RELEASE OF BIOACTIVE PEPTIDES FROM MILK PROTEINS BY *LACTOBACILLUS* SPECIES

A thesis submitted in completion of requirements

of the degree of Master of Science

By

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I. ABSTRACT

Proteolytic activity is very important characteristic of Lactic Acid Bacteria (LAB) They produce therapeutic benefits and also increase physiological activity of cultured dairy products by liberating a number of biologically active peptides. The main aim of this project was to determine the release of bioactive peptides from milk proteins by selected *Lactobacillus* species. Ten strains of *Lactobacillus* species (*Lactobacillus helveticus* 474, *Lactobacillus helveticus* 1188, *Lactobacillus helveticus* 1315, *Lactobacillus helveticus* 953, *Lactobacillus delbrueckii ssp. bulgaricus* 734, *Lactobacillus delbrueckii ssp. bulgaricus* 756, *Lactobacillus delbrueckii ssp. bulgaricus* 857, *Lactobacillus delbrueckii ssp. lactis* 1210, *Lactobacillus delbrueckii ssp. lactis* 1307, *Lactobacillus delbrueckii ssp. lactis* 1372 were assessed for growth characteristics, proteolytic activity and release of *in vitro* angiotensin-converting enzyme inhibitory peptides in reconstituted skim milk (RSM). One percent of each culture was initially propagated three times successfully in MRS broth at 37°C. After that, milk with14% total solid milk was inoculated with 1% of each activated strain and cell growth, pH changes, proteolytic and ACE-inhibitory activities were assessed during 0, 2, 4, 6, 8, 10 and 12 h of incubation at 37^{o}C. The viability of selected *Lactobacillus* species and their proteolytic activities were individually assessed in fermented milk at different incubation times stated. All selected strains achieved the desired level of 6.00 log cfu/g in the milk as growth medium. *Lactobacillus* strains showed good growth ability during incubation period regardless of termination pH. The cell counts of *L. helveticus* strains increased to 10^{9} by the end of incubation. The presence of selected LAB enhanced proteolysis significantly compared to the control. The proteolytic activity varied with changes in pH, but was also appeared to be strain dependent. The increase of proteolysis improved survival of all selected strains during incubation time resulting in further decrease in pH

compared to the control, whose pH was constant. All strains released bioactive peptides with ACE-inhibitory activities between 1.26 and 48.69%. Inhibition of angiotensin I-converting enzyme (ACE) activity results in an overall antihypertensive effect. Among ten strains used in this study, three strains of *L. helveticus* (474, 1188 and 1315) that showed high proteolytic and antihypertensive activity were selected for further studies. Their enzyme activity and effect of crude proteinase extract (CPE) on production of antihypertensive, antioxidative and immunostimulative peptides from milk proteins were evaluated. Aminopeptidase activity was found in both extracellular (EE) and intracellular extract (IE) in various extents in all three selected *L. helveticus* strains, while only oligopeptidase activity was observed in EE. Antioxidative activity was evaluated using DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical model system to determine the free radical scavenging ability of bioactive peptides produced by crude proteinases extracted from the selected strains. Antioxidative activity of all soluble freeze dried samples at 12 h was significantly ($P < 0.0001$) higher than samples at 0 and 6 h under the same condition, while the control had no activity was observed. The highest development of DPPH scavenging and ACE-I activity were observed in the soluble freeze dried peptides of CPE of *L. helveticus* 1188 compared to the other strains used at 12 h of incubation time. These activities appeared to be time and strain specific. The effects of soluble peptides produced by CPEs of individually selected *L. helveticus* strains on cytokine production by human peripheral blood mononuclear cells (PBMCs) were determine by ELISA (Enzyme-linked immunosorbent assay) method. Effects of soluble peptide samples on the stimulation of all tested cytokine Th2 Interleukin-10 and Th1 Interferon-γ production were detected in varied levels at 6 and 12 h. These bioactive peptides might have capability to drive immune responses in opposite directions *in vitro* and thus may bring about imbalance in the Th1/Th2 type cytokines.

II. Declaration

"I, , declare that the MSc thesis entitled "Release of Bioactive Peptides from Milk Proteins by *Lactobacillus* Species " is no more than 60,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature: Date:

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To my mother **Sabria** and wife, **Laila**

To my sons and daughters (**Malak, Ahmed, Alaa and Farah**)

To my brothers and sisters,

I dedicate this simple work

Khaled Elfahri

Werribee, Australia

Date:

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List of abbreviations

ACE-I = angiotensin-converting enzyme inhibitory
ATP = Adenosine triphosphate
ABC = A member of the Binding Cassette
BSA = blood serum albumin
BP = blood pressure
β- CN = beta casein
α- CN = alpha casein
Cfu = colony forming units
CPE = crude proteinase extract
Ca = calcium
CE = capillary electrophoresis
^{0}**C** = degree Celsius
CaCl = calcium chloride
CN = casein
CVD = cardiovascular diseases
DPPH = 1, 1-diphenyl-2-picrylhydrazyl
EE = extracellular extract
ELISA = enzyme-linked immunosorbent assay
EDTA = ethylene diamine tetra-acetic acid
FDA = food and drug administration
f = fragment
GIT = gastrointestinal tract
g = gram
HIV = human immune deficiency virus
HCl = hydrochloric acid
h = hour
HEPES = (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)
IL-10 = interleukin 10
IPP = Ile-Pro-Pro
IE = intracellular extract
IFN-γ = interferon gama
IMDM = Iscoves Modified Dulbeccos Medium
kDa = kilo Dalton
κ- CN = kappa- casein
LAB = Acid Bacteria
L = *Lactobacillus*
L. del = *Lactobacillus delbrueckii*
Lc = lactococcus
LPS = lactose permease
MRS = de Mann Rogosa and Sharpe
µ = micro
mM = millimolar
M = molar
mM = millimolar
mL = millilitre
min = minute
NH3 = amino groups
NaCl = sodium chloride

NK = natural killer
OPA = o-phthaldialdihyde
Opp = oligopeptides
PBMCs = human peripheral blood mononuclear cells
PEP-PTS = phosphoenolpyruvate dependent-phosphatotransferase system
PAGE = polyacrylamide gel electrophoresis
PrtP = proteinase
PepA = aminopeptidase A
PepC = aminopeptidase C
PepL = aminopeptidase L
PepN = aminopeptidase N
PepP = aminopeptidase P
PepX = aminopeptidase X
PepV = dipeptidase V
PepD = dipeptidase D
PepT = tripeptidase T
PepI = proiminopeptidase
PepQ = prolidase
PepR = prolinase
PepF = endopeptidase F
PepO = endopeptidase O
PepE = endopeptidase E
PepG = endopeptidase G
PNA = para-nitroanilide
rpm = revolution per minute
RSM = reconstituted skim milk
RP-HPLC = reverse phase- high performance liquid chromatography
sp. = species
ssp. = subspecies
St. = *streptococcus thermophilus*
S= *Saccharomyces cerevisiae*
Th = T helper cells
TCA = trichloroacetic acid
TFA = trifluoroacetic acid
UV = ultra violet
v/v = volume per volume
VPP = Val-Pro-Pro
VIC = Victoria
w/w = weight per weight
WHO = World Health Organization
x g = times gravitational force

Chapter 1

Introduction to Thesis

1.1. Background

Increased public consciousness of diet related health issues has resulted in a consumers' orientation towards healthy foods. Numerous scientific studies have confirmed that many chronic diseases including osteoporosis, cancer, coronary heart diseases and hypertension are linked to unbalanced diet. Furthermore, some reported that milk and other dairy products have long been recognized as a significant component of a balanced diet. Milk is a natural source which contains various essential nutrients and biologically active compounds with potential health benefits (Rogelj, 2000, Shah, 2000, Lourens-Hattingh and Viljoen, 2001b). Epidemiological studies have reported that individuals who constantly consumed milk were much less likely to suffer from heart attack than those who did not (Rogelj, 2000). Similarly some other studies have shown that people, who consumed dairy products had a lower incidence of diabetes type II (Korhonen, 2009a). Fairly recently, milk proteins have been recognized as one of the most significant sources of bioactive peptides. Upon consumption, peptides with potent physiological activities may be liberated from milk proteins by the action of proteolytic enzymes in the gut and thus influence the major body's systems including endocrine, nervous, digestive, cardiovascular and immune systems (Clare and Swaisgood, 2000, Pihlanto and Korhonen, 2003, Meisel, 2005, Silva and Malcata, 2005, Pihlanto, 2006b).

Milk is an excellent source of highly valuable proteins which are in general divided into caseins and whey proteins. Caseins and whey proteins comprise approximately 80% and 20%, respectively, of total milk proteins (Haque and Chand, 2006). Numerous health advantages of milk protein derived bioactive peptides have been claimed for commercial interests in the environment of health sustaining-functional foods (Pihlanto, 2006b). Möller et al (2008) defined bioactive peptides as substances that can affect the biological processes of

the body functions with beneficial effects. Dziuba and Darewicz (2007) reported that bioactive peptides are protein sequences that remain inactive in the native protein primary structure, but when released, for example by proteolytic enzymes, may regulate the most body's physiological functions. Bioactive peptides have been isolated from many protein sources such as soy proteins, gelatine, fish proteins; and maize, but milk proteins appear to be the most important sources of bioactive peptides identified thus far (Farnworth, 2003).

Milk proteins have been recognised as potential sources of biological active peptides that are latent and encrypted in their native form. These biologically active peptides can be generated and activated by different mechanisms including: (a) protein hydrolysis by digestive enzymes (b) food processing and (c) proteolytic activity by enzymes derived from microorganisms, especially lactic acid bacteria. Potent biologically active peptides have been isolated from a number of fermented dairy products such as cheese, fermented milk and yoghurt (Korhonen and Pihlanto, 2006). Due to growth requirements, dairy starter cultures have developed highly sophisticated proteolytic system capable of breaking down milk proteins, mainly α_{s1}- and β-caseins. The lactic acid bacteria (LAB) proteolytic structure and their activities in dairy products including yoghurt and cheese have been studied extensively (Christensen et al., 1999).

Lactobacillus (*L.*) strains are the most important starter cultures used in traditional fermented milk manufacturing. Their application mainly stems from two important properties: rapid utilization of lactose (milk sugar) leading to fast acidification of milk as growth medium, and highly developed proteolytic system capable of supplying essential amino acids required by a fast growing organism (Kunji et al., 1996). A number of small and oligo-peptides with different physiological functions has been released from milk proteins through microbial proteolysis and has been well recognized and assessed (Hannu, 2009). A number of scientific studies was conducted over the past several years and they have

confirmed that *L. helveticus* strains, in particular, were able to form antihypertensive peptides from milk proteins, including Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) with demonstrated *in vivo* antihypertensive activity in a rat model and human studies (Masuda et al., 1996, Seppo et al., 2003, Hirota et al., 2007). Also yoghurt starter cultures and commercial probiotic bacteria have been verified to form different bioactive peptides in milk during fermentation (Donkor et al., 2007b). Virtanen et al (2007) also showed that a single industrial dairy culture generated antioxidant activity in the whey protein fractions during milk fermentation. The activity was positively correlated with the degree of proteolysis suggesting that peptides were responsible for the antioxidative property. Similarly Chen et al (2007) observed that a commercial starter culture mixture consisting of five LAB strains released peptides that increased Angiotensine Converting Enzyme inhibitory (ACE-I) activity of the final hydrolyzate. A body of literature on this topic is extensive and covered into a greater detail in Chapter 2. For example, Donkor et al (2007b) studied growth, proteolytic and *in vitro* ACE inhibitory activities in milk fermented by several dairy LAB cultures and probiotic strains (*L. acidophilus*, *Bifidobacterium. lactis*, *L. casei*). Again they found that *Lactobacillus* strains showed the greatest ACE-inhibitory activity. Pihlanto-Leppala et al (1998) studied the potential of *in vitro* ACE-inhibitory peptides released from cheese whey and caseins during fermentation by the action of various commercial dairy starters used in the manufacture of yoghurt, ropy milk and sour milk. While there was no ACE-inhibitory activity detected initially, after adding pepsin and trypsin, as digestive enzymes, to the hydrolysates, several strong ACE-inhibitory peptides were produced and identified.

Much of the work has been conducted on detection and identification of bioactive peptides with various physiological properties. Furthermore sources of these peptides have also been suggested. Pihlanto-Leppälä et al (1998) found that ACE inhibitory peptides were primarily released from α_{s1}-casein and β-casein. However the role of cell wall bound

proteases and intracellular peptidases in liberation and further hydrolysis has not been assessed in a great detail. For instance, Kilpi et al. (2007) studied the influence of general aminopeptidase (PepN) and X-prolyl dipeptidyl aminopeptidase (PepX) activities of *L. helveticus* CNRZ32 strain on the ACE-inhibitory peptides produced in fermented milk by taking advantage of peptidase-negative derivatives of the same strain. They found that milk fermented by the peptidase deficient mutants, may increase ACE-inhibitory activity. These results suggest that PepN and PepX were involved in the release or degradation of ACE-inhibitory peptides during the fermentation process. Similarly Donkor et al (2007b) observed a decline in ACE inhibitory activity of yoghurt stored over a period of time, which suggested that bacterial peptidases were responsible for continuing hydrolysis and thus inactivation of previously released bioactive peptides. Therefore understanding the properties and function of different proteases and peptidases in relation to kinetics of bioactive peptide released, corresponding physiological activity and stability would be imperative for appropriate strain selection with a defined physiological benefit.

1.2 Research objectives

The main objective of the project was to assess the potential of highly proteolytic strains of *Lactobacillus* species (sp.) to liberate novel peptides encrypted in milk proteins, with potent physiological activities.

The specific objectives were:

(1) To assess proteolytic and peptidase activities of *Lactobacillus* sp. cultivated in milk.

(2) To establish the kinetics and character of liberated oligopeptides that served as bioactive peptides precursors.

(3) To investigate the released bioactive peptides with different physiological benefits from milk based system by selected proteolytic *Lactobacillus* strains during cultivation.

CHAPTER 2

Literature Review

2.1. Functional foods

Modern era migrations and industrialization have introduced new eating habits followed by innovative production and processing of foods, consumption of which have had substantial social and health impacts. Metabolic syndrome has been related to high energy dense foods, and thus an unbalanced diet has become a major health related challenge in most developed countries around the world. New eating habits in the European Union, for example, has caused a rise in modern era diet underlined diseases and conditions such as obesity, osteoporosis, cancer, diabetes, allergies and dental problems. For instance, more than 11 million American people in the United States have type-2 diabetes (Cordain et al., 2005). Moreover, as per a recent WHO report (2010), more than 1 billion adults have been deemed overweight globally, which reached epidemic levels. About 1/3 of them are clinically obese. Obesity is a medical condition diagnosed by genesis or increasing of body fat to the critical level that it may have an adverse effect on health, and subsequently lead to reduced life expectancy and/or increased health problems (Haslam and James, 2005). Almost 300,000 deaths each year and $117 billion in the United States alone are related to health ailments with obesity as underlining cause (Yanovski, 1996). Furthermore, Soedamah-Muthu et al (2011) and Erdmann et al (2008) reported that cardiovascular diseases are the primary cause of death in the Western countries.

Recently, a great deal of attention has been paid by food scientists, nutritionists and health professionals to functional foods and biological active compounds that can potentially reduce the risk of chronic diseases beyond their basic nutritional functions (Parvez et al., 2006). A wide variety of foods has been recognized as functional food with a range of components affecting a vast number of human physiological functions relevant to either a state of well-being and health and/or to the reduction of the risk of a disease. As a consequence, the term “functional foods” has been defined in a number of different ways.

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

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

a. It includes a food component (being nutrient or not) which have positive effects on one or a limited number of function(s) in the body.

b. It has physiological or psychological implication as a result of the traditional nutritional effect.

Collectively, functional foods should have a positive impact on well-being and health or lead to a reduction of risk of many diseases. The biological active substances in functional foods can be either an essential macronutrient if it has specific physiological effects or an essential micronutrient if its intake is over and above the daily recommendations. Additionally, it seems that food component can be functional even though some of its nutritive value is not listed as essential, such as some oligosaccharides, or it is of non-nutritive value, such as live microorganisms or plant chemicals (Roberfroid, 1999). The major types of functional foods are indicated in Table 2.1.

Table 2.1 Different types of functional foods (Source from Spence, 2006)

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

The demand for functional foods and bioactive components in the food industry has drown great attention from consumers, food scientists and nutritionist. Steady growth rates of market sales for functional foods have been reported (Marketresearch.com, 2008). In 2008, the global functional foods market occupied very important section in the food industry. Rapid growth is expected to continue in the following years. In 2010, functional foods are expected to represent 5% of the total global food market. Currently, the predicted sales of global functional foods market are between US $ 7 – 63 billion, depending on sources and definitions of functional foods (Marketresearch.com, 2008). It was expected that this market would reach US$ 167 billion by 2010 (Park, 2009) due to consumer demand for solutions that address long and short term health ailments.

Functional foods are also well-known as designer foods, medicinal or therapeutic foods (Shah, 2001). Nutritionally important foods, i.e. dairy products, may become functional if modified in a particular way such as by addition of LAB (Shah, 2007). Dairy products fermented by LAB are probably the most important among functional foods since Metchnikoff postulated underlining reasons for the relationship between long life of

Bulgarians peasants and their consumption of fermented milk containing LAB (Kailasapathy and Chin, 2000). Milk fermented by LAB has been known since thousands of years to preserve milk for prolonged storage. In addition to the preservation role from spoilage, fermented milk has been recognized to have other functionalities for human health. Degradation of milk proteins during fermentation is a potential means to improve their nutritional value for both humans and animals (Kilpi et al., 2007). Recently, a great attention has been paid to milk protein hydrolysis as potential ingredients to health-promoting functional foods targeting diet-related chronic diseases, such as cardiovascular disease, diabetes mellitus type 2 (Mensink, 2006, Korhonen, 2009b) and obesity (Korhonen, 2009b, Tudor et al., 2009). Rachid (2006) reported that a diet rich in cultured dairy products may inhibit the proliferation of many cancerogenous cells. The same author also stated that the epidemiological studies had suggested that the oral intake of LAB dairy products may minimize the incidence of colon cancer. Similarly, Mensink (2006) reported that the consumption of skimmed fermented dairy products such as yoghurt was associated with reducing the risk of development of type 2 diabetes. It has also been reported that there was a relationship between low fat dairy products consumption and the possibility of reducing the overweight syndrome (Korhonen, 2009b). Furthermore, oral administration of milk and milk products has been linked with the reduction of hypertension. All these health beneficial effects may be due to the biological compounds derived from milk proteins hydrolysis and other effectors, such as the weight control effects of milk calcium. These protein-derived compounds known as bioactive peptides may exert a number of activities affecting the digestive, endocrine, cardiovascular, immune and nervous systems under *in vitro* and *in vivo* conditions.

Bioactive peptides were first reported in 1950 when casein-derived phosphorylated peptides enhanced vitamin D-independent calcification in rachitic infants upon ingestion

(Hayes et al., 2007). Fitzgerald and Murray(2006) defined bioactive peptides as 'peptides with hormone- or drug-like activity that eventually regulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses'. In recent years, a number of *in vitro* studies has been provided evidence for the existence of biological active peptides and proteins derived from foods that might have beneficial effects on human health (Möller et al., 2008). These primary studies have opened a new scientific field to examine the production of bioactive peptides from many types of dietary proteins. Proteins in the diet have been increasingly acknowledged and confirmed by new scientific findings as a great value of vital source of amino acids and biologically active substances (Korhonen, 2009a). These biologically active peptides are hidden in their parent protein sequence and can be released by gastrointestinal tract (GIT) enzymes, food processing and fermentation. Various health benefits including anticarcinogenic, weight management, antithrombotic, antioxidative, immunomodulatory and antihypertensive properties, have been reported (Shah, 2000, Korhonen, 2009b).

2.2. Sources of bioactive peptides

In addition to milk proteins, as an important source of bioactive peptides, plants such as wheat, maize, soy, rice, mushroom, pumpkin and sorghum, as well as meat, fish, eggs from animals have been identified as other sources of bioactive peptides (Möller et al., 2008). Milk as a complete diet for infants consists of critical nutritive elements including lactose, fat and proteins, required for their growth and development. Milk proteins are the most important constituents of milk due to their nutritional, physiological and functional properties, which are extensively used in the food industry. These properties include:

- High heat stability - heat treatment allows dairy products to be sterilized without major changes in the physical property of milk.

- Coagulability with Ca^{++} following limited rennet-induced proteolysis, which is exploited in the manufacture of a wide range of cheeses and some functional proteins.
- Coagulability at their isoelectric point (pH 4.6), which is used in the making of many types of fermented dairy products (Fox, 2001).

Based on chemical, physical properties and their biological function, milk proteins can be classified in various ways. The old classification, which milk proteins grouping into casein, albumin and globulin, has given way to a more adequate classification system. Table 2.2 shows an abbreviated list of milk proteins according to a modern and widely accepted system.

Table 2.2 Modern classification of bovine milk proteins (Vasiljevic and Shah, 2009)

Type of protein	g/Kg
Total protein	35.1
Total Caseins	28.6
Alpha S_1	11.5
Alpha S_2	3.0
Beta	9.5
kappa	3.4
β casein	1.2
Total Whey Proteins	6.1
alpha lactalbumin	1.2
beta lactoglobulin	3.1
Proteose peptone	1.0
Immunoglobulin	0.8
Serum albumin	0.4

Caseins

In all mammals, milk caseins are a family of phosphoproteins. They exist in milk as complex micelles of the proteins and mineral calcium phosphate. About 80% of total milk proteins are casein proteins in bovine, ovine, caprine, and buffalo milk. α_{s1}- and α_{s2}-caseins

(CN), ß-CN and κ-CN are the principal casein fractions (Swaisgood, 1992, Fox et al., 2000). Moreover, bovine caseins contain minor proteins as a result of limited proteolysis by plasmin. The action of plasmin on αs_1-CN and β-CN produces λ-caseins and γ-caseins and proteose peptones, respectively (Swaisgood, 1992, Fox and McSweeney, 1997). The isoelectric point of casein is 4.6. Casein has a negative charge in milk at pH 4.6. The purified protein is not soluble in water. Even though it is insoluble in neutral salt solutions as well, with dilute alkalis and salt solutions such as sodium oxalate and sodium acetate, it is readily dispersible (from, http://en.wikipedia.org/wiki/Casein, 2010). It is important to note that many distinguishing properties of casein proteins are based on their charge distribution and as well as their sensitivity to calcium precipitation within the group of caseins. Most of milk caseins exist in a colloidal particle recognized as the casein micelle. The biological function of the casein micelle is to convey amounts of highly insoluble colloidal calcium phosphate (CCaP) to all mammalian young in liquid form and to form a clot in the stomach for required nutrition. Moreover, the micelle also contains enzymes such as lipase and plasmin enzymes, in addition to citrate, minor ions, and entrapped milk serum.

It is thought that there are two different kinds of casein sub micelle; with and without κ-casein. Aggregation of the submicelles occurs via calcium phosphate bridges, hydrophobic interaction, and hydrogen bonds. The hydrophobic core of the submicelles is composed of the calcium-sensitive caseins and the N terminus of κ-CN. The hydrophilic C-terminus of κ-CN protrudes from the micelle surface, forming a hairy layer that prevents further aggregation of the submicelles (Figure 2.1).

