation (Seppo et al., 2002; Jauhiainen, 2010; Gonzalez-Gonzalez et al., 2013). However, there were no reports regarding the identification of ACE-inhibitory peptides released from fermented skim milk with *L. helveticus* combined with Flavourzyme®.

Herein, the author investigated hydrolyses of skim milk proteins by using a combination of *L. helveticus* ASCC 8801315 and Flavourzyme® for the release of bioactive peptides with ACE-I properties. I isolated and identified these peptides from fermented reconstituted skim milk (RSM). The peptide and amino acid profiles were analysed.

6.2 Material and Methods

6.2.1 Substrates and Chemicals

Trichloroacetic acid (TCA), O-phthaldialdehyde (OPA), Hippuryl-Histidyl-Leucine, de Man Rogosa and Sharpe (MRS) bacteriological medium, sorbitol, bacteriological agar, trifluoroacetic acid (TFA), β-mercaptoethanol, Tris-HCl, glycerol, dithiothreitol, acetic acid were purchased from Sigma Chemical Company (St Louis, MO USA). Skim milk powder (52 % lactose, 37 % protein, 8.6 % ash and 1.2 % fat) was purchased from Murray Goulburn Co-operarative Co. Ltd. (Brunswick, Vic Australia). Acetonitrile was purchased from (Merck, Darmstadt, Germany) and bacteriological peptone was purchased from (Oxoid, West Heidelberg Australia). Flavourzyme®1000 L (EC 3.4.11.1, an amino peptidase with an activity of 1000 LAPU g^{-1}) was purchased from (Novozymes Australia, North Rocks, NSW Australia).

6.2.2 Propagation of cultures and preparation of fermented RSM

L. helveticus strain 881315 (Lh 881315) was obtained from (Dairy Innovation Australia Ltd, Werribee, Victoria, Australia) and stored at -80°C. Sterile 10 mL aliquots of MRS broth were inoculated with 1 % culture and incubated at 37°C for 18 h. Lh 881315 was inoculated at 1 % (v/v) into 10 mL aliquots of reconstituted skim milk (RSM, 12 % w/w) supplemented with 0.14 % Flavourzyme®. Following two successive transfers the cultures were finally transferred into sterile RSM. Reconstituted skim milk was

prepared by dissolving skim milk powder with distilled water. Reconstituted skim milk was heat-treated at 90°C for 20 min, cooled to 42°C.

6.2.2.1 Bioreactor assay of Low fat skim milk to increase the ACE-I % activity

Bioreactor (5-Litres) capacity (Bio-Stat® A plus, Germany) was employed to ferment 5 L of 12 % pasteurised RSM using a combination of Lh 881315 and Flavourzyme® at 37°C for 12 h. A jacketed thermostatic water bath bioreactor held at a constant temperature was used. Milk was continuously stirred by impellers (at 250 rpm). The pH during fermentation was measured using a sterile pH electrode (DPAS Ingold, Paris, France) connected to a transmitter (Demca 3B 1015; Alfortville, France). The pH electrode was calibrated before inoculating the medium. Bacterial growth, proteolytic and, ACE-I activities and pH were determined at 0, 2, 4, 6, 8 and 12 h of fermentation.

6.2.3 Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate studied (Ravallec-Plé et al., 2000) was calculated from the ratio of α-amino nitrogen (AN) to the total protein nitrogen (TPN) (Chen, Tao, & Li, 2003). Briefly, 60 mL of sample aqueous solution (0.05 mg mL^{-1}) was titrated with 0.05 M NaOH until its pH value reached 8.2. After adding 10 mL of 20 % (v/v) formaldehyde aqueous solution, the resulting solution was mixed and titrated again with 0.05 M NaOH until its pH value reached 9.2. The volume (mL) of 0.05 M NaOH added in the second-step titration was recorded as (V1). Deionised water was used as blank (V0) instead of a sample. The calculation formula used was α-amino nitrogen (%) = (V1–V0) C × 0.014/ M × 100 %, where C is the molar concentration of the aqueous NaOH used; M is the weight (g) of the sample used, using the equation:

$$\text{DH (\%)} = \frac{\alpha - \text{amino nitrogen (AN)}}{\text{total nitrogen (TN)}} \text{ x } 100$$

6.2.4 Determination of ACE inhibitor activity

A crude extract of the fermented sample (50 mL) was prepared by centrifugation at 4000 x g at 4°C for 30 min using Beckman Coulter (Avanti J-26S XPI) and the supernatant was freeze-dried (Freeze-drier model ALPHA 1-4 LSC plus; John Morris Scientific Pty. Ltd. Deepdene Australia) for 72 h. The freeze- dried extract (40 mg) was dissolved in 2 mL of Tris buffer (50 mM, pH 8.3) containing 300 mM Sodium chloride (Donkor et al., 2007) as previously reported (Ahtesh et al., 2016b) . Fifty µL of 1.25 mU ACE enzyme from rabbit lung in Tris buffer and 50 µL of 3.0 mM Hippuryl-Histidyl-Leucine (HHL) in Tris were added to 50 µL of sample and incubated at 37°C in a shaking water bath for 30 min. 150 µL of Glacial acetic acid was added to stop the reaction. The amount of Hippuric acid (HA) released was analysed by HPLC. The HPLC system consisted of a Varian 9012 solvent delivery, a Varian 9100 auto-sampler and a Varian 9050 variable wavelength ultraviolet-visible detector. An analysis was carried out using Gemini® C18 110 Å (100 mm x 4.60 mm, 3 µm) column (Phenomenex, NSW Australia) at room temperature (~22°C) with a mobile phase consisting of 12.5 % (v/v) Acetonitrile (Merck) in distilled water, pH adjusted to 3.0 using glacial acetic acid. The flow rate was set at 0.6 mL min^{-1} and the compounds were detected at 228 nm. The percentage ACE-I was calculated as follows:

$$\text{ACEI } \% = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100$$

A standard curve of HA was constructed using five predetermined concentrations (0.5 %, 1.0 %, 1.5 %, 2.0 %, and 2.5 %) for quantification of HA in the samples. ACE-I activity data were plotted against protein concentration in the sample in order to calculate IC_{50} value, defined as the protein concentration (µg mL^{-1}) needed to inhibit 50 % of ACE-I activity (Mullally et al., 1997).

6.2.5 Micro-fluidic Lab-on-a- chip electrophoresis (Loa C)

This method was performed on an Agilent 2100 bio-analyser (Agilent Technologies, Wald Bonn Germany), using High Sensitivity Protein 250 Reagents and the 2100 software. Samples, dye and the preparation of chip were carried out according to the manufacturer's protocol and as described by Nikolić, et al. (2012) with some modifications. Briefly, 0.5 µL of reconstituted dye solution added to 5 µL of protein ladder (5 - 240 kD), 5 µL of sample in micro tubes respectively, vortexed and incubated

for 30 min on ice. The samples and protein ladder in tubes were heated (95 °C, 5 min), all tubes were cooled for 15 s to recover the condensate of liquid and then briefly spun in a centrifuge (3000 x g) to ensure that the liquid sample and any condensate collected at the bottom of the tube. Distilled water (85 µL) added to the protein ladder and milk samples to give each a total volume of 90 µL. All samples were thoroughly mixed and incubated on ice before use. In a typical analysis, a new chip is primed with gel–matrix after which the protein ladder (6 µL) and samples (6 µL) are loaded and analysed.

6.2.6 Isolation and identification of peptides

6.2.6.1 Isolation of ACE-inhibitory peptides by RP-HPLC

Reconstituted skim milk (12 % w/v) was fermented with *L. helveticus* 881315 and Flavourzyme®, in a Bioreactor. Fermented RSM samples were centrifuged at 14,000-x g for 30 min (Sorvall RT7, Newtown, CT USA). The supernatant was freeze-dried (John Morris Scientific, Pty. Ltd. Deepdene, VIC Australia) and further analysed using reversed-phase high performance liquid chromatography (RP-HPLC, Varian Analytical Creek, CA USA). The freeze-dried supernatant (80 mg) was dissolved in 1 mL of solvent A (0.1 % Trifluoroacetic acid (TFA) in deionised water) and filtered through a 0.2 µm membrane filter (Schleicher & Schuell GmbH Germany). A sample (1 mL) was injected onto an RP-column C-18 Jupiter Proteo 90 A 250 mm x 10.0 mm, 10 micron (Phenomenex. Lane Cove NSW Australia Pty Ltd). The mobile phase was 0.05 % solvent A (0.1 % trifluoroacetic acid (TFA) in deionised water) and 60 % solvent B (0.1 % TFA in 90 % v/v acetonitrile in deionised water). Samples were eluted by a linear gradient from 0 -100 % solvent A and over 90 % solvent B at flow rate of 1 mL min^{-1}. Elution profiles of samples were detected by a UV detector set at 214 nm for 70 min. The RP-HPLC separation procedure was repeated 15 times to obtain higher concentrations and those, which were separated into six fractions. The fractions were concentrated using a vacuum evaporator (Speed Vac SC110 concentrator, Savant Instruments Inc. Farmingdale, NY USA) and stored at -80°C until further analysis. Aliquots of 50 µL of each concentrated fraction were used to determine ACE-I activity and fractions with the highest ACE-I were selected for further purification.

6.2.6.2 Identification of ACE-I peptides

6.2.6.2.1 Nano-LC/MS/MS analysis

The freeze-dried peptide extract fractions (F1 and F6) which gave the highest ACE-I activity were sent to the Australian Proteome Analysis Facility (APAF) for analyses of peptides (Appendixes II). Each peptide/s fraction was introduced into the QSTAR Elite Mass Spectrometer (AB Sciex, MA USA) coupled with the Exigent TEMPO Nano-LC (AB Sciex, CA USA). For each analysis, a sample was loaded into a commercial 0.5 mm × 1.3 mm, capillary trap column (0.5 μL, C18, Optimize Technologies, Inc., Oregon City, Oregon USA) and a 10 cm × 300 μ m analytical column (3 μ m particle sizes, Proteo Col G C18, SGE Analytical Science, Melbourne Australia). Eighty-three min LC gradient was used to separate the peptide mixtures with a flow rate of 500 nL min^{-1}. Each reverse phase (RP) began with 5 % mobile phase B, a gradient elution from 5 -10 % mobile phase B for 1 min, 10-40 % for 39 min, 40-100 % for 10 min, 100-5 % mobile phase B for 1 min, and then 5 % mobile phase B for 9 min for re-equilibration. For MS parameters, a full-mass scan was performed between *m/z* 400 and *m/z* 1600, 0.5 s accumulation time, followed by MS/MS scans of the top 3 high-intensity precursor ions (charge state +2 to +4, and ion count > 25) by Collision Induced Dissociation (CID). The dynamic exclusion duration was 20 s, switched after 1 spectrum.

For identification of peptides by Nano-LC/MS/MS, mascot generic format files (MGF) were generated from format files (wiff), (QSTAR Elite MS) with MASCOT script. These were searched against the in-house server of MASCOT Version 2.3.2, using the Swiss-Prot protein database (539 829 sequences, 2013). Other bacteria species were searched (13, 034 and 328 828 sequences in Swiss-Prot database, respectively) and chosen for taxonomic categorisation. Precursor and product ion-mass-tolerance were set at 300 ppm and ± 0.6 Da, respectively. Enzyme restriction was set as none, and a maximum of one missed cleavage was allowed. Methionine oxidation was set as a variable modification.

6.2.6.2.2 Matrix Assisted Laser Desorption Ionisation (MALDI)-MS/MS analysis

A matrix was prepared by dissolving alpha-cyano-4-hydroxycinnamic acid (1 mg mL^{-1} in 90:9, 9:0.1 acetonitrile: water: formic acid). Samples were zip-tip extracted (Perfect Pure, C18, Eppendorf Germany) and spotted onto a MALDI target plate (1 μL) prior to

analysis. A peptide mixture containing bradykinin, angiotensin-I and neurotising (Sigma), each was at 2 p mol μL^{-1}. Adrenocorticotropic hormones (ACTH) (clip 18-39) (Sigma) at 2 p mol μL^{-1} were spotted with matrix compositions for calibration of MS. Samples and calibration standards with the same matrix composition were spotted adjacent to each other on the target plate for optimal calibration and enhanced mass accuracy.

Matrix Assisted Laser Desorption Ionisation mass (MALDI) spectrometry was performed using the 4800 plus MALDI TOF/TOF Analyser (AB Sciex, MA USA). A Nd:YAG laser (355 nm) was used to irradiate the sample. Spectra were acquired in reflector MS scan mode in the mass range of 700 to 4000 Da. The instrument was then switched to MS/ MS mode where the eight strongest peptides from the MS scan were isolated and fragmented by CID, then re-accelerated to measure their masses and intensities. A near point calibration was applied and gave a typical mass accuracy of 50 ppm or better.

For identification of proteins by MALDI-MS/MS data, text files were generated from 2d files. These were searched against the in-house server of MASCOT Version 2.3.2, using the Swiss Prot protein database (539, 829 sequences, 2013). Other mammalian and bacteria species were searched (13, 034 and 328, 828 sequences in Swiss-Prot database, respectively) and chosen for taxonomic categorization. Precursor and product ion-mass-tolerance were set at 50 ppm and ± 0.6 Da, respectively. Enzyme restriction was set as none, and a maximum of one missed cleavage was allowed. Methionine oxidation was set as a variable modification.

6.3 Statistical analyses

Using Minitab 16, all the data were expressed as mean values of three replicates with the mean (± SEM). The differences between the experimental groups were determined by one way ANOVA with mean differences tested by Tukey- test and *P*-values less than 0.05 considered to be significant.

6.4 Results and Discussion

6.4.1 Degree of hydrolysis

The degree of hydrolysis (DH) was defined as the percentage ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate (Guo, Pan, & Tanokura, 2009). Therefore, DH is an important parameter to understand and interpret the effects and extent of the hydrolytic process of proteins and is useful to establish the relationships between proteolysis and improvement of the functional, bioactive and sensory properties of these biomolecules (Cheison et al., 2009). The hydrolysis of skim milk proteins by a combination of *L. helveticus* 881315 (Lh) with (0.14 %) Flavourzyme® compared to hydrolysis of Lh or Flavourzyme® separately as controls after 12 h of fermentation at 37 °C are shown in (Figure 6.1). The hydrolysis of the combination increased rapidly in the first 1 h of fermentation and continually increased after 8 h compared to the Flavourzyme® or strain separately as controls ($P < 0.05$). No apparent hydrolysis was observed after this period, whereas hydrolysis by Lh or Flavourzyme® separately increased slowly during 8 h fermentation. However, the DH (70.9 %) during the 12 h fermentation was the highest for the combination of Lh and Flavourzyme® (Figure 6.1) whilst DH was reached (~20 % and ~10 %) during the 12 h fermentation for Lh or Flavourzyme® respectively. Similarly, it was previously reported that the DH % of whey protein concentrate ranged between (13.3 % - 21 %) during 6 h fermentation using protease from *B. licheniformis* (Spellman et al., 2003). Here it was noted that DH significantly increased with a combination of Flavourzyme® and with increased fermentation time ($P < 0.05$) (Figure 6.1). The results suggested that Flavourzyme®-supplementation had the greatest effect on the DH of skim milk protein. The significant increase in the % DH by the combined activity was likely due to the complementary substrate specificities resulting in improved proteolytic activity (Ahtesh et al., 2016b). High proteolytic activity correlated with high DH and this was reflected in the performance of the combined activities of Lh and Flavourzyme® resulting in significant increased peptide production (Cheison et al., 2009; Pihlanto et al., 2010), (Figure 6.2C). Clearly, these results indicate that an increase in hydrolysis time of skim milk proteins by combination produced more small peptides, free amino acids and less large peptides.

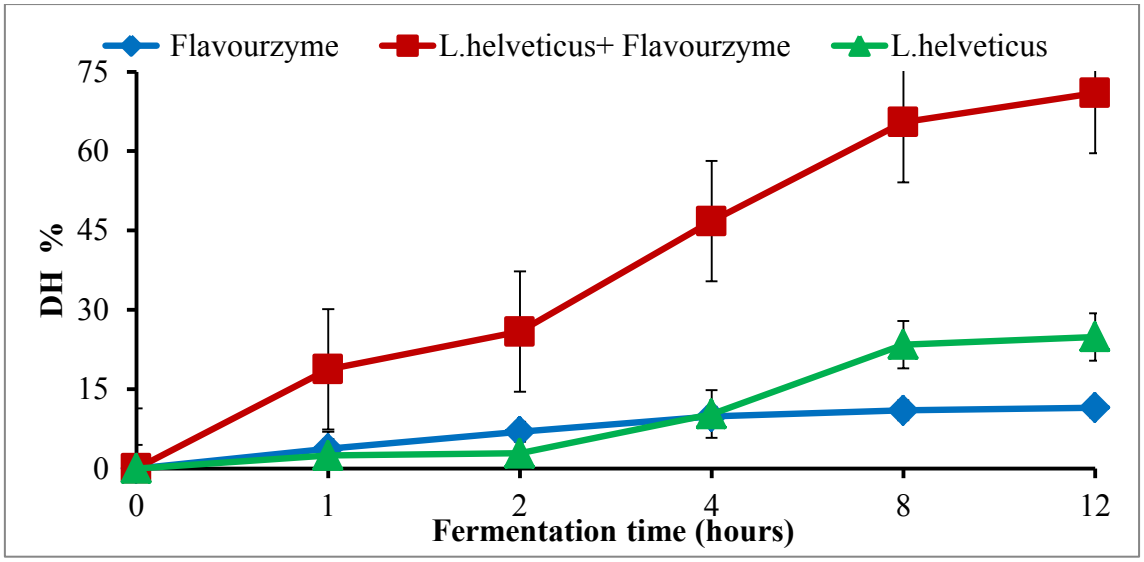
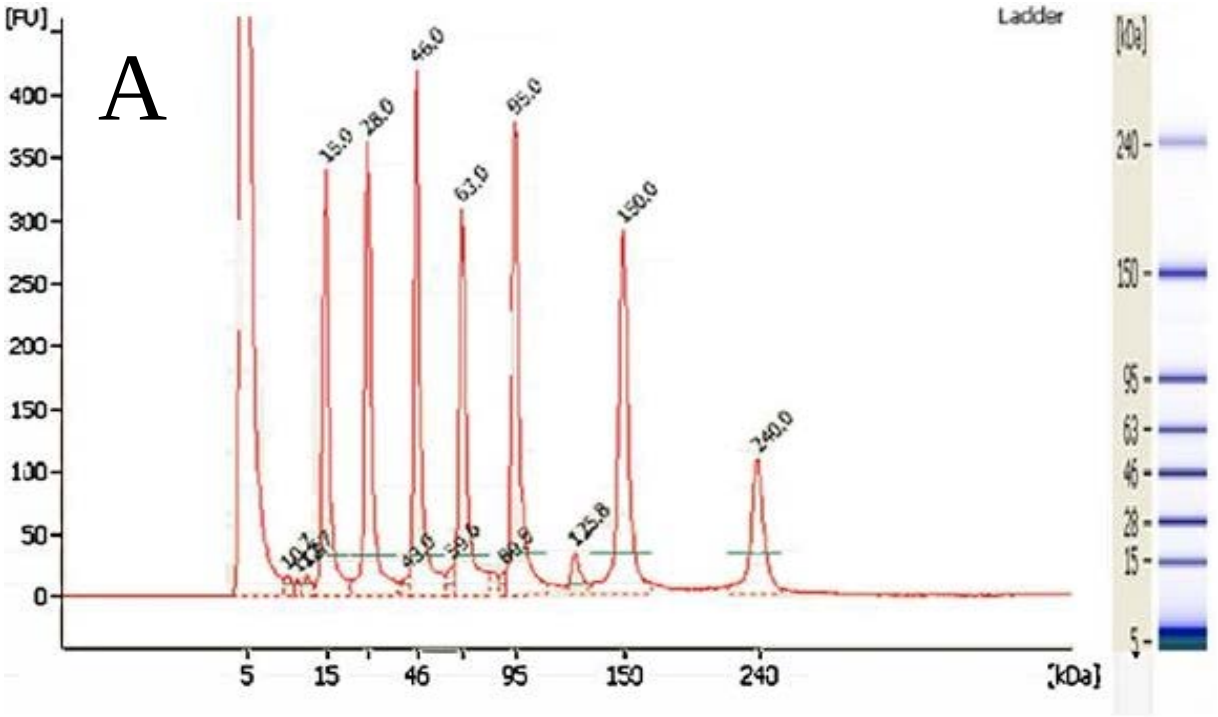
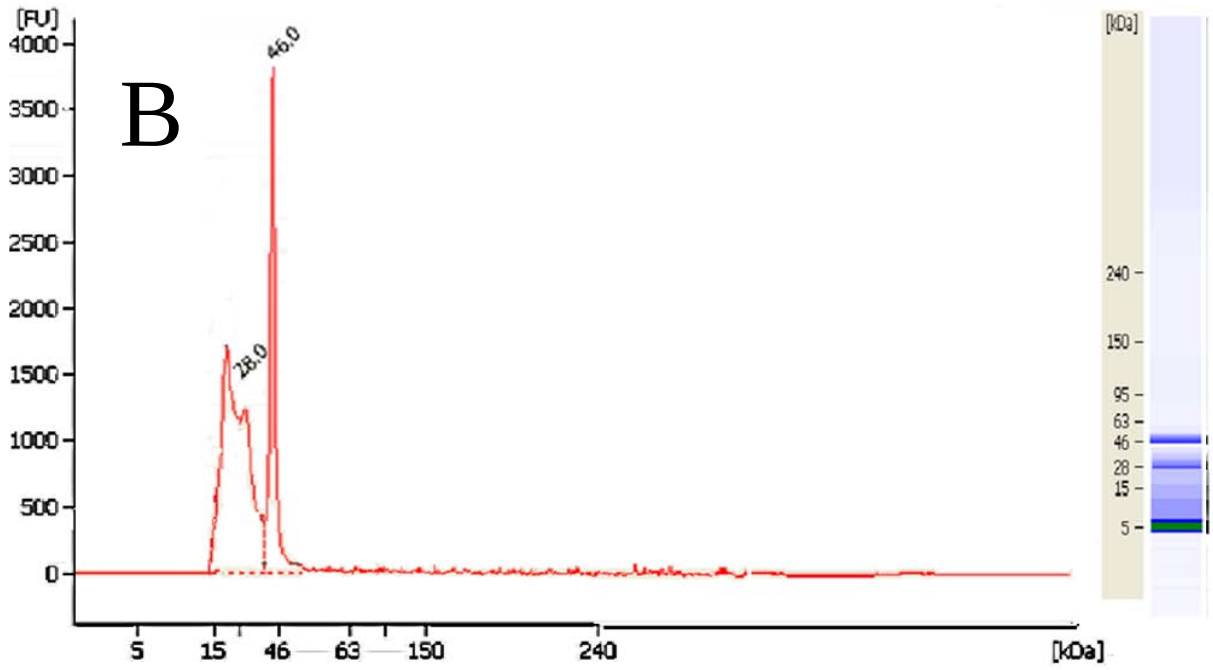


Figure 6.1 Degree of hydrolysis (DH %) in fermented skim milk generated with a combination of *L. helveticus* 881315 strain and Flavourzyme® (■) , *L. helveticus* 881315 alone (▲), and Flavourzyme® alone (◆) at 37°C for 0-12 h fermentation. Values are mean ± SD of three determinations for DH values. Error bars show standard error.

6.4.2 Loa C electrophoresis

The micro-fluidic 'lab-on-a-chip (Loa C)' technique provides an alternative method for simultaneous separation of major proteins in milk, as well as information on size, concentration and purity of milk protein in a single assay (Anema, 2009; Nikolić et al., 2012). The simulated gel patterns for fermented and untreated skim milk as control, obtained by Loa C with the elution profiles are shown in (Figure 0.2). The protein bands of fermented and untreated samples were in a different molecular weight (MW) range. Loa C uses internal lower and upper protein markers to correct for possible changes in the migration behaviour, thus enabling accurate and reproducible sizing. Preliminary investigation using the Agilent Protein 240 kit indicated its suitability for the separation of most milk proteins (Figure 6.2A) showing the migration pattern for proteins in the molecular weight ladder, and (Figures 6.2B, C) show the MW migration pattern of proteins in control RSM and fermented RSM respectively. Untreated RSM proteins presented two peaks of MWs 28 and 46 kDa (Figure 6.2B) indicating un-hydrolysed proteins (Anema, 2009). The migration pattern and profile of fermented milk shows a number of peaks ranging from 5 kDa to 240 kDa. This provides evidence for casein and other milk protein hydrolyses with varying MW of peptides.


A
[FU]
Ladder
[kDa]
15.0
28.0
46.0
63.0
95.0
150.0
240.0
125.8
5
15
46
95
150
240
[kDa]


B
[FU]
[kDa]
46.0
28.0
5
15
46
63
150
240
[kDa]

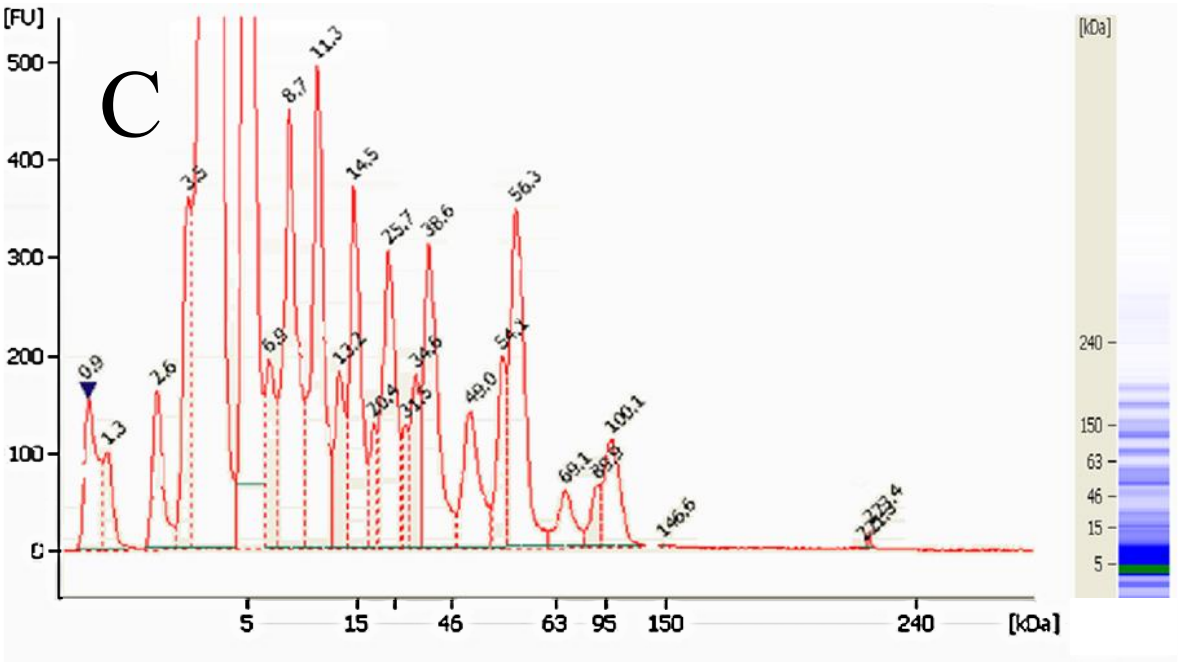


Figure 6.2 Lab-on-a-chip capillary electrophoresis and elution profiles with molecular weights, (**A**) Migration pattern for proteins MWs of the ladder, (**B**) migration pattern for untreated skim milk proteins as control and (**C**) migration pattern for fermented skim milk protein hydrolysis by combination of *L. helveticus* 881315 and Flavourzyme® at 37°C for 12 h.

6.4.3 Purification and identification of selected peptide fractions

Six fractions obtained from RP-HPLC, (F1and F6) showed the highest ACE-I activity and IC_{50} (95.51 % and 85.40 %) respectively (Table 6.1). The ACE-inhibitory activities of F6 and F1 were significantly higher with both having of IC_{50} 0.01 mg mL^{-1}, followed by F2 (72.04 % and IC_{50} 0.34 mg mL^{-1}). However, the activities of the remaining fractions were considered significantly low. Fractions F1 and F6 analysed by MS contained several peptide components as presented in (Tables 6.4, 6.5,) and (Figures 6.4A, B) and peptides fraction profiles are shown in (Figure 6.3). Several strategies were used in the current study including MS to identify peptides released from protein hydrolysis for which data sequences are known. The identification of peptide fractions was achieved using both MALDI MS/MS and Nano-LC/ MS/MS. LC.ESI.MS/MS enabled the determination of molecular weights and primary structures of peptides as a result of MALDI/MS however, these alone could not confirm the molecular weights of peptides. Results were subjected to database searches using bovine and bacterial databases. Sample F6 contained peptides which matched (109) identified peptides with 99 % confidence from the bovine database (Table 6.3) and showed the highest ACE-I

% activity (Table 6.1). For F1, (24) peptides were identified with higher than 99 % confidence (Table 6.5). When the bacteria database (Swiss Prot 2013) was searched, the mass error 55.7 ppm was higher than all peptides matched compared to the bovine database. The results showed that peptides greater than 1150 Da *f* (214-224) were isolated from F6 with casein origin (Table 6.3). The LC.MS/MS spectrum matched one sequence of the group of peptides selected by mass from milk casein (Figure 6.4). The major fragment ions were observed between m/z 903.30-2898.01 and 1702.96-2108.21 for F1 and F6 respectively, which were identified as b-type ions (b) adjacent to proline, in particular b3 and b5, respectively. This amino acid is associated with abundant y- and b-type fragment ions resulting from the cleavage of a peptide bond adjacent to proline. The resulting peptide originating from alpha and β-casein has been reported to decrease spontaneous hypertensive rats' (SHR) blood pressure (FitzGerald et al., 2004). Following this strategy, the majority of peptide components of each HPLC fraction identified and the results are summarised in (Table 6.4, 6.5). In (Figure 6.4) for example, a purified peptide from F6 TPVVVPPF was located between *f* (10-42). Most of the peptides in F6 contained proline amino acids, similar to captopril activity (Boutrou et al., 2013; Quirós et al., 2007) and high in ACE-I activity (Abd El-Salam, 2006). Peptide FFVAPFPGVFGK with antihypertensive properties was identified in F6 (Table 6.4). This was previously identified and has been shown to reduce SHR blood pressure (Hideaki et al., 1990). In addition, a novel casein-derived peptide sequence with ACE-I activity and antihypertensive activity was previously demonstrated in SHR (Contreras et al., 2009 and 2011). The peptides were obtained by enzymatic hydrolysis of total isoelectric casein with pepsin. To identify ACE-inhibitory peptides, the casein hydrolysate was fractionated by semi preparative HPLC, and 44 (CN) peptides contained in the active fractions were sequenced by using an ion-trap mass spectrometer. The identified peptide sequences, GPVRGPFPIIV, LHLPLPLL, RYLGY, AYFYPEL, and YQKFPQY, showed IC_{50} values between (0.71 mM - 6.58 mM) (Contreras et al., 2009 and 2011). These peptides exert antihypertensive activity when they were orally administered to SHR at a dose of 5 mg kg^{-1} of body weight (Contreras et al., 2009 and 2011). The activity of peptides RYLGY and AYFYPEL in SHR was similar to that found for tri-peptide VPP when orally administered (Contreras et al. 2009 and 2011). Similarly in our study, peptides GPVRGPFPIIV and LHLPLPLL were identified in F6 and F1 (Table 6.4, 6.5). In both fractions, a high amount of β-lacto globulin was present. β-lacto globulin has high biological importance as a source of

bioactive peptides (Yoshii et al., 2001; Madureira et al., 2010; Yu et al., 2012). Overall, bioactive peptides from F6 have the highest ACE-I activities compared to that from other fractions. Further studies are required to measure the ACE-I activity for each isolated peptide.

Table 6.1 The percentage ACE-inhibitory activity and IC_{50} mg mL^{-1} (means ± SE) of fermented skim milk peptides fractions.

Fractions	**ACE-I%**	**IC_{50} mg mL^{-1}**
F1	85.40±0.32[a]	0.01±0.00[d]
F2	72.04±0.91[b]	0.34±0.03c[d]
F3	42.39±1.20[c]	0.47±0.09b[c]
F4	17.31±2.21[d]	1.18±0.16[a]
F5	27.91±5.30[d]	0.78±0.11[b]
F6	90.31±0.21[a]	0.01±0.00[d]

Values followed by different letters indicated significant difference $P < 0{:}001$.

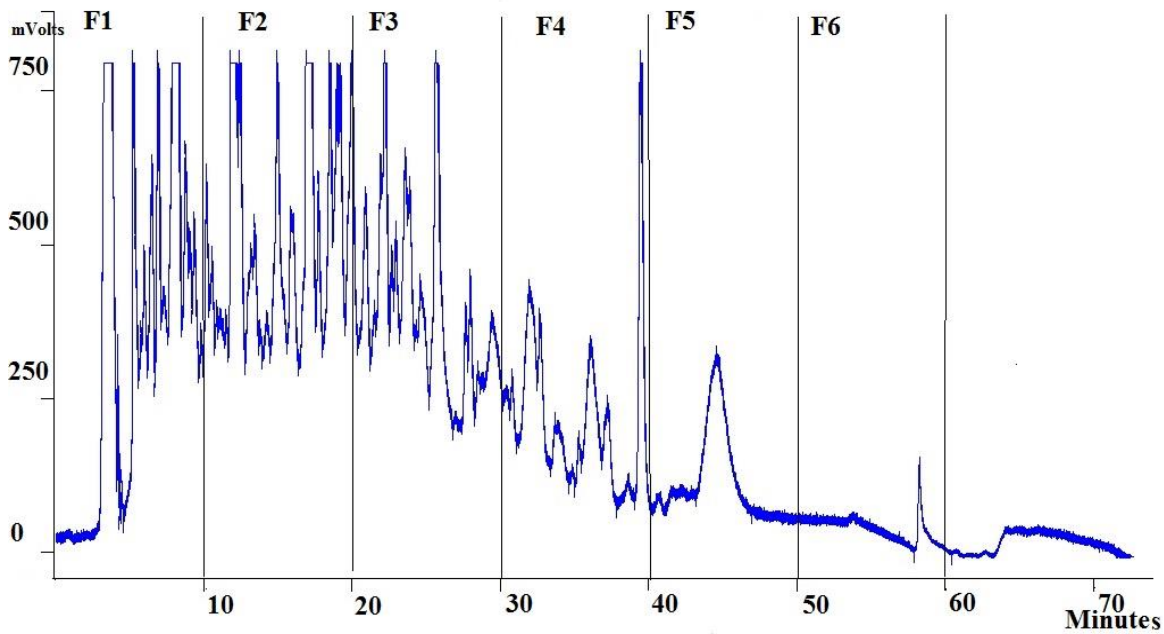


Figure 6.3 RP-HPLC purification of 6 peptide fractions from skim milk fermented by combination of *L. helveticus* 881315 and Flavourzyme®.

Table 6.2 Protein families of peptides identified in fraction (F1).

Protein family	**Mass (D a)**	**Sequence**
CASB_BOVIN	25091	Beta-casein
CASA1_BOVIN	24513	Alpha-S1-casein
CASA1_BUBBU	24311	Alpha-S1-casein
LACB_BOVIN	19870	Beta-lacto globulin
CAC1E_RABIT	254089	Voltage-dependent R-type calcium channel subunit alpha
CASA2_CAPHI	26372	Alpha-S2-casein
LALBA_BOSMU	16237	Alpha-lactoalbumin

Table 6.3 Protein families of peptides identified in fraction (F6).

Protein family	**Mass (D a)**	**Sequence**
CASB_BOVIN	25091	Beta-casein
CASA1_BOVIN	24513	Alpha-S1-casein
CASA1_BUBBU	24311	Alpha-S1-casein
LACB_BOVIN	19870	Beta-lacto globulin
CASA2_BOVIN	26002	Alpha-S2-casein
LALBA_BOSMU	16237	Alpha-lactoalbumin
CASK_BOVIN	21256	Kappa-casein OS
FETUA_BOVIN	38394	Alpha-2-HS-glycoprotein
GLCM1_BOVIN	17141	Glycosylation-dependent cell adhesion molecule

DDX56_BOVIN	61216	Probable ATP-dependent RNA helicase
BRAT1_AILME	88137	BRCA1-associated ATM activator
NIF3L_BOVIN	41880	NIF3-like protein

6.5 Conclusion

This study successfully identified 133 peptides with 99 % confidence from two fractions (F1 and F6). The highest ACE-inhibitory activity was in F6 (95.51 % with IC_{50} 0.01 mg mL^{-1}). The most potent ACE-inhibitory peptides found in this hydrolysate corresponded to FFVAPFPGVFGK, GPVRGPFPIIV and LHLPLPLL and showed significant antihypertensive activity. Those peptides were examined (Chapter 7) in spontaneously hypertensive rats (SHR) and their high blood pressure was successfully reduced during ten weeks oral administration.

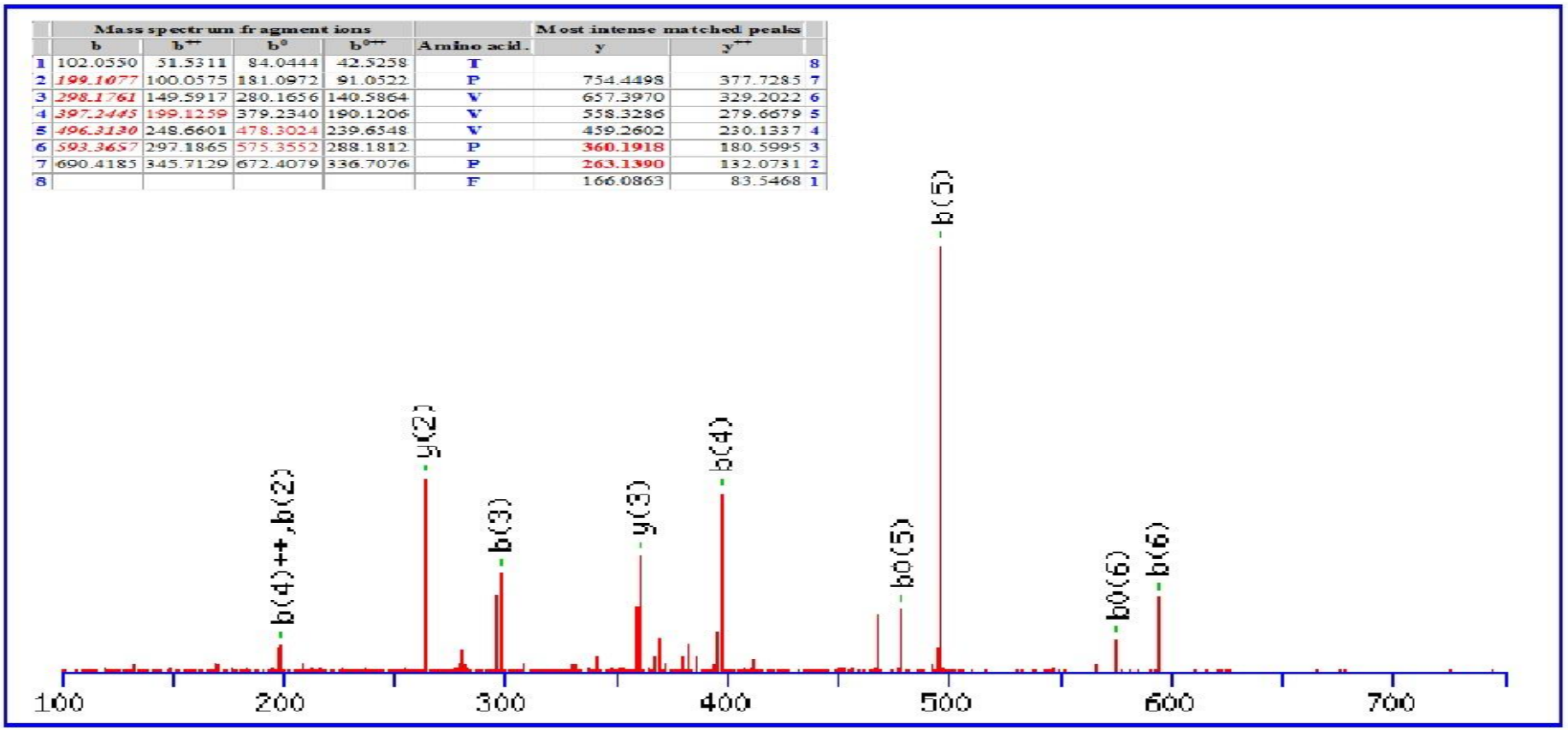
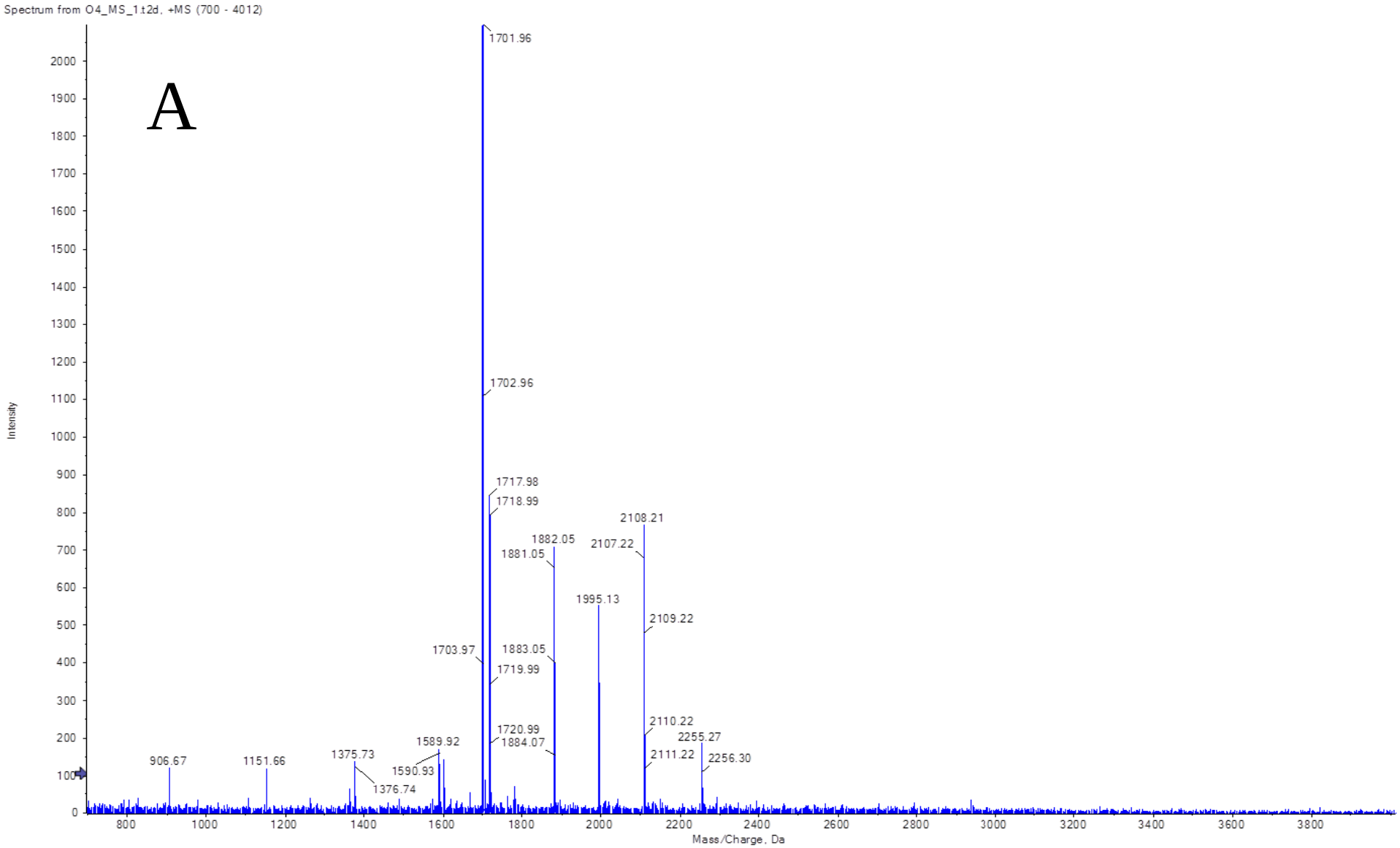


Figure 6.4 Peptide purified of Mono-isotopic mass of neutral peptide, Mr (calc):8854.4902, ions score :38, Expect :0.32 from fraction 6, (b) Molecular weight obtained with MALDI-TOF-MS .The first eight amino acids of the N-terminal was identified as TPVVVPPF. Following sequence interpretation and molecular weight determination, the peptide was identified as β-CN (*f*10-42) using 14 most intense peaks.


Spectrum from O4_MS_1.t2d, +MS (700 - 4012)
A
Intensity
Mass/Charge, Da
1701.96
1702.96
1717.98
1718.99
2108.21
1882.05
1881.05
2107.22
1995.13
2109.22
1703.97
1883.05
1719.99
2110.22
1720.99
1884.07
2255.27
2111.22
2256.30
1589.92
906.67
1151.66
1375.73
1590.93
1376.74

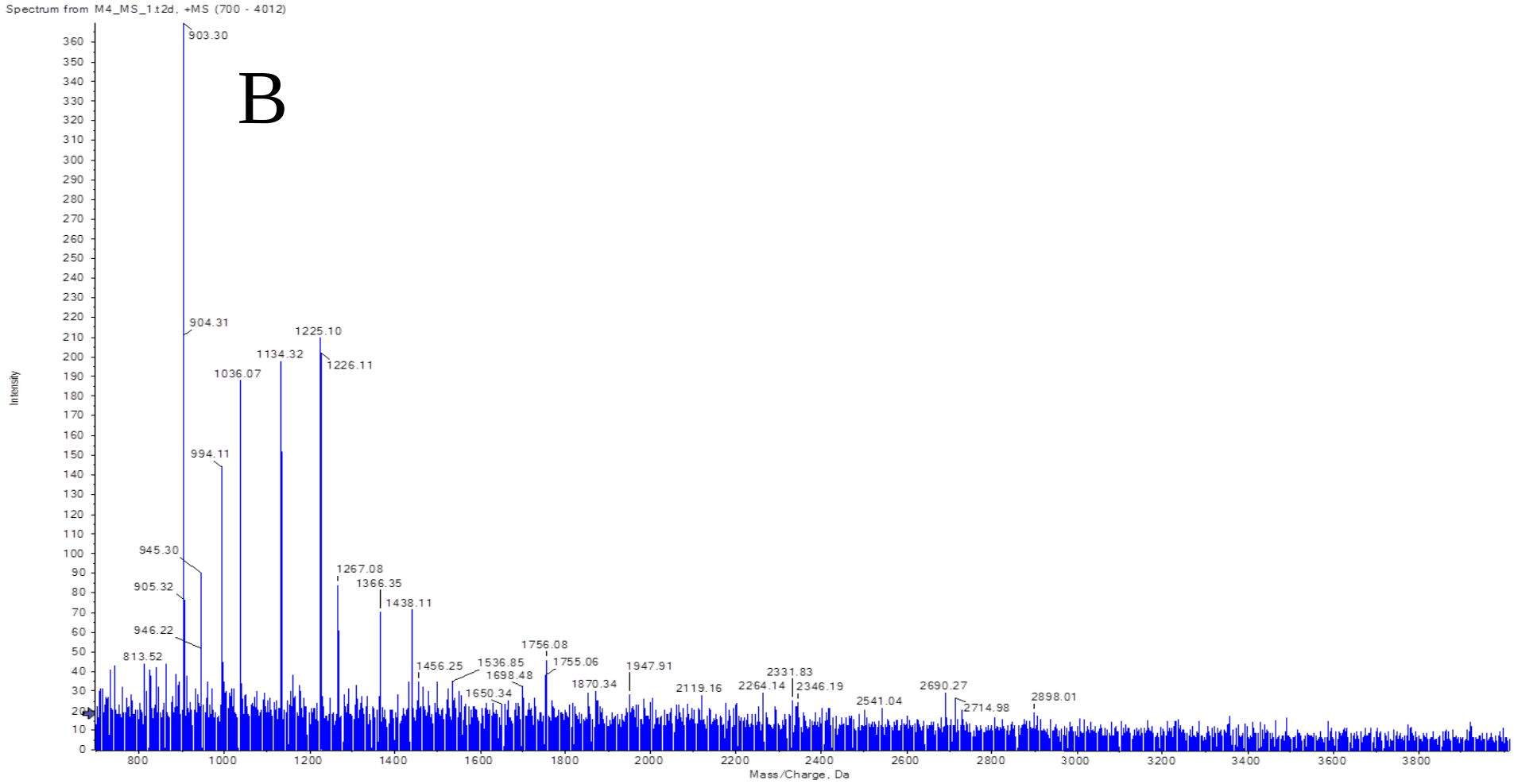


Figure 6.5 Identification of molecular mass and amino acid sequence of the fraction (**A**) F1 and (**B**) F2. MS/MS spectra of purified peptides were Q-TOF-MS/MS with an ESI source.

Table 6.4 Identification of peptides produced from fermented 12 % RSM in combination of *L. helveticus* 8801315 and Flavourzyme® ,and, contained in the RP-HPLC form F6, using MALDI-MS/MS analysis.

Protein Accession	**Protein Description**	**Peptide,*m/z* (Experimental)**	**Peptide mass, Da (Experimental)**	**Peptide mass,Da (Calculated)**	**Peptide Sequence**
CASB_BOVIN	Beta-casein	458.3093	914.6041	914.5953	LHLPLPLL
CASB_BOVIN	Beta-casein	522.3336	1042.6526	1042.6539	LHLPLPLLQ
CASB_BOVIN	Beta-casein	576.3528	1150.691	1150.6863	GPVRGPFPIIV
CASB_BOVIN	Beta-casein	577.8162	1153.6179	1153.5979	LTLTDVENLH
CASB_BOVIN	Beta-casein	578.3098	1154.605	1154.5931	RELEELNVPG
CASB_BOVIN	Beta-casein	602.8169	1203.6192	1203.5998	MPFPKYPVEP
CASB_BOVIN	Beta-casein	630.3618	1258.7091	1258.6921	DVENLHLPLPL
CASB_BOVIN	Beta-casein	641.8809	1281.7472	1281.7292	SLSQSKVLPVPQ
CASB_BOVIN	Beta-casein	675.3345	1348.6545	1348.6373	EMPFPKYPVEP
CASB_BOVIN	Beta-casein	692.4111	1382.8077	1382.7922	LLYQEPVLGPVR
CASB_BOVIN	Beta-casein	696.889	1391.7634	1391.7561	QEPVLGPVRGPFP
CASB_BOVIN	Beta-casein	718.427	1434.8395	1434.8235	VLPVPQKAVPYPQ
CASB_BOVIN	Beta-casein	745.9481	1489.8816	1489.8657	EPVLGPVRGPFPII
CASB_BOVIN	Beta-casein	750.9352	1499.8558	1499.8348	DVENLHLPLPLLQ
CASB_BOVIN	Beta-casein	778.4273	1554.8401	1554.8195	YQEPVLGPVRGPFP

CASB_BOVIN	Beta-casein	787.9526	1573.8907	1573.8716	LTLTDVENLHLPLP
CASB_BOVIN	Beta-casein	794.4532	1586.8918	1586.8668	DVENLHLPLPLLQS
CASB_BOVIN	Beta-casein	795.4854	1588.9562	1588.9341	EPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	819.9557	1637.8968	1637.8817	LVYPFPGPIPNSLPQ
CASB_BOVIN	Beta-casein	828.418	1654.8215	1654.7879	FPKYPVEPFTESQS
CASB_BOVIN	Beta-casein	832.4226	1662.8307	1662.7989	PVEPFTESQSLTLTD
CASB_BOVIN	Beta-casein	834.9656	1667.9166	1667.9035	LYQEPVLGPVRGPFP
CASB_BOVIN	Beta-casein	844.4942	1686.9739	1686.9556	LTLTDVENLHLPLPL
CASB_BOVIN	Beta-casein	844.508	1687.0014	1686.9556	LTLTDVENLHLPLPL
CASB_BOVIN	Beta-casein	858.0044	1713.9943	1713.9665	LTDVENLHLPLPLLQ
CASB_BOVIN	Beta-casein	859.5263	1717.0381	1716.9927	QEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	574.0205	1719.0396	1719.0195	KVLPVPQKAVPYPQR
CASB_BOVIN	Beta-casein	863.4663	1724.9181	1724.9138	SLVYPFPGPIPNSLPQ
CASB_BOVIN	Beta-casein	876.9755	1751.9365	1751.9247	LVYPFPGPIPNSLPQN
CASB_BOVIN	Beta-casein	887.4898	1772.965	1772.9461	DVENLHLPLPLLQSW
CASB_BOVIN	Beta-casein	891.5102	1781.0058	1780.9876	LLYQEPVLGPVRGPFP
CASB_BOVIN	Beta-casein	891.5243	1781.0341	1780.9876	YQEPVLGPVRGPFPII
CASB_BOVIN	Beta-casein	908.5265	1815.0385	1815.0142	TLTDVENLHLPLPLLQ
CASB_BOVIN	Beta-casein	612.3702	1834.0887	1834.0465	KVLPVPQKAVPYPQRD
CASB_BOVIN	Beta-casein	941.0501	1880.0856	1880.056	YQEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	944.5647	1887.1148	1887.0717	SLTLTDVENLHLPLPLL

CASB_BOVIN	Beta-casein	948.0568	1894.099	1894.0717	LYQEPVLGPVRGPFPII
CASB_BOVIN	Beta-casein	949.0648	1896.1151	1896.0721	SLPQNIPPLTQTPVVVPP
CASB_BOVIN	Beta-casein	641.3729	1921.0968	1921.0785	SKVLPVPQKAVPYPQRD
CASB_BOVIN	Beta-casein	965.0612	1928.1078	1928.0983	LTLTDVENLHLPLPLLQ
CASB_BOVIN	Beta-casein	660.3951	1978.1634	1978.1251	LSQSKVLPVPQKAVPYPQ
CASB_BOVIN	Beta-casein	997.5923	1993.17	1993.1401	LYQEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	1004.6051	2007.1957	2007.1557	LLYQEPVLGPVRGPFPII
CASB_BOVIN	Beta-casein	1008.5819	2015.1492	2015.1303	LTLTDVENLHLPLPLLQS
CASB_BOVIN	Beta-casein	1008.5909	2015.1672	2015.1303	SLTLTDVENLHLPLPLLQ
CASB_BOVIN	Beta-casein	1022.585	2043.1554	2043.1405	SLPQNIPPLTQTPVVVPPF
CASB_BOVIN	Beta-casein	689.4024	2065.1854	2065.1572	SLSQSKVLPVPQKAVPYPQ
CASB_BOVIN	Beta-casein	719.0915	2154.2527	2154.2241	FLLYQEPVLGPVRGPFPII
CASB_BOVIN	Beta-casein	771.7326	2312.1761	2312.1446	MHQPHQPLPPTVMFPPQSVL
CASB_BOVIN	Beta-casein	856.1683	2565.4829	2565.4723	IQAFLLYQEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	932.2084	2793.6032	2793.5656	MPIQAFLLYQEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	937.5362	2809.5867	2809.5605	MPIQAFLLYQEPVLGPVRGPFPIIV
CASB_BOVIN	Beta-casein	931.2757	3721.0736	3720.0266	AVPYPQRDMPIQAFLLYQEPVLGPVRGPF PIIV
CASA1_BOVIN	Alpha-S1-casein	552.8222	1103.6299	1103.6339	LGYLEQLLR
CASA1_BOVIN	Alpha-S1-casein	559.3271	1116.6396	1116.6291	VLNENLLRF
CASA1_BOVIN	Alpha-S1-casein	571.7766	1141.5387	1141.5251	SDIPNPIGSEN

CASA1_BOVIN	Alpha-S1-casein	609.3681	1216.7216	1216.7179	LGYLEQLLRL
CASA1_BOVIN	Alpha-S1-casein	610.3212	1218.6279	1218.6285	VAPFPEVFGKE
CASA1_BOVIN	Alpha-S1-casein	669.8782	1337.7419	1337.7191	GLPQEVLNENLL
CASA1_BOVIN	Alpha-S1-casein	683.8746	1365.7347	1365.6969	FFVAPFPEVFGKE
CASA1_BOVIN	Alpha-S1-casein	706.3481	1410.6816	1410.6667	YVPLGTQYTDAPS
CASA1_BOVIN	Alpha-S1-casein	733.9029	1465.7913	1465.7776	QGLPQEVLNENLL
CASA1_BOVIN	Alpha-S1-casein	743.8594	1485.7042	1485.6947	SDIPNPIGSENSEK
CASA1_BOVIN	Alpha-S1-casein	787.8862	1573.7579	1573.73	YYVPLGTQYTDAPS
CASA1_BOVIN	Alpha-S1-casein	840.4579	1678.9013	1678.893	VPSERYLGYLEQLL
CASA1_BOVIN	Alpha-S1-casein	865.9281	1729.8417	1729.8192	IPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	923.4429	1844.8712	1844.8462	DIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	636.0105	1905.0097	1904.9778	IHAQQKEPMIGVNQELA
CASA1_BOVIN	Alpha-S1-casein	958.9612	1915.9078	1915.8833	SDIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	966.9605	1931.9065	1931.8782	SDIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	1032.5039	2062.9932	2062.9517	FSDIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	1040.5046	2078.9947	2078.9466	FSDIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	873.4381	2617.2925	2617.237	APSFSDIPNPIGSENSEKTTMPLW
CASA1_BOVIN	Alpha-S1-casein	906.8165	2717.4278	2717.3523	IHAQQKEPMIGVNQELAYFYPEL
CASA1_BOVIN	Alpha-S1-casein	912.1428	2733.4066	2733.3472	IHAQQKEPMIGVNQELAYFYPEL
CASA1_BOVIN	Alpha-S1-casein	955.8351	2864.4834	2864.4207	IHAQQKEPMIGVNQELAYFYPELF
CASA1_BOVIN	Alpha-S1-casein	961.1507	2880.4303	2880.4156	IHAQQKEPMIGVNQELAYFYPELF

CASA1_BOVIN	Alpha-S1-casein	760.1399	3036.5306	3036.5167	IHAQQKEPMIGVNQELAYFYPELFR
LACB_BOVIN	Beta-lactoglobulin	565.8182	1129.6219	1129.6132	LDIQKVAGTW
LACB_BOVIN	Beta-lactoglobulin	607.3079	1212.6012	1212.5874	VEELKPTPEGD
LACB_BOVIN	Beta-lactoglobulin	617.7779	1233.5413	1233.6639	LIVTQTMKGLD
LACB_BOVIN	Beta-lactoglobulin	662.8843	1323.7541	1323.7286	KPTPEGDLEILL
LACB_BOVIN	Beta-lactoglobulin	663.8516	1325.6887	1325.6714	VEELKPTPEGDL
LACB_BOVIN	Beta-lactoglobulin	665.3539	1328.6932	1328.6823	SDISLLDAQSAPL
LACB_BOVIN	Beta-lactoglobulin	686.844	1371.6734	1371.6518	VRTPEVDDEALE
LACB_BOVIN	Beta-lactoglobulin	691.9059	1381.7972	1381.7929	ISLLDAQSAPLRV
LACB_BOVIN	Beta-lactoglobulin	728.3702	1454.7258	1454.714	VEELKPTPEGDLE
LACB_BOVIN	Beta-lactoglobulin	738.4126	1474.8105	1474.8065	LIVTQTMKGLDIQ
LACB_BOVIN	Beta-lactoglobulin	500.9245	1499.7516	1499.7467	VRTPEVDDEALEK
LACB_BOVIN	Beta-lactoglobulin	773.4391	1544.8637	1544.8562	ISLLDAQSAPLRVY
LACB_BOVIN	Beta-lactoglobulin	544.6167	1630.8281	1630.8202	RVYVEELKPTPEGD
LACB_BOVIN	Beta-lactoglobulin	549.9501	1646.8284	1646.8152	VRTPEVDDEALEKF
LACB_BOVIN	Beta-lactoglobulin	549.9567	1646.8484	1646.8152	VRTPEVDDEALEKF
LACB_BOVIN	Beta-lactoglobulin	841.4592	1680.9039	1680.8822	VEELKPTPEGDLEIL
LACB_BOVIN	Beta-lactoglobulin	874.4813	1746.948	1746.9152	SDISLLDAQSAPLRVY
LACB_BOVIN	Beta-lactoglobulin	898.0071	1793.9997	1793.9662	VEELKPTPEGDLEILL
CASA1_BUBBU	Alpha-S1-casein	552.8222	1103.6299	1103.6339	LGYLEQLLR
CASA1_BUBBU	Alpha-S1-casein	559.3271	1116.6396	1116.6291	VLNENLLRF

CASA1_BUBBU	Alpha-S1-casein	571.7766	1141.5387	1141.5251	SDIPNPIGSEN
CASA1_BUBBU	Alpha-S1-casein	609.3681	1216.7216	1216.7179	LGYLEQLLRL
CASA1_BUBBU	Alpha-S1-casein	610.3212	1218.6279	1218.6285	VAPFPEVFGKE
CASA1_BUBBU	Alpha-S1-casein	683.8746	1365.7347	1365.6969	FVAPFPEVFGKE
CASA1_BUBBU	Alpha-S1-casein	840.4579	1678.9013	1678.893	VPSERYLGYLEQLL
CASA1_BUBBU	Alpha-S1-casein	922.9485	1843.8825	1843.8622	SDIPNPIGSENSGKTTMP
CASA1_BUBBU	Alpha-S1-casein	930.9524	1859.8902	1859.8571	SDIPNPIGSENSGKTTMP
CASA1_BUBBU	Alpha-S1-casein	636.0105	1905.0097	1904.9778	IHAQQKEPMIGVNQELA
LALBA_BOSMU	Alpha-lactalbumin	553.305	1104.5955	1104.5815	DLKGYGGVSLP
LALBA_BOSMU	Alpha-lactalbumin	617.8273	1233.6401	1233.6241	DLKGYGGVSLPE
CASA2_BOVIN	Alpha-S2-casein	573.3589	1717.0547	1717.029	IQPKTKVIPYVRYL
CASA2_BOVIN	Alpha-S2-casein	680.7142	2039.1208	2039.0952	LYQGPIVLNPWDQVKRN
CASK_BISBO	Kappa-casein (Fragment)	771.9113	1541.8081	1541.7937	SPPEINTVQVTSTAV

Table 6.5 Identification of peptides produced from fermented 12 % RSM in combination of *L. helveticus* 8801315 and Flavourzyme®, and, contained in the RP-HPLC form F1, using MALDI-MS/MS analysis.

