Anticarcinogenic Peptides Released from Milk Proteins by *Lactobacillus* Strains

A thesis submitted for the degree of

Doctor of Philosophy

By

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Abstract

Bioactive compounds released by proteolytic cleavage of milk proteins during milk fermentation have a role beyond their nutritional importance. The first research chapter in this thesis assessed the proteolytic activity of *Lactobacillus helveticus* strains ASCC 953, ASCC 474, ASCC 1188 and ASCC 1315, and their ability to release bioactive compounds with antioxidative and *in vitro* anticarcinogenic properties during incubation at 37°C in reconstituted skim milk. The performance of these strains was not affected by the pH decline during fermentation. Soluble extracts of milk fermented by *L. helveticus* strain ASCC 474 showed the highest free radical (1,1-diphenyl-2-picrylhydrazyl) (DPPH) scavenging activity after 12 h of fermentation; this was followed by a significant reduction of activity at 24 h compared with the other strains and control (untreated milk). Skim milk fermented by *L. helveticus* contained compounds with anti-colon cancer activity at levels that differed throughout fermentation. Growth inhibition activity (19.03–50.98%) was greatest in the extract obtained after 12 h of fermentation but had markedly declined (5.40–9.94%) by the end of fermentation. *L. helveticus* ASCC 1315 released compounds into the skim milk supernatant that exerted greater growth inhibition (50.98%) on the HT-29 colon cancer cell line than did the other strains. More importantly, these compounds had no significant inhibitory effect on normal, primary colon cells T4056. Although these results suggest that milk fermented by *L. helveticus* may release bioactive compounds with important multifunctional properties, the characteristics and activities of these compounds appear highly strain and fermentation time dependent.

The second research chapter aimed to evaluate the effects of 28 days of cold storage on the release of antioxidative peptides in milk fermented by *L. helveticus* strain 1315 (*L.* 1315). Additional types of bioactivity including angiotensin-converting enzyme (ACE)

inhibition and antimicrobial activities were also assessed. Further, samples were subjected to *in vitro* digestion to assess the fate of peptides during gastrointestinal (GI) passage. The antioxidative properties of fermented milk exerted significantly higher radical scavenging activity using DPPH, $ABTS^{\bullet+}$ 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) reducing power and hydroxyl radical ($^{\bullet}OH$) assays after 14 days than at other time points, and were time dependent. However, these bioactivities diminished after exposure to *in vitro* digestive enzymes. Samples with the highest antioxidative activity were fractionated and purified, revealing the presence of nine peptides derived from beta casein (β-CN), as identified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The peptides KVLPVPQKAVPYPQ and SQSKVLPVPQKAVPYPQ exhibited the highest scavenging activity, in a dose-dependent manner.

The last research chapter in this thesis describes the isolation and identification of potential antiproliferative peptides from milk fermented by *L. helveticus* 1315 on HT-29, and evaluation of the antioxidant and anti-colon cancer activities of these peptides after *in vitro* GI digestion. The mechanism of anti-colon cancer activity (apoptotic activity, caspace-3 and cell cycle arrest) was also assessed. A peptide fraction derived from fermented milk after 14 days of cold storage at 4^{o}C had high anti-colon cancer activity on HT-29 cells using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Among the nine peptides identified in the fraction, KVLPVPQKAVPYPQ and SQSKVLPVPQKAVPYPQ derived from β-CN exhibited the highest antiproliferative activity. These two peptides were further subjected to *in vitro* GI digestion to determine their stability. The antioxidant activity of digested peptides was also assessed using DPPH and $ABTS^{\bullet+}$ assays, which revealed increased antioxidant activity and antiproliferative activity in HT-29 cancer cells through induction of apoptosis resulting in G2/M cell cycle

arrest. These results indicate that these peptides and their derivatives after digestion have potential physiological effects that may be harnessed to manage oxidation-related diseases and disorders including cancers.

Certificate

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This is to certify that the thesis entitled '**Anticarcinogenic Peptides Released from Milk Proteins by *Lactobacillus* Strains**' submitted by **Khaled Ramadan Elfahri** in partial fulfilment of the requirement for the award of the Doctor of Philosophy with specialization in Food Science and Technology at Victoria University is a record of bonafide research work carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Professor Todor Vasiljevic

(Principal supervisor)

Date:

Declaration

I, Khaled Elfahri, declare that this thesis entitled '**Anticarcinogenic Peptides Released from Milk Proteins by *Lactobacillus* Strains**' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

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To my mother and wife

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

To my brothers and sisters,

I dedicate this simple work

Khaled Elfahri

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List of Publications

Efahri, K., Vasiljevic, T., Yeager, T. & Donkor, O. 2015. Anti-colon cancer and antioxidant activities of bovine skim milk fermented by selected *Lactobacillus helveticus* strains. *Journal of Dairy Science*, 99, 31-40

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Manuscripts in preparation

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List of Abbreviations

AA	amino acid
ABTS	2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme-inhibitory
α-CN	alpha casein
Ala	alanine
AMP	antimicrobial peptide
ANOVA	analysis of variance
APAF-1	apoptotic protease-activating factor
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATCC	American Type Culture Collection
BAK	B-cell lymphoma-2 antagonist/killer
BAX	B-cell lymphoma-2-associated X protein
BCL-2	B-cell lymphoma-2
β-CN	beta casein
BCLB	B-cell lymphoma-2-like protein 10
BCLXL	B-cell lymphoma-X large
BCLXL1	B-cell lymphoma-X large1
BCLW	B-cell lymphoma-2-like protein 2
BFL1	B-cell lymphoma-2-related protein A1
B-Lf	bovine lactoferrin
B-Lfcin	bovine lactoferricin

β-Lg	beta lactoglobulin
BHA	butylated hydroxyl anisole
BHT	butylated hydroxyl toluene
BOK	B-cell lymphoma-2-related ovarian killer
BSA	bovine serum albumin
Caco-2	colon adenocarcinoma cell line
CARD	caspase activation and recruitment domain
CEP	cell envelope proteinase
Cfu	colony-forming unit
CPP	caseinphosphopeptide
Cys	cysteine
DNA	deoxyribonucleic acid
DE	digestive enzyme
DPP	dipeptidyl peptidase
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
ET	electron transfer
f	fragment
FBS	foetal bovine serum
FDA	Food and Drug Administration
FRAP	ferric ion-reducing antioxidant power
5FU	5-fluorouracil
Glu	glutamic acid
Gln	glutamine
GI	gastrointestinal

GIP	glucose-dependent insulinotropic polypeptide
GIT	gastrointestinal tract
Gly	glycine
HAT	hydrogen atom transfer
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
HRSA	hydroxyl radical scavenging activity
His	histidine
HT-29	human colonic epithelial carcinoma cell line
IFN-γ	interferon gamma
Ile	isoleucine
κ-CN	kappa casein
L.	*Lactobacillus*
LAB	lactic acid bacteria
Lc.	*Lactococcus*
LC–MS	liquid chromatography–mass spectrometry
Leu	leucine
LSD	least significant difference
Lys	lysine
MAPK	mitogen-activated protein kinase
MCL1	myeloid cell leukemia 1
Met	methionine
MMP	matrix metalloproteinase
MRS	de Man, Rogosa and Sharpe
MS	mass spectrometry

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NDE	nondigested enzyme
PBS	phosphate-buffered saline
Phe	phenylalanine
Pro	proline
PrtP	PII-type proteinase
OPA	o-phthaldialdihyde
PepA	aminopeptidase A
PepC	aminopeptidase C
PepL	aminopeptidase L
PepN	aminopeptidase N
PepP	aminopeptidase P
PepX	X-prolyl dipeptidyl aminopeptidase
PepV	dipeptidase V
PepD	dipeptidase D
PepT	tripeptidase T
PepI	proiminopeptidase
PepQ	prolidase
PepR	prolinase
PepF	endopeptidase F
PepO	endopeptidase O
PepE	endopeptidase E
PG	propyl gallate
ROS	reactive oxygen species

RP–HPLC	reverse-phase–high-performance liquid chromatography
RSM	reconstituted skim milk
Ser	serine
sp.	species
SPE	soluble peptide extract
ssp.	subspecies
St.	*Streptococcus*
TCA	trichloroacetic acid
Thr	threonine
TFA	trifluoroacetic acid
TKI	tyrosine kinase inhibitor
Trp	tryptophan
Tyr	tyrosine
UV	ultra violet
VIS	visible
Val	valine
VEGF	vascular endothelial growth factor