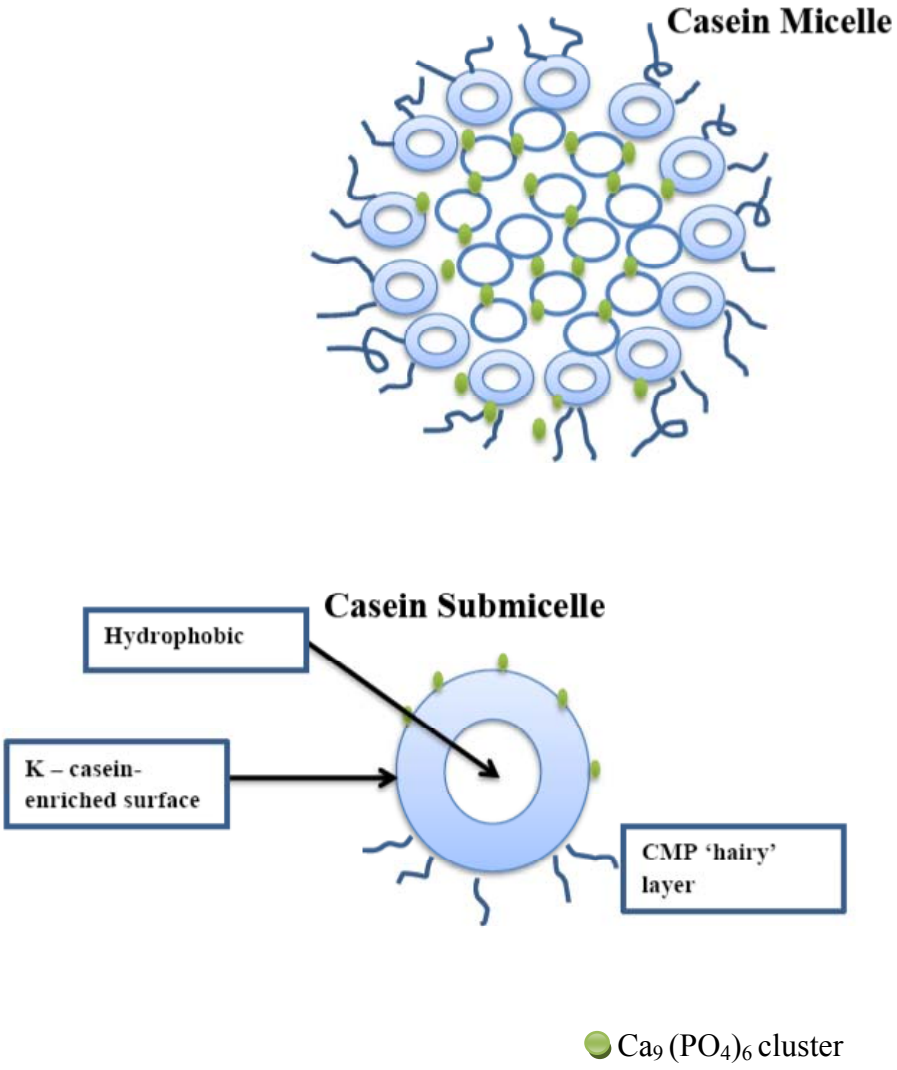


Figure 2.1 The structure of casein micelle in the sub-micelles model showing the protruding C-terminal parts of κ-casein as proposed by Walstra (adapted from Walstra, 1999).

In contrast to Walstra (1990), Holt (1992) suggested that calcium phosphate nanoclusters are the centres from which casein micelles grow. Caseins bind to the calcium phosphate via phosphoserine residues to form submicelles, which coalesce gradually due to hydrophobic interaction. The κ-CN has a tendency to be on the outside, while the minerals tend to be associated with the phosphoserine residues of the caseins. In this model, calcium

acts as a negative-charge neutralizer instead of a cross-linker. The resulting micelles have discontinuous distribution of caseins and calcium phosphate.

Although many types of sub-micelle models have been described, recent studies using improved microscopes have failed to confirm the presence of sub-micelles; in the irregularities were considered to be microtubules. Three alternatives to the sub-micelle models depict the micelle as being made up of casein molecules linked together by CCaP microcrystals and hydrophobic bonds but differ in detail. Further refinement of these models can be expected, especially as electron microscopes are improved (Fox and Brodkorb, 2008).

The following factors must be considered when assessing the stability of the casein micelle: (http://www.foodsci.uoguelph.ca/dairyedu/chem.html#protein2, 2010).

- Calcium - high percentage of calcium in skim milk (more than 90%) is linked with the casein micelle. Losing of Ca++ results in reversible dissociation of ß-CN without micellar disintegration, while adding Ca++ leads to aggregation.
- Hydrogen bonding - this happens between the individual caseins in the micelle but does not occur to a great extent due to lack of secondary structure in four milk caseins.
- Disulphide bonds - both αs_1 and ß-CN do not have any cysteine residues. If there is any creation of S-S bonds within the micelle, these would not be the driving force for stabilization.
- Hydrophobic interactions of caseins - as one of the most hydrophobic proteins, these interactions have a role in the stability of the micelle.
- Electrostatic interactions - some of the subunit interactions may be the result of ionic bonding, but the overall micellar structure is very loose and open.
- Salt content effects - salt content may affect the calcium activity in the serum in addition to calcium phosphate content of the micelles.

- Changing in pH - decreasing pH results in dissolution of calcium phosphate until at isoelectric point (pH 4.6), all phosphate is dissolved and the caseins precipitate.
- Temperature - at 4 °C, β-casein starts to split up from the micelle. It is important to note that there is no micellar aggregation at 0 °C; freezing produces a precipitate called cryo-casein.
- Heat treatment – during heat treatment whey proteins become denatured and interact with the micelle surface, altering the behaviour of the micelle.
- Dehydration - adding alcohol, ethanol for example, leads to aggregation of the micelles.

Whey proteins

In bovine milk, whey proteins comprise of four main types of proteins including β - lactoglobulin (β - Lg, 50%), α-lactalbumin (α-La, 20%), blood serum albumin (BSA, 10%), Lactoferrin (Lf) and immunoglobulins (Ig, 10%; mainly IgG1, with lesser amounts of IgG2, IgA and IgM). In human milk, there is no β -Lg and the principal Ig is IgA. The principal whey proteins are well characterized (Ha and Zemel, 2003). Whey proteins own secondary, tertiary and in most cases, quaternary structures in high levels. It has been reported that whey proteins are typical globular proteins and denature upon heating e.g. at 90°C for 10 min (Fox, 2001). Whey proteins are also not phosphorylated and insensible to Ca^{++} (Fox, 2001). They have the most important biological role, such as carrying of calcium, zinc, copper, iron and phosphate ions in the body. They also play a biological activity as an important source of a number of different bioactive peptides (Korhonen et al., 1998). Dropping pH at 4.6 by acidification or rennet coagulation allows keeping whey proteins in solution. However, other methods such as ultra-centrifugation, gel filtration as well as membrane technologies are ways that can be used to separate whole caseins from whey proteins (Léonil et al., 2000).

Milk and dairy products have been reported as an excellent source of biological active peptides. Table 2.3 and 2.4 list the main types of bioactive peptides with their physiological functions derived from different types of commercial fermented dairy products. Among these diversities of bioactive substances released, only a few of them have been acknowledged, such as hypotensive peptides, which have been assessed clinically in animal and human studies (Jauhiainen and Korpela, 2007, Murray and FitzGerald, 2007, Korhonen, 2009b). Therefore, these bioactive fractions serving antihypertensive effect may supply a healthy and natural alternative for antihypertensive medicines. These properties of lactic acid bacteria utilizing milk proteins to produce various chains of peptides during fermentation depend on their proteolytic system which is different from one species to another and also from strain to strain in the same species.

Table 2.3. Some examples of the identified bioactive peptides in fermented milk and their corresponding physiological activity (Vasiljevic and Shah, 2008)

Sequence	Microbial agent	Precursor	Bioactivity
Val-Pro-Pro Ile-Pro-Pro	*L. helveticus* CM4 & *Saccharomices cerevisae*	β -& κ-casein	Hypotensive
Val-Pro-Pro Ile-Pro-Pro	*L. helveticus LBK16H*	β -& κ-casein	Hypotensive
Phe-Pro-Glu-Val-Phe-Glu-Lys	Commercial products+ digestion	α_{s1}-casein	ACE inhibition
Lys-Val-Leu-Pro-Val-Pro-Glu	Commercial products+ digestion	β –casein	Antioxidative
Lys-Thr-Thr-Met-Pro-Leu-trp	Commercial products+ digestion	α_{s1}-casein	Possible Immunomodulation
Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu	*L. helveticus* NCC2765	β –casein	ACE inhibition
Tyr-Pro-Phe-Pro-Glu-Pro-Ile-Pro-Asn	*L. helveticus* NCC2765	β –casein	Opioid
Tyr-Pro	*L. helveticus* CPN4	Caseins	ACE inhibition
Leu-Asn-Val-Pro-Gly-glu-Ile-Val-glu	*L. delbrueckii ssp. bulgaricus* SS1	β –casein	ACE inhibition
Asn-Ile-Pro-Pro-Leu-Thr-Glu-Thr-Pro-Val	*Lc. lactis ssp. cremoris* FT4	β –casein	ACE inhibition

Table 2.4 Commercial dairy products and ingredients with health or function claims based on bioactive peptides (Korhonen, 2009a)

Brand name	Type of product	Bioactive peptides sequence	Health function claims	Manufacturer
Calpis	Sour milk	Vall-Pro-Pro, Ill-ProPro	Reduction of blood pressure	Calpis Co, Japan
Evolus	Ca enriched fermented milk drink	Vall-Pro-Pro, Ill-ProPro	Reduction of blood pressure	Valio, Finland
BioZate	Hydrolysed WPI	β-Lactoglobulin fragments	Reduction of blood pressure	Davisco, USA
BioPURE-GMP	WPI	κ-Casein f(106–169) (Glycomacropeptide)	Prevention of dental caries, influence the clotting of blood, protection against viruses and bacteria	Davisco, USA
PRODIET F200/Lactium	Flavoured milk drink	α_{s1}-casein f(91–100) (Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg)	Reduction of stress effects	Ingredia, France
Festivo	Fermented low-fat hard cheese	α_{s1}-casein f(1–6), f(1–7), f(1–9)	No health claim	MTT Agrifood Research Finland
Capolac	Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
PeptoPro	Ingredient/ hydrolysate	Casein derived peptide	Improves athletic performance and muscle recovery	DSM Food Specialties, the Netherlands
Vivinal Alpha	Ingredient/ hydrolysate	Whey derived peptide	Aids relaxation and sleep	Borculo Domo Ingredients (BDI), the Netherlands
Recaldent	Chewing gum	Calcium casein peptonecalcium Phosphate	Anticariogenic	Cadbury Adams, USA

2.3. Properties of lactic acid bacteria

The lactic acid bacteria are defined as Gram-positive cocci or rods with a low-GC. These are acid-tolerant, generally non-spore forming bacteria and associated by their common metabolic and physiological characteristics. Furthermore, LAB can be found in spoiling plants and lactic products which produce lactic acid as the major metabolic end-product as a result of carbohydrate fermentation. This characteristic has, throughout the history, associated LAB with fermented food industry, as acidification impedes the growth of other microbial spoilage organisms. Several strains of LAB produce proteinaceous bacteriocins that create an additional hurdle for spoilage and pathogenic microorganisms. Moreover, it seems that lactic acid and other metabolic activity products contribute to the organoleptic and textural profile of a food item. Due to their ubiquitous presence in fermented foods and their contribution to the intestinal microflora of human mucosal surfaces, the industrial importance of the LAB is more manifested by their generally recognized as safe (GRAS) status. The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order *Lactobacillales* (Sonomoto, 2011).

Two main pathways for hexose fermentation have been used to classify LAB genera. Under certain conditions (excess glucose and limited oxygen), homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas path to produce two moles of pyruvate. Intracellular redox balance can be maintained through the oxidation of NADH, associated with pyruvate reduction to produce lactic acid. This process yields two moles of ATP per

mole of glucose consumed. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and group I lactobacilli.

Heterofermentative LAB use the second pathway which is called pentose phosphoketolase pathway. In this pathway, one mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and afterward decarboxylated to yield one mole of CO_2. Pentose-5-phosphate formed is split into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP also subjected to further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. In theory, end-products (including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli (Sonomoto, 2011).

2.4. Lactose metabolism

The importance of LAB in the dairy industry for the production of fermented products has led to extensive research on the lactose metabolism of LAB (Figure 2.2). Two systems for lactose transport and metabolism have been established for dairy lactic acid bacteria: (i) a phosphoenolpyruvate-lactose phosphotransferase system (PEP-PTS) with a phospho-β-galactosidase enzyme, found in the *lactococci* and *L casei*, and (ii) a lactose permease system with a β-galactosidase, found in the thermophilic *L bulgaricus*, *L helveticus*, and *St thermophilus* and the mesophilic *Leuconostoc lactis* (Vaughan et al., 1996).

Lactose is taken inside the cell either via the PEP-PTS or by lactose permease systems. During the transport in the cell membrane, lactose translocated via PEP-PTS system is phosphorylated, and when inside the cell, cleaved by phospho-β-galactosidase. The glucose

formed is metabolized by enzymes through the glycolytic pathway, while resulting galactose is converted into tagatose and cleaved into trioses, entering the glycolytic pathway (Cocaign-Bousquet et al., 1996).

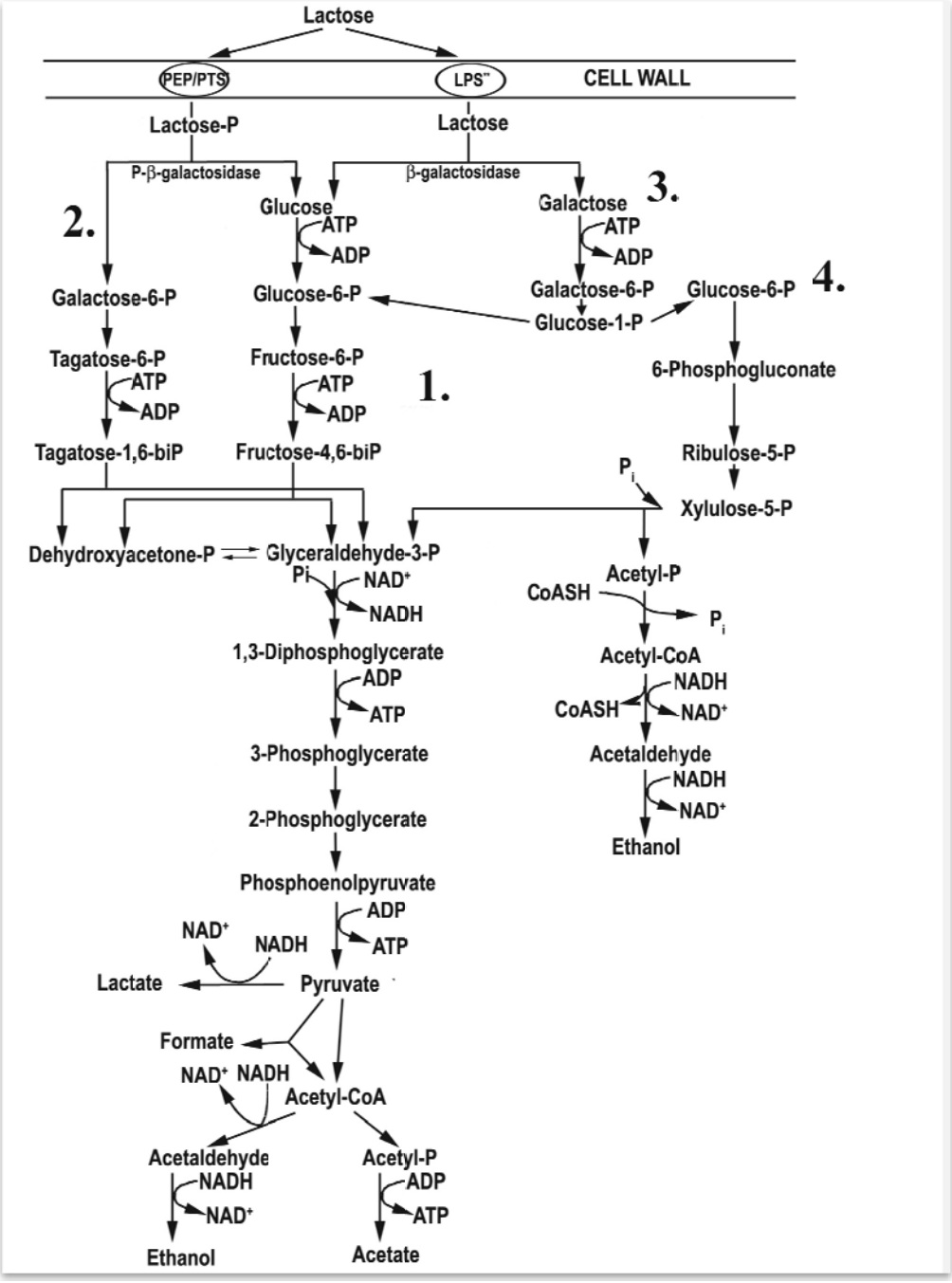


Figure 2.2 Lactose metabolism by lactic acid bacteria: 1. Embden-Meyerhof-Parnas pathway (glycolysis); 2. tagatose pathway; 3. LeLoir pathway; 4. phosphoketolase pathway; *PEP-PTS – phosphoenolpyruvate dependent-phosphatotransferase system; **LPS – lactose permease (Vasiljevic and Shah, 2009).

2.5. Genus *Lactobacillus*

Lactobacillus sp. have widely been used in animal feeds, milk and many dairy products. Many species of this genus are commercially used as starter cultures in fermented dairy production such as cheeses, sour milk and yoghurt. Although some *Lactobacillus* sp. have been recognized as aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, others have been identified as strictly anaerobic. The optimum pH for *lactobacilli* growth is between 5.5 and 5.8 and they have intricate nutritional needs for amino acids, peptides, vitamins, minerals, fatty acids and carbohydrates (Axelsson, 2004).

Based on fermentation patterns, the genus of lactobacilli can be divided into three groups including (1) homofermentative, this group produces more than 85% lactic acid from glucose; (2) facultative heterofermentativewhich produces only 50% lactic acid and considerable quantities of ethanol, acetic acid and carbon dioxide); and (3) obligate heterofermentative species, in this group, species produce DL-lactic acid, acetic acid and other chemicals such as formic acid, acetone, acetaldehyde, diacetyl, etc).

Lactobacilli are widely spread in nature and many species have been used in the food industry. The beneficial health roles of some *Lactobacillus* sp. have been confirmed in clinical studies. For example the ability of lactobacilli to convert lactose to lactic acid has been used as a preventive measure in the alleviation of lactose intolerance. *Lactobacillus* sp. also have ability to inhibit the growth of harmful pathogenic microorganisms through reduction of pH of the intestinal tract, some strains are able to produce bacteriocins and other metabolic products such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2) and diacetyl (Ouwehand and Vesterlund, 2004). The role of H_2O_2 as bactericidal effect has been attributed to its strong oxidizing effect on the microbial cells. Some of the H_2O_2 creating anaerobic condition that is unfavourable for certain organisms. It has also been suggested that H_2O_2

production by lactobacilli is important for colonization of the urinary and genital tract. Colonization of lactobacilli may have a role to reduce the acquisition of human immune deficiency virus (HIV) infection, gonorrhoea and urinary tract infections (Fontaine et al., 1999). It has been reported that production of CO_2 provides anaerobic atmosphere and in addition that CO_2 itself has an antimicrobial activity (Ouwehand and Vesterlund, 2004). Some strains of *Lactobacillus* such as *L. acidophilus*, *L. casei*, *L. helveticus*, *L. delbrueckii*, and *L. lactis* have been found to produce bacteriocins. This production of bacteriocins offers a more defined antimicrobial spectrum, ranging from only related strains to a wide variety of Gram positive and Gram negative bacteria (Ouwehand and Vesterlund, 2004). *Lactobacilli sp* have been used role restore intestinal flora in the digestive tract after antibiotic treatment. Some representatives of such bacteria include strains of *L. acidophilus* and *L. rhamnosus* and they have been approved as dietary supplements by Food and Drug Administration (FDA). Lactobacilli are normally found in the mouth, intestinal tract and vagina, where they play major part in preventing from mouth sores and vaginal infections caused by bacteria and yeast infection (Admin, 2010). *L. bulgaricus* is one of two bacterial cultures used for the production of yoghurt; it is homofermentative bacterium and producing lactic acid that leads to drop the pH of the medium to approximately 3.8 (Lim et al., 2000).

2.6. Proteolytic activity of LAB

Lactic acid bacteria isolated from fermented dairy products, need from 4 up to 14 amino acids for growth depending on the strain (Chopin, 1993). It has been confirmed that the quantity of free amino acids and short peptides in milk is very low. Therefore, LAB use developed proteolytic system allowing for degradation of milk proteins for their growth (Juillard et al., 1995b). Caseins are composed of all amino acids required for the growth of

lactic acid bacteria in milk to high cell density. Nevertheless, only less than 1% of the total casein constituents, is actually required (Kunji et al., 1996). It has been well established that a number of *Lactobacillus* sp. grow well in skim milk (Gilbert et al., 1996b).

Amino acids and peptides produced by enzymatic hydrolysis of milk proteins by LAB proteolytic system and utilization of these amino acids are a central and integral part of their metabolic activity. During fermentation, milk, as stated above cannot supply all essential amino acids required for LAB growth in free form; therefore, LAB have developed ability to degrade milk proteins, mainly caseins, by their proteolytic system producing initially peptides, and then amino acids needed for their growth (Savijoki et al., 2006). Milk proteins during fermentation are subjected to slight proteolytic degradation resulting in a number of potentially bioactive peptides which may vary between 2-20 amino acid residues. Many of them are known to express multi-functional physiological properties, some of which are presented in Table 2.5 (FitzGerald and Meisel, 2003). Proteolysis is a cascade process involving a number of steps including (i) an extracellular proteinase initiating degradation of casein into oligopeptides, (ii) transport systems that translocate peptides and amino acids across the cell wall, (iii) various intracellular peptidases for further degradation of peptides into amino acids, and (iv) different enzymes that convert liberated amino acids into various components (Kunji et al., 1996).

Lactobacillus helveticus have been especially reported as a species with very high proteolytic and peptidolytic activity in comparison to other LAB strains presumably due to the structure of their proteolytic system consisting of proteases, peptidases and a transport system. The proteolytic system of LAB is schematically presented in Figure 2.3. The protein hydrolysis by LAB and subsequently released peptides has been studied extensively. The proteolytic enzymes and their activity have been investigated using o-phthaldialdihyde (OPA) method (Donkor et al., 2007b), polyacrylamide gel electrophoresis (PAGE) (Hayaloglu et al.,

2004), RP-HPLC, and capillary electrophoresis (CE), mainly to assess the extent of hydrolysis and products of the degradation during growth and residence in various dairy products including yoghurt and cheeses.

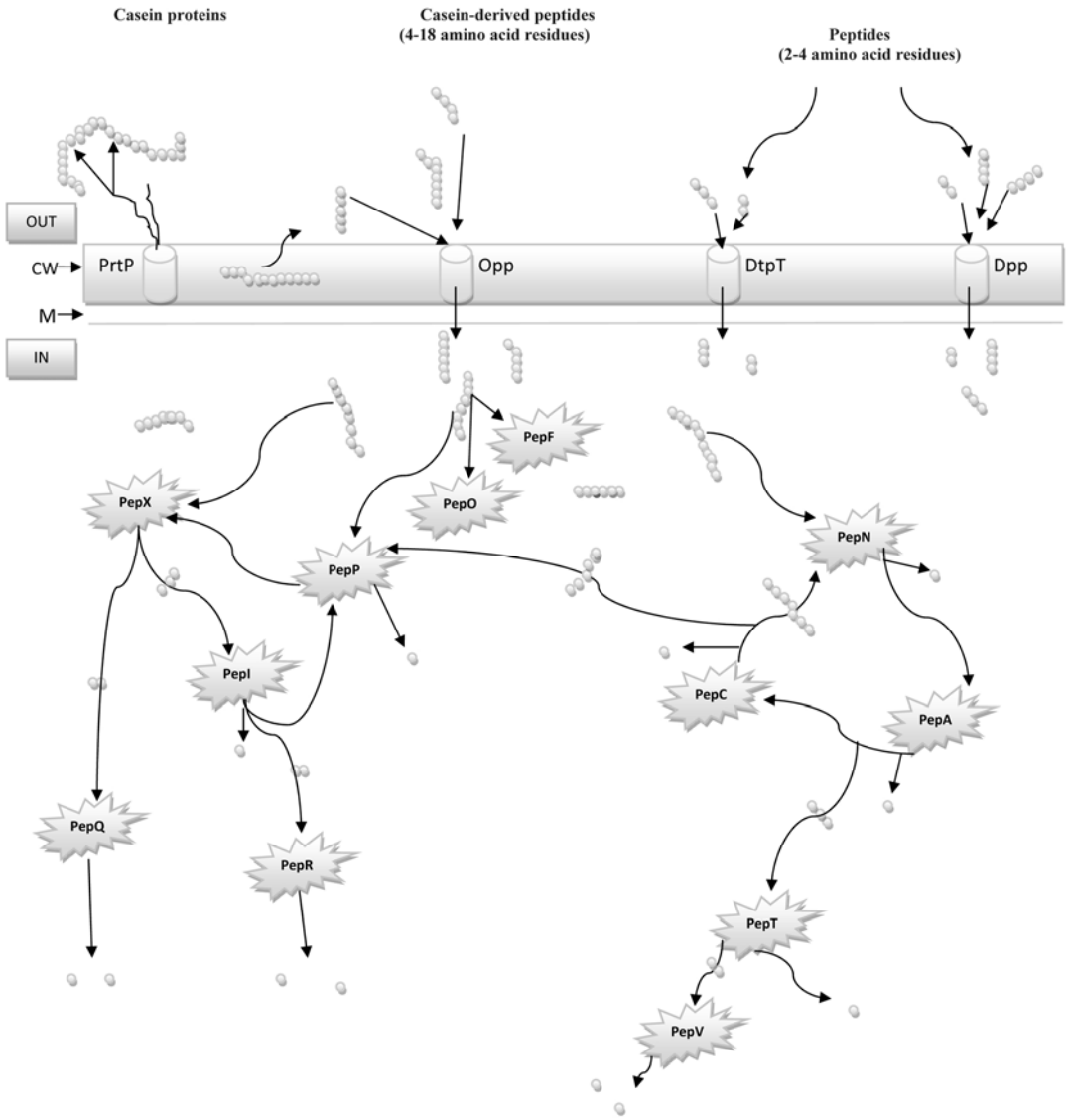


Figure 2.3 Schematic representation of the proteolytic system identified in LAB (Kunji et al., 1996)

2.7. Lactic acid bacteria proteinases

Numerous studies have shown that degradation of caseins starts with a single cell envelop proteinases (CEP) (Smid et al., 1991, Tan et al., 1993, Pritchard and Coolbear, 1993, Kok and Vos, 1994). In general, the proteinase is a monomeric serineproteinase with a molecular mass between 180-190 kDa (Laan and Konings, 1989). The extracellular location of PrtP has been well established. The proteinase can be liberated from the cell-wall with Ca^{2+}-free buffers (Tsakalidou et al., 1999) or lysozyme (Fernandez-Espla et al., 2000) . Treatment with lysozyme yields a product of 180 kDa. The electron microscopy of immuno-gold labelled proteinases has also confirmed a localization of the proteinase in the cell wall (Hugenholtz et al., 1987).