Protein Accession	Protein Description	Peptide,*m/z* (Experimental)	Peptide mass, (Experimental)	Peptide mass, Da (Calculated)	Peptide Sequence
CASA1_BOVIN	Alpha-S1-casein	24513	1828.8711	1828.8513	DIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	24513	997.5195	997.508	GLPQEVLNE
CASA1_BOVIN	Alpha-S1-casein	24513	1713.8169	1713.8243	IPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	24513	1729.8584	1729.8192	IPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	24513	1141.5341	1141.5251	SDIPNPIGSEN
CASA1_BOVIN	Alpha-S1-casein	24513	1228.5711	1228.5571	SDIPNPIGSENS
CASA1_BOVIN	Alpha-S1-casein	24513	1485.6939	1485.6947	SDIPNPIGSENSEK
CASA1_BOVIN	Alpha-S1-casein	24513	1586.7638	1586.7424	SDIPNPIGSENSEKT
CASA1_BOVIN	Alpha-S1-casein	24513	1687.8317	1687.7901	SDIPNPIGSENSEKTT
CASA1_BOVIN	Alpha-S1-casein	24513	1931.9241	1931.8782	SDIPNPIGSENSEKTTMP
CASA1_BOVIN	Alpha-S1-casein	24513	1915.9254	1915.8833	SDIPNPIGSENSEKTTMP
CASB_BOVIN	Beta-casein	25091	1258.6949	1258.6921	DVENLHLPLPL
CASB_BOVIN	Beta-casein	25091	1332.6589	1332.6424	EMPFPKYPVEP
CASB_BOVIN	Beta-casein	25091	1150.691	1150.6863	GPVRGPFPIIV
CASB_BOVIN	Beta-casein	25091	1356.8285	1356.8017	IPPLTQTPVVVPP
CASB_BOVIN	Beta-casein	25091	1503.8765	1503.8701	IPPLTQTPVVVPPF

CASB_BOVIN	Beta-casein	25091	1503.8817	1503.8701	IPPLTQTPVVVPPF
CASB_BOVIN	Beta-casein	25091	1363.7167	1363.6846	KEMPFPKYPVE
CASB_BOVIN	Beta-casein	25091	1380.7026	1380.6972	MHQPHQPLPPTV
CASB_BOVIN	Beta-casein	25091	1219.619	1219.5947	MPFPKYPVEP
CASB_BOVIN	Beta-casein	25091	1319.7531	1307.7085	PVVVPPFLQPEV
CASB_BOVIN	Beta-casein	25091	1391.7784	1359.7398	QEPVLGPVRGPFP
CASB_BOVIN	Beta-casein	25091	1717.0048	1880.056	QEPVLGPVRGPFPIIV

Chapter 7 Dietary supplementation with milk-derived angiotensin converting enzyme peptides decreases food intake, body weight and blood pressure in spontaneously hypertensive rats

This chapter has been submitted for publication. Ahtesh F., Stojanovska L., Mishra V. K & Michael M. (2016). Dietary supplementation with milk-derived angiotensin- converting enzyme peptides decreases food intake, body weight and blood pressure in spontaneously hypertensive rats. *Nutrition, metabolism and cardiovascular Disease*. Submitted. (Appendix. 1).

7.1 Introduction

Cardiovascular disease (CVD) is one of the leading causes of death among people worldwide (Quirós et al., 2007). Many drugs that are ACE inhibitors were developed as pharmaceuticals to treat hypertension, such as captopril and enalapril (Bellamy et al., 1992; FitzGerald, 1998; McCann et al., 2006; Saito et al., 2000; Takano, 2002; Yamamoto, 1997). While these drugs possess potent antihypertensive effects; they have side effects, such as organ damage (kidney and liver), taste disturbances and dry cough (Case et al., 1980; Cushman et al., 1980; Rizzello et al., 2008; Zhou et al., 2010).

Fermented dairy products are part of an important dietary strategy to reduce the risk of CVD (Yamamoto, 1997). Milk proteins have been identified as sources of bioactive peptides (Cheviron et al., 2000; Danilczyk & Penninger, 2006; Korhonen & Pihlanto, 2006; Mattu et al., 1995; O'Malley et al., 1998). Bioactive peptides can be produced from milk proteins by three methods: (1) enzymatic hydrolysis with a protease, (2) fermentation of milk by microorganisms with high protease activity, or (3) through the action of enzymes derived from proteolytic microorganisms (Saito, 2008). Several milk-derived peptides have been described to inhibit ACE *in vitro* (Saito, 2008). These peptides have considerable potential for the treatment and prevention of hypertension (Cheviron et al., 2000). To prepare bioactive peptides, milk proteins such as bovine casein are hydrolysed by lactic acid bacteria (LAB) (Gobbetti et al., 2002; Guan-Hong et al., 2004; Nakamura et al., 1995; Pihlanto-Leppälä, 2000). Fermentation with LAB involves the proteolytic processing of proteins to release peptides for use as a nitrogen source for bacteria. LAB is a suitable family of microorganisms for milk fermentation because they have a proteolytic system that decomposes casein, along with hydrolysing enzymes. It was shown that LAB such as *L. helveticus* have a higher proteolytic activity on milk proteins (Pan et al., 2005; Wakai & Yamamoto, 2012) compared with *Lactobacillus casei* (Ramchandran & Shah, 2011). Higher proteolytic activity leads to release of more antihypertensive peptides through higher extracellular proteinase activity in fermented milk (Griffiths & Tellez, 2013; Korhonen, 2009; Wakai & Yamamoto, 2012; Yamamoto et al., 1994). A variety of oligo-peptides released from casein by an extracellular proteinase of LAB has been reported. Recently, an antihypertensive effect related to ACE inhibitor peptides was found in sour milk produced by *L. helveticus*. Two kinds of bioactive short tri-peptides, Isoleucine-Proline-Proline (IPP) and Valine-Proline-Proline (VPP), with ACE inhibitor activity were

isolated and identified from sour milk, which had been fermented until pH 3.3 (Nakamura et al., 1995). The two tri-peptides termed 'lacto-tri-peptides' were confirmed as having antihypertensive activity using spontaneously hypertensive rats (SHR) (Nakamura et al., 1995). *In vitro*, the ACE inhibitory activities of the two peptides IPP and VPP were very high compared to other reported peptides, and the concentrations of peptides producing 50 % inhibition of ACE (IC_{50} value) were 9 and 5 mM, respectively. The amino acid sequences of VPP and IPP were found in the primary structure of bovine β-casein *f* (84–86) (74–76) and k- casein *f* (108–110), respectively (Mizuno & Yamamoto, 2004). They are cleaved from the casein molecule by an extracellular proteinase, followed by peptidase action during fermentation. The importance of the extracellular proteinase in the first decomposition of casein and the endo-peptidase in the carboxyl terminal processing has been confirmed (Yamamoto et al., 1994). The *L. helveticus* LBK-16H-fermented milk containing IPP and VPP, when consumed daily, had a blood pressure-lowering effect in hypertensive patients in Japan (Hata et al., 1996) and Finland (Seppo et al., 2003).

Several studies have been carried out to determine the anti-hypertensive effect of milk protein derived ACE inhibitors on SHR (Cam & de Mejia, 2012; FitzGerald & Murray, 2006; Hartmann & Meisel, 2007; Kawase et al., 2000; Pal et al., 2010; Shah, 2007). SHR are a naturally occurring breed, which develop essential hypertension and are frequently used for *in vivo* studies (Maeno et al., 1996; Pan et al., 2005; Yamamoto et al., 1994). Studies have shown that ACE-inhibitory peptides from milk protein hydrolysates reduce blood pressure in SHR (Yamamoto et al., 1999; Yamamoto et al., 1999; Gobbetti et al., 2000; LeBlanc et al., 2002; Quirós et al., 2007; Ramchandran & Shah, 2011; Wakai & Yamamoto, 2012; Boutrou et al., 2013; He et al., 2013). Furthermore, it has been reported that the SHR fed fermented milk with *L. helveticus* have shown a reduction in blood pressure (Leclerc et al., 2002; Griffiths & Tellez, 2013). A study using different types of proteases including Flavourzyme® has also shown the ability to produce bioactive peptides that lower blood pressure in SHR (He et al., 2013). The major antihypertensive peptides in the fermented milk, VPP and IPP, were detected in a heat-treated solubilized fraction from the abdominal aorta of rats fed with the fermented milk, but not in rats fed with unfermented milk (Saito, 2008). In addition to the various organs, ACE activity in the aorta was significantly lower in the animals fed with fermented milk than in the control group (Nakamura et al., 1996). The

major antihypertensive peptides in the fermented milk, VPP and IPP were detected in a heat-treated solubilized fraction from the abdominal aorta of rats fed with the fermented milk, but not in rats fed with unfermented milk (Masuda et al., 1996). Those results suggested that the lacto-tri-peptides pair were absorbed directly, without being decomposed by digestive enzymes, and transported to the abdominal aorta, where they inhibited ACE, producing an antihypertensive effect in SHR (Masuda et al., 1996).

A study reported that ACE-inhibition with perindopril for 14 weeks reduced body weight (~10 %) in SHR as well as reducing blood pressure and heart weight (Campbell et al., 1995). Reduction in ACE activity in a mouse ACE knock-out was also associated with reduction in body weight and a decreased accumulation of body fat, particularly in abdominal fat depots (Jayasooriya et al., 2008). The decreased body fat is independent of food intake and appears to be due to a high energy expenditure related to increased metabolism of fatty acids in the liver, with the additional effect of increased glucose tolerance (Jayasooriya et al., 2008). The control of blood pressure and food intake involves highly complex systems integrating peripheral and central signals, some of which can affect both energy homeostasis and blood pressure. The renin-angiotensin system is important in blood pressure control and a number of studies have advocated the involvement of the renin angiotensin system in obesity related to hypertension (Hall et al., 2000; Boustany et al., 2004). There is evidence that tissues in organs such as the liver, brain, kidney, heart and blood vessels represent major sites of production of angiotensin-II, the main vasoconstrictor product of renin-angiotensin system (Lapointe & Rouleau, 2002). It has been shown that adipose tissue is able to produce all the components of angiotensin converting enzyme (ACE), angiotensinogen and the angiotensin type 1 receptor (Cassis, Saye, & Peach, 1988; Crandall et al., 1994). The presence of this fully functioning local adipose tissue may contribute to the pathogenic mechanisms by which obesity increases the risk of cardiovascular disease (Goossens, Blaak, & Van Baak, 2003; Engeli et al., 2005).

Previous studies have investigated the effectiveness of milk-derived peptides on rats using short-term period and using different bacteria strains to release ACE-I peptides activity injecting the peptides into the rats' circulation to reduce BP (Campbell et al., 1995; Boutrou et al., 2013; He et al., 2013). There have been no studies in regard to energy homeostasis, with the relationship of body weight and peptides.

In light of the above, the present study was performed to evaluate the hypothesis that peptide extractions from fermented skim milk using a combination of Flavourzyme® and *Lactobacillus helveticus* ASCC 881315 significantly decreases rats' blood pressure and body weight; this combination of strain and Flavourzyme® has successfully increased ACE-inhibitory peptides activity (~95.5%) *in vitro* (Ahtesh et al., 2016). Following a three-week acclimatization period, animals were fed with a diet containing freeze-dried peptides for ten weeks. Blood pressure, heart rate, daily food intake and weekly body weight were recorded. The dietary treatments containing the freeze-dried peptides extracted from fermented skim milk were compared with two controls: control (1) chow with untreated skim milk and control (2) standard chow. Tissue weights were recorded post-mortem after 10 weeks experimental period at 24 weeks of age.

7.2 Material and Methods

7.2.1 Substrates and chemicals

Standard maintenance diet (AIN-93M), modified versions of the standard diet containing either skim milk(SF13-118) or peptides derived from skim milk(SF13-119), were manufactured by an experienced animal food pellet processor (Specialty Feeds, Glenvale, Western Australia). O-phthaldialdehyde (OPA), hippuryl–histidyl–leucine (HHL), trichloroacetic acid (TCA), bacteriological medium, bacteriological agar, trifluoroacetic acid (TFA) and ACE enzyme were purchased from Sigma Chemical Company (St Louis, MO USA). De Man Rogosa and Sharpe (MRS) and bacteriological peptone were purchased from Oxoid, Ltd., West Heidelberg Victoria, Australia. Flavourzyme®1000 L (EC 3.4.11.1, an amino peptidase with an activity of 1000 LAPU g^{-1}) purchased from Novozymes Australia, North Rocks, NSW Australia. Skim milk (SM) powder was purchased from (Murray Goulburn Co-operative Co. Ltd., Brunswick VIC Australia), while acetonitrile and reinforced clostridia agar (RCA) were purchased from Merck, Darmstadt Germany.

7.2.2 Determination of ACE inhibitor activity

ACE- inhibitory activity was measured according to the procedure described in section 3.2.6.

7.2.3 Animal diets and fermented skim milk preparation

The experimental design is shown in (Table 7.1). 12 % RSM (52 % lactose, 37 % protein, 8.6 % ash and 1.2 % fat) (160 litres) was fermented in bulk for 12 h using 1 % of *L. helveticus* 881315 (Dairy Innovation Australia Ltd. Australia) and 0.14 % (w/w) Flavourzyme® at 37°C in a Bioreactor Vessel system (bio Net® Bioreactor Vessel, Broadly-James Bioreactor Germany) with constant agitation at 120 rpm. Bioreactor assay was used for preparation of fermented RSM as described in section 6.2.2.1. The use of bioreactor improved production of ACE-I peptides. Fermented RSM was heat-treated to 85°C for 20 mins to stop the growth of probiotic bacteria and enzyme activity. Samples were then stored in a fridge at 4°C for freeze-dried. Fermented and non-fermented RSM were freeze-dried in bulk (Biotech Freeze Drying, Knox field, Melbourne, VIC Australia). The freeze-dried fermented and non-fermented RSM powders were incorporated into the manufacturing of rat feed pellets respectively by Specialty Feeds (Perth, Western Australia). The rat feed pellets were stored at 4°C. All the rat feed contained standard nutrients of vitamins, minerals, sugars and lipids as indicated in (Table 7.1). The SM powder and freeze- dried fermented SM containing peptides amounts in the formulated diets were limited by the pelleting process. It was observed that quantities above 44.7 % hindered the process of pellet formation due to the caking property of the lactose in SM powder. The SM powder and a freeze-dried fermented SM sample were incorporated and pelleted in the experimental diets.

Table 7.1 The table shows the type and frequency of experimental design measurement.
Group 1: Rats feed Chow (control) (NC).

parameters	**Rats groups**	**Experimental period (daily and weekly)**									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Daily											
Body weight/rat	Group1 Group2 Group3										
Feed intake (daily)/cage	Group1 Group2 Group3										
Weekly											
Blood pressure measurements	Group1 Group2 Group3										

Group 2: Rats feed Chow (control) + skim milk powder (NFC).
Group 3: Rats feed Chow + pelleted freeze dried fermented skim milk containing peptides (FC).
(N=9 each group).

7.2.4 Energy content measurements by bomb calorimeter

An isothermal (Static Jacket) bomb calorimeter system (Pty, Ltd, CAL2k) was used to determine the calorific value (energy content) of the pellet samples used in this study. A calorimeter is a device used for measuring the heat of the reaction, physical changes and heat capacity (Okoro et al., 2011). The calorimeter system uses a pellet of benzoic acid as a standard for calibration (1.216 g). The pellet is then tied to a 13 cm length cotton wire, placed inside the sample boat and the cotton wire was stretched between the electrodes. Oxygen is added through the gas inlet valve to give a pressure of 3000 K Pa and the system is ignited until the calorimeter indicates that the calibration is completed. The known mass of benzoic acid produces a standard amount of heat energy when burnt and this heat is transferred to the bomb. The increase in bomb temperature is measured accurately to calibrate the machine. Once this has been achieved, the bomb can then be cooled to baseline temperature using the water-cooler system. After that the calorimeter is ready to measure the pellets which have unknown energy content. The pre-weighed samples (chow, chow mixed with SM powder and chow mixed with peptide extract) are placed into a crucible; the firing cotton is placed in contact with the sample and the vessel pressurised with oxygen gas. The firing cotton is then ignited and the sample gets ignited because of contact with the firing cotton. The burning of the sample leads to a rise in temperature of the vessel which is then measured as the calorimetric value of the sample.

7.2.5 Animal care and experiments

This project was conducted with approval from the Animal Ethics Committee of Victoria University (AEC 12/009) (Appendixes II).

7.2.6 Experimental design spontaneously hypertensive rats (SHR)

The experimental design is shown in (Table 7.2). Twenty-seven male SHR were purchased from Animal Resource Centre in Western Australia at 14 weeks of age. These SHR were housed in animal care facilities at Victoria University. The weight of the SHR was recorded upon arrival (weight, 250 ± 5 g). The SHR were housed in three per stainless steel hanging wire mesh cages in 12 h dark light cycle with controlled environmental conditions (temperature 22.5°C - 23.5°C and humidity 32 % - 40 %). The rats were divided into three groups (n=9), and were allowed free access to feed and

tap water. The experimental group were fed the diets supplemented with freeze-dried peptide extract of fermented SM (FC), control group 1 were fed a diet with untreated SM (NFC) and control group 2 were fed standard chow (NC) (Table 7.2). Before starting the measurements, the rats were acclimatised for three weeks and trained on a blood pressure monitor system (Coda Non-Invasive Blood Pressure System, ODA NIBP Kent Scientific Corporation. Inc. U.S.A). The clear acrylic holders provided unrestricted breathing and allowed complete visibility to the researcher. During the three weeks, rats were fed on normal standard rats chow. SHR were fed with (40 to 70 g/box of pellets daily), with ad libitum tap water and food was changed daily. Blood pressure, heart rate, daily feed intake and weekly weights of all rats were recorded daily for ten weeks.

Table 7.2 Composition of the experimental groups feed.

Experimental group	Composition of the feed
FC	Freeze dried peptide extract of Fermented RSM
NFC	Non-fermented RSM control (1)
NC	Normal rat feed chow control (2)

7.2.7 Measurement of blood pressure

Rats were trained in Tail-Cuff (BP) Measurements for three weeks prior to the experiments and were familiarized with the procedures of tail-cuff BP monitoring, including regular handling and warming procedures. Correct SHR handling is critical for consistent and accurate blood pressure measurements. Nervous, stressed SHR may have diminished circulation in the tail. Systolic (SBP), diastolic (DBP), and mean blood pressure as well as heart rate (HR) and tail blood volume were measured in each animal weekly. This method used Volume Pressure Recording (VPR) sensor technology. (CODA® Non-Invasive Blood Pressure System, Kent Scientific Corporation. Inc. U.S.A). The VPR uses a specially designed differential pressure transducer to measure the blood flow and blood volume in the tail non-invasively. VPR actually measures six parameters simultaneously: systolic and diastolic blood pressure, mean calculated measure of blood pressure, heart pulse rate, tail blood volume and tail

blood flow. The pressure cuff device, similar to an arm blood pressure cuff used in humans, fits over the rodent's tail restricting inflow of blood to the tail when inflated. The unit then uses optical sensors to determine when the blood flow returns to the tail as the cuff is gradually deflated, which will be equivalent to SBP, DBP and HR, by recording the disappearance and reappearance of pulse signals in conjunction with measurements of cuff pressure. The SBP, DBP and HR were determined directly from the recordings. To make the pulsations of the tail artery detectable, rodents were taken to a quiet room and placed in an incubator at (30 ± 1°C for 10 min), and then gently walked into the restrainer. The warming chamber and the restrainer were pre-warmed to the appropriate temperature (30 ± 1°C). The SHR was allowed to enter the holder freely. After the rodent was placed in the holder, the nose cone was adjusted so the animal was comfortable but not able to move excessively. The tail was gently placed through the cuff and inflated by the computer system to a maximum pressure 300 mm mercury due to the breed of the rat. SHR are hypertensive and require a higher max cuff pressure than normal. After 5-10 minutes to acclimatise to the restrainer, 6 to 10 readings were taken from each rodent before the animal was removed from the restrainer and returned to its cages. For each measurement session, the animals were in the restrainer for around 15-25 minutes. Once rats were warmed, the VPR was attached to the tail of the animal, closer to the base of the tail. The five most consistent measurements of SBP, DBP and HR from 10 consecutive measurements were calculated as the mean of the measurements accepted by the CODA program and considered for statistical analysis. Mean arterial blood pressure (MAP) was calculated using the following equation (Leclerc et al., 2002):

$$\mathrm{MAP} = \mathrm{DBP} + \frac{\mathrm{SBP} - \mathrm{DBP}}{3}$$

7.2.8 Tissue collection

After the 13-weeks-period, SHR were euthanized using Sodium pentobarbital (100 mg/kg body weight). Blood samples were collected by cardiac puncture into heparinised tubes. The blood was centrifuged at 2000 x g for 10 min at 4°C (Beckman Coulter. Avanti J-265 XPI, Centrifuge). The blood and plasma samples were stored at -80°C. The kidney, heart, epididymal fat pad, liver, and left ventricular (LV) were then collected, weighed and stored at -80°C for further analysis.

7.3 Statistical analyses

Using Minitab 16, all the data were expressed as mean values and standard deviations of means of 5 closest measurements with the mean (± SEM). The differences between the experimental groups were determined by 2-way ANOVA and P-values less than 0.05 considered significant.

7.4 Results and Discussion

7.4.1 Feed intake, body weight, organ weights and energy contents

The food intake pattern and body weight of the SHR are shown in (Figures 7.1). Nutritional and energy content of diets are shown in (Table 7.3). Moreover, there were significant differences in energy content of the diets ($P < 0.05$). There were significant increases in food intake and body weight for the three different diet groups during ten weeks of feeding ($P < 0.05$) (Figures 7.1). All three groups, i.e. the group fed with freeze-dried peptide extract of Fermented RSM (FC), the group fed with non-fermented RSM control (1) (NFC) and group fed with normal rat food chow control (2) (NC) significantly increased food intake and body weight during the last seven weeks. However, the food intake was not consistent for all groups during the first four weeks. Food intake was initially lower in the FC group. By the end of the ten week period, the FC group consumed less food (~20 %), than the other groups (Figure 7.1B). However, there were no significant differences in total energy intake between diet groups (Table 7.5) because the FC diet was more energy-dense. The weight gained by rats in the three groups (Figure 7.1A) increased gradually over the course of the study, as expected. Similar results were found by Musoles et al., (2013) who conducted an acute study on the effect intake of a bovine lacto-ferrin hydrolysate enriched with low molecular weight peptides on the progression of hypertension SHR, and reported no differences in body weight between the two groups (Fernández-Musoles et al., 2013). The average body weight was less in the FC group compared with the groups fed with the diet containing skim milk (NFC) and standard rodent chow (NC). However, the calculated total energy intake showed no significant differences between the FC, NFC and NC groups (259.27; 212.77 and 238.14 MJ) ($P < 0.05$) respectively as presented in (Table 7.5). The study found that despite similar food energy consumption in all three groups, the FC group weighed less at the end of the study than the NC and NFC groups (Figures 7.1). There were no significant differences between the weights of organs of the three

different treatment groups related to the body weight (Table 7.4). This is similar to the results using SHR fed a diet containing milk fermented with *L. helveticus* and *Saccharomyces cerevisiae* where the weight of heart, liver, testes, kidney, and spleen were not significantly different between the control and fermented milk groups (Nakamura et al., 1996). However, a previous study showed a 5 % reduction in heart, body weight ratio in SHR treated with perindopril for 14 weeks (Campbell et al., 1995). In ACE-KO mice, it was found that the lower body fat was primarily due to increased energy expenditure and not related to differences in food intake or energy digestibility. The increase in energy expenditure was independent of locomotor activity and appears to be mediated by increased fatty acid oxidation in the liver, so it is possible that the differences in body fat and energy expenditure were due to differences in fat metabolism (Jayasooriya et al., 2008).

In conclusion, food intake and body weight were affected by ACE inhibitory peptides, but body fat (using epididymal fat as an index) was not significantly reduced. Further study is needed to determine whether the differences in body fat and energy expenditure are due to differences in fat metabolism and putatively higher energy expenditure.

Table 7.3 Nutrient composition of diet pellets prepared.

	Addition rate (g/100g)		
Ingredients	**Control feed (rats standard chow) (NC)**	**Skim Milk powder Control containing feed (NFC)**	**Fermented Skim Milk Peptides containing feed (FC)**
Sucrose	10.00	10.00	10.00
Freeze dried fermented SM containing Peptides (FSMP)	0.00	0.00	44.48
Skim milk powder (SMPOC)	0.00	44.48	0.00
Canola oil	4.00	3.50	3.50
Cellulose	5.00	5.00	5.00
Starch	19.26	19.26	19.16
Dextrinised Starch	15.50	15.50	15.50
DL- methionine	0.18	0.18	0.18
AIN-93-trace minerals	0.14	0.14	0.14
Calcium carbonate	0.13	0.06	0.06
Sodium chloride	0.26	0.18	0.18
Potassium sulphate	0.45	0.45	0.45
AIN-93-Vitamins	1.00	1.00	1.00
Choline chloride 75% W/W	0.25	0.25	0.25
Blue food colour (10%)	0.00	0.00	0.10
Energy content (MJ)*	15.1±0.012	18.4±0.002	16.9±0.011

*this represents the calorimetric value of the sample.

Table 7.4 Tissue weights of rats after 10 weeks treatments at 24 weeks of age.

Groups/tissue	Fat	Kidney	Heart	L.V
FC	3.49±0.45	2.16±0.23	1.11±0.15	0.96±0.72
NC	3.94±0.42	2.41±0.15	1.2±0.09	1.01±0.08
NFC	3.96±0.21	2.23±0.16	1.18±0.09	1.01±0.12

Table 7.5 Comparison of Food intake, body weight and total Energy intake of diet groups at the end of experiments.

Measurments	Diet groups		
	FC	NFC	NC
Food intake (g/cage)	42.00	48.10	51.5
Body weight (g/rat)	350.46±0.32*	399.82±2.05	400.78±1.03
Total Energy intake /each group (MJ)	238.14	259.27	212.77

*= means significant lower compared to the other groups

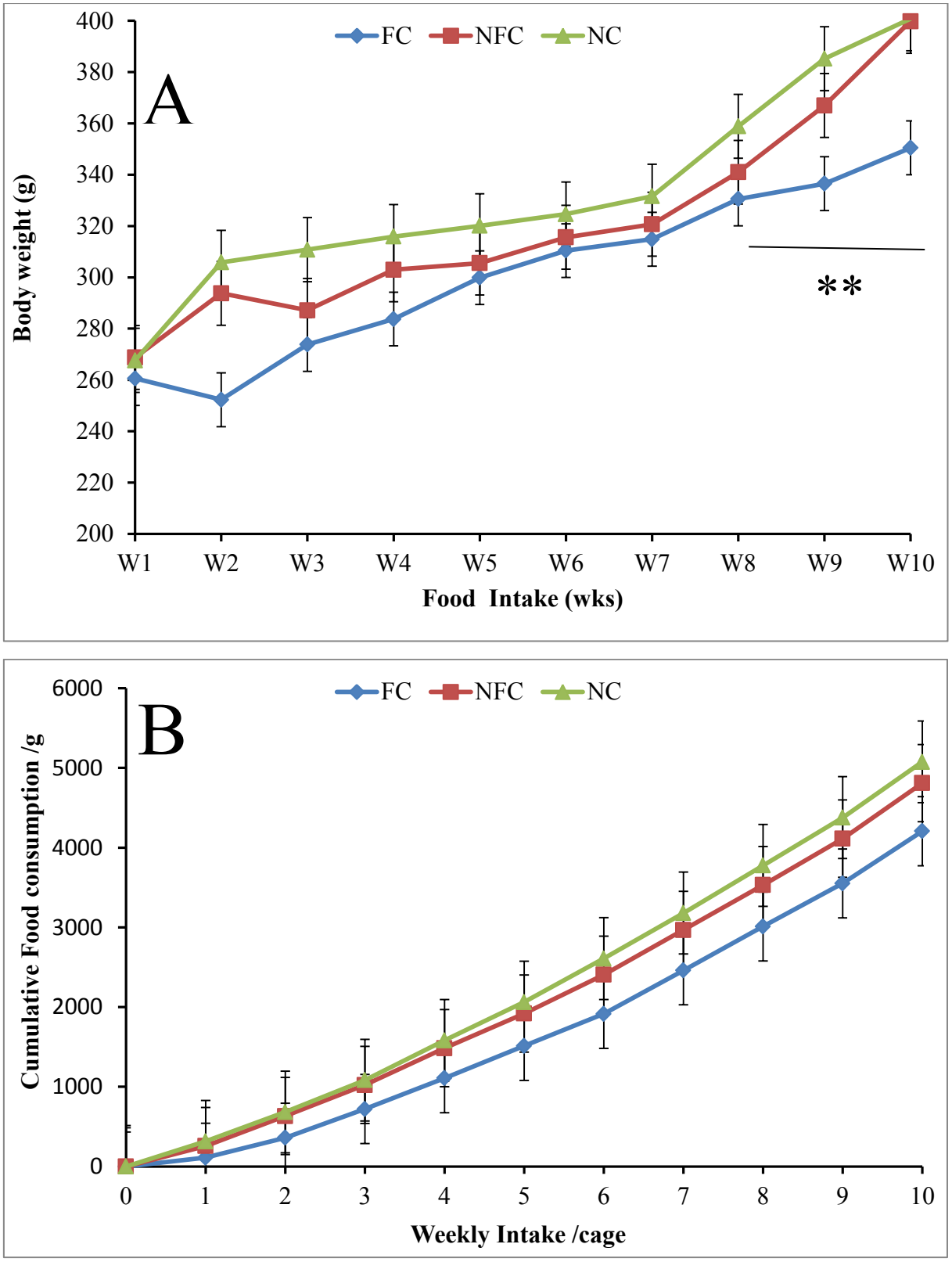


Figure 7.1 The body weight (A) and Cumulative feed consumption/g (B) of SHR orally administered with control 1 (NFC), control 2 (NC) and Fermented skim milk containing peptides (FC) during 10 weeks period feeding. All data were expressed as mean ±SEM (n-9).

7.4.2 Antihypertensive effects of dietary peptides in SHR

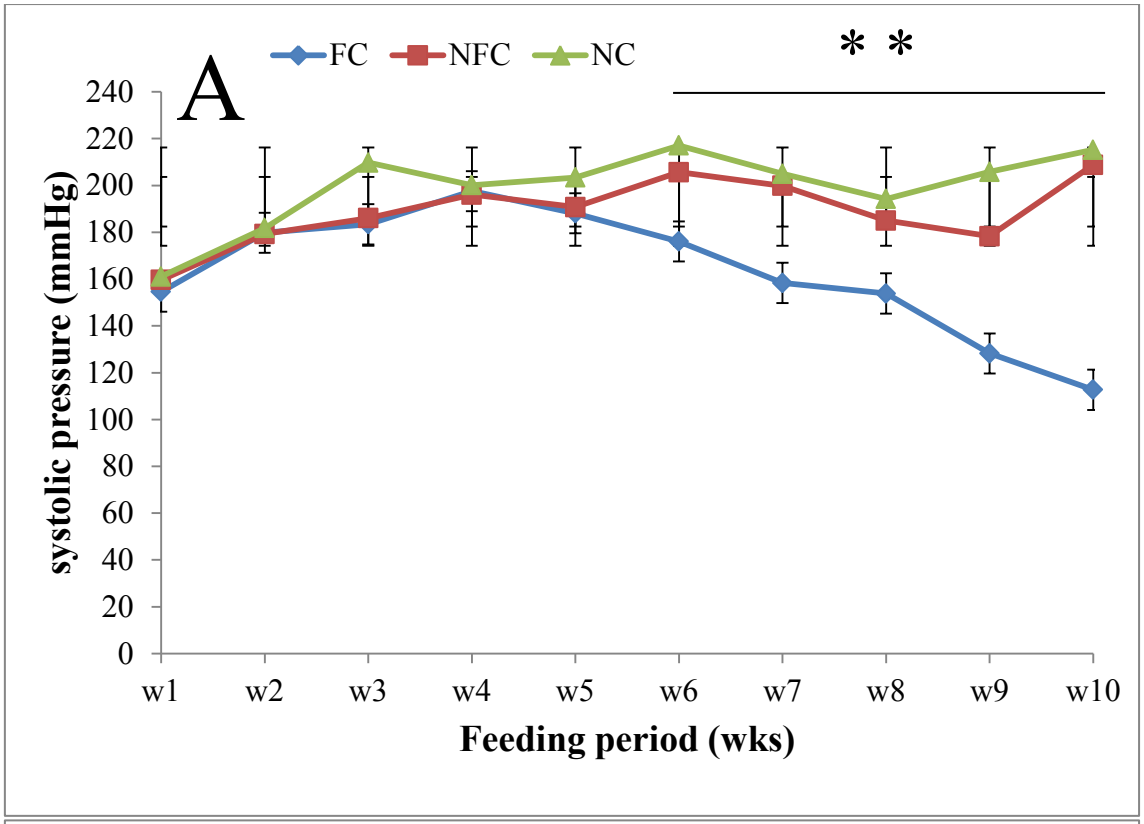
Mean blood pressure (MBP), SBP and DBP were measured in SHR during dietary supplementation for ten weeks, as shown in (Figure 7.2). The BP values of all groups were similar in week 0 (~155/120 mmHg). The SBP, DBP and MBP of all groups (Figure 7.2A) showed no significant changes of BP in the first two weeks of feeding ($P < 0.05$). There was a gradual decrease in SBP, DBP and MBP after oral administration of the peptide extract of fermented skim milk (FC), which became significant from week six ($P < 0.05$) and continued until the end of the feeding intervention (week ten), compared with the control groups. The BP values of the control groups were similar. At the end of the feeding period the reduction in systolic BP of rats fed on FC was 40 % (120 mm Hg) and diastolic BP was 30 % (65 mm Hg) which is normal for a rat. However, those groups fed NC and NFC showed increased SBP and DBP (220 mmHg /150 mmHg) and (220 mmHg /140 mmHg) respectively. The peptides derived from milk proteins having ACE inhibiting properties likely affected the FC-fed group positively by reducing BP (Yamamoto & Takano, 1999). In previous experiments, it was found that the ACE inhibition activity was higher in fraction 6 (95.5 %), which contains peptides TPVVVPPF, YPFPGPIP and SLPQNIPPLTQTPVVVPP with potent anti-hypertensive properties as described in section 6.4.3.1 (Table 6.1). These peptides were identified in the fermented SM. Similar peptides including tri-peptides have been reported to lower blood pressure similar to captopril (Iwaniak & Minkiewicz, 2008; Wang et al., 2012; Du et al., 2013). Nakamura et al., (1996) suggested that fermented milk containing peptides not only have a temporary antihypertensive effect by single oral administration but also a long-lasting effect on the hypertensive stage during long-term feeding in SHR. The authors further stated that unlike the effect on blood pressure, fermented milk did not alter the heart rate, body weight and organ weight. It is known that these small di- or tri-peptides are easily absorbed in the intestine (Adibi & Morse, 1971) and the Pro-Pro sequence is resistant to gut enzyme degradation (Kim, S. Y & Kim, 1972). One possible explanation for the decrease of ACE activity is that the tri-peptides are absorbed, they reach the systemic circulation and decrease ACE activity. Similarly, Fernández-Musoles et al., (2013) described that the long-term oral administration to SHR with low molecular weight peptides, attenuated and even reversed the progression of hypertension. The *in vitro* evidence of ACE-inhibitory properties was supported by reductions of ACE activity, angiotensin-II and aldosterone levels in the circulation, as well as a compensatory increase of renin activity in SHR,

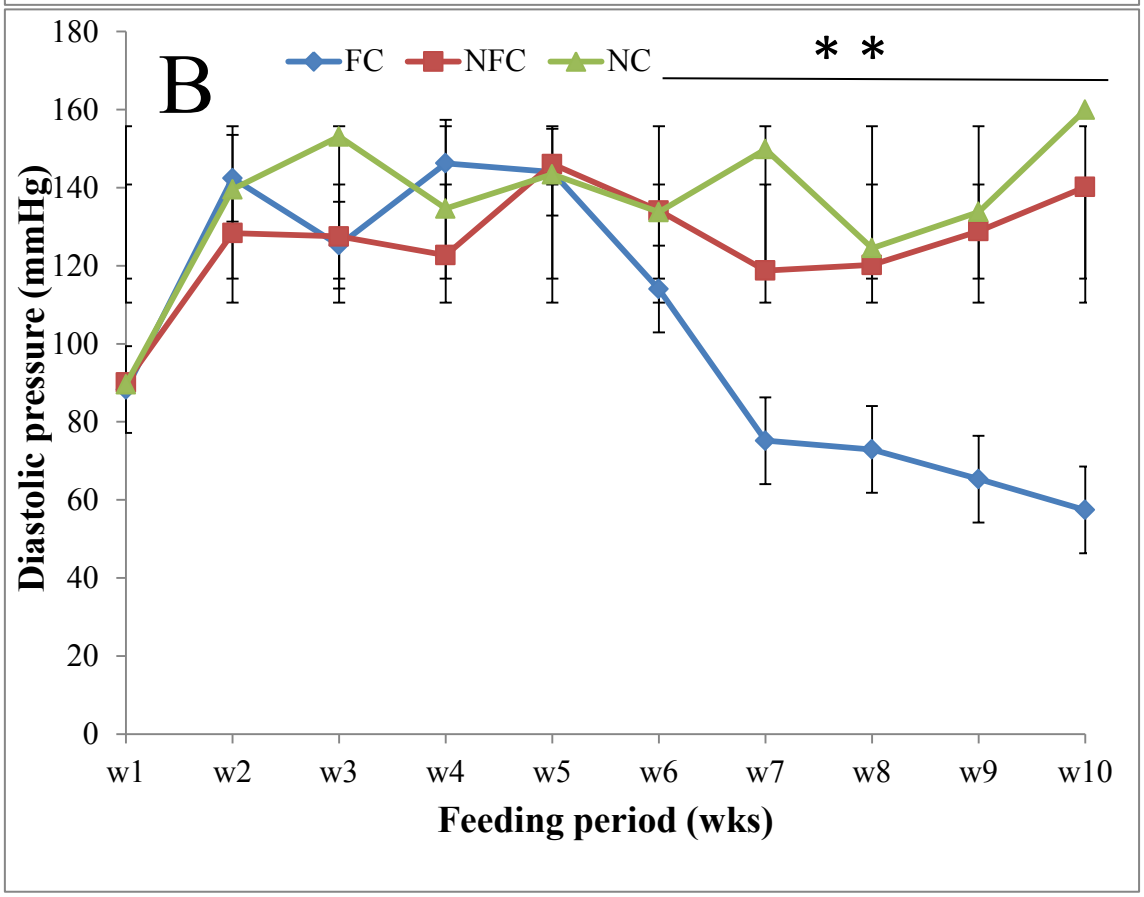
thus supporting ACE inhibition as an *in vivo* mechanism for the antihypertensive effects (Fernández-Musoles et al., 2013). Interestingly, during the period of dietary intervention, it was noticed that the FC group were more relaxed and calm when compared with SHR in the control groups, which were more active. Similarly, it has been reported that milk fermented by *L. helveticus* has a favourable effect on improving sleep in healthy, elderly people in the short-term (three-week) (Yamamura et al., 2009). This could have been as a result of the peptides effect on BP. It has been demonstrated that α-lactorphin, a tetra-peptide (YGLF) formed by *in vitro* proteolysis of α-lactalbumin with pepsin and trypsin, lowers blood pressure when administered subcutaneously in SHR (Nurminen et al., 2000) and produces an endothelium-dependent relaxation of their mesenteric arteries that is inhibited by an endothelial nitric oxide synthase inhibitor (Sipola et al., 2002). Although α-lactorphin interacts with opioid receptors, it does not elicit effects typical of centrally active opioids such as anti-nociception and sedation (Ijäs et al., 2004). It has also been suggested that these opioid peptides might lower blood pressure through receptors expressed in the gastrointestinal tract, which implies that no absorbance is required (Yamada et al., 2002). However, in this study, an effect of opioid peptides on blood pressure by treatment of the rats with naloxone was not measured (Nurminen et al., 2000).

According to Jauhiainen et al., (2005 and 2010), the mechanistic theory of ACE-inhibition of tri-peptides such as (IPP and VPP) remains to be confirmed and other effects have to be taken into consideration. These effects have been evaluated in animal models and clinical studies, plasma renin activity and levels have been found to be raised in SHR receiving IPP and VPP for 14 weeks (Jauhiainen et al., 2005; Jauhiainen et al., 2010). Another study has reported the protective effects exerted by these peptides on endothelial function of isolated mesenteric arteries of rats after 24 h incubation (Jäkälä et al., 2009). The administration of VPP and IPP on gene expression of SHR abdominal aorta (using DNA microarray) reported a significant increase in expression of the endothelial nitric oxide synthase gene (eNOS), which is involved in blood pressure regulation. In another long-term study with a product based on the casein hydrolysate, which contained the peptides (RYLGY and AYFYPEL) it was found that the development of hypertension in the rats' group treated with the casein hydrolysate product was attenuated (Fuglsang et al., 2003). In addition, the treatment improved aorta and mesenteric acetylcholine relaxations and increased the eNOS expression in the

aorta. The left ventricular hypertrophy decreased in treated SHR. Fuglsang et al., (2003) reported that ingestion of milk fermented with *L. helveticus* provokes a decrease of the response to an intravenous injection of angiotensin-I in unconscious normotensive rats, whereas the response to bradykinin was increased, confirming the inactivation of ACE (Fuglsang et al., 2003).

In conclusion, the reduction in systolic BP of rats fed FC was 40 % (120 mmHg) and diastolic BP was 30 % (65 mmHg) compared to BP before treatments, and this is effectively a normalisation of blood pressure. In comparison, the groups fed NC and NFC had elevated SBP and DBP (220 mmHg /150 mmHg) and (220 mmHg /140 mmHg) respectively. Further studies are necessary to demonstrate the absorbance of these peptides, and confirm that the mechanism underpinning the normalisation of blood pressure is due to a decrease in ACE activity in blood vessels. During the experimental period, there were no physiological side effects or toxicity from peptides on SHR (Health monitoring form in Appendixes-II). Further analyses in future is needed to measure the percentage of ACE-I activity peptides in rats' blood and tissue to understand the mechanism theory of ACE- inhibition peptides activities.


A
FC
NFC
NC
* *
systolic pressure (mmHg)
240
220
200
180
160
140
120
100
80
60
40
20
0
w1
w2
w3
w4
w5
w6
w7
w8
w9
w10
Feeding period (wks)


B
FC
NFC
NC
* *
Diastolic pressure (mmHg)
180
160
140
120
100
80
60
40
20
0
w1
w2
w3
w4
w5
w6
w7
w8
w9
w10
Feeding period (wks)

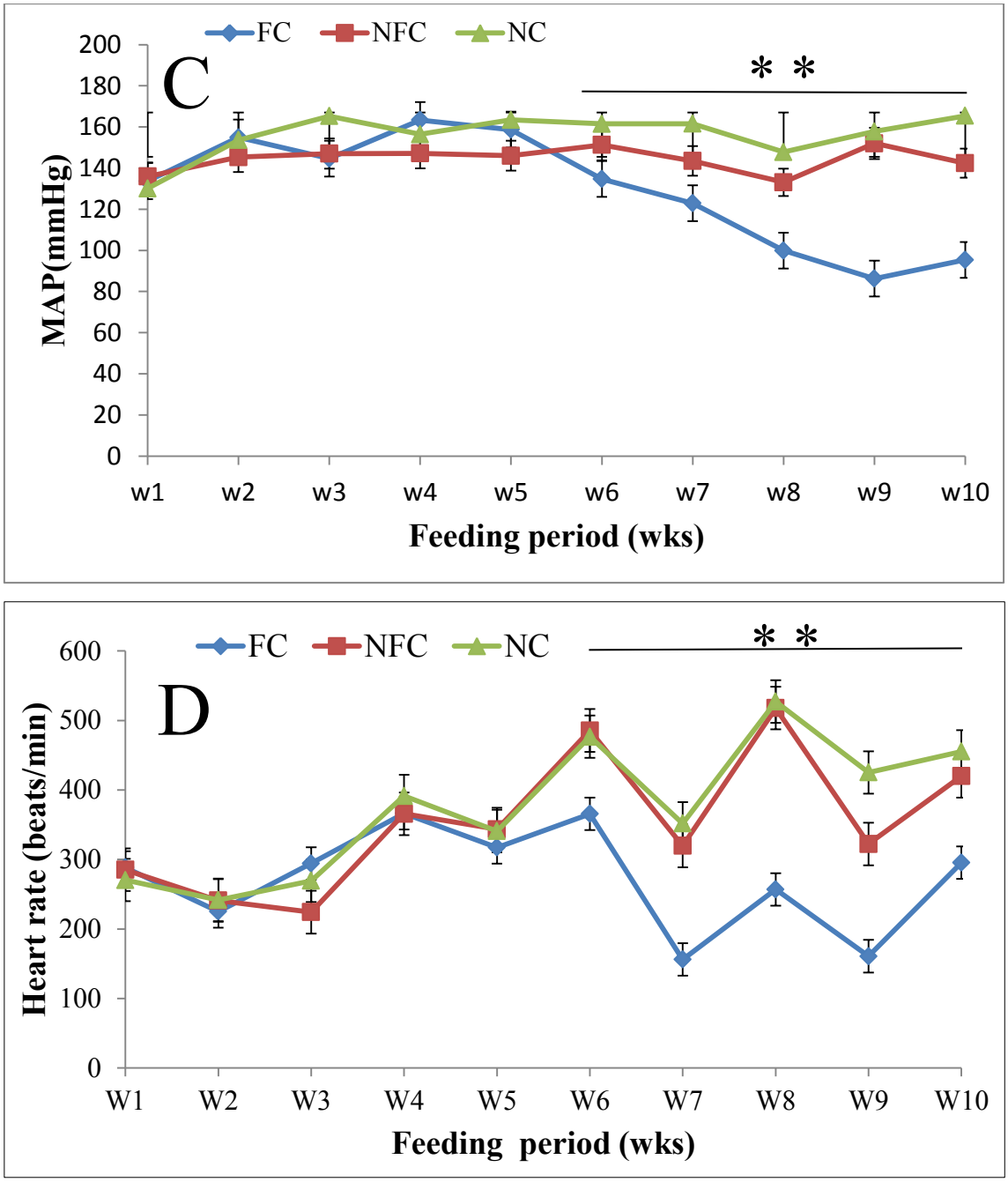


Figure 7.2 Decrease in systolic (**A**) , diastolic (**B**), mean arterial blood pressure (**C**) and Heart rate (**D**) of SHR Blood pressure lowering effect after oral administration of fermented skim milk containing peptides (FC), comparing with skim milk powder as control 1 (NFC), and diets Rats Chow as control 2 (NC). Data points are the mean ± SEM. ** indicate significant differences between group treatments ($P < 0.05$). No significant differences were found in first 5 weeks administration for all the groups.

7.4.3 Heart rate

The heart rate of the FC-treated group was lower ($P < 0.05$) than that of the other two control groups (Figure 7.2D), whereas there were no significant differences between groups fed control diets. Also, it was noted that there were no significant differences (*P*

< 0.05) between the three groups during the initial five week period (Figure 7.2D). However, there were significant ($P < 0.05$) differences of heart rate averages between treatment groups fed by peptides extract (FC, 290 beats/min) and the two control groups (NC and NFC, 432 and 460 beats/min, respectively) at the end of experiments, while the normal heart rate for rats is between (300 - 400 beats/min). It has been reported that heart rate was lowered after taking a dose of 240 mg/kg b.w. and 1200 mg/kg b.w. of peptides generated from fermented whey protein which can reduce cardiac stress (Liu et al., 2013; Wang et al., 2012). Gillman et al. (1993) stated that heart rate averages may be an independent risk factor for cardiovascular death in persons with hypertension. As heart rate increases there is a significant increase in oxygen consumption per beat (Gillman et al., 1993). The increased myocardial oxygen consumption associated with augmented heart rate leads to an increase in myocardial load and cardiac stress (Boerth et al., 1969).

7.5 Conclusion

These results show that there were positive effects of the peptides in reducing blood pressure in SHR. The elevated BP gradually decreased to a normal level ($P < 0.05$) from six to ten weeks in the FC group, compared with the other two control groups ($P < 0.05$) which remained hypertensive. While body weight was lower in the FC group, this could not be attributed to a change in energy consumption ($P < 0.05$). *In vitro*, the ACE-inhibitory activities of the experimental feeds (FC) were ~95.5 %. The control diets did not show any ACE-inhibitory activity. We can conclude that diets supplemented with peptides extracted from fermented skim milk exhibited potent antihypertensive effects in spontaneously hypertensive rats that normalised blood pressure. In addition, there was a significant reduction in body weight that may be as a result of increased metabolic rate due to inhibition of angiotensin converting enzyme activity.

Chapter 8 Effects of processing and sensory characteristics of a fermented skim milk drink as a functional milk product

8.1 Introduction

Fermented milk product is defined as a dairy product which, during the fermentation process, has its nutritional aspects as well as its physical and chemical sensory characteristics changed (World Health Organisation, 2002). This process is a result of the activities of lactic acid bacteria (LAB) that use milk as substrate as their main carbon source and growth factors (Oliveira et al., 2002). Numerous LAB used in the production of fermented milk products are considered as probiotics. Probiotics have been defined as 'live microorganisms that when administered in adequate amounts confer a health benefit on the host' (Saxelin et al., 2003). The scientific understanding in the field of probiotic bacteria and the processes of bacterial fermentation are improving. The genera of bacteria and dairy yeasts commonly used as probiotics are able to hydrolyse dairy proteins and carbohydrates to produce different types of fermented dairy products (Abbas, 2006; Ramesh. Chandan, 2013). Hydrolysed dairy proteins have several benefits over non-hydrolysed proteins as they have developed functionality in the food matrix and are rich sources of bioactive peptides (Hernández-Ledesma et al., 2011). Bioactive compounds in foods provide physiological benefits including reduced blood pressure (Chen, Remondetto, & Subirade, 2006; Chen et al., 2014; Pihlanto & Korhonen, 2015). Milk proteins have long been considered as an essential source of amino acid, and a potential media for the production of biologically active peptides (Hernández-Ledesma et al., 2011). There have been different processes employed to release bioactive peptides in short-time fermentation; some of which have used a stirred bioreactor system (Sodini et al., 1998; Stressler et al., 2013; Yadav et al., 2014). Furthermore, various bioactive peptides have been isolated from hydrolysates of casein, which include opioid agonists and angiotensin-converting enzyme inhibitor (ACE-I) peptides (Miyauchi et al., 1997; Gill et al., 2000; Ahtesh et al,. 2016). Peptides derived from milk fermentation appear to survive gastrointestinal digestion and have been identified in faeces (Ganjam et al., 1997; Gill et al., 2000). Among LAB, *L. helveticus* (Lh) has been reported to have high proteolytic activity (Yamamoto et al., 1999; Luoma et al., 2001; Griffiths & Tellez, 2013; Ahtesh et al., 2016) and has been used for milk fermentation, usually in cheese processing. These processes require long fermentation time to obtain the curd (Prevost & Divies, 1987). One of these processes is cell bioreactor technology, and this has been proposed for the continuous inoculation and acidification of fermented milk products (Prevost & Divies, 1987; Stressler et al., 2013; Yadav et al., 2014). An immobilised cell bioreactor may also be used to

inoculate and acidify milk simultaneously because of the growing activity of the immobilized culture and the resulting cell release into the bulk media (Prevost & Divies, 1987; Passos & Swaisgood, 1993; Sodini-Gallot et al., 1995). Bioreactor technology has optimal microbiological stability and a massive inoculation of milk with the starter culture of > 10^8 cfu mL^{-1} bieng observed (Sodini-Gallot et al., 1995). Continuous inoculation and milk acidification using four strains of mesophilic LAB that had been separately entrapped had very high productivity and good microbiological stability when operated with milk (Lacroix, 2005; Sodini-Gallot et al., 1995). Productivity increased further by 70 % when pH was controlled at 6.4 by adding fresh milk than when pH was controlled at 6.2 (Sodini et al., 1998).

Generally, dairy products, particularly fermented milks, are the most popular vehicles for delivery of bioactive peptides to the body due to their good compatibility, pleasant and attractive sensory profiles as well as high consumption around the world (Granato et al., 2010; Mohammadi & Mortazavian, 2011). However, bitterness of enzymatic hydrolysate may limit the use of these products (Favaro-Trindade et al., 2010; Spellman, O'Cuinn, & FitzGerald, 2009). Sensory evaluation is a method that provides integrated direct measurements of perceived intensities of target attributes (Bleibaum et al., 2002). The traditional method of evaluating the bitterness of fermented milk products is by sensory analysis using a human taste panel (Newman et al., 2014). Physicochemical characteristics have been used previously as predictors for bitterness in fermented foods, such as measuring poly- phenol content by HPLC analysis or by measuring peptide size and hydrophobicity using Urea-PAGE and RP-HPLC (Fallico et al., 2005; Newman et al., 2014). The consumption of fermented milk is widely associated with the presence of LAB due to their desirable sensory characteristics promoted by these microorganisms and the associated health benefits to the consumer. To the best of the author's knowledge, this work is the first to investigate the efficiency of agitation on ACE-I bioactive peptides by combination of *Lactobacillus helveticus* and Flavourzyme® using bioreactor. Therefore, the aims of this study were, (i) to reduce the bitterness of the fermented product by adding flavour and sucrose at the end of the fermentation processes and, (ii) to evaluate the chemical and sensory characteristics of the product using trained panellists. Furthermore, author's aim was to increase casein hydrolyses of fermented skim milk (SM) drink product in short fermentation time using a stirred bioreactor.

8.2 Material and Methods

8.2.1 Materials and Chemicals

Skim milk powder was obtained from (Murray Goulburn Co-operative Co. Ltd., Brunswick VIC Australia and United Milk Tasmania Ltd., TAS Australia), food acid, nature colour and flavour (Natural Strawberry, Flavouring Essence) were purchased from a local supermarket (Werribee, Victoria Australia), while MRS broth and sucrose were purchased from Oxoid (West Heidelberg, Vic Australia). Flavourzyme® [Flavourzyme®1000 L (EC 3.4.11.1, an amino peptidase with an activity of 1000 Leucine Amino-peptidase (LAPU g^{-1}) as quoted by Novozymes Australia] was purchased from Novozymes Australia, North Rocks, NSW, Australia, *Lactobacillus helveticus* ASCC 881315 strain was obtained from Dairy Innovation Australia Ltd. 20-Litres of bioreactor system was from (Bio-Stat® A plus, Germany). Bradford Reagent and Standard Bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St Louis, MO, USA.