Chapter 1: Introduction

1.1 Background

A great deal of interest has been directed by food researchers, nutritionists and health specialists towards functional foods and biologically active components that can potentially moderate the risk of chronic diseases, beyond their basic nutritional functions (Korhonen, 2009a; Gul et al., 2016). It is important that functional foods contain potentially beneficial substances including any altered food or food component that should be taken as part of the daily diet (Gul et al., 2016). Dairy products fermented by lactic acid bacteria (LAB) may have beneficial properties in functional foods, based on a postulation by Metchnikoff (2004) that there was a relationship between the long life of Bulgarian peasants and their consumption of fermented milk containing LAB (Culligan et al., 2009). The main purpose of using LAB in fermented dairy products is to convert lactose to lactic acid, thereby decreasing the pH (to ~4.5), which leads to the formation of a coagulum of casein proteins. As LAB have limited capacity to synthesise amino acids (AAs) for their optimal growth in milk, they must degrade proteins (mainly caseins) to create free AAs and small peptides during proteolysis. The proteolytic system in LAB, mainly of *Lactobacillus* strains, can release bioactive peptides from milk proteins that have potentially positive health properties such as antioxidant and anticancer activities (Elfahri et al., 2015; Tamang, 2015). Excessive levels of oxidants such as reactive oxygen and nitrogen species caused by an imbalance between oxidants and the ability of biological systems to neutralise them can cause cellular damage (Sosa et al., 2013). Oxidative damage to proteins, deoxyribonucleic acid (DNA) and membrane lipids is associated with chronic and degenerative diseases such as cardiovascular disease, diabetes (Sharma, 2014) and cancer (Tekiner-Gulbas et al., 2013). Cancer is a leading

cause of death worldwide and remains of primary concern. It is still considered as a major source of morbidity and mortality throughout the world (Jemal et al., 2011; Oh et al., 2016).

The use of chemical drugs, including radiation and chemotherapy, for cancer treatment is expensive and many have a number of negative side effects for cancer patients (Köhne & Lenz, 2009). Recently, there have been attempts to use natural anticancer compounds from milk proteins as an alternative method for cancer prevention and management (Ligo et al., 2009; Sah et al., 2015b). Potential anticancer peptides are contained in milk proteins and can be released by proteolytic enzymes or highly proteolytic cultures such as those involved in milk fermentation. Total intake of food proteins can be a risk influence for cancer incidence; however the type of protein may be the main factor determining any potential anticancer properties of milk (Parodi, 2007). Increasing evidence has been suggesting that cytomodulatory peptides derived from milk protein may act as specific signals that can initiate a decrease in the viability of cancer cells (Sah et al., 2015b). The antiproliferative effects of casein-derived peptides and other minor milk components on colon cancer cell lines suggests that beside prevention they might also have a role in colon cancer treatment (Asarat et al., 2015; Sah et al., 2016b). However, human clinical research has included only limited cross-sectional and cohort studies that have specifically examined the potentially beneficial anticancer effect of whole milk or modified milk products (Gill & Cross, 2000). These studies have reported contradictory results, either supporting or negating the role of milk in cancer therapies (Gill & Cross, 2000). Overall, the question of whether there is a causal correlation between milk consumption and cancer remains vague. The significance of potential anticarcinogenic peptides *in vitro* is that the peptides can be used on an industrial scale as dietary supplements and as pharmaceutical preparations. Although research has revealed new

possibilities for bioactive peptides as candidates for cancer therapy *in vitro* (Sah et al., 2016b), bioactive peptides may change their activity *in vivo* due to possible hydrolysis of proteolytic enzymes in the gastrointestinal tract (GIT). Therefore, an understanding of potential bioactive peptides released from fermented milk, their corresponding physiological activity (anticarcinogenic) *in vitro* and their stability in the GIT may offer a broad perspective for food scientists, producers and consumers.

1.2 Aims and Objectives of this Project

The aim of this project was to assess the potential of highly proteolytic strains of *Lactobacillus (L.) helveticus* to liberate peptides contained in milk proteins that show antioxidant and anti-colon cancer activities. The specific objectives were as follows:

1. Assess the growth and proteolytic activity of selected *L. helveticus* strains when cultivated in milk.
2. Investigate the antioxidant and anti-colon cancer activities of crude bioactive peptide extracts from selected strains during the cold storage period after fermentation and their stability in an *in vitro* GI digestion model.
3. Purify crude antioxidant and anti-colon cancer peptides to isolate and characterise the individual peptides.
4. Examine the antioxidant and anti-colon activities of each isolated peptide, and determine the AA sequences of the most potent bioactive peptides and their stability during *in vitro* GI digestion.
5. Determine *in vitro* biological mechanisms of cell suppression/death of colon cancer cells associated with peptides.
6. Examine the inhibitory activity of different peptide concentrations on cancer cells using apoptosis and cell cycle arrest assays.

Chapter 2: Literature Review

2.1 Cancer: Global Health Concerns

A cancer is described as a malignant growth caused by the nature of independent cell evolution (Beerenwinkel et al., 2014). The increase in cells occurs with loss of cell differentiation and commonly spreads to other tissues and organs, in a process known as metastasis. Cancer remains one the leading causes of mortality and morbidity across the world (Sah et al., 2015b). In 2012, more than 14 million new cases of cancer were diagnosed globally, leading to 8.2 million deaths (14.6% of all human deaths) (Ferlay et al., 2015). The most common categories of cancer are lung, prostate, stomach and colorectal cancers. Despite technological advancements in the medical sector, the outcomes remain unsatisfactory and depend on several factors, primarily the type of cancer and its location in the body. There are more than 100 categories of cancer (Hanahan & Weinberg, 2000). However, some types of cancer, such as skin, kidney, colon and prostate cancers, have continued to increase in prevalence in recent years (Jemal et al., 2011). Six specific changes to a cell are necessary for it to become cancerous. For instance, cells can secrete their own growth signals and also act on weak external growth signals that do not affect normal cell division (Leber & Efferth, 2009). In addition, cells can develop mechanisms that overcome their antiproliferative properties (Evan & Vousden, 2001). When cells are not affected by cellular suicide mechanisms, they become prone to cancer progression. Similarly, cells that become capable of unchecked cellular replication and can initiate angiogenesis that attracts new blood vessels to the site, allowing cancer expansion. Finally, during malignancy, cells can invade other tissues and spread to various parts of the body (Brábek et al., 2010). Some locally contained cancers, in particular solid tumours, can be treated by surgery.

However, chemotherapy remains the primary method of dealing with the most advanced types of cancers (Weeks et al., 2012).

2.2 Colon Cancer, Causes and Progression

2.2.1 The Causes of Colon Cancer

Colorectal cancer (also known as bowel cancer) is the most commonly diagnosed cancer in both males and females, and has the highest incidences in North America, New Zealand, Europe and Australia (Jemal et al., 2011). According to Ferlay et al. (2015), colon cancer was the third most common cancer in 2008, when it accounted for nearly 12.1% and 7.8% respectively of all cancers in the developed and less developed regions of the world. Colorectal cancer occurs in the large intestine, which is the lower part of the digestive system of the body (Ferlay et al., 2015). It also includes rectal cancer, the last several inches of the colon.

It could be argued that there is no specific cause of colon cancer (Haggar & Boushey, 2009) although its development is associated with many factors ranging from hereditary to various external factors including diet and health status (Heald et al., 2012; Johannesdottir et al., 2012). A family history of certain types of cancer may point to the development of the disease due to heredity. However, some diets may be associated with the development of colon cancer (Kim et al., 2010; Itano et al., 2012). For example, a high fat diet that contains a great deal of red meat may increase the risk of the disease. According to published research, countries in which less red meat is consumed have fewer colon cancer cases reported (Heald et al., 2012; Johannesdottir et al., 2012). In Japan, red meat is not consumed regularly and the colon cancer rate is the lowest in the world (Aykan, 2015). Further, excess use of alcohol and smoking have been reported to increase the risk of colon cancer (Schottenfeld & Fraumeni Jr, 2006). Some health factors

such as diabetes, obesity and lack of exercise also result in increased colon cancer risk (Frezza et al., 2006). Inflammatory disorders such as ulcerative colitis enhance the growth of colon cancers (Haggar & Boushey, 2009).

To reduce the incidence of colon cancer, it is important to minimise the factors leading to colon cancer, and to identify additional regimes with reduced toxicity and expense for cancer patients (Mariadason et al., 2004). One proposed strategy for combatting colon cancer is through primary prevention, such as avoiding negative environmental and dietary factors (Palmirotta et al., 2009). The application of natural drugs as chemopreventative agents to suppress or prevent the development of cancer has been on the rise (Dunn & Jankowski, 2008). These drugs are designed to minimise the incidence of, and thus the death rate from, colon cancer and may be an attractive substitute for mass screening of the general population or particular groups who may be at increased risk of colon cancer. Underlying mechanisms that cause colon cancer, indicated by mouse models, can be modulated by dietary means (Nyström & Mutanen, 2009). Several studies have proposed that colon cancer can be managed by eating plenty of nutritious foods such as low-fat milk, yoghurts and vegetables as well as exercising regularly (Zhang & Kesteloot, 2005; Annema et al., 2011; Corpet, 2011).