Generally, LAB proteinases are classified into two groups based on their specificity of the casein degradation (Pritchard and Coolbear, 1993). The P_I type proteinase degrades β-casein (β-CN) readily with slight tendency to act upon on α_{s1}-CN and κ-CN. P_{III} type proteinase also cleaves β-casein, but at different sites from the P_I type and shows a greater tendency towards κ-CN and α_{s1}-CN (Law and Haandrikman, 1997, Stepaniak, 2004). Two regions in the PrtP contribute to the differentiation in substrate specificity of P_I type and P_{III}-type PrtP (Kunji et al., 1996). The first region is around the centre of the active site and is homologous with substrate-binding site of subtilising (Kunji et al., 1996). The second region is the amino acid residues at 747-748 positions. The β-CN cleavage site of P_I-type is characterized by glutamine and serine residues and is usually located in the region having low charge, high hydrophobicity, and high proline content (Tan et al., 1993). P_{III}-type PrtP cleaves β-CN at GL-X-X or X-GL-X peptide bonds, where X is generally a hydrophobic residue such as methionine, phenylanine, leucine or tyrocine.

Exterkate et al (1993) reported another scheme of lactococcal PrtP classification based on specificity of hydrolysis of αs1-CN f1-23. The scheme classified PrtP into 7 groups, i.e. group a to group g. Group a in this classification was formerly reported as PIII-type PrtP, while group e was formerly PI-type PrtP (Table 2.5). Broadbent et al. (1998) added group h PrtP to the Exterkate et al. (1993) classification. The latter PrtP was reported to produce αs1-CN f1-9 that was responsible for bitterness in Cheddar cheese (Broadbent et al., 2002, Broadbent et al., 1998). However, specificity of proteinase identified in Lactococcus sp. is different from proteinases in lactobacilli (Bockelmann, 1995, Hébert et al., 2002, Oberg et al., 2002). Tsakalidou et al. (1999) isolated a PrtP from *L. delbrueckii* ssp. *lactis* ACA-DC178, which is similar to PI type lactococcal PrtP. Orbeg et al. (2002) reported the specificity of PrtP from 14 strains of L. delbrueckii ssp. bulgaricus and 8 strains of *L. helveticus* on f1-23 of αs1-CN. The results have shown 6 groups of lactobacilli PrtP specificities based on the primary and secondary products from this fraction. Table 2.5 represents 6 groups of lactobacilli PrtP specificities based on the primary and secondary products of αs1-CN f1-23 hydrolysis. Undetectable amounts of di- and tripeptides have been produced by Lactic acid bacteria Proteinase activity, and only traces of phenylalanine were measured. More than 50% of polypeptides originated from the C-terminal part of β-casein, while about half of them remaining were derived from the 60-105 regions (Kunji et al., 1996). Degradation of β-casein by bacterial proteinases has been analyzed in vitro by using purified enzymes from different *Lc. lactis* and *L. helveticus* strains (Monnet et al., 1989, Yamamoto et al., 1993). According to Kunji et al (1996), the same peptides might be visible by using HPLC profiles in each hydrolysate, although in different amounts. The degradation of αs1-and αs2 –CN is hydrolysed by PIII-type and an intermediate-type proteinase yielding various peptides, out of which 25 major oligopeptides were identified with about 50% originating from the C terminal (Kunji et al., 1996).

Table 2.5 Specificities of *lactobacilli* PrtP on α_{s1}-CN f1-23 (Oberg et al., 2002)

Group	Species	Strains	Primary products	Secondary products
I	*Ld*	1,2,26	f 1-13 + f 14-23	f 1-9 + f 10-13
	Lh	29	f 1-16 + f 17-23	f 1-6 + f 7-13
II	*Lh*	10,12,36	f 1-9 + f 10-23	f 1-6 + f 7-13
			f 1-13 + f 14-23	
III	*Ld*	4,5,6,8	f 1 f 1-13 + f 14-	f 1-6 + f 7-13
			23-9 + f 10-23	f 1-9 + f 10-13
			f 1-17	
IV	*Ld*	7	f 1-13 + f 14-23	f 1-9 + f 10-13
				f 1-6 + f 7-13
				f 1-7
V	*Ld*	13	f 1-9 + f 10-23	f 1-6 + f 7-13
	Lh	9,11	f 1-16 + f 17-23	
VI	*Lh*	3,37,41	f 1-8 + f 9-23	f 1-6 + f 7-13
	Ld	38	f 1-9 + f 10-23	
			f 1-13 + f 14-23	
			f 1-16 + f 17-23	

2.8. Amino acid and peptide transport systems

In order to utilize amino acids as cellular building blocks, bacterial cells must import degradation products derived from the caseins into the cell. Early studies showed that *Lactococcus* sp. possess at least 10 amino acid transport systems with a high specificity for structurally similar amino acids, e.g. Glu/Gln, Leu/Ile/Val, Ser/Thr, Ala/Gly, Lys/Arg/Orn (Kunji et al., 1996). It appears that the translocation is driven either by hydrolysis of ATP or by the proton motive force, depending on the type of amino acids. For example, ATP driven transport is used for Glu/Gln, Ash and Pro/Glycine-Betaine (Konings et al., 1989, Molenaar et al., 1993, Poolman, 1993). On the other hand, the proton motive force is used for translocation of Leu/Val/Ile, Ala/Gly, Ser/Thr and Met (Konings et al., 1989). A study on peptide importers in *Lc. lactis* has shown that relatively hydrophilic di- and tripeptides are transported by a proton motive force-driven transport system (Smid et al., 1989).

Furthermore, it has been shown that *Lc. lactis* also possesses a transporter that is specific for oligopeptides (Opp) (Kunji et al., 1993). This Opp system is capable of transporting peptides up to 8 residues (Tynkkynen et al., 1993) and appears to be ATP rather than the proton motive force driven (Kunji et al., 1993). On the basis of gene sequence comparisons the system has been classified as a member of the Binding Cassette (ABC) superfamily (Higgins, 1992), containing five subunits including a peptide binding protein (OppA), two integral membrane proteins (OppB and OppC), and two ATP-binding proteins (OppD and OppF) (Kunji et al., 1996). OppA act as a receptor protein which captures and brings peptides to the membrane-bound proteinase. The function of OppA is to bind peptides with high attraction properties, which depend on size and amino acid composition (Tame et al., 1994). OppB and OppC recognised as highly hydrophobic proteins and likely involved in the pathway that enables the translocation of oligopeptides through the membrane. OppD and

OppF are homologous to the ATP binding protein(s) domains of the ABC-transporter superfamily (Higgins, 1992). These proteins most likely couple the hydrolysis of ATP to conformational changes in OppB/C that allow passage of the peptides across the membrane. Generally, the OPP systems of other LAB are not widely investigated. However, oligopeptide systems of *streptococcus thermophilus* (*St. thermophiles*) (Garault et al., 2002) and *L. bulgaricus* (Peltoniemi et al., 2002) were reported that similar to that described for *Lactococcus* sp.

2.9. Peptidases of LAB

After the casein degradation by cell envelop proteinases (CEP), peptides released are taken up by the LAB cells and further degraded by action of peptidases with different specificities (Kunji et al., 1996). Generally, peptidases of LAB are classified into aminopeptidases, proline-specific peptidases, dipeptidases, tripeptidases, and endopeptidases (Christensen et al., 1999). Table 2.6 represents the collated information about the intracellular peptidases purified and characterized from LAB. Intracellular peptidases such as PepN, PepC, PepO, PepO2, PepF, PepV, PepX, and PepQ were detected and isolated from *Lactococcus* and *Lactobacillus* sp. (Christensen et al., 1999, Chen et al., 2003). It has been however reported that PepA and PepP were found only in *Lactococcus sp*., whereas PepD, PepR, PepL, PepE, PepO3, and PepG were identified only in *Lactobacillus* sp (Christensen et al., 1999). Dako et al. (1995) and Sasaki et al. (1995) first noted that the peptidases activity of lactobacilli was higher than that in lactococci.

Table 2.6 Peptidases of Lactic acid bacteria (Hutkins, 2001)

Peptidase	Abbreviation	Specificity
Aminopeptidase A	PepA	Glu/Asp↓(X)n
Aminopeptidase C	PepC	X↓(X)n
Aminopeptidase L	PepL	Leu↓X or Leu↓X−X
Aminopeptidase N	PepN	X↓(X)n
Aminopeptidase P	PepP	X↓Pro−(X)n
Aminopeptidase X	PepX	X−Pro↓(X)n
Dipeptidase V	PepV	X↓X
Dipeptidase D	PepD	X↓X
Tripeptidase T	PepT	X↓X−X
Proiminopeptidase	PepI	Pro↓X−(X)n
Prolidase	PepQ	X↓Pro
Prolinase	PepR	Pro↓X
Endopeptidase F	PepF	(X)n−X−X↓X−(X)n
Endopeptidase O	PepO	(X)n−X ↓ X−(X)n
Endopeptidase E	PepE	(X)n−X ↓ X−(X)n
Endopeptidase G	PepG	(X)n−X ↓ X−(X)n

The position of the hydrolysed peptide bonds is shown by arrows.

While the cellular location of the proteinases has been confirmed by a number of studies, the location of the identified peptidases still remains a subject of controversies. In their comprehensive review, Kunji et al. (1996) have shown that some LAB peptidases are present in cell-wall fractions. They also reported that most immunological, biochemical, and genetic data suggest an intracellular position for most peptidases studied to date. According to previous assertions which emphasize that peptidases of LAB are required for the release of essential amino acids, this theory has been changed since oligopeptides transport system (Opp) able to convey several large casein derived peptides (Kunji et al., 1996).

2.10. Peptidase specificity

Aminopeptidase C

Aminopeptidase C (PepC) has been categorized and purified from number of strains including *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus* and *St. thermophilus* (Wohlrab and Bockelmann, 1993). Significant activity of PepC has been found on residues that are acidic (Glu and Asp), hydrophobic/uncharged (Ala and Leu), basic (Arg, His, and Lys), and aromatic (Phe) by using AA-βNAP substrates. Similar activity was determined for the corresponding AA-ρNA substrates, including Gly- and Met-ρNA. The activity of PepC on Pro-ρNA, Pro-βNAP, Xaa-Pro-ρNA, or Xaa-Pro-βNAP substrates was not found. PepC activity was also reported for a variety of di- and tripeptides with basic or uncharged residues in the amino terminal position (Kunji et al., 1996; Christensen et al., 1999)

Aminopeptidase N

The purification and characterization of aminopeptidase N (PepN) have been carried out from strains of *L. casei*, *L. delbrueckii*, *L. helveticus*, *Lc. lactis* and *St. thermophilus* (Christensen et al., 1999). Generally, the highest specificity of PepN on *p*NA-AA substrates is for the basic amino acids Lys and Arg, followed by the hydrophobic/uncharged residues Leu and Ala. Significant activity is also observed for Met and *p*NA-Ph, while unclear activity for Asp-, Glu-, and *p*NA-Gly was reported. General augment in activity with increase in the hydrophobicity of the carboxyl terminal residue of an Arg-Xaa dipeptide was reported (Niven et al., 1995). Additionally, a study using cell-free extracts prepared from peptidase mutants of *L. helveticus* has shown that PepN is predominant in the liberation of amino-terminal Tyr residue from β- casein f193-209 (Christensen et al., 1999). The proportion of total aminopeptidase activity increases nearly six-fold during the same time frame. The fact that PepN activity remains constant for 12 h into the stationary phase while PepN transcription levels decrease suggests the enzyme is relatively stable and/or there is an elongated balance of turnover and expression under pH controlled conditions. In contrast, a study of PepN activity from MRS broth grown *L. helveticus* without pH control indicates a decrease in PepN activity from end of exponential phase (pH ~4.8) and reduction by four-fold during transition to stationary phase (Christensen et al., 1999). The activity of *L. helveticus* PepN accounts for greater than 99% of the hydrolysis of the substrates (Lys-, Leu-, Met-, and Ala- *p*NA) used to measure total aminopeptidase activity in the previously mentioned studies (Christensen et al., 1999).

X-prolyl dipeptidyl aminopeptidase

X-prolyl dipeptidyl aminopeptidase (PepX) has been purified and characterized from strains of *L. acidophilus*, *L. delbrueckii*, *L. casei, L. helveticus*, *Lc. lactis* and *St. thermophilus* (Christensen et al., 1999). The capability of PepX is to cleave Xaa-Pro dipeptides from the N-terminus of peptides (Gatti et al., 2004, Pan et al., 2005). It has been reported that the highest activities for PepX on Xaa-Pro-ρNA substrates are when the N-terminal residues are uncharged (Ala-, Gly-) or basic (Arg-) (Christensen et al., 1999). While liberation of amino acids from dipeptides has not been detected to date, PepX releases Xaa-Pro dipeptides from peptides ranging from three to seven amino acid residues. No kinetics data is reported for comparison with respect to substrate size (Booth et al., 1990, Miyakawa et al., 1991, Pan et al., 2005). Liberated Xaa-Pro dipeptides contain residues that are basic (Arg-, His-, Lys-), aromatic (Phe-, Tyr-), and hydrophobic/uncharged (Ala-, Ile-, Val-, Gly-). PepX is also capable of hydrolyzing Pro-Pro-(Xaa) *n* substrates, but little or no hydrolysis is observed for Xaa-Pro-Pro (including when Xaa is Pro) (Miyakawa et al., 1994, Pan et al., 2005).

Amino peptidases (PepL, PepP) and Endopeptidase PepO

A peptidase, PepL, has been cloned and characterized from *L. delbrueckii* that displays high specificity for Leu- *p*NA and Ala- *p*NA (Klein et al., 1995). Growth tests with a Leu / Pro auxotrophic *E. coli* strain expressing recombinant PepL suggest hydrolysis occurs with several di- and tripeptides with N-terminal Leu residues. Growth was also obtained with Pro-Pro, Pro-Ser, and Gln-Pro as the sole source of proline (Christensen et al., 1999)

The aminopeptidase, PepP, has been purified and characterized from *Lc. lactis* (Mars and Monnet, 1995). PepP liberates the N-terminal amino acid from peptides with general specificity for Xaa-Pro-Pro-(Yaa) *n* sequences (Mars and Monnet 1995). The rate of hydrolysis was highest for the pentapeptides Arg-Pro-Pro- Gly-Phe (bradykinin f1-5) and

Leu-Pro-Pro-Ser-Arg. Relatively high activity was observed with peptides ranging from three to nine residues, but the dipeptides tested were not hydrolyzed. The specificity was observed for the N terminal (Xaa-) residues Arg, Met, Lys, Leu and Tyr (Christensen et al., 1999).

Endopeptidase PepO has been purified and characterized from *Lc. lactis* (Lian et al., 1996). PepO is able to hydrolyse oligopeptides ranging in length from five (Met- and Leu-enkephalin) to thirty-five residues (α_{s1}-casein f165-199) (Tan et al., 1991, Pritchard et al., 1994, Stepaniak and Fox, 1995). Although PepO has capability to hydrolyze several large casein derived fragments, no activity was detected on the native caseins (Tan et al., 1991, Stepaniak and Fox, 1995).

Tripeptidase (PepT)

Tripeptidases have been isolated and characterized from *L. delbrueckii*, *L. sake*, and *Lc. Lactis* (Sahlstrom et al., 1993, Bockelmann et al., 1997, Sanz et al., 1998). PepT has ability to hydrolyse tripeptides from wide range amino acids including substrates consisting of hydrophobic/uncharged, aromatic, basic, acidic, and sulfur-containing residues. This activity is observed for Pro-Gly-Gly and Leu-Xaa-Pro (-Gly-, -Ala-), but no hydrolysis was reported for any di-, tetra-, or larger oligopeptides (Christensen et al., 1999).

2.11. Viability of LAB

Viability and culture performance (growth rate, acid production, proteolytic acitivity) are important detriments of the culture selection in industrial applications. It has been suggested that the concentration of dairy lactic acid bacteria should be at least 10^7 colony forming units (cfu) per gram of a medium to obtain desired health benefits (Rybka and Fleet, 1997, Gomes and Malcata, 1999a). A consumer should consume about 10^8 cfu/g of product daily to recompense the possible losses during the transit in the gastro intestinal tract (GIT) as well as regular washouts due to poor adhesion of these bacteria. Some of LAB such as *L. acidophilus and Bifidobacterium sp*. have shown poor survival and viability in acidified products including yoghurt (Shah et al., 1995, Dave and Shah, 1998).

2.12. Factors affecting viability of LAB

Many factors, such as species specificity, interactions between strains, acidity and hydrogen peroxide production as a result of bacterial metabolism, have been reported to exert their effect on the culture activity and viability (Donkor et al., 2007b). These factors can affect the viability of fermented lactic acid bacteria even during fermentation (Dave and Shah, 1998, Lankaputhra and Shah, 1996). Moreover, storage temperature, concentration of produced organic acids in the medium, growth promoters and inhibitors, inoculation level, fermentation time and post-acidification and oxygen content can also affect the viability and growth of LAB during fermentation and storage in dairy products (Dave and Shah, 1998, Shah and Ravula, 2000, Tamime et al., 2007). Similarly, Lourens-Hattingh and Viljoen (2001a) reported that adding enhancers with appropriate LAB strains can improve the viability and survival of LAB in fermented dairy products.

2.13. Physiological functions of dairy derived bioactive peptides

Fermented dairy foods, in addition to providing energy and nutrients, also are a source of physiologically important peptides that have a positive impact on body's functions. These potential health benefits may be due to the production of microbial metabolites, such as cell wall components (Nakajima et al., 1995), bacteriocins (Hernández et al., 2005) and the hydrolysis of cell-free extracts containing proteinase and peptidase activities on milk proteins substrates (Pan et al., 2005). Research carried out during the last 10-15 years has shown that the caseins and whey proteins can be an important source of biologically active peptides or bioactive peptides. Bioactive peptides are described as 'food derived components that in addition to their nutritional value exert a physiological effect in the body' (Vermeirssen et al., 2004). Bioactive peptides usually contain 3 to 20 amino acid residues per molecule. They have been found to have specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opiod or mineral-binding activities. Many milk-derived bioactive peptides reveal multifunctional properties, i.e., specific peptide sequences may exert two or more different biological activities. Due to their physiological and physicochemical versatility, milk-borne bioactive peptides are regarded as important ingredients for health-promoting functional foods (Korhonen and Pihlanto-Leppa¨la¨, 2004). The major means for producing biologically active peptides from milk peptides is shown in Figure 2.4.

Production of bioactive peptides derived from fermented milk with starter cultures has been studied extensively (Gobbetti et al., 2002, Fitzgerald and Murray, 2006). Such peptides are inactive in their parent protein sequences, and these biologically active peptides can be released by three ways including digestive enzymes during passage through the gastrointestinal tract, during processing of foods and by fermentation. Upon consumption of microbially fermented milk, bioactive peptides may affect many human physiological

systems including digestive, nervous, endocrine, immune and cardiovascular (Korhonen, 2009). Fitzgerald and Meisel (2003) reported that some of the well-recognized activities of known bioactive peptides are antihypertensive and immunomodulatory activities. Moreover, risks of acquiring some of chronic diseases and metabolic disorders that are associated with unbalanced diet may be reduced by consumption of fermented dairy products. Some of the explored risk lowering effects involved cancer, osteoporosis, coronary heart diseases, hypertension and obesity.

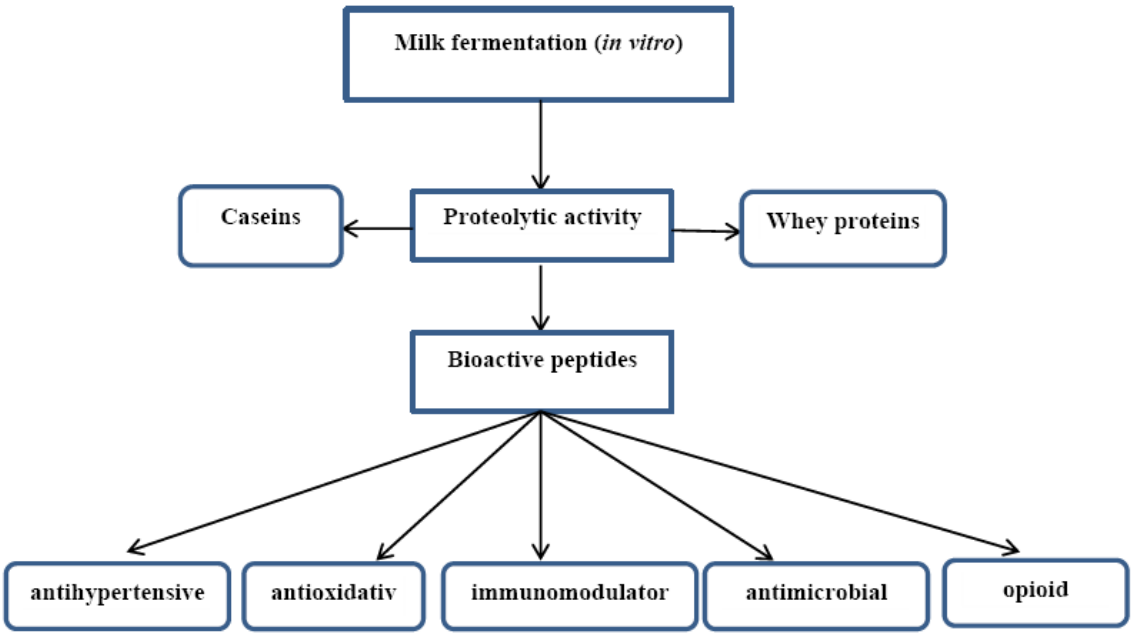


Figure 2.4 Possible pathways for the release of milk derived bioactive peptides (Ramchandran, 2009)

2.14. Angiotensin converting enzyme inhibitory peptides

Milk protein derived antihypertensive peptides are considered to be angiotensin converting enzyme (ACE) inhibitors. This particular enzyme, which is a part of the renin-angiotensin system, converts angiotensin-I into angiotensin-II, thus regulates the peripheral blood pressure due to vasoconstricting action (Figure 2.5). Inhibition of ACE exerts an antihypertensive effect through a decrease of angiotensin II and an increase of bradykinin. Due to the multifunctional property of ACE, its inhibition may also have an effect on other regulatory systems involved in immuno-defense and nervous system activities (Fitzgerald and Meisel, 2003) and even affect the lipogenesis thus slowing down a fat deposition (Medenieks and Vasiljevic, 2008).