8.2.2 Ethics procedure

This study was approved by the Human Research Ethics Committee (HRECs) of Victoria University, under application ID number HRE 13-079, for the conduct of sensory evaluation. All participants signed consent forms before taking part in the sensory test (Appendix III)**.**

8.2.3 Bacteria storage, culture conditions and propagation

The propagation was observed for Lh strain in RSM as described in section 6.2.2.

8.2.4 Preparation of Fermented skim milk drink

Reconstituted skim milk (RSM 12 %) was prepared by mixing skim milk powder (SMP; Murray Goulburn Co-operative Co. Ltd., Brunswick VIC., Australia) in distilled water (20 Litters) total volume, and pasteurised at 90°C for 20 min. The media was then inoculated with a combination of *L. helveticus* (1 % level) and Flavourzyme® (0.14 % w/w) (Novozymes Australia, North Rocks NSW Australia) and incubated at 37°C for 12 h with agitation. Flavourzyme® was added to improve proteolysis in milk. After the fermentation process, the samples were heat treated at 85°C for 20 min in water bath to kill and inactivate probiotic bacteria and enzyme activities. The product was cooled to room temperature, and strawberry flavour and sugar were added.

8.2.5 Bioreactor assay of low fat skim milk to increase the ACE-I% activity

Bioreactor assay was used for the preparation of fermented RSM as described in section 6.2.2.1.

8.2.6 Measurement of bacterial growth

Growth was assessed every 4 h up to 12 h during fermentation in 12 % RSM as described in the procedure in section 3.2.3.

8.2.7 Determination of proteolytic activity

Proteolytic activity during fermentation was determined according to the procedure described in section 3.2.5.

8.2.8 Determination of ACE-Inhibitory activity

ACE- inhibitory activity was measured according to the procedure described in section 3.2.6.

8.2.9 Chemical Measurements

Protein content of samples, ash and moisture, were examined according to the Association of Official Agricultural Chemists (AOAC) International (1995) methods. For protein concentration, the Bradford method (Bradford, 1976) was used. Three mL Bradford Reagent (Sigma) and 0.1 mL protein sample were added to a test-tube and vortexed to mix. The sample was then incubated at room temperature for 25 min and absorbance was measured at 595 nm using a Pharmacia spectrophotometer (LKB Novaspec II, LKB Biochrom St Albans U.K). Ash and total solids content were obtained using the muffle furnace method; approximately 5 g of fermented RSM was placed in a stainless steel crucible and evaporated to dryness in an oven at 100°C. The dry sample was placed in a muffle furnace at 550°C for 16 h, until it was free of carbon. Once ash temperature was the same as room temperature, the crucible containing the ash was weighed and the results calculated using the equation below:

$$\text{Ash } \% = \frac{\text{weight of residue x100}}{\text{weight of sample}}$$

All samples were in triplicate using the same equipment and conditions. For pH measurements, a calibrated digital pH meter (Meter Lab, Pacific Laboratory Products, and Blackburn Victoria Australia) was used.

The percentage moisture content was determined by the oven-drying method at 102°C, using the equation according to the AOAC (1998):

$$\text{Moisture } \% = \frac{\text{A} - \text{B} - \text{C X } 100}{\text{D}}$$

A= Sample and dish weight/g
B=Blank average/g
C=Empty dish weight
D=Sample weight/g

8.2.10 Sensory analyses of the fermented skim milk drink

Sensory properties of the fermented skim milk and control batches were assessed by 20 trained panellists recruited from staff members and students from the College of Health and Biomedicine at Werribee campus, Victoria University. The panellists were first trained for perception of flavour by giving them standard solutions of lactose 5 %, for sweetness judgement (normal sweetness) and 0.19 g/dl L-leucine for bitterness (extreme bitterness). They were presented with samples coded as (A) fermented skim milk drink containing peptides (FSMP) (Appendixes III); (B) final product of fermented skim milk containing peptides and 5 % sucrose (FSMPC) (to mask the bitterness) and (C) 15 % sucrose with 5 % strawberry flavor and aroma, FSMPCF; (D) Reference fermented milk commercially available-Yakult as control 1; and (E) unfermented skim milk (UN.F.SM) as control 2. The lighting and environmental conditions for the test were in accordance with international standards (Standard 8589; ISO, 1988). Samples in 30 mL white plastic cups coded with three digits at room temperature (~25°C), were presented to each panellist. Water and crackers were given to panellists for palate cleansing between samples allowing 15-min breaks between sessions. Panellists were advised not to swallow the product. Each panellist evaluated four samples for flavour (bitterness), texture, colour, and appearance, using a 10-point hedonic scale (0 = dislike extremely to 10 = like extremely) and compared them to the two controls. The test was repeated three times over the next three weeks as replicates.

The results of this assessment will help the researchers understand how fermentation has influenced texture, flavour and aroma of fermented skim milk containing peptides and an insight into how to develop a product accepted by consumers. Three sensory evaluation sessions in 3 weeks were performed by the same group of panellists in order to assess the acceptability of the products compared to the controls as affected by supplementation with sucrose and/or peptides. The scores were analysed statistically using one-way ANOVA test.

8.2.11 Statistical analysis

All data were expressed as mean values of three replicates with standard deviation. One-way ANOVA was performed to investigate the significant differences in the treatments; by Minitab 16 software. The level of significance was tested at $P < 0.01$. The test was used to investigate significant differences among the treatment means.

8.3 Results and Discussion

8.3.1 Efficiency of mechanical agitation on ACE-I peptides activity, proteolytic activities, growth and pH

Several studies on *Lactobacillus species* have focused on the enhancement of lactic acid and biomass production using bioreactor rather than on bioactive peptides production (Bury, Hajsmanova, & Jelen, 2000; Altiok, Tokatli, & Harsa, 2006; Tobajas et al., 2007). This study reports on the development of an efficient fermentation process, with respect to effect of agitation, along with ACE-inhibition (ACE-I) peptide activity production during the 12 h fermentation and strategies like fed-batch and semi-continuous fermentation in the 20 L bioreactor (Parente & Zottola, 1991; Bury, Hajsmanova & Jelen, 2000).

The effect of using a bioreactor on skim milk fermentation with the combination of Lh 881315 and Flavourzyme® was compared to traditional fermentation presented in (Figure 8.1). There were sharp increases ($P < 0.01$) in ACE-I activity using stirred bioreactor between 0 and 2 h fermentation (~ 60 %) correlated to the protein hydrolysates (Figure 8.3) and compared to the traditional fermentation method in the same fermentation time (~10 %). This may due to the mechanical process of the bioreactor that has led to the improvement at the casein hydrolysis. ACE-I activity between 4 h and 12 h fermentation increased further from 60 % to 95 % at pH 3.5 using

stirred bioreactor, whereas ACE-I activity using traditional fermentation was 82 % at 12 h fermentation and the pH was 4.9 (Figure 8.1). This could be attributed to self-digestion of the enzyme (Lin et al., 1997). However, it has been reported that casein may act as a protecting agent against self-digestion and subsequent loss of enzyme activity (Boudrant & Cheftel, 1976). Results show that mechanical treatment in bioreactor actually aided membrane damage to bacteria and resulted in greater accessibility of enzyme hydrolyses of substrates and consequently yielded higher peptide production (Choonia & Lele, 2013; Stressler et al., 2013). The suggested concentration for probiotic bacteria providing health benefits was at least $\log_6$ CFU mL^{-1} of a product during its shelf life (Shah, 2000; Betoret et al., 2003). Probiotic fermented skim milk drink revealed populations of Lh of $\log_6$ cfu mL^{-1} using bioreactor fermentation at 37°C during 12 h, whilst $\log_{5.6}$ cfu mL^{-1} during 12 h with a traditional fermentation (Figure 8.2). There was an increase in growth during 12 h fermentation in both methods (agitation and non- agitation) with significantly higher growth in the agitated system ($P < 0.01$) (Figure 8.2). The growth correlated with a drop in pH measured during fermentation and was due to the lactic acid production which increased between 2 to 12 h fermentation in the bioreactor (pH dropped from ~ 6.5 to ~ 3.9 at 12 h) (Figure 8.2). There was no significant decrease in pH in the fermentation without agitation during the first 6 h; however, there was a significant decrease in pH between 6 - 12 h fermentation time ($P < 0.01$) (Figure 8.2). Overall, the bioreactor system hydrolyses with improved ACE-I activity in a shorter fermentation time compared to the traditional fermentation.

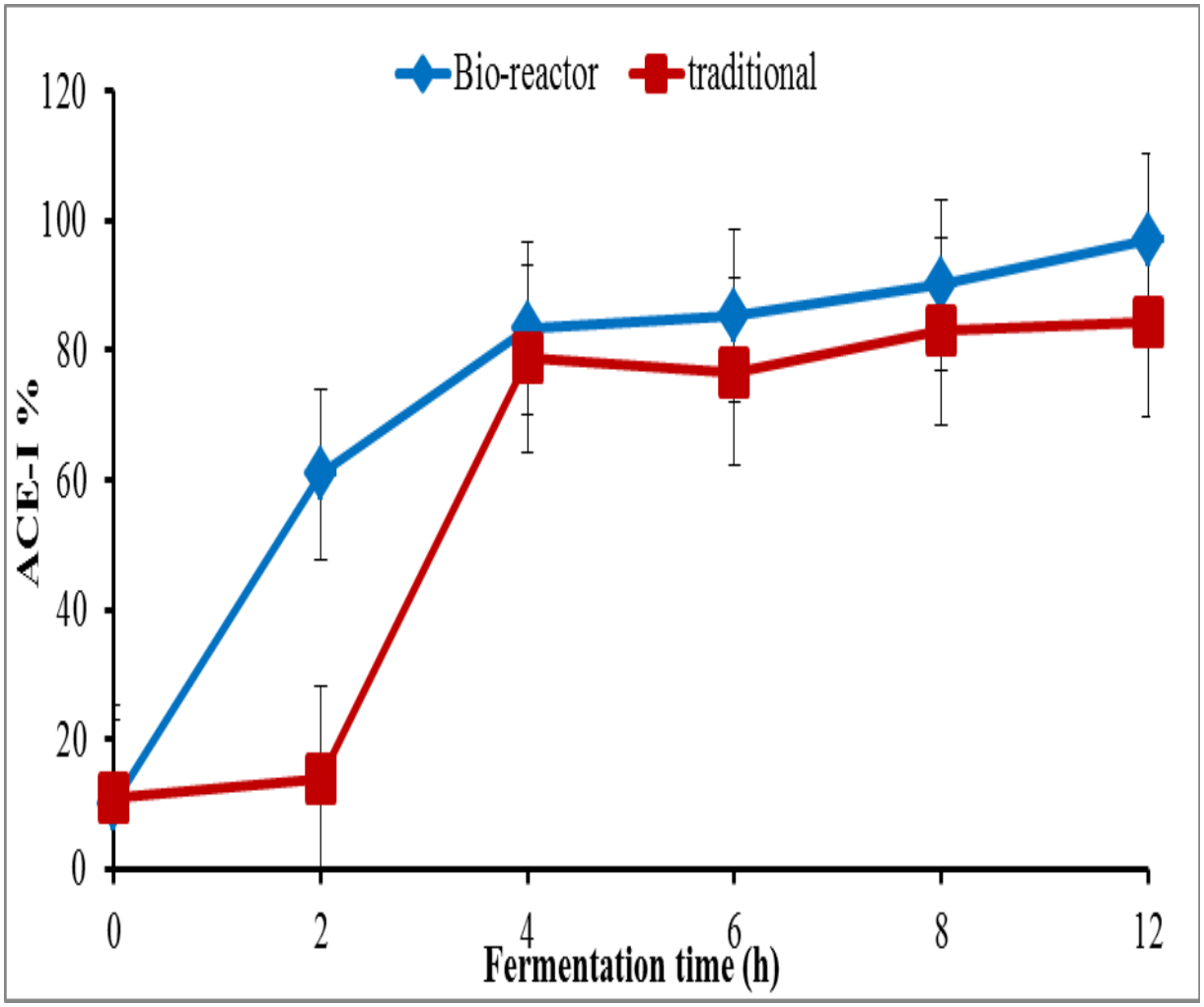


Figure 8.1 Comparison was between Bio-reactor fermentation system and the traditional fermentation (without agitation) of 12 % RSM by combination of *L. helveticus* 8801315 and Flavourzyme® at 37°C.

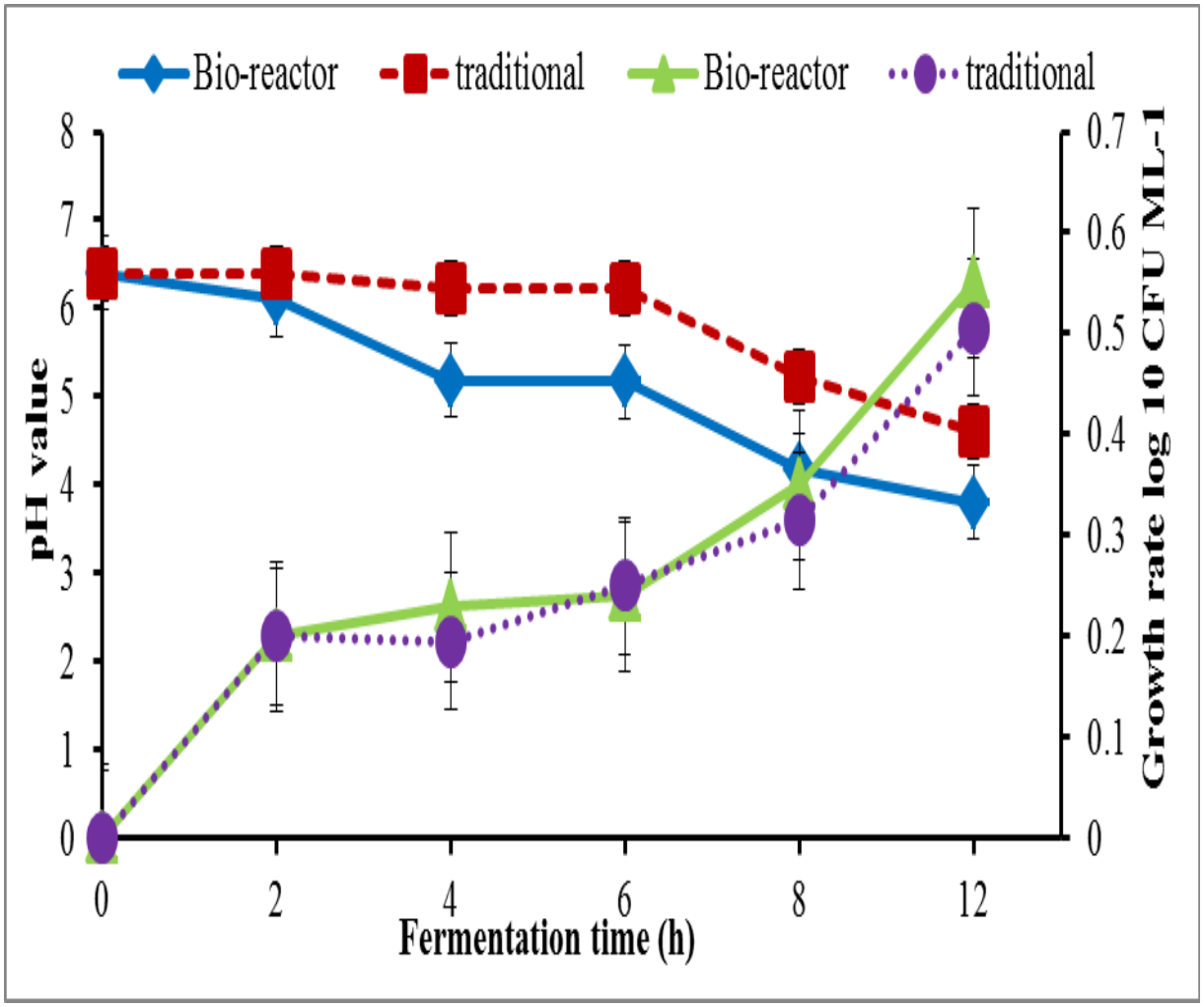


Figure 8.2 Effect of Bio-reactor fermentation system (solid line) on pH value and Bactrial growth of 12 % RSM by combination of *L. helveticus* 8801315 and Flavourzyme® at 37°C, compared with traditional fermentation (without agitation; dotted line).

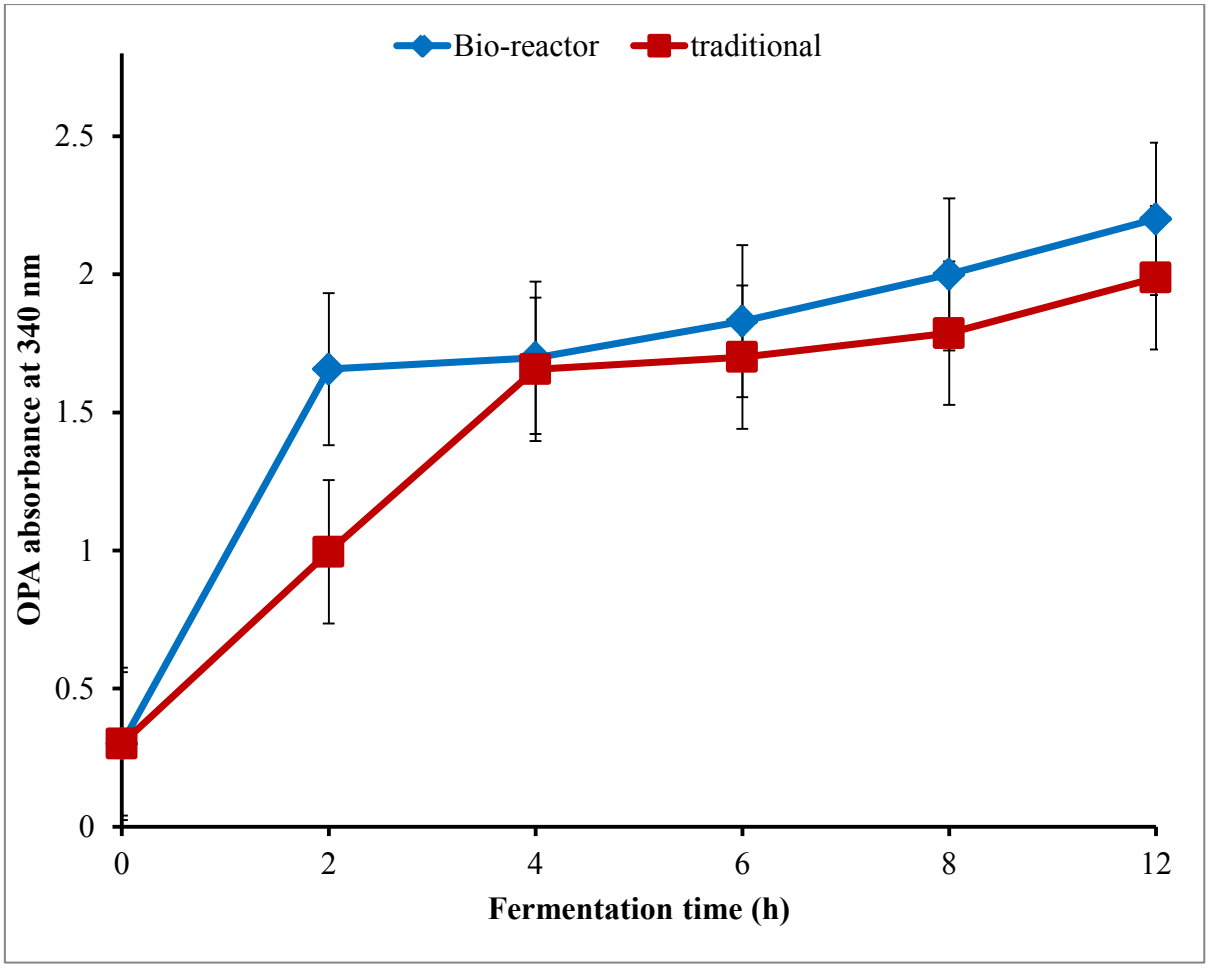


Figure 8.3 The proteolytic activity of *L. helveticus* 8801314 combined with Flavourzyme® at 37°C using Bioreactor system compared with traditional fermentation without agitation.

8.3.2 Chemical measurements

The nutritional ingredients of low-fat fermented SM drink compared to unfermented SM and commercial dairy product (Yakult) are shown in (Table 8.1). The ash content, sugar, moisture and protein were not significantly different between fermented RSM containing peptides compared to Yakult, whereas results of fermented SM drink were significantly different ($P < 0.05$) from untreated SM. Similarly, the use of *Lactobacillus plantarum* to ferment 10 % SM for 8 h at 37°C (Souza et al., 2013) were reported. Most analyses involving the development of milk-based fermented beverages, like fermented milk, yoghurts and milk drink, have reduced content or even absence of fat (Thamer & Penna, 2006; Venturoso et al., 2007). Overall, there is similarity between commercial dairy product (Yakult) compared to fermented SM drink;

however, the protein content was less in fermented SM drink (0.1/100g) compared to Yakult (1.9/100g) due to the protein hydrolyses during the fermentation process (Table 8.1). In general, most of the nutrition ingredients specially Total minerals of fermented drink have been declined into the about half compared to the untreated skim milk powder and reconstituted skim milk due to the bacterial strains consumed it for growth.

Table 8.1 Nutrition ingredients of the developed low fat fermented skim milk drink by combination of *L. helveticus* 8801315 and Flavourzyme® compared with skim milk powder, reconstituted skim milk and Yakult as commercial products containing probiotic strains.

Ingredients	**Skim milk powder**	**(12%) (RSM)**	**Fermented RSM (final product)**	**Yakult (control)**
Protein/100g	3.5±0.01	3.2±0.14	0.1±0.29	1.9±0.75
Fat/100g	0.1±0.06	0.01±0.05	0.01±0.09	0.1±0.96
Moisture (%)	3.00±0.21	88±0.54	89.9±0.01	82.4±0.02
Total minerals (%) ash	0.8±0.28	0.8±0.43	0.4±0.03	0.3±0.04
Sugar (%)/100g	5.3±0.043	0.62±0.07	15±0.17	16±0.01

8.3.3 Sensory analyses

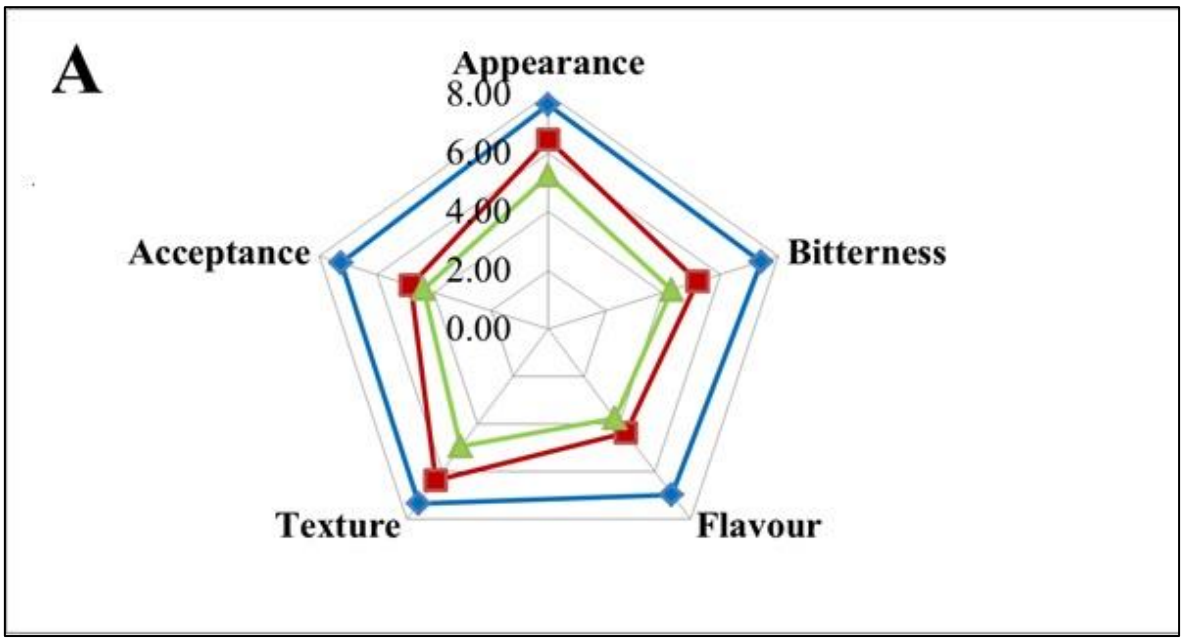
In these studies, sensory evaluation was fundamental to observe the behaviour of different types of SM drinks before and after the fermentation process. As such, the addition of sucrose that characterises the resulting products in relation to appearance, flavour, taste and/or texture, during their shelf-life, beyond verifying the acceptability by consumers was key to the evaluation. Comparisons of samples were conducted by means of sensory evaluation using the hedonic scale (Lawless & Heymann, 1999). Sensory evaluations were carried out after 12 h fermentation time at 37°C and cooled to 5°C. Twenty trained Panellists were asked to taste and compare three different fermented SM drink samples; fermented SM drink containing peptides (FSMP), fermented SM drink containing peptides and 5 % sucrose (FSMPC), and fermented SM drink containing peptides and 15 % sucrose with 5 % flavor (FSMPCF). Yakult and unfermented SM (UN.F.SM) were used as controls. The attributes described were bitterness, flavour and texture compared to the control samples (Figure 8.4). It has been reported that the production of quality of fermented milk products depends on the proteolytic activity of the strains used, since the amino acids and peptides formed have a

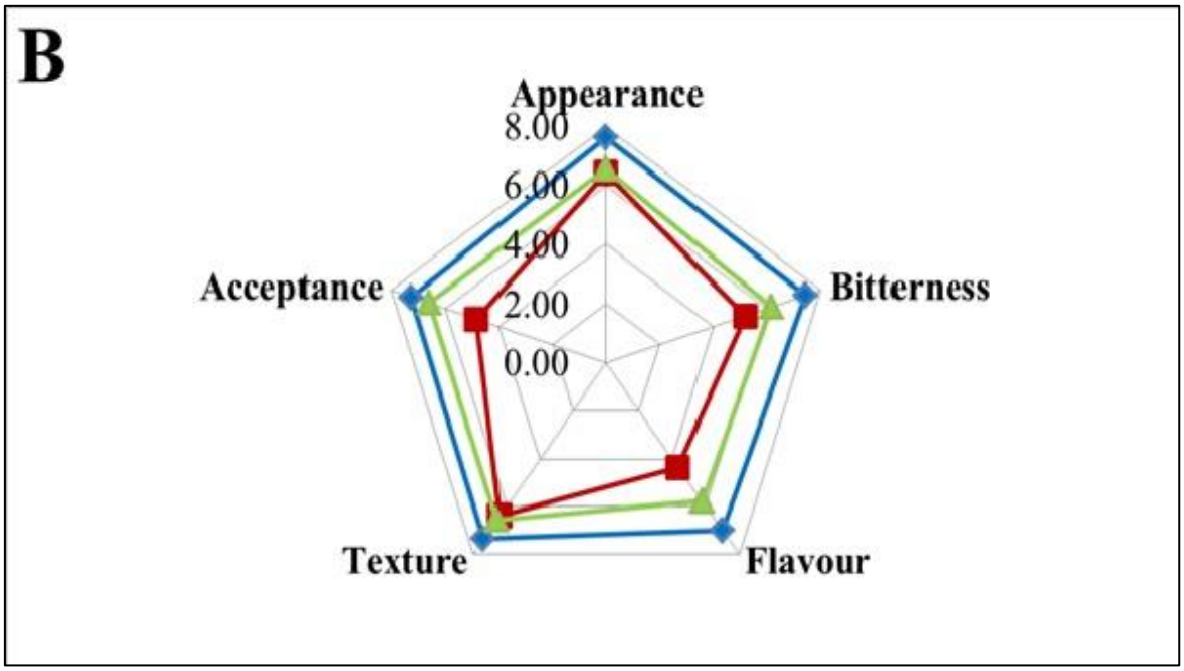
direct impact on flavour (Williams & Banks, 1997). In addition, a study reported that bitterness was generated by peptides containing phenylalanine (Akira Kawakami, 1995). There was a significant difference ($P < 0.05$) verified between the initial and final mean values of the attributes: flavour, bitterness, appearance and overall acceptance of the product (Figure 8.4 A).

Adding flavor and aroma are considered to be important parameters for consumer acceptance (Williams & Banks, 1997). Koksoy & Kilic., (2004) reported that the addition of fruit flavor and sugar can mask the sour taste in the formulations of fermented dairy products. Only FSMCPF was noted as having small, visible differences in texture and appearance compared to untreated SM. Four from 20 panellists were able to accept the bitterness test of fermented SM containing peptides without flavor. Eighteen panellists preferred Yakult and FSMCPF, with no differences between them being reported. Hence, the addition of adding 15 % sucrose and 5 % flavour to fermented SM positively affected the product and masked the bitter taste. The acceptability of 5 samples were significantly different, whilst there were no significant differences of the acceptability between 15 % sucrose FSMPC and Yakult as control ($P < 0.05$). However, there were significant differences ($P < 0.05$) in acceptance, bitterness, texture and appearance of FSMPC compared to Yakult (Figure 8.4 B). UN.F.SM and un-flavoured fermented SM was not preferred. Similarly, what was observed in the present study for the fermented SM drink, on addition of sweetness (sucrose) in the fermented dairy products, has been shown to be related with improvement of sensory behaviour. In fact, fermented milk products by *L. casei* subsp. *rhamnosus* LBC 80 combined with *Lactococcus lactis* subsp. *lactis* and one strain of *Lactococcus lactis* subsp. *cremoris* produce positive sensory changes in relation to texture and flavour in low-fat cheese, when compared with control unfermented low-fat cheese (Katsiari et al., 2002). In addition, Menendez et al., (2000) obtained improvement of sensory parameters of cheeses by the reduction of bitter taste, in relation to control.

Generally, markets for functional dairy products have reached a significant level and are expected to grow in the future. However, it is important to point out that the maximum expressions of the real functional properties of these products must be reconciled with the sensory acceptance of the dairy that is being developed (Castro et al., 2004).

Several dairy products were tested as vehicle of probiotic cultures, which showed functionally and sensorial appropriateness (Oliveira et al., 2002). According to these results, the acceptable sensory quality and the nutritional and health claims may be used for the promotion of the products and increasing the marketing appeal of functional dairy products.





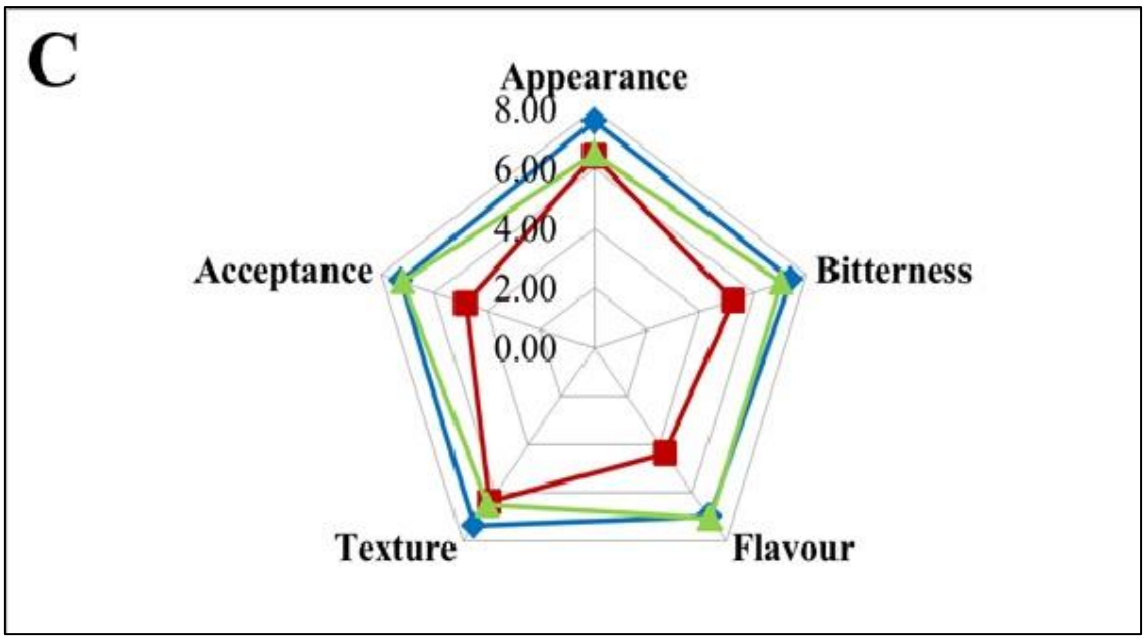


Figure 8.4 A graphic representation of the mean of sensory evaluation by quantitative descriptive analysis (QDA) of unfermented skim milk (UN.F.SM ■) as control, commercial product Yakult (♦) as control 2 and (**A**) fermented low fat skim milk drink containing 95.5 % peptides, FSMP (▲), fermented low fat skim milk drink containing peptides and 5 % sucrose FSMPC (▲) (**B**), and (**C**) fermented low fat skim milk drink containing peptides and 15 % sucrose with 5 % flavor, FSMPCF(▲).

8.4 Conclusion

The efficiency of a bioreactor was improved with mechanical agitation during fermentation and resulted in increased cell viability and ACE-I activity from 90.3 % to 95.5 %, using *L. helveticus* 8801315 and Flavourzyme®. Fermented skim milk containing bioactive peptides was developed with acceptable sensory characteristics. However, increased acidity, as well as bioactive peptides, led to increased bitterness of the fermented skim milk drink. The addition of 15 % sucrose and 5 % strawberry flavour provided positive changes to the fermented product in terms of being accepted by consumers.

Chapter 9 Overall Conclusions and Future Research Directions

9.1 Overall Conclusions

RSM was a better growth promoter of probiotic bacteria than WPC. In fact, all strains were capable of growing well in RSM due to their proteolytic activities which resulted in increased ACE-inhibition activity, compared to WPC. When strains were incubated in combination with protease in RSM, the proteolytic and ACE-I activities of Lc210, Bb12, Lb11842, La2410 were higher in RSM media compared with WPC media. Bb12 and La2410 demonstrated higher ACE-inhibitory and proteolytic activities compared to Lb11842 and Lc210. The supplementation of media with Flavourzyme® increased proteolysis and thus production of ACE-I peptides of all four bacterial strains. Flavourzyme® supplementation of media reduced fermentation time from 12 h to 8 h. The increase in growth translated to the corresponding decrease in pH value.

Differences in the production of ACE-I peptides by *L. helveticus* (Lh 881315, Lh 881188, Lh 880474 and Lh 880953) varied between the strains due to differences in proteolytic activity. Casein-rich RSM supported higher growth of *L. helveticus* strains, higher proteolytic activity and higher production of ACE-I peptides. Beneficial effects of protease supplementation were more pronounced in the first 8 h of fermentation. The highest proteolytic and ACE-I activity was observed for Lh 881315 combined with Flavourzyme® in RSM. In fermented RSM, enhanced proteolytic activity by probiotic organisms and protease improved the production of ACE-I peptides, which, in turn, caused appreciable *in vitro* ACE-I activity.

The combination of LAB strains with *Kluyveromyces marxianus* LAF4 (*K. marxianus*) led to reduced bacterial growth and ACE-I activity after 8 h fermentation most likely due to alcohol production compared to LAB separately. It was suggested that the *K. marxianus* or LAB preferred separately to release more ACE-I peptides in fermented dairy products other than in combination forms.

The selected strains produced a range of bioactive peptides with varying degrees of ACE-inhibition. In this study, has been successfully identified 133 peptides with 99 % confidence from two fractions (F1 and F6). The highest ACE-I activity was in F6 (90.31 % with IC_{50} 0.01 mg mL^{-1}). The most potent ACE-I peptides found in this hydrolysate corresponded to FFVAPFPGVFGK, GPVRGPFPIIV, and LHLPLPLL and showed significant antihypertensive activity.

Elevated blood pressure (BP) of spontaneously hypertensive rats (SHR) fed with peptide extraction gradually decreased to normal levels in six to ten weeks for the rats group, compared with the other 2 control groups, which remained hypertensive. While body weight was lower in the FC group, it could not be attributed to a change in energy consumption. The control group's feed did not show any effects on SHR blood pressure. Feeding diets supplemented with peptide extract from fermented skim milk drink exhibited potent antihypertensive effects on SHR blood pressure. There was a significant reduction in body weight that may be a result of increased metabolic rate due to inhibition of angiotensin converting enzyme activity.

The efficiency of a bioreactor improved with mechanical agitation during fermentation and resulted in increased cell viability and ACE-I activity from 90.3 % to ~95.5 % using *L. helveticus* 8801315 and Flavourzyme®. Fermented skim milk containing bioactive peptides was developed with acceptable sensory characteristics. However, increased acidity as well as bioactive peptides led to increased bitterness of the fermented skim milk. The addition of 15 % sucrose and 5 % of natural strawberry flavour proved more acceptable to consumers in terms of flavour.

9.2 Future Research Direction

The project results showed that probiotic organism (*Lactobacillus helveticus* 881315) used in this research released more bioactive compounds (peptides) in combination with Flavourzyme® during fermentation of low-fat skim milk in a bioreactor system. This research finding has raised some important questions that need to be addressed in future research studies which can be classified under two major areas of research:

A) Stability, mechanism of the ACE-I peptides and human trials,

B) Large scale production of biologically active peptides and the potential use as nutraceutical additives in functional foods.

Chapter 10 References

Abbas, C. (2006). Production of antioxidants, aromas, colours, flavours, and vitamins by yeasts. In A. Querol & G. Fleet (Eds.), *Yeasts in Food and Beverages* (pp. 285-334): Springer Berlin Heidelberg.

Abdelgadir, W. S., Ahmed, T. K., & Dirar, H. A. (1998). The traditional fermented milk products of the Sudan. *International Journal of Food Microbiology, 44*(1–2), 1-13. doi: http://dx.doi.org/10.1016/S0168-1605(98)00090-7.

Acharya, K. R., Sturrock, E. D., Riordan, J. F., & Ehlers, M. R. W. (2003). Ace revisited: A new target for structure-based drug design. *Nat Rev Drug Discov, 2*(11), 891-902.

Adibi, S. A., & Morse, E. L. (1971). Intestinal transport of dipeptides in man: relative importance of hydrolysis and intact absorbance. *Journal of clinical investegation, 50*(11), 2266-2275. doi: 10.1172/JCI106724.

Agyei, D., Potumarthi, R., & Danquah, M. K. (2015). Food-derived multifunctional bioactive proteins and peptides *Biotechnology of Bioactive Compounds* (pp. 507-524): John Wiley & Sons, Ltd.

Ahn, C.-B., Jeon, Y.-J., Kim, Y.-T., & Je, J.-Y. (2012). Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis. *Process Biochemistry, 47*(12), 2240-2245. doi: http://dx.doi.org/10.1016/j.procbio.2012.08.019.

Ahtesh, F. B., Stojanovska, L., Mathai, M. L., Apostolopoulos, V., & Mishra, V. K. (2016a). Proteolytic and angiotensin-converting enzyme-inhibitory activities of selected probiotic bacteria. *International Journal of Food Science & Technology*.

Ahtesh, F., Stojanovska, L., Shah, N., & Mishra, V. K. (2016b). Effect of Flavourzyme® on Angiotensin-Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by *Lactobacillus helveticus*. *Journal of food science*, 81(1), M135-M143.

Aihara, K., Kajimoto, O., Hirata, H., Takahashi, R., & Nakamura, Y. (2005). Effect of powdered fermented milk with *Lactobacillus helveticus* on subjects with high-normal blood pressure or mild hypertension. *Journal of the American College of Nutrition, 24*(4), 257-265.

Akira Kawakami, H. K. (1995). Taste evaluations of angiotensin I converting enzyme inhibitors, Leu-Lys-Tyr analogues. Bioscience, biotechnology, and biochemistry, 59, 709–710.

Alhaj, O. A., Kanekanian, A. D., & Peters, A. C. (2007). Investigation on whey proteins profile of commercially available milk-based probiotics health drinks using fast protein liquid chromatography (FPLC). *British Food Journal, 109*(6), 469-480.

Altiok, D., Tokatli, F., & Harsa, Ş. (2006). Kinetic modelling of lactic acid production from whey by *Lactobacillus casei* (NRRL B-441). *Journal of Chemical Technology and Biotechnology*, 81(7), 1190-1197.

Amerine, M. A., Pangborn, R. M., & Roessler, E. B. (2013). *Principles of sensory evaluation of food*: Elsevier.

Amrane, A., & Prigent, Y. (1998b). Influence of yeast extract concentration on batch cultures of Lactobacillus helveticus: Growth and production coupling. *World Journal of Microbiology and Biotechnology, 14*(4), 529-534.

Amrane, A., & Prigent, Y. (1998a). Lactic acid production rates during the different growth phases of *Lactobacillus helveticus* cultivated on whey supplemented with yeast extract. *Biotechnology Letters, 20*(4), 379-383.

Anema, S. G. (2009). The use of "lab-on-a-chip" microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. *International DairyJournal,19*(4),198-204.doi:http://dx.doi.org/10.1016/j.idairyj.2008.11.001.

Antalis, T. M., Shea-Donohue, T., Vogel, S. N., Sears, C., & Fasano, A. (2007). Mechanisms of disease: Protease functions in intestinal mucosal pathobiology. *Nature Clinical Practice Gastroenterology and Hepatology, 4*(7), 393-402.

AOAC, International. (1995). *AOAC Official Methods of Analysis.* . Arlington, VA: AOAC International. (Accessed, 15.Sep. 2014).

Appel, L. J., Moore, T. J., Obarzanek, E., Vollmer, W. M., Svetkey, L. P., Sacks, F. M., Harsha, D. W. (1997). A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure. *New England Journal of Medicine, 336*(16), 1117-1124. doi: doi:10.1056/NEJM199704173361601.

Banks, J. M., Law, A. J. R., Leaver, J., & Horne, D. S. (1995). Maturation profiles of cheddar-type cheese produced from high heat treatment milk to incorporate whey protein. *Advances in Experimental Medicine and Biology, 367*, 221-236.

Barbana, C., & Boye, J. I. (2010). Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. *Food Research International, 43*(6), 1642-1649. doi: 10.1016/j.foodres.2010.05.003.

Bayoumi, M. A., & Griffiths, M. W. (2012). In vitro inhibition of expression of virulence genes responsible for colonization and systemic spread of enteric pathogens usingBifidobacterium bifidum secreted molecules. *International*

Journal of Food Microbiology, 156 (3), 255-263.doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2012.03.034.

Belem, M. A. F., & Lee, B. H. (1999). Fed-batch fermentation to produce oligonucleotides from Kluyveromyces marxianus grown on whey. Process Biochemistry, 34(5), 501-509. doi: http://dx.doi.org/10.1016/S0032-9592(98)00118-6.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1121(1–2), 130-136. doi: http://dx.doi.org/10.1016/0167-4838(92)90346-F

Bertrand-Harb, C., Baday, A., Dalgalarrondo, M., Chobert, J. M. & Haertle, T. (2002). Thermal modifications of structure and codenaturation of α-lactalbumin and β-lactoglobulin induce changes of solubility and susceptibility to proteases. *Food/Nahrung*, 46, 283-289.

Betoret, N., Puente, L., Dı´az, M.J., Paga´ n, M.J., Garcı´a, M.J., Gras, M.L., et al. (2003). Development of probiotic-enriched dried fruits by vacuum impregnation. *Journal of Food Engineering*, 56, 273-277.

Bleibaum, R. N., Stone, H., Tan, T., Labreche, S., Saint-Martin, E., & Isz, S. (2002). Comparison of sensory and consumer results with electronic nose and tongue sensors for apple juices. *Food Quality and Preference, 13*(6), 409-422. doi: http://dx.doi.org/10.1016/S0950-3293(02)00017-4.

Boerth, R. C., covell, J. W., pool, P. E., & ross, J. (1969). Increased myocardial oxygen consumption and contractile state associated with increased heart rate in dogs. *Circulation Research, 24*(5), 725-734.

Bopp, P. A. (1997). *Consumer Testing*. Access date,10. Sep. 2014. Doi: http://food.oregonstate.edu/sensory/phil2.html .

Boudrant, J. and Cheftel, C. 1976. Continuous proteolysis with a stabilized protease. II Continuous experiments. Biotechnol. Bioeng. 18: 1735–1749. Cheftel, C., Ahern, M., Wang, D.I.C., and Tannenbaum, S.R. 1971. Enzymatic solubilization of fish protein concentrate: Batch studies applicable to continuous enzyme recycling processes. *Journal of Agriculture and Food Chem*. 19: 155–161.

Boustany, C. M., Bharadwaj, K., Daugherty, A., Brown, D. R., Randall, D. C., & Cassis, L. A. (2004). Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 287(4), R943-R949. doi: 10.1152/ajpregu.00265.2004

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition, 97*(6), 1314-1323.

Brew, K. (2003). Structure of human ACE gives new insights into inhibitor binding and design. *Trends in Pharmacological Sciences*, 24(8), 391-394. doi: http://dx.doi.org/10.1016/S0165-6147(03)00196-2

Brew, K., Castellino, F. J., Vanaman, T. C., & Hill, R. L. (1970). The Complete Amino Acid Sequence of Bovine α-Lactalbumin. *Journal of Biological Chemistry, 245*(17), 4570-4582.

Brown, N. J., & Vaughan, D. E. (1998). Angiotensin-Converting Enzyme Inhibitors. *Circulation, 97*(14), 1411-1420. doi: 10.1161/01.cir.97.14.1411.

Burke, V., Hodgson, J. M., Beilin, L. J., Giangiulioi, N., Rogers, P., & Puddey, I. B. (2001). Dietary protein and soluble fiber reduce ambulatory blood pressure in treated hypertensives. Hypertension, 38(4), 821-826.

Cameron, A. J., Welborn, T. A., Zimmet, P. Z., Dunstan, D. W., Owen, N., Salmon, J., Shaw, J. E. (2003). Overweight and obesity in Australia: the 1999-2000 Australian diabetes, obesity and lifestyle study (AusDiab). *Medical Journal of Australia, 178*(9), 427-432.

Cam, A., & de Mejia, E. G. (2012). Role of dietary proteins and peptides in cardiovascular disease. *Molecular Nutrition & Food Research*, 56(1), 53-66. doi: 10.1002/mnfr.201100535.

Case, D., Atlas, S., Laragh, J., Sealey, J., Sullivan, P., & McKinstry, D. (1980). Clinical experience with blockade of the renin-angiotensin-aldosterone system by an oral converting enzyme inhibitor (SQ 14,225, Captopril) in hypertensive Patients. In D. Case, E. Sonnenblick, & J. Laragh (Eds.), *Captopril and Hypertension* (pp. 211-230): Springer US.

Cassis, L. A., Saye, J., & Peach, M. J. (1988). Location and regulation of rat angiotensinogen messenger rna. *Hypertension*, 11(6 Pt 2), 591-596.

Castro, I.A., Tirapegui, J., Silva, R.S.S.F. & Cutrim, A.J.S. (2004). Sensory evaluation of a milk formulation supplemented with n3 polyunsaturated fatty acids and soluble fibres. *Food Chemistry*, 85, 503–512.

Castro, S., Vazquez Peyronel, D., & Cantera, A. M. B. (1996). Proteolysis of whey proteins by a Bacillus subtilis enzyme preparation. *International Dairy Journal*, 6(3), 285-294.

Champagne, C. P., Girard, F., & Gardner, N. (1989). Growth of yeast contaminants in an immobilized lactic acid bacteria system. *Letters in Applied Microbiology*, 8(6), 207-210. doi: 10.1111/j.1472-765X.1989.tb00249.x.

Chatterton, D. E. W., Smithers, G., Roupas, P., & Brodkorb, A. (2006). Bioactivity of β-lactoglobulin and α-lactalbumin-Technological implications for processing. International Dairy Journal, 16(11), 1229-1240. doi: http://dx.doi.org/10.1016/j.idairyj.2006.06.001.

Chaves-López, C., Serio, A., Paparella, A., Martuscelli, M., Corsetti, A., Tofalo, R., & Suzzi, G. (2014). Impact of microbial cultures on proteolysis and release of bioactive peptides in fermented milk. *Food Microbiology, 42*(0), 117-121. doi: http://dx.doi.org/10.1016/j.fm.2014.03.005.

Chaves-López, C., Tofalo, R., Serio, A., Paparella, A., Sacchetti, G., & Suzzi, G. (2012). Yeasts from Colombian Kumis as source of peptides with Angiotensin I converting enzyme (ACE) inhibitory activity in milk. *International Journal of Food Microbiology*, 159(1), 39-46.

Chaves-López, C., Serio, A., Martuscelli, M., Paparella, A., Osorio-Cadavid, E., & Suzzi, G. (2011). Microbiological characteristics of kumis, a traditional fermented Colombian milk, with particular emphasis on enterococci population. *Food Microbiology*, 28(5), 1041-1047.

Cheison, S. C., Zhang, S.-B., Wang, Z., & Xu, S.-Y. (2009). Comparison of a modified spectrophotometric and the pH-stat methods for determination of the degree of hydrolysis of whey proteins hydrolysed in a tangential-flow filter membrane reactor. *Food Research International*, 42(1), 91-97.

Cheison, S. C., Wang, Z., & Xu, S.-Y. (2007). Multivariate strategy in screening of enzymes to be used for whey protein hydrolysis in an enzymatic membrane reactor. *International Dairy Journal*, 17(4), 393-402. doi: http://dx.doi.org/10.1016/j.idairyj.2006.05.006.

Chen, Y., Liu, W., Xue, J., Yang, J., Chen, X., Shao, Y., Kwok, L.y., Bilige, M., Mang, L. & Zhang, H. (2014). Angiotensin-converting enzyme inhibitory activity of *Lactobacillus helveticus* strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9. *Journal of Dairy Science*, 97, 6680-6692.

Chen, G.W., Tsai, J.-S., & Sun Pan, B. (2007). Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. *International Dairy Journal*, 17(6), 641-647. doi: http://dx.doi.org/10.1016/j.idairyj.2006.07.004.

Chen, L., Remondetto, G. E., & Subirade, M. (2006). Food protein-based materials as nutraceutical delivery systems. *Trends in Food Science & Technology*, 17(5), 272-283. doi: http://dx.doi.org/10.1016/j.tifs.2005.12.011.

Chen, J. H., Tao, L., & Li, J. (2003). The Formol titration method. Experiments of Biochemistry. *Beijing: Science Press*, 57–58.

Cheviron, N., Rousseau-Plasse, A., Lenfant, M., Adeline, M.-T., Potier, P., & Thierry, J. (2000). Coumarin-Ser-Asp-Lys-Pro-OH, A fluorescent substrate for determination of angiotensin-converting enzyme activity via high-performance Liquid chromatography. *Analytical Biochemistry*, 280(1), 58-64. doi: http://dx.doi.org/10.1006/abio.2000.4484.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the cooh-terminal dipeptide sequence. *Journal of Biological Chemistry, 255*(2), 401-407.

Choonia, H. S., & Lele, S. S. (2013). Kinetic modeling and implementation of superior process strategies for β-galactosidase production during submerged fermentation in a stirred tank bioreactor. *Biochemical Engineering Journal*, 77, 49-57. doi: http://dx.doi.org/10.1016/j.bej.2013.04.021.

Christensen, J. E., Dudley, E. G., Pederson, J. A., & Steele, J. L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek, 76*(1), 217-246. doi: 10.1023/a:1002001919720.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins. *Journal of Dairy Science*, 66(6), 1219-1227. doi: 10.3168/jds.S0022-0302(83)81926-2.

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus1. *Journal of Dairy Science, 83*(6), 1187-1195.

Commission, T. C. D. (2012). *Fermented Milk Products*. *Canadian Dairy Commission*. http://www.milkingredients.ca/index-eng.php?id=180.

Contreras, M. d. M., Sevilla, M. A., Monroy-Ruiz, J., Amigo, L., Gómez-Sala, B., Molina, E., Recio, I. (2011). Food-grade production of an antihypertensive casein hydrolysate and resistance of active peptides to drying and storage. *International Dairy Journal*, 21(7), 470-476. doi: 10.1016/j.idairyj.2011.02.004

Contreras, M. d. M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal, 19*(10), 566-573. doi: http://dx.doi.org/10.1016/j.idairyj.2009.05.004.

Contor, L. (2001). Functional food science in europe. *Nutrition, metabolism, and cardiovascular diseases: NMCD, 11*(4 Suppl), 20-23.

Coulter, D., & Edwards, R. (1987). Cough associated with captopril and enalapril. *British Medical Journal, 294*(6586), 1521-1523.

Cushman, D., Cheung, H., Sabo, E., & Ondetti, M. (1980). Design of New Antihypertensive Drugs. In D. Case, E. Sonnenblick, & J. Laragh (Eds.), *Captopril and Hypertension* (pp. 103-113): Springer US.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977). Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry, 16*(25), 5484-5491. doi: 10.1021/bi00644a014.

Cruz, A. G., Castro, W. F., Faria, J. A. F., Lollo, P. C. B., Amaya-Farfán, J., Freitas, M. Q., Godoy, H. T. (2012). Probiotic yogurts manufactured with increased glucose oxidase levels: Postacidification, proteolytic patterns, survival of probiotic microorganisms, production of organic acid and aroma compounds. *Journal of Dairy Science, 95*(5), 2261-2269. doi: http://dx.doi.org/10.3168/jds.2011-4582.

Damodaran, S., Parkin, K. L., & Fennema, O. R. (2008). *Fennema's Food Chemistry,* (4th ed.). Boca Raton FL: CRC press.

Danilczyk, U., & Penninger, J. M. (2006). Angiotensin-converting enzyme II in the heart and the kidney. *Circulation Research, 98*(4), 463-471. doi: 10.1161/01.RES.0000205761.22353.5f.

Dave, R. I., & Shah, N. P. (1998). Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *Journal of Dairy Science, 81*(11), 2804-2816. doi: http://dx.doi.org/10.3168/jds.S0022-0302(98)75839-4.

Davies, E. A., Bevis, H. E., Potter, R., Harris, J., Williams, G. C., & Delves-Broughton, J. (1998). Research note: The effect of pH on the stability of nisin solution during autoclaving. *Letters in Applied Microbiology, 27*(3), 186-187. doi: 10.1046/j.1472-765X.1998.t01-1-00401.x.

Degraeve, P., & Martial-Gros, A. (2003). Purification and partial characterisation of X-prolyl dipeptidyl aminopeptidase of *Lactobacillus helveticus* ITG LH1. *International Dairy Journal, 13*(7), 497-507. doi: http://dx.doi.org/10.1016/S0958-6946(03)00057-8.

Del Mar Contreras, M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal, 19*(10), 566-573.

Dissanayake, M., Ramchandran, L., Donkor, O. N., & Vasiljevic, T. (2013). Denaturation of whey proteins as a function of heat, pH and protein concentration. *International Dairy Journal, 31*(2), 93-99. doi: http://dx.doi.org/10.1016/j.idairyj.2013.02.002.

Dissanayake, M., Ramchandran, L., Piyadasa, C., & Vasiljevic, T. (2013). Influence of heat and pH on structure and conformation of whey proteins. *International Dairy Journal,* 28 (2), 56-61. doi: http://dx.doi.org/10.1016/j.idairyj.2012.08.014.

Dissanayake, M., Liyanaarachchi, S., & Vasiljevic, T. (2012). Functional properties of whey proteins microparticulated at low pH. *Journal of Dairy Science, 95*(4), 1667-1679. doi: http://dx.doi.org/10.3168/jds.2011-4823.

Dolan, E., Thijs, L., Li, Y., Atkins, N., McCormack, P., McClory, S., Stanton, A. V. (2006). Ambulatory arterial stiffness index as a predictor of cardiovascular mortality in the Dublin Outcome Study. *Hypertension*, 47(3), 365-370.

Domingues, L., Guimarães, P. M. R., & Oliveira, C. (2010). Metabolic engineering of *Saccharomyces cerevisiae* for lactose/whey fermentation. *Bioengineered Bugs*, 1(3), 164-171.

Donkor, O., Henriksson, A., Singh, T. K., Vasiljevic, T., & Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal, 17*(11), 1321-1331. doi: 10.1016/j.idairyj.2007.02.009.

Donkor, O., Henriksson, A., Vasiljevic, T., & Shah. (2007). Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait, 87*(1), 21-38.

Donkor, O., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. *International Dairy Journal, 16*(10), 1181-1189. doi: 10.1016/j.idairyj.2005.10.008.

Donkor, O., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2005). Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. *Journal of Food Science*, 70(8), m375-m381. doi: 10.1111/j.1365-2621.2005.tb11522.x.

Doolittle, R. (1983). Angiotensinogen is related to the antitrypsin-antithrombin-ovalbumin family. *Science, 222*(4622), 417-419. doi: 10.1126/science.6604942.

Du, L., Fang, M., Wu, H., Xie, J., Wu, Y., Li, P., Wei, D. (2013). A novel angiotensin I-converting enzyme inhibitory peptide from Phascolosoma esculenta water-

soluble protein hydrolysate. *Journal of Functional Foods, 5*(1), 475-483. doi: http://dx.doi.org/10.1016/j.jff.2012.12.003.

Ebner, J., Aşçı Arslan, A., Fedorova, M., Hoffmann, R., Küçükçetin, A. & Pischetsrieder, M. (2015). Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains. *Journal of Proteomics,* 117**,** 41-57.

Eisele, T., Stressler, T., Kranz, B., & Fischer, L. (2013). Bioactive peptides generated in an enzyme membrane reactor using bacillus lentus alkaline peptidase. *European Food Research and Technology, 236*(3), 483-490.

Engeli, S., Böhnke, J., Gorzelniak, K., Janke, J., Schling, P., Bader, M., Sharma, A. M. (2005). Weight loss and the renin-angiotensin-aldosterone system. *Hypertension*, 45(3), 356-362.

Erdos, E. G. (1975). Angiotensin I converting enzyme. *Circulation Research, 36*(2), 247-255.

Erdmann, K., Cheung, B. W., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654.

Fallico, V., McSweeney, P. L. H., Horne, J., Pediliggieri, C., Hannon, J. A., Carpino, S., & Licitra, G. (2005). Evaluation of Bitterness in Ragusano Cheese. *Journal of Dairy Science, 88*(4), 1288-1300. doi: http://dx.doi.org/10.3168/jds.S0022-0302(05)72795-8.

Farrell Jr, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Swaisgood, H. E. (2004). Nomenclature of the Proteins of Cows' Milk—Sixth Revision. Journal of Dairy Science, 87(6), 1641-1674. doi: http://dx.doi.org/10.3168/jds.S0022-0302(04)73319-6.