2.2.2 Development of Colon Cancer

Colon cancer often begins as small and noncancerous clumps of cells called adenomatous polyps; after some time, these polyps may progress to colon cancer (Nyström & Mutanen, 2009). Colon cancer usually develops slowly over a period of 10–15 years, beginning as a small growth from the epithelial lining of the colon (stage 0) and developing to almost completely blocking the colon (stage IV) (Figure 2.1) (Centelles, 2012).

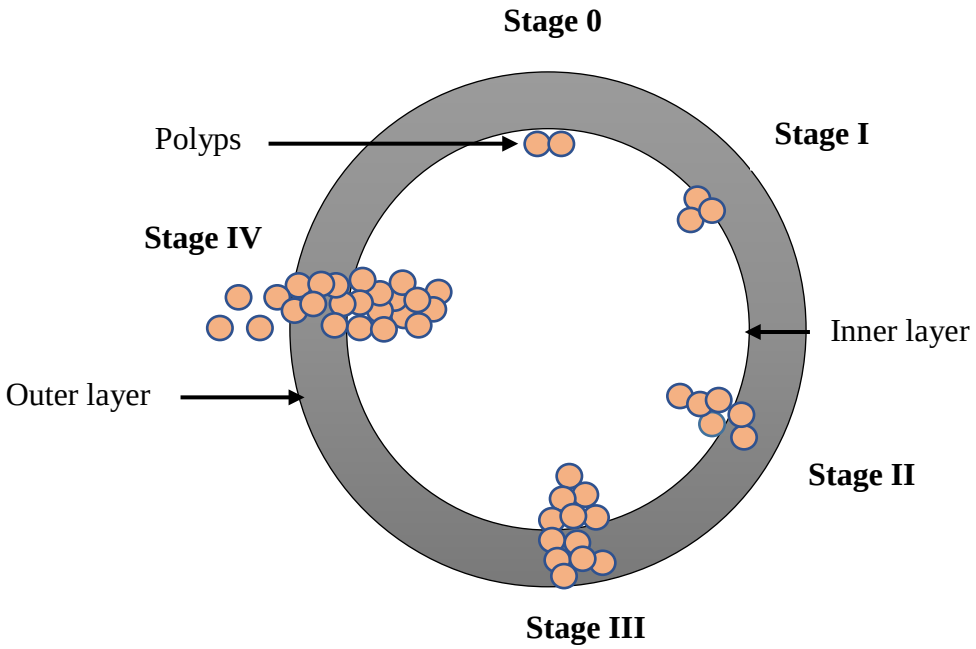

Figure 2.1 Colon cancer stages/progression

(Centelles, 2012)

Usually, polyps generate few or no symptoms, which is why regular colon screening via colonoscopy is recommended by doctors. This test allows for the identification and removal of polyps at an early stage to prevent cancer progression.

2.2.3 Signs and Symptoms of Colon Cancer

The signs and symptoms of colon disorders are not specific. These symptoms can also vary from one person to the next depending on a number of factors (Vega et al., 2015). Such factors may include the specific location of the tumour. However, it is not easy to detect the symptoms of cancer at the early stages of development. Symptoms of the disease (Nyström & Mutanen, 2009) include:

- dark-coloured stool
- constipation changes in stool consistency
- rectal bleeding as evident in the stool
- change in bowel habit

- diarrhoea
- narrow stools.

2.3 Colon Cancer, Treatments and Cost

Surgery, chemotherapy, radiotherapy and monoclonal antibodies are the most frequent treatment regimens for patients with colon cancer (Woude & Klein, 2009). The use of each type of therapy depends on the location and progression (colon, rectum or anus) of the disease. Cancer-preventative agents block the conversion of primary cells (healthy) to carcinogenic cells and inhibit the progression of premalignant cells to malignant cells. One of the earliest cancer cytotoxic drugs commonly used as a chemotherapy treatment for colon cancer is 5-fluorouracil (5FU) (Centelles, 2012). Although this treatment agent is able to induce differentiation in several types of cancer cell lines including HT-29 (Srimuangwong et al., 2012), its effectiveness is limited by the increase of drug resistance, which is one of the key aspects affecting the use of cancer drugs (Centelles, 2012). The cancer inhibitor staurosporine, a kinase inhibitor from a *Streptomyces* bacterium, is also among the most favourable anti-carcinogen treatments through its apoptosis-promoting effect on cancer cells (Ding et al., 2017). At low concentrations, staurosporine has the ability to decrease colon cancer cell proliferation (Zong et al., 2001) with moderate side effects on healthy cells (Nurdiani et al., 2016).

Table 2.1 Contemporary colon cancer treatment modalities and their side effects and efficacy

Current treatment modalities	Drug	Stage at which treatment is used	Efficacy and side effects	Reference
Surgery			~40% of patients relapse	Chau & Cunningham, 2002; Roselli, 2009
Chemotherapy	Irinotecan	1st line combination and 2nd line monotherapy for advanced disease	5-year survival rate ~50% Arterial and venous (thromboembolic) clots	Giacchetti et al., 1999; Chau & Cunningham, 2002
	Oxaliplatin	Treatment of metastatic colorectal cancer	5-year survival rate ~50% Hematologic toxicity, gastrointestinal tract toxicity and neuropathy	Giacchetti et al., 1999; Cassidy & Misset, 2002
	Capecitabine	Treatment of developed or metastatic colon cancer alone or in combination	5-year survival rate ~77.6% Diarrhoea, vomiting and hair loss	Saif et al., 2008; Haller et al., 2011
	5-flurouracil (5FU)	At any stage of colon cancer, commonly merged with folinic acid (leucovorin calcium)	5-year survival rate 33–50% No benefit for 80% of patients	Guo et al., 2006; Folprecht et al., 2010
Monoclonal antibodies	Cetuximab (Erbitux)	Applied in patients with epidermal growth factor receptor (EGFR)-expressing, wild-type KRAS+ developed or metastatic colon cancer when merged with chemotherapy	5-year survival rate 33–60% Gastrointestinal haemorrhage, cardiovascular disorders and sepsis; dermatological toxicities experienced in 45–100% of patients	Xiong et al., 2004; Adam et al., 2007; Köhne & Lenz, 2009
	Panitumumab (Vectibix)	As a main therapy for the remedy of patients with EGFR-expressing metastatic colon carcinoma with wild-type KRAS+ after failure of irinotecan regimes	5-year survival rate ~77.6%. Skin rash, chest pain, diarrhoea and constipation; 90% chance of developing skin toxicity	Köhne & Lenz, 2009
	Bevacizumab (Avastin)	Used for first and later treatment lines of developed or	5-year survival rate increased by ~10%	Di Nicolantonio et al., 2008;

Current treatment modalities	Drug	Stage at which treatment is used	Efficacy and side effects	Reference
		metastatic colon cancer in merging with a fluoropyrimidine-based chemotherapy. Use of bevacizumab is not dependent on KRAS status	Gastrointestinal perforation, haemorrhage and rectal haemorrhage	Köhne & Lenz, 2009; Koukourakis & Sotiropoulou, 2011
Radiation			Not yet shown to effectively improve outcomes for patients Diarrhoea, narrowing of the bowel and inability of nutrients to be absorbed efficiently; short- and long-term effects	Chau & Cunningham, 2002

The prediction of colon cancer therapy depends on the stage and type of the disease and the age of the patient (Woude & Klein, 2009). There is a distinct correlation between survival rate and stage of prognosis (Table 2.1). A stage I diagnosis of colon cancer is associated with a 90% five-year survival rate. This drops to 87% with stage II, 57% with stage III and 10% with stage IV of the cancer (McLeish et al., 2002). Despite advances in chemotherapy regimens, over half of all colon cancer patients receiving treatment do not display significant reduction of the tumour. Significant side effects and high costs have created a keen interest in developing alternative chemotherapeutic agents, especially as the cost of chemotherapy is estimated to rise between 180% and 980% over 12 years (O'Leary et al., 2004) (Table 2.2).

Table 2.2 Estimated chemotherapy costs in the last 12 years

Stage	Current estimates	Previous estimates (1999 costs)	Increase (%)	Reference
1	$30,890	$17,148	46	O'Leary et al., 2004
2	$47,534	$33,364	17	
3	$74,225	$25,771	180	
4	$61,423	$6,264	710	

2.4 Possible Targets for Colon Cancer Treatment and Prevention

A great deal of information exists regarding cancer control strategies designed to suppress or reverse the progression of precancerous lesions to reduce the occurrence and recurrence of this disease (Watson & Preedy, 2010). Many agents, such as 5FU, 5FU plus levamisole, or 5FU plus leucovorin have been identified to inhibit colon cancer development (Centelles, 2012). Previously, the hypothesis was that misregulation of apoptosis was one of the key steps leading to the growth of cancer (Glinsky, 1997). If valid, this concept has significant implications for the avoidance of carcinogenesis and may lead to development of new types of cancer control approaches for the restoration of competence to regulate tissue size. Further, this will essentially increase the number of cells that can be killed in the tumourigenic process. As apoptosis is highly conserved, specified and selected only for the means of controlling tissue mass and shape, this also suggests that it can be exploited for the prevention or control of cancer (Wong, 2011).