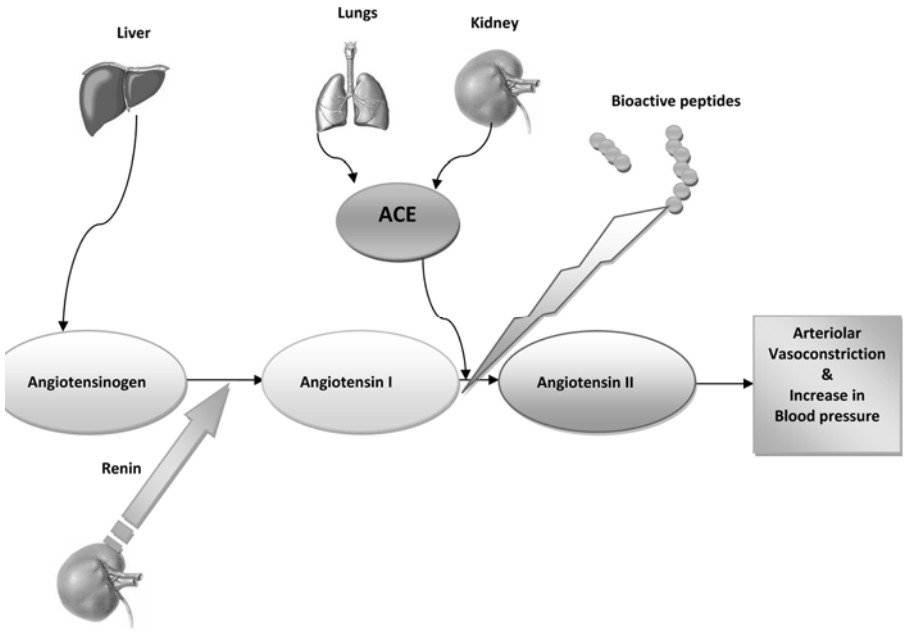


Figure 2.5 Regulation of blood pressure: role of angiotensin-I-converting enzyme (adapted from Li et al., 2004)

Angiotensin-I converting enzyme-inhibitors are presumably competitive substrates for ACE. The primary structural feature governing this inhibitory response is the C-terminal

tripeptide sequence and thus these peptides may interact with subsites s_1, s_1' and s_2' at the active site of ACE (Hong et al., 2008). ACE preferentially interacts with substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions (Cheung et al., 1980). Generally, aliphatic, basic and aromatic residues are preferred in the penultimate positions, while aromatic proline and aliphatic residues are preferred in the ultimate positions. The positive charge of Arg or the ε-amino group of Lys at the C-terminus has also been shown to contribute to the ACE-inhibitory potential of several peptides (Cheung et al., 1980).

Lactic acid bacteria possess the proteolytic system that hydrolyses milk proteins which present a potential for release of ACE-inhibitory peptides (Yamamoto et al., 1993). ACE-inhibitory and antihypertensive peptides originating from milk usually contain up to 10 amino acids. The majority of milk protein derived ACE-inhibitors have moderate inhibitory potencies, usually within an IC_{50} range from 100 – 500 μmol/L (Hayes et al., 2007) and it appears to be highly strain specific (Donkor et al., 2007b). Additionally these peptides may be released by action of extracellular proteolytic and human digestive enzymes. Bioactive peptides exerting ACE inhibitory activity derived from fermented milk proteins by micro-organisms are presented in Table 2.7.

Table 2.7 Summary of ACE-inhibitory activities of milk protein fermented with LAB (Hayes et al, 2007)

Microorganism	Substrate	Activity
L. helveticus	Milk	ACE-I
L. helveticus	Whey protein	ACE-I
L. helveticus LBK 16H	Milk	ACE-I
L. helveticus CPN 4	Milk	ACE-I
L. helveticus CH CC637	Milk	ACE-I
L. helveticus R389	Milk	ACE-I
L. helveticus CP 790 proteinase	β-casein	ACE-I
L. helveticus CM4 endopeptidase	Val-Pro-Pro-Leu	ACE-I
L. helveticus JCM 1004 cell-free extract	Skim milk	ACE-I
L. helveticus R211 and R389	Casein enriched milk	ACE-I
L. delbrueckii ssp. *bulgaricus* SS1	Milk	ACE-I
L. delbrueckii ssp. *Bulgaricus*	Milk	ACE-I
Commercial dairy starters from yoghurt and ropy milk treated with enzymes	Whey, casein	ACE-I
Aspergillus oryzae protease	Skim Milk	ACE-I

Among these cultures that are commonly used in milk fermentations, *L. helveticus* has shown the highest proteolytic activity during manufacturing of dairy fermented products. Calpis, a fermented products produced by using *Lactobacillus helveticus* which is capable of releasing antihypertensive peptides such as VPP and IPP (Nakamura et al., 1995, Seppo et al., 2003). Similarly, *L. delbrueckii* sp. also showed the ability to produce ACE-I during 72 h fermentation (Gobbetti et al., 2000). A study reported that fermented milk with *L. delbrueckii* ssp *bulgaricus* was able to release oligo anti-hypertensive peptides such as Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-IIe derived from β-casein. Importantly, most of bioactive

peptides expressing ACE-I activity have a short chain with proline residue at the end of C-terminal end (2004). Proline is known to escape digestive enzyme degradation and therefore, these short sequences of bioactive peptides may cross to the blood circulation and exert an antihypertensive activity (Yamamoto et al., 2003)

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

Fermented milk produced by a mixture containing several types of microorganisms may contain a wider variety of functional substances than milk cultured with a single strain (Kuwabara et al., 1995). Inclusion of probiotics to yogurt has been shown to enhance *in vitro* ACE-inhibitory activity due to improved proteolytic activity (Donkor et al., 2007b). Nielsen et al. (2009) found that the pH at the end of fermentation influences the ACE-inhibitory

activity of fermented milk which varies with the strain of LAB used. As a result of intercellular peptidase activity, the residual levels of peptides with bioactivity, such as ACE-inhibitory activity increase in fermented milks. The oligopeptides derived from milk proteins as a result of fermentation can be an additional source for the liberation of bioactive peptides when further degraded by intracellular peptidases after the lysis of bacterial cells. The digestive enzymes in the gastrointestinal tract may also further degrade long oligopeptides leading to possible release of bioactive peptides. Once liberated in the intestine, bioactive peptides may act locally or pass through the intestinal wall into the blood circulation and end up at a target organ.

2.15. Antihypertensive effect of bioactive peptides

Hypertension is defined as a sustained increase in blood pressure (BP) and is a controllable risk factor in the development of a number of cardiovascular diseases (CVD) such as stroke and coronary infarction. Uncontrolled high BP increases the risk for CVD, stroke, heart failure and kidney disease. Even a small decrease in BP results in a significant reduction in the risk of CVD and a 5 mm Hg reduction in diastolic BP reduces the risk of heart disease by approximately 16% in hypertensive subjects(FitzGerald et al., 2004). Such a reduction in diastolic BP corresponds to a decrease in systolic BP by 9-10 mm Hg. It has been estimated that a decrease in systolic BP by 3 mm Hg would reduce the risk of stroke by about 10-13% (Tuomilehto et al., 2004). The high cost of and potential adverse side effects associated with pharmacological therapy for hypertension have encouraged individuals to adopt lifestyle modifications such as weight reduction, low-fat dairy products and dietary sodium reduction.(Miller et al., 2007) The blood pressure lowering effects of specific casein and whey protein hydrolysates or fermented dairy products provide compelling evidence for a

beneficial role of dairy peptides to induce clinically significant reductions in systolic BP and diastolic BP with no reported adverse effects (Huth et al., 2006).

The bioactive peptides such as ACE-inhibitory peptides must reach their target organ intact to exert their effects *in vivo*. Degradation of peptides in the acidic environment of the stomach, alkaline conditions of the small intestines as well as hydrolysis by the brush border peptidases can either activate or deactivate ACE-inhibitory peptides before they reach the portal circulation. Therefore, only those ACE-inhibitors that are not affected by the action of angiotensin-II and gastrointestinal enzymes or those that are converted to stronger ACE-inhibitors exert antihypertensive effects *in vivo* (Korhonen and Pihlanto, 2003a)

Due to the incomplete and often unknown bioavailability of the ACE-inhibitory peptides following oral administration, it is difficult and unreliable to predict the *in vivo* antihypertensive effect based on inhibitory activity *in vitro* (Erdmann et al., 2008). Although valuable information can be obtained from *in vitro* model systems regarding the proteolytic/peptideolytic stability and susceptibility to intracellular passage of these peptides, the actual hypotensive effects can be reliably assessed only through *in vivo* studies (FitzGerald and Meisel, 2003). The *in vivo* effects are tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension (López-Fandiño et al., 2006). Numerous rat studies have been performed to determine the hypotensive effect of milk protein derived ACE-inhibitors. The maximal decrease in systolic BP achieved in SHRs using various peptides from milk proteins is summarized in Table 2.8.

In general ACE-inhibitory peptides that have been found to have antihypertensive activity in SHR have IC_{50} values lower than 150 μM. However, in some cases, the extent of ACE-inhibitory activity of the peptide is not correlated with the antihypertensive activity (Matar et al., 2003). Some peptides show strong antihypertensive activity at a low dose even though they possess a low ACE-inhibitory activity (Maeno et al., 1996). Thus, there appears

to be no direct relationship between the extent of systolic BP decrease and the IC_{50} values for the different peptides tested to date (FitzGerald et al., 2004). This implies that apart from ACE-inhibition, milk peptides may exert antihypertensive effect through other mechanisms also, such as inhibition of the release of endothelin-I by endothelial cells (Maes et al., 2004), stimulation of bradykinin activity (Perpetuo et al., 2003), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002), enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000) and a vascular relaxing mechanism (Miguel et al., 2007). Most LAB produce ACE-inhibitors during milk fermentation. However, the activity, and thus the *in vivo* potential of the fermented milk, varies with the strain (Fuglsang et al., 2003). Some studies have recorded lowering of blood pressure in animals and human subjects fed on fermented milks. Seppo et al. (2002) found that daily consumption of *L. helveticus* LBK-16H fermented milk reduced systolic and diastolic BP by 6.7 and 3.6 mm Hg in hypertensive patients while Tuomilehto et al. (2004) found a 11-16 mm Hg decrease in systolic BP but no difference in diastolic BP when subjects with mild hypertension were fed sour milk fermented with *L. helveticus*. Chen et al. (2007) found that the whey separated from low-fat milk fermented with five mixed LAB when fed orally to SHR for 8 wks reduced the systolic and diastolic BP by 22 and 21.5 mm Hg. Tsai et al. (2008) observed reduction in diastolic and systolic BP of SHR by 15.9 and 15.6 mm Hg respectively, when fed with whey of milk fermented with *St. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Costa et al. (2007) reported a reduction in systolic BP of 25 mm Hg when SHR were intraperitoneally administered whey protein hydrolysate.

Table 2.8 Reported antihypertensive effects of milk protein derived bioactive peptides

Milk protein	Peptide fraction	Maximum decrease in systolic BP (mm Hg)	Reference
α_{s1}-Casein	f(1-9)	-9.3	Saito et al. (2000)
	f(23-24)	-34.0	Karaki et al. (1990)
	f(90-94)	-25.0	del Mar Contreras et al. (2009)
	f(104-109)	-13.0	Maeno et al (1996)
	f(143-149)	-20	del Mar Contreras et al. (2009)
	f(146-147)	-32.1	Yamamoto et al. (1999)
α_{s2}-Casein	f(89-95)	-15.0	del Mar Contreras et al. (2009)
	f(198-202)	-9.0	Maeno et al. (1996)
β-Casein	f(59-61)	-21.0	Abubakar et al. (1998)
	f(59-64)	-22.0	Abubakar et al. (1998)
	f(60-68)	-7.0	Saito et al. (2000)
	f(74-76)	-28.3	Nakamura et al. (1995)
	f(80-90)	-8.0	Abubakar et al. (1998)
	f(84-86)	-32.1	Nakamura et al. (1995)
	f(169-174)	-32.2	Maeno et al. (1996)
	f(169-175)	-31.5	Maeno et al. (1996)
	f(177-183)	-10.0	(Hideaki et al., 1990)
α-Lactalbumin	f(50-53)	-23.0	Mullally et al. (1997)
β-Lactoglobulin	f(58-61)	-20.0	Hernández-Ledesma et al. (2008)
	f(78-80)	-31.0	Abubakar et al. (1998)
	f(103-105)	-20.0	Hernández-Ledesma et al. (2008)
Bovine serum albumin	f(221-222)	-27.0	Abubakar et al. (1998)
β_2-Microglobulin	f(18-20)	-26.0	Abubakar et al. (1998)

2.16. Antioxidant activity of fermented milks generated using LAB

Oxidative metabolism is crucial for the survival of human cells (Pihlanto, 2006a). However, the risk associated with this activity is that the production of free radicals may cause oxidative changes. For example, in some of the age specific diseases, oxidative stress has been reported to play an important role in the genesis of these diseases (Pihlanto, 2006a). Free radicals have also been linked with many other pathological conditions such as atherosclerosis, diabetes, rheumatoid arthritis (Abuja and Albertini, 2001, Gutteridge and Halliwell, 2000, Halliwell and Whiteman, 2004). Inhibition of the free radicals formed in the living body and foodstuff is an important way to protect body from these serious diseases. Many artificial antioxidative agents are prohibited in some countries because of the risk associated with their consumption (Pihlanto, 2006a). A number of natural antioxidative agents have been produced from plants, and some dietary proteins have also been reported to have antioxidant activity (Okada and Okada, 1998). Several food protein hydrolysates have been found to exhibit antioxidant activity (Saiga et al., 2003, Dávalos et al., 2004). To date, only a few details are available about antioxidant peptides derived from fermented dairy products. Milk proteins have been suggested to have possible free radical scavenging by amino acids such as tyrosine and cysteine (Pihlanto, 2006a). Peptides derived from casein hydrolysis have been reported to have antioxidant activity (Cervato et al., 1999, Wong and Kitts, 2003). Peptide produced from β-casein f (177–183), for example, which known as ACE-I, it has been reported to have antioxidant activity . Furthermore, a potent antioxidant activity was found in the peptide Tyr–Phe–Tyr–Glu-Pro–Leu. Casein hydrolysates were reported to have higher concentration of histidine, lysine, proline and tyrosine, which are able to react with free radical and serve as scavengers(Suetsuna et al., 2000). However, few antioxidant peptides have been observed in microbial fermented milk. A κ-casein derived

peptide with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity has been found in milk fermented with *L. delbrueckii* ssp. *bulgaricus* (Kudoh et al., 2001).Liu et al (2005) assessed the antioxidative activity of milk-kefir and soymilk- kefir and found that the DPPH radical-scavenging activity of milk-kefir and soymilk-kefir was significantly higher than that of milk and soymilk. These results suggest that this activity may be, in part, attributed to the peptides derived from degradation of milk and soybean proteins. These results of scavenging properties of free radicals by dairy cultures might be useful in food manufacturing and can present additional sources of health enhancing antioxidants (Osuntoki and Korie, 2010).

2.17. Immunoregulatory activity

The physiology of the immune system

The reactions of the immune system can be influenced by various factors. Many reports demonstrate that bioactive peptides produced from milk proteins may interact with the immune system at different levels. The immune system is a wide and complicated network of cells, tissues, and organs that has evolved to defend the body against pathogens and foreign material, generally called as "non-self". Pathogens include infectious organisms as viruses, bacteria and parasites and foreign material include such as toxins. All the non-self-substances capable of triggering an immune response are known as antigens (from the National Cancer Institute of USA, www.cancer.gov/cancertopics/understandingcancer/immunesystem/). The immune system network is positioned throughout the body, and includes the bone marrow that is involved in the production of the immune cells. Moreover, the thymus, where T lymphocytes mature, the spleen and the lymph nodes contain specialized compartments where immune cells gather and deal with antigens. Beside these organs, clusters of lymphoid tissue are found in many parts of the body, especially in the linings of the digestive tract

which called Gut-Associated Lymphoid Tissue, the airways (Bronchus-Associated Lymphoid Tissue) and the various mucosal compartments of the body (Mucosa-Associated Lymphoid Tissue) (from the National Cancer Institute of USA). It has been considered that the cells of the immune system are of various natures and each group has a particular function. Neutrophils for example, are particularly active against bacteria. Circulation of monocytes in the blood takes about one to three days, after which they typically move into tissues throughout the body, where they differentiate into tissue resident macrophages or dendritic cells. Circulating monocytes are responsible for phagocytosis of antigens. Basophils are granulocytic cells and they release granules containing histamine and hence play a role in both parasitic infections and allergies. Mast cells are similar to the basophil cells in morphology and function but they resident cells of several types of tissues. Eosinophils are granulocites with the main role of combating multicellular parasites and some infections. Finally, Natural killer (NK) cells are a kind of cytotoxic lymphocyte; they play an important role in the rejection of cancers and cells infected by viruses. The mechanism of this activity is by liberating the proteins named perforin and granzyme, resulting in the target cell to die by apoptosis (cell programmed death) (from http://en.wikipedia.org/wiki/Immune_system). In particular, these cells populations constitute the first line defense against antigens. In fact they are involved in the recruitment of the immune cells to the infection sites, through the production of chemical factors. In addition they promote the clearance of dead cell and they activate the process of inflammation that is one of the first reactions of the immune system defenses to the infection sites or positions. The first response to an antigen is rapid and important but it is not selective against the antigen and it is called innate immune response. This means that the cells of the innate system recognize and respond to pathogens in a generic way, not conferring long-lasting or protective immunity to the host (from http://en.wikipedia.org/wiki/Immune_system). While adaptative immune system is the other

part of the immune system which composes of highly specialized, systemic cells and processes that recognize and "remember" specific pathogens. In this way the response to the pathogen is more selective and efficient each time the pathogen is encountered. The most important cells intervening in this system are lymphocytes (Figure 2.6).

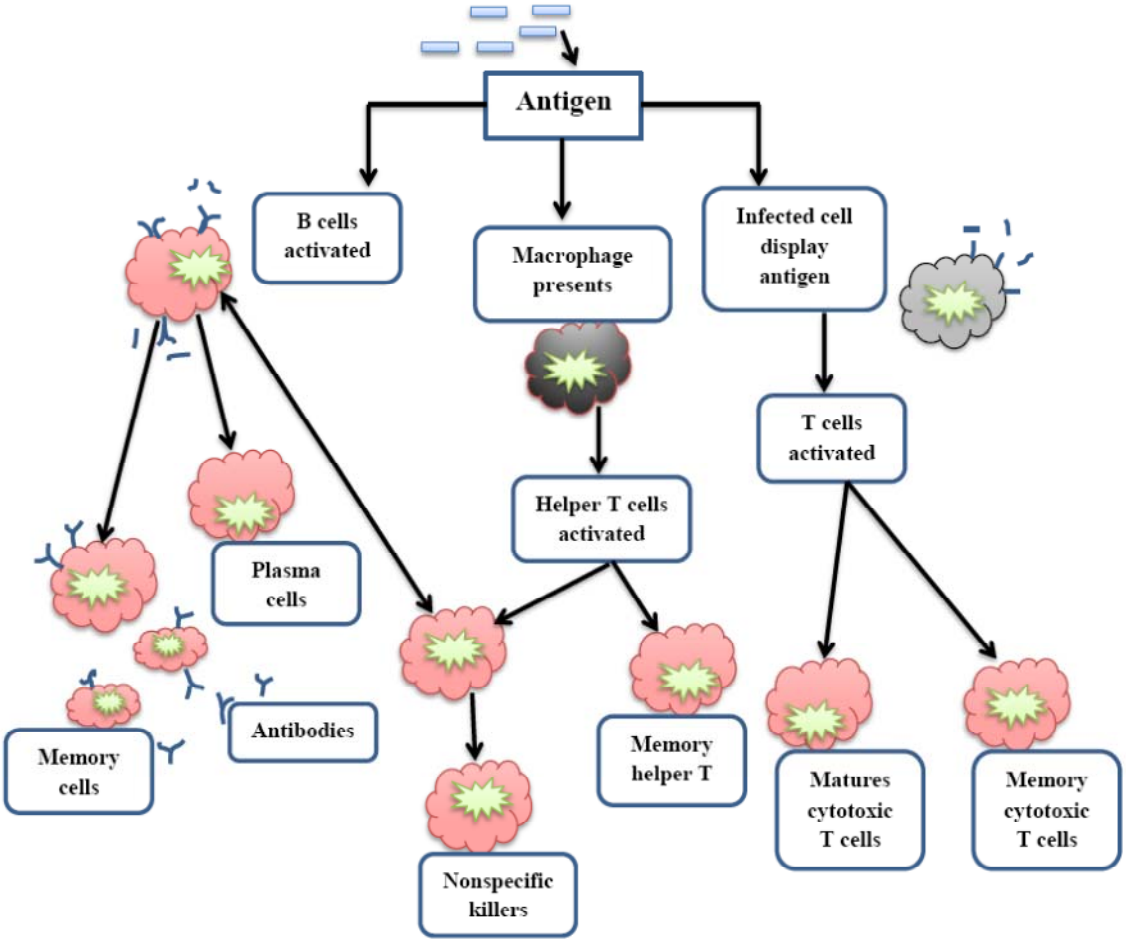


Figure 2.6 Overview of the human immune response system (adapted from http://www.uta.edu/chagas/html/biolImS1.html.)

These cells attack the pathogens after antigen-presenting cells such as dendritic cells (or macrophages) display the foreign substance in the form of antigen fragments. Lymphocytes can be divided in different subgroups, T lymphocytes (T cells) and B lymphocytes (B cells). When B cell turns into a plasma cell, thousands of specific antibodies

release into the bloodstream. Antibodies are large soluble proteins used to identify, recognize and neutralize specific antigens. There are different categories of antibodies, differing in their biological aspects; each one has developed to handle different types of antigens (from http://en.wikipedia.org/wiki/Immune_system). The T cells organize the whole immune reaction, they eliminate the viruses that hiding in diseased cells and contribute to the immune defenses in a cell-mediated way. The T cells can be sub-grouped as follows - T helper cells (TH cells) - these cells give help to the other white blood cells in immunologic developments, which including maturating of B cells into plasma cells and triggering cytotoxic T cells and macrophages, among other functions. Cytotoxic T cells (TC cells) destroy virus-related infected cells and cancer cells, they are also involved in transplant rejection. After the infection has resolved, another subset of antigen-specific T cells persist and they are called Memory T cells. These cells increase rapidly in large amounts of effector T cells upon re-contact to their cognate antigen, and hence providing the immune system with "memory" against past infections. There are two subtypes of memory T cells: central memory T cells (TCM cells) and effector memory T cells (TEM cells). Finally, regulatory T cells (Treg cells), which are also known as suppressors. This type of cells is essential for the upkeep of immunological tolerance. The main function of these cells is to shut down T cell-mediated immunity in the direction of the end of an immune reaction.(from http://en.wikipedia.org/wiki/T_cell). The efficient components of the immune system act cooperatively to eliminate the infection. The messengers that responsible for the communication between the different parts are specialized chemical mediators, called cytokines. Many types of cytokines have been recognized as potent chemical messengers. These cytokines are secreted by the immune cells. Cytokines include interleukins, interferons, and growth factors. Cytokines secreted by lymphocytes, including both T cells and B cells, are named lymphokines, while the cytokines secreted by monocytes and macrophages are

called monokines. Number of cytokines secreted is known as interleukins because they act as a messenger between leukocytes. When receptors bind with specific substrates on the targeted cells, cytokines employ number of different subsets of the immune system. Cytokines have a role to cell growth, cell activation, direct cellular traffic, and terminate diseased cells including cancer cells. Moreover, it is important to note that different kinds of cells may secrete the same kinds of cytokines or single cytokine to act on several cell types. Cytokines are redundant in their activity; this signifies that the same function might be triggered by different types of cytokines. In particular, two important cytokines involved in lymphocytes proliferation and activation are IL2 and INFγ. INFγ is an important macrophage-activating lymphokine and it is involved in other cytokines induction, particularly T helper 2 cytokines, such as IL4, IL5, and IL10. Because of its role in mediating macrophages and NK cell activation, INFγ is essential in the immune system against intracellular pathogens and viruses and against tumors responses thus influencing downstream immunological responses (http://microvet.arizona.edu/courses/MIC419/ Tutorials/ cytokines.html).