Favaro-Trindade, C. S., Santana, A. S., Monterrey-Quintero, E. S., Trindade, M. A., & Netto, F. M. (2010). The use of spray drying technology to reduce bitter taste of casein hydrolysate. *Food Hydrocolloids, 24*(4), 336-340. doi: http://dx.doi.org/10.1016/j.foodhyd.2009.10.012.

Fernández-Musoles, R., Manzanares, P., Burguete, M. C., Alborch, E., & Salom, J. B. (2013). In vivo angiotensin I-converting enzyme inhibition by long-term intake of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats. *Food Research International, 54*(1), 627-632. doi: http://dx.doi.org/10.1016/j.foodres.2013.07.063.

Fiat, A.M., Migliore-Samour, D., Jollès, P., Drouet, L., Sollier, C. B. D., & Caen, J. (1993). Biologically Active Peptides from Milk Proteins with Emphasis on Two

Examples Concerning Antithrombotic and Immunomodulating Activities. *Journal of Dairy Science, 76*(1), 301-310 .

FitzGerald, C., Gallagher, E., Tasdemir, D., & Hayes, M. (2011). Heart health peptides from macroalgae and their potential use in functional foods. Journal of Agricultural and Food Chemistry, 59(13), 6829-6836.

FitzGerald, R. J., & Murray, B. A. (2006). Bioactive peptides and lactic fermentations. International Journal of Dairy Technology, 59(2), 118-125. doi: 10.1111/j.1471-0307.2006.00250.

FitzGerald, R., Murray, B., & Walsh, D. (2004). Hypotensive peptides from milk proteins. The Journal of Nutrition, 134(4), 980-988.

FitzGerald, R. J., & Meisel, H. (2003). Milk protein hydrolysates and bioactive peptides. In P. F. Fox & P. L. H. McSweeney (Eds.), Advanced Dairy Chemistry—1 Proteins (pp. 675-698): Springer US.

FitzGerald, R., & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. British Journal of Nutrition, 84, 33-37. 5.

FitzGerald, R. J. (1998). Potential Uses of Caseinophosphopeptides. *International Dairy Journal*, 8(5–6), 451-457. doi: http://dx.doi.org/10.1016/S0958-6946(98)00068.

Fitzsimons, S. M., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids, 21*(4), 638-644. doi: http://dx.doi.org/10.1016/j.foodhyd.2006.07.007.

Food and Agriculture Organization of the United Nations, & World Health Organization. (2002). Guidelines for the evaluation of probiotics in food: Report of a joint FAO/WHO working group on drafiting guidelines for the evaluation of probiotics in food. Accessed 2 February, 2013, fromhttp ://www.fda.gov/ohrms/dockets/dockets/95s0316/95s-0316-rpt0282-tab-03-ref-19-joint-faowho-vol219.pdf.

Fox, P. F. (2003). *Milk Proteins: General and Historical Aspects*. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry-1 Proteins* (pp. 1-48): Springer US.

Fuglsang, A., Rattray, F., Nilsson, D., & Nyborg, N. (2003). Lactic acid bacteria: inhibition of angiotensin converting enzyme; in vitro and in vivo. Antonie Van Leeuwenhoek, 83(1), 27-34. doi: 10.1023/a:1022993905778.

Fuller, R. (2012). Probiotics: the scientific basis (R. Fuller Ed.): *Springer Science & Business Media*.

Galat, A., Dufresne, J., Combrisson, J., Thépaut, J., Boumghar-Bourtchai, L., Boyer, M., & Fourmestraux, C. (2015). Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milkproducts. *Food Microbiology*. doi:http://dx.doi.org/10.1016/j.fm.2015.11.005.

Ganjam, L. S., Thornton Jr, W. H., Marshall, R. T., & MacDonald, R. S. (1997). antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *Journal of Dairy Science, 80*(10), 2325-2329. doi: http://dx.doi.org/10.3168/jds.S0022-0302(97)76183-6.

García-Tejedor, A., Padilla, B., Salom, J. B., Belloch, C., & Manzanares, P. (2013). Dairy yeasts produce milk protein-derived antihypertensive hydrolysates. *Food Research International,* 53(1), 203208. doi:http://dx.doi.org/10.1016/j.foodres.2013.05.005.

Gaudichon, C., Roos, N., Mahé, S., Sick, H., Bouley, C., & Tomé, D. (1994). Gastric Emptying Regulates the Kinetics of Nitrogen Absorbance from 15N-Labeled Milk and 15N-Labeled Yogurt in Miniature Pigs. *The Journal of Nutrition, 124*(10), 1970-1977.

Gauthier, S. F., & Pouliot, Y. (2003). Functional and biological properties of peptides obtained by enzymatic hydrolysis of Whey proteins. *Journal of Dairy Science, 86, Supplement*(0), E78-E87. doi: http://dx.doi.org/10.3168/jds.S0022-0302(03)74041-7.

Ghiadoni, L., Bruno, R., Stea, F., Virdis, A., & Taddei, S. (2009). Central blood pressure, arterial stiffness, and wave reflection: New targets of treatment in essential hypertension. *Current Hypertension Reports, 11*(3), 190-196. doi: 10.1007/s11906-009-0034-5.

Gill, H. S., Doull, F., Rutherfurd, K. J., & Cross, M. L. (2000). Immunoregulatory peptides in bovine milk. *British Journal of Nutrition, 84*(SupplementS1), 111-117. doi: doi:10.1017/S0007114500002336.

Gillman, M. W., Kannel, W. B., Belanger, A., & D'Agostino, R. B. (1993). Influence of heart rate on mortality among persons with hypertension: the Framingham Study. *American heart journal, 125*(4), 1148-1154.

Gobbetti, M., Minervini, F., & Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology, 57*(2-3), 173-188.

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., & Di Cagno, R. (2002). Latent bioactive peptides in milk proteins: Proteolytic activation and

significance in dairy processing. *Critical Reviews in Food Science and Nutrition*, 42(3), 223-239.

Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., & Addeo, F. (2000). Production of Angiotensin-I-Converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii subsp. bulgaricus SS1* and *Lactococcus lactis* subsp. *cremoris* FT4. *Applied and Environmental Microbiology*, 66(9), 3898-3904. doi: 10.1128/aem.66.9.3898-3904.2000.

Gomes, A. M. P., Malcata, F. X. & Klaver, F. A. M. (1998). Growth Enhancement of *Bifidobacterium lactis Bo* and *Lactobacillus acidophilus Ki* by Milk Hydrolyzates. Journal of Dairy Science, 81, 2817-2825.

Gómez-Ruiz, J. Á., Ramos, M. & Recio, I. (2002). Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. *International Dairy Journal*, 12, 697-706.

Gonzalez-Gonzalez, C., Gibson, T., & Jauregi, P. (2013). Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by *Bifidobacterium bifidum* MF 20/5. *International Journal of Food Microbiology, 167*(2), 131-137.

Gonzalez-Gonzalez, C. R., Tuohy, K. M., & Jauregi, P. (2011). Production of angiotensin-I-converting enzyme (ACE) inhibitory activity in milk fermented with probiotic strains: Effects of calcium, pH and peptides on the ACE-inhibitory activity. *International Dairy Journal, 21*(9), 615-622.

Goossens, G., Blaak, E., & Van Baak, M. (2003). Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders. *Obesity Reviews*, 4(1), 43-55.

Graham H, F. (2006). The commercial and community significance of yeasts in food and beverage production. Book, 2(3), 540. doi: DOI: 10.1007/978-3-540-28398.

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. d. A. F., & Shah, N. P. (2010). probiotic dairy products as functional foods. Comprehensive Reviews in Food Science and Food Safety, 9(5), 455-470. doi: 10.1111/j.1541-4337.2010.00120.

Crandall, D. L., Herzlinger, H. E., Saunders, B. D., Armellino, D. C., & Kral, J. G. (1994). Distribution of angiotensin ii receptors in rat and human adipocytes. *Journal of Lipid Research*, 35(8), 1378-1385.

Grigorov, M., Germond, J.-E., Tournade, S., & Affolter, M. (2014). *Lactobacillus helveticus* strains for producing hypotensive peptides: Google Patents.

Griffiths, M. W., & Tellez, A. M. (2013). *Lactobacillus helveticus*: the proteolytic system. *Frontiers in Microbiology, 4*(30). doi: 10.3389/fmicb.2013.00030.

Guan-Hong, L., Guo-Wei, L., Yong-Hui, S., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research, 24*(7), 469-486. doi: http://dx.doi.org/10.1016/j.nutres.2003.10.014.

Guo, S., Goetze, J. P., Jeppesen, J. L., Burnett, J. C., Olesen, J., Jansen-Olesen, I., & Ashina, M. (2015). Effect of natriuretic peptides on cerebral artery blood flow in healthyvolunteers.*Peptides*,74,33-42.doi:http://dx.doi.org/10.1016/j. peptides. 2015.09.008.

Guo, Y., Pan, D., & Tanokura, M. (2009). Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. *Food Chemistry*, 114(1), 328-333.

Halken, S. & Host, A. (1997). How hypoallergenic are hypoallergenic cow's milk-based formulas? *Allergy,* 52**,** 1175-1183.

Hall, J., Brands, M., Hildebrandt, D., Kuo, J., & Fitzgerald, S. (2000). Role of sympathetic nervous system and neuropeptides in obesity hypertension. *Brazilian Journal of Medical and Biological Research*, 33(6), 605-618.

Hamme, V., Sannier, F., Piot, J.-M., Didelot, S., & Bordenave-Juchereau, S. (2009). Crude goat whey fermentation by *Kluyveromyces marxianus* and *Lactobacillus rhamnosus*: contribution to proteolysis and ACE inhibitory activity. *Journal of Dairy Research, 76*(02), 152-157. doi: doi:10.1017/S0022029908003877.

Hansson, L., Lindholm, L. H., Niskanen, L., Lanke, J., Hedner, T., & Niklason, A. (1999). Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: The Captopril Prevention Project (CAPPP) randomised trial. *The Lancet, 353*(9153), 611-616.

Hansen, E. B. (2002). Commercial bacterial starter cultures for fermented foods of the future. International Journal of Food Microbiology, 78(1–2), 119-131. doi: http://dx.doi.org/10.1016/S0168-1605(02)00238-6.

Hansen, T. K., & Jakobsen, M. (2001). Taxonomical and technological characteristics of Saccharomyces spp. associated with blue veined cheese. *International Journal of Food Microbiology, 69*(1–2), 59-68. doi: http://dx.doi.org/10.1016/S0168-1605(01)00573-6.

Hansen, M., Sandstrom, B., & Lonnerdal, B. (1996). The effect of casein phosphopetides on zinc and calcium absorbance from high phytate infant diets assessed in rat pups and Caco-2 Cells. *Pediatr Res, 40*(4), 547-552.

Hansson, L., Lindholm, L. H., Niskanen, L., Lanke, J., Hedner, T., & Niklason, A. (1999). Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: The Captopril Prevention Project (CAPPP) randomised trial. *The Lancet, 353*(9153), 611-616.

Hara, H., funabiki, R., iwata, M. & yamazaki, K.-I. (1984). Portal absorbance of small peptides in rats under unrestrained conditions. *The Journal of Nutrition,* 114**,** 1122-1129.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology, 18*(2), 163-169. doi: http://dx.doi.org/10.1016/j.copbio.2007.01.013.

Hata, Y., Yamamoto, N., Ohni, M., Nakajima, K., Nakamura, Y., & Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *The American Journal of Clinical Nutrition, 64*, 767-771.

Haug, A., Hostmark, A. T., & Harstad, O. M. (2007). Bovine milk in human nutrition–a review. *Lipids Health Dis, 6*(25), 1-16.

Hayes, M., Stanton, C., FitzGerald, G. F., & Ross, R. P. (2007). Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnology Journal, 2*(4), 435-449. doi: 10.1002/biot.200700045.

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D., Ju, X., & Aluko, R. E. (2013). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry, 141*(1), 153-159. doi: http://dx.doi.org/10.1016/j.foodchem.2013.02.087.

Hébert, E. M., Raya, R. R., & De Giori, G. S. (1999). Characterisation of a cell-envelope proteinase from Lactobacillus helveticus. Biotechnology Letters, 21(9), 831-834.

Hekmat, S., Soltani, H., & Reid, G. (2009). Growth and survival of Lactobacillus reuteri RC-14 and Lactobacillus rhamnosus GR-1 in yogurt for use as a functional food. Innovative food science & emerging technologies, 10(2), 293-296.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). Dairy protein hydrolysates: Peptides for health benefits.

International Dairy Journal,38(2),82-100.doi:http://dx.doi.org/10.1016/j.idairyj. 2013.11.004.

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science,165*(1),23-35.doi:http://dx.doi.org/10.1016/j.cis. 2010.11.001.

Hernández-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Angiotensin-I converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 52*(6), 1504-1510. doi: 10.1021/jf034997.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). Dairy protein hydrolysates: Peptides for health benefits. *International Dairy Journal,38*(2),82-100.doi:http://dx.doi.org/10.1016/j. idairyj. 2013.11.004.

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science,165*(1),23-35.doi:http://dx.doi.org/10.1016/j.cis. 2010.11.001.

Heyman, M. (1999). Evaluation of the impact of food technology on the allergenicity of cow's milk proteins. *Proceedings of the Nutrition Society*, 58, 587-592.

Hideaki, K., Kunio, D., Shigeru, S., Hideyo, U., Ryuji, S., Umeji, M., & Shizume, T. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 96*(2), 367-371. doi: http://dx.doi.org/10.1016/0742-8413(90)90023-3.

Hiroyuki, K., Hirohito, M., Tsutomu, M., & Akira, N. (2013). Angiotensin II Blockade and Renal Protection. *Current pharmaceutical design*, 19(17), 3033-3042. doi: http://dx.doi.org/10.2174/1381612811319170009.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*(6), 1062-1071. doi: http://dx.doi.org/10.1016/j.peptides.2008.02.005.

Huppertz, T., Fox, P. F., de Kruif, K. G., & Kelly, A. L. (2006). High pressure-induced changes in bovine milk proteins: A review. *Biochimica et Biophysica Acta (BBA)* Proteins and Proteomics, 1764(3), 593-598.doi: http://dx.doi.org/10.1016/j. 2005.11.010.

Ijäs, H., Collin, M., Finckenberg, P., Pihlanto-Leppälä, A., Korhonen, H., Korpela, R., Nurminen, M.-L. (2004). Antihypertensive opioid-like milk peptide α-lactorphin: lack of effect on behavioural tests in mice. *International Dairy Journal, 14*(3), 201-205.

Illanés, A. (2011). Whey upgrading by enzyme biocatalysis. *Electronic Journal of Biotechnology, 14*(6).

Isono, Y., Shingu, I., & Shimizu, S. (1994). Identification and characteristics of Lactic acid bacteria isolated from masai fermented milk in northern tanzania. *Bioscience, biotechnology, and biochemistry, 58*(4), 660-664. doi: 10.1271/.58.660.

Iwaniak, A., & Minkiewicz, P. (2008). Biologically active peptides derived from proteins-a review. *Polish Journal of Food and Nutrition Sciences, 58*(3), 289-294.

Jakobsen, M., & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. *International Dairy Journal, 6*(8–9), 755-768. doi: http://dx.doi.org/10.1016/0958-6946(95)00071-2.

Jäkälä, P., Jauhiainen, T., Korpela, R., & Vapaatalo, H. (2009). Milk protein-derived bioactive tripeptides Ile-Pro-Pro and Val-Pro-Pro protect endothelial function in vitro in hypertensive rats. *Journal of Functional Foods, 1*(3), 266-273.

Jauhiainen, T., Rönnback, M., Vapaatalo, H., Wuolle, K., Kautiainen, H., Groop, P. H., & Korpela, R. (2010). Long-term intervention with Lactobacillus helveticus fermented milk reduces augmentation index in hypertensive subjects. European Journal of Clinical Nutrition, 64(4), 424-431.

Jauhiainen, T., Collin, M., Narva, M., Cheng, Z. J., Poussa, T., Vapaatalo, H., & Korpela, R. (2005). Effect of long-term intake of milk peptides and minerals on blood pressure and arterial function in spontaneously hypertensive rats. Milchwissenschaft-Milk Science International, 60(4), 358-362.

Jauhiainen, T., Vapaatalo, H., Poussa, T., Kyrönpalo, S., Rasmussen, M., & Korpela, R. (2005). *Lactobacillus helveticus* fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. *American Journal of Hypertension, 18*(12), 1600-1605.

Jauregi, P., & Welderufael, F. (2010). Added-value protein products from whey. *Nutrafoods, 9*(4), 13-23. doi: 10.1007/BF03223344.

Jespersen, L. (2003). Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods

and beverages. *FEMS Yeast Research, 3*(2), 191-200. doi: 10.1016/s1567-1356(02)00185-x.

Juillard, V., Guillot, A., Le Bars, D., & Gripon, J.-C. (1998). Specificity of milk Peptide utilization by *Lactococcus lactis*. *Applied and Environmental Microbiology, 64*(4), 1230-1236.

Katsiari, M.C., Voutsinas, L.P. & Kondyli, E. (2002). Improvement of sensory quality of low-fat Kefalograviera-type cheese with commercial adjunct cultures. *International Dairy Journal*, 12, 757–764.

Kawase, M., Hashimoto, H., Hosoda, M., Morita, H., & Hosono, A. (2000). Effect of administration of fermented milk containing Whey protein concentrate to rats and healthy men on serum lipids and blood pressure. *Journal of Dairy Science, 83*(2), 255-263.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., & He, J. (2005). Global burden of hypertension: analysis of worldwide data. *The Lancet, 365*(9455), 217-223. doi: http://dx.doi.org/10.1016/S0140-6736(05)17741-1.

Kebede, A., Viljoen, B. C., Gadaga, T. H., Narvhus, J. A., & Lourens-Hattingh, A. (2007). The effect of container type on the growth of yeast and lactic acid bacteria during production of Sethemi, South African spontaneously fermented milk. Food Research International, 40(1), 33-38. doi: http://dx.doi.org/10.1016/j.foodres.2006.07.012.

Kenny, O., FitzGerald, R. J., O'Cuinn, G., Beresford, T., & Jordan, K. (2003). Growth phase and growth medium effects on the peptidase activities of *Lactobacillus helveticus*. *International Dairy Journal*, 13(7), 509-516. doi: http://dx.doi.org/10.1016/S0958-6946(03)00073-6.

Khan, R. S., Grigor, J., Winger, R., & Win, A. (2013). Functional food product development–Opportunities and challenges for food manufacturers. *Trends in Food Science & Technology, 30*(1), 27-37.

Kilpi, E. E. R., Kahala, M. M., Steele, J. L., Pihlanto, A. M., & Joutsjoki, V. V. (2007). Angiotensin I-converting enzyme inhibitory activity in milk fermented by wild-type and peptidase-deletion derivatives of *Lactobacillus helveticus* CNRZ32. *International Dairy Journal,17*(8),976-984.doi:http://dx.doi.org/10.1016/j. idairyj. 2006. 12.001.

Kim, Y. S., & Kim, Y. W. (1972). Peptide hydrolases in the brush border and soluble fractions of small intestinal mucosa of rat and man. Journal of clinical investigation, 51(6), 1419–1430. . doi: 10.1172/JCI106938.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current pharmaceutical design, 9*(16), 1309-1323.

Koksoy, A., & Kilic, M. (2004). Use of hydrocolloids in textural stabilization of a yoghurt drink, ayran. *Food Hydrocolloids, 18*(4), 593-600. doi: http://dx.doi.org/10.1016/j.foodhyd.2003.10.002.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods*, 1(2), 177-187.

Korhonen, H., & Pihlanto, A. (2007). Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Current pharmaceutical design, 13*(8), 829-843.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16(9), 945–960. doi: 10.1016/j.idairyj.2005.10.012.

Korhonen, H., and Pihlanto-Leppälä. (2004). Milk-derived bioactive peptides: Formation and prospects for health .*Book, 6*, 109.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides - opportunities for designing future foods. *Current pharmaceutical design, 9*(16), 1297-1308. doi: 10.2174/1381612033454892.

Kuwabara, Y., Nagai, S., Yoshimitsu, N., Nakagawa, I., Watanabe, Y., & Tamai, Y. (1995). Antihypertensive effect of the milk fermented by culturing with various lactic acid bacteria and a yeast. *Journal of Fermentation and Bioengineering, 80*(3), 294-295. doi: http://dx.doi.org/10.1016/0922-338X(95)90834.

Kunji, E. R. S., Fang, G., Jeronimus-Stratingh, C. M., Bruins, A. P., Poolman, B., & Konings, W. N. (1998). Reconstruction of the proteolytic pathway for use of β-casein by Lactococcus lactis. *Molecular Microbiology, 27*(6), 1107-1118. doi: 10.1046/j.1365-2958.1998.00769.

Lacroix, C., Grattepanche, Franck, Doleyres, Yann,Bergmaier, Dirk,. (2005). *Immobilised cell technologies for the dairy industry*. In V. Nedović & R. Willaert (Eds.), *Applications of Cell Immobilisation Biotechnology* (Vol. 8B, pp. 295-319): Springer Netherlands.

Lahtinen, S., Ouwehand, A. C., Salminen, S., & von Wright, A. (2011*). Lactic Acid Bacteria*: Microbiological and Functional Aspects, Fourth Edition: Taylor & Francis.

Lamoureux, L., Roy, D., & Gauthier, S. F. (2002). Production of oligosaccharides in yogurt Containing *Bifidobacteria* and yogurt cultures. *Journal of Dairy Science, 85*(5), 1058-1069. doi: DOI: 10.3168/jds.S0022-0302(02)74166-0.

Lapointe, N., & Rouleau, J. L. (2002). Activation of vascular tissue angiotensin-converting enzyme (ACE) in heart failureEffects of ACE inhibitors. *Journal of the American College of Cardiology*, 39(5), 776-779. doi: 10.1016/S0735-1097(01)01830-7.

Lapre, V. d. M. R. J. (1991). Calcium and colon cancer. *Bulletin of the International Dairy Federation, 255*, 55-59.

Lawless, H. T., Heymann, Hildegarde,. (2010). *Sensory evaluation of food: principles and practices* (Vol. 5999): Springer Science & Business Media

Lawless, H.T. & Heymann, H. (1999). *Sensory Evaluation of Foods: Principles and Practices*. Pp.827. Gaithersburg: Aspen.

LeBlanc, J. G., Matar, C., Valdéz, J. C., LeBlanc, J., & Perdigon, G. (2002). Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. *Journal of Dairy Science, 85*(11), 2733-2742.

Leclerc, P.-L., Gauthier, S. F., Bachelard, H., Santure, M., & Roy, D. (2002). Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. *International Dairy Journal, 12*(12), 995-1004. doi: 10.1016/s0958-6946(02)00125-5.

Lehtinen, R., Jauhiainen, T., Kankuri, E., Lindstedt, K., Kovanen, P. T., Kerojoki, O., Vapaatalo, H. (2010). Effects of milk casein-derived tripeptides Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro on enzymes processing vasoactive precursors in vitro. *Arzneimittelforschung, 60*(04), 182-185. doi: 10.1055/s-0031-1296270.

Lemarié, C. A., & Schiffrin, E. L. (2010). The angiotensin II type 2 receptor in cardiovascular disease. *Journal of Renin-Angiotensin-Aldosterone System, 11*(1), 19-31. doi: 10.1177/1470320309347785.

Leroy, F., & De Vuyst, L. (2001). Growth of the Bacteriocin-Producing *Lactobacillus sakei* Strain CTC 494 in MRS Broth Is Strongly Reduced Due to Nutrient Exhaustion: a Nutrient Depletion Model for the Growth of Lactic Acid Bacteria. *Applied and Environmental Microbiology, 67*(10), 4407-4413. doi: 10.1128/aem.67.10.4407-4413.2001.

Li, S., & Shah, N. P. (2015). Effects of pleurotus eryngii polysaccharides on bacterial growth, texture properties, proteolytic capacity, and angiotensin-i-converting

enzyme–inhibitory activities of fermented milk. *Journal of Dairy Science*, 98(5), 2949-2961. doi: http://dx.doi.org/10.3168/jds.2014-9116.

Lim, S. M., Lee, N. K., Park, K. K., Yoon, Y. C., & Paik, H. D. (2011). ACE-inhibitory effect and physicochemical characteristics of yogurt beverage fortified with whey protein hydrolysates. *Korean Journal for Food Science of Animal Resources, 31*(6), 886-892.

Lin, S.-B., Chiang, W.-D., Cordle, C. T., & Thomas, R. L. (1997). Functional and Immunological Properties of Casein Hydrolysate Produced from a Two-Stage Membrane System. *Journal of Food Science*, 62(3), 480-483.

Liu, X., Zhang, M., Jia, A., Zhang, Y., Zhu, H., Zhang, C., Liu, C. (2013). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from jellyfish Rhopilema esculentum. *Food Research International, 50*(1), 339-343. doi: http://dx.doi.org/10.1016/j.foodres.2012.11.002.

Liu, K. (2012). *Soybeans: chemistry, technology, and utilization* (C. Hall Ed.). New York Springer.

López-Expósito, I., Amigo, L., & Recio, I. (2012). A mini-review on health and nutritional aspects of cheese with a focus on bioactive peptides. *Dairy Science & Technology, 92*(5), 419-438. doi: 10.1007/s13594-012-0066-5.

López Expósito, I., & Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal, 16*(11), 1294-1305.

López-Fandiño, R., Otte, J., & van Camp, J. (2006). Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal, 16*(11), 1277-1293. doi: http://dx.doi.org/10.1016/j.idairyj.2006.06.004.

Losito, I., Carbonara, T., De Bari, M. D., Gobbetti, M., Palmisano, F., Rizzello, C. G. & Zambonin, P. G. (2006). Identification of peptides in antimicrobial fractions of cheese extracts by electrospray ionization ion trap mass spectrometry coupled to a two-dimensional liquid chromatographic separation. *Rapid Communications in Mass Spectrometry,* 20**,** 447-455.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1-17. doi: 10.1016/s0958-6946(01)00036.

Luoma, S., Peltoniemi, K., Joutsjoki, V., Rantanen, T., Tamminen, M., Heikkinen, I., & Palva, A. (2001). Expression of six peptidases from *Lactobacillus helveticus* in *Lactococcus lactis*. *Applied and Environmental Microbiology, 67*(3), 1232-1238. doi: 10.1128/aem.67.3.1232-1238.2001.

Madureira, A. R., Tavares, T., Gomes, A. M. P., Pintado, M. E., & Malcata, F. X. (2010). Invited review: Physiological properties of bioactive peptides obtained from whey proteins. Journal of Dairy Science, 93(2), 437-455.

Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., & Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. Biochemical and Biophysical Research Communications, 365(4), 851-855. doi: http://dx.doi.org/10.1016/j.bbrc.2007.11.070.

Maeno, M., Yamamoto, N., & Takano, T. (1996). Identification of an antihypertensive peptide from casein hydrolysate produced by a Proteinase from *Lactobacillus helveticus* CP790. *Journal of Dairy Science*, 79(8), 1316-1321. doi: http://dx.doi.org/10.3168/jds.S0022-0302(96)76487-1.

Maganha, L. C., Rosim, R. E., Corassin, C. H., Cruz, A. G., Faria, J. A. F., & Oliveira, C. A. F. (2014). Viability of probiotic bacteria in fermented skim milk produced with different levels of milk powder and sugar. International Journal of Dairy Technology, 67(1), 89-94. doi: 10.1111/1471-0307.12087.

Marteau, P., Flourie, B., Pochart, P., Chastang, C., Desjeux, J.-F., & Rambaud, J.-C. (1990). Effect of the microbial lactase (EC 3.2. 1.23) activity in yoghurt on the intestinal absorbance of lactose: an in vivo study in lactase-deficient humans. *British Journal of Nutrition, 64*(01), 71-79.

Marinik, E. L., Frisard, M. I., Hulver, M. W., Davy, B. M., Rivero, J. M., Savla, J. S., & Davy, K. P. (2013). Angiotensin ii receptor blockade and insulin sensitivity in overweight and obese adults with elevated blood pressure. Therapeutic Advances in Cardiovascular Disease, 7(1), 11-20. doi: 10.1177/1753944712471740.

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., Rodríguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. The Journal of Pediatrics,143(6),754-758.doi:http: //dx.doi.org/10.1016/j. jpeds. 2003.09.028.

Maruyama, S., Nakagomi, K., Tomizuka, N., & Suzuki, H. (1985). Angiotensin converting enzyme inhibitor derived from an enzymatic hydrolysate of casein.. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. Agricultural and Biological Chemistry, 49(5), 1405-1409. doi: 10.1271/bbb1961.49.1405.

Maruyama, S., & Suzuki, H. (1982). A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agricultural and Biological Chemistry, 46(5), 1393-1394.

Masuda, O., Nakamura, Y., & Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. The Journal of Nutrition, 126(12), 3063-3068.

Matar, C., Amiot, J., Savoie, L., & Goulet, J. (1996). The Effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during in vitro digestion. *Journal of Dairy Science, 79*(6), 971-979.

Matar, C., & Goulet, J. (1996). β-Casomorphin 4 from milk fermented by a mutant of *Lactobacillus helveticus*. *International Dairy Journal, 6*(4), 383-397.

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K., & Holzapfel, W. H. (2004). Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *International Journal of Food Microbiology, 94*(3), 269-278. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2004.01.008.

Mattu, R. K., Needham, E. W. A., Galton, D. J., Frangos, E., Clark, A. J. L., & Caulfield, M. (1995). A DNA Variant at the Angiotensin-converting enzyme gene locus associates With coronary artery disease in the caerphilly heart Study. *Circulation, 91*(2), 270-274. doi: 10.1161/01.cir.91.2.270.

Maubois, J., L., & Léonil, J. (1989). Peptides du lait à activité biologique. *Lait, 69*(4), 245-269.

Mavromoustakos, T. (2004). Modern aspects in the design and discovery of novel antihypertensive drugs-Preface. *Current Topics in Medicinal Chemistry*, 4(4), 1.

Mavromoustakos, T., Apostolopoulos, V., & Matsoukas, J. (2001). Antihypertensive Drugs that Act on Renin-Angiotensin System with Emphasis in AT1 Antagonists. *Mini-Reviews in Medicinal Chemistry*, 1(2), 207-217. doi: http://dx.doi.org/10.2174/1389557013407115.

Maxime, D., marcotte, M. & arcand, Y. (2006). Development of eco-efficiency indicators for the Canadian food and beverage industry. *Journal of Cleaner Production,* 14**,** 636-648.

McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H., & Coventry, M. J. (2006). Isolation and characterisation of a novel antibacterial peptide from bovine αS1-casein. *International Dairy Journal, 16*(4), 316-323. doi: http://dx.doi.org/10.1016/j.idairyj.2005.05.005.

McIntosh, G. H., Regester, G. O., Le Leu, R. K., Royle, P. J., & Smithers, G. W. (1995). Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *The Journal of Nutrition, 125*(4), 809-816.

McKinley, M. C. (2005). The nutrition and health benefits of yoghurt. *International Journal of Dairy Technology, 58*(1), 1-12. doi: 10.1111/j.1471-0307.2005.00180.

McLeod A, F. W., Ozimek L. (1996). Binding of retinoic acid to lactoglobulin variants A and B:effect of hepatic and tryptic digestion on the protein-ligand complex. *Milchwissenschaft, 51*, 3-7.

McNeil, B., & Harvey, L. M. (2008). *Practical fermentation technology*: Wiley Online Library.

Medicine, I. o. (1997). Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, DC: The National Academies Press.Washington: Institute of Medicine.

Meena, K., Kapila, S., Chand, R., Haque, E., Singh, S., & Aggarwal, P. K. (2008). Immunomodulatory effect of oral administration of milk fermented with *Lactobacillus helveticus* in mice. *Milchwissenschaft, 63*(1), 27-29.

Meilgaard, M. C., Carr, B. T., & Civille, G. V. (2006). *Sensory Evaluation Techniques*, Fourth Edition: Taylor & Francis.

Meisel, H., & Bockelmann, W. (1999). Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie van Leeuwenhoek*, 76(1-4), 207-215. doi: 10.1023/a:1002063805780.

Meisel, H. (1998). Overview on Milk Protein-derived Peptides. *International Dairy Journal, 8*(5–6), 363-373. doi: http: //dx.doi. org/10.1016/S0958-6946(98) 00059-4.

Meisel, H., & Schlimme, E. (1990). Milk proteins: precursors of bioactive peptides. *Trends in Food Science & Technology, 1*(0), 41-43. doi: http://dx.doi.org/10.1016/0924-2244(90)90029.

Menendez, S., Centeno, J.A., Godı´nez, R. & Rodrı´guez-Otero, J.L. (2000). Effect of Lactobacillus strains on the ripening and organoleptic characteristics of Arzu´ a-Ulloa cheese. *International Journal of Food Microbiology*, 59, 37–46.

Merrifield, D. L., Bradley, G., Baker, R. T. M., & Davies, S. J. (2010). Probiotic applications for rainbow trout (Oncorhynchus mykiss Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquaculture Nutrition*, 16(5), 496-503. doi: 10.1111/j.1365-2095.2009.00688.

Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., & Gobbetti, M. (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. *Applied and Environmental Microbiology, 69*(9), 5297-5305.

Miura, S.-i., Karnik, S. S., & Saku, K. (2011). Review: Angiotensin II type 1 receptor blockers: class effects versus molecular effects. *Journal of Renin-Angiotensin-Aldosterone System, 12*(1), 1-7. doi: 10.1177/1470320310370852.

Miyauchi, H., Kaino, A., Shinoda, I., Fukuwatari, Y., & Hayasawa, H. (1997). Immunomodulatory effect of bovine lactoferrin pepsin hydrolysate on murine splenocytes and peyer's patch cells. *Journal of Dairy Science, 80*(10), 2330-2339. doi: http://dx.doi.org/10.3168/jds.S0022-0302(97)76184-8.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T., & Yamamoto, N. (2004). Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. *Journal of Dairy Science, 87*, 3183-3188.

Mohammadi, R., & Mortazavian, A. M. (2011). Review Article: Technological aspects of prebiotics in probiotic fermented milks. *Food Reviews International, 27*(2), 192-212. doi: 10.1080/87559129.2010.535235.

Morgan, T. O., Anderson, A. I. E., & MacInnis, R. J. (2001). Ace inhibitors, beta-blockers, calcium blockers, and diuretics for the control of systolic hypertension. *American Journal of Hypertension, 14*(3), 241-247. doi: http://dx.doi.org/10.1016/S0895-7061(00)01266-8.

Morr, C., & Ha, E. (1993). Whey protein concentrates and isolates: processing and functional properties. *Critical Reviews in Food Science & Nutrition, 33*(6), 431-476.

Muguerza, B., Ramos, M., Sánchez, E., Manso, M. A., Miguel, M., Aleixandre, A., Recio, I. (2006). Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. *International Dairy Journal, 16*(1), 61-69.

Mullally, M. M., Meisel, H., & FitzGerald, R. J. (1997). Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *International Dairy Journal, 7*(5), 299-303. doi: http://dx.doi.org/10.1016/S0958-6946(97)00018-6.

Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Yadav, H. (2011). Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & Function, 2*(1), 18-27. doi: 10.1039/c0fo00016g.

Nakamura, Y., MASUDA, O., & TAKANO, T. (1996). Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. *Bioscience, biotechnology, and biochemistry, 60*, 488–489. doi: http://dx.doi.org/10.1271/bbb.60.488.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of Angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*, 777-783.

Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from It that are inhibitors to Angiotensin I-Converting Enzyme. *Journal of Dairy Science, 78*(6), 1253-1257. doi: http://dx.doi.org/10.3168/jds.S0022-0302(95)76745-5.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*(4), 777-783. doi: http://dx.doi.org/10.3168/jds.S0022-0302(95)76689-9.

Nakamura, H. (1987). Effects of antihypertensive drugs on plasma lipid. American Journal of Cardiology, 60, 24E-28E.

Narva, M., Collin, M., Jauhiainen, T., Vapaatalo, H., & Korpela, R. (2004). Effects of *Lactobacillus helveticus* fermented milk and its bioactive peptides on bone parameters in spontaneously hypertensive rats. *Milchwissenschaft, 59*(7-8), 359-363.

Newman, J., Egan, T., Harbourne, N., O′Riordan, D., Jacquier, J. C., & O′Sullivan, M. (2014). Correlation of sensory bitterness in dairy protein hydrolysates: Comparison of prediction models built using sensory, chromatographic and electronic tongue data. *Talanta, 126*(0), 46-53. doi: http://dx.doi.org/10.1016/j.talanta.2014.03.036.

Nielsen, M. S., Martinussen, T., Flambard, B., Sørensen, K. I., & Otte, J. (2009). Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time. *International Dairy Journal, 19*(3), 155-165. doi: http://dx.doi.org/10.1016/j.idairyj.2008.10.003.

Nikolić, Z., Đorđević, V., Torbica, A., & Mikić, A. (2012). Legumes seed storage proteins characterization by SDS-PAGE and Lab-on-a-Chip electrophoresis. *Journal of Food Composition and Analysis, 28*(2), 75-80. doi: http://dx.doi.org/10.1016/j.jfca.2012.08.005.

Nurminen, M.-L., Sipola, M., Kaarto, H., Pihlanto-Leppälä, A., Piilola, K., Korpela, R., Vapaatalo, H. (2000). α-Lactorphin lowers blood pressure measured by

radiotelemetry in normotensive and spontaneously hypertensive rats. *Life sciences, 66*(16), 1535-1543.

Oberg, C. J., Weimer, B. C., Moyes, L. V., Brown, R. J., & Richardson, G. H. (1991). Proteolytic Characterization of *Lactobacillus delbrueckii ssp.*{Bounous, 2000 #670}{Bounous, 2000 #670}{Bounous, 2000 #670} Strains by the o-Phthaldialdehyde Test and Amino Acid Analysis. *Journal of Dairy Science, 74*(2), 398-403. doi: http://dx.doi.org/10.3168/jds.S0022-0302(91)78181-2.

Oliveira, M.N., Sivieri, K., Alegro, J.H.A. & Saad, S.M.I. (2002). Aspectos tecnolo´ gicos de alimentos funcionais contendo probio´ ticos. *Brazilian Journal of Pharmaceutical Sciences*, 38, 1–21.

O'Malley, J. P., BS, Maslen, C. L., & Illingworth, D. R. (1998). Angiotensin-I-converting enzyme DD genotype and cardiovascular disease in heterozygous familial hypercholesterolemia. Circulation, 97(18), 1780-1783. doi: 10.1161/01.cir.97.18.1780.

Ono, H., Yamamoto, N., Maeno, M., Takano, T., & Momose, H. (1997). Purification and characterization of a cell-wall associated proteinase of *Lactobacillus helveticus* CP53. *Milchwissenschaft, 52*(7), 373-376.

Otte, J., Lenhard, T., Flambard, B., & Sørensen, K. I. (2011). Influence of fermentation temperature and autolysis on ACE-inhibitory activity and peptide profiles of milk fermented by selected strains of *Lactobacillus helveticus* and *Lactococcus lactis*. *International Dairy Journal*, 21(4), 229-238. doi: 10.1016/j.idairyj.2010.12.008.

Otte, J., Shalaby, S. M., Zakora, M., Pripp, A. H., & El-Shabrawy, S. A. (2007). Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis. *International Dairy Journal, 17*(5), 488-503. doi: http://dx.doi.org/10.1016/j.idairyj.2006.05.011.

Otte, J., Shalaby, S. M. A., Zakora, M., & Nielsen, M. S. (2007). Fractionation and identification of ACE-inhibitory peptides from α -lactalbumin and β -casein produced by thermolysin-catalysed hydrolysis. *International Dairy Journal*, 17(12), 1460-1472. doi: http://dx.doi.org/10.1016/j.idairyj.2007.04.008.

Otagiri, K., Nosho, Y., Shinoda, I., Fukui, H., & Okai, H. (1985). Studies on a Model of Bitter Peptides Including Arginine, Proline and Phenylalanine Residues. I. Bitter Taste of Di- and Tripeptides, and Bitterness Increase of the Model Peptides by Extension of the Peptide Chain. *Agricultural and Biological Chemistry, 49*(4), 1019-1026. doi: 10.1080/00021369.1985.10866857.

Okoro, L. N., Sambo, F. I., Lawal, M., & Nwaeburu, C. (2011). Thermodynamic and viscometric evaluation of biodiesel and blends from olive oil and cashew nut oil. *Research journal of chemical sciences, 1*(4), 90-97.

Ozer, B., Kirmaci, H. A., Oztekin, S., Hayaloglu, A., & Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal, 17*(3), 199-207.

Pal, S., Ellis, V., & Dhaliwal, S. (2010). Effects of whey protein isolate on body composition, lipids, insulin and glucose in overweight and obese individuals. *British Journal of Nutrition, 104*(05), 716-723. doi: doi:10.1017/S0007114510000991.

Panagiotopoulos, D., Matsoukas, J. M., Alexopoulos, K., Zebeki, A., Mavromoustakos, T., Saifeddine, M., & Hollenberg, M. D. (1996). Synthesis and activities of cyclic thrombin-receptor-derived peptide analogues of the Ser42-Phe-Leu-Leu-Arg46 motif sequence containing D-Phe and/or D-Arg. *Letters in Peptide Science,* 3(4), 233-240.

Pan, D., Luo, Y., & Tanokura, M. (2005). Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of *Lactobacillus helveticus* JCM1004. *Food Chemistry,91*(1),123-129.doi:10.1016/j.foodchem.2004.05.055.

Pan, D., & Guo, Y. (2010). Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. *International Dairy Journal*, 20, 472-479.

Park, Y. W. (2009). Overview of bioactive components in milk and dairy products. *Bioactive components in milk and dairy products*, 3-5.

Paola Ortiz-Chaoa, J. A. G.-R., 1, Robert A. Rastalla, Davinia Millsb, Rainer Cramerb, Anne Pihlantoc, Hannu Korhonenc, Paula Jauregi. (2009). Production of novel ACE inhibitory peptides from β-lactoglobulin using Protease N Amano. *International Dairy Journal, 19* (2), 69-76.

Parker, R. (2007). *Yakult: Targeting US demand for functional health, convenience,*(Online).Available:http://www.drinks-business review.com/article_feature.asp?guid=58B1CB95-5BBB-40E4-883B-29F347924DF1 ,(Accessed 8th/07/ 2015).

Pariza, MW1, P. Y., Cook ME. (1999). Conjugated linoleic acid and the control of cancer and obesity*., 52*(2), 107-110.

Parente, E., & Zottola, E. A. (1991). Growth of Thermophilic Starters in Whey Permeate Media1. Journal of Dairy Science, 74(1), 20-28.

Passos, F. M. L., & Swaisgood, H. E. (1993). Development of a spiral mesh bioreactor with immobilized *lactococci* for continuous inoculation and acidification of milk. *Journal of Dairy Science, 76*(10), 2856-2867. doi: http://dx.doi.org/10.3168/jds.S0022-0302(93)77624-9.

Patel, HA, Creamer LK. 2008. High-pressure-induced interactions involving whey proteins. Chapter 7.In: A. T. B. Singh editor. Milk Proteins. San Diego: *Academic Press*; p. 205-238.

Penner, R., Fedorak, R. N., & Madsen, K. L. (2005). Probiotics and nutraceuticals: non-medicinal treatments of gastrointestinal diseases. *Current Opinion in Pharmacology, 5*(6),596-603. doi: http://dx.doi.org/10.1016/j.coph.2005.06.009.

Pepe, G., Tenore, G. C., Mastrocinque, R., Stusio, P., & Campiglia, P. (2013). Potential Anticarcinogenic Peptides from Bovine Milk. *Journal of Amino Acids, 2013*, 7. doi: 10.1155/2013/939804.

Pescuma, M., Hébert, E. M., Mozzi, F., & Font de Valdez, G. (2008). Whey fermentation by thermophilic lactic acid bacteria: Evolution of carbohydrates and protein content. *Food Microbiology, 25*(3), 442-451. doi: 10.1016/j.fm.2008.01.007.

Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. (2009). Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal, 19*(11), 643-654. doi: http://dx.doi.org/10.1016/j.idairyj.2009.06.001.

Phelan, M., & Kerins, D. (2011). The potential role of milk-derived peptides in cardiovascular disease. *Food & Function, 2*(3-4), 153-167. doi: 10.1039/c1fo10017c.

Pihlanto, A. & Korhonen, H. (2015). Bioactive peptides from fermented foods and health promotion. In: Holzapfel, W. (ed.) *Advances in Fermented Foods and Beverages*. Woodhead Publishing, 39-74. doi:10.1016/B978-1-78242-015-6.00003-7.

Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. *International Dairy Journal*, 20(1), 3-10. doi: 10.1016/j.idairyj.2009.07.003.

Pihlanto, A., & Korhonen, H. (2003). Bioactive peptides and proteins. Advances in food and nutrition research, 47, 175-276.

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science & Technology, 11*(9–10), 347-356. doi: http://dx.doi.org/10.1016/S0924-2244(01)00003-6.

Pihlanto-Leppälä, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal, 8*, 325-331.

Plessas, S., Fisher, A., Koureta, K., Psarianos, C., Nigam, P., & Koutinas, A. A. (2008). Application of *kluyveromyces marxianus*, *lactobacillus delbrueckii ssp. bulgaricus* and *L. Helveticus* for sourdough bread making. *Food Chemistry*, 106(3), 985-990.

Poste, L. M., Mackie DA, Butler G, Larmond E,. (1991). Laboratory methods for sensory analysis of food. Canada: *Canada Communication Group*, Pub. Centre. *1864/E.*

Pripp, A. H., & Ardö, Y. (2007). Modelling relationship between angiotensin-(I)-converting enzyme inhibition and the bitter taste of peptides. *Food Chemistry, 102*(3), 880-888. doi: http://dx.doi.org/10.1016/j.foodchem.2006.06.026.

Pripp, A. H., Isaksson, T., Stepaniak, L., & Sørhaug, T. (2004). Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. *European Food Research and Technology, 219*(6), 579-583.

Prevost, H., & Divies, C. (1987). Fresh fermented cheese production with continuous pre-fermented milk by a mixed culture of *mesophilic lactic streptococci* entrapped in Ca-Al ginate. *Biotechnology Letters, 9*(11), 789-794. doi: 10.1007/BF01028285.

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., & Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. *International Dairy Journal, 17*(1), 33-41. doi: http://dx.doi.org/10.1016/j.idairyj.2005.12.011.

Rajapakse, N., Mendis, E., Byun, H.-G., & Kim, S.-K. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. The Journal of Nutritional Biochemistry, 16(9), 562-569. doi: http://dx.doi.org/10.1016/j.jnutbio.2005.02.005.

Ramesh C. Chandan, A. K. (2013). Manufacturing Yogurt and Fermented Milks *Technology & Engineering*, 496.

Ramesh, V., Kumar, R., Singh, R. R. B., Kaushik, J. K., & Mann, B. (2012). Comparative evaluation of selected strains of lactobacilli for the development of antioxidant activity in milk. Dairy Science and Technology, 92(2), 179-188.

Ramchandran, & Shah, N. P. (2011). Yogurt can beneficially affect blood contributors of cardiovascular health status in hypertensive rats. *Journal of Food Science*, 76(4), H131-H136. doi: 10.1111/j.1750-3841.2011.02127.

Ramchandran, & Shah, N. P. (2010). Influence of addition of Raftiline HP® on the growth, proteolytic, ACE- and α-glucosidase inhibitory activities of selected lactic acid bacteria and *Bifidobacterium*. *LWT - Food Science and Technology, 43*(1), 146-152. doi: http://dx.doi.org/10.1016/j.lwt.2009.06.022.

Ramchandran, L., & Shah, N. P. (2008). Proteolytic Profiles and Angiotensin-I Converting enzyme and α-Glucosidase inhibitory activities of selected Lactic acid bacteria. *Journal of Food Science, 73*(2), M75-M81. doi: 10.1111/j.1750-3841.2007.00643.

Ramos, Ó. L., Pereira, J. O., Silva, S. I., Amorim, M. M., Fernandes, J. C., Lopes-da-Silva, J. A., Malcata, F. X. (2012). Effect of composition of commercial whey protein preparations upon gelation at various pH values. Food ResearchInternational,48(2),681-689.doi: http://dx.doi.org/10.1016/j. foodres. 2012.06.004.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, 62(3), 597-635.

Rasic, J. L., & Kurmann, J. A. (1983). *Bifidobacteria and their role. Microbiological, nutritional-physiological, medical and technological aspects and bibliography* (Vol. 39).

Ravallec-Plé, R., Gilmartin, L., Van Wormhoudt, A., & Le Gal, Y. (2000). Influence of the hydrolysis process on the biological activities of protein hydrolysates from cod (Gadus morhua) muscle. *Journal of the Science of Food and Agriculture, 80*(15), 2176-2180. doi: 10.1002/1097-0010(200012)80:15<2176::AID-JSFA763>3.0.CO;2-G.

Rijkers, G. T., de Vos, W. M., Brummer, R.-J., Morelli, L., Corthier, G., & Marteau, P. (2011). Health benefits and health claims of probiotics: bridging science and marketing. *British Journal of Nutrition, 106*(09), 1291-1296. doi: doi:10.1017/S000711451100287.

Rizzello, C. G., Cassone, A., Di Cagno, R., & Gobbetti, M. (2008). Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. *Journal of Agricultural and Food Chemistry, 56*(16), 6936-6943. doi: 10.1021/jf800512.

Roberfroid, M. B. (2000). Prebiotics and probiotics: are they functional foods? *The American Journal of Clinical Nutrition, 71*(6), 1682s-1687.

Roberfroid, M. B. (1999). Concepts in functional foods: the case of inulin and oligofructose. *The Journal of Nutrition, 129*(7), 1398S-1401.

Roostita, R., & Fleet, G. H. (1996). Growth of yeasts in milk and associated changes to milk composition. *International Journal of Food Microbiology, 31*(1-3), 205-219.

Roy, M. K., Watanabe, Y., & Tamai, Y. (2000). Yeast protease B-digested skimmed milk inhibits angiotensin-I-converting-enzyme activity. *Biotechnology and Applied Biochemistry, 31*(2), 95-100. doi: 10.1042/ba19990081.

Roy, M. K., Watanabe, Y., & Tamai, Y. (1999). Induction of apoptosis in human leukemia (HL-60) cells by skimmed milk digested with a proteolytic enzyme purified from *Saccharomyces cerevisiae*. *Biotechnology Techniques, 13*(11), 727-734.

Saito, T. (2008). Antihypertensive peptides derived from bovine casein and Whey proteins. In Z. Bösze (Ed.), *Bioactive Components of Milk* (Vol. 606, pp. 295-317): Springer New York.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and structural analysis of antihypertensive peptides that exist naturally in gouda cheese. *Journal of Dairy Science, 83*(7), 1434-1440. doi: http://dx.doi.org/10.3168/jds.S0022-0302(00)75013-2.

Salminen, S., & von Wright, A. (2004). *Lactic Acid Bacteria: Microbiological and Functional Aspects*. In. Salminen, S (3rd Ed): CRC Press.New York: Marcel Dekker,INC.656-656.

Salminen, S., & Wright A. (1998). *Lactic acid bacteria: Microbiology and functional aspects*. In. Salminen, S (2nd Ed.). New York: Malcr Dekker.14-19.

Sanders, M. E. (2008). Probiotics: definition, sources, Selection, and uses. Clinical Infectious Diseases, 46(Supplement 2), S58-S61. doi: 10.1086/523341.

Santos, A., San Mauro, M., & Diaz, D. M. (2006). Prebiotics and their long-term influence on the microbial populations of the mouse bowel. *Food Microbiology, 23*(5), 498-503.

SAS, II. (2008). SAS V9.0 software.

Savijoki, K., Ingmer, H., & Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology, 71*(4), 394-406. doi: 10.1007/s00253-006-0427-1.

Saxelin, M., Korpela, R., & Mäyrä-Mäkinen, A. (2003). 1 - Introduction: classifying functional dairy products. In T. Mattila-Sandholm & M. Saarela (Eds.), *Functional Dairy Products* (pp. 1-16): Woodhead Publishing.

Schanbacher, F. L., Talhouk, R. S., Murray, F. A., Gherman, L. I., & Willett, L. B. (1998). Milk-borne bioactive peptides. *International Dairy Journal, 8*(5-6), 393-403.

Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Food/Nahrung*, *39*(1), 1-20.

Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *American Journal of Clinical Nutrition*, 77(2), 326-330.

Seppo, L., Kerojoki, O., Suomalainen, T., & Korpela, R. (2002). The effect of a *Lactobacillus helveticus* LBK-16 H fermented milk on hypertension - A pilot study on humans. *Milchwissenschaft*, 57(3), 124-127.

Sesoko, S., & Kaneko, Y. (1985). Cough associated with the use of Captopril. *Internal Medicine, 145*(8), 1524.

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal, 17*(11), 1262-1277. doi: http://dx.doi.org/10.1016/j.idairyj.2007.01.014.

Shah, N. P., & Ravula, R. (2000). Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. *The Australian Journal of Dairy Technology, 55*, 127–131.

Shah, N. P. (2001). Functional foods, probiotics and prebiotics. *Food Technology*, 55, 46–53.

Shah, N. P. (2000). Effects of milk-derived bioactives: an overview. *British Journal of Nutrition, 84*(SupplementS1), 3-10. doi: doi:10.1017/S000711450000218.

Shah, N. P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science*, 83, 894–907.

Shihata, A., & Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal, 10*(5–6), 401-408. doi: 10.1016/s0958-6946(00)00072-8.

Silva, S. V., & Malcata, F. X. (2005). Caseins as source of bioactive peptides. *InternationalDairyJournal,15*(1),1-15. doi:http://dx.doi.org/10.1016/j. idairyj. 2004.04.009.

Singh, H., & Thompson, A. (2014). Milk Proteins: From Expression to Food,Hardback *Food Science and Technology,2nd Revised edition*. *Book, 10*(13), 622.

Singh, B., Chand, R., & Singh, R. (2011). Angiotensin-1 converting enzyme (ACE) inhibitory activity of peptides isolated from bovine milk fermented with *Lactobacillus helveticus* NCDC- 288. *Milchwissenschaft, 66*(4), 429-432.

Singh, B., & Singh, R. (2011). Antimicrobial effect of peptides isolated from bovine milk fermented with *Lactobacillus helveticus*. *Milchwissenschaft, 66*(3), 262-265.

Sipola, M., finckenberg, P., korpela, R., vapaatalo, H., & nurminen, M.-L. (2002). Effect of long-term intake of milk products on blood pressure in hypertensive rats.*Journal of Dairy Research,69*(01), 103-111.doi:doi: 10. 1017/ S0022-0299(01) 0052-6.

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance A review. *Appetite, 51*(3), 456-467. doi: 10.1016/j.appet.2008.05.060.

Smacchi, E., & Gobbetti, M. (2000). Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiology, 17*(2), 129-141.

Sobol, C. V. (2014). How Microbiome Impact on the Cardiovascular System. *J Clin Trial Cardiol*, 1(1), 1-4.

Sodini, Lagace, L., Lacroix, C., & Corrieu, G. (1998). Effect of continuous prefermentation of milk with an immobilized cell bioreactor on fermentation kinetics and curd properties. *Journal of Dairy Science*, 81(3), 631-638. doi: http://dx.doi.org/10.3168/jds.S0022-0302(98)75617-6.

Sodini-Gallot, Corrieu, G., Boquien, C. Y., Latrille, E., & Lacroix, C. (1995). Process performance of continuous inoculation and acidification of milk with immobilized lactic acid bacteria. *Journal of Dairy Science*, 78(7), 1407-1420. doi: http://dx.doi.org/10.3168/jds.S0022-0302(95)76763-7.

Souza, A. H. P. d., Costa, G. A. N., Miglioranza, L. H. d. S., Furlaneto-Maia, L., & Oliveira, A. F. d. (2013). Microbiological, physical, chemical and sensory characteristics of milk fermented with *Lactobacillus plantarum*. Acta Scientiarum. *Health Sciences*, 35(1).

Spellman, D., O'Cuinn, G., & FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins. *Food Chemistry, 114*(2), 440-446. doi: http://dx.doi.org/10.1016/j.foodchem.2008.09.067

Spellman, D., McEvoy, E., O'cuinn, G., & FitzGerald, R. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13(6), 447-453.

Standard. Australian Food Standards. Act. (2003). Food standards code Australian New Zealand: http: Accessed from // www.foodstandards.gov.au /code/ Pages/ default. aspx.

Steenson, L. R., Klaenhammer, T. R., & Swaisgood, H. E. (1987). Calcium alginate-immobilized cultures of *lactic streptococci* are protected from bacteriophages. *Journal of Dairy Science, 70*(6), 1121-1127.

Stone, H., Bleibaum, R., & Thomas, H. A. (2012). *Sensory evaluation practices* (B. Herbert Stone, Heather Thomas, & Eds. Chapter 3.4th ed. London: *Academic press*.p. 1-178.

Stressler, T., Eisele, T., Schlayer, M., Lutz-Wahl, S., & Fischer, L. (2013). Characterization of the recombinant exopeptidases PepX and PepN from *Lactobacillus helveticus* ATCC 12046 important for food protein hydrolysis. *PLoS ONE, 8*(7), e70055. doi: 10.1371/journal.pone.0070055.

Stanton, C., Ross, R. P., FitzGerald, G. F., & Van Sinderen, D. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology*, 16(2), 198-203.

Stanton, C., Desmond, C., FitzGerald, G., & Ross, R. (2003). Probiotic health benefits: reality or myth? *Australian journal of dairy technology, 58*(2), 107-113.

Sun, T., Zhao, S., Wang, H., Cai, C., Chen, Y., & Zhang, H. (2009). ACE-inhibitory activity and gamma-aminobutyric acid content of fermented skim milk by *Lactobacillus helveticus* isolated from Xinjiang koumiss in China. *European Food Research and Technology, 228*(4), 607-612.

Swedish, i. (2007). Arla Foods. *Foodservice*. Arla Foods växel: 08 - 789 50 00. Doi: http://www.arlafoodservice.se/produkter.

Takano, D. T. (2002). Anti-hypertensive activity of fermented dairy products containing biogenic peptides. *Antonie Van Leeuwenhoek, 82*(1-4), 333-340. doi: 10.1023/a:1020600119907.

Tamime, A. (2002). Fermented milks: a historical food with modern applications—a review. *European Journal of Clinical Nutrition, 56*(Suppl 4), s2-s15.

Tamime, A. Y., & Robinson, R. K. (1999). *Yoghurt: science and technology*: Woodhead Publishing.

Tauzin, J., Miclo, L., & Gaillard, J.-L. (2002). Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine αS2-casein. *FEBS Letters, 531*(2), 369-374. doi: http://dx.doi.org/10.1016/S0014-5793(02)03576-7.