2.4.1 Matrix Metalloproteinase Inhibitors

Matrix metalloproteinases (MMPs), particularly MMP-14 (pro-MMP-2's primary activator), MMP-9 (gelatinase B) and MMP-2 (gelatinase A) are involved in several physiological and pathological processes such as stem cell differentiation and proliferation, apoptosis, tissue degeneration and cancer (Ligi & Mannello, 2016). MMPs have important roles in carcinogenesis through their capability to inactivate or activate proteins that play essential roles in tumour initiation, progression and metastases Therefore, MMP inhibitors may be development targets for new anticancer medication because they play an essential role in angiogenesis, carcinogenesis and growth of the tumour at both primary and metastatic locations (Hua et al., 2011).

2.4.2 Therapeutics

Angiogenesis, the establishment of new blood vessels, has been a key therapeutic target for cancer treatment because it forms a hallmark for the development of cancer. Vascular endothelial growth factor (VEGF), which performs an essential function in angiogenesis, is a signalling molecule and is highly abundant in tumours. Therefore, clinical research appears to focus on developing antiangiogenic therapies through the inhibition of VEGF (Welti et al., 2013).

2.4.3 Tyrosine Kinase Inhibitors

Among the most recent and significant mechanisms, or rather classes of treatment, of cancer are tyrosine kinase inhibitors (TKIs). Nevertheless, aberrant activation, particularly when linked to tyrosine kinase pathways, remains the most common human cancer dysregulated molecular pathway; thus, the majority of tyrosine kinases might serve as useful molecular targets (Natoli et al., 2010).

2.4.4 Immunomodulatory Agents

Interferon gamma (IFN-γ) is an immunoregulatory molecule that is a key cytokine in tumour immunology and is involved in carcinogenesis inhibition. According to Ossina et al. (1997) and Blankenstein and Qin (2003), it is also involved in inhibition of cancer cell proliferation in tumour microenvironments. Absence of IFN-γ secretion was observed to inhibit tumour rejection (Gansbacher et al., 1990; Blankenstein and Qin, 2003). Interleukin-2, -6 and -12 (Conlon & Bird 2014), and TNF-α play a key role in antitumour and immunoregulatory activities, and are considered cancer therapy targets for blocking tumour progression and/or deterring established tumours (Byun et al., 2010).

2.4.5 Antimutagenic Agents

The development of cancer proceeds via multiple stages and DNA damage (i.e., mutations) can be an essential step in cancer progression. Tumour protein *p53* (tumour suppressor gene) is muted in most human malignancies (Hussain et al., 2001). This gene has a crucial role in regulating cellular functions, for example, it is able to induce cell cycle arrest, apoptosis regulation and DNA repair. Thus, mutations in the *p53* gene may result in its inability to repair DNA mutations and consequently cause instability in various genes leading to cancer progression (Rivlin et al., 2011). Therefore, antimutagenic drugs may target cancer cells via *p53* reactivation.

2.4.6 Antioxidants

Deleterious effects of oxidants include oxidative DNA damage associated with early stages of carcinogenesis (Choudhari et al., 2014). Therefore, reducing oxidative damage may be significant in cancer prevention/treatment as antioxidant molecules are able to abort free radical formation and prevent oxidising chain reactions (Sah et al., 2015b). Although the main use of chemotherapy agents is to deliver cytotoxic effects to colon cancer cells, their non-selective action affects even healthy cells; development of drug resistance and drug clearance are concerns with conventional chemotherapy agents (Shapira et al., 2014). Therefore, a great deal of attention has been focused on the use of natural bioactive compounds such as peptides to improve antiproliferative efficacy and overcome the limitations of conventional therapeutic agents.

The use of peptides from food proteins has the potential for treating colon cancer (Sah et al., 2016b) as they may target cancer cells without damaging healthy cells. Peptides derived from foods may also be less toxic and cause fewer side effects in healthy cells. Moreover, peptides may be used directly as cytotoxic agents or indirectly as carriers for

cytotoxic agents, such as 5FU and capecitabine, on cancer cells (Shapira et al., 2014). Food proteins are an important source of AAs that primarily help the body to build needed proteins (Korhonen, 2009a). Recent research has shown that through *in vitro* digestion of dietary proteins, a wide range of short and oligopeptides are released from inactive protein sequences and may act as physiological modulators, both in the gut and throughout the body (Sah et al., 2016b, c). These peptides might alter biological functions of certain target proteins in the event of their release by proteolytic enzymes (Muheem et al., 2016).

2.5 Food Bioactive Peptides

Food peptides can be obtained from both animal and vegetable sources. Bioactive peptides have been reported from numerous food sources (Table 2.3) including eggs, fish, some cereal grains and soybeans; however, milk and other dairy fermented foods have been considered the most effective sources of these peptides. In fermented milk, the proteolytic enzymes in dairy starter cultures including LAB and probiotics play an important role in protein hydrolysis and formation of bioactive peptides. The most common milk bioactive peptides range in size between 2 and 20 AAs (Mohanty et al., 2016). The peptides may be present as independent entities or as part of the parent protein and are typically resistant to digestion by peptidases. Since the discovery of opioid peptide fragments isolated from bovine beta casein (β-CN) by enzymatic protein hydrolysis, various studies have identified bioactive peptides from dairy proteins (Korhonen, 2009b). Subsequently, many AA sequences and their physiological functions have also been identified (Korhonen, 2009a).

Table 2.3 Some bioactive peptides obtained from food

Bioactivity	Source	Type of protein	Reference
ACE inhibitory/hypotensive	Soy	Soy protein	Zhu et al., 2008
	Fish	Cuttlefish protein	Balti et al., 2010
	Meat	Meat muscle protein	Toldrá et al., 2012
	Milk	α-La, β-Lg	Murray & FitzGerald, 2007
		α-, β-, κ-CN	
	Eggs	White protein	
	Wheat	Wheat germ	Liu et al., 2010
	Broccoli	Plant protein	Motoi & Kodama, 2003
			Lee et al., 2006
Immunomodulatory	Mushroom	Mushroom proteins	Xu et al., 2011
	Egg	Egg albumin	Mine & Kovacs, 2006
	Milk	α-, β-, κ-CN, α-La	Meisel, 2005
	Wheat	Wheat gluten	Horiguchi et al., 2005
Cytomodulatory	Milk	α-, β-CN	Kampa et al., 1997
	Rice	de-fatted rice bran	Kannan et al., 2010
Opioid agonist	Wheat	Wheat gluten	Takahashi et al., 2000
			Fukudome & Yoshikawa, 1993
	Milk	α -La, β-Lg	Silva & Malcata, 2005
		α-, β-CN	
Opioid antagonist	Milk	Lactoferrin (Lf)	Clare & Swaisgood, 2000
		κ-CN	
Antimicrobial	Egg	Ovotransferrin	Mine & Kovacs, 2006
		Lysozyme	
	Milk	Lactoferrin	McCann et al., 2006
		α-, β-, κ-CN	Hayes et al., 2006
	Symbiotic yoghurt	β-CN	Sah et al., 2016c
Antithrombotic	Milk	κ-CN (glycomacropeptide)	Chabance et al., 1995
Mineral binding, anticarcinogenic	Milk	α-, β-CN	Walker et al., 2006
Antioxidant	Fish	Sardine muscle	Erdmann et al., 2006
	Wheat	Wheat germ protein	Zhu et al., 2006
	Milk	α-La, β-La	Hernández et al., 2005b
	Symbiotic yoghurt	β-CN	Sah et al., 2016b

In vitro digestion systems and microbial enzymes have been utilised in the formation of bioactive peptides through hydrolysis of milk proteins. These mechanisms allow small

and oligopeptide fragments to be released and characterised. The potential of biologically active peptides arising from casein hydrolysis was inferred from the kinetics of proteolytic enzymes and the AA components of peptides. It may be possible to predict the physiological function of a given peptide fragment based on its AA sequence (Sah et al., 2016c). However, some bioactive peptides may contain multifunctional activity and thus interact with more than one target site in the body. The role of nutrition in the cancer process has been broadly investigated (Cencic & Chingwaru, 2010). According to reports by the World Cancer Research Fund and American Institute for Cancer Research, ~30–40% of cancers can be avoided by maintaining a balanced diet in addition to regular physical activity and body weight management (Donaldson, 2004).

Recent human clinical studies have examined the association between milk/dairy consumption and the risk of growing several malignancies, such as colon cancer (Thorning et al., 2016). However, limited information has been generated with respect to colon cancer incidence and the intake of milk and other dairy products. One study reported that individuals with the highest intake of dairy foodstuffs had the lowest likelihood of developing colon cancer (Aune et al., 2012). Another study suggested that the protective effect of milk and dairy products may be related to certain compounds, including milk peptides (Thorning et al., 2016). However, Aune et al. (2012) reported an increased chance of rectal cancer related to higher consumption of dairy products. The problem with epidemiological studies is that it is exceedingly difficult to distinguish the effects of various components and other lifestyle factors. Major milk proteins might have preventative effects on colon cancer based on rat models, which have shown that certain types of milk proteins are protective (Davoodi et al., 2013).