Th1/Th2 balance

The equilibrium between Th1/Th2 is essential for the stability of immune system. The polarization of immune reactions towards Th1 or Th2 underlies the development of many immunoinflammatory syndromes. Allergies, as an example, are motivated by excesses of activation of Th2 immune responses and inflammatory bowel disease (IBD) and autoimmune disorders such as type 1 diabetes are mainly motivated by Th1 type immune responses. According to suggestions from recent immune studies, malfunctioning of Treg cell activity might be the crucial cause for the concurrent rise in Th1 and Th2-mediated diseases observed

within the past few decades. People who suffering type 1 diabetes, multiple sclerosis and those who have a predisposition to allergy development are known to have malfunction of Treg cell activity (Guarner et al., 2006). Th1 cells produce pro-inflammatory cytokines such as interferon-γ (IFNγ), TNFα, interleukin- (IL) 2, and IL-12, while IL-4, IL-5, IL-6 and IL-13 cytokines are produced by Th2 cells. The functions of cytokines produced by Th1 cells are to motivate the phagocytosis operation and destroy pathogenic microorganisms, while Th2 cytokines such as IL-4 are basically to stimulate the production of antibodies targeted to extracellular parasites. IL-5 stimulates eosinophil reactions, and it's also part of the immune response against extracellular parasites (Donkor et al., 2010).

Immunomodulatory peptides derived from milk

Immunomodulatory milk peptides act on the immune system and cell proliferation responses thus influencing downstream immunological responses and cellular functions. Indeed, Jollés et al (1981) discovered that a tryptic hydrolysate of human milk possessed *in vitro* immunostimulatory activity (more specifically, stimulation of phagocytosis of sheep red blood cells and production of hemolytic antibodies against the same cells). In the following years, a number of potentially immunoregulatory peptides were identified encrypted in bovine caseins (Otani and Hata, 1995, Meisel, 1997) and whey proteins (Mattsby-Baltzer et al., 1996, Shinoda et al., 1996), which can manifest different effects. Some casein-derived peptides (residues 54-59 of human β-casein and residues 194-199 of αs_1-casein) can stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages (Parker et al., 1984), exert a protective effect against *Klebsiella pneumoniae* (Migliore-Samour et al., 1989) or modulate proliferative responses and immunoglobulin production in mouse spleen cell cultures (fragment 1-28 of bovine β-casein, (Otani and Hata, 1995, Otani et al., 2001). More recently, lactoferricin B, obtained by hydrolysis of lactoferricin by pepsin, was found to

promote phagocytic activity of human neutrophils (Miyauchi et al., 1998). Others fragments (fragment 18-20 of β-casein, fragment 90-96 of αs_1-casein) can either stimulate or inhibit lymphocyte proliferation depending upon the concentration used (Kayser and Meisel, 1996), while some whey-derived peptides can affect cytokine production from leucocytes (Mattsby-Baltzer et al., 1996, Shinoda et al., 1996).

It seems, therefore, that the immunomodulatory potential of bovine milk and bovine milk bioactive peptides is not restricted to the cells of bovine derivation. Although the precise effects of these milk components may be different on target cells of different species, however, the mechanisms by which these milk-derived peptides exert their immunomodulatory effects or influence cell proliferation are not currently fully elucidated. Some immunomodulatory peptides are multifunctional peptides and may modulate cell proliferation by interacting with opioid receptors. This is the case of the opioid peptide β-casomorphin derived from human β-casein that *in vitro* inhibits the proliferation of human lamina propria lymphocytes via opiate receptor (Elitsur and Luk, 1991). Indeed, immune system and opioid peptides are related and it has already been demonstrated that opioid receptors are expressed on T lymphocytes (Carr et al., 1989, Wybran et al., 1979). Other milk-derived peptides with immunomodulatory activity belong to the caseinophosphopeptides class. For example, caseinophosphopeptide preparation CPP-III that is commercially available and comprising mainly of the oligopeptides extracted from bovine caseins αs_2- CN (1-32) and β-CN (1–28), improves the proliferative response stimulated by lipopolysaccharide, phytohaemagglutinin and concanavalin A (conA) stimulation, and immunoglobulin production in cell cultures from mouse spleen (Hata et al., 1999, Hata et al., 1998); this stimulation of immune activity was resulted from the o-phospho-L-serine residue, and hence, there is a suggestion that this bioactivity is relatively resistant to proteinase action in the intestinal tract (Otani et al., 2000). Furthermore, the study of Otani et al. (2003), which

aiming to determine the effects of CPP-III on serum and intestinal immunoglobulin G and immunoglobulin A secretion in mice, showed that oral consumption of caseinophosphopeptide is beneficial toward improvement of the mucosal immunity. In addition, an alternative hypothesis involves a possible immunomodulatory action via ACE-inhibitory mechanism. ACE-inhibitory peptides are well known as antihypertensive properties through their role in inhibiting the conversion of angiotensin I to angiotensin II. These peptides are also found to prevent hydrolysis of bradykinin that is mediated by ACE. Bradykinin is able to stimulate macrophages, and thus enhance the migration of lymphocyte cells and stimulate the secretion of cytokines from lymphocyte in culture as a mediator of the acute inflammatory process. It should be noted that amino acid sequences of some ACE-inhibitory and immunomodulatory peptides have arginine as the C-terminal residue. Immunomodulatory milk-derived peptides may contribute to the overall immune response and may ameliorate immune system function. Migliore-Samour (1989) suggested that bioactive peptides derived from casein are involved in the stimulation of the immune system of neonates. It cannot be excluded that these peptides may act as anti pathogenic bacteria and viral infection in adults.

A recent study reported that peptides derived from casein led to Th1 response by enhancing ConA-induced IL-2 production while no activity was observed on IL-10 (Phelan et al., 2009). In vitro results also have shown that fractions obtained from casein degradation have effect on the production of Th1 cytokines and, thus able to influence on cellular immunity. It has been observed that human consumption of casein degraded by *Lactobacillus* GG down-regulated IL-4 production but showed no effect on IFN-γ production (Sütas et al., 1996). Dairy peptides have been demonstrated to modulate antibody production during infancy (Juvonen et al., 1999). These results suggested that some peptides derived from casein may have a function for the control of allergic responses.

Although the effects of LAB on stimulation of Th1/Th2 cells in cytokine production are well characterized, studies of immunomodulatory effects of peptides derived from milk proteins has not been widely investigated due to lack of characterization of such peptides (Gauthier et al., 2006). Therefore this project further explored the *in vitro* immunomodulatory effects of hydrolysed milk proteins by LAB on buffy coat PBMCs.

CHAPTER 3

MATERIALS AND METHODS

3.1. The cultures and their propagation

Lactobacillus helveticus ASCC474, *L. helveticus* ASCC118, *L. helveticus* ASCC1315, *L. helveticus* ASCC953, *L. delbrueckii subsp. bulgaricus* ASCC734, *L. delbrueckii subsp. bulgaricus* ASCC756, *L. delbrueckii subsp. bulgaricus* ASCC857, *L. delbrueckii subsp. lactis* ASCC1210, *L. delbrueckii subsp. lactis* ASCC1307, and *L. delbrueckii subsp. lactis* ASCC1372 were obtained from the Australian Starter Culture Collection (Dairy Innovation Australia, Werribee, Australia). The organisms were stored at -80 °C. The propagation for each strain was performed according to Donker et el (2007b). Briefly, sterile 10 mL aliquots of de Mann Rogosa Sharpe (MRS) broth (Oxoid West Heidelberg, Australia) were inoculated with 1% (100 µl) of each strain individually and incubated at 37°C for 24h. After two successive transfers of 24 h incubations, the pre-inocula cultures were prepared by transferring 1% (v/v) of activated culture to 10 mL aliquots of reconstituted sterile skim milk (RSM) containing 14% (w/w) total milk solid. The skim milk was autoclaved following the standard procedure (121°C for 15 min).

3.2. Culture performance during cultivation in milk

The fermentation trials were conducted as described by (Donkor et al., 2007a). Five hundred millilitres of reconstituted skim milk (14% total solids) was prepared by dissolving of skim milk powder (SMP) (Bonlac Foods Limited, Australia) in Milli Q water and heat treated at 85°C for 30 min followed by cooling to approximately 40°C. This milk was aseptically inoculated with 1% (v/v) of each single strains in this study and incubated at 37°C for 12 h. Samples were taken for analysis at 2 h intervals: 0, 2, 4, 6, 8, 10 and 12 h during the fermentation time. The pH change was estimated by a pH metre (model 8417; HANNA Instruments, Singapore) at the times mentioned above.

Cell populations of each culture *L. helveticus* ASCC474, *L. helveticus* ASCC118, *L. helveticus* ASCC1315, *L. helveticus* ASCC953, *L. delbrueckii subsp. bulgaricus* ASCC734, *L. delbrueckii subsp. bulgaricus* ASCC756, *L. delbrueckii subsp. bulgaricus* ASCC857, *L. delbrueckii subsp. lactis* ASCC1210, *L. delbrueckii subsp. lactis* ASCC1307, and *L. delbrueckii subsp. lactis* ASCC1372 were assessed using pour plate technique (Donkor et al., 2006). Briefly, one mL of each fermented sample taken was added to 9 mL of sterile 0.15% (w/v) bacteriological peptone (Oxoid) and water diluent followed by vortexing using a MT 19 auto vortex mixer (Chiltern Scientific, Biolab Ltd., Auckland, New Zealand) for 30 s. The resulting suspension was serially diluted in 0.15% of sterile peptone water (v/v) (Oxoid) and one mL of the appropriate dilution was selected for enumeration by the pour plate technique. All samples were enumerated on MRS-sorbitol agar under anaerobic incubation using anaerobic kits at 37°C for 72 h. Plates containing 25–250 colonies were enumerated and the colony forming units (cfu) per g of the product was calculated

3.3. Determination of proteolytic activity

The extent of proteolytic activity at time stated previously during fermentation of milk was measured by assessing free amino groups using the o-phthaldialdehyde (OPA) method (Donkor et al., 2007b). Briefly, three millilitre aliquots of the samples at each time point selected were mixed with 3-mL of 1% (w/v) TCA (trichloroaceticacid) and vacuum-filtered using an Advantec #231 filter paper (MFS Inc., Dublin, CA, USA). The permeate was collected and approximately 150 µl was added to 3 mL of OPA reagent. The mixture was held at room temperature (~ 20°C) for 2 minutes and the absorbance of each solution was measured by using a spectrophotometer (Cary IE UV/visible spectrophotometer, Varian Australia Pty. Ltd., Melbourne, Australia) at 340 nm. The proteolytic activity of the bacterial cultures was expressed as the absorbance of OPA derivatives at 340 nm. A relative degree of

proteolysis was determined as the difference between proteolytic activities in fermented milk to that of untreated milk.

3.4. Preparation of released soluble peptides and chromatographic determination

These samples were prepared according to the method of Donkor et al. (2007a). Briefly, 50 mL of each fermented milk sample was taken at regular time intervals 0, 2, 4, 6, 8, 10 and 12 h during fermentation and subsequently pH was adjusted to 4.6 using either 1M HCl or NaOH. All samples were centrifuged at 15,000 x g (JA20 rotor, Beckman Instruments Inc., Palo Alto, CA, USA) for 30 min at 4°C and the resulting supernatant was subsequently filtered separately through a 0.45 mm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) and stored at -20°C for further analysis.

The filtered soluble peptides from all individually strains in RSM samples were profiled using a reversed-phase HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with C-18 monomeric column (5 µm, 300A, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA) according to the method of Donkor et al. (2007a) with some modifications. Filtered samples were applied using a 50 µl injection loop. The peptides were eluted by a linear gradient from 100% to 0% of solvent A (0.1% trifluoroacetic acid (TFA) in deionised water) in solvent B (0.1% TFA in 90%, v/v acetonitrile in deionised water) over 40 min. Separations were performed at room temperature (~20 °C) at a flow rate of 0.75 mL min^{-1}. The eluted peptides were detected using a Varian 9050 variable wavelength UV/Vis detector at 214 nm. All solvents and samples were filtered throughout a 0.45 mm membrane filter.

3.5 ACE inhibitory activity

The Angiotensin Converting Enzyme inhibitory (ACE-I) activity was determined according to the method of Ramchandran et al. (2008). Briefly, 0.005 M Hip-His-Leu was diluted in 0.1 M borate buffer containing 0.3 M NaCl at pH 8.3, as a substrate for the rabbit lung ACE (0.1 units/mL). Approximately, 60 µl of the borate buffer was added to 200 µl of the substrate solution, followed by the addition of 30 µl of the filtered sample and pre-incubated at 37 °C for 5 min. The next step was addition of exactly 20 µl of the enzyme solution (ACE) and incubation at 37 °C for 30 min. The reaction was stopped by adding 250 µl of 1 M HCl solution and then mixing with 1.7 mL ethyl acetate. After 10 min at room temperature, 1.4 mL of the ethyl acetate layer was removed into a new test tubeand dried on a boiling water bath and then in an oven at 80°C for 30 min. The remaining residue after the removal of 1.4 mL ethyl acetate was discarded. The dried hippuric acid in the test tube was dissolved in 1 mL of deionised water and the absorbance of the resulting solution was measured at 228 nm using a UV/Vis spectrophotometer (Pharmacia, LKB-UltrospecIII). The percent inhibition was calculated using the following formula:

$$ACE - inhibition\ (\%) = 1 - (\frac{C - D}{A - B}) \cdot 100$$

where A is the absorbance in the presence of ACE and without sample, B is the absorbance without ACE and sample, C is the absorbance with ACE and ACE- inhibitory component, and D is the absorbance with sample but without ACE.

3.6. Preparation of intracellular and cell wall extracts

The cultures, which exhibited high proteolytic and ACE inhibition activities (*L. helveticus* ASCC474, *L. helveticus* ASCC118 and *L. helveticus* ASCC1315), were selected for further analysis. The preparation of intracellular (IE) and extracellular (EE) extracts was done as described by Shihata and Shah (2000). Briefly, three selected strains were propagated three successive times in RSM and finally in MRS broth. The latter was performed to avoid a carryover of milk proteins and their interference with the assay. Thus after two subculturing in MRS medium for 18 h at 37°C, cultures were cultivated separately in 100 mL batches of MRS broth at 37°C. After that, cells were collected by centrifugation at 4000× *g* at 4°C for 30 min. The supernatant obtained was designated as the cell-free extracellular enzymatic extract (EE). The cell pellet gained was washed two times using 0.9% (w/v) NaCl solution and centrifuged each time to the NaCl solution. The washed pellet was dissolved in 1 mL 0.05 M Tris-HCl buffer, pH 8.5. The resulting cell dispersion was sonicated for 5 min at 30 s intervals at 4°C. The supernatant obtained after centrifugation at 4000× *g* for 30 min at 4°C was designated as the intracellular enzymatic extract (IE).

3.7. Aminopeptidase activity

The aminopeptidase activity of the extracts of all the selected cultures separately was determined by the methods of Donkor et al. (2007a) and Ramchandran et al. (2008) using chromogenic substrates of *p*-nitroanilide (*pNA)* derivatives of glycine-*pNA*, arginin-*pNA*, glycine-prolin- *pNA* and succinyl-alanin-alanin-prolin-phenylanin-*pNA* (Gly-*pNA*, Arg-*pNA*, Gly-Pro-*pNA* and Suc-Ala-Ala-Pro-Phe-*pNA*) (Sigma-Aldrich Pty. Ltd. Australia). Each assay mixture consisted of 100 µL of the IE, 400 µL of 0.05M Tris HCl buffer (pH 7.0), and 50 µL of 0.01M of each substrate. The mixture was incubated at 37 °C for 20 min and the reaction was after that stopped by adding 1 mL (30%) of acetic acid. The concentration of p-

nitroanilide released was determined by measuring the absorbance at 410 nm using NovaSpec®-II UV-Spectrophotometer. The enzyme activity was calculated using a molar absorption coefficient of 8100 $mol^{-1}cm^{-1}$ and expressed as micromoles of substrate hydrolyzed per milliliter of extract per minute. The specific activity was expressed per milligram of protein. The protein content of the EE and IE extracts was estimated using the method of Bradford (Bradford, 1976). A 0.1 mL aliquot of the enzyme solution and 3 mL aliquot of the Bradford reagent (Sigma) were vortexed gently to mix thoroughly and the samples were incubated at room temperature for 30 min after which the absorbance was measured at 595 nm. Bovine serum albumin (Sigma-Aldrich Pty. Ltd, Australia) was used as a standard. The protein concentration of the samples was determined by comparing the net absorbance values obtained at 595 nm against the standard curve.

3.8. Assessment of X-prolyl-dipeptidyl aminopeptidase activity

X-prolyl-dipeptidyl aminopeptidase (PepX) activity of dairy cultures is a very important feature required for production of fermented milk products due to high proline content in milk protein. This activity was evaluated according to Pan et al. (2005) and Donkor et al. (2007a) with glycyl-prolyl p-nitroanilide (Gly-Pro-pNA) as a substrate from Sigma Aldrich (Australia). Briefly, 50 µL of 0.0064 M of chromogenic substrate was added to 2.85 mL of 0.05 M Tris-HCl buffer pH 7.0, and 100 µL of cell-free intracellular extract (IE) in 0.05M Tris-HCl buffer pH 7.0. The mixture was incubated at 37°C for 20 min and the reaction after that was stopped by adding 500 µL of 30% acetic acid. The extent of hydrolysis was measured with the Cary IE UV/visible spectrophotometer (Varian Australia Pty. Ltd., Melbourne, Australia) at 410 nm. The same experiment was also carried out with cell-free extracellular (EE) enzymatic extracts. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitroanilide per min under assay conditions. The

enzyme activity reported is expressed as specific Pep X activity defined as units of enzyme activity per milligram of proteins. A unit of activity is derived from the enzyme content required to liberate 1 μmol of p-nitroanilide per min under assay conditions.

3.9. Extraction of crude proteinase

The extraction of crude cell wall bound proteases was conducted according to the method of Nas and Nissen-Meyer (1992). Briefly, cells of the three highly proteolytic *L helveticus* strains were selected and individually harvested from 200 mL culture by centrifugation at 10,000xg (JA20 rotor, Beckman Instruments Inc., Palo Alto, CA, USA) for 30 min. The cells obtained as a pellet were washed twice with 0.05-M of β-glycerophosphate at pH 7.0 containing 0.02 M $CaCl_2$ in order to minimize the release of the cell wall bound proteinases. After washing, the extraction of the cell-wall proteinase was carried out by resuspending the cells three successive times in 0.02M Bistris buffer adjusted to pH 6-0 with using concentrated HCl, containing 0.01 M EDTA. The reason of adding EDTA with this buffer was to optimize the release of proteinase (Nas and Nissen-Meyer, 1992). Each extraction took an hour at room temperature (~20 °C). The supernatants obtained from the extractions were filtered through 0.45 μ (Millipore) and the solution filtrated was designated crude proteinase extract (CPE).

3.10. Hydrolysis of milk proteins by *L. helveticus* crude proteinase extract for the release of oligopeptides

The crude proteinases extracts (CPE) obtained from the highly proteolytic *L. helveticus* strains were kept frozen at -20 °C after extraction. Each single selected strain proteinase was mixed with 200 mL (14% total solid) of reconstituted skim milk (RSM) and incubated for 12 h at 37°C. During this incubation, samples were taken at 0, 6 and 12 h followed by centrifugation at 15,000xg (Beckman Instruments Inc., Palo Alto, CA, USA) and 4°C for 30 min. Supernatants likely containing oligopeptides were poured out and freeze-dried (Dynavac freeze drier; Dynavac Eng. Pty. Ltd., Melbourne, Australia). The freeze dried samples were stored at -20 °C for further analysis.

3.11. Determination of radical scavenging activity of oligopeptides

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was used according to the method of (Bhandari et al., 2010) to determine the free radical scavenging ability of oligopeptides released by crude proteinase extracts in section 3.10. Briefly, 0.3 mL of a sample was added to two millilitre of 0.1 mM DPPH dissolved in methanol in test tubes and dissolved solution was shaken vigorously. The tubes were then incubated in the dark at room temperature for 30 min. A DPPH blank sample was prepared in free extract and methanol was used for the baseline correction. Reduction of the absorbance at 517 nm was measured using a UV-Vis spectrophotometer. All experiments were carried out in triplicate. The radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{Radical scavenging activity } (\%) = \frac{Control - Sample}{Control} \cdot 100$$

3.12. Isolation of human peripheral blood mononuclear cells from buffy coat

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donor buffy coats (Australian Red Cross Blood Services (ARCBS), Melbourne, Australia) by Ficoll gradient. PBMCs were isolated according to the methods described in Laffineur et al (1996) with minor modifications. Briefly, buffy coat was diluted with an equal volume of PBS and layered on Ficoll-Paque Plus (GE Healthcare, Bio-Sciences, Uppsala, Sweden) in a 50 mL centrifuge tube. Cells at the interphase were collected after centrifugation (680xg) for 25 min at 18°C (Sorvall® RT7 centrifuge, DuPont, Newtown, Ct. USA). Blood lymphocytes were then washed once in cold PBS and after centrifugation at 18°C for 10 min at 680xg, the pellet was resuspend in 2 mL ACK lysing buffer (8.024g/L NH_4Cl, 1.001g/L $KHCO_3$ and 0.003722g/L EDTA $Na_2.2H_2O$) to lyse any remaining erythrocytes and incubated for 2 min at room temperature. The volume was then adjusted to 30 mL via the addition of sterile PBS and centrifuged at 18 °C for 10 min at 680 x g. The above washing step with PBS was repeated two times and the resulting pellet was resuspended in Iscoves Modified Dulbeccose Medium (IMDM) (Sigma) supplemented with 10% fetal bovine serum (FBS, GIBCO, Mulgrave, Australia), and antibiotic antimycotic solution (Sigma) at 10 mL/L ready for further experimentation.

3.13. Immunomodulatory activity of peptides

Immunomodulatory activity of peptides was performed according to the method of Laffineur et al (1996) to determine the *in vitro* immunostimulation of soluble peptides. Briefly, PBMC count was obtained using 0.4% trypan blue stain and haemocytometer. The average number of cells in one large square was determined by the dilution factor x 10^4 cells. PBMCs (1 x $l0^6$ cells/ml) were co-cultured with 100 µL of each resuspended soluble peptide sample in IMDM (20 µg protein) onto 24-well flat bottomed plate (Greiner bio-one, Cellstar)

and incubated for 72 h at 37°C in a humidified 5% CO_2 incubator. PBMCs alone were cultured for period as a control. Cytokine production by PBMCs were analysed from supernatants collected after 72 h co-culture and centrifuged at 14,000 rpm for 10 min. Interleukin (IL)-10 and interferon gama (IFN-γ) were measured by commercially available ELISA kits (BD biosciences, Australia).

3.14. Statistical analysis

All the experiments were carried out in triplicate for each bacterial culture. Results obtained were analysed as a split plot in time design with 2 main factors: strains and replications as the main plot and time as a subplot. The statistical evaluations of the data were performed using the General Linear Model (SAS, 1996). Significant differences between treatments were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed by Fisher's least significant difference (LSD) method, with a level of significance of $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. The growth performance of the selected strains

The strains selected for the study have been recognized as highly proteolytic and fast growing cultures. The culture performance was assessed as the ability of the strains to produce organic acids as the primary metabolites, which was measured by pH decline shown in Figure 4.1. The growth was also assessed by determining viable cell counts. For appropriate culture performance in milk, the cells require highly developed proteolytic and glycolytic systems capable of providing essential compounds for the culture growth. As it appears the performance was highly strain dependant. During 12 hours of incubation of selected LAB strain in sterile milk, these cultures induced pH reduction in various degrees ranging from 5.72 to 3.9 (Figure. 4.1A,B and C). All *L. helveticus* strains reached and even passed ultimate pH of 4.6 as illustrated in Figure 4.1A. (Donkor et al., 2007b). This performance was not followed by the strains belonging to *L. delbrueckii* ssp. *bulgaricus* or *lactis*, which in majority failed to reach the ultimate pH (Figure 4.1.B, C). The only exception among these strains was *L. bulgaricus* 1307, which decreased pH of milk down to 4.54 at the end of the fermentation significantly ($P< 0.05$) lower than any other strain of this species. The best performer among all the strain was *L. helveticus* 1315, which brought pH down to 3.92 at the end of the fermentation at 37°C. On the other hand, *L. bulgaricus* 1210 which growth in milk resulted in pH decline only down to 5.72.