Tellez, A., Corredig, M., Turner, P. V., Morales, R., & Griffiths, M. (2011). A peptidic fraction from milk fermented with *Lactobacillus helveticus* protects mice against Salmonella infection. *International Dairy Journal, 21*(9), 607-614.

Tharmaraj, N., & Shah, N. P. (2003). Selective Enumeration of *Lactobacillus delbrueckii ssp*. *bulgaricus , Streptococcus thermophilus, Lactobacillus acidophilus*, *Bifidobacteria*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and Propionibacteria. *Journal of Dairy Science, 86*(7), 2288-2296. doi: http://dx.doi.org/10.3168/jds.S0022-0302(03)73821-1.

Thamer, K. G., & Penna, A. L. B. (2006). Caracterização de bebidas lácteas funcionais fermentadas por probióticos e acrescidas de prebiótico. Food Science and Technology (Campinas), 26, 589-595.

Thomas, T. D., & Crow, V. L. (1984). Selection of galactose-fermenting Streptococcus thermophilus in lactose-limited chemostat cultures. *Applied and Environmental Microbiology, 48*(1), 186-191.

Thomas, J. (2014). *Functional foods market increases in size*. Retrieved from(https://www.leatherheadfood.com/functional-foods-market-increases-in size#sthash.Fvjn91qF.dpuf). Online access 4.Jun.2013.

Tiina Jauhiainen, T. P., Zhong Jian Cheng, Hannu Kautiainen, Dominik N. Müller, Heikki Vapaatalo, Riitta Korpela, and Eero Mervaala. (2010). Milk products containing bioactive tripeptides have an antihypertensive effect in double transgenic rats (dTGR) harbouring human renin and human angiotensinogen Genes. *Journal of Nutritional Metabolism,, 287030.*

Tobajas, M., Mohedano, A. F., Casas, J. A., & Rodríguez, J. J. (2007). A kinetic study of reuterin production by *Lactobacillus reuteri* PRO 137 in resting cells. *Biochemical Engineering Journal*, 35(2), 218-225.

Toledo, E., Delgado-Rodríguez, M., Estruch, R., Salas-Salvadó, J., Corella, D., Gomez-Gracia, E., Arós, F. (2009). Low-fat dairy products and blood pressure: follow-up of 2290 older persons at high cardiovascular risk participating in the PREDIMED study. *British Journal of Nutrition, 101*(01), 59-67.

Tsai, J.-S., Chen, T.-J., Pan, B. S., Gong, S.-D., & Chung, M.-Y. (2008). Antihypertensive effect of bioactive peptides produced by protease-faciliated lactic acid fermentation of milk. *Food Chemistry, 106*, 552-558.

Turner, A. J., & Hooper, N. M. (2002). The angiotensin-converting enzyme gene family: Genomics and pharmacology. *Trends in Pharmacological Sciences, 23*(4), 177-183.

Tomasik, P. J., & Tomasik, P. (2003). Probiotics and prebiotics. *Cereal Chemistry, 80*(2), 113-117.

Tuohy, K. M., Probert, H. M., Smejkal, C. W., & Gibson, G. R. (2003). Using probiotics and prebiotics to improve gut health. *Drug discovery today, 8*(15), 692-700.

Unal, G., & Akalin, A. S. (2012). Antioxidant and angiotensin-converting enzyme inhibitory activity of yoghurt fortified with sodium calcium caseinate or whey protein concentrate. *Dairy Science and Technology, 92*(6), 627-639.

Unger, T., Ganten, D., Lang, R. E., & Schölkens, B. A. (1985). Persistent Tissue Converting Enzyme Inhibition Following Chronic Treatment with Hoe498 and MK421 in Spontaneously Hypertensive Rats. *Journal of Cardiovascular Pharmacology, 7*(1), 36-41.

Unger, T., Ganten, D., Lang, R. E., & Schölkens, B. A. (1984). Is tissue converting enzyme inhibition a determinant of the antihypertensive efficacy of converting enzyme inhibitors? Studies with the two different compounds, Hoe498 and MK421, in spontaneously hypertensive rats. *Journal of Cardiovascular Pharmacology, 6*(5), 872-880.

Van Beresteijn, E. C. H., & Alting, A. C. (2002). Method for the selective degradation of milk protein in the presence of other milk proteins: Google Patents.

Van der Burg-Koorevaar, M. C., & Schalk, J. (2010). Hydrolysed casein product comprising tripeptides IPP and/or VPP: Google Patents.

Van der Ven, C., Gruppen, H., de Bont, D. B. A., & Voragen, A. G. J. (2002). Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *International Dairy Journal, 12*(10), 813-820. doi: 10.1016/s0958-6946(02)00077-8.

Van Mierlo, L., Arends, L., Streppel, M., Zeegers, M., Kok, F., Grobbee, D., & Geleijnse, J. (2006). Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials. *Journal of human hypertension, 20*(8), 571-580.

Venturoso, R. C., Almeida, K. E. d., Rodrigues, A. M., Damin, M. R., & Oliveira, M. N. d. (2007). Determinação da composição físico-química de produtos lácteos: estudo exploratório de comparação dos resultados obtidos por metodologia oficial e por ultra-som. *Revista Brasileira de Ciências Farmacêuticas, 43*, 607-613.

Verheul, M., & Roefs, S. P. F. M. (1998). Structure of whey protein gels, studied by permeability, scanning electron microscopy and rheology. *Food Hydrocolloids, 12*(1), 17-24. doi: http://dx.doi.org/10.1016/S0268-005X(98)00041-1.

Vermeirssen, V., Van Camp, J., Decroos, K., Van Wijmelbeke, L., & Verstraete, W. (2003). The impact of fermentation and in vitro digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and Whey Protein. *Journal of Dairy Science,86*(2),429-438.doi: http://dx.doi.org/10.3168/jds. S0022-0302(03)73621-2.

Vierhele, T. (2006). Functional 'add-ins' boost yogurt consumption. *Food technology, 60*(v. 60, no. 7), 44-46, 48.

Virginie, Amilien, Hanne, & Vittersø, T. a. G. (2005). Tjukkmjølk, the traditional thick sour milk from Røros, Norway. *Anthropology of food*.

Vioque, M., Gómez, R., Sánchez, E., Mata, C., Tejada, L., & Fernández-Salguero, J. (2000). Chemical and microbiological characteristics of ewes' milk cheese manufactured with extracts from flowers of cynara cardunculus and cynara humilis as coagulants. *Journal of Agricultural and Food Chemistry*, 48(2), 451-456. doi: 10.1021/jf990326v.

Wakai, T., Shinoda, T., Uchida, N., Hattori, M., Nakamura, Y., Beresford, T., Yamamoto, N. (2013). Comparative analysis of proteolytic enzymes need for processing of antihypertensive peptides between *Lactobacillus helveticus* CM4 and DPC4571. *Journal of Bioscience and Bioengineering, 115*(3), 246-252. doi: http://dx.doi.org/10.1016/j.jbiosc.2012.09.020.

Wakai, T., Yamaguchi, N., Hatanaka, M., Nakamura, Y., & Yamamoto, N. (2012). Repressive processing of antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, in *Lactobacillus helveticus* fermented milk by added peptides. *Journal of Bioscience and Bioengineering, 114*(2), 133-137.

Wakai, T., & Yamamoto, N. (2012). Antihypertensive peptides specific to *Lactobacillus helveticus* fermented milk. Biotechnology-Molecular Studies and Novel Applications for Improved Quality of Human Life. Biochemistry, Genetics and Molecular Biology. InTech, 159-172.

Walstra, P., Walstra, P., Wouters, J. T., & Geurts, T. J. (2005). *Dairy science and technology* (Seppo Salminen, and & Ouwehand Eds. 2th ed. Vol. 13). USA: CRC press.

Wang, X., Wang, L., Cheng, X., Zhou, J., Tang, X., & Mao, X.-Y. (2012). Hypertension-attenuating effect of whey protein hydrolysate on spontaneously hypertensive rats. *Food Chemistry, 134*(1), 122-126. doi: http://dx.doi.org/10.1016/j.foodchem.2012.02.074.

Wanasundara, P. K. J. P. D., Ross, A. R. S., Amarowicz, R., Ambrose, S. J., Pegg, R. B., & Shand, P. J. (2002). Peptides with Angiotensin I-Converting enzyme (ACE) inhibitory activity from defibrinated, hydrolyzed bovine plasma. *Journal of Agricultural and Food Chemistry, 50*(24), 6981-6988. doi: 10.1021/jf025592.

Welderufael, F. T., Gibson, T., & Jauregi, P. (2012). Production of angiotensin-I-converting enzyme inhibitory peptides from β-lactoglobulin- and casein-derived peptides: An integrative approach. *Biotechnology Progress, 28*(3), 746-755. doi: 10.1002/btpr.1541.

Williams, C. M., & Jackson, K. G. (2002). Inulin and oligofructose: effects on lipid metabolism from human studies. *British Journal of Nutrition, 87*(S2), S261-S264.

World Health Organization. (2013). *A global brief on hypertension: Silent killer, global public health crisis*. *Cardiovascular disease.* Retrieved 2 February, 2013, from http://www.who.int/cardiovascular_diseases/publications/global_brief_hypertension/en/.

World Health Organization. (2003). diet, nutrition and the prevention of chronic diseases. *WHO Library Cataloguing-in-Publication Data.* (WHO technical report series ; 916), from http://whqlibdoc.who.int/trs/who_trs_916.pdf.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural Requirements of Angiotensin I-Converting Enzyme Inhibitory Peptides: Quantitative Structure−Activity Relationship Study of Di- and Tripeptides. *Journal of Agricultural and Food Chemistry, 54*(3), 732-738. doi: 10.1021/jf051263l.

Wyss, J. & Carlson, S. (2001). The role of the nervous system in hypertension. (3)**,** 255-62.

Yamada, Y., Matoba, N., Usui, H., ONISHI, K., & YOSHIKAWA, M. (2002). Design of a highly potent anti-hypertensive peptide based on ovokinin (2-7). *Bioscience, biotechnology, and biochemistry, 66*(6), 1213-1217.

Yadav, J. S. S., Bezawada, J., Ajila, C. M., Yan, S., Tyagi, R. D., & Surampalli, R. Y. (2014). Mixed culture of Kluyveromyces marxianus and Candida krusei for single-cell protein production and organic load removal from whey. Bioresource Technology,164(0),119-127.doi:http://dx.doi.org/10.1016/j.biortech.2014.04.06.

Yamaguchi, N., Kawaguchi, K., & Yamamoto, N. (2009). Study of the mechanism of antihypertensive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis. *European Journal of Pharmacology, 620*(1–3), 71-77. doi: http://dx.doi.org/10.1016/j.ejphar.2009.08.005.

Yamamoto, N., Mine, Y., Li-Chan, E., & Jiang, B. (2010). Functional food products with antihypertensive effects. Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals, 29, 169.

Yamamoto, N., Maeno, M., & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yoghurt-like product fermented by *Lactobacillus helveticus* CPN4. *Journal of Dairy Science, 82*, 1388-1393.

Yamamoto, N., & Takano, T. (1999). Antihypertensive peptides derived from milk proteins. *Die Nahrung, 43*(3), 159-164.

Yamamoto, N. (1997). Antihypertensive peptides derived from food proteins. *Peptide Science, 43*(2), 129-134. doi:10.1002/(sici)1097-0282(1997)43:2<129:aid-bip5>3.0.co;2.

Yamamoto, N., Akino, A., & Takano, T. (1994). Antihypertensive Effect of the Peptides Derived from Casein by an Extracellular Proteinase from *Lactobacillus helveticus* CP790. *Journal of Dairy Science, 77*(4), 917-922. doi: http://dx.doi.org/10.3168/jds.S0022-0302(94)77026-0.

Yamamura, S., Morishima, H., Kumano-Go, T., Suganuma, N., Matsumoto, H., Adachi, H., Takeda, M. (2009). The effect of *Lactobacillus helveticus* fermented milk on sleep and health perception in elderly subjects. *European Journal of Clinical Nutrition*, 63(1), 100-105.

Yoshii, H., Tachi, N., Ohba, R., Sakamura, O., Takeyama, H., & Itani, T. (2001). Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 128*(1), 27-33. doi: 10.1016/s1532-0456(00)00172-1.

Zhou, F., Xue, Z., & Wang, J. (2010). Antihypertensive effects of silk fibroin hydrolysate by alcalase and purification of an ACE inhibitory dipeptide. *Journal of Agricultural and Food Chemistry, 58*(11), 6735-6740. doi: 10.1021/jf101101.

Ziadi, M., Bergot, G., Courtin, P., Chambellon, E., Hamdi, M., & Yvon, M. (2010). Amino acid catabolism by *Lactococcus lactis* during milk fermentation. *International Dairy Journal, 20*(1), 25-31.

APPENDIX-I Published papers

Effect of Flavourzyme® on Angiotensin-Converting Enzyme Inhibitory Peptides Formed in Skim Milk and Whey Protein Concentrate during Fermentation by *Lactobacillus helveticus*

Fatah Ahtesh, Lily Stojanovska, Nagendra Shah, and Vijay Kumar Mishra

Abstract: Angiotensin-converting enzyme inhibitory (ACE-I) activity as affected by *Lactobacillus helveticus* strains (881315, 881188, 880474, and 880953), and supplementation with a proteolytic enzyme was studied. Reconstituted skim milk (12% RSM) or whey protein concentrate (4% WPC), with and without Flavourzyme® (0.14% w/w), were fermented with 4 different *L. helveticus* strains at 37 °C for 0, 4, 8, and 12 h. Proteolytic and *in vitro* ACE-I activities, and growth were significantly affected ($P < 0.05$) by strains, media, and with enzyme supplementation. RSM supported higher growth and produced higher proteolysis and ACE-I compared to WPC without enzyme supplementation. The strains *L. helveticus* 881315 and 881188 were able to increase ACE-I to >80% after 8 h of fermentation when combined with Flavourzyme® in RSM compared to the same strains without enzyme supplementation. Supplementation of media by Flavourzyme® was beneficial in increasing ACE-I peptides in both media. The best media to release more ACE-I peptides was RSM with enzyme supplementation. The *L. helveticus* 881315 outperformed all strains as indicated by highest proteolytic and ACE-I activities.

Keywords: ACE inhibition, flavourzyme, *Lactobacillus helveticus*, skim milk

Practical Application: *Lactobacillus helveticus* in combination with Flavourzyme® was used for producing ACE-I peptides from reconstituted skim milk and whey protein concentrate. Fermentation of skim milk by *L. helveticus* in combination with Flavourzyme® resulted in >80% ACE-I after 8 h. These conditions can be used for developing a functional drink with antihypertensive activity.

Introduction

Hypertension is considered a risk factor for coronary heart disease, such as myocardial infarction and stroke (FitzGerald and others 2004). According to the World Health Organization, nearly one billion people worldwide suffer from hypertension (World Health Organization 2013). Angiotensin-converting enzyme (peptidyl dipeptidase A; EC 3.4.15.1) catalyses converstion of angiotensin-I to angiotensin-II (a vasoconstrictor), which contributes to hypertension and heart failure. Hypertension is usually controlled by a number of drugs, the most common being synthetic angiotensin-converting enzyme inhibitory (ACE-I) drugs such as captopril and enalapril (Hansson and others 1999; Turner and Hooper 2002). ACE-I drugs decrease active angiotensin-II production from inactive angiotensin-I (Erdos 1975; FitzGerald and others 2004). Angiotensin-II receptor antagonists are agents used to modify the renin–angiotensin–aldosterone system through blocking angiotensin receptors, resulting in a decrease in blood pressure (Miura and others 2011). Long-term use of synthetic ACE-I drugs, however, may result in side effects such as, cough, skin rash or development of impaired renal function (Sesoko and Kaneko 1985; Coulter and Edwards 1987). Peptides such as Val-Pro-Pro and Ile-Pro-Pro derived from milk proteins (FitzGerald and Meisel 2000; Pihlanto-Leppälä 2000; Pan and others 2005; Tsai and others 2008; Nielsen and others 2009; Pihlanto and others 2010; Phelan and Kerins 2011) have been identified to have similar effects of ACE-I action opening possibilities of replacing or complementing synthetic drugs (FitzGerald and Meisel 2000; Pan and others 2005; Tsai and others 2008; Nielsen and others 2009; Pihlanto and others 2010; Phelan and Kerins 2011). Lactic acid bacteria (LAB) used to produce fermented dairy products (yoghurt, fermented milk, cheeses) have shown to produce peptides with varied but significant ACE-I activities as reported in several studies (Korhonen and Pihlanto 2003, 2006, 2007; Korhonen 2009; Phelan and Kerins 2011; Hernández-Ledesma and others 2011). The use of specific LAB or proteases for producing ACE-I peptides from various milk media have been reported (van der Ven and others 2002; Donkor and others 2005; Pan and others 2005; Kilpi and others 2007; Meena and others 2008; Tsai and others 2008; Korhonen 2009; Hamme and others 2009; Ramchandran and Shah 2010, 2011; Tellez and others 2011; Chaves-López and others 2012;

MS 20151290 Submitted 7/30/2015, Accepted 11/8/2015. *Authors Ahtesh and Stojanovska are with College of Health and Biomedicine, Center for Chronic Disease, Victoria Univ., Werribee Campus, P.O. Box 14428, Melbourne, VIC 8001, Australia. Author Shah is with Food and Nutritional Science, School of Biological Sciences, Hong Kong Univ., Hong Kong. Author Mishra is with Inst. of Sustainability and Innovation, Victoria Univ., Werribee Campus, P.O. Box 14428, Melbourne, VIC 8001, Australia. Direct inquiries to author Mishra (E-mail: Vijay.Mishra@vu.edu.au).*

doi: 10.1111/1750-3841.13177

International Journal of Food Science +Technology

International Journal of Food Science and Technology 2016

Original article

Proteolytic and angiotensin-converting enzyme-inhibitory activities of selected probiotic bacteria

Fatah B. Ahtesh, Lily Stojanovska, Michael L. Mathai, Vasso Apostolopoulos & Vijay K. Mishra*

Center for Chronic Disease, College of Health and Biomedicine, Victoria University, Werribee Campus, PO Box 14428, Melbourne, Vic., 8001, Australia

(Received 26 October 2015; Accepted in revised form 14 December 2015)

Summary This study was carried out to examine the proteolytic and angiotensin-converting enzyme (ACE-I) activities of probiotic lactic acid bacteria (LAB) as influenced by the type of media, fermentation time, strain type and media supplementation with a proteolytic enzyme (Flavourzyme®). *Lactobacillus casei (Lc210)*, *Bifidobacterium animalis* ssp12 (Bb12), *Lactobacillus delbrueckii subsp. bulgaricus (Lb11842)* and *Lactobacillus acidophilus* (La2410) were grown in 12% of reconstituted skim milk (RSM) or 4% of whey protein concentrates (WPC-35) with or without combination (0.14%) of Flavourzyme® for 12 h at 37 °C. All the strains were able to grow in both media depending on type of strains used and fermentation time. All the strains showed higher proteolytic activity and produced more antihypersensitive peptides when grown in RSM medium at 12 h, when compared to WPC. Combination with Flavourzyme® also increased LAB growth and proteolytic and ACE-I activities. Of the four strains used, Bb12 *and* La2410 outperformed Lc210 and *Lb11842*. The highest ACE-I activity and proteolytic activity were found in *B. animalis* ssp12 combined with Flavourzyme®. Flavourzyme® led to an increase in the production of bioactive peptides with ACE-I activity during 12 h at 37 °C.

Keywords Angiotensin-converting enzyme, Flavourzyme®, proteolytic activity, reconstituted skim milk, whey protein concentration.

Introduction

The most extensively studied microorganisms are lactic acid bacteria (LAB), *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Bifidobacterium* (Castro *et al.*, 1996; Christensen *et al.*, 1999; Ziadi *et al.*, 2010). LAB including probiotic organisms are fastidious in nature, demanding several essential growth factors (Donkor *et al.*, 2007b). The proteolytic systems of LAB have been studied widely, and the enzymes involved have been isolated and characterised (Shihata & Shah, 2000). However, *Bifidobacterium* strains were not as proteolytic as other LAB, which explains why *Bifidobacterium* grow slowly in milk and may require supplementation from external sources (Dave & Shah, 1998; Gomes *et al.*, 1998). Milk products, such as skim milk, although they are rich growth media, contain low concentration of free amino acids and peptides to efficiently support growth of LAB (Shihata & Shah, 2000). Therefore, through proteolytic activity of LAB, bioactive peptides and amino acids are released from parent proteins in milk to support growth (Gobbetti *et al.*, 2000). There are two methods of releasing milk peptides, namely by milk fermentation with LAB and by enzymatic hydrolysis of proteins. The cell wall of LAB is able to hydrolyse caseins into peptides by extracellular proteinases and intracellular peptidases (Korhonen & Pihlanto, 2006; Otte *et al.*, 2007). Some of these peptides are classified as having angiotensin-converting enzyme (ACE-I inhibition) activity (Yamamoto *et al.*, 1994). Angiotensin-I-converting enzyme (ACE) plays a role in the regulation of blood pressure by catalysing the production of vasoconstrictor angiotensin II and inactivating the vasodilator and bradykinin (Doolittle, 1983; Brown & Vaughan, 1998). Hypertension is defined as persistent systolic blood pressure (≥140 mmHg) and diastolic pressure (≥90 mmHg) (Lollo *et al.*, 2015). Consumption of cheese with probiotics exhibits significantly lower blood pressure and improved blood lipids (triglycerides and cholesterol) when compared to consumption of cheese without probiotics (traditional cheese) (Lollo *et al.*, 2015). Hence, consumption of probiotics may potentially be useful in improving cardiovascular health parameters (Lollo *et al.*, 2015). Several factors are related to the development of hypertension, includ-

*Correspondent: E-mail: Vijay.Mishra@vu.edu.au

doi:10.1111/ijfs.13054

APPENDIX-II Identification of peptides

australian proteome analysis facility

Report:
MALDI TOF/TOF MS analysis for gel spots

Project code: **PROJ15328**

Client: Fatah Ahtesh
Address: Victoria University
Building 2, Rm 21.68
Hoppers Lane
Werribee VIC 3030

Contact tel: 0469808940
E-mail: **fateh.ahtest@live.vu.edu.au**

Supervisor's name: Vijay Mishba

Report date: 1st July 2013

Report prepared by: Matthew Fitzhenry
E-mail: **mfitzhenry@proteome.org.au**

(a) **Date samples received:**

7th June 2013

(b) **Sample names:**

Fraction 1 and 6

(c) **Instrument used**

4800 plus MALDI TOF/TOF Analyser (AB Sciex)

(d) **Sample preparation**

Sample was reconstituted in formic acid, then zip-tipped with C18 PerfectPure zip-tips (Millipore) with spotted onto a MALDI target plate with alpha cyano cinnamic acid matrix.

(e) **Data acquisition**

Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectroscopy was performed using the 4800 plus MALDI TOF/TOF Analyser. A Nd:YAG laser (355 nm) was used to irradiate the sample. Spectra were acquired in reflectron MS scan mode in the mass range of 700 to 4000 Da. The instrument was then switched to MS/MS mode where the twelve strongest peptides from the MS scan were isolated and fragmented by collision-induced dissociation, then re-accelerated to measure their masses and intensities. A near point calibration was applied and will give a typical mass accuracy of 50 ppm or better.

(f) **Data processing**

The data were exported in a format suitable for submission to the database search program, Mascot (Matrix Science Ltd, London UK). Peaklists were searched against *Other Mammals and Bacteria* in the SwissProt database. High scores in the database search indicate a likely match, confirmed or qualified by operator inspection.

(g) **Results:**

The monoisotopic peak list in text format and MS scan spectra and Mascot search result PDF files are attached.

Sample	ID	ID	Score	MS	MSMS	Coverage
Fraction 6	CASB_BOVIN	Beta-casein	196	14	7	37%
Fraction 1	-	-	-	-	-	-

Acknowledgment

Any publication arise from this work should be acknowledged the contribution of APAF with the words ***"This work was undertaken at APAF the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS)."***

Report:
1D nanoLC ESI MS/MS analysis

Project code: **PROJ15328**

Client: Fatah Ahtesh
Address: Victoria University
Building 2, Rm 21.68
Hoppers Lane
Werribee VIC 3030

Contact tel: 0469808940
E-mail: **fateh.ahtest@live.vu.edu.au**

Supervisor's name: Vijay Mishba

Report date: 1st July 2013

Report prepared by: Matthew Fitzhenry
E-mail: **mfitzhenry@proteome.org.au**

(a) **Date samples received:**

7th June 2013

(b) **Sample names:**

Fraction 1 and 6

(c) **Instrument used**

Mass Spectrometer: Q Star Elite (AB Sciex)

NanoLC system: Exigent TEMPO nanoflow

Analytical Column: SGE ProteCol C18, 300A, 3µm, 150µm x 10 cm

(d) **Sample preparation**

Solid sample was reconstituted with formic acid, then run.

(e) **Data acquisition**

(f) Solution was made up to 40 µL in ESI loading buffer then was injected onto a peptide trap (Michrome peptide Captrap) for preconcentration and desalted with 0.1% formic acid, 2% ACN, at 8µL/min.

The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O:CH_3CN (100:0, + 0.1% formic acid) to H_2O:CH_3CN (10:90, + 0.1% formic acid) at 500nL/min over an 80 min period. The LC eluent was subject to positive ion nanoflow electrospray MS analysis on QSTAR which was operated in an information dependant acquisition mode (IDA).

In IDA mode a TOFMS survey scan was acquired (m/z 400-1600, 0.5s), with the three largest multiply charged ions (counts >25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100-1600).

(g) **Data processing**

The data was exported in a format suitable for submission to the database search program, Mascot (Matrix Science Ltd, London UK). Peaklists were searched against *Other Mammals and Bacteria* in the SwissProt database. High scores in the database search indicate a likely match, confirmed or qualified by operator inspection. Search results were generated with a significance threshold of $P < 0.01$ with minimum cut-off score of 54 for all samples except Fraction 1 Bacteria ($P<0.05$ cut-off 60)

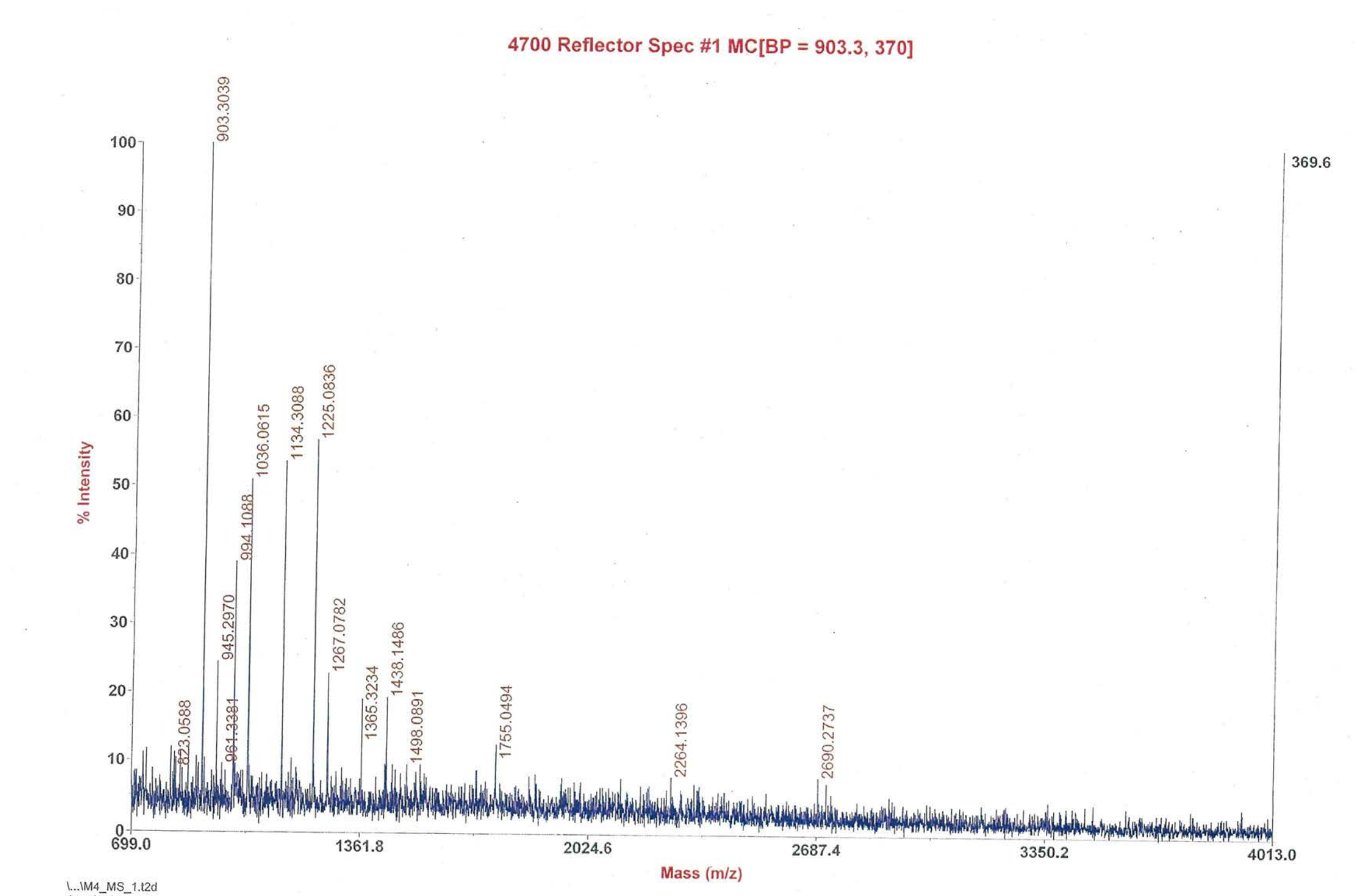
```
Database : SwissProt 2013 (539829 sequences; 191670831 residues)
Taxonomy : Other mammalia (13034 sequences)
Taxonomy : Bacteria (Eubacteria) (328828 sequences)

Type of search : MS/MS Ion Search
Enzyme : None
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 300 ppm
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages : 1
Instrument type : ESI-QUAD-TOF
```

Results:

Following files are attached:
Peak list in MGF format
Mascot search results in PDF

Acknowledgment

Any publication that arises from this work should be acknowledged the contribution of APAF with the words ***"This work was undertaken at APAF the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS)."***

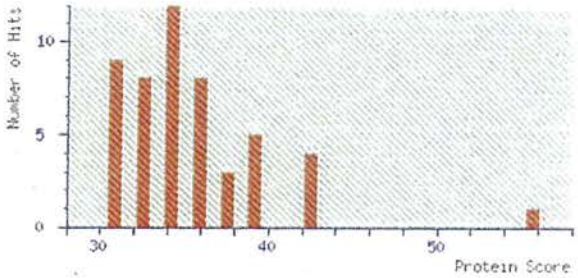

4700 Reflector Spec #1 MC[BP = 903.3, 370]
903.3039
823.0588
945.2970
961.3381
994.1088
1036.0615
1134.3088
1225.0836
1267.0782
1365.3234
1438.1486
1498.0891
1755.0494
2264.1396
2690.2737
369.6
100
90
80
70
60
50
40
30
20
10
0
% Intensity
699.0
1361.8
2024.6
2687.4
3350.2
4013.0
Mass (m/z)
\...\M4_MS_1.t2d
Acquired:

Mascot Search Results

```
User            :
Email           :
Search title    : Project: Users\2013.06, Spot Set: Users\2013.06\MF170613, Label: O4, Spot Id: 871775, Peak List Id: 666430, MS Job Run Id: 26909
MS data file    : \\apaf-hpv-file\projects\External\e_15328_VictoriaUni_FatahAhtesh_20130607\1_MassSpec\4800\Run1\RawData\Run1\MF170613\O4_MF170613.txt
Database        : SwissProt 2013 (539829 sequences; 191670831 residues)
Taxonomy        : Bacteria (Eubacteria) (328828 sequences)
Timestamp       : 27 Jun 2013 at 05:45:26 GMT
Warning         : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score       : 56 for RL23_CHLPN, 50S ribosomal protein L23 OS=Chlamydia pneumoniae GN=rplW PE=3 SV=1
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 68 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

Format As Protein Summary (deprecated) Help

Significance threshold p< 0.05 Max. number of hits AUTO

Re-Search All Search Unmatched

Index

	Accession	Mass	Score	Description
1.	RL23_CHLPN	12417	56	50S ribosomal protein L23 OS=Chlamydia pneumoniae GN=rplW PE=3 SV=1

Results List

1. RL23_CHLPN **Mass:** 12417 **Score:** 56 **Expect:** 0.89 **Matches:** 22

50S ribosomal protein L23 OS=Chlamydia pneumoniae GN=rplW PE=3 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6505	5.72	85	93	0	---	R.MFRGRRKGK.T + Oxidation (M)
1151.6644	1150.6571	1150.6346	19.6	46	56	0	---	I.VSHDATKPLIA.Q
1363.8259	1362.8186	1362.8208	-1.57	89	100	0	---	G.RRKGKTSGFKKA.I
1375.7285	1374.7212	1374.7442	-16.68	10	20	0	---	K.RHYVTEKAKML.E
1375.7285	1374.7212	1374.7442	-16.68	10	20	0	---	K.RHYVTEKAKML.E
1589.9221	1588.9148	1588.9188	-2.51	58	71	0	---	Q.ALEAIYVDKNVKVK.S
1589.9221	1588.9148	1588.8824	20.4	57	70	0	---	A.QALEAIYVDKNVKV.K
1601.9026	1600.8953	1600.8977	-1.50	41	54	0	---	P.KFVFIVSHDATKPL.I
1669.8888	1668.8815	1668.8624	11.5	95	109	0	---	T.SGFKKAIVTFYQGHS.V
1700.9590	1699.9517	1699.9529	-0.67	80	93	0	---	K.PQPARMFRGRRKGK.T + Oxidation (M)
1700.9590	1699.9517	1699.8934	34.3	39	53	0	---	K.DPKFVFIVSHDATKP.L
1717.9808	1716.9735	1716.9774	-2.25	57	71	0	---	A.QALEAIYVDKNVKVK.S
1717.9808	1716.9735	1716.9345	22.7	6	19	0	---	Y.DVIKRHYVTEKAKM.L
1781.9735	1780.9662	1780.9982	-17.93	70	85	0	---	K.VKSVNTINVKPQPARM.F
1881.0486	1880.0413	1879.9978	23.1	5	19	0	---	P.YDVIKRHYVTEKAKM.L
1881.0486	1880.0413	1879.9978	23.1	5	19	0	---	P.YDVIKRHYVTEKAKM.L
1994.1243	1993.1170	1993.0819	17.6	5	20	0	---	P.YDVIKRHYVTEKAKML.E
2107.2170	2106.2097	2106.2161	-3.02	64	82	0	---	Y.VDKNVKVKSVNTINVKPQP.A
2107.2170	2106.2097	2106.1295	38.1	4	20	0	---	D.PYDVIKRHYVTEKAKML.E + Oxidation (M)
2254.2810	2253.2737	2253.2521	9.58	38	57	0	---	C.KDPKFVFIVSHDATKPLIAQ.A
2254.2810	2253.2737	2253.1616	49.8	32	51	0	---	K.KKGSFCKDPKFVFIVSHDAT.K
2938.5374	2937.5301	2937.5204	3.30	16	42	0	---	E.KAKMLEHLSAGTGEGKKKGSFCKDPKF.V + Oxidation (M)

No match to: 906.6738

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : None
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 50 ppm
Fragment Mass Tolerance : ± 0.6 Da
Max Missed Cleavages    : 1
Instrument type         : MALDI-TOF-TOF
   Query1 (906.6738,1+): <no title>
  Query2 (1151.6644,1+): <no title>
  Query3 (1151.6644,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666432, MSMS Job_Run_Id: 26910, Comment:
  Query4 (1363.8259,1+): <no title>
  Query5 (1375.7285,1+): <no title>
  Query6 (1375.7285,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666433, MSMS Job_Run_Id: 26910, Comment:
  Query7 (1589.9221,1+): <no title>
  Query8 (1589.9221,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666435, MSMS Job_Run_Id: 26910, Comment:
  Query9 (1601.9026,1+): <no title>
 Query10 (1669.8888,1+): <no title>
 Query11 (1700.9590,1+): <no title>
 Query12 (1700.9590,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666439, MSMS Job_Run_Id: 26910, Comment:
```

```
Query13 (1717.9808,1+): <no title>
Query14 (1717.9808,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666438, MSMS Job_Run_Id: 26910, Comment:
Query15 (1781.9735,1+): <no title>
Query16 (1881.0486,1+): <no title>
Query17 (1881.0486,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666436, MSMS Job_Run_Id: 26910, Comment:
Query18 (1994.1243,1+): <no title>
Query19 (2107.2170,1+): <no title>
Query20 (2107.2170,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666437, MSMS Job_Run_Id: 26910, Comment:
Query21 (2254.2810,1+): <no title>
Query22 (2254.2810,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666434, MSMS Job_Run_Id: 26910, Comment:
Query23 (2938.5374,1+): <no title>
```

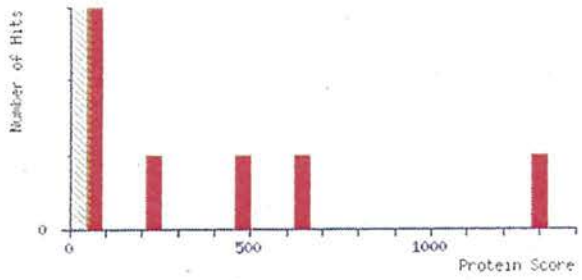
Mascot Search Results

User	:	
Email	:	
Search title	:	Submitted from VU-Bovine by Mascot Daemon on APAF-WS-08
MS data file	:	\\apaf-hpv-file\projects\External\e_15328_VictoriaUni_FatahAhtesh_20130607\1_MassSpec\QStarElite\Run1\Results\F6.mgf
Database	:	SwissProt 2013 (539829 sequences; 191670831 residues)
Taxonomy	:	Other mammalia (13034 sequences)
Timestamp	:	21 Jun 2013 at 03:46:07 GMT
Protein hits	:	CASB_BOVIN Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2
		CASA1_BOVIN Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2
		LACB_BOVIN Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3
		CASA1_BUBBU Alpha-S1-casein OS=Bubalus bubalis GN=CSN1S1 PE=2 SV=2
		LALBA_BOSMU Alpha-lactalbumin OS=Bos mutus grunniens GN=LALBA PE=2 SV=1
		CASA2_BOVIN Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2
		CASK_BISBO Kappa-casein (Fragment) OS=Bison bonasus GN=CSN3 PE=2 SV=1

	SwissProt	Decoy	False discovery rate
Peptide matches above identity threshold	103	0	0.00 %
Peptide matches above homology or identity threshold	144	0	0.00 %

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 54 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As Peptide Summary — Help

Significance threshold p< 0.01 — Max. number of hits AUTO — Show Percolator scores

Standard scoring — MudPIT scoring — Ions score or expect cut-off 54 — Show sub-sets 0

Show pop-ups • Suppress pop-ups Sort unassigned Decreasing Score Require bold red •

Select All Select None Search Selected **Error tolerant** Archive Report

1. CASB_BOVIN **Mass:** 25091 **Score:** 1299 **Matches:** 53(53) **Sequences:** 51(51) **emPAI:** 1135.85
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	102	458.3093	914.6041	914.5953	9.65	0	60	0.0029	1	U	N.LHLPLPLL.Q
✓	174	522.3336	1042.6526	1042.6539	-1.22	0	66	0.00072	1	U	N.LHLPLPLLQ.S
✓	221	576.3528	1150.6910	1150.6863	4.06	0	54	0.0098	1	U	L.GPVRGPFPIIV.-
✓	223	577.8162	1153.6179	1153.5979	17.4	0	71	0.0002	1	U	S.LTLTDVENLH.L
✓	224	578.3098	1154.6050	1154.5931	10.3	0	58	0.0037	1	U	A.RELEELNVPG.E
✓	240	602.8169	1203.6192	1203.5998	16.1	0	57	0.0053	1		E.MPFPKYPVEP.F
✓	291	630.3618	1258.7091	1258.6921	13.5	0	58	0.0047	1	U	T.DVENLHLPLPL.L
✓	299	641.8809	1281.7472	1281.7292	14.0	0	61	0.0021	1	U	L.SLSQSKVLPVPQ.K
✓	330	675.3345	1348.6545	1348.6373	12.7	0	60	0.0025	1		K.EMPFPKYPVEP.F + Oxidation (M)
✓	362	692.4111	1382.8077	1382.7922	11.2	0	62	0.0017	1		F.LLYQEPVLGPVR.G
✓	365	696.8890	1391.7634	1391.7561	5.22	0	56	0.007	1		Y.QEPVLGPVRGPFP.I
✓	378	718.4270	1434.8395	1434.8235	11.2	0	68	0.00044	1	U	K.VLPVPQKAVPYPQ.R
✓	400	745.9481	1489.8816	1489.8657	10.7	0	72	0.00018	1	U	Q.EPVLGPVRGPFPII.V
✓	403	750.9352	1499.8558	1499.8348	14.0	0	83	1.3e-005	1	U	T.DVENLHLPLPLLQ.S
✓	418	778.4273	1554.8401	1554.8195	13.3	0	68	0.00043	1		L.YQEPVLGPVRGPFP.I
✓	427	787.9526	1573.8907	1573.8716	12.1	0	77	5.7e-005	1	U	S.LTLTDVENLHLPLP.L
✓	429	794.4532	1586.8918	1586.8668	15.7	0	63	0.0013	1	U	T.DVENLHLPLPLLQS.W
✓	430	795.4854	1588.9562	1588.9341	13.9	0	57	0.0057	1	U	Q.EPVLGPVRGPFPIIV.-
✓	443	819.9557	1637.8968	1637.8817	9.18	0	88	4.1e-006	1	U	S.LVYPFPGPIPNSLPQ.N
✓	450	828.4180	1654.8215	1654.7879	20.3	0	61	0.0024	1		P.FPKYPVEPFTESQS.L
✓	454	832.4226	1662.8307	1662.7989	19.1	0	86	6.6e-006	1		Y.PVEPFTESQSLTLTD.V
✓	455	834.9656	1667.9166	1667.9035	7.81	0	71	0.00024	1		L.LYQEPVLGPVRGPFP.I
✓	460	844.4942	1686.9739	1686.9556	10.8	0	93	1.4e-006	1	U	S.LTLTDVENLHLPLPL.L
✓	461	844.5080	1687.0014	1686.9556	27.1	0	(88)	4.9e-006	1	U	S.LTLTDVENLHLPLPL.L
✓	470	858.0044	1713.9943	1713.9665	16.2	0	89	3.2e-006	1	U	T.LTDVENLHLPLPLLQ.S
✓	472	859.5263	1717.0381	1716.9927	26.5	0	83	1.4e-005	1	U	Y.QEPVLGPVRGPFPIIV.-
✓	474	574.0205	1719.0396	1719.0195	11.7	0	75	9.9e-005	1	U	S.KVLPVPQKAVPYPQR.D
✓	479	863.4663	1724.9181	1724.9138	2.50	0	69	0.00038	1	U	Q.SLVYPFPGPIPNSLPQ.N
✓	489	876.9755	1751.9365	1751.9247	6.78	0	87	5.4e-006	1	U	S.LVYPFPGPIPNSLPQN.I
✓	498	887.4898	1772.9650	1772.9461	10.6	0	63	0.0014	1	U	T.DVENLHLPLPLLQSW.M
✓	502	891.5102	1781.0058	1780.9876	10.2	0	71	0.00022	1		F.LLYQEPVLGPVRGPFP.I
✓	503	891.5243	1781.0341	1780.9876	26.1	0	60	0.0026	1	U	L.YQEPVLGPVRGPFPII.V
✓	513	908.5265	1815.0385	1815.0142	13.4	0	100	2.6e-007	1	U	L.TLTDVENLHLPLPLLQ.S
✓	533	612.3702	1834.0887	1834.0465	23.0	0	83	1.5e-005	1	U	S.KVLPVPQKAVPYPQRD.M
✓	552	941.0501	1880.0856	1880.0560	15.7	0	56	0.0066	1	U	L.YQEPVLGPVRGPFPIIV.-
✓	553	944.5647	1887.1148	1887.0717	22.9	0	77	5.3e-005	1	U	Q.SLTLTDVENLHLPLPLL.Q

✓	555	948.0568	1894.0990	1894.0717	14.4	0	65	0.00085	1	U	L.LYQEPVLGPVRGPFPII.V
✓	556	949.0648	1896.1151	1896.0721	22.7	0	59	0.004	1	U	N.SLPQNIPPLTQTPVVVPP.F
✓	561	641.3729	1921.0968	1921.0785	9.54	0	58	0.0046	1	U	Q.SKVLPVPQKAVPYPQRD.M
✓	563	965.0612	1928.1078	1928.0983	4.93	0	81	2.1e-005	1	U	S.LTLTDVENLHLPLPLLQ.S
✓	568	660.3951	1978.1634	1978.1251	19.4	0	56	0.0076	1	U	S.LSQSKVLPVPQKAVPYPQ.R
✓	571	997.5923	1993.1700	1993.1401	15.0	0	66	0.00079	1	U	L.LYQEPVLGPVRGPFPIIV.-
✓	575	1004.6051	2007.1957	2007.1557	19.9	0	64	0.0014	1	U	F.LLYQEPVLGPVRGPFPII.V
✓	578	1008.5819	2015.1492	2015.1303	9.38	0	78	5e-005	1	U	S.LTLTDVENLHLPLPLLQS.W
✓	579	1008.5909	2015.1672	2015.1303	18.3	0	98	4.4e-007	1	U	Q.SLTLTDVENLHLPLPLLQ.S
✓	583	1022.5850	2043.1554	2043.1405	7.31	0	66	0.00075	1	U	N.SLPQNIPPLTQTPVVVPPF.L
✓	587	689.4024	2065.1854	2065.1572	13.7	0	59	0.0034	1	U	L.SLSQSKVLPVPQKAVPYPQ.R
✓	605	719.0915	2154.2527	2154.2241	13.2	0	63	0.0015	1	U	A.FLLYQEPVLGPVRGPFPII.V
✓	629	771.7326	2312.1761	2312.1446	13.6	0	64	0.0012	1	U	W.MHQPHQPLPPTVMFPPQSVL.S + 2 Oxidation (M)
✓	658	856.1683	2565.4829	2565.4723	4.14	0	89	4.8e-006	1	U	P.IQAFLLYQEPVLGPVRGPFPIIV.-
✓	672	932.2084	2793.6032	2793.5656	13.5	0	84	2.1e-005	1	U	D.MPIQAFLLYQEPVLGPVRGPFPIIV.-
✓	674	937.5362	2809.5867	2809.5605	9.33	0	(80)	5.2e-005	1	U	D.MPIQAFLLYQEPVLGPVRGPFPIIV.- + Oxidation (M)
✓	700	931.2757	3721.0736	3720.0266	281	0	63	0.0037	1	U	K.AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV.-

---

2. CASA1_BOVIN **Mass:** 24513 **Score:** 634 **Matches:** 25(25) **Sequences:** 21(21) **emPAI:** 35.42

Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2

Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	192	552.8222	1103.6299	1103.6339	-3.56	0	62	0.0016	1		Y.LGYLEQLLR.L
✓	200	559.3271	1116.6396	1116.6291	9.39	0	69	0.00038	1		E.VLNENLLRF.F
✓	217	571.7766	1141.5387	1141.5251	11.9	0	82	1.5e-005	1		F.SDIPNPIGSEN.S
✓	261	609.3681	1216.7216	1216.7179	3.06	0	66	0.00062	1		Y.LGYLEQLLRL.K
✓	263	610.3212	1218.6279	1218.6285	-0.46	0	58	0.0046	1		F.VAPFPEVFGKE.K
✓	325	669.8782	1337.7419	1337.7191	17.1	0	73	0.00014	1	U	Q.GLPQEVLNENLL.R
✓	353	683.8746	1365.7347	1365.6969	27.6	0	68	0.00048	1		F.FVAPFPEVFGKE.K
✓	372	706.3481	1410.6816	1410.6667	10.5	0	64	0.0011	1	U	Y.YVPLGTQYTDAPS.F
✓	387	733.9029	1465.7913	1465.7776	9.32	0	82	1.7e-005	1	U	H.QGLPQEVLNENLL.R
✓	398	743.8594	1485.7042	1485.6947	6.39	0	75	9.4e-005	1	U	F.SDIPNPIGSENSEK.T
✓	426	787.8862	1573.7579	1573.7300	17.7	0	77	5.2e-005	1	U	W.YYVPLGTQYTDAPS.F
✓	458	840.4579	1678.9013	1678.8930	4.97	0	55	0.0092	1		D.VPSERYLGYLEQLL.R
✓	482	865.9281	1729.8417	1729.8192	13.0	0	78	4.5e-005	1	U	D.IPNPIGSENSEKTTMP.L + Oxidation (M)
✓	537	923.4429	1844.8712	1844.8462	13.6	0	77	5e-005	1	U	S.DIPNPIGSENSEKTTMP.L + Oxidation (M)
✓	557	636.0105	1905.0097	1904.9778	16.7	0	62	0.0018	1		G.IHAQQKEPMIGVNQELA.Y
✓	558	958.9612	1915.9078	1915.8833	12.8	0	(65)	0.00084	1	U	F.SDIPNPIGSENSEKTTMP.L
✓	564	966.9605	1931.9065	1931.8782	14.6	0	88	4.2e-006	1	U	F.SDIPNPIGSENSEKTTMP.L + Oxidation (M)
✓	586	1032.5039	2062.9932	2062.9517	20.1	0	(69)	0.00039	1	U	S.FSDIPNPIGSENSEKTTMP.L
✓	590	1040.5046	2078.9947	2078.9466	23.1	0	83	1.3e-005	1	U	S.FSDIPNPIGSENSEKTTMP.L + Oxidation (M)
✓	662	873.4381	2617.2925	2617.2370	21.2	0	59	0.0041	1	U	D.APSFSDIPNPIGSENSEKTTMPLW.-
✓	667	906.8165	2717.4278	2717.3523	27.8	0	(68)	0.0006	1	U	G.IHAQQKEPMIGVNQELAYFYPEL.F

✓	668	912.1428	2733.4066	2733.3472	21.7	0	71	0.0003	1	U	G.IHAQQKEPMIGVNQELAYFYPEL.F + Oxidation (M)
✓	675	955.8351	2864.4834	2864.4207	21.9	0	88	6.6e-006	1	U	G.IHAQQKEPMIGVNQELAYFYPELF.R
✓	676	961.1507	2880.4303	2880.4156	5.11	0	(70)	0.00041	1	U	G.IHAQQKEPMIGVNQELAYFYPELF.R + Oxidation (M)
✓	683	760.1399	3036.5306	3036.5167	4.58	0	57	0.0085	1	U	G.IHAQQKEPMIGVNQELAYFYPELFR.Q + Oxidation (M)

3. LACB_BOVIN **Mass:** 19870 **Score:** 468 **Matches:** 18(18) **Sequences:** 17(17) **emPAI:** 19.00

Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3

Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	209	565.8182	1129.6219	1129.6132	7.70	0	85	9.4e-006	1		G.LDIQKVAGTW.Y
✓	249	607.3079	1212.6012	1212.5874	11.4	0	57	0.0057	1		Y.VEELKPTPEGD.L
✓	269	617.7779	1233.5413	1233.6639	-99.33	0	72	0.00016	1	U	A.LIVTQTMKGLD.I + Oxidation (M)
✓	317	662.8843	1323.7541	1323.7286	19.3	0	57	0.0052	1		L.KPTPEGDLEILL.Q
✓	318	663.8516	1325.6887	1325.6714	13.0	0	87	5.1e-006	1		Y.VEELKPTPEGDL.E
✓	319	665.3539	1328.6932	1328.6823	8.19	0	67	0.00055	1		A.SDISLLDAQSAPL.R
✓	354	686.8440	1371.6734	1371.6518	15.7	0	73	0.00015	1		L.VRTPEVDDEALE.K
✓	361	691.9059	1381.7972	1381.7929	3.12	0	73	0.00013	1		D.ISLLDAQSAPLRV.Y
✓	384	728.3702	1454.7258	1454.7140	8.12	0	89	3.1e-006	1		Y.VEELKPTPEGDLE.I
✓	393	738.4126	1474.8105	1474.8065	2.73	0	85	8.1e-006	1	U	A.LIVTQTMKGLDIQ.K + Oxidation (M)
✓	402	500.9245	1499.7516	1499.7467	3.26	0	71	0.00025	1		L.VRTPEVDDEALEK.F
✓	417	773.4391	1544.8637	1544.8562	4.84	0	70	0.00028	1		D.ISLLDAQSAPLRVY.V
✓	441	544.6167	1630.8281	1630.8202	4.84	0	68	0.00044	1		L.RVYVEELKPTPEGD.L
✓	444	549.9501	1646.8284	1646.8152	8.04	0	84	1.2e-005	1		L.VRTPEVDDEALEKF.D
✓	445	549.9567	1646.8484	1646.8152	20.2	0	(81)	2.5e-005	1		L.VRTPEVDDEALEKF.D
✓	459	841.4592	1680.9039	1680.8822	13.0	0	79	3.7e-005	1		Y.VEELKPTPEGDLEIL.L
✓	488	874.4813	1746.9480	1746.9152	18.8	0	84	1.2e-005	1		A.SDISLLDAQSAPLRVY.V
✓	507	898.0071	1793.9997	1793.9662	18.7	0	69	0.00033	1		Y.VEELKPTPEGDLEILL.Q

4. CASA1_BUBBU **Mass:** 24311 **Score:** 251 **Matches:** 10(10) **Sequences:** 9(9) **emPAI:** 3.27

Alpha-S1-casein OS=Bubalus bubalis GN=CSN1S1 PE=2 SV=2

Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
	192	552.8222	1103.6299	1103.6339	-3.56	0	62	0.0016	1		Y.LGYLEQLLR.L
	200	559.3271	1116.6396	1116.6291	9.39	0	69	0.00038	1		G.VLNENLLRF.F
	217	571.7766	1141.5387	1141.5251	11.9	0	82	1.5e-005	1		F.SDIPNPIGSEN.S
	261	609.3681	1216.7216	1216.7179	3.06	0	66	0.00062	1		Y.LGYLEQLLRL.K
	263	610.3212	1218.6279	1218.6285	-0.46	0	58	0.0046	1		F.VAPFPEVFGKE.K
	353	683.8746	1365.7347	1365.6969	27.6	0	68	0.00048	1		F.FVAPFPEVFGKE.K
	458	840.4579	1678.9013	1678.8930	4.97	0	55	0.0092	1		D.VPSERYLGYLEQLL.R
✓	535	922.9485	1843.8825	1843.8622	11.0	0	(73)	0.00016	1	U	F.SDIPNPIGSENSGKTTMP.L
✓	539	930.9524	1859.8902	1859.8571	17.8	0	100	3.3e-007	1	U	F.SDIPNPIGSENSGKTTMP.L + Oxidation (M)
	557	636.0105	1905.0097	1904.9778	16.7	0	62	0.0018	1		G.IHAQQKEPMIGVNQELA.Y

5. LALBA_BOSMU Mass: 16237 Score: 81 Matches: 2(2) Sequences: 2(2) emPAI: 0.53
Alpha-lactalbumin OS=Bos mutus grunniens GN=LALBA PE=2 SV=1
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
193	553.3050	1104.5955	1104.5815	12.6	0	64	0.0011	1	U	K.DLKGYGGVSLP.E
270	617.8273	1233.6401	1233.6241	13.0	0	71	0.0002	1	U	K.DLKGYGGVSLPE.W

Proteins matching the same set of peptides:
LALBA_BOVIN Mass: 16236 Score: 81 Matches: 2(2) Sequences: 2(2)
Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2

6. CASA2_BOVIN Mass: 26002 Score: 77 Matches: 2(2) Sequences: 2(2) emPAI: 0.31
Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
473	573.3589	1717.0547	1717.0290	15.0	0	58	0.004	1	U	W.IQPKTKVIPYVRYL.-
582	680.7142	2039.1208	2039.0952	12.6	0	62	0.0017	1	U	Y.LYQGPIVLNPWDQVKRN.A

7. CASK_BISBO Mass: 14895 Score: 67 Matches: 1(1) Sequences: 1(1) emPAI: 0.26
Kappa-casein (Fragment) OS=Bison bonasus GN=CSN3 PE=2 SV=1
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
413	771.9113	1541.8081	1541.7937	9.36	0	67	0.00052	1	U	E.SPPEINTVQVTSTAV.-

Proteins matching the same set of peptides:
CASK_BOVIN Mass: 21256 Score: 67 Matches: 1(1) Sequences: 1(1)
Kappa-casein OS=Bos taurus GN=CSN3 PE=1 SV=1

Peptide matches not assigned to protein hits: (no details means no match)

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
431	531.3205	1590.9396	1590.9246	9.43	0	54	0.011	1		VLPVPQKAVPYPQR
448	550.9955	1649.9645	1649.9505	8.52	0	54	0.012	1		SKVLPVPQKAVPYPQ
534	612.9954	1835.9643	1835.9458	10.1	0	54	0.013	1		FVAPFPEVFGKEKVNE
439	813.4296	1624.8447	1624.8308	8.56	0	54	0.012	1		RELEELNVPGEIVE
356	687.9005	1373.7865	1373.7595	19.6	0	53	0.012	1		FLLYQEPVLGPV
296	634.8468	1267.6791	1267.6561	18.2	0	53	0.013	1		VLSRYPSYGIN
157	499.3103	996.6061	996.5757	30.5	0	53	0.012	1		FHVGKTPIV