The search for nutraceutical agents (foods containing health-giving additives with medicinal advantages) has driven investigations into the prevention of colon cancer by

dietary means. Of particular interest is the potential effect of peptides derived from fermented milk on antiproliferation of colon cancer cells by encouraging apoptosis, resistant to breakdown by the cellular machinery and lack toxicity to healthy cells (Sah et al., 2015b, 2016b).

2.6 Fermented Milk and Colon Cancer

Approximately 70% of colon cancer cases are related to environmental factors, the main one being diet (Haggar & Boushey, 2009; Mármol et al., 2017). There is a great deal of interest in the likely defensive function of fermented milk and probiotic (live bacteria with health benefits) cultures against colon cancer. Interventional studies have indicated a change in intermediary indicators of colon cancer risk in human bodies from high to low hazard following probiotic ingestion (Conlon & Bird, 2014). Animal research has repeatedly indicated a decrease in chemically prompted colorectal growth occurrence and abnormal crypt development in response to consumption of probiotics (Conlon & Bird, 2014). Probiotics might beneficially modulate various major intestinal functions, such as detoxification, colonic fermentation, transit and immune condition, which may accompany advancement in the growth of colon cancer (Rafter, 2003). Although more research is required, the reviewed studies constitute an encouraging element of proof that the defensive function of milk fermented with probiotic cultures could decrease the risk of cancer in the large intestine (Rafter, 2003).

Fermented milk products have been used both to benefit human health and to improve the revenue stream of companies. Partial milk protein hydrolysis during fermentation by LAB has improved the nutritional value of milk for humans. Milk proteins are potential components of health-enhancing efficient foods aiming to treat diet-linked chronic disorders such as obesity, diabetes mellitus type 2 and cardiovascular disease (Korhonen,

2009a; Tudor et al., 2009). Research has also shown that diets rich in dairy products might hinder the proliferation of numerous cancerous cell types (Parodi, 2007). Peptides released from bovine milk by LAB proteolytic enzymes have been shown to improve the immune response. Further, milk peptides with possible antiproliferative activity have been reported as bioactive compounds that might affect the viability of cancer cells by inducing apoptosis (Meisel, 2004; Sah et al., 2016b). These peptides may be important in controlling malignant cell proliferation (Parodi, 1998, 2007). Thus, potential antiproliferative peptides may provide a protective effect against cancer progression. Peptide fractions extracted from cell-free supernatants of *L. helveticus*-fermented milk have been reported to cause a significant reduction in fibrosarcoma proliferation *in vivo* (LeBlanc et al., 2004).

2.7 The Proteolytic Systems of Lactic Acid Bacteria

One of the most broadly investigated biochemical pathways is the proteolytic system of *Lactococcus (Lc.) lactis*. This system is essential for significant and rapid growth of the bacterium in milk (Kunji et al., 1996; Christensen et al., 1999). LAB, mainly *Streptococcus* (*St.*) *thermophilus*, *Lc. lactis* and some *Lactobacillus* species, have become commercially important as starter strains in an assortment of fermented dairy products (Savijoki et al., 2006). Several *Lactobacillus* species are used as components of starter cultures for the production of fermented dairy products and various types of cheese (Ummadi & Curic, 2010). The AAs required for LAB growth in milk differ between species and even between strains within the same species (Morishita et al., 1981; Elfahri et al., 2014). The proteolytic system of lactococci has been thoroughly studied due to of their importance in the cheese and dairy industries and the extent of knowledge transferred to some degree to other LAB (Christensen et al., 1999). The hydrolysis of

milk proteins by LAB proteolytic enzymes begins with the action of extracellular proteinases. Cell envelope proteinases (CEPs) are critical for the growth of LAB in milk because they hydrolyse casein into more than 100 different oligopeptides, the majority having 4–10 residues (Kunji et al., 1996).

2.8 Genus *Lactobacillus*

Some *Lactobacillus* species have been described as aerotolerant, capable of using oxygen through the flavoprotein oxidase, while others are described as being strictly anaerobic (Griffiths & Tellez, 2013). In addition, *Lactobacillus* spp. have an optimum pH 5.5–5.8 with numerous nutritional requirements for carbohydrates, peptides and AAs (Axelsson, 2004). The genus *Lactobacillus* has been divided into three groups: homofermentative species, which ferment glucose almost exclusively (over 85%) to lactic acid (Fooks & Gibson, 2002); facultative heterofermentative species, which produce only half of the lactic acid along with a smaller quantity of ethanol, carbon dioxide and acetic acid (Lahtinen et al., 2012); and obligate heterofermentative species, which are capable of producing DL-lactic acid, acetic acid and carbon dioxide.

Lactobacilli are widespread in nature with most known species utilised in the food industry. Some species have also been used in clinical research (Lahtinen et al., 2012). One example is the capacity of lactobacilli to convert lactose to lactic acid, which has been utilised in the prevention of lactose intolerance (Reid, 2008). Additionally, some species of *Lactobacillus* act as inhibitors of some common pathogenic bacteria by reducing pH in the intestinal tract, thus creating an environment that is not suitable for the growth of pathogens (Šušković et al., 2010). Further, certain strains of *Lactobacillus* spp. possess the capability to produce compounds such as hydrogen peroxide, carbon dioxide, diacetyl or bacteriocins during metabolism, which also result in an environment

that is unfavourable for the growth of some pathogenic organisms (Šušković et al., 2010). Some strains, including *L. rhamnosus* and *L. acidophilus*, have been commercially utilised as dietary supplements following United States Food and Drug Administration approval (Vanderhoof & Young, 2008).

2.8.1 *Lactobacillus helveticus*

L. helveticus has been used mainly in the manufacturing of fermented milk beverages. The versatile nature of this bacterium is based on it having the most effective proteolytic system, which is composed of cell envelop proteinases and a number of peptidases (Widyastuti & Febrisiantosa, 2014). Additionally, the utilisation of *L. helveticus* in cheese and fermented milk manufacturing has become more significant in the dairy industry since bioactive peptides have been linked with health benefits (Widyastuti & Febrisiantosa, 2014). *L. helveticus* strains grow exceptionally well in milk as they are capable of hydrolysing milk proteins by exo- and endogenous peptidolytic and proteolytic enzymes (Griffiths & Tellez, 2013), and thus derive peptides and AAs required for their growth (Christensen & Steele, 2003; Griffiths & Tellez, 2013). Studies by Elli et al. (2000) and Chervaux et al. (2000) used a chemically defined medium that contained 21 AAs and other nutrients to characterise the nutrient requirements of 22 *Lactobacillus* strains. For example, 14 AAs (Arg, Glu, His, lie, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr, Val, and either Asp or Asn; Christensen & Steele, 2003) appear to be important for *L. helveticus* CNRZ 32 growth. Generally, to reach optimal growth and viability, these strains require fermentation media enhanced with plentiful carbon and nitrogen, vitamins, micro and macronutrients and nucleotide bases. Different levels of growth among some *Lactobacillus* spp. have been observed (Horn et al., 2007) and it is assumed that this could be based on their nutritional requirements.

Apart from providing important and essential AAs, this metabolic activity releases simultaneously a wide range of peptides with many important bioactivities such as immune boosting, radical scavenging and angiotensin-converting enzyme (ACE) inhibition activities (LeBlanc et al., 2002; Hernández et al., 2005b; Chen et al., 2014). Understanding pathways involved in their release is important as they may be numerous; thus, appropriate strain selection with the aim of expressing a particular activity could be drawn upon to identify key enzymes involved in the liberation, or even engineering an enzymatic cascade that would liberate particular peptides.

2.9 The Proteolytic System of *Lactobacillus helveticus*

The high proteolytic activity of *L. helveticus* strains in milk depends on several factors including pH, temperature, fermentation stage and storage (Elfahri et al., 2015; Mohanty et al., 2016). These factors are associated with release or hydrolysis of bioactive peptides. As mentioned above, the proteolytic system has three main components (Fig 2.2): CEPS, peptide transport system and intracellular peptidases (Griffiths & Tellez, 2013; Pihlanto, 2013). The following section will focus on the rules of the proteolytic scheme of *L. helveticus* in releasing bioactive peptides.