The rate of pH decline is indicative of the culture activity and performance when it comes to the culture applications in the industry. Closer data analysis by curve fitting has suggested that the rate of acid production and thus pH decline was the strain dependent (Figure. 4.2 A, B and C). The greatest rate of the pH decrease was observed for *L. helveticus* 1315, which was -0.243 units h^{-1}. All *L. helveticus* strains performed substantially better than other *Lactobacillus* species assessed in this study, recording pH lowering rate on average of -0.217 units h^{-1}. Similarly, the same *Lactobacillus* strains showed very high specific growth

rate, with *L. helveticus* 1315 having the highest. Correlational analysis between specific growth rate and pH decrease rate showed a weak relationship of -0.479. Surprisingly, *L. bulgaricus* 1210 and 1372 showed appreciable specific growth rate of 0.235 and 0.221 cfu h^{-1}, respectively, although the rate by which they lowered pH was substantially lower than any other strains. This observation could be only explained in the differences in the metabolic ability and growth requirements. The pH decline depends on the amount of lactic acid and other organic acids released, which is directly linked to the culture metabolic capacity. Certain lactic acid bacteria strains can utilize lactose fully as opposed to some others than can mainly convert a part of lactose, namely glucose, into lactic acid which could have been a reason for a slow pH decline (Donkor et al., 2007b).

To efficiently modulate the balance of intestinal microflora and potentially improve the health and wellbeing of the recipients, it is important that dairy starter cultures should be alive in adequate numbers in fermented milk when consumed by consumers at the time of consumption (Donkor et al., 2007a). To provide therapeutic effects, dairy starter cultures should be presented in cultured milk to the minimum level of 10^6 log colony forming unit per gram (cfu g^{-1}) of fermented milk (Gomes and Malcata, 1999b).

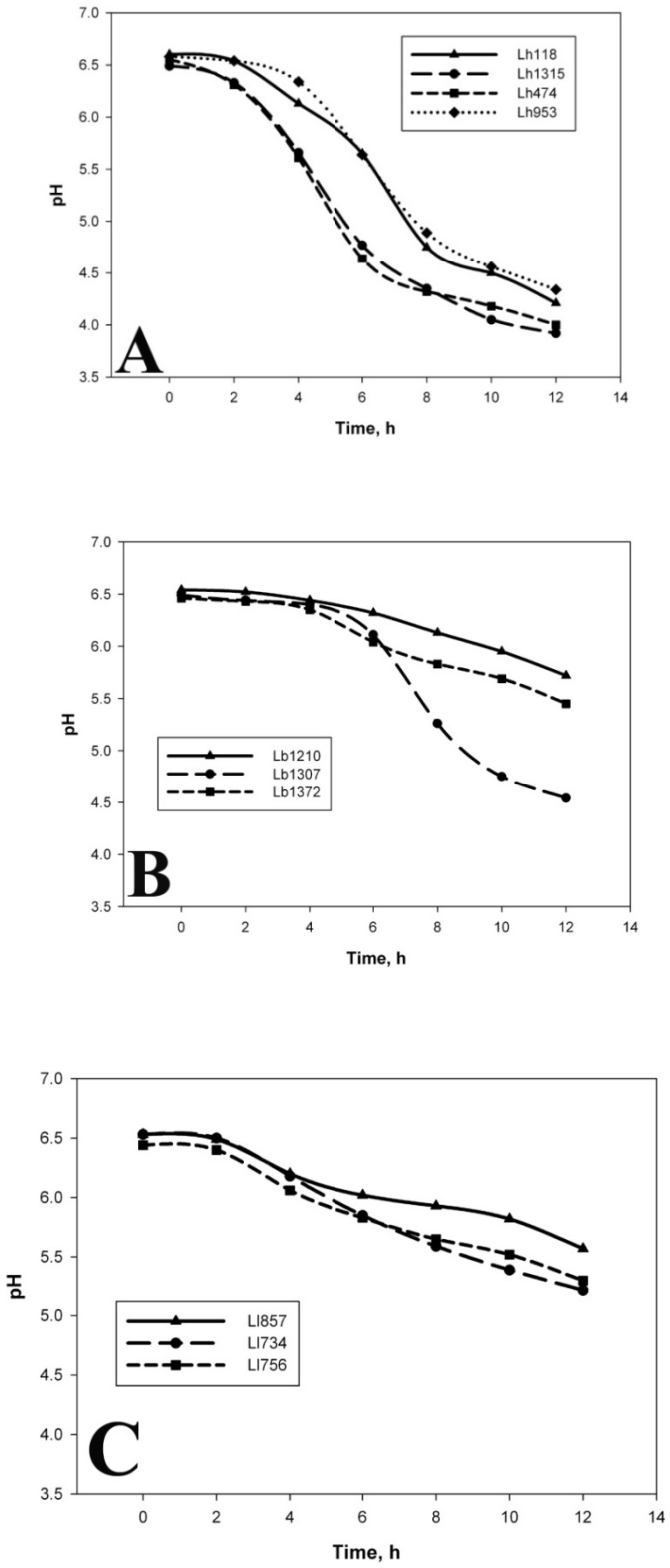


Figure 4.1 pH decline during growth of selected *Lactobacillus* species in sterile skim milk for 12 h at 37ºC. Legend A = *L. helveticus* strains; B = *L. bulgaricus* strains, C = *L. lactis*. Standard error of the mean for all samples was 1.537.

A population of all lactic acid bacteria (LAB) used (*L. helveticus* 474, *L. helveticus* 118, *L. helveticus* 1315, *L. helveticus* 953, *L. bulgaricus* 734, *L. bulgaricus* 756, *L. bulgaricus* 857, *L. lactis* 1210, *L. lactis* 1307, *L. lactis* 1372) successfully achieved the desired level, reaching at least 7.03 log cfu mL^{-1} at the end of incubation time in milk as the growth medium, which is shown in Figure 4.2A, B and C. During the first half of the incubation period, *L. helveticus* 474 and *L. lactis* 1372 reached the highest ($P< 0.005$) growth level (8.55 and 8.08 cfu/g, respectively) compared with all other selected strains at the same time of incubation. However, at the end of incubation time, the population of *L. helveticus* 474 and *L. lactis* 1210 were significantly higher ($P< 0.005$), which increased to 9.84 and 9.0 cfu/g, respectively, than other fermented cultures, which showed a consistent and gradual increase in their cell concentration during incubation time stated. The decrease of the pH during fermentation time did not affect the increase of growth level of these strains. This desired observation could be probably due to their stability and ability to survive at various pH during fermentation time. Donkor et al (2006) reported that pH is one of the most important factors that can affect the growth of microorganisms. The growth of *L. bulgaricus* strains were rather more inferior to other species examined during milk fermentation. These results could be due to a weak ability of these strains to release or required amino acids from milk proteins and probably attributed to the decline of pH changes during fermentation time. On the other hand, nutrients such as free NH_3 groups and peptides produced may also be other factors affecting the viability of LAB growth (Donkor et al., 2007b). Chandan and O'Rell (2006) found that *L. bulgaricus* has a poor ability to degrade peptides to free amino acids in comparison to *S. thermophilus.* These results also suggest that *L. delbrueckii* ssp. *bulgaricus* are more fastidious than other *Lactobacillus* species tested in this study and need extra sources of amino acids needed for their growth. Hence, *L. delbrueckii* ssp. *bulgaricus* growing in milk as the growth medium may take longer time to establish desired levels in

such a system that contains complex proteins as a source of nitrogen(Ramchandran and Shah, 2008). The growth of *L. helveticus* 474 in fermented milk reached the highest level among those strains used in this study. More importantly, even though pH of some *Lactobacillus* strains (*L. lactis* 1210, *L. helveticus* 315, *L. helveticus* 474, *L. helveticus* 118, *L. lactis* 1372 and *L. helveticus* 953) declined below 4.6, these strains in the milk as growth medium appeared to maintain more appreciable growth than other strains tested under the same conditions.

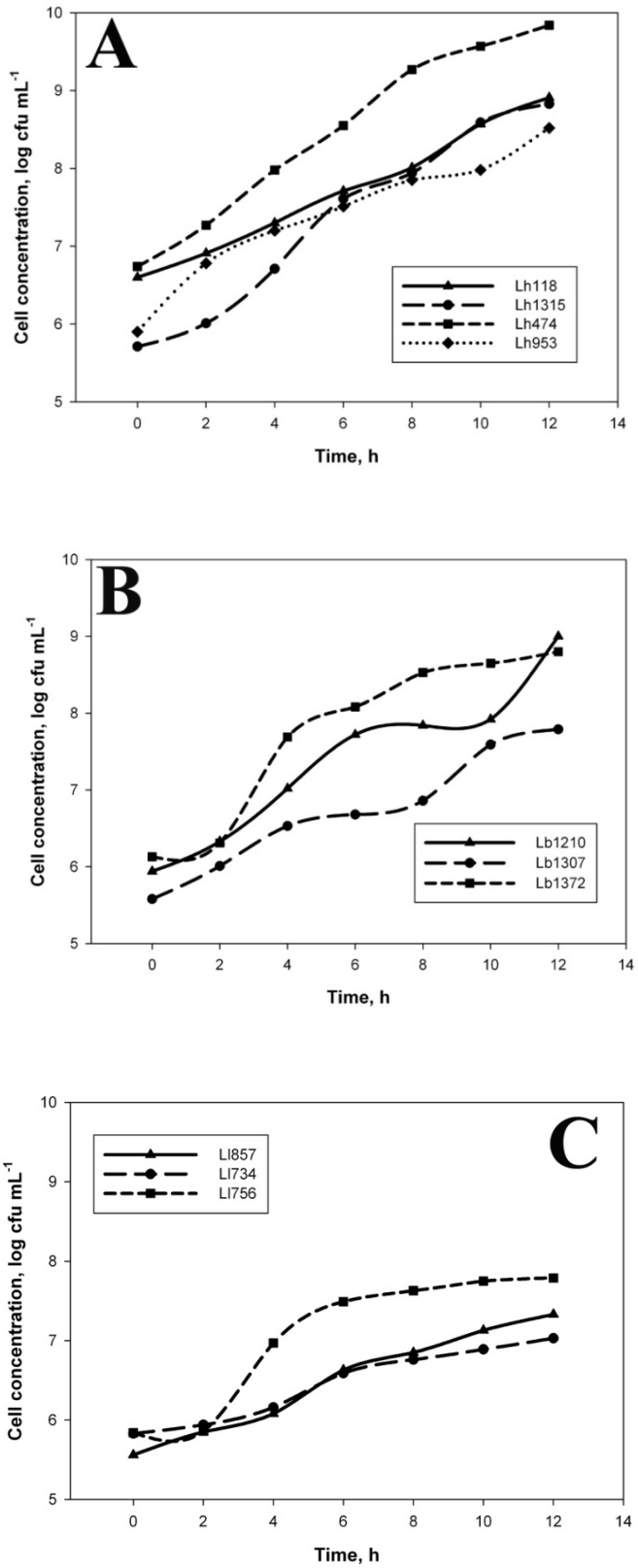


Figure 4.2 Change of cell concentration during growth of selected *Lactobacillus* species in sterile skim milk for 12 h at 37ºC. Legend A = *L. helveticus* strains; B = *L. bulgaricus* strains, C = *L. lactis*. Standard error of the mean for all samples was 0.02.

4.2. Proteolytic activity

Production of amino acids and peptides from the degradation of milk proteins by LAB enzymes and utilization of these amino acids are a central metabolic activity of LAB (Gobbetti et al 2002). Lactic acid bacteria, isolated from milk products, require from 4 up to 14 amino acids depending on the strain (Chopin, 1993). However, the amount of free amino acids and peptides in milk is very low. Therefore, lactic acid bacteria depend on a proteolytic system allowing degradation of milk proteins for the growth (Juillard et al., 1995a). Casein, comprising the major part of milk proteins, contains all amino acids necessary for the growth of lactic acid bacteria in milk to high cell density, yet only a minor fraction, less than 1% of the total casein content, is actually needed (Kunji et al., 1996).

Amino acids and peptides produced by enzymatic hydrolysis of milk proteins by LAB proteolytic system and utilization of these amino acids are a central and integral part of their metabolic activity. During fermentation, milk, as stated in literature review, cannot supply all essential amino acids needed for LAB growth in free form; therefore, these microorganisms have developed their capability to degrade milk proteins, mainly caseins, by their proteolytic system producing initially peptides and then amino acids needed for their growth(Donkor et al., 2007a). It has been well established that a number of *Lactobacillus* sp. grow well in skim milk (Gilbert et al., 1996a). Proteolysis is a cascade of processes involving a number of steps including an extracellular proteinase initiating degradation of casein into oligopeptides, transport systems that translocate peptides and amino acids across the cell wall, various intracellular peptidases for further degradation of peptides into amino acids, and different enzymes that convert liberated amino acids into various components (Kunji et al., 1996).

Proteolytic activity of all fermented strains used in this study was assessed during 12 h of incubation time at 37°C and is shown in figure 4.3.A, B and C. The free amino acid content in all fermented milk samples was variable due to proteolytic system of these

microorganisms in the milk as the growth medium (Donkor et al., 2007b). Proteolysis as assessed by the release of free NH_3 groups by using OPA method, increased during the 12 h of the incubation time. During fermentation, milk proteins were degraded by *Lactobacillus* proteinases and peptidases resulting in producing a number of free amino groups and various forms of peptides. As depicted in Figure 4.3 A, B and C, the amount of liberated amino groups and peptides increased significantly during fermentation from 0 to 12 h for all strains tested in this study. The extent of proteolysis varied among strains examined and showed to be the time and strain dependant (Donkor et al., 2007a). The extent of proteolysis in the *L. helveticus* strains fermented milk was significantly higher ($P< 0.05$) than that of the other fermented strains in this study at the first half period of incubation time. The level of proteolytic activity remained substantially higher ($P< 0.05$) than those of *L. bulgaricus* and *L. lactis* strains, and that degree of proteolysis was depended significantly on incubation time and strain. These results were similar as those reported by Leclerc et al. (2002), who demonstrated a linear increase in the extent of proteolysis with fermentation time for *L. helveticus* among the species of LAB studied. The primary enzymes in LAB responsible for the hydrolysis of the proteins are proteinases and peptidases (Law and Haandrikman, 1997, Shihata and Shah, 2000). *L. helveticus* 118 achieved the highest ($P< 0.05$) proteolytic activity among strains studied followed by *L. helveticus* 474, *L. helveticus* 1315 and *L. helveticus* 953, of which absorbance were 2.226, 1.841 and 1.754 respectively. These results indicate that the proteolytic activity of *L. helveticus* strains under the dropping of the pH, had a strong effect on bacterial growth ($P< 0.05$) compared to other strains examined (Leclerc et al., 2002). On the other hand, *L. bulgaricus* 1210 had the lowest ($P< 0.05$) proteolytic activity comparing with *L. helveticus* strains in this study which only reached to 0.592 of absorbance. As indicated previously *L. bulgaricus* 1210 showed appreciable growth in the medium, however the levels of free amino acids and peptides in milk remained low. This strain may

have proteolytic enzymes affected by decreasing the pH ($P< 0.05$) during fermentation time and thus might need longer time to adapt to the growth medium for proteolytic system development. These differences in the amounts of amino groups released during fermentation of milk observed could probably relate to the different proteinases and peptidases of the strains and appeared to be strain dependent (Shihata and Shah, 2000).

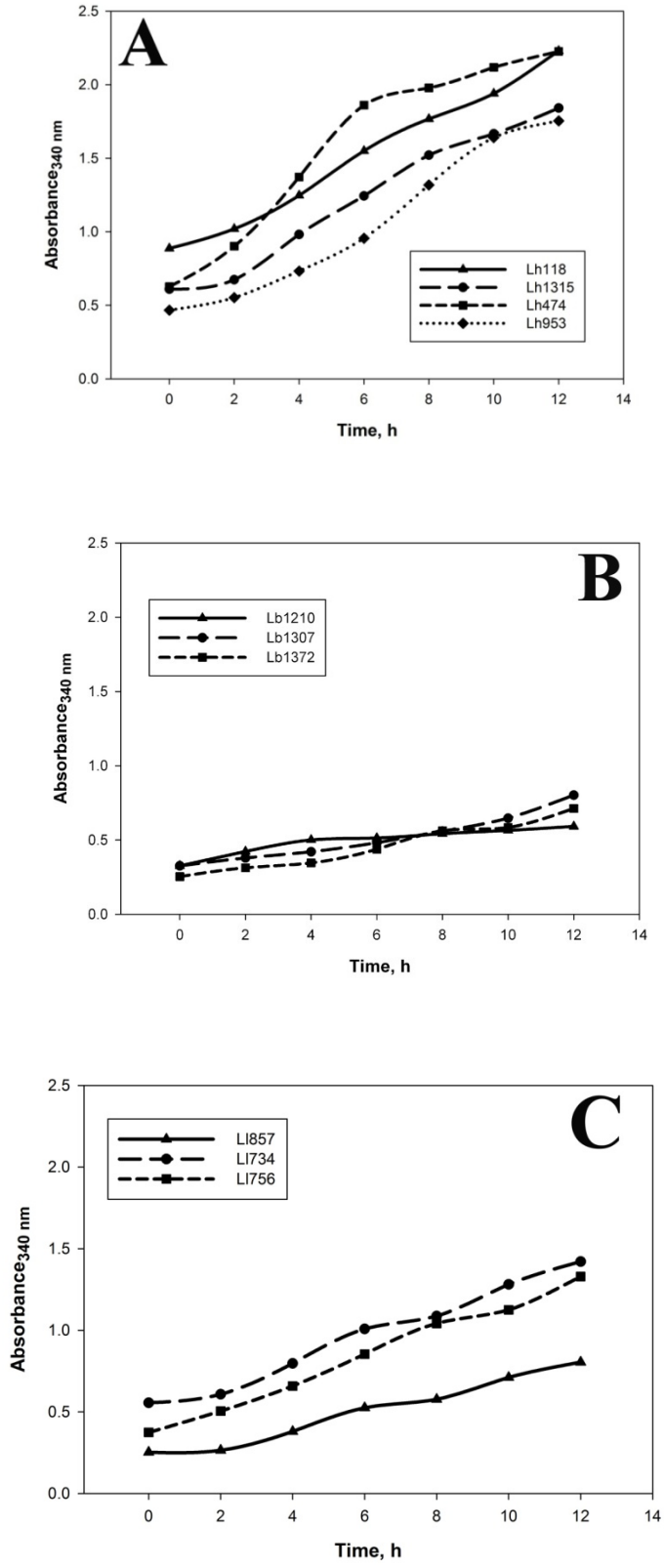


Figure 4.3 Extent of proteolysis measured using OPA method during growth of selected *Lactobacillus* species in sterile skim milk for 12 h at 37ºC. Legend A = *L. helveticus* strains; B = *L. bulgaricus* strains, C = *L. lactis*. Standard error of the mean for all samples was 1.54.

4.3. R P-HPLC profiling peptides

The data obtained from RP-HPLC (reverse phase- high performance chromatography) show the profile of the peptides released during the growth of selected cultures in milk over 12 h period at 37 °C. Initial increase in the released peptides was due to the developing proteolytic activity of the selected *Lactobacillus* strains. At the beginning, only one peak was observed (Fig. 4.4-4.6) at 10 min elution time for almost all *L. helveticus* strains. This peak was not detected for *L. helveticus* 953. Similarly, there were two peaks detected for *L. bulgaricus* strains indicating peptides at 14 and 30 min for *L. bulgaricus* 734 and one small peak at 18 min for *L. bulgaricus* 756. Peptide profiles of *L. lactis* strains were similar to the previous strains with the exception of *L. lactis* 1372 where two peaks were observed at 12 and 30 min. These peptides produced at zero time could probably be due to proteinases and peptidase activities of these organisms to provide amino acids initially required for LAB growth (Dnkor et al., 2007b), or might probably be due to small peptides existed as a result of milk enzymes activity such as plasmin. The principal substrate for plasmin is β-CN. Alfa s_2,-Casein in solution is also hydrolysed very rapidly by plasmin at certain bonds(Crudden et al., 2005). A number of peaks for all *L. helveticus* strains increased substantially after 6h comparing with the beginning of the fermentation time. Seven peaks were detected at first 20min of elution for *L. helveticus* 474 followed by *L. helveticus* 118, which indicate that high proteolytic activity by proteinases and peptidases of these strains. This is another evidence that these activities are crucial for the performance of these strains in milk; for example, the greatest pH decline and highest cell concentration (Donkor et al., 2007b). On the other hand, the extent of protein hydrolysis showed as the breakdown of peptides by *L. bulgaricus* and *L. lactis* strains did not change substantially in comparison to *L. helveticus* strains during the same period. Some of the initially detected peaks released by *L. bulgaricus* and *L. lactis* strains disappeared and were probably hydrolysed after 6 h of fermentation. It appears from

these findings that these strains may require longer time to adapt to develop their proteolytic system for more amino acids and peptides. The fermentation temperature can be another factor affecting proteolytic activity during incubation and in the current study was not changed but maintained constant. At the end of the fermentation, the number of peaks increased significantly for all the strains examined with the exception of *L. bulgaricus* 1372 with only a few sporadic peaks. These findings demonstrate again that the release of the peptides is a very dynamic process and strictly strain specific (Ong et al., 2007). It can be concluded from the chromatograms that milk fermented by *L. helveticus* strains showed substantially higher levels of liberated peptides compared with those of *L. delbrueckii* sp under the same conditions and *L. helveticus* strains were not affected by the acidity and incubation time at 37 ^{0}C. These peptides produced might have bioactive functions such as ACE-I inhibitory, antioxidant and immunomodulatory activity. The peptide pattern of *Lactobacillus* strains also substantially differed from one to another which indicated differences in the proteolytic activity among these organisms.

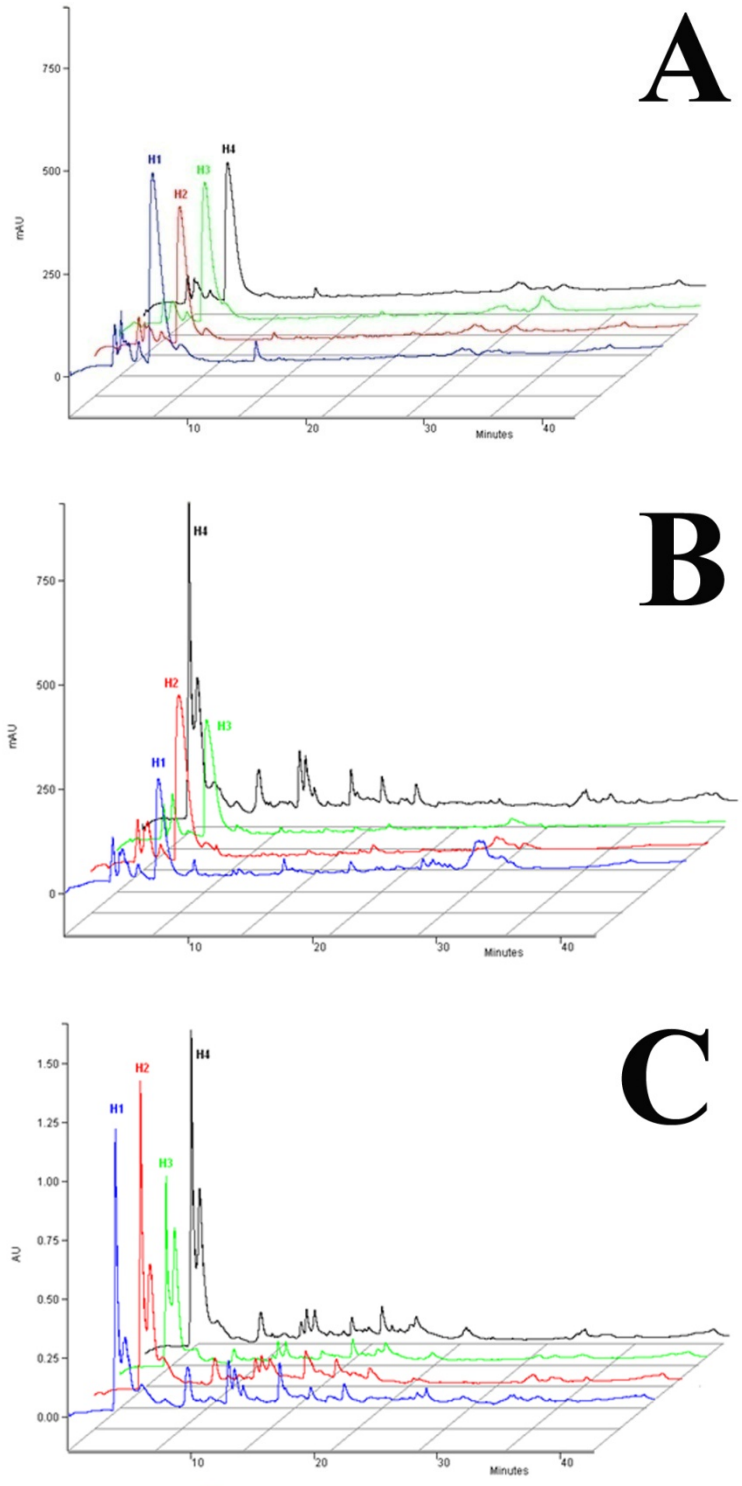


Figure 4.4 RP HPLC profile of the water-soluble peptides released in milk during growth of *L. helveticus* 118 (H1), *L. helveticus* 1315 (H2), *L. helveticus* 953 (H3) and *L. helveticus* 474 (H4) cultures at zero (A) h, 6 (B) and 12 (C) h at 37ºC by using a linear gradient from 100% to 0% solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%, v/v acetonitrile in water) over 40 min at a flow rate of 0.75 $mLmin^{-1}$. The eluted peptides were detected at 214 nm.