✓	210	565.8520	1129.6894	1129.6859	3.05	0	52	0.016	1	LHLPLPLLQS
✓	624	761.0739	2280.2000	2280.1548	19.8	0	52	0.018	1	MHQPHQPLPPTVMFPPQSVL
✓	407	757.4012	1512.7879	1512.7653	15.0	0	52	0.017	1	FFVAPFPEVFGKE
✓	225	579.3590	1156.7035	1156.6968	5.74	0	52	0.016	1	NLHLPLPLLQ
✓	293	632.9010	1263.7874	1263.7703	13.5	0	52	0.017	1	LGPVRGPFPIIV
✓	462	563.9700	1688.8883	1688.8774	6.48	0	52	0.02	1	VAPFPEVFGKEKVNE
✓	352	455.5794	1363.7165	1363.7136	2.09	0	51	0.02	1	SLFSHAFEVVKT
✓	433	802.4268	1602.8391	1602.8365	1.58	0	51	0.023	1	HQGLPQEVLNENLL
✓	422	521.9834	1562.9284	1562.9184	6.34	0	51	0.024	1	KVLPVPQKAVPYPQ
✓	168	515.3328	1028.6511	1028.6382	12.5	0	51	0.023	1	NLHLPLPLL
✓	355	686.9109	1371.8072	1371.7762	22.6	0	50	0.028	1	DVENLHLPLPLL
✓	238	598.3565	1194.6984	1194.6972	0.97	0	49	0.031	1	LSQSKVLPVPQ
✓	268	616.8402	1231.6658	1231.6561	7.86	0	49	0.035	1	LDAQSAPLRVY
✓	178	526.7860	1051.5574	1051.5379	18.5	0	49	0.035	1	FVAPFPEVF
✓	385	730.9715	1459.9285	1459.8915	25.3	0	49	0.038	1	PVLGPVRGPFPIIV
✓	140	485.2710	968.5275	968.5179	10.00	0	48	0.032	1	LKPTPEGDL
✓	320	667.3343	1332.6540	1332.6424	8.74	0	48	0.041	1	EMPFPKYPVEP
✓	359	689.4024	1376.7903	1376.7816	6.26	0	48	0.044	1	EPVLGPVRGPFPI
✓	446	824.9176	1647.8206	1647.8079	7.69	0	48	0.046	1	AVPYPQRDMPIQAF + Oxidation (M)
✓	442	816.9219	1631.8292	1631.8130	9.96	0	48	0.047	1	AVPYPQRDMPIQAF
✓	406	752.9481	1503.8817	1503.8701	7.69	0	48	0.046	1	IPPLTQTPVVVPPF
✓	145	487.7875	973.5604	973.5484	12.3	0	47	0.049	1	LLYQEPVL
✓	562	643.0222	1926.0448	1926.0112	17.5	0	47	0.061	1	YQGPIVLNPWDQVKRN
✓	608	738.4203	2212.2390	2212.1991	18.0	0	47	0.063	1	RVYVEELKPTPEGDLEILL
✓	203	560.7867	1119.5589	1119.5389	17.8	0	46	0.06	1	FYQKFPQY
✓	204	561.3178	1120.6210	1120.6168	3.71	0	46	0.063	1	FLLYQEPVL
✓	595	703.0910	2106.2511	2106.2241	12.8	0	46	0.073	1	LLYQEPVLGPVRGPFPIIV
✓	16	403.7137	805.4128	805.4010	14.6	0	46	0.069	1	FVAPFPE
✓	380	719.4162	1436.8179	1436.8126	3.69	0	46	0.071	1	LKPTPEGDLEILL
✓	12	403.2310	804.4475	804.4494	-2.33	0	46	0.073	1	SAPLRVY
✓	576	1007.5397	2013.0648	2012.9877	38.3	0	45	0.084	1	IPNPIGSENSEKTTMPLW
✓	87	449.7829	897.5512	897.5436	8.42	0	45	0.072	1	RGPFPIIV
✓	467	569.6636	1705.9689	1705.9515	10.2	0	45	0.096	1	VLPVPQKAVPYPQRD
✓	307	646.3224	1290.6303	1290.6172	10.1	0	44	0.1	1	ELAYFYPELF
✓	637	798.7812	2393.3218	2393.2930	12.1	0	44	0.12	1	VLPVPQKAVPYPQRDMPIQAF
✓	632	775.7929	2324.3569	2324.3297	11.7	0	44	0.13	1	AFLLYQEPVLGPVRGPFPIIV
✓	440	543.9568	1628.8485	1628.8344	8.61	0	44	0.11	1	KAVPYPQRDMPIQA + Oxidation (M)
✓	315	441.9388	1322.7946	1322.7922	1.84	0	44	0.1	1	LSQSKVLPVPQK
✓	264	610.8098	1219.6050	1219.5947	8.41	0	44	0.11	1	MPFPKYPVEP + Oxidation (M)
✓	630	771.7379	2312.1919	2312.1446	20.4	0	44	0.13	1	MHQPHQPLPPTVMFPPQSVL + 2 Oxidation (M)
✓	202	560.3253	1118.6361	1118.6369	-0.71	0	44	0.12	1	IIVTQTMKGL + Oxidation (M)
✓	591	695.6933	2084.0581	2084.0360	10.6	0	44	0.13	1	IHAQQKEPMIGVNQELAY + Oxidation (M)
✓	510	901.9689	1801.9232	1801.8927	16.9	0	43	0.14	1	IGVNQELAYFYPELF
✓	191	552.7933	1103.5721	1103.5710	0.99	0	43	0.14	1	SLTLTDVENL
✓	401	498.9447	1493.8123	1493.7919	13.7	0	43	0.14	1	FVAPFPEVFGKEK

✓	386	487.9233	1460.7480	1460.7374	7.32	0	43	0.15	1	KEMPFPKYPVEP
✓	273	622.3171	1242.6197	1242.6092	8.46	0	42	0.15	1	VRTPEVDDEAL
✓	604	716.7393	2147.1962	2147.1851	5.16	0	42	0.19	1	IKHQGLPQEVLNENLLRF
✓	350	682.4331	1362.8517	1362.8388	9.48	0	42	0.19	1	VLGPVRGPFPIIV
✓	613	752.1203	2253.3391	2253.2926	20.6	0	42	0.22	1	FLLYQEPVLGPVRGPFPIIV
✓	187	549.7837	1097.5529	1097.5604	-6.91	0	41	0.2	1	ELKPTPEGDL
✓	198	555.2872	1108.5598	1108.5594	0.43	0	41	0.2	1	FVAPFPEVFG
✓	348	682.2923	1362.5700	1362.5398	22.1	0	41	0.24	1	EDPESLGFEHMG + Oxidation (M)
✓	456	557.2953	1668.8639	1668.8664	-1.49	0	41	0.24	1	RFFVAPFPEVFGKE
✓	214	568.3006	1134.5867	1134.5710	13.9	0	40	0.25	1	SLFSHAFEVV
✓	337	678.3715	1354.7285	1354.7074	15.6	0	40	0.26	1	RFFVAPFPEVF
✓	250	607.3428	1212.6711	1212.6543	13.9	0	40	0.27	1	LVYPFPGPIPN
✓	404	751.3837	1500.7528	1500.7395	8.89	0	40	0.28	1	AVPYPQRDMPIQA + Oxidation (M)
✓	346	680.8870	1359.7594	1359.7398	14.4	0	40	0.29	1	LTDVENLHLPLP
✓	390	736.4371	1470.8596	1470.8446	10.2	0	40	0.29	1	NIPPLTQTPVVVPP
✓	271	619.3378	1236.6611	1236.6543	5.47	0	39	0.32	1	FVAPFPEVFGK
✓	594	703.0910	2106.2511	2106.2241	12.8	0	39	0.38	1	LLYQEPVLGPVRGPFPIIV
✓	349	455.2322	1362.6748	1362.6779	-2.30	0	39	0.37	1	IQKEDVPSERY
✓	394	493.2579	1476.7518	1476.7323	13.2	0	39	0.37	1	KEMPFPKYPVEP + Oxidation (M)
✓	244	606.3408	1210.6670	1210.6445	18.6	0	39	0.33	1	KPTPEGDLEIL
✓	54	428.2530	854.4914	854.4902	1.32	0	38	0.32	1	TPVVVPPF
✓	313	650.8517	1299.6888	1299.6863	1.87	0	38	0.38	1	SLVYPFPGPIPN
✓	451	828.4344	1654.8542	1654.8242	18.1	0	38	0.41	1	IGVNQELAYFYPEL
✓	494	881.4607	1760.9069	1760.8920	8.48	0	38	0.42	1	AVPYPQRDMPIQAFL + Oxidation (M)
✓	185	545.7962	1089.5778	1089.6117	-31.11	0	38	0.4	1	MSPRLRAFL
✓	614	752.1203	2253.3391	2253.2926	20.6	0	38	0.51	1	FLLYQEPVLGPVRGPFPIIV
✓	142	487.2528	972.4910	972.4705	21.1	0	38	0.41	1	YQKFPQY
✓	515	607.0375	1818.0906	1817.8229	147	0	38	0.46	1	SNHWLMAWMGLEINT + Oxidation (M)
✓	241	403.2500	1206.7282	1206.7271	0.98	0	38	0.43	1	LKALPMHIRL + Oxidation (M)
✓	190	551.7729	1101.5313	1101.5277	3.23	0	38	0.46	1	VPYPQRDMP
✓	612	749.7561	2246.2465	2246.2245	9.79	0	37	0.55	1	VLPVPQKAVPYPQRDMPIQA
✓	147	489.2511	976.4876	976.4800	7.76	0	37	0.47	1	RDMPIQAF
✓	437	809.9771	1617.9397	1617.9131	16.5	0	37	0.56	1	NIPPLTQTPVVVPPF
✓	179	526.8229	1051.6312	1051.6179	12.7	0	37	0.49	1	GPVRGPFPII
✓	619	755.0912	2262.2516	2262.2195	14.2	0	37	0.6	1	VLPVPQKAVPYPQRDMPIQA + Oxidation (M)
✓	569	662.0273	1983.0601	1983.0142	23.1	0	37	0.61	1	FFVAPFPEVFGKEKVNE
✓	86	448.7362	895.4579	895.5015	-48.70	0	36	0.5	1	DPLPDKLV
✓	436	538.6284	1612.8634	1612.8395	14.8	0	36	0.72	1	KAVPYPQRDMPIQA
✓	295	634.3702	1266.7259	1266.5836	112	0	36	0.67	1	MADDTCLEKLL + Oxidation (M)
✓	230	587.2985	1172.5824	1172.5648	15.0	0	36	0.68	1	AVPYPQRDMP
✓	14	403.7061	805.3976	805.3970	0.69	0	36	0.71	1	SFNPTQL
✓	290	628.8261	1255.6376	1255.6278	7.85	0	35	0.75	1	FFVAPFPEVFG
✓	428	792.4600	1582.9054	1582.9554	-31.57	0	35	0.81	1	IIPLLIMLICNAKI + Oxidation (M)
✓	438	809.9798	1617.9451	1617.9243	12.9	0	35	0.9	1	QEPVLGPVRGPFPII
✓	397	743.3821	1484.7497	1484.7446	3.46	0	35	0.89	1	AVPYPQRDMPIQA

✓	220	575.8578	1149.7010	1149.6910	8.69	0	34	0.95	1	KVIPYVRYL
✓	536	615.6894	1844.0465	1844.0156	16.8	0	34	1.2	1	IKHQGLPQEVLNENLL
✓	663	659.6368	2634.5182	2634.4720	17.6	0	34	1.8	1	KVLPVPQKAVPYPQRDMPIQAFL
✓	15	403.7080	805.4014	805.4010	0.41	0	33	1.2	1	APFPEVF
✓	639	804.1186	2409.3340	2409.2879	19.1	0	33	1.5	1	VLPVPQKAVPYPQRDMPIQAF + Oxidation (M)
✓	499	592.9885	1775.9437	1775.9029	23.0	0	33	1.4	1	KAVPYPQRDMPIQAF + Oxidation (M)
✓	376	715.3623	1428.7100	1428.7038	4.35	0	33	1.4	1	YQGPIVLNPWDQ
✓	371	470.9515	1409.8325	1409.8242	5.90	0	33	1.4	1	SLSQSKVLPVPQK
✓	409	506.2805	1515.8197	1515.7933	17.4	0	33	1.5	1	RVYVEELKPTPEG
✓	327	672.3485	1342.6824	1342.6703	8.96	0	32	1.6	1	VPYPQRDMPIQ
✓	316	662.8770	1323.7395	1323.7286	8.29	0	32	1.6	1	LKPTPEGDLEIL
✓	447	825.9069	1649.7992	1649.8182	-11.53	0	32	1.8	1	LDESVSLETMIERI + Oxidation (M)
✓	135	481.7577	961.5009	961.4909	10.4	0	32	1.7	1	VAPFPEVFG
✓	396	743.3718	1484.7291	1484.7187	6.98	0	32	1.8	1	VNQELAYFYPEL
✓	493	587.6516	1759.9330	1759.9079	14.2	0	31	2.1	1	KAVPYPQRDMPIQAF
✓	666	903.4156	2707.2248	2707.4367	-78.25	0	31	3.4	1	GALKIKEITYMHSEGILAGELKHGP + Oxidation (M)
✓	173	521.2639	1040.5133	1040.5219	-8.23	0	31	2	1	FLFPEFEI
✓	340	679.4117	1356.8088	1356.8017	5.22	0	31	2.2	1	IPPLTQTPVVVPP
✓	392	737.4309	1472.8473	1472.8239	15.9	0	31	2.2	1	LTDVENLHLPLPL
✓	274	623.8378	1245.6611	1245.6182	34.4	0	31	2.2	1	FYPELFRQF
✓	373	706.8862	1411.7579	1411.7289	20.5	0	31	2.2	1	RFFVAPFPEVFG
✓	164	509.3695	1016.7244	1016.4419	278	0	31	2.2	1	MNPAGNPVAM + Oxidation (M)
✓	119	468.2332	934.4519	934.4396	13.1	0	31	2.3	1	SFNPTQLE
✓	329	673.8456	1345.6766	1345.6911	-10.80	0	31	2.4	1	QELTKNSSVMLP
✓	538	930.0088	1858.0030	1857.9811	11.8	0	30	2.6	1	AVPYPQRDMPIQAFLL
✓	239	600.3157	1198.6169	1198.6063	8.81	0	30	2.5	1	FFVAPFPEVF
✓	411	761.4550	1520.8955	1520.7844	73.0	0	30	2.9	1	MTQVVLRGGGFLPM + Oxidation (M)
✓	410	760.8260	1519.6375	1519.6977	-39.60	0	30	2.8	1	IDISKNSFHSMPE + Oxidation (M)
✓	156	499.2638	996.5131	996.5128	0.32	0	30	2.5	1	QETDPLPVV
✓	284	625.4000	1248.7854	1248.6788	85.4	0	30	2.7	1	LTADIFLALCIG
✓	195	553.8436	1105.6727	1105.6383	31.1	0	30	2.8	1	PLDPLPDKLV
✓	627	766.4097	2296.2074	2296.1497	25.1	0	29	3.4	1	MHQPHQPLPPTVMFPPQSVL + Oxidation (M)
✓	516	607.1000	1818.2782	1818.7883	-280.47	0	29	3.7	1	NPAIENWGSDFLCPEQ
✓	186	547.8446	1093.6746	1093.6648	8.97	0	29	3	1	PVRGPFPIIV
✓	159	501.2718	1000.5290	1000.5077	21.3	0	29	3.1	1	DVGLDTLTPA
✓	228	582.7658	1163.5170	1163.5757	-50.42	0	29	3.2	1	KEFANRCLSP
✓	68	434.2702	866.5259	866.3882	159	0	29	2.8	1	QSHAADAAP
✓	19	409.2389	816.4632	816.2960	205	0	28	3.8	1	QDYSGTM + Oxidation (M)
✓	364	692.8742	1383.7338	1383.7227	8.00	0	28	4.2	1	FFVAPFPEVFGK
✓	626	765.0825	2292.2258	2292.2001	11.2	0	28	4.7	1	IQKEDVPSERYLGYLEQLL
✓	487	873.4689	1744.9232	1744.8970	15.0	0	28	4.5	1	AVPYPQRDMPIQAFL
✓	550	938.0286	1874.0427	1873.9760	35.6	0	28	4.5	1	AVPYPQRDMPIQAFLL + Oxidation (M)
✓	610	559.5000	2233.9709	2234.2609	-129.80	0	28	4.9	1	HQSLSLLIFGIVHLLNKICS
✓	547	625.4000	1873.1782	1872.8829	158	0	28	4.7	1	SQFIIMYSLDGRNWQ + Oxidation (M)
✓	108	462.3503	922.6861	922.5124	188	0	28	2.9	1	PELLQPLN

✓	634	780.6962	2339.0667	2339.2822	-92.11	0	28	5.1	1	LQQQLEKKRRQAPPPGPNGPT
✓	7	401.2332	800.4519	800.4466	6.65	0	28	4.2	1	NLIMTPL
✓	656	631.3526	2521.3814	2521.3879	-2.57	0	28	5.7	1	KVLPVPQKAVPYPQRDMPIQAF
✓	641	607.1000	2424.3709	2424.1413	94.7	0	28	5.8	1	IQGPEDKQIPHMQGNMINLET + 2 Oxidation (M)
✓	625	764.7764	2291.3075	2291.2273	35.0	0	28	5.4	1	NHLRKVPDGLPSALEQLYLE
✓	113	464.2460	926.4775	926.4684	9.80	0	28	4	1	YMLPPGLH
✓	628	575.4000	2297.5709	2298.0788	-221.02	0	28	9.6	1	LPLHIINCFTFFCPECPHAP
✓	573	668.3757	2002.1054	2002.1979	-46.19	0	28	5	1	FKKYIDNPKLRELLII
✓	546	625.3627	1873.0664	1872.9920	39.7	0	28	5.2	1	KAVPYPQRDMPIQAFL
✓	520	607.4594	1819.3564	1818.8168	297	0	28	6.6	1	QDLCEMLGKFGSSLEF + Oxidation (M)
✓	678	739.1711	2952.6552	2952.5895	22.2	0	28	9.7	1	LSQSKVLPVPQKAVPYPQRDMPIQAF + Oxidation (M)
✓	640	607.0445	2424.1487	2424.1413	3.06	0	28	5.6	1	IQGPEDKQIPHMQGNMINLET + 2 Oxidation (M)
✓	560	641.3705	1921.0897	1920.9537	70.8	0	27	5.2	1	LPSSITMSQGGMVTVIPAT + 2 Oxidation (M)
✓	648	624.1000	2492.3709	2492.1165	102	0	27	6	1	CEENLFSDYISEVERTFGNLQ
✓	389	735.9545	1469.8945	1469.8858	5.97	0	27	5	1	IDPVVKALVGLFSL
✓	412	763.9343	1525.8540	1525.8789	-16.34	0	27	4.9	1	LPMLLGNSILVDLL + Oxidation (M)
✓	606	720.7312	2159.1717	2159.0768	43.9	0	27	5.7	1	PLGKRPCEMQAFRIWDVN
✓	469	429.1000	1712.3709	1711.9178	265	0	27	5.8	1	KPENVKMLGGEVDALL
✓	48	425.2454	848.4763	848.4314	53.0	0	27	5.1	1	DTMGALLL + Oxidation (M)
✓	671	698.3982	2789.5635	2789.5262	13.4	0	27	9.5	1	LSQSKVLPVPQKAVPYPQRDMPIQA
✓	620	756.3186	2265.9340	2266.0787	-63.85	0	27	7.1	1	KPPESDDEEMKEAAGSLLHLA
✓	688	1027.4763	3079.4071	3078.6098	259	0	27	12	1	AAPELPVPTSGPLAGSREQALAVSRNYLSQ
✓	528	609.3751	1825.1035	1824.8061	163	0	27	6.2	1	YAAAGGGGAGGVSGGSGGGLAAMG + Oxidation (M)
✓	294	633.3675	1264.7205	1264.6815	30.8	0	27	5.8	1	KVDPAAKYLFN
✓	105	459.7281	917.4416	917.3702	77.8	0	26	6	1	PTFQECHG
✓	381	724.4158	1446.8170	1446.6991	81.5	0	26	6.4	1	VEDPVNQNGYTLV
✓	336	678.3099	1354.6053	1354.7820	-130.41	0	26	6.3	1	VLRLEEQEVLK
✓	162	507.8100	1013.6054	1013.5467	57.9	0	26	6.2	1	LPVTEPIVM + Oxidation (M)
✓	167	511.8106	1021.6066	1021.5960	10.3	0	26	6.4	1	VIPYVRYL
✓	432	801.4571	1600.8996	1600.8032	60.2	0	26	7	1	FTGMIQGGLQDGHKI
✓	435	806.8786	1611.7426	1611.8104	-42.02	0	26	6.8	1	QPEKKEEQVLEQQ
✓	3	576.4073	575.4000	575.2551	252	0	26	6.2	1	EGLSGN
✓	128	474.7714	947.5283	947.4647	67.1	0	26	6.6	1	LAPMHLGHG + Oxidation (M)
✓	213	567.7888	1133.5631	1133.5546	7.57	0	26	7	1	YFYPELFR
✓	391	737.3513	1472.6880	1472.7407	-35.81	0	26	7.4	1	PMIPPPPPICPDSL
✓	171	516.8135	1031.6124	1031.6015	10.6	0	26	7.3	1	EKTKIPAVF
✓	483	577.9891	1730.9454	1730.9349	6.07	0	26	8.1	1	MNLNLSVVVGAKSDRL + Oxidation (M)
✓	287	627.3420	1252.6694	1252.7067	-29.78	0	26	7	1	VLYDEIKKFV
✓	10	402.2514	802.4883	802.4800	10.3	0	26	7.3	1	AATTTLLL
✓	698	832.4827	3325.9015	3326.5720	-201.55	0	25	19	1	YSLIIREDGKSISSIMVKDMENETTYGMT + Oxidation (M)
✓	585	515.5000	2057.9709	2057.8571	55.3	0	25	8.4	1	RTASEPYHMDNFQDKTC + Oxidation (M)
✓	103	458.7854	915.5562	915.5542	2.23	0	25	7.6	1	NLHLPLPL
✓	314	652.3150	1302.6154	1302.5802	27.0	0	25	7.9	1	FQIISMDDVYG + Oxidation (M)
✓	188	550.2873	1098.5601	1098.5498	9.36	0	25	7.7	1	FYPELFRQ
✓	26	413.2867	824.5589	824.5120	56.9	0	25	5.9	1	LIQPGAVK

✓	351	455.5471	1363.6195	1363.5602	43.5	0	25	7.5	1	VSSENFDEYMK + Oxidation (M)
✓	80	444.2367	886.4589	886.4589	0.02	0	25	7.8	1	YPFPGPIP
✓	581	678.0537	2031.1393	2031.1364	1.44	0	25	8.9	1	LYKERLSQVDAKLQEVI
✓	256	607.8115	1213.6085	1213.5979	8.72	0	25	8.1	1	YETQPVPNPVA
✓	21	409.2714	816.5282	816.5109	21.2	0	25	8.2	1	ALLAVLAF
✓	405	502.2804	1503.8195	1503.7457	49.1	0	25	8.3	1	ELDSYPVVNLINE
✓	652	625.4000	2497.5709	2497.1849	155	0	25	17	1	EHFRQQCFFIKPTVFGGVQDD
✓	323	668.8234	1335.6322	1335.7697	-102.92	0	25	8.3	1	NKVRVKISYVM
✓	283	625.3202	1248.6257	1248.5809	36.0	0	25	8.3	1	YQSVCEPGAAPK
✓	334	676.3896	1350.7646	1350.7547	7.30	0	25	8.5	1	DKIAKYIPIQY
✓	679	989.1308	2964.3704	2964.5516	-61.10	0	25	17	1	EKELQRKSQAAVAQAAKEVEPEVVAEGA
✓	199	556.2997	1110.5848	1110.5961	-10.13	0	25	7.8	1	DPKSYLILY
✓	544	624.4000	1870.1782	1869.8084	198	0	25	10	1	LCRDSGGETESMLLNET + Oxidation (M)
✓	434	803.9335	1605.8525	1605.8297	14.2	0	25	9.5	1	PGQMLLSGGPRGPVPQ + Oxidation (M)
✓	374	707.8649	1413.7153	1413.6816	23.8	0	25	9.5	1	IGFLNIYEPDYA
✓	689	779.4306	3113.6934	3113.6292	20.6	0	24	20	1	PKKPGLSQTPSPPHPQLKVIMDSSRASSAG + Oxidation (M)
✓	255	607.7882	1213.5618	1213.5107	42.1	0	24	9.5	1	NNPSFIMGSMT + Oxidation (M)
✓	425	786.4716	1570.9286	1570.8640	41.1	0	24	11	1	KMVTSEALDGVPILV
✓	522	607.7649	1820.2728	1819.8821	215	0	24	12	1	AASRNARLPMCASSLWA + Oxidation (M)
✓	657	635.3665	2537.4370	2537.3828	21.4	0	24	14	1	KVLPVPQKAVPYPQRDMPIQAF + Oxidation (M)
✓	151	490.2874	978.5602	978.4407	122	0	24	9.3	1	THSAGPPPDT
✓	651	624.4000	2493.5709	2493.2396	133	0	24	21	1	HPKLLMSGGGGYLLSGFTVAMDNL + Oxidation (M)
✓	172	518.3760	1034.7374	1034.4458	282	0	24	9.4	1	YTSPHWGST
✓	644	609.1000	2432.3709	2432.2623	44.7	0	24	14	1	FAATELILLYIMFEATLIPTM + 2 Oxidation (M)
✓	514	607.0118	1818.0137	1817.8229	105	0	24	12	1	SNHWLMAWMGLEINT + Oxidation (M)
✓	132	476.2877	950.5608	950.4895	74.9	0	24	10	1	LQLFMGNL + Oxidation (M)
✓	123	472.2786	942.5426	942.5022	42.9	0	24	11	1	NLDLENLI
✓	615	752.1255	2253.3546	2253.2926	27.6	0	24	14	1	FLLYQEPVLGPVRGPFPIIV
✓	247	607.1000	1212.1854	1212.5267	-281.44	0	24	2.6	1	ASKLHSNMMY + 2 Oxidation (M)
✓	399	744.9253	1487.8361	1487.7555	54.2	0	24	13	1	RRMPEVPGLYSAP + Oxidation (M)
✓	242	604.8276	1207.6407	1207.6390	1.41	0	24	11	1	RFFVAPFPEV
✓	682	1011.4650	3031.3732	3031.4968	-40.75	0	23	26	1	GSGPGAGQQQAAPGALLQAGPPRCSSLQAPIM + Oxidation (M)
✓	379	718.8632	1435.7119	1435.8082	-67.07	0	23	12	1	KIHLVPCSLRDR
✓	653	625.4000	2497.5709	2498.2809	-284.21	0	23	24	1	MMLTLFIMGTIMILNTHFTLAS
✓	333	676.3182	1350.6219	1350.7945	-127.80	0	23	12	1	LVLVIMPLLHTS + Oxidation (M)
✓	673	702.4013	2805.5762	2805.5211	19.7	0	23	22	1	LSQSKVLPVPQKAVPYPQRDMPIQA + Oxidation (M)
✓	75	439.2582	876.5018	876.4593	48.5	0	23	12	1	FPIDISSV
✓	146	488.3664	974.7182	974.4314	294	0	23	11	1	SVGCHMTKP + Oxidation (M)
✓	360	690.4087	1378.8029	1378.8799	-55.89	0	23	13	1	LVVVIGLENVLVL
✓	646	620.3529	2477.3825	2477.3489	13.5	0	23	16	1	EQSGLIQAGDIILAVNGRPLVDLS
✓	643	607.4000	2425.5709	2425.0561	212	0	23	26	1	SPLEYMMKCYPEIKEKEEM + 3 Oxidation (M)
✓	686	763.4428	3049.7421	3049.6787	20.8	0	23	31	1	LSQSKVLPVPQKAVPYPQRDMPIQAFL
✓	650	624.4000	2493.5709	2494.3002	-292.38	0	23	27	1	EMYVAKFAAKGEGQLGPAERAKK + Oxidation (M)
✓	543	624.4000	1870.1782	1869.9944	98.3	0	23	15	1	TNIIMMKALTTTLFLGA + 2 Oxidation (M)
✓	377	715.8608	1429.7070	1429.8293	-85.50	0	23	14	1	FIVAKAIRDGVIE

✓	649	624.4000	2493.5709	2493.0626	204	0	23	27	1	FDFGLGYMMCTAFRTFDPCKQ + Oxidation (M)
✓	540	624.0363	1869.0871	1868.9343	81.8	0	23	15	1	FQSPTPNTLMPVVPAASP + Oxidation (M)
✓	222	576.7957	1151.5768	1151.5459	26.9	0	23	13	1	QTPGALPAPEGD
✓	91	452.2902	902.5658	902.5589	7.56	0	23	15	1	KTKIPAVF
✓	207	563.7814	1125.5482	1125.6506	-91.00	0	23	14	1	SNVTILPLRN
✓	548	625.4000	1873.1782	1872.9680	112	0	23	17	1	QSIEAPLTASDTAILSGSL
✓	60	431.2180	860.4215	860.4062	17.8	0	23	16	1	GSCPSNALI
✓	165	510.3798	1018.7451	1018.5117	229	0	23	13	1	REIIGGEMV + Oxidation (M)
✓	45	422.6957	843.3769	843.3871	-12.05	0	23	16	1	YMGLVVM + 2 Oxidation (M)
✓	366	697.4210	1392.8275	1392.6781	107	0	22	16	1	TQWKMMDPLLV + 2 Oxidation (M)
✓	116	465.2861	928.5577	928.4728	91.4	0	22	15	1	TLAMIAFF + Oxidation (M)
✓	697	823.1335	3288.5048	3288.5512	-14.11	0	22	38	1	LSTAQCTNGHLMCAGCFIHLLADARLKEEQ + Oxidation (M)
✓	251	607.4000	1212.7854	1212.5267	213	0	22	15	1	ASKLHSNMMY + 2 Oxidation (M)
✓	163	509.2798	1016.5450	1016.5940	-48.16	0	22	16	1	MALLTGSLVL
✓	478	575.6557	1723.9454	1723.8240	70.5	0	22	16	1	IFQASDIGASMTIHAF + Oxidation (M)
✓	463	845.6905	1689.3664	1689.8430	-282.02	0	22	18	1	GEMKGSAITGPVIKECA
✓	681	1009.4443	3025.3110	3024.6356	223	0	22	36	1	AEKPQVTGPIEVPVARTEERKASGPPKGP
✓	297	424.8125	1271.4156	1271.4862	-55.53	0	22	15	1	SGNSGGSSSSGSSTN
✓	197	554.7390	1107.4634	1107.4841	-18.75	0	22	15	1	NTMNMAWQI
✓	680	756.9197	3023.6496	3023.6266	7.61	0	22	32	1	SLSQSKVLPVPQKAVPYPQRDMPIQAF
✓	253	607.7765	1213.5385	1213.5107	22.9	0	22	16	1	NNPSFIMGSMT + Oxidation (M)
✓	416	515.5000	1543.4782	1543.8280	-226.57	0	22	16	1	KGIDVIIEMLANVN + Oxidation (M)
✓	660	861.7604	2582.2593	2582.1957	24.6	0	22	20	1	DSDSDSESQSRVRGQGGLPGGAAPGPV
✓	154	496.3575	990.7004	990.4692	233	0	22	16	1	ISPIDVMNS + Oxidation (M)
✓	358	688.5358	1375.0571	1374.8268	167	0	22	14	1	MSLKSLTKKLLN
✓	183	542.8145	1083.6144	1083.5965	16.6	0	22	16	1	TQTPVVVPPF
✓	115	465.2779	928.5413	928.5052	38.9	0	22	17	1	MLPPLTKN + Oxidation (M)
✓	158	501.2654	1000.5163	1000.4648	51.5	0	22	17	1	MNPSTLQPN
✓	597	704.3080	2109.9023	2110.2361	-158.19	0	22	20	1	GIEKLAQKGKSLSPLASITGI
✓	130	475.3240	948.6335	948.5505	87.5	0	22	17	1	RNKPLPPQ
✓	542	624.4000	1870.1782	1869.9407	127	0	22	20	1	PNLYGNIVNNVCAGLVGGP
✓	517	607.1000	1818.2782	1818.8202	-297.99	0	22	22	1	MAEYTNIIMMNALTAT + 2 Oxidation (M)
✓	524	608.7728	1823.2965	1822.8919	222	0	22	23	1	ILLTTTFFMMLMLNS + 3 Oxidation (M)
✓	219	575.4106	1148.8067	1148.5284	242	0	22	17	1	RGAEPAAPAMY + Oxidation (M)
✓	521	607.5000	1819.4782	1819.9075	-235.92	0	22	26	1	CFGVLGVLFMAISLGFM + Oxidation (M)
✓	592	698.3257	2091.9551	2092.0324	-36.93	0	22	21	1	ADAQVLGSPEKEEVAPEIPN
✓	257	608.7868	1215.5590	1215.5983	-32.30	0	21	20	1	AEQLDALLTNE
✓	326	670.8653	1339.7160	1339.5714	108	0	21	18	1	DMTRDEYNALP + Oxidation (M)
✓	525	609.0249	1824.0528	1823.7003	193	0	21	21	1	LRQEEMDESDDDLDD
✓	655	836.4669	2506.3789	2506.3770	0.76	0	21	25	1	VLPVPQKAVPYPQRDMPIQAFL
✓	9	402.2363	802.4580	802.3457	140	0	21	20	1	LNAANDAD
✓	669	691.9034	2763.5845	2763.5332	18.6	0	21	39	1	ILMIGLVWRSMEHPGKLLFAPNLL + Oxidation (M)
✓	248	607.1000	1212.1854	1212.5267	-281.44	0	21	4.7	1	ASKLHSNMMY + 2 Oxidation (M)
✓	363	692.8567	1383.6989	1383.7187	-14.28	0	21	22	1	DLLWVTWIGGQP
✓	692	791.7172	3162.8396	3162.5873	79.8	0	21	51	1	MGKRSIEDRVQEEARCLVEELRKTNAS + Oxidation (M)

✓	125	474.3428	946.6710	946.5157	164	0	21	20	1	KLDMLVASA
✓	285	625.4000	1248.7854	1248.7230	50.0	0	21	21	1	VNGKLFLPKYA
✓	519	607.4000	1819.1782	1818.8202	197	0	21	24	1	MAEYTNIIMMNALTAT + 2 Oxidation (M)
✓	85	448.3215	894.6284	894.3906	266	0	21	17	1	GWTTGGAMV + Oxidation (M)
✓	218	575.4000	1148.7854	1148.5900	170	0	21	21	1	PLMFAASIANV + Oxidation (M)
✓	357	688.3819	1374.7493	1374.6099	101	0	21	23	1	RNSGNHCGIASYP
✓	670	929.1204	2784.3395	2784.3063	11.9	0	21	32	1	KGEQGEAGQKGDAGAPGPQGPSGAPGPQGPT
✓	618	754.4544	2260.3414	2260.0324	137	0	21	28	1	YNAITLPEEFHDFDQPLPD
✓	243	604.8393	1207.6640	1207.5431	100	0	21	21	1	MTEEFPTLPQ + Oxidation (M)
✓	588	519.1344	2072.5085	2071.9024	293	0	21	43	1	AHSDWGYDSPNGPQEWVK
✓	233	594.4706	1186.9267	1186.7074	185	0	21	15	1	PLPPVAPEVLR
✓	642	607.1000	2424.3709	2424.0948	114	0	21	29	1	NLLLYILMTTSMFMMFMYN + 3 Oxidation (M)
✓	572	667.3490	1999.0250	1999.0197	2.67	0	21	26	1	GPEGIGKPGAPGIPGQPGIPGM + Oxidation (M)
✓	495	883.4756	1764.9367	1764.9662	-16.71	0	21	25	1	VENGSIIFSLSGVAFLL
✓	120	468.3008	934.5870	934.4872	107	0	20	23	1	ASAFGKQLN
✓	492	586.3131	1755.9176	1756.0822	-93.75	0	20	25	1	RSSEKSLALLKTVIIV
✓	466	853.6761	1705.3377	1704.9370	235	0	20	28	1	KTTKTVEQETRKEK
✓	32	416.3038	830.5931	830.4286	198	0	20	23	1	AGYAHVLT
✓	321	445.3000	1332.8782	1332.6959	137	0	20	25	1	VVITGMSGKLPES + Oxidation (M)
✓	131	476.2794	950.5442	950.4822	65.3	0	20	23	1	TGPQGPQGIP
✓	383	484.8002	1451.3788	1451.7330	-244.02	0	20	22	1	EPGLPVVAPMLDSQ
✓	695	1080.6324	3238.8753	3239.2909	-128.28	0	20	63	1	QESNKMHSMNGFMYGNQPGLSMCQGDSVM + 2 Oxidation (M)
✓	216	570.7976	1139.5806	1139.4490	115	0	20	24	1	MMPMTFYFG + Oxidation (M)
✓	141	485.3294	968.6442	968.3766	276	0	20	21	1	MQGMPMLN + 3 Oxidation (M)
✓	677	962.4592	2884.3557	2884.5626	-71.72	0	20	43	1	PIKNGKKNKVIGVCQLVNKMEENTGK + Oxidation (M)
✓	126	474.3469	946.6793	946.5276	160	0	20	23	1	FWILAANL
✓	83	446.2748	890.5350	890.4345	113	0	20	26	1	ATLASPGSTS
✓	497	886.9516	1771.8886	1771.8345	30.5	0	20	27	1	SGPAGQELGPGEKRACCI
✓	345	680.5464	1359.0782	1358.7632	232	0	20	16	1	MNPLLILAFLGAA + Oxidation (M)
✓	51	426.2350	850.4555	850.3531	120	0	20	23	1	MYSPQPE
✓	395	495.5843	1483.7310	1483.7031	18.9	0	20	27	1	WMHQPHQPLPPT + Oxidation (M)
✓	490	585.3011	1752.8815	1752.8909	-5.35	0	20	28	1	IYVHYGFPNMSLTLV
✓	511	603.0306	1806.0699	1806.0516	10.2	0	20	29	1	SKVLPVPQKAVPYPQR
✓	177	526.3193	1050.6240	1050.5458	74.4	0	20	25	1	LNLAADLAHN
✓	477	575.4000	1723.1782	1722.9167	152	0	20	30	1	PLSPPPAGWFCVLAGVL
✓	114	464.3337	926.6528	926.5450	116	0	20	23	1	VKKPHRY
✓	659	644.0589	2572.2066	2572.3683	-62.86	0	20	36	1	KINQVFHGSCITEGNELTKTLIK
✓	74	439.2542	876.4939	876.4076	98.4	0	20	27	1	SVEVSLDE
✓	527	609.1000	1824.2782	1823.8189	252	0	20	33	1	FSSRYPFDSPQVMFT + Oxidation (M)
✓	523	607.7835	1820.3288	1820.8300	-275.27	0	20	39	1	MATTWGAAFFMLVASCV + Oxidation (M)
✓	139	483.3308	964.6470	964.5997	49.1	0	20	26	1	YLAIFILL
✓	280	624.8117	1247.6088	1247.6034	4.30	0	20	28	1	VLDLFANDPTGS
✓	636	598.5948	2390.3502	2390.1345	90.2	0	20	36	1	VQISLPSTMSMTTSDGTQYLAK + 2 Oxidation (M)
✓	623	570.3343	2277.3083	2277.3096	-0.58	0	20	35	1	ALALALALGPAATLAGPAKSPYQL
✓	144	487.3551	972.6956	972.4851	216	0	20	27	1	MHKAGFGPL + Oxidation (M)

✓	137	482.3559	962.6972	962.5073	197	0	19	27	1	LDPKFESK
✓	208	564.3206	1126.6266	1126.6962	-61.76	0	19	29	1	IDLSLLGAKVV
✓	484	580.3134	1737.9185	1738.0254	-61.50	0	19	33	1	LLVVHEHRGTPVGLIV
✓	84	446.3000	890.5854	890.4610	140	0	19	31	1	AVSFPGRAS
✓	90	450.8508	899.6871	899.4600	252	0	19	21	1	ATDLPDVLG
✓	654	625.4000	2497.5709	2498.0995	-211.60	0	19	63	1	FSDVDLIPMNDHNTYRCFSQP
✓	106	460.3023	918.5900	918.4779	122	0	19	32	1	AACLCLVAR
✓	50	425.2610	848.5075	848.4392	80.5	0	19	30	1	APAPAASPPA
✓	596	703.4196	2107.2370	2107.0918	68.9	0	19	35	1	MRHALLQEVDIVVAPCQGL + Oxidation (M)
✓	262	609.3704	1216.7263	1216.5045	182	0	19	33	1	FSPNFMWMAA + Oxidation (M)
✓	166	511.3142	1020.6138	1020.4409	169	0	19	31	1	KCGIQMYY + Oxidation (M)
✓	278	624.4000	1246.7854	1246.5902	157	0	19	31	1	NQLREERTDS
✓	593	526.3127	2101.2219	2101.1432	37.4	0	19	37	1	GPDAKLRGALSWPSLAAAIHA
✓	423	785.4293	1568.8441	1568.8497	-3.58	0	19	34	1	PSKAKIKSHPQCVF
✓	599	708.7688	2123.2845	2123.1561	60.4	0	19	39	1	QTFGGKKFKAVVNMEGGKVV
✓	452	415.0367	1656.1177	1655.6670	272	0	19	34	1	STGDCPFIVCMSYAF + Oxidation (M)
✓	603	715.7393	2144.1961	2144.0473	69.4	0	19	38	1	NIYRYDERKETWCLAGK
✓	633	779.0134	2334.0183	2334.0434	-10.74	0	19	43	1	TLEGFADPVTGIADASQSSMHNA + Oxidation (M)
✓	508	900.4496	1798.8846	1799.0529	-93.60	0	19	37	1	TKNSRPKVASRFPKLA
✓	215	570.3140	1138.6135	1138.5580	48.7	0	19	34	1	IVYMLQEID + Oxidation (M)
✓	122	470.3317	938.6488	938.4201	244	0	19	29	1	LMIMASQN + 2 Oxidation (M)
✓	306	430.8881	1289.6424	1289.6112	24.1	0	19	36	1	APGESEFRNGRA
✓	449	552.2730	1653.7972	1653.6577	84.3	0	19	37	1	DGAPAAAGDGPGPGEPSEC
✓	501	445.3000	1777.1709	1776.8545	178	0	19	39	1	AFLNTAVHVVMYSYY
✓	169	515.4037	1028.7928	1028.5688	218	0	19	26	1	KLAKAICADP
✓	530	915.0135	1828.0125	1827.9124	54.8	0	19	38	1	YPVVVKMGHAHSGMGKV + 2 Oxidation (M)
✓	693	795.7122	3178.8196	3179.5301	-223.45	0	19	89	1	SEFSMNSKEALGGGKFGAVCTCTEKSTGLKL
✓	304	430.8861	1289.6365	1289.6980	-47.66	0	19	37	1	RIFGETIDIAVG
✓	532	612.3257	1833.9553	1834.0618	-58.05	0	19	40	1	WVVGRLFLHPKLQEL
✓	471	859.5208	1717.0270	1716.9927	20.0	0	18	40	1	QEPVLGPVRGPFPIIV
✓	150	490.2874	978.5602	978.4869	74.9	0	18	35	1	KTTEETASL
✓	347	681.8525	1361.6904	1361.5809	80.4	0	18	38	1	LFPTTGPGCEDEP
✓	408	757.8856	1513.7566	1513.7201	24.1	0	18	39	1	SGGKFTEEWKAQF
✓	551	626.2975	1875.8707	1875.8388	17.0	0	18	41	1	GDPDNYTPANPLNTPPHG
✓	36	419.2874	836.5602	836.4136	175	0	18	32	1	CLSLTMVA
✓	505	595.9616	1784.8628	1784.9282	-36.61	0	18	40	1	PAGPPGLRGGPGSRGLPGAD
✓	310	432.8894	1295.6464	1295.6074	30.1	0	18	38	1	GCAKATRSRMES
✓	424	524.2487	1569.7243	1569.6949	18.7	0	18	39	1	QRMLAEMQSAMNL + 3 Oxidation (M)
✓	475	431.0687	1720.2459	1719.9230	188	0	18	43	1	HVIVGTLIPLVDGQME
✓	328	672.5374	1343.0602	1342.7220	252	0	18	25	1	ILNRWGPLMPF
✓	118	467.3350	932.6555	932.4087	265	0	18	38	1	ALPGEDSSAS
✓	11	402.2837	802.5528	802.4371	144	0	18	39	1	AAGKAMGVV
✓	160	503.3686	1004.7227	1004.4961	226	0	18	36	1	TKSMQTVPN
✓	702	1009.3451	4033.3513	4033.1129	59.1	0	18	1.3e+002	1	QSSGTWTSLSKIIALCNRAEFKPGEESVPIMKRVVVG + Oxidation (M)
✓	500	445.1204	1776.4527	1776.8547	-226.28	0	18	55	1	PPCPPKPCVKSCPPKCP

✓	121	469.2757	936.5369	936.4400	103	0	18	38	1	TDSSIGLSAS
✓	211	566.4327	1130.8508	1130.6812	150	0	18	36	1	QVVKPHTPLI
✓	227	581.8139	1161.6132	1161.6315	-15.72	0	18	42	1	DILGALIMIST + Oxidation (M)
✓	234	595.2897	1188.5649	1188.6060	-34.59	0	18	44	1	LGPKELSCTEL
✓	638	803.6948	2408.0626	2408.3786	-131.22	0	18	55	1	LSPELNGPMVTMALAVVLLALLK + Oxidation (M)
✓	566	485.4000	1937.5709	1937.9413	-191.13	0	18	67	1	MVALNFQTVDLAMQINM
✓	266	613.3257	1224.6368	1224.6026	27.9	0	18	40	1	VPASVNLSEYF
✓	645	609.1000	2432.3709	2433.0553	-281.30	0	18	58	1	EYAAGPFAMFFMAEYANIIMM + Oxidation (M)
✓	298	638.8431	1275.6716	1275.6612	8.20	0	18	46	1	KTVGYPQGVAAW
✓	570	664.0637	1989.1693	1989.0183	75.9	0	18	49	1	LLPDFCHKFLPGYVGGVQ
✓	485	581.0110	1740.0111	1739.8288	105	0	18	48	1	KEPVLENSTMVVYTD + Oxidation (M)
✓	47	424.8164	847.6182	847.4287	224	0	18	40	1	ALLSDDSK
✓	181	535.3074	1068.6002	1068.6179	-16.53	0	18	45	1	QPAILSQTIV
✓	481	865.5079	1729.0012	1728.9960	2.99	0	18	49	1	LEANRYCALLLPLLK
✓	509	601.2942	1800.8608	1800.8321	16.0	0	18	51	1	NCLMRAIEIYTDMGR + Oxidation (M)
✓	302	429.1000	1284.2782	1284.5802	-235.13	0	18	26	1	MTAVSSQMATRA + 2 Oxidation (M)
✓	276	624.4000	1246.7854	1246.5944	153	0	18	46	1	CLFFFLDETL
✓	28	414.7612	827.5077	827.3484	193	0	18	43	1	YVCGKGDS
✓	175	523.3848	1044.7551	1044.5927	155	0	18	44	1	SSRKSPASLL
✓	617	754.1427	2259.4061	2259.0664	150	0	17	70	1	SMQELPPPVHLSKPGEHMDV + 2 Oxidation (M)
✓	369	704.2980	1406.5815	1406.8061	-159.68	0	17	49	1	TFLLFDLEIAIL
✓	690	786.3761	3141.4752	3141.6564	-57.68	0	17	1.1e+002	1	VSLKVPSPRSSASHKSLSLQSRCSVSKASE
✓	621	757.7814	2270.3224	2270.0573	117	0	17	60	1	QFDAKGGGPGPMGLMGPRGPPGAS + 2 Oxidation (M)
✓	309	432.8874	1295.6404	1295.5187	94.0	0	17	47	1	ENVSDLSMEEQ + Oxidation (M)
✓	23	412.2738	822.5331	822.4599	89.0	0	17	45	1	KISYGKGA
✓	136	482.2333	962.4521	962.4168	36.7	0	17	48	1	VEMHPAYT + Oxidation (M)
✓	44	422.6782	843.3419	843.4888	-174.16	0	17	52	1	LMLKPLN + Oxidation (M)
✓	541	624.1000	1869.2782	1868.9012	202	0	17	59	1	CIMQLFGKKVDDGSELS
✓	453	554.4000	1660.1782	1659.7787	241	0	17	56	1	HQGIKGMVRDENYN
✓	457	559.5000	1675.4782	1675.7849	-183.03	0	17	55	1	KTFGGGGGGARSNLNMH + Oxidation (M)
✓	288	628.3873	1254.7601	1254.6754	67.5	0	17	49	1	KKSIHVSCPKE
✓	554	631.6355	1891.8847	1891.9179	-17.53	0	17	57	1	FVMGFVGFSSKPSPIYGG + Oxidation (M)
✓	691	789.4430	3153.7429	3153.6607	26.1	0	17	1.2e+002	1	KNGVITGVYPASPSSWLIVVVGVMSTMYAK
✓	464	846.4114	1690.8082	1690.8811	-43.10	0	17	55	1	QKALEKGIEILTDMS + Oxidation (M)
✓	20	409.2561	816.4976	816.3800	144	0	17	53	1	CAPRLEE
✓	584	683.0412	2046.1017	2045.9768	61.0	0	17	59	1	NVEPLPDFSQYIEMHIV + Oxidation (M)
✓	480	432.4000	1725.5709	1725.6862	-66.80	0	17	57	1	NPTDEYLDAMMNEAP + Oxidation (M)
✓	344	680.3441	1358.6735	1358.7843	-81.52	0	17	54	1	GIVVLTMKASVIE
✓	76	439.2740	876.5335	876.3461	214	0	17	53	1	DDDAGGTLN
✓	303	429.2696	1284.7869	1284.5698	169	0	17	57	1	INMIMKQMMN + 2 Oxidation (M)
✓	665	663.6347	2650.5098	2650.4669	16.2	0	17	94	1	KVLPVPQKAVPYPQRDMPIQAFL + Oxidation (M)
✓	232	590.7515	1179.4885	1179.4866	1.55	0	17	57	1	WASGCPSGTLSD
✓	281	624.8117	1247.6088	1247.5930	12.6	0	17	58	1	AGSFLLALYMM + 2 Oxidation (M)
✓	322	445.8332	1334.4779	1334.7459	-200.78	0	17	58	1	HKIVLEWASPR
✓	607	729.9974	2186.9704	2187.0444	-33.85	0	17	66	1	VVLGDHNLSQNDGTEQYISV

✓	182	540.3994	1078.7843	1078.5295	236	0	17	51	1	TSIPSAYGGQV
✓	684	761.1287	3040.4858	3040.5474	-20.25	0	17	1.1e+002	1	NSGPVVAMVWEGLNVVKTGRVMLGETNPA + Oxidation (M)
✓	275	624.1000	1246.1854	1246.5322	-278.16	0	16	9.8	1	SKLNSNMMYQ + 2 Oxidation (M)
✓	34	417.2791	832.5437	832.4728	85.2	0	16	59	1	SLMSGLIL
✓	272	619.8747	1237.7348	1237.6927	34.1	0	16	60	1	VLMIMPLLHTA
✓	279	624.8093	1247.6040	1247.5930	8.84	0	16	61	1	AGSFLLALYMM + 2 Oxidation (M)
✓	421	781.8675	1561.7205	1561.8239	-66.19	0	16	66	1	EPPLELTIEPPLSQ
✓	468	429.0854	1712.3123	1712.8046	-287.42	0	16	71	1	FQLFSTEGSPATWDK
✓	82	445.3000	888.5854	888.5029	92.9	0	16	67	1	ATLTGLTRG
✓	382	725.3913	1448.7680	1448.7446	16.2	0	16	64	1	MLAPLQTGAARFSS
✓	342	679.5177	1357.0209	1356.6166	298	0	16	53	1	PRPCTPAMTPSPS + Oxidation (M)
✓	138	482.8650	963.7155	963.4807	244	0	16	50	1	CEARSALSK
✓	282	624.8164	1247.6182	1247.5969	17.1	0	16	63	1	THMGSLFGAAGLS
✓	609	554.4000	2213.5709	2214.2273	-296.44	0	16	1.3e+002	1	WSYGAGIVLRLGNIARLELN
✓	486	582.3388	1743.9947	1743.9804	8.15	0	16	67	1	ALAIAAGQSVVSIATLLM + Oxidation (M)
✓	496	590.9861	1769.9363	1769.8988	21.2	0	16	67	1	PEYAYGLAGSPPKIPPN
✓	129	475.1941	948.3736	948.4335	-63.11	0	16	62	1	LSNAGAVMGN + Oxidation (M)
✓	259	609.1000	1216.1854	1216.5401	-291.50	0	16	11	1	DGQSGFFPLSY
✓	65	432.2480	862.4814	862.4153	76.7	0	16	66	1	ACRILACN
✓	101	455.3129	908.6113	908.4644	162	0	16	58	1	APEFSIFV
✓	46	424.2998	846.5851	846.3832	239	0	16	66	1	NTTGNGANV
✓	110	463.2091	924.4037	924.4705	-72.27	0	16	60	1	PYGLSFNK
✓	301	428.8875	1283.6406	1283.5894	39.8	0	16	71	1	AGNYYNQGEIR
✓	226	581.8002	1161.5859	1161.6144	-24.57	0	16	70	1	YFSIFIILM + Oxidation (M)
✓	701	935.2985	3737.1649	3737.9383	-206.90	0	16	2e+002	1	PILIIIMATIMTGTMIVMLSSHWLLIWIGFEM + 4 Oxidation (M)
✓	258	608.8031	1215.5917	1215.7339	-117.00	0	16	75	1	KQALWLTKTK
✓	55	428.2627	854.5109	854.4610	58.4	0	16	62	1	PAPSSLQR
✓	64	432.2382	862.4618	862.3789	96.1	0	16	71	1	PNCDMKR
✓	277	624.4000	1246.7854	1246.5944	153	0	16	70	1	CLFFFLDETL
✓	647	1241.7121	2481.4096	2480.9889	170	0	16	1e+002	1	YNGEQAPYASCHMFELYQEDS
✓	589	519.1473	2072.5602	2072.0699	237	0	16	1.5e+002	1	DKKCPGSLHIIKWIQGCF
✓	31	416.2923	830.5700	830.4498	145	0	16	73	1	APTGSGKTL
✓	465	851.0055	1699.9964	1699.9695	15.8	0	16	77	1	PISMVLPQVIGYRLV + Oxidation (M)
✓	370	705.3629	1408.7112	1408.7059	3.74	0	15	74	1	RPGPVGGPGSSGAKGE
✓	73	438.3149	874.6153	874.4661	171	0	15	75	1	AAPSFRQV
✓	124	472.3217	942.6289	942.5022	134	0	15	74	1	VSISELAQP
✓	33	416.3077	830.6008	830.4610	168	0	15	69	1	KAAQAGSAK
✓	61	431.2357	860.4569	860.3876	80.5	0	15	80	1	ASGESPKEG
✓	143	487.2966	972.5787	972.5280	52.1	0	15	76	1	KPLYTSAPP
✓	611	746.9742	2237.9007	2238.1467	-109.91	0	15	99	1	EPQNSLPDIVIWMLQGDKR
✓	42	421.2325	840.4504	840.3721	93.1	0	15	66	1	VGTMASLM + 2 Oxidation (M)
✓	245	607.0351	1212.0557	1211.7067	288	0	15	13	1	VGFHLLAFVPL
✓	49	425.2454	848.4763	848.4141	73.4	0	15	75	1	SLHGGGAPGP
✓	531	611.3476	1831.0210	1830.8458	95.7	0	15	87	1	ILFPTTGPGCEDEPEAR
✓	13	403.2329	804.4513	804.4341	21.3	0	15	78	1	RSLTLDT

✓	72	436.7774	871.5403	871.5167	27.1	0	15	78	1	IILTWIGG
✓	127	474.3490	946.6834	946.4066	293	0	15	72	1	SEEGCIPNV
✓	491	585.9948	1754.9625	1754.9349	15.7	0	15	85	1	GAQVCELAQALRDGVLL
✓	254	607.7859	1213.5572	1213.5842	-22.27	0	15	80	1	LFCLGFGFDVP
✓	545	624.7975	1871.3706	1871.9062	-286.16	0	15	1.2e+002	1	LYNRIGDVGFIMAMAW + Oxidation (M)
✓	598	707.0874	2118.2405	2118.0656	82.6	0	15	90	1	GFHVCAAGWMAKGRVGYPIV
✓	332	450.8508	1349.5306	1349.5349	-3.12	0	15	83	1	MMASQNLMCGHQ
✓	77	439.2859	876.5573	876.4011	178	0	15	82	1	VAMVGDGVN + Oxidation (M)
✓	246	607.0398	1212.0650	1211.7278	278	0	15	12	1	KTPLLALALWS
✓	529	914.4213	1826.8280	1826.8621	-18.66	0	15	91	1	HCLEKVDVFEEQHKS
✓	149	489.3599	976.7052	976.4899	220	0	15	75	1	LDKMLQDV + Oxidation (M)
✓	414	514.9506	1541.8301	1541.8395	-6.08	0	15	91	1	NPPFYGFLGKKFK
✓	300	641.8857	1281.7568	1281.5837	135	0	15	83	1	YGKNTLSSDPSN
✓	622	759.4385	2275.2937	2275.2578	15.8	0	15	1.1e+002	1	KMLSKMVNNKDLKLPRMLT + Oxidation (M)
✓	635	594.5906	2374.3332	2374.1084	94.7	0	15	1.1e+002	1	SFGSRMPGTSGRQRATPDAPPAD + Oxidation (M)
✓	664	880.7250	2639.1533	2639.2762	-46.57	0	15	1.5e+002	1	LHSSGNNPAFSAAVNNPKMPVSQKE + Oxidation (M)
✓	574	669.4131	2005.2175	2005.0199	98.6	0	15	98	1	KTLKICANHYITPMMEL
✓	331	675.3443	1348.6741	1348.7094	-26.18	0	15	87	1	KTCLMLLPSVQT + Oxidation (M)
✓	577	672.4000	2014.1781	2014.0405	68.3	0	15	97	1	QLCGRDPTLTDELLNILT
✓	17	405.2395	808.4644	808.3967	83.8	0	15	74	1	INIESFS
✓	694	806.3816	3221.4971	3222.4074	-282.50	0	15	2.1e+002	1	DNYDEVIDLSDYEGLMDYGDQLPEAKVT + Oxidation (M)
✓	4	597.3249	596.3176	596.2298	147	0	15	52	1	CALMGS + Oxidation (M)
✓	526	609.1000	1824.2782	1823.9828	162	0	15	1.1e+002	1	NKKRIERFYNCLQL
✓	39	420.2584	838.5022	838.4912	13.1	0	15	74	1	HISEKII
✓	375	711.3652	1420.7158	1420.7708	-38.73	0	15	91	1	MRSTVKSSVSLGGI
✓	92	452.3364	902.6583	902.4280	255	0	15	90	1	KSVAMHTN + Oxidation (M)
✓	661	650.1000	2596.3709	2596.1537	83.6	0	14	1.2e+002	1	LTGLLMYRSHMMSSLLCLEGMM + 5 Oxidation (M)
✓	161	504.3220	1006.6295	1006.4719	157	0	14	92	1	NINYQLEN
✓	89	450.8488	899.6831	899.4600	248	0	14	70	1	ATDLPDVLG
✓	96	453.2805	904.5465	904.5204	28.8	0	14	93	1	RPFMLLL + Oxidation (M)
✓	631	773.6785	2318.0138	2318.2416	-98.27	0	14	1.2e+002	1	GTLTVRENLQFSAALRLPTTM
✓	100	453.3470	904.6794	904.4324	273	0	14	75	1	PKVTSGMDA
✓	343	679.7520	1357.4894	1357.6038	-84.24	0	14	1e+002	1	VVYEAQDVYTGD
✓	506	895.5057	1788.9969	1788.7303	149	0	14	1.1e+002	1	MSCTCLGNGKGEFKCDP
✓	104	458.7935	915.5724	915.4087	179	0	14	1e+002	1	HQSPVFDS
✓	601	711.7714	2132.2925	2132.2457	22.0	0	14	1.3e+002	1	VPALPSSLVSLSLSHTSILVL
✓	133	477.2833	952.5520	952.4688	87.4	0	14	90	1	MNSLSLFAA
✓	78	441.2669	880.5192	880.3055	243	0	14	92	1	QCENGECV
✓	152	492.3402	982.6658	982.4066	264	0	14	89	1	FETMENVN
✓	699	835.1460	3336.5548	3336.6698	-34.48	0	14	2.7e+002	1	KVSGEVHTARPLQGARPGDSYTVLVEAQDADA
✓	184	545.3211	1088.6277	1088.6376	-9.07	0	14	1.1e+002	1	LEKAMKKKGG
✓	305	430.8861	1289.6365	1289.5823	42.1	0	14	1.1e+002	1	AGHTAPMRPSYS + Oxidation (M)
✓	339	679.4043	1356.7940	1356.6885	77.8	0	14	1.1e+002	1	KGPNGELTIDTVN
✓	25	413.2040	824.3935	824.3698	28.7	0	14	87	1	TMSLDRS + Oxidation (M)
✓	338	678.3888	1354.7630	1354.6803	61.1	0	14	1.1e+002	1	SQMPTVAPEPVAL + Oxidation (M)