2.9.1 Cell Envelope Proteinases

Unlike most LAB proteolytic systems, which have only one CEP, *L. helveticus* possesses at least two types of CEPs (PrtH2 and PrtH), with some others having PrtH3 and PrtH4 (Griffiths & Tellez, 2013). Based on the action of the CEPs of *L. helveticus* strain CNRZ32 on an alpha s1-casein (αs1-CN) fragment (1–23) with short incubation (5–15 min), eight fragments of peptides (1–9, 1–6, 1–17, 1–16, 17–23, 18–23, 9–23 and 10–23) are released. This action revealed specific cleavage for the following peptide bonds; His_8–

Gln_9, Ile_6–Lys_7, Gln_9–Gly_{10}, Asn_{17}–Glu_{18} and Leu_{16}–Asn_{17} (Pederson et al., 1999). Conversely, the cleavage bonds linked to similar substrates that have been hydrolysed by PrtH release only four fragments (18–23, 1–17, 17–23 and 1–16) of peptide. Although these fragments might not be noticed in PrtH deletion, four other fragments of peptides have been detected (9–23, 1–6, 10–23, and 1–9) (Pederson et al., 1999). This indicates that the strain has at least two extracellular proteinases that are responsible for releasing peptides from αs1-CN f (1–23) (Pederson et al., 1999).

2.9.2 Cleavage Sites of Cell Envelope Proteinases on Milk Proteins

2.9.2.1 Beta Casein

Studies by Hébert et al. (2002) on four *L. helveticus* strains (ATCC 15009, CRL 1062, CRL 1178 and CRL 1177) revealed the hydrolysis patterns of purified β-CN. Each of these strains showed a different hydrolysis pattern, with the exception of CRL 1062 and ATCC 15009, which showed a similar pattern of protein hydrolysis. Tables 2.4, 2.5 and 2.6 provide further information on several CEP cleavage bonds common for three *L. helveticus* strains assessed on β-CN whereas other bonds are strain specific (Sadat-Mekmene et al., 2011a).

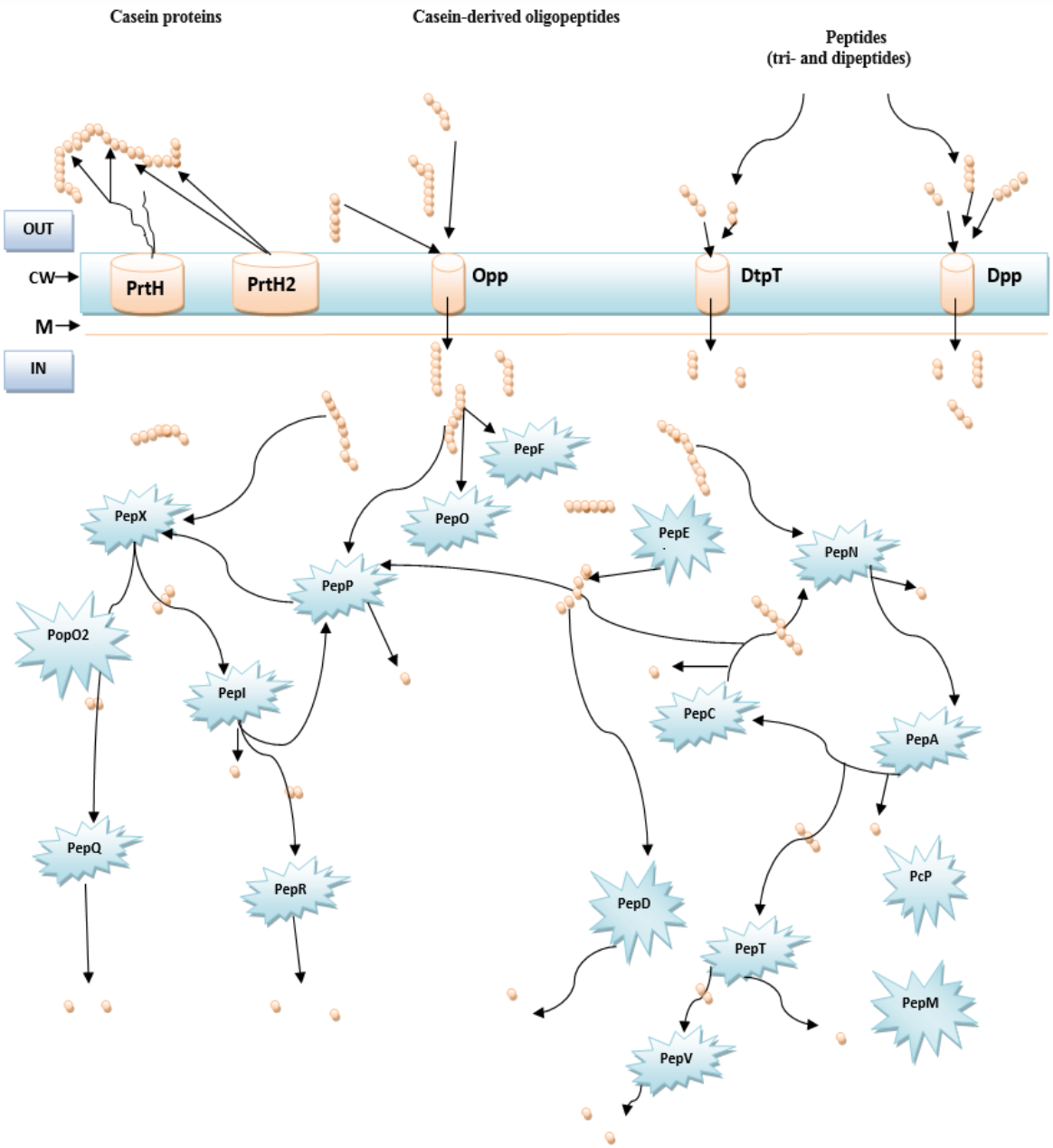

Figure 2.2 General schematic of the proteolytic systems identified in *Lactobacillus helveticus.* M indicates the cell membrane and CW denotes the cell wall membrane (Sadat-Mekmene et al., 2011a)

Further, other studies revealed various proteolytic activities and specificities among 15 strains of *L. helveticus* examined on pure caseins or on casein micelles directly in milk (Sadat-Mekmene et al., 2011a). The results showed more rapid hydrolysis of β-CN for all 15 strains compared to the kinetics involved in the hydrolysis of αs1-CN taking place in the presence of the two CEPs. The number of AA residues in the resulting peptides

varied from 6 to 33, showing vast specificity in cleavage bonds (Sadat-Mekmene et al., 2011a). In contrast, the growth of bacteria in milk provides varying results revealing massive reduction in the number of peptides released, indicating the accessibility of CEPs on casein micelle in milk compared with pure caseins.

Table 2.4 Amino acid cleavage sites for *Lactobacillus helveticus* CNR303 cell envelope proteinases in beta casein

1 RELEE**L**↓**N**VPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY
61 PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAP**K**↓**H**KEMP FPKYPVQP**F**↓**T**
121 ESQSLTLTDV ENLHLPPLLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVP**E**↓**K**AVPY
181 P**Q**↓ **R**DMPIQA**F**↓**L**↓**L**↓**Y**QQPVLGP VRGPFPIIV

Table 2.5 Amino acid cleavage sites for *Lactobacillus helveticus* CP790 envelope proteinases in beta casein

1 RELEELNVPG EIVE**S**↓**L**SSSE ESITRINKKI EKFQSEEQQQ T**E**↓**D**ELQDKIH PFAQTQSLVY
61 PFPGPIPN**S**↓**L** PQNIPPLTQT PVVVPPFLQP EVMGVS**K**↓**V**KE AMAP**K**↓**H**KEMP F**P**↓**K**YPVQP**F**↓**T**
121 E**S**↓**Q**SLT**L**↓**T**DV ENLHLPPLLL **Q**↓**S**WMHQP**H**↓**Q**P LPPTVM**F**↓**P**PQ SVLSLS**Q**↓**S**KV LPVP**E**↓**K**↓**A**VPY
181 P**Q**↓**R**DMPIQA**F**↓ **L**↓**L**↓**Y**QQPVLGP VRGPFPIIV

Table 2.6 Amino acid cleavage sites for *Lactobacillus helveticus* CP53 envelope proteins in beta casein

1 RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY
61 PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVQPFT
121 ESQSLTLTDV ENLHLPPLLL **Q↓S**W**M↓H**QP**H↓Q**P LPPTVMFPPQ SVLSLSQSKV LPVPEKAVPY
181 PQ RDMPIQA**F↓L**L YQQPV**L↓G**P VRGPFPIIV

2.9.2.2 Alpha s1 Casein

Tables 2.7 and 2.8 show the locations of cleavage sites for some CEPs on pure αs1-CN in *L. helveticus*. The bonds indicate cleavage sites (Ile_6–Lys_7, Gln_9–Gly_{10} and Leu_{142}–Ala_{143}) shared among some *L. helveticus* strains, while other bonds are strain specific. Further, Hébert et al. (2002) reported the ability of four *L. helveticus* strains (ATCC 15009, CRL 1177, CRL 1178 and CRL 1062) to release peptides from αs1-CN as a substrate after 3 h of incubation. In addition, Scolari et al. (2006) found that the proteinase of *L. helveticus* Zuc2 exhibits a low affinity for αs1-CN, cleaving the Arg_{22}–Phe_{23} bond after incubation for 2 h. Martín-Hernández et al. (1994) showed that αs1-CN (1–9) was the main peptide released from this type of casein using *L. helveticus* L89 CEP, while αs1-CN (1–14) occurred to a lesser extent. Oberg et al. (2002) observed four patterns of specificity for eight *L. helveticus* strains on αs1-CN (1–23). Hydrolysis of the fragment αs1-CN (1–23) by the whole cell of *L. helveticus* CNRZ 32 and the PrtH mutant reveals that this strain has the ability to synthesise two active CEPs. Each has a particular specificity in terms of cleavage: for example, PrtH cleaves for αs1-CN (1–16, 18–23, 1–17 and 17–23), whereas PrtH2 releases αs1-CN (1–9, 9–23, 1–16 and 10–23) (Pederson et al., 1999). Sadat-Mekmene et al. (2011b) reported that the cleavage sites on purified

αs1-casein are located not only between 1 and 40 residues of the N-terminus, but also in the middle of the sequence of residues ranging from 80 to 150. The hydrolysis of residues 170 and 199 at the C-terminus of αs1-CN was also observed by Jensen et al. (2009).