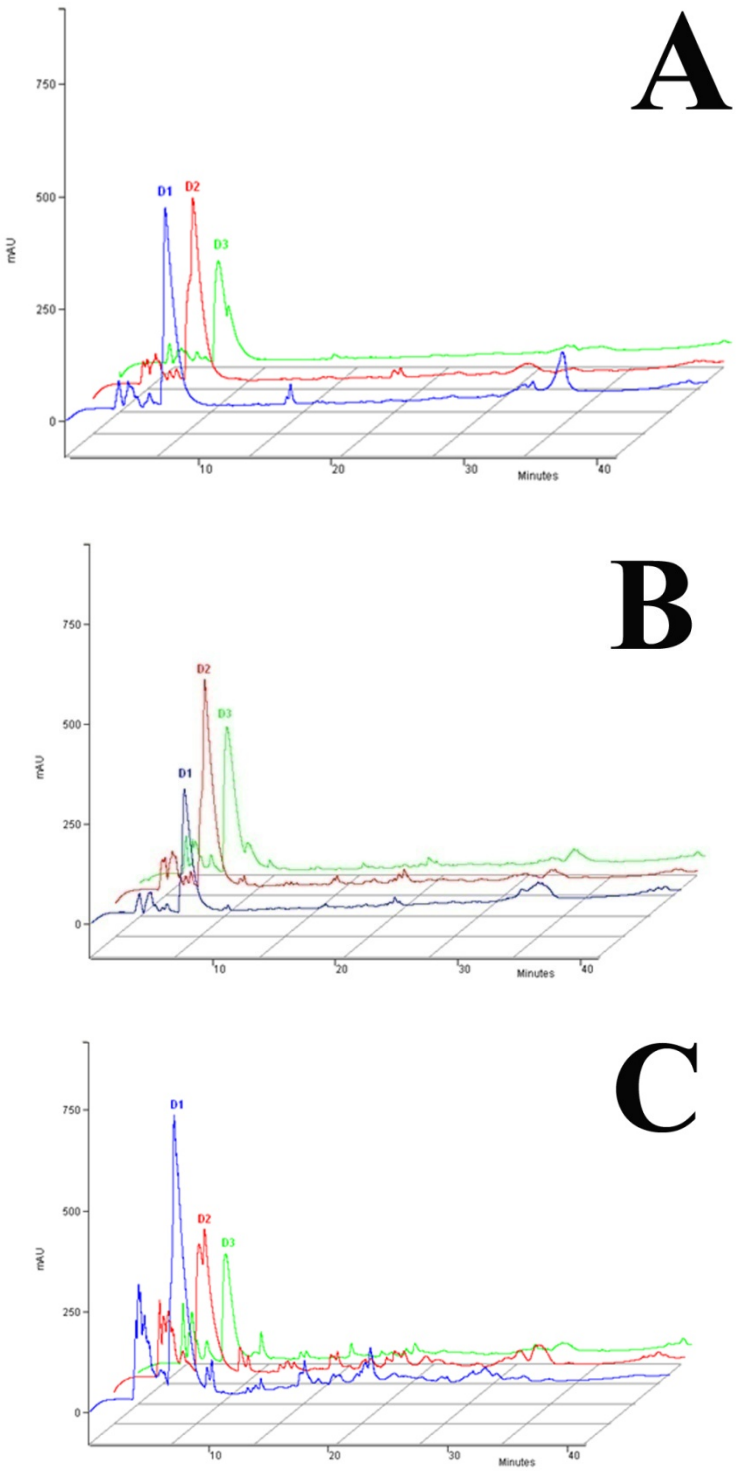


Figure 4.5 RP HPLC profile of the water-soluble peptides released in milk during growth of *L. bulgaricus* 734 (D1), *L. bulgaricus* 756 (D2) and *L. bulgaricus* 857 (D3) cultures at zero (A) h, 6 (B) and 12 (C) h at 37ºC by using a linear gradient from 100% to 0% solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%, v/v acetonitrile in water) over 40 min at a flow rate of 0.75 $mLmin^{-1}$. The eluted peptides were detected at 214 nm.

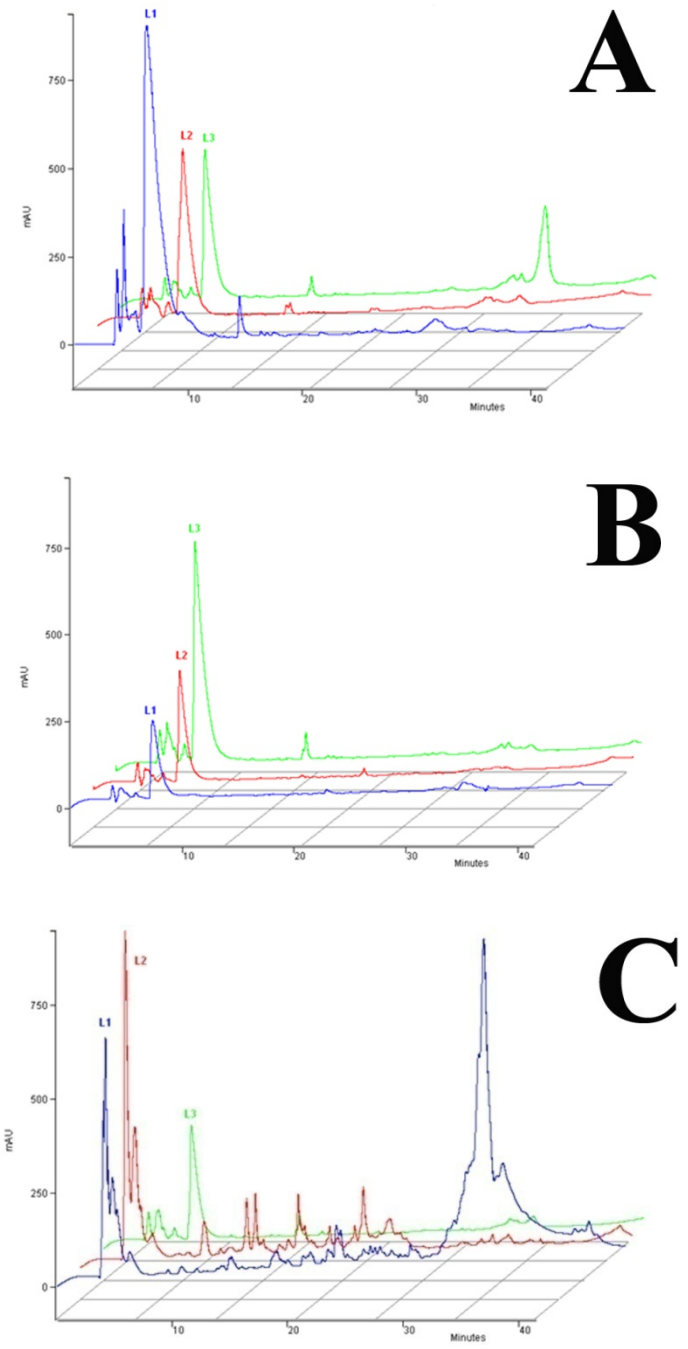


Figure 4.6 RP HPLC profile of the water-soluble peptides released in milk during growth of *L. lactis* 1210 (L1), *L. lactis* 1307 (L2) and *L. lactis* 1372 (L3) cultures at zero (A) h, 6 (B) and 12 (C) h at 37ºC by using a linear gradient from 100% to 0% solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%, v/v acetonitrile in water) over 40 min at a flow rate of 0.75 $mLmin^{-1}$. The eluted peptides were detected at 214 nm.

4.4. *In vitro* ACE inhibitory activities of fermented milk

The increase in blood pressure (BP) is a controllable risk factor in the development of a number of cardiovascular diseases (CVD) such as stroke and coronary infarction. Uncontrolled high BP results in more risk in the body such as CVD, stroke, heart failure and kidney disease. The high cost of and potential adverse side effects associated with pharmacological therapy for hypertension have encouraged individuals to adopt lifestyle modifications such as weight reduction, low-fat dairy products, dietary sodium reduction and regular physical activity to combat hypertension(FitzGerald et al., 2004). Uncontrolled high BP can be also reduced by consumption of microbial fermented milk exhibiting angiotensin-converting enzyme-inhibitory (ACE-I) (Lye et al., 2009).

Specific milk protein hydrolysates or fermented dairy products have been shown to induce clinically significant reductions in systolic BP and diastolic BP with no reported adverse effects (von Huth Smith et al., 2007). Bioactive peptides such as ACE-inhibitory peptides must reach their target organ intact to exert their effects *in vivo*. Degradation of peptides in the acidic environment of the stomach, alkaline conditions of the small intestines as well as hydrolysis by peptidases can affect the activation or deactivation of ACE-inhibitory peptides before they reach the portal circulation. Therefore, only those ACE-inhibitors that are not affected by the action of angiotensin-II and gastrointestinal enzymes or those that are converted to stronger ACE-inhibitors exert antihypertensive effects *in vivo* (Korhonen and Pihlanto, 2003b, Vermeirssen et al., 2003). Proteolytic strains of the *Lactobacillus* species have been demonstrated to produce products containing a high number of bioactive peptides including ACE-inhibitory and antihypertensive peptides (López-Fandiño et al., 2006). Some ACE-inhibitory peptides are products of extracellular proteases alone such as the large β-casein fragments produced by the extracellular proteases from *L.*

helveticus CP790 (Yamamoto et al., 1993) whereas others are most likely the result of the concerted action of both proteases and peptidases (Yamamoto et al., 1999).

In the current study, Figure 4.7 A, B and C depicts the *in vitro* ACE inhibitory activity of filtered soluble peptides fractions from milk fermented by all bacterial strains at various time intervals (0, 2, 4, 6, 8, 10 or 12 h) during incubation at 37°C. Ten strains of *Lactobacillus* species were screened for their ACE inhibitory effect. The measured inhibitory activities varied from 1.26 to 48.69% during the fermentation time. Among LAB tested in this study, only *L. helveticus* strains have shown to possess strong proteolytic activity in milk-based media and were also known to produce potent *in vitro* ACE-inhibitory peptides during milk fermentation. This relationship has been reported earlier by (Pihlanto et al., 2010).The higher ACE inhibitory activity ($P< 0.05$) in fermented milk was observed by *L. helveticus* 1315, which increased from 6.33% at the beginning to 48.69% at the end of the fermentation period. This could be associated with the proteolytic activity of this strain and its ability to produce ACE inhibitory peptides stronger than others (Fuglsang et al., 2003). However, ACE inhibitory activity in filtered peptides from *L. helveticus* 474 increased remarkably ($P< 0.05$) to 48.66% at 10 h and then followed by a significant reduction ($P< 0.05$) at the end of the incubation time, declining to 24.34%. Similar fluctuation was observed in all strains tested at different incubation times, with the exception of *L. helveticus* 1315. This instability in ACE-I peptide production could be attributed to the activity of *Lactobacillus* proteinases and peptidases which cause some ACE-I peptides to disappear and other new peptides to appear (Donkor et al., 2007b). All milk fermented by *L. bulgaricus* and *L. lactis* strains showed a weak and substantially lower ($P<0.05$) inhibition of ACE in comparison to *L. helveticus* strains. These activities varied between 5.06 with *L. lactis* 1372 to 32% with *L. lactis* 1307. The incubation time and pH reduction that affect the release of various peptides observed in this study might have important consequences on the extent of *in vitro* ACE inhibitory

activity in fermented milk, which need more investigation (Donkor et al., 2007a). To sum up, the ability of *Lactobacillus* strains to generate ACE inhibitory peptides during milk fermentation was observed to be a strain specific characteristic (Pihlanto et al., 2010) which might be connected to many factors such as bacterial growth, organic acid production and proteolytic activity of these strains. Furthermore, the time-dependent release of various peptides observed in our study might have important consequences on the extent of in vitro ACE inhibitory activity in fermented milk, which deserves further elaboration.

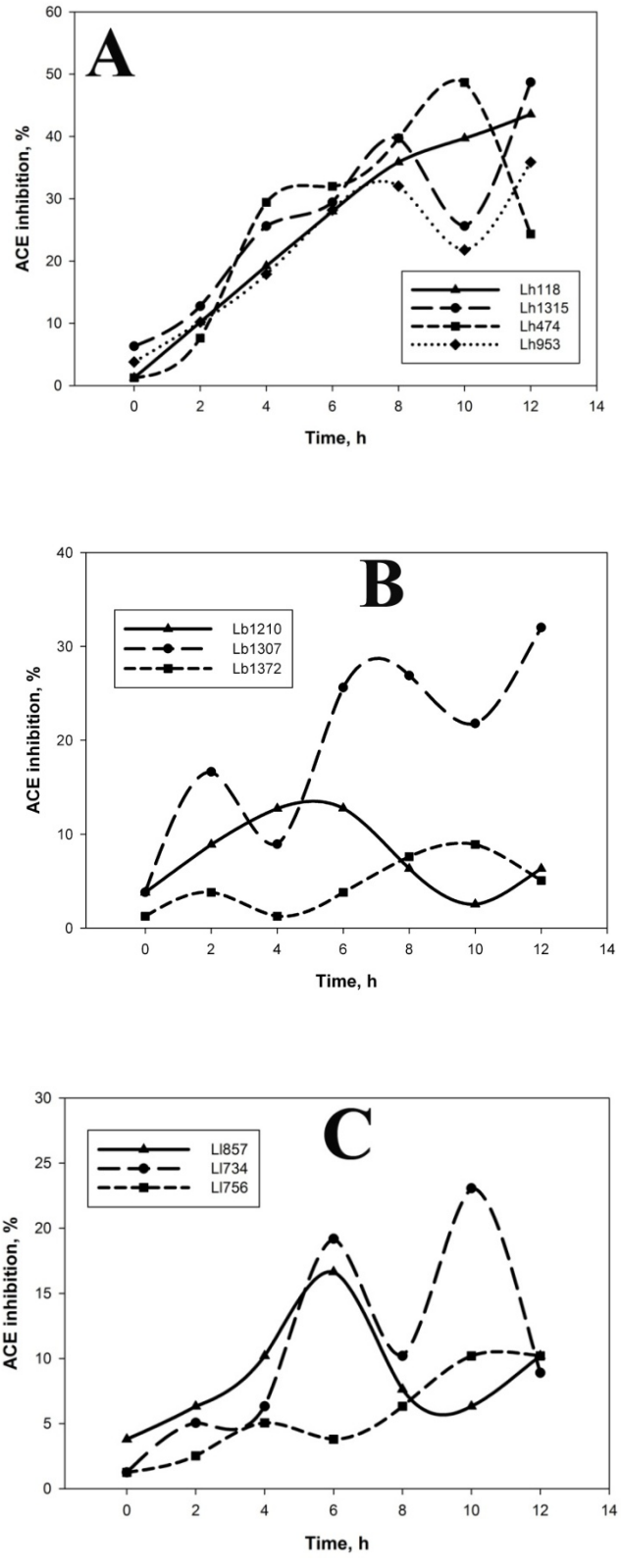


Figure 4.7 *In vitro* ACE inhibitory activity during growth of selected *Lactobacillus* species in sterile skim milk for 12 h at 37ºC. Legend A = *L. helveticus* strains; B = *L. bulgaricus* strains, C = *L. lactis*. Standard error of the mean for all samples was 1.73.

4.5 Strain selection for further investigation

In the following part, three strains of *L. helveticus* (474, 118 and 1315), which have shown the highest proteolytic activity and expressed greatest ACE-I activity than other strains used in the previous phase, were selected. These selected strains were subjected to further analysis to determine their enzyme activity using chromogenic substrates after successively growing in sterile reconstituted skim milk (RSM) and finally in MRS to avoid the carryover of proteins. More investigations were done to determine the role of crude cell proteinases of these selected strains to produce bioactive oligopeptides serving as ACE-I, antioxidant and immuno regulation activity.

4.6. Aminopeptidase activity

The specific activity of aminopeptidases in the extracellular and intracellular extracts of all *L. helveticus* strain selected is shown in Table 4.1. All differences in the specific activity of aminopeptidases were observed among the various strains in this species used and also for the various chromogenic substrates. All strains of *L. helveticus* showed high aminopeptidase activity (sum of IE and EE) to all chromogenic substrates used with exception of Succinyl-Alanin-Alanin-Proline-Phenyline *PNA*. Among selected strains, EEs of *L. helveticus* 118 and 1315 were able to hydrolyse *p*-nitroanilide derivatives of Glycine, indicative of *PepC* activity. These EE activities were significantly higher ($P < 0.05$) than IE of all strains with exception of *L. helveticus* 474, which peptidolytic activity was higher in IE than in EE. *L. helveticus* 118, 1315 and 474 showed similar activity to *para*-nitroanilide derivatives of Arginine, as substrate for *PepN*, in both IE and EE. Furthermore, specific activity of aminopeptidase toward Arginin-*PNA* was significantly higher ($P < 0.05$) in the EE of *L. helveticus* 474 and 118 than IE which might indicate the presence of aminopeptidase

activity includes *PepN* and *PepC*. These observations of extracellular enzyme activity could be attributed to the cell lysis and hence release of intracellular enzymes into the medium (Donkor et al., 2007a). The EE activity may further indicate that the extent of cell lysis may be high with these cultures and likely lead to erroneous conclusions (Donkor et al., 2007a).

Amino peptidases activity at both IE and EE that was observed with all selected *L. helveticus* strains maybe because that these strains secreted some general amino peptidases such as PepC and PepN to release essential amino acids for *L. helveticus* strain growth(Donkor et al., 2007a). Shihata and Shah (2000) found similar aminopeptidase activity, which were detected for all bacterial strains used at both extracellular and intracellular levels.

The activity of X-prolyl-dipeptidyl aminopeptidase (PepX) is very important for the selection of dairy starter cultures due to high proline content in milk proteins. PepX cleaves Xaa-Pro dipeptides from the N-terminus of peptides (Gatti et al., 2004, Pan et al., 2005). The highest activity of this enzyme on Xaa-Pro-ρNA substrates particularly when N-terminal residues are uncharged (Ala-Gly-) or basic (Arg-) (Christensen et al., 1999). All selected *L. helveticus* strains exhibited PepX activity towards glycyl-prolyl *p*-nitroanilide (Gly-Pro-pNA) as substrate in various extents of intracellular and extracellular extracts, which was significantly higher ($P< 0.05$) at EE level of *L. helveticus* 474 and 118 than IE with exception of *L. helveticus* 1315. Once again, these differences of the activity of this aminopeptidase location could probably contribute to the production of free amino acids in fermented milk, which are required for LAB growth as growth factors, and peptides which may have biological function activity (Pan et al., 2005). These findings have shown clearly the significance of aminopeptidases for the release of amino acids for growth of microorganisms through the hydrolysis of peptides in the growth medium. Christensen and Steele (2003) showed that the loss of aminopeptidases such as PepC, PepN, and PepX activities led to

significant impairment of growth rate in milk. Table 4.1 also shows the activity of extracellular and intracellular peptidases towards Succinyl-Alanin-Alanin-Proline-Phenyline *PNA*. All selected *L. helveticus* cultures appeared to have produced enzymes capable of hydrolysing large peptides only at extracellular level. This activity might indicate that some enzymes involved in milk peptides hydrolysis may include proteinases.

Table 4.1 Specific enzyme activity in extracellular (EE) and intracellular (IE) extracts of selected *L. helveticus* strains.

Strain	Enzyme location activity	Succinyl-Ala-Ala-Pro-Phe *P*NA	Gly *P*NA	Gly-Pro *P*NA	Arg *P*NA
Lh474	IE	ND	0.042	0.140	0.140
	EE	0.255	0.039	0.057	0.304
Lh118	IE	ND	0.047	0.054	0.028
	EE	0.162	0.114	0.072	0.044
Lh1315	IE	ND	0.030	0.034	0.347
	EE	0.163	0.101	0.073	0.304
SEM		0.005	0.005	0.007	0.097

4.7 Protein hydrolysis by crude proteinase extract of selected *Lb. helveticus*

The role of LAB proteinases is to degrade latent milk proteins at specific bonds to produce more than one hundred different oligopeptides ranging from 4 to 18 amino acid residues with most of them containing 4-8 amino acids residues (Kunji et al., 1996). Only short peptides are able to cross the cell wall into the cytoplasm for further degradation by peptidases to release amino acids required for nutrition. Longer peptides that remain in the medium could serve as biological active peptides (Meisel and Bockelmann, 1999). In this study, after cells were harvested, cell wall bound proteinases which were designed as crude protease extract (CPE) were extracted and milk proteins served as substrates for CPE under defined conditions (pH, temperature and incubation time). The profiles of released oligopeptides were determined using RP-HPLC as shown in Figure 4.8. A number of different peaks were detected for all milk batches inoculated with CPE of selected *L. helveticus* strains while comparing with untreated milk. All peaks appeared between 15 to 40 min and were mostly of the similar size. Comparing the extent of protein hydrolysis for these treated batches was difficult due to the similarities in the number of peaks and total area. These results indicate that these cultures could probably have similar types of proteinases which are the first in line for milk proteins degradation. Theses peptides detected could probably exhibit bioactivity such as ACE-I, antioxidant and immuno regulatory activity. Similar bio activities have been observed by Qian et al (2011) who evaluated the *in vitro* antihypertensive, antioxidant and immunomodulatory peptides derived from skim milk fermented with *L. bulgaricus* LB340. They found that peptides produced exhibited a good antioxidant, angiotensin I-converting enzyme inhibition and good immunomodulatory peptides. Further research is needed to evaluate the biofunctional activity of these peptides produced *in vivo*.