✓	252	607.7695	1213.5245	1213.6165	-75.80	0	14	1.2e+002	1	LIQSFQKMGY
✓	69	434.2801	866.5456	866.3658	208	0	14	96	1	SEPSFEAT
✓	194	553.3985	1104.7824	1104.6192	148	0	14	1.1e+002	1	ALWIGHGRVP
✓	559	640.3527	1918.0363	1917.8745	84.4	0	14	1.3e+002	1	IEDFTAYGGVFGNKQDSA
✓	201	559.5000	1116.9854	1116.6767	276	0	14	11	1	LYLGRRQLV
✓	420	520.2774	1557.8103	1557.8110	-0.48	0	14	1.2e+002	1	EKRAETSRPEDIK
✓	206	562.4008	1122.7871	1122.5768	187	0	14	1.1e+002	1	SSVSISVISSSA
✓	549	625.4000	1873.1782	1872.8155	194	0	14	1.3e+002	1	RNNGFFSYHMPNWFG
✓	176	524.8250	1047.6355	1047.4720	156	0	13	1.2e+002	1	QVELEGESSA
✓	71	435.2683	868.5220	868.4364	98.5	0	13	1e+002	1	LMTYVGAV + Oxidation (M)
✓	341	453.3000	1356.8782	1356.6422	174	0	13	1.2e+002	1	QGATPLHYAAQSN
✓	99	453.3208	904.6270	904.4515	194	0	13	1.1e+002	1	APAPAEHGR
✓	18	406.2246	810.4347	810.5215	-107.10	0	13	92	1	PTLAVVVL
✓	286	625.4000	1248.7854	1248.5710	172	0	13	1.2e+002	1	HTGWAGKFCDK
✓	504	446.3000	1781.1709	1780.7873	215	0	13	1.4e+002	1	QKMVDDGSGEVQMWR + Oxidation (M)
✓	229	584.4203	1166.8261	1166.5311	253	0	13	1.1e+002	1	MASMIENNLL + 2 Oxidation (M)
✓	687	767.4367	3065.7176	3066.4718	-245.96	0	13	2.8e+002	1	SLGDPDKNCSRAASVMINCLLKERGNML + 2 Oxidation (M)
✓	324	446.3000	1335.8782	1335.6228	191	0	13	1.2e+002	1	MSIPSTTSSLPSE
✓	88	450.2806	898.5466	898.3161	257	0	13	1.2e+002	1	AACTEASCSG
✓	600	711.0801	2130.2184	2130.1433	35.3	0	13	1.5e+002	1	VKLTQAAVETHLQHLGLSGE
✓	93	453.2443	904.4740	904.4389	38.7	0	13	1.3e+002	1	EDVETIIS
✓	155	496.3596	990.7046	990.5175	189	0	13	1.3e+002	1	LFTPTWIGG
✓	236	597.4197	1192.8248	1192.5580	224	0	13	1.3e+002	1	IVSGGAGCGMVLN + Oxidation (M)
✓	107	461.3034	920.5922	920.4392	166	0	13	1.3e+002	1	FPSNISAW
✓	117	467.3023	932.5900	932.4459	155	0	13	1.4e+002	1	NPMMTLLN
✓	335	677.3839	1352.7532	1352.7160	27.5	0	13	1.3e+002	1	AHVVVSSRKQEN
✓	53	427.2395	852.4644	852.4085	65.6	0	13	1.2e+002	1	SIMGGMII + 2 Oxidation (M)
✓	512	453.3000	1809.1709	1809.0658	58.1	0	13	1.5e+002	1	MLGSLVSKRTAPAPRLL
✓	685	762.8836	3047.5051	3046.6311	287	0	13	2.5e+002	1	YDAKKKAAHAAKKVKHGAGAEISTVNPEQ
✓	312	650.3980	1298.7815	1298.6830	75.8	0	13	1.4e+002	1	NDLLKLNNELN
✓	267	613.8388	1225.6631	1225.6303	26.8	0	13	1.4e+002	1	GGVGIIEESKHT
✓	476	861.9881	1721.9617	1721.9927	-18.03	0	13	1.5e+002	1	QLLLTPNAVVIVEDAK
✓	70	435.2624	868.5102	868.3974	130	0	13	1.3e+002	1	RSSCFGGR
✓	97	453.2966	904.5787	904.4290	165	0	13	1.4e+002	1	ALSQDFAGP
✓	111	463.3069	924.5992	924.4408	171	0	13	1.3e+002	1	SSLAAKMMS
✓	8	401.2579	800.5012	800.3664	168	0	12	1.4e+002	1	HQSSLTE
✓	35	419.2448	836.4750	836.4504	29.3	0	12	1.3e+002	1	NKAAHPLS
✓	260	609.1000	1216.1854	1216.5376	-289.43	0	12	25	1	VGAAAWWFMY + Oxidation (M)
✓	196	554.4000	1106.7854	1106.6409	131	0	12	1.4e+002	1	ILMLGTFLIA + Oxidation (M)
✓	112	463.3130	924.6114	924.3971	232	0	12	1.3e+002	1	GTSDSIRSC
✓	109	462.3544	922.6943	922.4430	272	0	12	1.1e+002	1	NTTMTILN + Oxidation (M)
✓	134	480.8052	959.5958	959.4131	190	0	12	1.8e+002	1	LTHGNSAMN + Oxidation (M)
✓	22	409.2714	816.5282	816.3865	174	0	12	1.8e+002	1	ALSLDPDS
✓	79	442.7964	883.5782	883.3415	268	0	12	1.5e+002	1	ELQSMCAS + Oxidation (M)
✓	696	819.1378	3272.5221	3272.6360	-34.81	0	12	4.4e+002	1	RKVVGEFRDALRNSGGDGLGQMSLEFYQK + Oxidation (M)

✓	616	752.4342	2254.2809	2254.2910	-4.45	0	12	2.2e+002	1	KSILSRHLNTQVKKTTSKW
✓	265	611.3593	1220.7041	1220.6626	34.0	0	12	1.8e+002	1	DLRKRSNSVF
✓	518	607.3149	1818.9227	1818.9550	-17.71	0	12	2e+002	1	IDMVGGDIFLEALRSLA
✓	98	453.3000	904.5854	904.4324	169	0	11	1.8e+002	1	MLQAGALDS
✓	24	412.7965	823.5784	823.4262	185	0	11	1.5e+002	1	YLKGMAAA
✓	237	597.8637	1193.7128	1193.5784	113	0	11	2e+002	1	KNLDVMKEAM + Oxidation (M)
✓	308	432.4000	1294.1782	1294.5499	-287.18	0	11	33	1	QESSEYIISCH
✓	419	520.2752	1557.8038	1557.8767	-46.76	0	11	2.2e+002	1	VGAALTNVLSVFGLPT
✓	30	415.5977	829.1808	829.4082	-274.17	0	11	1.1e+002	1	AAQHSVFA
✓	59	430.8861	859.7577	859.5015	298	0	11	26	1	ATKDSLII
✓	81	445.2901	888.5657	888.3825	206	0	11	2.4e+002	1	GKSSSYSSS
✓	63	431.3025	860.5904	860.3738	252	0	11	2.4e+002	1	MPYPSPGP + Oxidation (M)
✓	567	650.1000	1947.2782	1947.8416	-289.28	0	11	2.7e+002	1	MDISCVYSGSYPMAITPN
✓	94	453.2563	904.4981	904.4477	55.8	0	11	2.2e+002	1	ILLGWGGCS
✓	40	420.6793	839.3441	839.3814	-44.42	0	11	1.8e+002	1	AFGSFSQP
✓	289	628.4347	1254.8549	1254.6828	137	0	11	2.2e+002	1	IIAAMVMQHLL + Oxidation (M)
✓	62	431.2691	860.5237	860.4174	123	0	10	2.5e+002	1	APSRNGMI + Oxidation (M)
✓	148	489.2762	976.5378	976.4138	127	0	10	2.3e+002	1	SGEPGSPYSP
✓	235	595.3243	1188.6341	1188.6754	-34.72	0	10	2.6e+002	1	LAAELPKVSYV
✓	95	453.2725	904.5304	904.4250	116	0	10	2.4e+002	1	ATAATAAGGTGG
✓	27	414.1815	826.3484	826.4007	-63.34	0	10	2.3e+002	1	KGFMSNK + Oxidation (M)
✓	368	701.3061	1400.5977	1400.5766	15.1	0	10	2.7e+002	1	PPDTEAVDCKDPD
✓	388	735.4131	1468.8117	1468.7231	60.3	0	10	2.7e+002	1	KEVYRLEEMEK + Oxidation (M)
✓	58	429.1000	856.1854	856.3927	-241.95	0	10	1.3e+002	1	APTAASDQP
✓	180	532.3960	1062.7775	1062.5305	232	0	10	2.3e+002	1	AADDSKRTVT
✓	189	550.2984	1098.5823	1098.4726	99.9	0	10	2.8e+002	1	SEFQPVMVM + 2 Oxidation (M)
✓	415	771.9297	1541.8449	1541.7595	55.4	0	10	3e+002	1	GGNMKEVFRRFCV
✓	153	495.8096	989.6047	989.3913	216	0	10	3e+002	1	PGQEMQWD
✓	565	645.3416	1933.0029	1932.8921	57.3	0	10	3.2e+002	1	TQLMQTTSSPGPPMVQNT + Oxidation (M)
✓	43	421.2577	840.5009	840.3721	153	0	9	2.6e+002	1	AMKMETV + 2 Oxidation (M)
✓	38	420.2448	838.4751	838.3353	167	0	9	2.5e+002	1	CFAGCLPE
✓	367	698.6532	1395.2918	1395.6518	-257.92	0	9	1.9e+002	1	ISNIQSYIGASED
✓	57	429.0873	856.1601	856.3749	-250.84	0	9	1.2e+002	1	CPSPGPAAAS
✓	66	432.4000	862.7854	862.5501	273	0	9	9.1	1	LRKYKR
✓	205	562.3492	1122.6839	1122.4724	188	0	9	3.4e+002	1	SAQQMSNERG + Oxidation (M)
✓	37	420.2371	838.4596	838.3933	79.0	0	9	2.9e+002	1	LSGSQHPN
✓	292	631.0175	1260.0205	1259.7098	247	0	9	1.8e+002	1	HVKPRAPQNLT
✓	231	589.3498	1176.6851	1176.6655	16.6	0	8	3.8e+002	1	VHLKKSGYVF
✓	602	1071.2088	2140.4030	2140.9407	-251.15	0	8	6e+002	1	MEFCMVYYALKEEEVEI + Oxidation (M)
✓	52	426.2468	850.4790	850.3742	123	0	8	3.8e+002	1	EMNELIS + Oxidation (M)
✓	67	432.8874	863.7603	863.5341	262	0	8	40	1	AHILALRA
✓	212	567.3359	1132.6572	1132.5295	113	0	8	4.6e+002	1	KSRMGPSGGEGA
✓	580	1009.5825	2017.1505	2016.9687	90.1	0	7	6.5e+002	1	DRTAGWNIPMGLIANQTGS + Oxidation (M)
✓	2	541.3961	540.3888	540.2292	295	0	6	1.7e+002	1	DVNHG
✓	41	420.7938	839.5731	839.4613	133	0	6	5.1e+002	1	LAGSHSLR

✓	311	650.1000	1298.1854	1298.5343	-268.66	0	5	1.4e+002	1	MASNRSGNSSCGL + Oxidation (M)
✓	6	400.1728	798.3310	798.3193	14.7	0	4	8.5e+002	1	CCFWLAG
✓	56	428.8953	855.7760	855.5430	272	0	3	1.4e+002	1	VPKVTVIT
✓	29	415.0386	828.0627	828.2993	-285.71	0	2	38	1	ASMISSDC + Oxidation (M)
✓	5	607.2123	606.2050	606.1890	26.4	0	1	2e+003	1	THCCSG
✓	1	*427.3060*	*426.2987*							
✓	170	*515.5000*	*1028.9854*							

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : None
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 300 ppm
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages    : 1
Instrument type         : ESI-QUAD-TOF
Number of queries       : 702
```

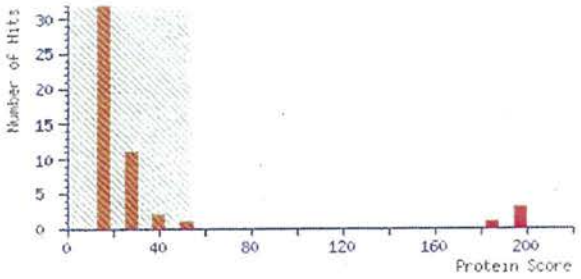
Mascot: http://www.matrixscience.com/

Mascot Search Results

```
User           :
Email          :
Search title   : Project: Users\2013.06, Spot Set: Users\2013.06\MF170613, Label: O4, Spot Id: 871775, Peak List Id: 666430, MS Job Run Id: 26909
MS data file   : \\apaf-hpv-file\projects\External\e_15328_VictoriaUni_FatahAhtesh_20130607\1_MassSpec\4800\Run1\RawData\Run1\MF170613\O4_MF170613.txt
Database       : SwissProt 2013 (539829 sequences; 191670831 residues)
Taxonomy       : Other mammalia (13034 sequences)
Timestamp      : 27 Jun 2013 at 05:44:48 GMT
Warning        : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score      : 197 for CASB_CAPHI, Beta-casein OS=Capra hircus GN=CSN2 PE=2 SV=1
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 54 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

Format As Protein Summary (deprecated) Help

Significance threshold p< 0.05 Max. number of hits AUTO

Re-Search All Search Unmatched

Index

	Accession	Mass	Score	Description
1.	CASB_CAPHI	24849	197	Beta-casein OS=Capra hircus GN=CSN2 PE=2 SV=1
2.	CASB_SHEEP	24859	197	Beta-casein OS=Ovis aries GN=CSN2 PE=1 SV=3
3.	CASB_BOVIN	25091	196	Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2
4.	CASB_BUBBU	25090	182	Beta-casein OS=Bubalus bubalis GN=CSN2 PE=2 SV=1
5.	ATP8_ORYAF	8057	56	ATP synthase protein 8 OS=Orycteropus afer GN=MT-ATP8 PE=3 SV=1
6.	ATP8_HALGR	7878	42	ATP synthase protein 8 OS=Halichoerus grypus GN=MT-ATP8 PE=3 SV=1

Results List

1. CASB_CAPHI Mass: 24849 Score: 197 Expect: 2.6e-016 Matches: 22

Beta-casein OS=Capra hircus GN=CSN2 PE=2 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6863	-25.34	212	222	0	---	L.GPVRGPFPILV.-
1151.6644	1150.6571	1150.6863	-25.34	212	222	0	29	L.GPVRGPFPILV.-
1363.8259	1362.8186	1362.8388	-14.78	210	222	0	---	P.VLGPVRGPFPILV.-
1375.7285	1374.7212	1374.6878	24.3	20	32	0	---	E.ELNVVGETVESLS.S
1375.7285	1374.7212	1374.6878	24.3	20	32	0	---	E.ELNVVGETVESLS.S
1589.9221	1588.9148	1588.9188	-2.53	173	187	0	---	F.PPQSVLSLSQPKVLP.V
1589.9221	1588.9148	1588.9341	-12.14	208	222	0	21	Q.EPVLGPVRGPFPILV.-
1601.9026	1600.8953	1600.8977	-1.49	202	215	0	---	Q.AFLLYQEPVLGPVR.G
1669.8888	1668.8815	1668.8682	7.97	12	26	0	---	A.LAIAREQEELNVVGE.T
1700.9590	1699.9517	1699.8790	42.8	113	126	0	---	K.VKETMVPKHKEMPF.P
1700.9590	1699.9517	1699.8790	42.8	113	126	0	---	K.VKETMVPKHKEMPF.P
1717.9808	1716.9735	1716.9927	-11.16	207	222	0	---	Y.QEPVLGPVRGPFPILV.-
1717.9808	1716.9735	1716.9927	-11.16	207	222	0	50	Y.QEPVLGPVRGPFPILV.-
1781.9735	1780.9662	1780.9876	-12.00	206	221	0	---	L.YQEPVLGPVRGPFPIL.V
1881.0486	1880.0413	1880.0560	-7.81	206	222	0	---	L.YQEPVLGPVRGPFPILV.-
1881.0486	1880.0413	1880.0560	-7.81	206	222	0	42	L.YQEPVLGPVRGPFPILV.-
1994.1243	1993.1170	1993.1401	-11.57	205	222	0	---	L.LYQEPVLGPVRGPFPILV.-
2107.2170	2106.2097	2106.2241	-6.84	204	222	0	---	F.LLYQEPVLGPVRGPFPILV.-
2107.2170	2106.2097	2106.2241	-6.84	204	222	0	41	F.LLYQEPVLGPVRGPFPILV.-
2254.2810	2253.2737	2253.2926	-8.36	203	222	0	---	A.FLLYQEPVLGPVRGPFPILV.-
2254.2810	2253.2737	2253.2926	-8.36	203	222	0	7	A.FLLYQEPVLGPVRGPFPILV.-
2938.5374	2937.5301	2937.5166	4.60	103	127	0	---	F.LQPEIMGVPKVKETMVPKHKEMPFP.K + 3 Oxidation (M)

No match to: 906.6738

2. CASB_SHEEP Mass: 24859 Score: 197 Expect: 2.6e-016 Matches: 22

Beta-casein OS=Ovis aries GN=CSN2 PE=1 SV=3

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6863	-25.34	212	222	0	---	L.GPVRGPFPILV.-
1151.6644	1150.6571	1150.6863	-25.34	212	222	0	29	L.GPVRGPFPILV.-
1363.8259	1362.8186	1362.8388	-14.78	210	222	0	---	P.VLGPVRGPFPILV.-
1375.7285	1374.7212	1374.6878	24.3	20	32	0	---	E.ELNVVGETVESLS.S
1375.7285	1374.7212	1374.6878	24.3	20	32	0	---	E.ELNVVGETVESLS.S
1589.9221	1588.9148	1588.9188	-2.53	173	187	0	---	F.PPQSVLSLSQPKVLP.V
1589.9221	1588.9148	1588.9341	-12.14	208	222	0	21	Q.EPVLGPVRGPFPILV.-
1601.9026	1600.8953	1600.8977	-1.49	202	215	0	---	Q.AFLLYQEPVLGPVR.G
1669.8888	1668.8815	1668.8682	7.97	12	26	0	---	A.LALAREQEELNVVGE.T
1700.9590	1699.9517	1699.8790	42.8	113	126	0	---	K.VKETMVPKHKEMPF.P
1700.9590	1699.9517	1699.8790	42.8	113	126	0	---	K.VKETMVPKHKEMPF.P
1717.9808	1716.9735	1716.9927	-11.16	207	222	0	---	Y.QEPVLGPVRGPFPILV.-
1717.9808	1716.9735	1716.9927	-11.16	207	222	0	50	Y.QEPVLGPVRGPFPILV.-
1781.9735	1780.9662	1780.9876	-12.00	206	221	0	---	L.YQEPVLGPVRGPFPIL.V

1881.0486	1880.0413	1880.0560	-7.81	206 - 222	0	---	L.YQEPVLGPVRGPFPILV.-
1881.0486	1880.0413	1880.0560	-7.81	206 - 222	0	42	L.YQEPVLGPVRGPFPILV.-
1994.1243	1993.1170	1993.1401	-11.57	205 - 222	0	---	L.LYQEPVLGPVRGPFPILV.-
2107.2170	2106.2097	2106.2241	-6.84	204 - 222	0	---	F.LLYQEPVLGPVRGPFPILV.-
2107.2170	2106.2097	2106.2241	-6.84	204 - 222	0	41	F.LLYQEPVLGPVRGPFPILV.-
2254.2810	2253.2737	2253.2926	-8.36	203 - 222	0	---	A.FLLYQEPVLGPVRGPFPILV.-
2254.2810	2253.2737	2253.2926	-8.36	203 - 222	0	7	A.FLLYQEPVLGPVRGPFPILV.-
2938.5374	2937.5301	2937.5166	4.60	103 - 127	0	---	F.LQPEIMGVPKVKETMVPKHKEMPFP.K + 3 Oxidation (M)

No match to: 906.6738

3. CASB_BOVIN Mass: 25091 Score: 196 Expect: 3.3e-016 Matches: 22

Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6863	-25.34	214 -	224	0	---	L.GPVRGPFPIIV.-
1151.6644	1150.6571	1150.6863	-25.34	214 -	224	0	29	L.GPVRGPFPIIV.-
1363.8259	1362.8186	1362.8388	-14.78	212 -	224	0	---	P.VLGPVRGPFPIIV.-
1375.7285	1374.7212	1374.7363	-10.97	108 -	120	0	---	V.MGVSKVKEAMAPK.H
1375.7285	1374.7212	1374.7363	-10.97	108 -	120	0	---	V.MGVSKVKEAMAPK.H
1589.9221	1588.9148	1588.9341	-12.14	210 -	224	0	---	Q.EPVLGPVRGPFPIIV.-
1589.9221	1588.9148	1588.9341	-12.14	210 -	224	0	21	Q.EPVLGPVRGPFPIIV.-
1601.9026	1600.8953	1600.8977	-1.49	204 -	217	0	---	Q.AFLLYQEPVLGPVR.G
1669.8888	1668.8815	1668.8458	21.4	17 -	31	0	---	R.ELEELNVPGEIVESL.S
1700.9590	1699.9517	1699.9297	12.9	77 -	92	0	---	P.FPGPIPNSLPQNIPPL.T
1700.9590	1699.9517	1699.9297	12.9	77 -	92	0	6	P.FPGPIPNSLPQNIPPL.T
1717.9808	1716.9735	1716.9927	-11.16	209 -	224	0	---	Y.QEPVLGPVRGPFPIIV.-
1717.9808	1716.9735	1716.9927	-11.16	209 -	224	0	50	Y.QEPVLGPVRGPFPIIV.-
1781.9735	1780.9662	1780.9876	-12.00	208 -	223	0	---	L.YQEPVLGPVRGPFPII.V
1881.0486	1880.0413	1880.0560	-7.81	208 -	224	0	---	L.YQEPVLGPVRGPFPIIV.-
1881.0486	1880.0413	1880.0560	-7.81	208 -	224	0	42	L.YQEPVLGPVRGPFPIIV.-
1994.1243	1993.1170	1993.1401	-11.57	207 -	224	0	---	L.LYQEPVLGPVRGPFPIIV.-
2107.2170	2106.2097	2106.2241	-6.84	206 -	224	0	---	F.LLYQEPVLGPVRGPFPIIV.-
2107.2170	2106.2097	2106.2241	-6.84	206 -	224	0	41	F.LLYQEPVLGPVRGPFPIIV.-
2254.2810	2253.2737	2253.2926	-8.36	205 -	224	0	---	A.FLLYQEPVLGPVRGPFPIIV.-
2254.2810	2253.2737	2253.2926	-8.36	205 -	224	0	7	A.FLLYQEPVLGPVRGPFPIIV.-
2938.5374	2937.5301	2937.5294	0.23	25 -	50	0	---	P.GEIVESLSSSEESITRINKKIEKFQS.E

No match to: 906.6738

4. CASB_BUBBU Mass: 25090 Score: 182 Expect: 8.2e-015 Matches: 20

Beta-casein OS=Bubalus bubalis GN=CSN2 PE=2 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6863	-25.34	214 -	224	0	---	L.GPVRGPFPIIV.-
1151.6644	1150.6571	1150.6863	-25.34	214 -	224	0	29	L.GPVRGPFPIIV.-
1363.8259	1362.8186	1362.8388	-14.78	212 -	224	0	---	P.VLGPVRGPFPIIV.-
1375.7285	1374.7212	1374.7363	-10.97	108 -	120	0	---	I.MGVSKVKEAMAPK.H
1375.7285	1374.7212	1374.7363	-10.97	108 -	120	0	---	I.MGVSKVKEAMAPK.H
1589.9221	1588.9148	1588.9341	-12.14	210 -	224	0	---	Q.EPVLGPVRGPFPIIV.-
1589.9221	1588.9148	1588.9341	-12.14	210 -	224	0	21	Q.EPVLGPVRGPFPIIV.-

1601.9026	1600.8953	1600.8977	-1.49	204 - 217	0	---	Q.AFLLYQEPVLGPVR.G
1669.8888	1668.8815	1668.8458	21.4	17 - 31	0	---	R.ELEELNVPGEIVESL.S
1717.9808	1716.9735	1716.9927	-11.16	209 - 224	0	---	Y.QEPVLGPVRGPFPIIV.-
1717.9808	1716.9735	1716.9927	-11.16	209 - 224	0	50	Y.QEPVLGPVRGPFPIIV.-
1781.9735	1780.9662	1780.9876	-12.00	208 - 223	0	---	L.YQEPVLGPVRGPFPII.V
1881.0486	1880.0413	1880.0560	-7.81	208 - 224	0	---	L.YQEPVLGPVRGPFPIIV.-
1881.0486	1880.0413	1880.0560	-7.81	208 - 224	0	42	L.YQEPVLGPVRGPFPIIV.-
1994.1243	1993.1170	1993.1401	-11.57	207 - 224	0	---	L.LYQEPVLGPVRGPFPIIV.-
2107.2170	2106.2097	2106.2241	-6.84	206 - 224	0	---	F.LLYQEPVLGPVRGPFPIIV.-
2107.2170	2106.2097	2106.2241	-6.84	206 - 224	0	41	F.LLYQEPVLGPVRGPFPIIV.-
2254.2810	2253.2737	2253.2926	-8.36	205 - 224	0	---	A.FLLYQEPVLGPVRGPFPIIV.-
2254.2810	2253.2737	2253.2926	-8.36	205 - 224	0	7	A.FLLYQEPVLGPVRGPFPIIV.-
2938.5374	2937.5301	2937.5732	-14.67	6 - 33	0	---	I.LACLVALALARELEELNVPGEIVESLSS.S

No match to: 906.6738, 1700.9590, 1700.9590

5. ATP8_ORYAF Mass: 8057 Score: 56 Expect: 0.037 Matches: 19

ATP synthase protein 8 OS=Orycteropus afer GN=MT-ATP8 PE=3 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6672	-8.72	12 -	21	0	---	I.TILSMIITLF.I
1151.6644	1150.6571	1150.6672	-8.72	10 -	19	0	---	W.FITILSMIIT.L
1375.7285	1374.7212	1374.7547	-24.35	30 -	40	0	---	S.KYLYPLEPQPK.T
1375.7285	1374.7212	1374.7547	-24.35	30 -	40	0	---	S.KYLYPLEPQPK.T
1589.9221	1588.9148	1588.8864	17.9	31 -	43	0	---	K.YLYPLEPQPKTLK.T
1589.9221	1588.9148	1588.8864	17.9	30 -	42	0	---	S.KYLYPLEPQPKTL.K
1601.9026	1600.8953	1600.8402	34.4	51 -	62	0	---	P.WETKWTKIYLPH.S
1669.8888	1668.8815	1668.8221	35.6	21 -	33	0	---	L.FILFQSNMSKYLY.P + Oxidation (M)
1717.9808	1716.9735	1716.9814	-4.59	30 -	43	0	---	S.KYLYPLEPQPKTLK.T
1717.9808	1716.9735	1716.9814	-4.59	30 -	43	0	---	S.KYLYPLEPQPKTLK.T
1781.9735	1780.9662	1781.0413	-42.13	11 -	25	0	---	F.ITILSMIITLFILFQ.S + Oxidation (M)
1881.0486	1880.0413	1880.0005	21.7	5 -	20	0	---	L.DTTPWFITILSMIITL.F + Oxidation (M)
1881.0486	1880.0413	1880.0005	21.7	4 -	19	0	---	Q.LDTTPWFITILSMIIT.L + Oxidation (M)
1994.1243	1993.1170	1993.0846	16.3	4 -	20	0	---	Q.LDTTPWFITILSMIITL.F + Oxidation (M)
2107.2170	2106.2097	2106.1051	49.7	49 -	65	0	---	N.APWETKWTKIYLPHSLH.L
2107.2170	2106.2097	2106.1051	49.7	49 -	65	0	---	N.APWETKWTKIYLPHSLH.L
2254.2810	2253.2737	2253.2371	16.3	5 -	23	0	---	L.DTTPWFITILSMIITLFIL.F + Oxidation (M)
2254.2810	2253.2737	2253.2371	16.3	4 -	22	0	---	Q.LDTTPWFITILSMIITLFI.L + Oxidation (M)
2938.5374	2937.5301	2937.5424	-4.19	8 -	31	0	---	T.PWFITILSMIITLFILFQSNMSKY.L + 2 Oxidation (M)

No match to: 906.6738, 1363.8259, 1700.9590, 1700.9590

6. ATP8_HALGR Mass: 7878 Score: 42 Expect: 0.75 Matches: 18

ATP synthase protein 8 OS=Halichoerus grypus GN=MT-ATP8 PE=3 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1151.6644	1150.6571	1150.6022	47.7	53 -	61	0	---	E.EKWTKIYSP.L
1151.6644	1150.6571	1150.6022	47.7	53 -	61	0	---	E.EKWTKIYSP.L
1375.7285	1374.7212	1374.6568	46.9	47 -	57	0	---	K.NSAPWEEKWTK.I
1375.7285	1374.7212	1374.6568	46.9	46 -	56	0	---	L.KNSAPWEEKWT.K
1601.9026	1600.8953	1600.8937	1.04	34 -	47	0	---	F.PTNPEPKHTLLLKN.S

1669.8888	1668.8815	1668.8875	-3.60	32 -	45	0	---	H.YFPTNPEPKHTLLL.K
1700.9590	1699.9517	1699.8929	34.6	8 -	21	0	---	S.TWLIMISSMILTLF.I + 2 Oxidation (M)
1700.9590	1699.9517	1699.8929	34.6	8 -	21	0	---	S.TWLIMISSMILTLF.I + 2 Oxidation (M)
1717.9808	1716.9735	1716.9352	22.3	22 -	35	0	---	F.ITFHLKVSKHYFPT.N
1717.9808	1716.9735	1716.9352	22.3	22 -	35	0	---	F.ITFHLKVSKHYFPT.N
1781.9735	1780.9662	1780.9871	-11.72	9 -	23	0	---	T.WLIMISSMILTLFIT.F
1881.0486	1880.0413	1880.0349	3.43	19 -	33	0	---	L.TLFITFHLKVSKHYF.P
1881.0486	1880.0413	1880.0349	3.43	19 -	33	0	---	L.TLFITFHLKVSKHYF.P
1994.1243	1993.1170	1993.1189	-0.96	18 -	33	0	---	I.LTLFITFHLKVSKHYF.P
2107.2170	2106.2097	2106.2030	3.19	17 -	33	0	---	M.ILTLFITFHLKVSKHYF.P
2107.2170	2106.2097	2106.1700	18.9	16 -	32	0	1	S.MILTLFITFHLKVSKHY.F + Oxidation (M)
2254.2810	2253.2737	2253.2384	15.7	16 -	33	0	---	S.MILTLFITFHLKVSKHYF.P + Oxidation (M)
2254.2810	2253.2737	2253.1942	35.3	7 -	25	0	---	T.STWLIMISSMILTLFITFH.L

No match to: 906.6738, 1363.8259, 1589.9221, 1589.9221, 2938.5374

Search Parameters

```
Type of search         : MS/MS Ion Search
Enzyme                 : None
Variable modifications : Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 50 ppm
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages   : 1
Instrument type        : MALDI-TOF-TOF
   Query1 (906.6738,1+): <no title>
  Query2 (1151.6644,1+): <no title>
  Query3 (1151.6644,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666432, MSMS Job_Run_Id: 26910, Comment:
  Query4 (1363.8259,1+): <no title>
  Query5 (1375.7285,1+): <no title>
  Query6 (1375.7285,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666433, MSMS Job_Run_Id: 26910, Comment:
  Query7 (1589.9221,1+): <no title>
  Query8 (1589.9221,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666435, MSMS Job_Run_Id: 26910, Comment:
  Query9 (1601.9026,1+): <no title>
 Query10 (1669.8888,1+): <no title>
 Query11 (1700.9590,1+): <no title>
 Query12 (1700.9590,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666439, MSMS Job_Run_Id: 26910, Comment:
 Query13 (1717.9808,1+): <no title>
 Query14 (1717.9808,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666438, MSMS Job_Run_Id: 26910, Comment:
 Query15 (1781.9735,1+): <no title>
 Query16 (1881.0486,1+): <no title>
 Query17 (1881.0486,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666436, MSMS Job_Run_Id: 26910, Comment:
 Query18 (1994.1243,1+): <no title>
 Query19 (2107.2170,1+): <no title>
 Query20 (2107.2170,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666437, MSMS Job_Run_Id: 26910, Comment:
 Query21 (2254.2810,1+): <no title>
 Query22 (2254.2810,1+): Label: O4, Spot_Id: 871775, Peak_List_Id: 666434, MSMS Job_Run_Id: 26910, Comment:
 Query23 (2938.5374,1+): <no title>
```

Mascot: http://www.matrixscience.com/

MASCOT Search Results

Protein View: CASB_BOVIN

Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2

Database:	SwissProt
Score:	196
Expect:	3.3e-016
Nominal mass (M_r):	25091
Calculated pI:	5.26
Taxonomy:	Bos taurus

Sequence similarity is available as an NCBI BLAST search of CASB_BOVIN against nr.

Search parameters

MS data file:	\\apaf-hpv-file\projects\External\e_15328_VictoriaUni_FatahAhtesh_20130607\1_MassSpec\4800\Run1\RawData\Run1\MF170613\04_MF170613.txt
Enzyme:	No enzyme cleavage specificity.
Variable modifications:	Oxidation (M)

Protein sequence coverage: 37%

Matched peptides shown in ***bold red***.

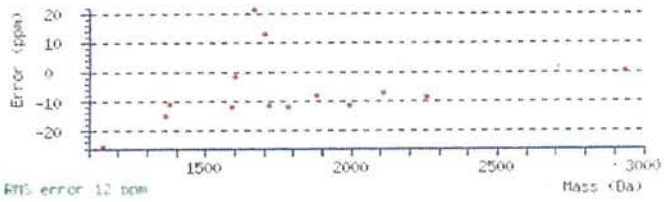
```
  1 MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
 51 EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
101 PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
151 PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
201 PIQAFLLYQE PVLGPVRGPF PIIV
```

Unformatted sequence string: 224 residues (for pasting into other applications).

Sort peptides by • Residue Number Increasing Mass Decreasing Mass

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
10	17 – 31	1669.8888	1668.8815	1668.8458	21.4	0		R.ELEELNVPGEIVESL.S
23	25 – 50	2938.5374	2937.5301	2937.5294	0.23	0		P.GEIVESLSSSEESITRINKKIEKFQS.E
11	77 – 92	1700.9590	1699.9517	1699.9297	12.9	0		P.FPGPIPNSLPQNIPPL.T
12	77 – 92	1700.9590	1699.9517	1699.9297	12.9	0	6	P.FPGPIPNSLPQNIPPL.T
5	108 – 120	1375.7285	1374.7212	1374.7363	-11.0	0		V.MGVSKVKEAMAPK.H
6	108 – 120	1375.7285	1374.7212	1374.7363	-11.0	0		V.MGVSKVKEAMAPK.H
9	204 – 217	1601.9026	1600.8953	1600.8977	-1.49	0		Q.AFLLYQEPVLGPVR.G
21	205 – 224	2254.2810	2253.2737	2253.2926	-8.36	0		A.FLLYQEPVLGPVRGPFPIIV.-
22	205 – 224	2254.2810	2253.2737	2253.2926	-8.36	0	7	A.FLLYQEPVLGPVRGPFPIIV.-
19	206 – 224	2107.2170	2106.2097	2106.2241	-6.84	0		F.LLYQEPVLGPVRGPFPIIV.-

Query	Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
20	206 - 224	2107.2170	2106.2097	2106.2241	-6.84	0	41	F.LLYQEPVLGPVRGPFPIIV.-
18	207 - 224	1994.1243	1993.1170	1993.1401	-11.6	0		L.LYQEPVLGPVRGPFPIIV.-
15	208 - 223	1781.9735	1780.9662	1780.9876	-12.0	0		L.YQEPVLGPVRGPFPII.V
16	208 - 224	1881.0486	1880.0413	1880.0560	-7.81	0		L.YQEPVLGPVRGPFPIIV.-
17	208 - 224	1881.0486	1880.0413	1880.0560	-7.81	0	42	L.YQEPVLGPVRGPFPIIV.-
13	209 - 224	1717.9808	1716.9735	1716.9927	-11.2	0		Y.QEPVLGPVRGPFPIIV.-
14	209 - 224	1717.9808	1716.9735	1716.9927	-11.2	0	50	Y.QEPVLGPVRGPFPIIV.-
7	210 - 224	1589.9221	1588.9148	1588.9341	-12.1	0		Q.EPVLGPVRGPFPIIV.-
8	210 - 224	1589.9221	1588.9148	1588.9341	-12.1	0	21	Q.EPVLGPVRGPFPIIV.-
4	212 - 224	1363.8259	1362.8186	1362.8388	-14.8	0		P.VLGPVRGPFPIIV.-
2	214 - 224	1151.6644	1150.6571	1150.6863	-25.3	0		L.GPVRGPFPIIV.-
3	214 - 224	1151.6644	1150.6571	1150.6863	-25.3	0	29	L.GPVRGPFPIIV.-




```
ID   CASB_BOVIN              Reviewed;         224 AA.
AC   P02666; A1YQZ8; A6N8V0; Q2TA13; Q5EEQ6; Q5EEQ7; Q6UN63; Q9BDG5;
AC   Q9TSD5;
DT   21-JUL-1986, integrated into UniProtKB/Swiss-Prot.
DT   01-JUL-1989, sequence version 2.
DT   03-APR-2013, entry version 119.
DE   RecName: Full=Beta-casein;
DE   Contains:
DE     RecName: Full=Casoparan;
DE   Contains:
DE     RecName: Full=Antioxidant peptide;
DE   Contains:
DE     RecName: Full=Casohypotensin;
DE   Flags: Precursor;
GN   Name=CSN2;
OS   Bos taurus (Bovine).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;
OC   Pecora; Bovidae; Bovinae; Bos.
OX   NCBI_TaxID=9913;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANTS LEU-108; PRO-152 AND LEU-153.
RX   PubMed=3814153; DOI=10.1016/0006-291X(87)90318-4;
RA   Jimenez-Flores R., Kang Y.C., Richardson T.;
RT   "Cloning and sequence analysis of bovine beta-casein cDNA.";
RL   Biochem. Biophys. Res. Commun. 142:617-621(1987).
RN   [2]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANT HIS-82.
RA   Baev A.A., Smirnov I.K., Gorodetsky S.I.;
RT   "Primary structure of bovine beta-casein cDNA.";
```

```
RL   Mol. Biol. (Mosk.) 21:214-222(1987).
RN   [3]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RX   PubMed=2833669;
RA   Stewart A.F., Bonsing J., Beattie C.W., Shah F., Willis I.M.,
RA   Mackinlay A.G.;
RT   "Complete nucleotide sequences of bovine alpha S2- and beta-casein
RT   cDNAs: comparisons with related sequences in other species.";
RL   Mol. Biol. Evol. 4:231-241(1987).
RN   [4]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA], AND VARIANT HIS-82.
RX   PubMed=3271384;
RA   Bonsing J., Ring J.M., Stewart A.F., Mackinlay A.G.;
RT   "Complete nucleotide sequence of the bovine beta-casein gene.";
RL   Aust. J. Biol. Sci. 41:527-537(1988).
RN   [5]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANT A3 GLN-121.
RC   TISSUE=Mammary gland;
RX   PubMed=8248100; DOI=10.1093/protein/6.7.763;
RA   Simons G., van den Heuvel W., Reynen T., Frijters A., Rutten G.,
RA   Slangen C.J., Groenen M., de Vos W.M., Siezen R.J.;
RT   "Overproduction of bovine beta-casein in Escherichia coli and
RT   engineering of its main chymosin cleavage site.";
RL   Protein Eng. 6:763-770(1993).
RN   [6]
RP   NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA], AND VARIANTS HIS-82 AND
RP   ARG-137.
RC   STRAIN=Crossbred X Angus; TISSUE=Liver;
RG   NIH - Mammalian Gene Collection (MGC) project;
RL   Submitted (DEC-2005) to the EMBL/GenBank/DDBJ databases.
RN   [7]
RP   NUCLEOTIDE SEQUENCE [MRNA] OF 1-101.
RC   TISSUE=Mammary epithelium;
RX   PubMed=16624358; DOI=10.1016/j.rvsc.2006.02.002;
RA   Anaya-Lopez J.L., Contreras-Guzman O.E., Carabez-Trejo A.,
RA   Baizabal-Aguirre V.M., Lopez-Meza J.E., Valdez-Alarcon J.J.,
RA   Ochoa-Zarzosa A.;
RT   "Invasive potential of bacterial isolates associated with subclinical
RT   bovine mastitis.";
RL   Res. Vet. Sci. 81:358-361(2006).
RN   [8]
RP   PROTEIN SEQUENCE OF 16-224 (VARIANT A2), AND VARIANT LEU-108.
RX   PubMed=4557764; DOI=10.1111/j.1432-1033.1972.tb01722.x;
RA   Ribadeau-Dumas B., Brignon G., Grosclaude F., Mercier J.-C.;
RT   "Primary structure of bovine beta casein. Complete sequence.";
RL   Eur. J. Biochem. 25:505-514(1972).
RN   [9]
RP   PROTEIN SEQUENCE OF 16-224 (VARIANT A2).
RX   PubMed=3278933; DOI=10.1016/0014-5793(88)81138-4;
RA   Carles C., Huet J.-C., Ribadeau-Dumas B.;
RT   "A new strategy for primary structure determination of proteins:
RT   application to bovine beta-casein.";
RL   FEBS Lett. 229:265-272(1988).
RN   [10]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA] OF 18-57, PROTEIN SEQUENCE OF 16-224
RP   (VARIANT H), AND VARIANT D LYS-33.
RC   STRAIN=Korean; TISSUE=Milk;
RX   PubMed=10690361; DOI=10.1046/j.1365-2052.2000.00582.x;
```

RA Han S.K., Shin Y.C., Byun H.D.;
RT "Biochemical, molecular and physiological characterization of a new
RT beta-casein variant detected in Korean cattle.";
RL Anim. Genet. 31:49-51(2000).
RN [11]
RP PROTEIN SEQUENCE OF 41-71; 113-157 AND 180-224, AND VARIANT GLN-132.
RX PubMed=1804413; DOI=10.1002/rcm.1290050410;
RA Jones D.S., Heerma W., van Wassenaar P.D., Haverkamp J.;
RT "Analysis of bovine beta-casein tryptic digest by continuous-flow
RT fast-atom bombardment mass spectrometry.";
RL Rapid Commun. Mass Spectrom. 5:192-195(1991).
RN [12]
RP PROTEIN SEQUENCE OF 41-45, AND FUNCTION.
RX PubMed=15545057; DOI=10.1080/09629350400003068;
RA Lebrun I., Cavallaro V., Juliano L., Juliano M.A.,
RA de Sousa e Silva M.C.C.;
RT "Effects of 'casoparan', a peptide isolated from casein hydrolysates
RT with mastoparan-like properties.";
RL Mediators Inflamm. 13:263-268(2004).
RN [13]
RP PROTEIN SEQUENCE OF 48-63, AND VARIANT E LYS-51.
RX PubMed=4411121; DOI=10.1016/0014-5793(74)80796-9;
RA Grosclaude F., Mahe M.-F., Voglino G.-F.;
RT "The beta E variant and the phosphorylation code of bovine caseins.";
RL FEBS Lett. 45:3-5(1974).
RN [14]
RP NUCLEOTIDE SEQUENCE [GENOMIC DNA] OF 58-223.
RA Otaviano A.R., Lima A.L.F., Laureano M.M.M., Albuquerque L.G.,
RA Tonhati H., Sena J.A.D.;
RT "Polymorphisms in beta and kappa casein genes in bubaline and
RT bovine.";
RL Submitted (NOV-2006) to the EMBL/GenBank/DDBJ databases.
RN [15]
RP NUCLEOTIDE SEQUENCE [GENOMIC DNA] OF 58-223.
RA Shahla M.N., Cheema F.R., Naeem M.K., Riazuddin S.;
RT "Polymorphism in the cattle beta casein gene.";
RL Submitted (MAY-2007) to the EMBL/GenBank/DDBJ databases.
RN [16]
RP NUCLEOTIDE SEQUENCE [GENOMIC DNA] OF 63-208.
RC TISSUE=Mammary gland;
RA Klotz A., Buchberger J., Krause I., Einspanier R.;
RT "Characterization of milk proteins.";
RL Submitted (JAN-2001) to the EMBL/GenBank/DDBJ databases.
RN [17]
RP NUCLEOTIDE SEQUENCE [MRNA] OF 68-105.
RX PubMed=6397405; DOI=10.1016/0378-1119(84)90013-1;
RA Ivanov V.N., Kershulite D.R., Bayev A.A., Akhundova A.A.,
RA Sulimova G.E., Judinkova E.S., Gorodetsky S.I.;
RT "Identification of bacterial clones encoding bovine caseins by direct
RT immunological screening of the cDNA library.";
RL Gene 32:381-388(1984).
RN [18]
RP NUCLEOTIDE SEQUENCE [MRNA] OF 68-95.
RX PubMed=3900695;
RA Ivanov V.N., Kershulite D.R., Bayev A.A., Akhundova A.A.,
RA Silimova G.E.;
RT "Identification of bacterial clones that encode cow's caseins by
RT direct immunological screening of the cDNA library.";

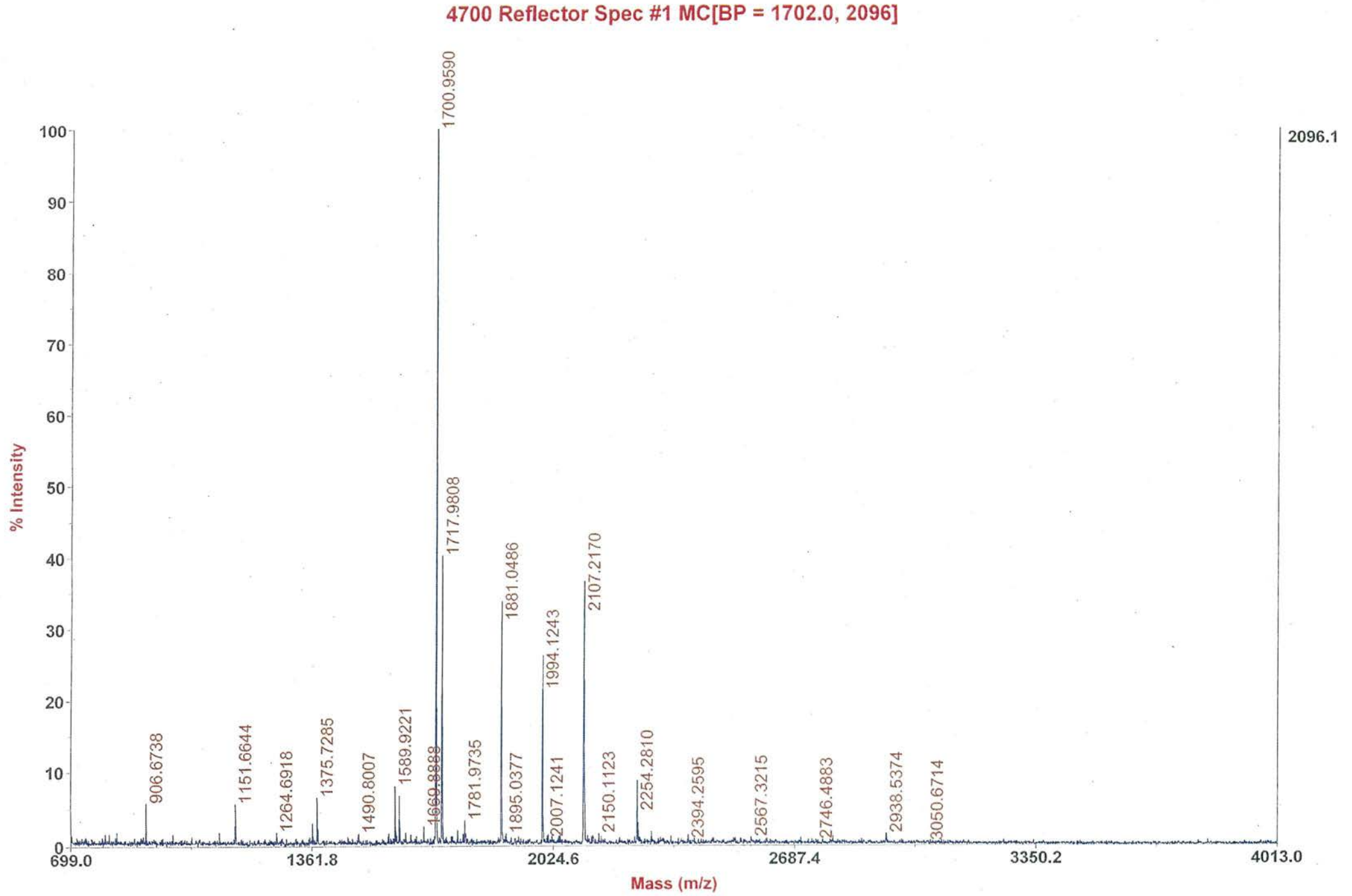
RL Mol. Biol. (Mosk.) 19:955-963(1985).
RN [19]
RP PROTEIN SEQUENCE OF 74-108, VARIANT LEU-108, PHOSPHORYLATION, AND MASS SPECTROMETRY.
RX PubMed=17720176; DOI=10.1016/j.chroma.2007.08.015;
RA Schmelzer C.E.H., Schoeps R., Reynell L., Ulbrich-Hofmann R.,
RA Neubert R.H.H., Raith K.;
RT "Peptic digestion of beta-casein: Time course and fate of possible
RT bioactive peptides.";
RL J. Chromatogr. A 1166:108-115(2007).
RN [20]
RP NUCLEOTIDE SEQUENCE [GENOMIC DNA] OF 80-143, AND VARIANT LEU-108.
RA Jann O., Ceriotti G., Caroli A., Erhardt G.;
RT "A new variant in exon VII of bovine beta-casein gene (CSN2) and its
RT contribution among European cattle breeds.";
RL J. Anim. Breed. Genet. 119:65-68(2002).
RN [21]
RP PROTEIN SEQUENCE OF 113-120, FUNCTION, AND MASS SPECTROMETRY.
RA Gupta A., Mann B., Kumar Bajaj R., Sangwan R.B.;
RT "Studies on antioxidative peptides generated in cheddar cheese.";
RL Submitted (JAN-2008) to UniProtKB.
RN [22]
RP PROTEIN SEQUENCE OF 118-124, AND VARIANT A3 GLN-121.
RX PubMed=4997616;
RA Ribadeau-Dumas B., Grosclaude F., Mercier J.-C.;
RT "Localization in the peptide chain of bovine beta casein of the His-
RT Gln substitution differentiating the A2 and A3 genetic variants.";
RL C. R. Hebd. Seances Acad. Sci., D, Sci. Nat. 270:2369-2372(1970).
RN [23]
RP PROTEIN SEQUENCE OF 125-195 (VARIANTS A1 AND G).
RX AGRICOLA=IND22004684; DOI=10.1016/S0958-6946(99)00019-9;
RA Dong C., Ng-Kwai-Hang K.F.;
RT "Characterization of a non-electrophoretic genetic variant of beta-
RT casein by peptide mapping and mass spectrometric analysis.";
RL Int. Dairy J. 8:967-972(1998).
RN [24]
RP PROTEIN SEQUENCE OF 129-136, FUNCTION, AND VARIANT GLN-132.
RX PubMed=7600458;
RA Lebrun I., Lebrun F.L.A.S., Henriques O.B., Carmona A.K., Juliano L.,
RA Camargo A.C.M.;
RT "Isolation and characterization of a new bradykinin potentiating
RT octapeptide from gamma-casein.";
RL Can. J. Physiol. Pharmacol. 73:85-91(1995).
RN [25]
RP PROTEIN SEQUENCE OF 129-136, AND FUNCTION.
RX PubMed=14714726; DOI=10.1023/B:JOPC.0000008724.98339.ff;
RA Perpetuo E.A., Juliano L., Lebrun I.;
RT "Biochemical and pharmacological aspects of two bradykinin-
RT potentiating peptides obtained from tryptic hydrolysis of casein.";
RL J. Protein Chem. 22:601-606(2003).
RN [26]
RP PROTEIN SEQUENCE OF 160-171 (VARIANT F).
RX PubMed=7496485; DOI=10.1016/0021-9673(95)00058-U;
RA Visser S., Slangen C.J., Lagerwerf F.M., Van Dongen W.D.,
RA Haverkamp J.;
RT "Identification of a new genetic variant of bovine beta-casein using
RT reversed-phase high-performance liquid chromatography and mass
RT spectrometric analysis.";

```
RL   J. Chromatogr. A 711:141-150(1995).
RN   [27]
RP   NUCLEOTIDE SEQUENCE [MRNA] OF 170-184.
RX   PubMed=6897774;
RA   Willis I.M., Stewart A.F., Caputo A., Thompson A.R., McKinlay A.G.;
RT   "Construction and identification by partial nucleotide sequence
RT   analysis of bovine casein and beta-lactoglobulin cDNA clones.";
RL   DNA 1:375-386(1982).
RN   [28]
RP   PHOSPHORYLATION AT SER-30; SER-32; SER-33; SER-34 AND SER-50, AND MASS
RP   SPECTROMETRY.
RX   PubMed=16083266; DOI=10.1021/pr050113n;
RA   Wu S.L., Kim J., Hancock W.S., Karger B.;
RT   "Extended Range Proteomic Analysis (ERPA): a new and sensitive LC-MS
RT   platform for high sequence coverage of complex proteins with extensive
RT   post-translational modifications-comprehensive analysis of beta-casein
RT   and epidermal growth factor receptor (EGFR).";
RL   J. Proteome Res. 4:1155-1170(2005).
RN   [29]
RP   PHOSPHORYLATION AT SER-30; SER-32; SER-33; SER-34 AND SER-50, AND MASS
RP   SPECTROMETRY.
RX   PubMed=17510049; DOI=10.1074/mcp.M600480-MCP200;
RA   Imanishi S.Y., Kochin V., Ferraris S.E., de Thonel A., Pallari H.M.,
RA   Corthals G.L., Eriksson J.E.;
RT   "Reference-facilitated phosphoproteomics: fast and reliable
RT   phosphopeptide validation by micro LC-ESI-Q-TOF MS/MS.";
RL   Mol. Cell. Proteomics 6:1380-1391(2007).
RN   [30]
RP   VARIANTS A1; B AND C.
RX   PubMed=5064450; DOI=10.1111/j.1432-1033.1972.tb01771.x;
RA   Grosclaude F., Mahe M.-F., Mercier J.-C., Ribadeau-Dumas B.;
RT   "Characterization of genetic variants of alpha-S1 and beta bovine
RT   caseins.";
RL   Eur. J. Biochem. 26:328-337(1972).
CC   -!- FUNCTION: Important role in determination of the surface
CC       properties of the casein micelles.
CC   -!- FUNCTION: Casoparan acts as a macrophage activator, increasing the
CC       phagocytic activity of macrophages and peroxide release from
CC       macrophages. It also acts as a bradykinin-potentiating peptide.
CC   -!- FUNCTION: Casohypotensin acts as a bradykinin-potentiating
CC       peptide. Induces hypotension in rats. Acts as a strong competitive
CC       inhibitor of endo-oligopeptidase A.
CC   -!- FUNCTION: Antioxidant peptide has antioxidant activity.
CC   -!- INTERACTION:
CC       O43464:HTRA2 (xeno); NbExp=2; IntAct=EBI-5260183, EBI-517086;
CC   -!- SUBCELLULAR LOCATION: Secreted.
CC   -!- TISSUE SPECIFICITY: Mammary gland specific. Secreted in milk.
CC   -!- MASS SPECTROMETRY: Mass=872.51; Method=Electrospray; Range=113-
CC       120; Source=Ref.21;
CC   -!- POLYMORPHISM: Leu-152 is present in the variants F and G; Gln-190
CC       and Glu-210 are present in the variant H. The sequence shown is
CC       the A2 variant.
CC   -!- SIMILARITY: Belongs to the beta-casein family.
CC   -!- SEQUENCE CAUTION:
CC       Sequence=AAW84270.1; Type=Erroneous initiation;
CC       Sequence=AAW84271.1; Type=Erroneous initiation;
CC       Sequence=ABL74247.1; Type=Frameshift; Positions=65, 71;
CC   -!- WEB RESOURCE: Name=Protein Spotlight; Note=Of buttons, digestion
```

```
CC       and glue - Issue 16 of November 2001;
CC       URL="http://web.expasy.org/spotlight/back_issues/sptlt016.shtml";
CC   ---------------------------------------------------------------------
CC   Copyrighted by the UniProt Consortium, see http://www.uniprot.org/terms
CC   Distributed under the Creative Commons Attribution-NoDerivs License
CC   ---------------------------------------------------------------------
DR   EMBL; M15132; AAA30430.1; -; mRNA.
DR   EMBL; X06359; CAA29658.1; -; mRNA.
DR   EMBL; M16645; AAA30480.1; -; mRNA.
DR   EMBL; M55158; AAA30431.1; -; Genomic_DNA.
DR   EMBL; S67277; AAB29137.1; -; mRNA.
DR   EMBL; BC111172; AAI11173.1; -; mRNA.
DR   EMBL; AY899917; AAW84270.1; ALT_INIT; mRNA.
DR   EMBL; AY899918; AAW84271.1; ALT_INIT; mRNA.
DR   EMBL; AH007287; AAD09813.1; -; Genomic_DNA.
DR   EMBL; EF123100; ABL74247.1; ALT_FRAME; Genomic_DNA.
DR   EMBL; EF628290; ABR10906.1; -; Genomic_DNA.
DR   EMBL; AJ296330; CAC37028.1; -; Genomic_DNA.
DR   EMBL; M64756; AAB59254.1; -; mRNA.
DR   EMBL; AY366419; AAR14677.1; -; Genomic_DNA.
DR   EMBL; K01087; AAA30481.1; -; mRNA.
DR   IPI; IPI00697085; -.
DR   PIR; A59068; A59068.
DR   PIR; I45873; KBBOA2.
DR   RefSeq; NP_851351.1; NM_181008.2.
DR   UniGene; Bt.53272; -.
DR   DisProt; DP00329; -.
DR   ProteinModelPortal; P02666; -.
DR   DIP; DIP-46257N; -.
DR   IntAct; P02666; 1.
DR   STRING; 9913.ENSBTAP00000003409; -.
DR   Allergome; 10199; Bos d 11.0101.
DR   Allergome; 167; Bos d 8.
DR   Allergome; 2736; Bos d 11.
DR   PaxDb; P02666; -.
DR   PRIDE; P02666; -.
DR   Ensembl; ENSBTAT00000003409; ENSBTAP00000003409; ENSBTAG00000002632.
DR   GeneID; 281099; -.
DR   KEGG; bta:281099; -.
DR   CTD; 1447; -.
DR   eggNOG; NOG45871; -.
DR   GeneTree; ENSGT00390000001890; -.
DR   HOVERGEN; HBG004973; -.
DR   InParanoid; P02666; -.
DR   OMA; VVPYPQR; -.
DR   OrthoDB; EOG41NTND; -.
DR   NextBio; 20805172; -.
DR   PMAP-CutDB; P02666; -.
DR   GO; GO:0005576; C:extracellular region; IEA:UniProtKB-SubCell.
DR   GO; GO:0016209; F:antioxidant activity; IEA:UniProtKB-KW.
DR   GO; GO:0008191; F:metalloendopeptidase inhibitor activity; IEA:UniProtKB-KW.
DR   GO; GO:0005215; F:transporter activity; IEA:InterPro.
DR   GO; GO:0043086; P:negative regulation of catalytic activity; IEA:GOC.
DR   GO; GO:0008217; P:regulation of blood pressure; IEA:UniProtKB-KW.
DR   InterPro; IPR001588; Casein.
DR   InterPro; IPR016345; Casein_beta.
DR   PANTHER; PTHR11500; PTHR11500; 1.
DR   Pfam; PF00363; Casein; 1.
```

```
DR   PIRSF; PIRSF002372; Beta-casein; 1.
DR   PROSITE; PS00306; CASEIN_ALPHA_BETA; 1.
PE   1: Evidence at protein level;
KW   Antioxidant; Complete proteome; Direct protein sequencing;
KW   Hypotensive agent; Metalloenzyme inhibitor; Metalloprotease inhibitor;
KW   Milk protein; Phosphoprotein; Polymorphism; Protease inhibitor;
KW   Reference proteome; Secreted; Signal.
FT   SIGNAL        1     15
FT   CHAIN        16    224       Beta-casein.
FT                                 /FTId=PRO_0000004470.
FT   PEPTIDE      41     45       Casoparan.
FT                                 /FTId=PRO_0000292031.
FT   PEPTIDE     113    120       Antioxidant peptide.
FT                                 /FTId=PRO_0000320153.
FT   PEPTIDE     129    136       Casohypotensin.
FT                                 /FTId=PRO_0000308464.
FT   MOD_RES      30     30       Phosphoserine.
FT   MOD_RES      32     32       Phosphoserine.
FT   MOD_RES      33     33       Phosphoserine.
FT   MOD_RES      34     34       Phosphoserine.
FT   MOD_RES      50     50       Phosphoserine; in variant A1, variant A2,
FT                                 variant A3, variant B, variant E, variant
FT                                 F, variant G and variant H.
FT   VARIANT      33     33       S -> K (in variant D).
FT   VARIANT      40     40       R -> C (in variant H).
FT   VARIANT      51     51       E -> K (in variant E).
FT   VARIANT      52     52       E -> K (in variant C).
FT   VARIANT      82     82       P -> H (in variants A1, B, C, F and G).
FT   VARIANT     103    103       L -> I (in variant H).
FT   VARIANT     108    108       M -> L.
FT   VARIANT     121    121       H -> Q (in variant A3).
FT   VARIANT     132    132       E -> Q (in variants A1 and G).
FT   VARIANT     137    137       S -> R (in variant B).
FT   VARIANT     152    152       L -> P (in variants A1 and H).
FT   VARIANT     153    153       P -> L (in variants A1, G and H).
FT   VARIANT     167    167       P -> L (in variant F).
FT   VARIANT     190    190       Q -> E (in variants A1 and G).
FT   CONFLICT     50     50       S -> Z (in Ref. 11; AA sequence).
FT   CONFLICT     69     69       Q -> R (in Ref. 14; ABL74247).
FT   CONFLICT    112    112       K -> R (in Ref. 14; ABL74247).
FT   CONFLICT    208    208       Y -> V (in Ref. 16; CAC37028).
FT   CONFLICT    209    210       QE -> EQ (in Ref. 11; AA sequence).
FT   CONFLICT    210    210       E -> Q (in Ref. 1; AAA30430 and 8; no
FT                                 nucleotide entry).
FT   CONFLICT    212    212       V -> A (in Ref. 15; ABR10906).
SQ   SEQUENCE   224 AA;  25107 MW;  F0BBDD8148A238AE CRC64;
     MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS EEQQQTEDEL
     QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP PFLQPEVMGV SKVKEAMAPK
     HKEMPFPKYP VEPFTESQSL TLTDVENLHL PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL
     SQSKVLPVPQ KAVPYPQRDM PIQAFLLYQE PVLGPVRGPF PIIV
```