2.9.2.3 Alpha s2 Casein

Hydrolysis of purified αs2-CN has not been reported, which may be due to the level of difficulty involved in αs2-CN purification. The specificity of CEPs to this casein in assembled micelles in milk has been demonstrated (Sadat-Mekmene et al., 2011a). Cleavage sites are restricted to certain regions (positions 1–25 and 97–162), indicating that other cleavage sites are probably not accessible.

Table 2.7 Amino acid cleavage sites for *Lactobacillus helveticus* CNRZ303 cell envelope proteinases in alpha S1-casein

1 RPKHP**I**↓ **K**H**Q**↓**G** LPQEVLNENL LRFFVAPFPQ VFGKEKVNEL SKDIGSESTE DQAMEDIKQM
61 EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR LKKYKVPQLE IVPNSAEERL
121 HSMKEGIHAQ QKEPMIGVNQ E**L**↓**A**YFYP**E**↓**L**F RQFYQLDAYP SGAWYYVPLG TQYTDAPSFS
181 DIPNPIGSEN SEKTTMPLW

Table 2.8 Amino acid cleavage sites for *Lactobacillus helveticus* CP790 cell envelope proteinases in alpha S1-casein

1 RPKHP**I**↓**K**H**Q**↓**G** LPQEVLNENL LR**F**↓**F**VAPFPQ **V**↓**F**GKEKVNEL SKDIGSESTE DQAMEDIKQM
61 EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR LKKYKVPQLE IVPNSAEERL
121 HS**M**↓**K**EGIHAQ **Q**↓**K**EPMIGVNQ E**L**↓**A**YFYP**E**↓**L**F RQF**Y**↓**Q**L**D**↓**A**YP **S**↓**G**AWYY**V**↓**P**↓**L**↓**G** TQYTDAPSFS
181 DIPNP**I**↓**G**SE**N**↓**S**E**K**↓**T**TMPLW

2.9.2.4 Kappa Casein

The degree of hydrolysis of κ-casein by *L. helveticus* CEPs provides little or no activity compared to the other caseins as reported by Hébert et al. (2000) and Scolari et al. (2006). After 30 minutes of κ-casein incubation with *L. helveticus* Zuc CEP, κ-casein was cleaved into glycomacropeptides, that is, κ-CN (106–169). The released peptides included κ-CN (147–169), κ-CN (106–111), κ-CN (112–116), κ-CN (112–149), κ-CN (106–113) and κ-CN (117–146) (Scolari et al., 2006). In contrast, this hydrolytic pattern was not observed by CEP of *L. helveticus* CP790, again indicating diversity in CEPs (Laloi et al., 1991; Yamamoto et al., 1993).

2.9.3 Transport Systems of Peptides

After caseins (mainly β-CN) are degraded by CEPs to short and oligopeptides, the second stage is transport of these peptides via the cell wall of the bacterial cell with the aid of the peptide transport system, based on peptide size and sequence of AA (Griffiths & Tellez, 2013). According to a 1998 study on the proteolytic activity of *L. helveticus,* Val, Thr, Ile

and Lys are conveyed by a secondary transport system, while the other AAs (Tyr, Arg, His, Glu and Asp) appear to be conveyed by a system driven by ATP (Nakajima et al., 1998). Moreover, research confirms that three systems of peptide transportation are contained in the *L. helveticus* DPC4571 strain: the oligopeptide (Fabian et al., 2005), tri-(DtpT) and di-(DPP) (Callanan et al., 2008) peptide transport systems shown in Figure 2.2. Only two transport systems, DtpT and oligopeptides, have been determined in *L. helveticus* H10, demonstrating dissimilarity in the proteolytic systems of strains of *L. helveticus* (Zhao et al., 2011).

2.9.4 Peptidases

As far as proteolytic activity (Table 2.9) is concerned, the peptidase step is significant, particularly with regard to LAB growth due to the fact that peptidases primarily produce AAs. In the last two decades, more peptidases have been recognised (Christensen et al., 1999; Savijoki & Palva, 2000; Kenny et al., 2003; Pan & Tanokura, 2004), including five proline-specific peptidases, seven oligoendopeptidases, eight tri- or dipeptidases and three aminopeptidases (Broadbent et al., 2011). Some di- and tripeptidases such as dipeptidase A (PepDA) and prolinase (PepR) are not necessary for the growth of *L. helveticus* strains in milk (Dudley et al., 1996; Shao et al., 1997). For example, deletion of the *pepDA* gene does not affect *L. helveticus* CNRZ23 growth, indicating that PepDA has no essential role in releasing the AAs required for *L. helveticus* growth (Dudley et al., 1996). However, other peptidases such as aminopeptidases (PepN and PepC) and X-prolyl dipeptidyl aminopeptidase (PepX), are important for bacterial growth (Griffiths & Tellez, 2013). One study showed that *L. helveticus* strains exhibit amino peptidase activity with de Man, Rogosa and Sharpe (MRS) medium or milk as the growth medium, using specific enzymes (Griffiths & Tellez, 2013).

2.9.4.1 Endopeptidase Genes

Callanan et al. (2008) showed that the *L. helveticus* DPC4571 genome carries seven endopeptidase genes (*pepO, pepO3, pepO2, pepF, ydiC, gcp* and *pepE2*). These have been characterised separately and their biological significance established (Christensen et al., 1999).

2.9.4.2 Endopeptidase E

This enzyme was isolated from *L. helveticus* CNRZ32 and found to be identical to aminopeptidase C isolated from *L. delbrueckii* ssp. *lactis* DSM7290 and other LAB, indicating that they all result from the same family of proteolytic enzymes. PepE has the ability to hydrolyse internal peptide bonds to create smaller peptides such as bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg) and Met-enkephalin (Tyr–Gly–Gly–Phe–Met) at the Gly_4–Phe_5 and Gly_3–Phe_4 bonds, respectively, but not intact caseins (Fenster et al., 1997; Christensen et al., 1999).

2.9.4.3 Endopeptidase F

PepF can hydrolyse peptides with number of AAs ranging from 7 to 11, with broad specificity. The enzyme contains zinc-binding sites, leading to its characterisation as a metalloenzyme. Sridhar et al. (2005) showed that PepF can cleave X–Phe and Ilu–Phe bonds of β-CN (f193–209) when X is proline.

2.9.4.4 Endopeptidase PepO

Chen and Steele (1998) revealed the existence of 40% similarity between PepO from *L. helveticus* CNRZ32 and that of *Lc. lactis* P8-2-47. An enzymatic study of *L. helveticus* CNRZ32 reported significant reduction (79% and >94%) in endopeptidase activity when

a PepO-deficient mutant strain was used for substrates N–benzoyl–Phe–Val–Arg–pNA and N–benzoyl–Val–Gly–Arg–pNA, respectively. However, this reduction in the PepO-deficient mutant had no effect on strain growth or acid production in milk as the growth medium, indicating that this peptidase is not growth related in this strain (Chen & Steele, 1998). It has been reported that $PepO_2$ is able to hydrolyse β-CN (f193–209) (Chen et al., 2003).

2.9.4.5 Di-and Tripeptidases

Callanan et al. (2008) reported different dipeptidases in the *L. helveticus* DPC4571 strain, including PepD, PepD3, PepD4 and PepA, as well as a tripeptidase, PepT. The specificity of peptidolytic enzyme PepD is similar to that of PepV and it is able to cleave Met–Ala. Further, in growth studies, deletion of PepDA, a general dipeptidase isolated from *L. helveticus* CNRZ32, does not have any detectable effects on the production of acid or the growth rate in milk or MRS medium. PepDA therefore does not play an essential role in the liberation of AAs from casein-derived peptides (Dudley et al., 1996).