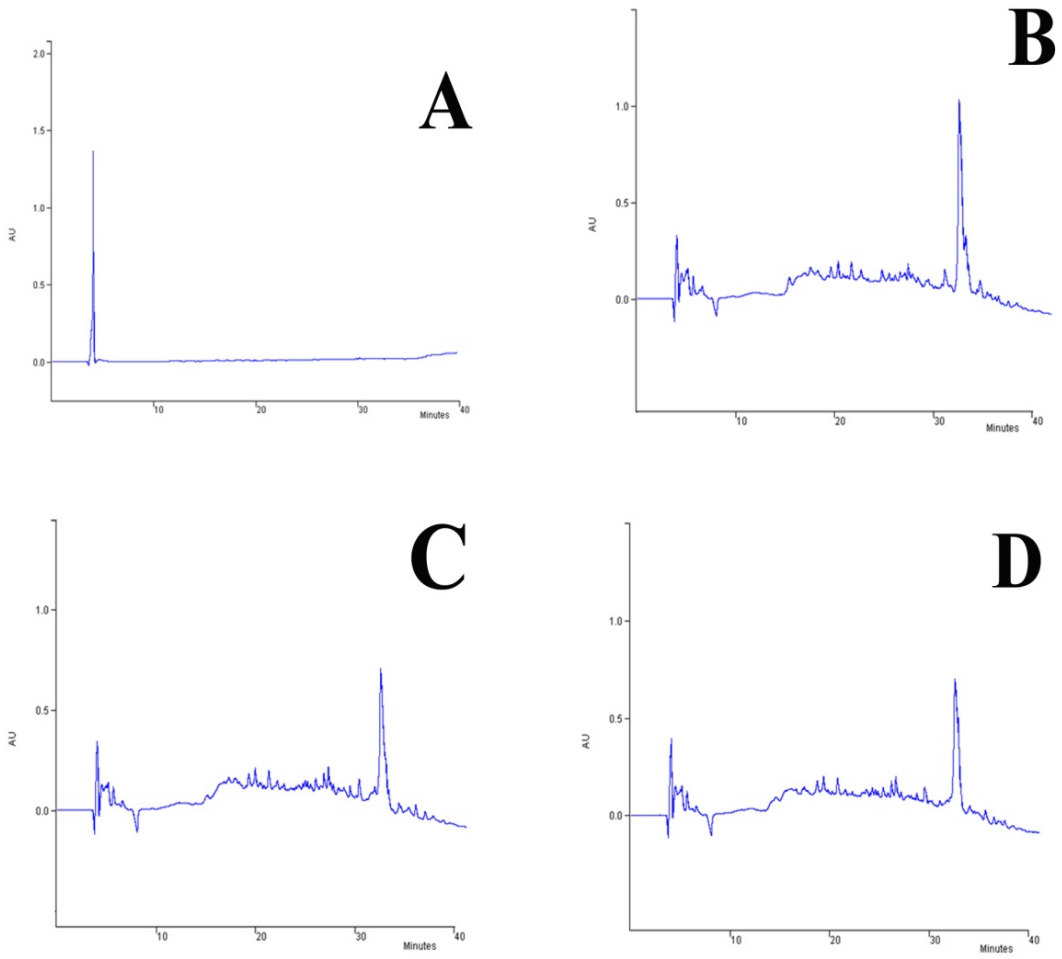


Figure 4.8 RP-HPLC profile of the water-soluble peptides released during incubation of milk after 12 h and at 37°C with individual crude proteinase extracts obtained from *L. helveticus* 474 (B), *L. helveticus* 118 (C) or *L. helveticus* 1315 (D). Untreated milk (A) served as a control. The chromatographs were obtained eluting samples using a linear gradient from 100% to 0% solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%, v/v acetonitrile in water) over 40 min at a flow rate of 0.75 $mLmin^{-1}$. The eluted peptides were detected at 214 nm.

4.8. In vitro ACE inhibitory activities from soluble peptides produced by CPE of selected *Lb helveticus* strains in milk

Most LAB produce ACE-inhibitors during milk fermentation. However, this activity of the fermented milk varies with the strain (Fuglsang et al., 2003). In preliminary *in vitro* experiments, inoculation of milk with CPEs of *L. helveticus* 474, 118 and 1315 strains as illustrated in Figure 4.9 produced a weak ACE inhibitory activity comparing with their activity in the first part in this study. The percentage of ACE inhibition activity of *L. helveticus* 474 increased significantly ($P< 0.05$) at 6 h which reached to 10.3% followed by significant reduction ($P< 0.05$) at 12 h declining to 7.1%. This reduction may indicate that some ACE-I peptides appeared and others disappeared due to action by CPE which may also contains some peptidases. However, there was no fluctuation in the activity of soluble peptides of *L. helveticus* 118 and 1315 throughout the same period of incubation. Although the ACE-I activity of the sample with CPE of *L. helveticus* 118 increased significantly ($P< 0.05$) at 6 and 12 h comparing with zero time and control (untreated milk). No significant changes with *L. helveticus* 1315 were observed. These results could be attributed to the production of ACE-I oligopeptides derived from milk proteins hydrolysis. The oligopeptides produced might be unable to transport into the cell and can remain in the medium to exhibit bioactivity (Meisel and Bockelmann, 1999). Interestingly, ACE-I activity of soluble peptides of *L. helveticus* 118 CPE at 6 and 12 h of incubation was remarkably higher ($P< 0.05$) than *L. helveticus* 474 and 1315 at the same time of incubation.

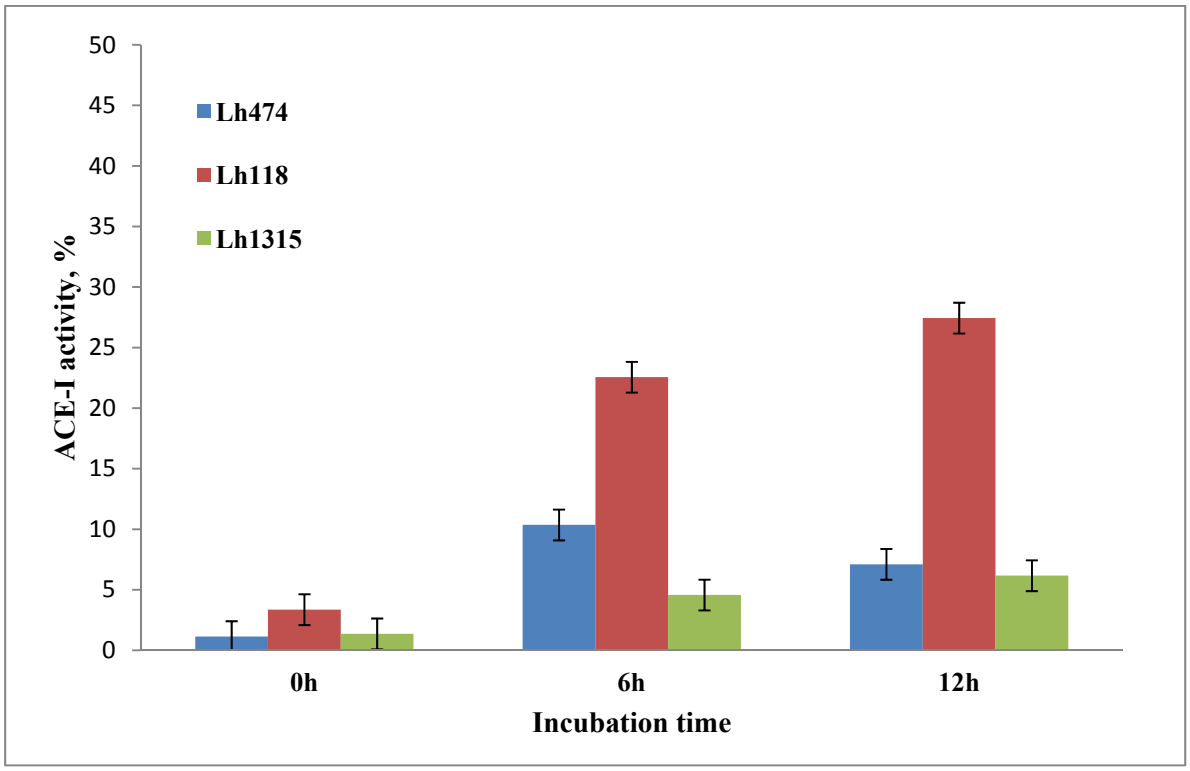


Figure 4.9 Angiotensin I-converting enzyme inhibitory (ACE-I) activity of liberated peptides samples obtained after 0, 6 or 12h incubation of milk at 37°C with individual crude proteinase extracts obtained from *L. helveticus* 474, *L. helveticus* 118 or *L. helveticus* 1315. Error bars indicate a standard error of the mean (1.27%) of triplicate determinations.

4.9. Determination of antioxidative capacity

Oxidative metabolism is crucial for the survival of human body's cells. The risk of this activity is that the production of free radicals causes oxidative changes (Pihlanto, 2006a). Free radicals have been linked with many pathological conditions such as atherosclerosis, diabetes, rheumatoid arthritis (Abuja and Albertini, 2001, Gutteridge and Halliwell, 2000, Halliwell and Whiteman, 2004). Inhibition of the free radicals formed in the living body and foodstuff is the important way to protect body from above serious diseases.

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a popular indicator for showing free radical scavenging activity of biological samples. In this study, peptides derived from milk proteins by crude proteinases of selected highly proteolytic *L. helveticus* strains were assessed separately for their antioxidant activity. The antioxidant activity of all peptides produced from milk by using crude proteinase extract (CPE) from selected *L. helveticus* strains was determined by $DPPH^{\bullet+}$ radical cation assay. This method is a type of inhibition assay in which the extent of scavenging of a preformed free radical relative to that of a standard antioxidant compound corresponds to the value of antioxidant activity. A more apparent increase in the antioxidant activity was observed in the milk inoculated with individual CPE from selected *L. helveticus* strains and incubated for 12 h at 37°C as shown in Figure 4.10. The free radical scavenging activity in all samples changed significantly ($P< 0.05$) from zero to 6 and 12 h comparing with untreated milk as a control, where no antioxidative activity was detected (data not shown). Milk with crude proteinase from *L. helveticus* 118 had the highest antioxidant capacity which increased from 18.8% at zero time to 23.5, 86.7 at 6 and 12 h respectively, whereas similar trends were observed with *L. helveticus* 474 and 1315 during the same incubation time which reached to 84.2% and 81.6%, respectively.

The variations of these activities may be attributed to the production of different bioactive peptides, which may or may not have antioxidant properties and it is likely to be

strain dependent (Donkor et al., 2007b). It has been reported that DPPH radical-scavenging activity of skim milk increased by fermentation with a *L. casei* strain and also suggested that peptides produced in the fermented milk might be one of the factors enhancing radical-scavenging activity (Nishino et al., 2000). A study published by Suetsuna et al (2000) also showed that some peptides derived from the pepsinic hydrolysate of casein demonstrated DPPH radical scavenging activities. Liu et al (2005) found that the DPPH radical-scavenging activity of milk-kefir and soymilk-kefir was significantly higher than that of milk and soymilk and they suggested that this activity may be, in part, attributed to the peptides deriving from degradation of milk and soybean proteins. These results of scavenging properties of free radicals by dairy cultures might be useful in food manufacturing and can present additional sources of health enhancing antioxidants.

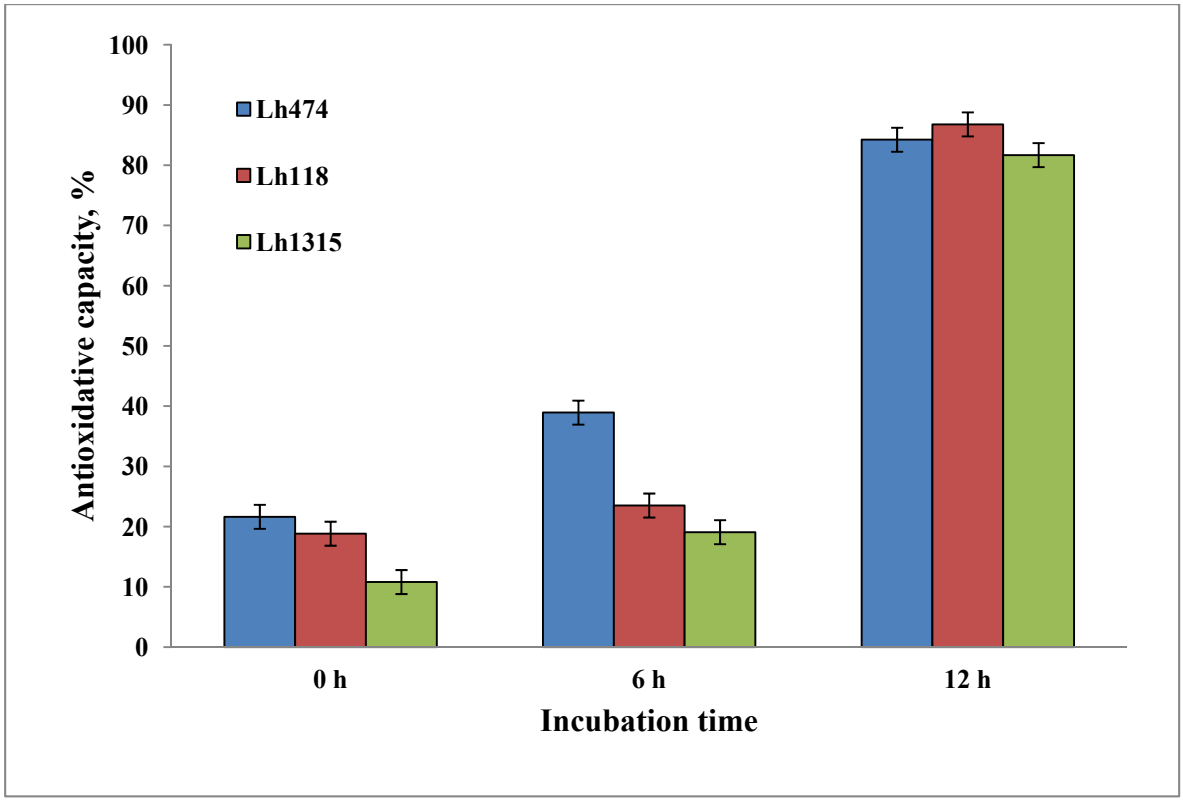


Figure 4.10 Antioxidative capacity of liberated peptides samples obtained after 0, 6 or 12 h incubation of milk at 37°C with individual crude proteinase extracts obtained from *L. helveticus* 474, *L. helveticus* 118 or *L. helveticus* 1315. Error bars indicate a standard error of the mean (1.99%) of triplicate determinations.

4.10. Effects of fermented milk derived peptides on cytokine production

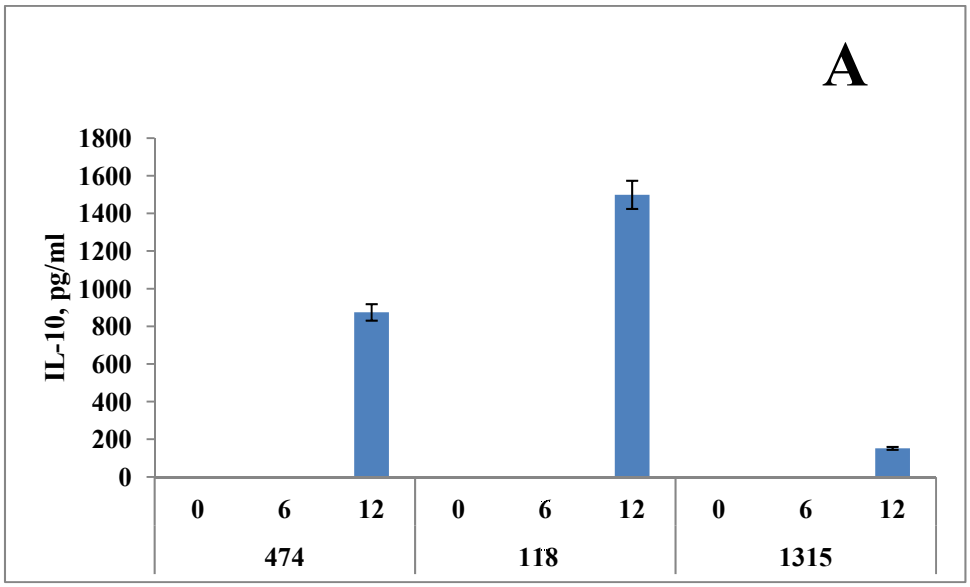
Inflammation has been reported as a host defence process that occurs at injured sites and is strictly regulated. The organization of pro-inflammatory and anti-inflammatory moderators is necessary in the regulation of the inflammatory response (Lee et al., 2002). Regulation of host immunity is one of the most commonly proposed benefits of the consumption of foods containing *Lactobacillus* strains. The main purpose of their use is to prevent and treat the consumers from many diseases such as acute gastrointestinal infections, allergy and inflammatory bowel diseases (Gill and Prasad, 2008). The beneficial effects of these strains are based partly on their ability to regulate differentially the production of anti- and pro-inflammatory cytokines and the balance of Th1/Th2 activity (Delcenserie et al., 2008). Several milk proteins have been reported to modulate lymphocyte proliferation *in vitro* (Phadke et al., 2005)such as whole casein, α-, β-, κ-casein (Phadke et al., 2005, Sütas et al., 1996, Araujo et al., 2000). The actions of fermented LAB on immune system regulation may also be strongly dependent on the lactic acid bacteria species, or on their metabolites such as peptides released from proteins hydrolysis, which may or may not interact with immune cells to induce distinct immunomodulatory response (LeBlanc et al., 2004).

In this study, the results of the effects of soluble peptides produced by CPEs of individually selected *L. helveticus* strains on cytokine production by human peripheral blood mononuclear cells (PBMCs) are shown in Figure 4.11A and B. The effects of soluble peptide samples were detected in the stimulation of production of all tested cytokines. At 12 h of incubation, all samples induced the production of cytokine IL-10 higher than the nonstimulated PBMCs, comparing to untreated milk as a control and peptide samples at zero hour, were no stimulation was detected (data not shown). The stimulation of PBMCs by peptides at 37°C for 72 h from crude proteinase extract of *L. helveticus* 118 showed highest production of IL-10 followed by *L. helveticus* 474 and 1315. However, peptides produced by

CPE from *L. helveticus* 474 induced the production of IFN-γ higher than other strains, followed by *L. helveticus* 1315 and 118. In contrast, *L. helveticus* 118 was among the strains inducing the production of the lowest levels of the Th1 cytokines IFN-γ, while inducing higher IL-10 production. *L. helveticus* 474 induced a similar cytokine profile regarding IFN-γ and higher production of IL-10. Therefore, the peptides produced of the same CPE species are potentially able to drive immune responses into opposite directions *in vitro*.

These results clearly showed (Figure 4.11) that the soluble peptides derived from degradation of milk proteins by selected *L. helveticus* strains stimulated the production of both pro- and anti-inflammatory cytokines (IFN-γ and IL-10) in varying concentrations compared with untreated milk or unstimulated PBMCs. The results also indicated that soluble peptides may have induced Th1 and Th2 responses leading to the production of these cytokines. It could be suggested from this study, that different peptides produced from the same CPE may potentially be able to drive immune responses in opposite directions *in vitro* and thus bring about an imbalance of Th1/Th2 type cytokines.

To sum up, the results showed that the *lactobacillus* strains used in this research may release ACE-I, antioxidant and immunostimulative peptides during fermentation in bovine milk. This bioactivity might rely on the growth level, proteolytic enzymes and pH changes in addition to other factors that could affect the production of bioactive peptides such as incubation time and the inoculation level of the fermented bacteria. Oligopeptides produced by *L helveticus* proteinases and under certain conditions may serve as ACE-I, antioxidant and immunostimulatory activity in different degrees.



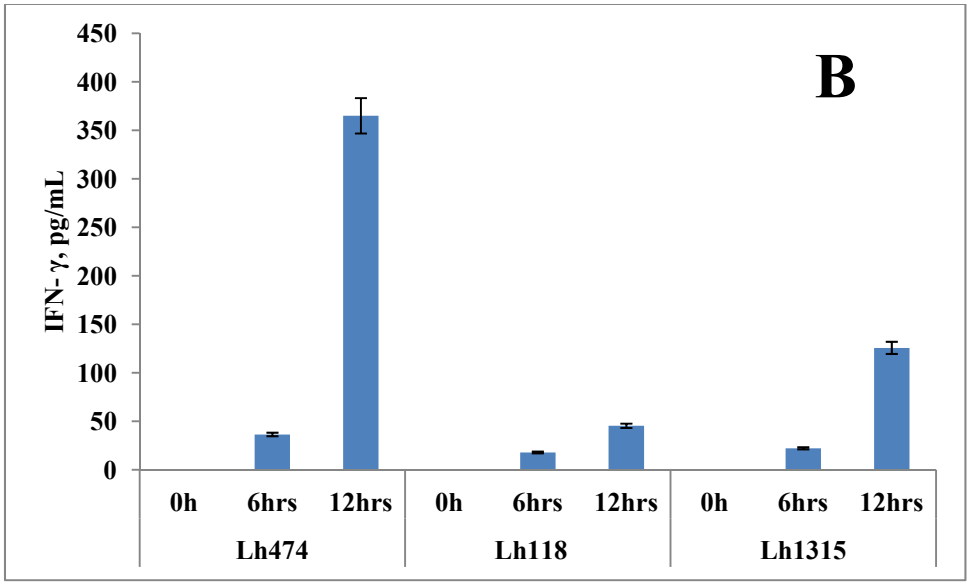


Figure 4.11 IL-10 (A) and IFN- γ (B) cytokine production induced by stimulation of human (PBMCs) with soluble milk peptides incubated for 72 h at 37°C in a humidified 5% CO_2 incubator. The peptide samples were obtained after 0, 6 or 12 h incubation of milk at 37°C with individual crude proteinase extracts obtained from *L. helveticus* 474, *L. helveticus* 118 or *L. helveticus* 1315.

CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Overall Conclusions

Ten selected strains of *Lactobacillus helveticus* 474, *Lactobacillus helveticus* 118, *Lactobacillus helveticus* 1315, *Lactobacillus helveticus* 953, *Lactobacillus delbrueckii ssp. bulgaricus* 734, *Lactobacillus delbrueckii ssp. bulgaricus* 756, *Lactobacillus delbrueckii ssp. bulgaricus* 857, *Lactobacillus delbrueckii ssp. lactis* 1210, *Lactobacillus delbrueckii ssp. lactis* 1307 and *Lactobacillus delbrueckii ssp. lactis* 1372 were assessed for their growth and metabolic performance in reconstituted skim milk. All the cultures showed the appreciable growth in RSM due to their proteolytic activities and some of them were not affected by the rate of pH decrease or the final pH. The growth and the rate of acid production indirectly showed as a pH decline was strictly strain dependant. *L. helveticus* 474 showed highest growth among strains examined. The increase in growth was correlated with the time of incubation and the decrease in pH during milk fermentation. The growth and metabolic activity was also dependant on the extent of the proteolytic activity, which varied to a great extent among the strains. Among all these organisms, *L. helveticus* strains exerted exemplary proteolytic activity in comparison to other strains. On the other hand, *L. bulgaricus* and *L. lactis,* as poor proteolytic activity strains, may need a longer time or different temperature or even growth enhancers to establish similar cell concentrations to those of *L. helveticus* strains. Due to their proteolytic activities, all organisms in this study were able to release a number of bioactive peptides from milk proteins. These peptides were assessed for ACE inhibitory activity *in vitro* and their potency varied with the strains. *L. helveticus* strains, as highly proteolytic strains, released either more potent or more peptides that had higher ACE-I activity than other strains. The time of fermentation, temperature, pH and as well as proteolytic enzymes activity for each strain influenced the increase and decrease of ACE-I activity for *Lactobacillus* strains during milk fermentation with exception of *L. helveticus* 118 which showed a minimal effect on ACE inhibition. Based on these findings, *L. helveticus*

474, 118 and 1315 were further examined. Extracellular and intracellular extracts of these strains exhibited aminopeptidase activities towards all chromogenic substrates tested. On the other hand, extracellular activity was characterised by the oligopeptidase activity. These activities reflected the appreciable growth and proteolytic activity for these strains. The crude proteinase extracts of these strains in milk produced a very low percentage ACE-I activity while high levels of antioxidative activity. *L. helvericus* 118 produced the highest activity of *in vitro* ACE-I and antioxidative activity at the end of fermentation. These findings showed that bioactive peptides produced may rely on many factors such as temperature, inoculation level of the crude proteinase extracts of selected *L. helvericus* strains. Furthermore, the incubation time and strain used were the main possible factors influencing these bioactivities. However in order to maintain high levels of these activities it would be necessary to prevent further degradation. The oligopeptides produced were also assessed for their potential to induce different cytokines. They induced release of interleukin-10 and interferon-γ, as main representatives of anti- and pro-inflammatory cytokines, respectively. These bioactive peptides might have capability to drive immune responses in opposite directions *in vitro* and thus may bring about imbalance in the Th1/Th2 type cytokines.

5.2. Future Research Directions

Our results revealed that *Lactobacillus* species particularly *L. helveticus* strains used in this study could release bioactive peptides from bovine milk proteins during fermentation. Furthermore, the dairy industry which uses *Lactobacillus* species as starter cultures in dairy products, should work closely with the medical professionals in order to substantiate the health claims associated with these beneficial micro-organisms. A number of *Lactobacillus* strains have been identified that are capable to release different peptides with varying ACE-inhibitory, antioxidative and immunostimulatory activity from milk proteins. Although these

observed activities in this study were strictly strain and time dependant during fermentation, their stability during storage of fermented dairy products by these strains and their resistance to digestive enzymes need to be investigated. *L. helveticus* strains revealed higher proteolytic and ACE-I activity than other strains studied. More research is required to develop and enhance the proteolytic activity of *Lactobacillus* species to obtain products enriched with bioactive peptides of specific or multi-functions that might be used to optimize the nutritional and health effect of these compounds as therapeutic additives in dairy products.

CHAPTER 6

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