Mascot: http://www.matrixscience.com/



\...\O4_MS_1.I2d
Acquired:

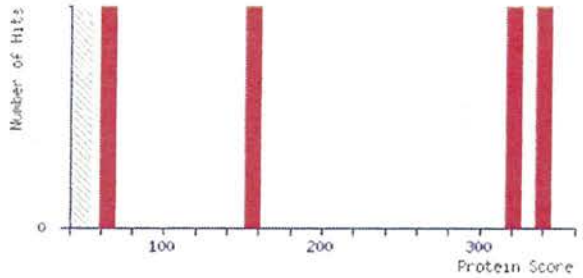
Mascot Search Results

User :
Email :
Search title : Submitted from VU-Bovine by Mascot Daemon on APAF-WS-08
MS data file : \\apaf-hpv-file\projects\External\e_15328_VictoriaUni_FatahAhtesh_20130607\1_MassSpec\QStarElite\Run1\Results\F1.mgf
Database : SwissProt 2013 (539829 sequences; 191670831 residues)
Taxonomy : Other mammalia (13034 sequences)
Timestamp : 21 Jun 2013 at 03:40:51 GMT
Protein hits : CASA1_BOVIN Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2
CASB_BOVIN Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2
CASA1_CAPHI Alpha-S1-casein OS=Capra hircus GN=CSN1S1 PE=1 SV=2
LACB_BOVIN Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3

	SwissProt	Decoy	False discovery rate
Peptide matches above identity threshold	33	0	0.00 %
Peptide matches above homology or identity threshold	43	1	2.33 %

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 54 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As Peptide Summary Help

Significance threshold p< 0.01 Max. number of hits AUTO Show Percolator scores
Standard scoring MudPIT scoring Ions score or expect cut-off 54 Show sub-sets 0
Show pop-ups Suppress pop-ups Sort unassigned Decreasing Score Require bold red

Select All Select None Search Selected **Error tolerant** Archive Report

1. CASA1_BOVIN Mass: 24513 Score: 340 Matches: 11(11) Sequences: 9(9) emPAI: 3.86
Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	112	499.7670	997.5195	997.5080	11.5	0	56	0.0059	1	U	Q.GLPQEVLNE.N
✓	154	571.7743	1141.5341	1141.5251	7.90	0	82	1.6e-005	1		F.SDIPNPIGSEN.S
✓	173	615.2928	1228.5711	1228.5571	11.4	0	59	0.0033	1		F.SDIPNPIGSENS.E
✓	252	743.8542	1485.6939	1485.6947	-0.56	0	93	1.4e-006	1	U	F.SDIPNPIGSENSEK.T
	268	794.3892	1586.7638	1586.7424	13.5	0	60	0.0027	2	U	F.SDIPNPIGSENSEKT.T
✓	283	844.9231	1687.8317	1687.7901	24.7	0	72	0.00017	1	U	F.SDIPNPIGSENSEKTT.M
✓	292	857.9157	1713.8169	1713.8243	-4.31	0	68	0.0004	1	U	D.IPNPIGSENSEKTTMP.L
✓	304	865.9365	1729.8584	1729.8192	22.7	0	(63)	0.0014	1	U	D.IPNPIGSENSEKTTMP.L + Oxidation (M)
✓	313	915.4428	1828.8711	1828.8513	10.8	0	78	4.2e-005	1	U	S.DIPNPIGSENSEKTTMP.L
✓	319	958.9700	1915.9254	1915.8833	22.0	0	(65)	0.00087	1	U	F.SDIPNPIGSENSEKTTMP.L
✓	320	966.9693	1931.9241	1931.8782	23.8	0	94	1.1e-006	1	U	F.SDIPNPIGSENSEKTTMP.L + Oxidation (M)

2. CASB_BOVIN Mass: 25091 Score: 321 Matches: 19(19) Sequences: 17(17) emPAI: 9.94
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	136	550.8001	1099.5856	1099.5702	13.9	0	55	0.0085	1	U	L.VYPFPGPIPN.S
✓	157	576.3528	1150.6910	1150.6863	4.06	0	59	0.0031	1	U	L.GPVRGPFPIIV.-
✓	169	610.8168	1219.6190	1219.5947	19.9	0	58	0.0046	1		E.MPFPKYPVEP.F + Oxidation (M)
✓	170	611.3570	1220.6994	1220.6805	15.5	0	56	0.0073	1		T.PVVVPPFLQPE.V
✓	182	630.3547	1258.6949	1258.6921	2.18	0	57	0.0054	1	U	T.DVENLHLPLPL.L
✓	206	654.8689	1307.7232	1307.7085	11.2	0	57	0.0048	1	U	N.SLPQNIPPLTQT.P
✓	208	660.8838	1319.7531	1319.7489	3.12	0	61	0.0021	1	U	T.PVVVPPFLQPEV.M
✓	212	667.3367	1332.6589	1332.6424	12.4	0	65	0.00087	1		K.EMPFPKYPVEP.F
✓	217	672.3436	1342.6726	1342.6703	1.66	0	56	0.0072	1	U	A.VPYPQRDMPIQ.A
✓	223	679.4215	1356.8285	1356.8017	19.8	0	57	0.0049	1	U	N.IPPLTQTPVVVPP.F
✓	225	680.8895	1359.7644	1359.7398	18.1	0	84	1.1e-005	1	U	L.TDVENLHLPLPL.L
✓	228	682.8656	1363.7167	1363.6846	23.6	0	59	0.0033	1		H.KEMPFPKYPVE.P
✓	233	691.3586	1380.7026	1380.6972	3.84	0	74	0.00011	1	U	W.MHQPHQPLPPTV.M
✓	234	696.8965	1391.7784	1391.7561	16.0	0	64	0.0011	1		Y.QEPVLGPVRGPFP.I
✓	257	752.9455	1503.8765	1503.8701	4.24	0	(55)	0.0076	1	U	N.IPPLTQTPVVVPPF.L
✓	258	752.9455	1503.8765	1503.8701	4.24	0	67	0.00057	1	U	N.IPPLTQTPVVVPPF.L
✓	259	752.9481	1503.8817	1503.8701	7.69	0	(63)	0.0013	1	U	N.IPPLTQTPVVVPPF.L
✓	294	859.5097	1717.0048	1716.9927	7.08	0	70	0.00026	1	U	Y.QEPVLGPVRGPFPIIV.-
✓	318	941.0443	1880.0740	1880.0560	9.54	0	56	0.0068	1	U	L.YQEPVLGPVRGPFPIIV.-

3. CASA1_CAPHI Mass: 24274 Score: 160 Matches: 4(4) Sequences: 3(3) emPAI: 0.79
Alpha-S1-casein OS=Capra hircus GN=CSN1S1 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
	154	571.7743	1141.5341	1141.5251	7.90	0	82	1.6e-005	1		F.SDIPNPIGSEN.S
	173	615.2928	1228.5711	1228.5571	11.4	0	59	0.0033	1		F.SDIPNPIGSENS.G
✓	314	922.9629	1843.9112	1843.8622	26.6	0	(74)	0.00011	1	U	F.SDIPNPIGSENSGKTTMP.L
✓	316	930.9610	1859.9075	1859.8571	27.1	0	83	1.5e-005	1	U	F.SDIPNPIGSENSGKTTMP.L + Oxidation (M)

Proteins matching the same set of peptides:
CASA1_BUBBU Mass: 24311 Score: 160 Matches: 4(4) Sequences: 3(3)
Alpha-S1-casein OS=Bubalus bubalis GN=CSN1S1 PE=2 SV=2

---

4. LACB_BOVIN Mass: 19870 Score: 64 Matches: 1(1) Sequences: 1(1) emPAI: 0.19
Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3
Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	210	663.8492	1325.6838	1325.6714	9.34	0	64	0.001	1	U	Y.VEELKPTPEGDL.E

Proteins matching the same set of peptides:
LACB_BUBBU Mass: 20010 Score: 64 Matches: 1(1) Sequences: 1(1)
Beta-lactoglobulin OS=Bubalus bubalis GN=LGB PE=1 SV=2

Peptide matches not assigned to protein hits: (no details means no match)

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓	268	794.3892	1586.7638	1586.8126	-30.78	0	66	0.00066	1		APPMNPLNPLNPLSP + Oxidation (M)
✓	155	572.8425	1143.6705	1143.6652	4.64	0	54	0.011	1		VENLHLPLPL
✓	255	745.3746	1488.7347	1488.7348	-0.04	0	53	0.012	1		YVEELKPTPEGDL
✓	116	514.7467	1027.4789	1027.4822	-3.20	0	53	0.012	1		SDIPNPIGSE
✓	74	451.2285	900.4425	900.4301	13.8	0	52	0.017	1		VPNSAEER
✓	221	676.3551	1350.6957	1350.6682	20.3	0	51	0.021	1		MPFPKYPVEPF
✓	220	675.3419	1348.6692	1348.6373	23.7	0	50	0.025	1		EMPFPKYPVEP + Oxidation (M)
✓	174	617.8273	1233.6401	1233.6241	13.0	0	50	0.029	1		DLKGYGGVSLPE
✓	152	569.8013	1137.5881	1137.5819	5.45	0	49	0.033	1		GPIVLNPWDQ
✓	269	795.4827	1588.9509	1588.9341	10.5	0	48	0.046	1		EPVLGPVRGPFPIIV
✓	244	487.9191	1460.7355	1460.7374	-1.26	0	48	0.049	1		KEMPFPKYPVEP
✓	266	778.4221	1554.8296	1554.8195	6.51	0	47	0.055	1		YQEPVLGPVRGPFP
✓	251	743.3899	1484.7652	1484.7446	13.9	0	46	0.066	1		AVPYPQRDMPIQA
✓	151	568.8059	1135.5973	1135.5736	20.9	0	46	0.06	1		KEMPFPKYP
✓	77	453.2201	904.4256	904.4250	0.73	0	46	0.063	1		TEEEKNR
✓	277	809.9637	1617.9128	1617.9131	-0.16	0	46	0.071	1		NIPPLTQTPVVVPPF

✓	134	549.7926	1097.5706	1097.5604	9.26	0	46	0.069	1	ELNVPGEIVE
✓	137	551.7774	1101.5402	1101.5277	11.3	0	44	0.1	1	VPYPQRDMP
✓	138	553.2894	1104.5643	1104.5604	3.55	0	44	0.11	1	INNQFIPYP
✓	177	416.8908	1247.6506	1247.6550	-3.55	0	43	0.12	1	APFPEVFGKEK
✓	247	736.4474	1470.8802	1470.8446	24.2	0	43	0.14	1	NIPPLTQTPVVVPP
✓	237	700.3445	1398.6744	1398.6627	8.41	0	43	0.15	1	DIPNPIGSENSEK
✓	163	602.8099	1203.6053	1203.5998	4.55	0	42	0.16	1	MPFPKYPVEP
✓	93	480.2473	958.4801	958.4720	8.52	0	42	0.16	1	KEDVPSER
✓	239	715.3673	1428.7201	1428.7038	11.4	0	42	0.18	1	YQGPIVLNPWDQ
✓	204	651.3296	1300.6446	1300.6452	-0.43	0	41	0.21	1	YQGPIVLNPWD
✓	114	501.2739	1000.5332	1000.5229	10.3	0	41	0.22	1	YQEPVLGPV
✓	160	587.2962	1172.5779	1172.5648	11.1	0	41	0.23	1	AVPYPQRDMP
✓	156	576.3414	1150.6682	1150.6598	7.35	0	40	0.23	1	VPEVTQGIPLV
✓	242	727.4004	1452.7862	1452.7711	10.3	0	40	0.25	1	ELKPTPEGDLEIL
✓	248	493.2537	1476.7392	1476.7323	4.69	0	40	0.3	1	KEMPFPKYPVEP + Oxidation (M)
✓	162	602.8099	1203.6053	1203.6023	2.49	0	39	0.3	1	YVEELKPTPE
✓	270	795.4907	1588.9669	1588.9341	20.6	0	39	0.33	1	EPVLGPVRGPFPIIV
✓	240	715.8760	1429.7374	1429.7024	24.5	0	39	0.33	1	AVPYPQRDMPIQ + Oxidation (M)
✓	176	621.8146	1241.6146	1241.7707	-125.72	0	38	0.39	1	KSLVGKGILVQT
✓	64	434.2643	866.5140	866.5226	-9.82	0	38	0.35	1	SKVLPVPQ
✓	175	618.8106	1235.6066	1235.6584	-41.89	0	38	0.41	1	FLKVLNNMEI + Oxidation (M)
✓	110	496.2690	990.5234	990.5175	5.94	0	38	0.45	1	APFPEVFGK
✓	39	413.7330	825.4515	825.4497	2.08	0	36	0.54	1	GPVRGPFP
✓	180	627.8231	1253.6316	1253.5710	48.3	0	36	0.66	1	ETEGIMVHPNQ
✓	184	632.8677	1263.7208	1263.6976	18.4	0	36	0.69	1	EPVLGPVRGPFP
✓	11	402.2154	802.4162	802.4185	-2.80	0	36	0.72	1	GKEKVNE
✓	109	492.2898	982.5650	982.5488	16.5	0	35	0.66	1	QTPVVVPPF
✓	324	661.4000	1981.1782	1981.0157	82.0	0	35	0.9	1	PVHESLSIENTLWASTVV
✓	82	464.2419	926.4693	926.4684	1.00	0	34	0.98	1	YMLPPGLH
✓	238	707.8674	1413.7203	1413.7075	9.10	0	34	1.2	1	AVPYPQRDMPIQ
✓	69	442.2710	882.5274	882.4377	102	0	33	1.3	1	MIKTMML + Oxidation (M)
✓	72	448.7322	895.4499	895.5201	-78.44	0	32	1.3	1	LVIPMLNP
✓	181	630.3262	1258.6379	1258.6405	-2.09	0	32	1.6	1	QDKTEIPTINT
✓	47	422.6821	843.3497	843.3320	20.9	0	31	2.1	1	FVEESSM + Oxidation (M)
✓	305	580.3180	1737.9322	1737.9348	-1.52	0	31	2.2	1	MYAARNGHPQVVALLV
✓	78	453.2463	904.4780	904.4695	9.44	0	31	2.1	1	FVAPFPEV
✓	120	526.8207	1051.6269	1051.6179	8.55	0	30	2.3	1	GPVRGPFPII
✓	96	491.2095	980.4044	980.5113	-109.10	0	30	2.6	1	VPKHKEMP + Oxidation (M)
✓	197	648.3683	1294.7220	1294.7034	14.3	0	30	2.8	1	QEPVLGPVRGPF
✓	84	470.2783	938.5421	938.5338	8.86	0	30	2.4	1	GPVRGPFPI
✓	262	761.4654	1520.9163	1520.7844	86.8	0	30	3.2	1	MTQVVLRGGGFLPM + Oxidation (M)
✓	36	412.8003	823.5861	823.4552	159	0	29	2.1	1	INHALKE
✓	199	432.8855	1295.6345	1295.7489	-88.26	0	29	3	1	EILLKGPDWIL
✓	286	850.9972	1699.9798	1699.9695	6.07	0	29	3.3	1	PISMVLPQVIGYRLV + Oxidation (M)
✓	315	462.1510	1844.5750	1844.9744	-216.51	0	29	3.8	1	IIDNFNQQKKKLGGQD

✓	322	652.2884	1953.8434	1953.9023	-30.16	0	29	3.6	1	NSLDCEIVSAKSSEMNVL + Oxidation (M)
✓	141	557.8135	1113.6124	1113.6070	4.84	0	29	3.1	1	LYQEPVLGPV
✓	50	428.2490	854.4835	854.4902	-7.84	0	29	2.9	1	TPVVVPPF
✓	111	499.2596	996.5046	996.5426	-38.12	0	29	3.2	1	TVIIIMNHG
✓	290	428.9000	1711.5709	1711.8464	-160.96	0	29	3.6	1	HAQSVTCLRWGGDGLL
✓	158	579.3294	1156.6442	1156.6241	17.5	0	29	3.5	1	YQEPVLGPVR
✓	32	405.3081	808.6015	808.5058	118	0	29	2.3	1	IEPLLPK
✓	145	560.7912	1119.5679	1119.5601	6.97	0	28	3.7	1	APFPEVFGKE
✓	150	566.3066	1130.5987	1130.5819	14.8	0	28	4.1	1	DKTEIPTINT
✓	159	584.3357	1166.6569	1166.6448	10.4	0	28	4.1	1	EPVLGPVRGPF
✓	153	570.8021	1139.5896	1139.3925	173	0	28	4	1	DEKMGGEDDE + Oxidation (M)
✓	203	433.3000	1296.8782	1296.6285	193	0	28	4.3	1	FSPVGMGHLHVT + Oxidation (M)
✓	12	402.2173	802.4200	802.4185	1.93	0	28	4.4	1	GKEKVNE
✓	62	434.2209	866.4273	866.2964	151	0	28	3.7	1	DMNDLDE + Oxidation (M)
✓	27	403.7080	805.4014	805.4010	0.41	0	27	5.3	1	APFPEVF
✓	76	453.2141	904.4136	904.3774	40.0	0	27	5.3	1	TEQESGASP
✓	185	635.3141	1268.6136	1268.6877	-58.44	0	27	5.5	1	IDLRGPPGPPGPP
✓	279	408.1000	1628.3709	1628.7823	-252.61	0	27	5.3	1	GDMMTLLMKKDTLT + 2 Oxidation (M)
✓	293	859.5097	1717.0048	1716.9927	7.08	0	27	6.2	1	QEPVLGPVRGPFPIIV
✓	161	590.7469	1179.4793	1179.6759	-166.71	0	26	6.3	1	MKFLCILLLA + Oxidation (M)
✓	41	416.1977	830.3808	830.3294	61.9	0	26	6.7	1	EDVPNEE
✓	332	903.4184	2707.2334	2707.4367	-75.10	0	26	10	1	GALKIKEITYMHSEGILAGELKHGP + Oxidation (M)
✓	7	402.2078	802.4010	802.4297	-35.73	0	26	6.9	1	QGEKVSR
✓	211	666.3376	1330.6607	1330.4871	130	0	26	7.3	1	DEEEMLYGDSGS
✓	113	501.2612	1000.5078	1000.5018	5.99	0	26	6.9	1	YPFPGPIPN
✓	209	661.4000	1320.7854	1320.6642	91.8	0	26	7.1	1	KMVREANMKQA + Oxidation (M)
✓	135	549.7948	1097.5750	1097.6444	-63.21	0	26	7	1	KNIQQLIEL
✓	139	554.2890	1106.5634	1106.5140	44.6	0	25	7.2	1	KPYPMEPMV + Oxidation (M)
✓	70	444.2407	886.4669	886.3821	95.7	0	25	7.6	1	DIGGWENP
✓	264	764.8654	1527.7162	1527.7062	6.57	0	25	7.8	1	DCLEVVDVHTCKAP
✓	31	405.1938	808.3730	808.3603	15.8	0	25	6.6	1	DKESSFP
✓	168	609.3331	1216.6516	1216.5369	94.3	0	25	8.3	1	PFSGMLHMGQP + Oxidation (M)
✓	280	818.8806	1635.7467	1635.8515	-64.07	0	25	9	1	DIFGDLRKMNKRQ + Oxidation (M)
✓	148	564.2734	1126.5322	1126.5982	-58.63	0	25	8.3	1	SLLNAQGVQPT
✓	289	428.9000	1711.5709	1711.7216	-88.03	0	25	9.4	1	QPTCSTSSTCQATCVPV
✓	241	720.8556	1439.6967	1439.6932	2.39	0	25	9.1	1	DVPSERYLGYLE
✓	86	472.2190	942.4234	942.4732	-52.89	0	24	9.5	1	VPEIMPEL + Oxidation (M)
✓	274	536.9502	1607.8287	1607.8058	14.3	0	24	10	1	KEMPFPKYPVEPF
✓	9	402.2135	802.4124	802.4297	-21.54	0	24	9.7	1	QGEKVSR
✓	317	467.2000	1864.7709	1864.9867	-115.73	0	24	11	1	ETSHRRGPEKRSVIDV
✓	312	604.9649	1811.8728	1811.8764	-1.95	0	24	11	1	YIISCHPVGIDEEPLQ
✓	107	491.2178	980.4211	980.4927	-73.00	0	24	9.9	1	VPDPQAQNL
✓	188	428.2197	1281.6372	1281.6288	6.52	0	24	10	1	MHQPHQPLPPT
✓	166	607.3219	1212.6292	1212.6213	6.48	0	24	11	1	LPPTVMFPPQS
✓	288	853.6595	1705.3045	1704.8352	275	0	24	13	1	KSMGLPTSDEQKKQE

✓	299	432.8855	1727.5127	1727.9318	-242.55	0	24	13	1	LARTIEYLQPNPASR
✓	118	521.2704	1040.5263	1040.5219	4.22	0	24	11	1	FLFPEFEI
✓	273	803.9362	1605.8578	1605.7920	41.0	0	24	12	1	GVSPVLAAIDKSSSME + Oxidation (M)
✓	207	658.3255	1314.6364	1314.7184	-62.30	0	24	12	1	QTQLLFGAVELP
✓	282	838.3996	1674.7847	1674.9192	-80.35	0	23	14	1	TLKDIFKDLNVGVVD
✓	101	491.2157	980.4169	980.3757	42.1	0	23	12	1	QDTDINME + Oxidation (M)
✓	103	491.2157	980.4169	980.4671	-51.14	0	23	12	1	CLKVKEMD + Oxidation (M)
✓	99	491.2136	980.4127	980.5477	-137.67	0	23	13	1	VVRPTPCLP
✓	254	744.9305	1487.8464	1487.8209	17.1	0	23	14	1	PGPQGIGGQRGVVGLP
✓	97	491.2095	980.4044	980.5403	-138.66	0	23	13	1	VPREPVER
✓	291	429.0912	1712.3358	1712.7927	-266.75	0	23	16	1	SSALEDNCKTFSTTLP
✓	198	432.8855	1295.6345	1295.5128	93.9	0	23	14	1	ENWGSDFLCPE
✓	229	688.3745	1374.7344	1374.6099	90.6	0	23	15	1	RNSGNHCGIASYP
✓	189	428.9000	1283.6782	1283.5816	75.2	0	23	15	1	APEICQEHSGTL
✓	133	542.8123	1083.6100	1083.5271	76.6	0	23	14	1	TLMTLNVGGY + Oxidation (M)
✓	67	440.2362	878.4578	878.4605	-3.15	0	22	14	1	DIMKMLL + Oxidation (M)
✓	230	688.3844	1374.7543	1374.6099	105	0	22	16	1	RNSGNHCGIASYP
✓	325	1021.5567	2041.0989	2041.0269	35.3	0	22	17	1	TLNISPNLWVPPGGHVEPD
✓	297	430.9000	1719.5709	1719.7444	-100.86	0	22	17	1	PNKEAAAGSSDLDPSMM
✓	222	676.3969	1350.7793	1350.7547	18.2	0	22	16	1	DKIAKYIPIQY
✓	219	673.8554	1345.6962	1345.6911	3.80	0	22	17	1	QELTKNSSVMLP
✓	75	452.1232	902.2318	902.4241	-213.13	0	22	14	1	PMLEMAVP + Oxidation (M)
✓	147	563.7791	1125.5437	1125.7319	-167.19	0	22	16	1	RKRKRIRN
✓	307	585.9994	1754.9762	1754.9766	-0.22	0	22	19	1	RTLLLHPAIFGLHHM
✓	167	607.3358	1212.6571	1212.6543	2.33	0	22	18	1	LVYPFPGPIPN
✓	232	690.4211	1378.8277	1378.6286	144	0	22	18	1	VILNSGSDMVEAE + Oxidation (M)
✓	100	491.2136	980.4127	980.4927	-81.57	0	22	18	1	VPAQPGQTSP
✓	25	402.2400	802.4655	802.4259	49.4	0	22	18	1	VVACAVIE
✓	275	403.1000	1608.3709	1608.6218	-156.00	0	22	16	1	GDMAELMGVQDQHM + 3 Oxidation (M)
✓	330	661.4000	2641.5709	2641.3138	97.3	0	22	35	1	YPCLSPKSDQMMEKNKGLRTKR + 2 Oxidation (M)
✓	146	561.2909	1120.5672	1120.4239	128	0	21	19	1	EYAMMVSMGA + 2 Oxidation (M)
✓	216	669.8562	1337.6978	1337.5888	81.5	0	21	18	1	NNFSGNSLPEYP
✓	61	433.3000	864.5854	864.4085	205	0	21	18	1	LCDCTVLV
✓	271	533.2000	1596.5782	1597.0000	-264.17	0	21	20	1	ARIIAIVSVMVILIS
✓	267	783.4290	1564.8434	1564.8357	4.91	0	21	20	1	MDMLFPGSIALKKV + Oxidation (M)
✓	336	882.8000	3527.1709	3527.7044	-151.24	0	21	51	1	HRNIAYDEGFIIRHFAGAVCYETTQFVEKN
✓	311	601.2965	1800.8678	1800.8788	-6.13	0	21	22	1	NDTELVACLRTRPAQD
✓	95	491.2053	980.3960	980.5542	-161.40	0	21	19	1	VPLNLPESL
✓	272	801.4597	1600.9049	1600.7944	69.0	0	21	22	1	LEPEQSTSNLNEKI
✓	17	402.2230	802.4314	802.3933	47.5	0	21	22	1	RLDSQNA
✓	224	680.5365	1359.0585	1358.7194	250	0	21	16	1	NFAKDSVNPGVLV
✓	42	416.2131	830.4117	830.3480	76.7	0	21	23	1	PESACVPE
✓	65	437.2244	872.4342	872.3521	94.1	0	21	24	1	CLRGMDY + Oxidation (M)
✓	48	423.2268	844.4391	844.4654	-31.11	0	20	25	1	TAALQAASL
✓	63	434.2446	866.4746	866.3878	100	0	20	20	1	VSMSMAPL + 2 Oxidation (M)

✓	303	433.3000	1729.1709	1728.9397	134	0	20	26	1	NHPLHFRQNRRAAL
✓	329	806.6000	2416.7782	2417.2737	-204.99	0	20	56	1	KGNPGLPGKPGLTGPPGLKGSIGDMG + Oxidation (M)
✓	144	559.2644	1116.5143	1116.5676	-47.70	0	20	27	1	KGPEEPAPRH
✓	235	466.1231	1395.3475	1395.7445	-284.48	0	20	21	1	GHCVLVTLALHHP
✓	309	882.8000	1763.5854	1763.8229	-134.63	0	20	28	1	FSSSWLQMFVAEAVF + Oxidation (M)
✓	287	851.0110	1700.0074	1699.8161	113	0	20	29	1	PDIDAEKLMCPQEIV
✓	125	533.1712	1064.3278	1064.5906	-246.90	0	20	26	1	IDLFVPKFS
✓	121	533.1668	1064.3191	1064.5576	-224.09	0	20	26	1	FITNLMDVL
✓	122	533.1668	1064.3191	1064.5576	-224.09	0	20	26	1	FITNLMDVL
✓	149	564.2756	1126.5367	1126.4965	35.7	0	20	28	1	PTAACQDVEPP
✓	123	533.1690	1064.3234	1064.5179	-182.67	0	19	27	1	NDIVFDPFV
✓	130	533.1777	1064.3409	1064.5365	-183.71	0	19	27	1	KKGYLFMY + Oxidation (M)
✓	202	433.2000	1296.5782	1296.7264	-114.29	0	19	31	1	YCYARILAVLL
✓	60	433.2000	864.3854	864.3825	3.42	0	19	30	1	QSSLTSQD
✓	129	533.1777	1064.3409	1064.5576	-203.57	0	19	29	1	FITNLMDVL
✓	98	491.2115	980.4085	980.4927	-85.83	0	19	31	1	VPDPQAQNL
✓	73	450.2665	898.5185	898.4106	120	0	19	30	1	FVTMSGEL + Oxidation (M)
✓	334	989.1278	2964.3615	2964.6106	-84.02	0	19	67	1	QKLIQQKEYAKQVKEYNMKALSIPS
✓	83	467.2000	932.3854	932.4096	-25.86	0	19	34	1	AMGPSACPSL
✓	18	402.2230	802.4314	802.4185	16.1	0	19	33	1	LVGDGGTGK
✓	59	433.2000	864.3854	864.4705	-98.39	0	19	32	1	SKSLNIFG
✓	308	880.4808	1758.9471	1758.8909	31.9	0	19	37	1	IVATTCMGINHPIFSR
✓	19	402.2230	802.4314	802.3821	61.4	0	19	34	1	KELGGGDGA
✓	190	430.8802	1289.6188	1289.6108	6.23	0	19	34	1	AACAVKPQEVQM + Oxidation (M)
✓	227	682.4306	1362.8467	1362.8388	5.85	0	19	38	1	VLGPVRGPFPIIV
✓	276	806.6000	1611.1854	1610.9369	154	0	19	37	1	RWAGVAKGVGTQRIL
✓	327	1121.0284	2240.0422	2240.3117	-120.31	0	19	42	1	HRKATGPVVEQAVRALVLVPT
✓	186	639.8477	1277.6809	1277.5566	97.3	0	18	37	1	AENEMPGLMRM
✓	49	425.2201	848.4256	848.4062	22.9	0	18	36	1	DIMNNKV + Oxidation (M)
✓	326	533.2000	2128.7709	2128.1754	280	0	18	55	1	LRALAFLACAFLLTTALYGT
✓	56	431.6739	861.3332	861.4749	-164.43	0	18	40	1	ALFVGWVA
✓	81	463.2132	924.4118	924.4705	-63.46	0	18	34	1	PYGLSFNK
✓	300	433.1473	1728.5600	1728.8829	-186.77	0	18	42	1	PQLVAAVVRTMQNTSD
✓	104	491.2157	980.4169	980.6382	-225.68	0	18	38	1	VPVILRALT
✓	196	648.2791	1294.5437	1294.6088	-50.30	0	18	40	1	RLGFRDGSCDGL
✓	117	515.4037	1028.7928	1028.5138	271	0	18	29	1	QQLAEAEAAV
✓	195	430.9057	1289.6954	1289.5057	147	0	18	40	1	MHYSSDTMTAF
✓	20	402.2230	802.4314	802.3531	97.6	0	18	42	1	LDCELGPG
✓	22	402.2249	802.4352	802.3933	52.2	0	18	42	1	RSVENQA
✓	231	688.5284	1375.0422	1374.7215	233	0	18	38	1	SAGDGARVTRLSVS
✓	323	657.2774	1968.8103	1969.0732	-133.50	0	18	47	1	IDLIDSLRSLGVEQDLAL
✓	214	446.2508	1335.7306	1335.7108	14.8	0	18	43	1	FLVMELVKGGDL + Oxidation (M)
✓	128	533.1755	1064.3365	1064.5179	-170.36	0	18	40	1	NDIVFDPFV
✓	164	403.1000	1206.2782	1206.5490	-224.45	0	18	32	1	RSEPSDHSRH
✓	193	430.8900	1289.6483	1289.6463	1.53	0	18	44	1	VTSKKPKGESDD

✓	263	508.8935	1523.6586	1523.7687	-72.30	0	18	46	1	SVKETKMLMAAGDK + Oxidation (M)
✓	249	495.5843	1483.7310	1483.6072	83.5	0	18	46	1	CNMSFTSAVVADSH + Oxidation (M)
✓	142	559.2533	1116.4920	1116.5564	-57.71	0	18	47	1	VVFSHLSAGNS
✓	23	402.2249	802.4352	802.4450	-12.18	0	18	46	1	PAKFRGQ
✓	126	533.1734	1064.3322	1064.5178	-174.42	0	18	42	1	LVEYLNEW
✓	37	413.2136	824.4127	824.4466	-41.06	0	18	37	1	YLLKMAA + Oxidation (M)
✓	284	845.6712	1689.3279	1688.8628	275	0	17	55	1	CLNNITNRTAKGQKE
✓	66	437.2303	872.4460	872.3521	108	0	17	49	1	CLRGMDY + Oxidation (M)
✓	246	734.3797	1466.7448	1466.7551	-7.05	0	17	48	1	RSMFLRGEEILT + Oxidation (M)
✓	301	433.2000	1728.7709	1728.8352	-37.22	0	17	51	1	IAMTPPNATEASKPQGT + Oxidation (M)
✓	183	422.2174	1263.6304	1263.6711	-32.17	0	17	48	1	LIDLPDNPPASL
✓	331	882.8000	2645.3782	2645.3853	-2.71	0	17	63	1	SVLIVSSVGAYHPFPNLGPYNVSKT
✓	35	408.2195	814.4244	814.4800	-68.20	0	17	50	1	ASVIALEL
✓	16	402.2211	802.4276	802.4086	23.7	0	17	51	1	QSRAVGW
✓	88	476.2071	950.3997	950.4354	-37.53	0	17	48	1	MLQCNVFP
✓	321	486.0507	1940.1738	1940.1968	-11.85	0	17	56	1	MLAGGGLKVRLLKKALEK + Oxidation (M)
✓	236	467.2000	1398.5782	1398.6892	-79.36	0	17	54	1	HNDHASTPLLPTP
✓	256	500.5731	1498.6974	1498.7814	-56.00	0	17	59	1	VSVEHVAEMLRTI + Oxidation (M)
✓	91	476.2587	950.5029	950.4532	52.4	0	17	52	1	AVMYGGGPIS
✓	171	408.1000	1221.2782	1221.6064	-268.66	0	17	38	1	NAMDVVVQFAI + Oxidation (M)
✓	106	491.2178	980.4211	980.6131	-195.77	0	17	55	1	VPRNLVGKV
✓	108	491.2178	980.4211	980.5542	-135.76	0	17	56	1	VPDPILLTN
✓	205	434.8815	1301.6228	1301.6139	6.83	0	17	58	1	YNIDYITSSIN
✓	245	733.8312	1465.6478	1465.5928	37.5	0	17	60	1	QTMSFCIPTEYM + Oxidation (M)
✓	6	401.2143	800.4140	800.4181	-5.09	0	17	57	1	WGAAAVGLG
✓	132	533.2000	1064.3854	1064.4808	-89.58	0	16	55	1	AAAVGAMEDKS + Oxidation (M)
✓	302	433.2000	1728.7709	1728.8950	-71.76	0	16	64	1	FVVYTLLPFSMWGAV
✓	102	491.2157	980.4169	980.4671	-51.15	0	16	59	1	MLLVGCKSD + Oxidation (M)
✓	194	430.9000	1289.6782	1289.6173	47.2	0	16	62	1	KDPETQETVLM
✓	201	433.2000	1296.5782	1296.5656	9.69	0	16	61	1	LAMHYTADTSTA + Oxidation (M)
✓	226	681.8451	1361.6756	1361.5809	69.5	0	16	62	1	LFPTTGPGCEDEP
✓	44	421.2189	840.4232	840.4705	-56.26	0	16	53	1	GAKLQPLD
✓	71	448.2594	894.5043	894.5763	-80.52	0	16	54	1	RLAPRLGI
✓	328	773.6785	2318.0138	2318.1361	-52.74	0	16	77	1	FQDTTMIMIGMVSFGSGALLLA + Oxidation (M)
✓	85	471.2184	940.4222	940.5593	-145.81	0	16	60	1	IIVKSSPTP
✓	38	413.2867	824.5589	824.5120	56.9	0	16	51	1	LNPAGILK
✓	281	829.3985	1656.7825	1656.8107	-17.04	0	16	66	1	LFVQYETSVNTSRN
✓	191	430.8861	1289.6365	1289.6736	-28.81	0	16	66	1	MKFPMSHLRK + Oxidation (M)
✓	26	403.1000	804.1854	804.3688	-227.89	0	16	45	1	QPMEVSV + Oxidation (M)
✓	21	402.2230	802.4314	802.4297	2.10	0	16	67	1	QGEKVSR
✓	253	744.5820	1487.1494	1486.8620	193	0	16	52	1	IGNKVNIFSRQLV
✓	13	402.2192	802.4238	802.4072	20.7	0	16	68	1	DENLILS
✓	140	555.2850	1108.5554	1108.5764	-19.01	0	16	65	1	LLVHPVTDSE
✓	295	859.5263	1717.0381	1716.9232	66.9	0	16	75	1	LPLILMHPKPSNEAAS
✓	68	441.2411	880.4676	880.5018	-38.89	0	16	62	1	APQLPVGIS

✓	265	767.9242	1533.8338	1533.8014	21.2	0	16	74	1	PFPCPLDSLRVPPP
✓	58	433.1492	864.2839	864.4453	-186.74	0	16	69	1	KHAINGPE
✓	178	625.3320	1248.6494	1248.6271	17.8	0	15	73	1	DLLSVIEEMGK + Oxidation (M)
✓	192	430.8900	1289.6483	1289.5412	83.1	0	15	76	1	ESGSSVPWSEEP
✓	8	402.2116	802.4086	802.3933	19.1	0	15	78	1	AGEGGRKE
✓	250	743.1552	1484.2958	1484.6929	-267.48	0	15	47	1	SQCDIGHGIILNSE
✓	215	446.2508	1335.7306	1335.6857	33.6	0	15	80	1	KNSMIWINTTL + Oxidation (M)
✓	115	504.7513	1007.4880	1007.4574	30.4	0	15	75	1	GGGWGQPHVGG
✓	14	402.2192	802.4238	802.3933	38.0	0	15	82	1	AGEGGRKE
✓	124	533.1690	1064.3234	1064.6342	-291.91	0	15	76	1	AKLLKADLHG
✓	200	432.8874	1295.6404	1295.5486	70.9	0	15	84	1	EPMSSRISNME + Oxidation (M)
✓	143	559.2577	1116.5009	1116.4724	25.6	0	15	91	1	PVPSSDHSYE
✓	10	402.2135	802.4124	802.2981	143	0	15	92	1	QEGDPEE
✓	4	400.1936	798.3727	798.3074	81.7	0	14	81	1	MSGGICIC + Oxidation (M)
✓	285	846.3866	1690.7587	1690.7587	-0.02	0	14	1e+002	1	GAYPSPGQPSGAGAYPGAS
✓	119	523.3870	1044.7595	1044.4546	292	0	14	89	1	KCVDLEHSD
✓	80	456.2137	910.4128	910.4695	-62.28	0	14	80	1	PRGPMPSGI
✓	278	541.9465	1622.8176	1622.8535	-22.16	0	14	98	1	RSMLDARSRFRGR + Oxidation (M)
✓	105	491.2178	980.4211	980.4637	-43.44	0	14	96	1	IVNNMFID + Oxidation (M)
✓	89	476.2071	950.3997	950.4565	-59.76	0	14	94	1	ALQMGVSVM + Oxidation (M)
✓	94	489.2762	976.5378	976.4138	127	0	14	96	1	SGEPGSPYSP
✓	172	614.3217	1226.6289	1226.5457	67.8	0	14	97	1	MASREDLMKM + Oxidation (M)
✓	5	401.2067	800.3989	800.3851	17.2	0	14	97	1	PLCAAAAQG
✓	218	672.5374	1343.0602	1342.8118	185	0	14	64	1	AARLGITMKAKLA
✓	45	421.2247	840.4349	840.4130	26.0	0	14	87	1	FSGFRDL
✓	24	402.2306	802.4466	802.4185	35.0	0	14	1e+002	1	TVGGQLGTA
✓	213	446.2508	1335.7306	1335.6306	74.8	0	14	1.1e+002	1	QDGAELYEAVKN
✓	29	404.1776	806.3406	806.3480	-9.18	0	14	1.1e+002	1	LASMGEPS + Oxidation (M)
✓	306	581.3280	1740.9621	1740.9192	24.6	0	14	1.2e+002	1	AILPRSNMINTGPTLQ + Oxidation (M)
✓	52	430.1716	858.3287	858.3616	-38.28	0	14	1.2e+002	1	MVFSMQT + Oxidation (M)
✓	57	432.8815	863.7485	863.4939	295	0	14	13	1	AFCLALKV
✓	46	421.2305	840.4465	840.5685	-145.08	0	14	97	1	VVVTVIVL
✓	298	862.0048	1721.9950	1721.8947	58.2	0	14	1.2e+002	1	EEKAEDKEVKKAYR
✓	335	806.6000	3222.3709	3222.5855	-66.60	0	14	2.9e+002	1	KDRECNPVKATRAFALFFFPFNSCGTTR
✓	296	430.8900	1719.5310	1719.9155	-223.56	0	14	1.2e+002	1	SYTLAALGDLAQTLGRA
✓	260	504.2371	1509.6894	1509.8514	-107.33	0	13	1.2e+002	1	KVKLAPEEEARLQ
✓	165	606.2919	1210.5692	1210.5005	56.7	0	13	1.1e+002	1	SSCMGGMNRRP + Oxidation (M)
✓	30	404.1928	806.3710	806.3996	-35.48	0	13	1.2e+002	1	PNGIYLM
✓	310	595.9708	1784.8906	1784.8767	7.76	0	13	1.4e+002	1	QPLTLEQHKGYMVEP + Oxidation (M)
✓	333	678.0981	2708.3631	2708.2460	43.2	0	13	1.7e+002	1	GSSNSGGGSGGGSSSNMPASVAHVPAAVLPP + Oxidation (M)
✓	243	485.4000	1453.1782	1452.7687	282	0	13	67	1	TAWVTLQVLLYM + Oxidation (M)
✓	261	505.5592	1513.6558	1513.7343	-51.86	0	13	1.4e+002	1	SVGGVCYLSMGMVVL
✓	87	476.1968	950.3791	950.4206	-43.70	0	13	1.3e+002	1	ASGPDRDPH
✓	15	402.2192	802.4238	802.4953	-89.06	0	13	1.4e+002	1	AQFVLLL
✓	28	404.1700	806.3254	806.4538	-159.24	0	12	1.5e+002	1	PISPTPVP

✓	34	408.1000	814.1854	814.4185	-286.10	0	12	93	1	AVQAINEA
✓	187	640.7933	1279.5720	1279.6628	-71.00	0	11	1.9e+002	1	TRSLSMCLLLQ + Oxidation (M)
✓	79	454.2155	906.4165	906.4368	-22.46	0	11	2e+002	1	MDLSVETL
✓	127	533.1734	1064.3322	1064.4995	-157.15	0	11	2.1e+002	1	ITMNGQVIAC + Oxidation (M)
✓	179	626.3046	1250.5947	1250.5788	12.7	0	11	2.2e+002	1	MENKMSFIVH + Oxidation (M)
✓	92	476.7172	951.4198	951.4774	-60.53	0	10	2.2e+002	1	AIAQVHAEN
✓	131	533.1777	1064.3409	1064.5325	-179.94	0	10	2.5e+002	1	IGNQINMFL + Oxidation (M)
✓	40	415.1793	828.3441	828.3357	10.1	0	10	2.7e+002	1	LMSMSASS + Oxidation (M)
✓	54	430.8881	859.7616	859.5201	281	0	10	23	1	LLKLVMAG + Oxidation (M)
✓	90	476.2113	950.4080	950.3764	33.3	0	10	2.7e+002	1	RAVCGEDDS
✓	33	407.1060	812.1974	812.4392	-297.64	0	10	1.7e+002	1	DAREPII
✓	43	417.2308	832.4471	832.3749	86.7	0	9	3e+002	1	GGGQALMSP + Oxidation (M)
✓	55	430.9000	859.7854	859.5378	288	0	7	14	1	IIISSSLK
✓	51	428.9000	855.7854	855.5290	300	0	4	1e+002	1	KALQGLRA
✓	53	430.8861	859.7577	859.5167	280	0	4	1.4e+002	1	LLVAAFNL
✓	1	*446.1169*	*445.1096*							
✓	2	*446.1209*	*445.1136*							
✓	3	*446.1209*	*445.1136*							

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : None
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 300 ppm
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages    : 1
Instrument type         : ESI-QUAD-TOF
Number of queries       : 336
```

Mascot: http://www.matrixscience.com/

APPENDIX-III Animal Ethics

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

MEMO

TO A/Professor Michael Mathai
HES School of Biomedical and Health Sciences
St Albans Campus
Victoria University

DATE 19 July 2013

FROM A/Prof Alan Hayes
Deputy Chair
Victoria University AEEC

SUBJECT **Ethics Application AEETH 09/12**

Dear Michael

AEETH 09/12 Assessment of antihypertensive activity of fermented milk peptides (AEEC 12/52)

The revised application was assessed and the Executive Committee resolved to **approve** the application between 19th July 2013 and 19th July 2015.

Continued approval of the project is conditional upon the following:

- Any variation proposed to the project, and the reasons for that change, must be submitted to the AEEC for approval and must not be implemented until approval is granted.
- Annual and Final Reports should be supplied promptly to the Secretary of the AEEC.
- The project should only be conducted in approved premises nominated on the Licence SPPL 77. Use of other premises would constitute a variation and relevant details are to be notified to the AEEC for approval as "field work".
- The AEEC must be notified in writing of:
  - Any changes to the following approved personnel listed on the application
  - Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.
- Should the numbers of animals treated exceed that estimated for the first year of the ethics application, the primary investigator should submit a request for a minor amendment to update the numbers accordingly.

On behalf of the Committee, I wish you all the best for the conduct of the project.

If you have any further queries, please do not hesitate to contact me.

Kind Regards,

A/Professor Alan Hayes
Deputy Chair,
Animal Experimentation Ethics Committee
Victoria University

Clinical Animal Monitoring Sheet AEETH No. 09/12 Project Title: Assessment of antihypertensive activity of fermented milk peptides

Animal Species: **Strain:** **Sex:** **Age:**

Emergency Contacts	Name	Phone number(s)
Chief Investigator	Michael Mathai	Office: +61 3 99192211 Mobile: 0414718748
Other Primary contacts	Fatah Ahtesh	Office AND Mobile: 0469808940

Each animal will be examined and observed for abnormalities daily. As they are in a shared cage there will be a general check for food and water consumption by weighing the food and water. We do not expect these animals to become unwell on the proposed diets. A weekly body weight and measurement of blood pressure will be performed. Animals will also be given plastic tubes and shredded paper for nest-building and cardboard boxes for enrichment.

Observations will be recorded in the table below. Normal clinical signs are recorded as 0 Abnormalities are recorded as a number reflecting the score given see clinical signs severity score below

Intervention Points and Humane endpoints

Clinical symptoms	Action
Intervention Points: Symptoms requiring increased observation **OR** treatment **OR** removal from study.	We will indicate on check list if animal is considered Normal or Abnormal by writing, 0 or the score if an abnormality is detected Additionally, comments will be included for any abnormal results and the actions that were taken (e.g. Stopping procedure on animal). Both the cumulative and single score is considered and the intervention relates to the highest score (a single score of 2 or 3 will override a cumulative score of 1-3
Single score 1	Single score 1 Place in a separate box – weigh, review and rescore twice daily. Supportive care such as jelly/mash to be provided Monitor food and water consumption by weighing daily the food and water – this will be compared with the known expected food and water consumption based on other animals within the study as food and water is weighed daily for each box of animals. If the animal deteriorates it will be euthanased. If the animal improves and no longer requires individual monitoring it will be returned to group housing.
Single score 2	Single score 2 Place in a separate box –review and rescore every hour . Weigh twice

Cumulative score 1-3 Cumulative score 3-8	daily. Supportive care such as jelly/mash to be provided Monitor food and water consumption by weighing daily the food and water – this will be compared with the known expected food and water consumption based on other animals within the study as food and water is weighed daily for each box of animals. If the animal deteriorates it will be euthanased. If the animal fails to show improvement after 24 hours it will be euthanased Cumulative score 1-3 Place in a separate box – weigh, review and rescore twice daily. Supportive care such as jelly/mash to be provided Monitor food and water consumption by weighing daily the food and water – this will be compared with the known expected food and water consumption based on other animals within the study as food and water is weighed daily for each box of animals. If the animal deteriorates it will be euthanased. If the animal improves and no longer requires individual monitoring it will be returned to group housing. Cumulative score 3-8 Place in a separate box –review and rescore every hour day. Supportive care such as jelly/mash to be provided Weigh twice daily. Monitor food and water consumption by weighing daily the food and water – this will be compared with the known expected food and water consumption based on other animals within the study as food and water is weighed daily for each box of animals. If the animal deteriorates it will be euthanased. If the animal fails to show improvement after 24 hours it will be euthanased
<u>Humane Endpoints</u>: Single score 3 **Single score 2 for >24 hrs** **Cumulative score greater than or equal to 9**	animal will be immediately euthanased Anaesthetic overdose with I.P. Pentabarbitone (100 mg/kg) followed by cardiac puncture.

LIST any **SAMPLES** (blood /tissues) required if emergency euthanasia is performed. ...Nil

Rat Box#	Groups	Starting Weight:	Week No.		Week No.	
Rat #			Date		Date	
			Weight		Weight	

Daily Check	Date													
Clinical Observation Score General Clinical Signs														
Coat –,														
Activity –														
Breathing -														
Movement/gait -														
Eating & Drinking														
Alertness-														
Body weight loss-														
Diarrhoea														

CLINICAL SIGNS SEVERITY SCORE

SIGNS	0 – Normal	1 minor change from normal	2 moderate change from normal	3 severe – immediate euthanasia
Coat	Normal Smooth Clean	Coat rough	Unkempt; wounds, hair thinning	Severe Hair loss, Bleeding, Infected Wounds
Activity	Normal Movement & Response	Isolated, abnormal posture	Huddled/inactive or overactive	Moribund or fitting
Breathing	Normal Easy, unhindered	Rapid and shallow	Rapid, abdominal breathing, Noisy	Laboured, irregular, skin blue
Movement/gait	Normal, free to move, stretch	Abnormal gait, limited stretch.	Limited movement, Unable to stretch	Immobile

Eating & Drinking-Water and food consumption weighed Daily	Diet may result in altered Food Consumption	Increased or decreased intake over 24 hrs	Increased or decreased intake over 48 hrs	Constantly drinking or not drinking over 24 hours, obese or in appetence
Alertness	Normal Eyes Bright, Observant	dull or depressed	Little response to handling	unconscious
Body weight loss – referenced to highest recoded weight	Normal weight/growth rate	Less than 10%	10-15%	Greater than 15%
Diarrhoea	Normal Formed Dry Faeces	Soft, moist faeces	unformed, prolonged over 3days	Liquid with or without blood

APPENDIX-IV Human Ethics

Ethics Application - Approved

Quest.Noreply@vu.edu.au [Quest.Noreply@vu.edu.au]

You forwarded this message on 29/07/2013 12:09 PM.

Sent: Friday, 7 June 2013 9:37 AM
To: Vijay.Mishra@vu.edu.au
Cc: fateh.ahtesh@live.vu.edu.au

Dear DR VIJAY KUMAR MISHRA,

Your ethics application has been formally reviewed.

Application ID: HRE13-079
Application Title: Assessing the sensory characteristics of skim milk fermented by proteolytic dairy cultures

The application has been accepted and deemed to meet the requirements of the National Health and Medical Research Council (NHMRC) 'National Statement on Ethical Conduct in Human Research (2007)' by the Victoria University Human Research Ethics Committee. Approval has been granted for two (2) years from the approval date; 07/06/2013.

Continued approval of this research project by the Victoria University Human Research Ethics Committee (VUHREC) is conditional upon the provision of a report within 12 months of the above approval date or upon the completion of the project (if earlier). A report proforma may be downloaded from the Office for Research website at: http://research.vu.edu.au/hrec.php.

Please note that the Human Research Ethics Committee must be informed of the following: any changes to the approved research protocol, project timelines, any serious events or adverse and/or unforeseen events that may affect continued ethical acceptability of the project. In these unlikely events, researchers must immediately cease all data collection until the Committee has approved the changes. Researchers are also reminded of the need to notify the approving HREC of changes to personnel in research projects via a request for a minor amendment. It should also be noted that it is the Chief Investigators' responsibility to ensure the research project is conducted in line with the recommendations outlined in the National Health and Medical Research Council (NHMRC) 'National Statement on Ethical Conduct in Human Research (2007).'

On behalf of the Committee, I wish you all the best for the conduct of the project.