PepT purified from *L. helveticus* is a trimeric metallopeptidase exhibiting maximum activity in the presence of tripeptides with hydrophobic properties; and showing high activity with Met–Gly–Gly tripeptide. However, slow hydrolysis of hydrophobic dipeptides and undetectable activity towards tetrapeptides or AA *ρ*-nitroanilide has been reported (Savijoki & Palva, 2000).

Table 2.9 Peptidases of *L. helveticus*; the arrows show hydrolysed peptide bond positions

Peptidase	*L. helveticus* strain	Specificity
Oligoendopeptidases		
PepO, PepO2, PepO3	CNRZ32	NH_2–Xn↓Xn–COOH
PepE, PepE2	CNRZ32	NH_2–Xn↓Xn–COOH
PepF	CNRZ32	NH_2–Xn↓Xn–COOH
Aminopeptidases		
PepN	53/7 CNRZ32	NH_2–X↓Xn–COOH
PepC	53/7 CNRZ32	NH_2–X↓Xn–COOH
Tripeptidases		
PepT	53/7	NH_2–X↓X–X–COOH
Dipeptidases		
PepD	53/7 CNRZ32	NH_2–X↓X–COOH
Proline-specific		
PepI	53/7	NH_2–Pro↓Xn–COOH
PepR	53/7 CNRZ32	NH_2–Pro↓X–COOH
PepX	53/7 CNRZ32	NH_2–X–Pro↓Xn–COOH

2.9.4.6 Proline-specific Peptidases

According to genomic data from *L. helveticus* DP4571, five types of proline-specific peptidases have been reported (Callanan et al., 2008). These peptidases (PepP, PepQ, PepI, PepR and X-prolyl dipeptidyl aminopeptidase (PepX)) can hydrolyse peptides containing proline in the second position, whereas other aminopeptidases (di- and tripeptidases) cannot hydrolyse proline-containing peptides due to imido bonding (Kunji

et al., 1996). Aminopeptidase P (PepP) is another proline peptidase that has been revealed to exist in lactobacilli (Christensen et al., 1999). It cleaves the AA–proline bond from the N-terminus of peptides, but cannot hydrolyse dipeptides containing proline. Proline iminopeptidase (PepI) catalyses the release of proline from peptides with proline at the N-terminus and with hydrophobic/uncharged AAs such as Ala, -Gly, -Ile, -Leu and -Val. Another proline peptidase is PepX (Savijoki et al., 2006). It releases dipeptide from N-terminal polypeptides X–Ala–Y– and X–Pro–Y as long as the penultimate residue is Ala or Pro and the cleavage bond is on the carboxyl side of the proline or alanine residue (Vesanto et al., 1995).

Proline iminodipeptidase (PepR) is another dipeptidase, which acts as a catalyst to release dipeptides (Pro–X) with hydrophobic/uncharged AAs (-Ala, -Ile, -Leu, -Val) at the C-terminus and proline at the N-terminus (Shao et al., 1997). PepR was also found to hydrolyse Thr–Leu, Pro–Met and Ser–Phe peptide bonds (Shao et al., 1997). Imidodipeptidase (PepQ) is a dipeptidase that strictly releases proline at the C-terminus from dipeptides (X–Pro) with more specificity for Met–Pro, Leu–Pro and Phe–Pro, but shows no activity on other dipeptides such as Gly–Pro and Pro–Pro (Christensen et al., 1999).

2.9.5 The Role of Proteolytic Enzymes in Production of Bioactive Peptides

When bioactive peptides are released from milk proteins by *L. helveticus* strains, the level of protein degradation during fermentation is an essential factor in production of bioactive peptides and needs to be considered during the fermentation process. This is mainly because proteolytic enzymes under these conditions would remain active and may further hydrolyse proteins and peptides, including those with important bioactivities. Therefore, the released bioactive peptides may or may not be subjected to further hydrolysis that

may increase, decrease or even destroy bioactivity due to continuous degradation of the peptides (Agyei et al., 2016).

Evaluation of the responsibility of each *L. helveticus* CEP or peptidase in terms of the bioactive peptides released from milk proteins is a complex process that has not been characterised in several studies (Elfahri et al., 2014, 2015). For example, ACE-inhibitory (ACE-I) peptides released by the action of *L. helveticus* strains on milk caseins are the most well-studied peptides (Yamamoto et al., 1993; Fuglsang et al., 2003; Kilpi et al., 2007). Among these peptides, VPP and IPP released from β-CN by *L. helveticus* display the highest ACE-I activity. *L. helveticus* proteinases are probably responsible for this activity in fermented milk as no activity was observed with a proteinase-deficient mutant strain (Yamamoto et al., 1994). In contrast, an increase in ACE-I activity was observed in milk fermented by a *L. helveticus* peptidase-deficient mutant (*pepN* and *pepX*) (Kilpi et al., 2007), suggesting that blocking *pepN* or *pepX* gene expression may delay or prevent hydrolysis of the released ACE-I peptides and allow their accumulation in milk.

Milk fermented with highly proteolytic *L. helveticus* strains can also release anticarcinogenic peptides *in vitro* and in animal models (Elfahri et al., 2015; Pessione & Cirrincione, 2016). However, it is difficult to propose or establish pathways by which these peptides are released during fermentation. One of the possible ways to investigate this is by knowing the structure and AA sequence of potential anticancer peptides (Sah et al., 2015b), and the specificity of each strain of *L. helveticus* proteolytic enzyme(s) on milk proteins under controlled conditions (Sadat-Mekmene et al., 2011a; Griffiths & Tellez, 2013; Pihlanto, 2013). For example, identified anticancer peptides derived from caseins are short and long AA residues mainly derived from β- and α_{s1}-CN (Griffiths & Tellez, 2013). This means that the enzymes responsible for the release may belong to proteinase(s) or may be combined with some specific peptidase(s) activities. Anticancer

peptides ^{63}Pro–Gly–Pro–Ile–Pro–Asn^{68} and ^{41}Ile–Asn–Lys–Lys–Ile^{45} isolated from β-CN, ^{158}Tyr–Val–Pro–Phe–Pro^{162} (casomorphin), ^{1}Arg–Pro–Lys^{5}, ^{101}Leu–Lys–Lys^{103} and ^{104}Tyr–Lys^{105} asecidin peptides from αs1-CN, and ^{17}Phe–Phe–Ser–Asp–Lys^{21} (κ-casecidin peptides) from κ-CN might be released by *L. helveticus* proteinases and/or a number of specific peptidases (Table 2.10).

Table 2.10 Possible roles for *L. helveticus* proteolytic enzymes in release of predicted anticancer peptides from caseins

Type of protein	PrtH/PrtH2	Possible peptidase(s)	Anticancer peptide
β-CN (^{1}RELEELNVPG^{10})-	^{1}RELEE**L**↓ **N**VPG^{10} →	PepN or PepC ^{1}**R** ↓ **E**LEEL^{6} →	^{2}ELEEL^{6}
β-CN – (^{171}LPVPEKAVPYPQ RDMPIQAF^{180}) –	^{171}LPVP**E** ↓ **K**AVPYP**Q** ↓ **D**MPIQAF^{180} →	PepO/PepE/PepF ^{176}KAVP**Y** ↓ **P**Q^{182} →	^{176}KAVPY^{180}
β-CN f (^{191}LLQQPVLGP VRGPFPIIV^{209})	^{191}LLQQPV**L** ↓ **G**PVRGPFPIIV^{209} →	PepX ^{198}G**P** ↓ **V**RGPFPIIV^{209} →	^{200}VRGPFPIIV^{209}
β-CN f (^{91}EVMGVSK VKE AMAPK HKEMP^{110})	^{91}EVMGVS**K** ↓ **V**KE AMAP**K** ↓**H**KEMP^{110} →	PepN/PepC, and PepO/PepE/PepF ^{98}**V** ↓ **KE** ↓ MAPK^{105} →	^{101}AMAPK^{105}
αS1-CN f (^{21}LRF FVAPFPQ V FGKEKVNEL^{40})	^{21}LR**F** ↓ **F**VAPFPQ **V** ↓**F**GKEKVNEL^{40} →	PepO/PepE/PepF ^{24}FVAP**F** ↓ **P**QV^{31} →	^{24}FVAPF^{28}
K-CN f (^{111}A QPTTM ARHP^{120})	^{111}**A** ↓ **Q**PTT**M** ↓ **A**RHP^{120} →	No peptidases required ^{112}QPTTM^{116}	^{112}QPTTM^{116}

Therefore, it appears possible to release bioactive peptides that would be resistant to further degradation by *L. helveticus* in milk. By engineering genetically modified strains, or applying pure proteinase or peptidase sequentially, it may be possible to alleviate or avoid further hydrolysis. However, designing these models is more expensive than milk

fermented by unaltered strains. In addition, the bioactivity of peptides may increase in milk during fermentation due to their synergistic effects with other components produced during fermentation, because of the metabolic activity of the fermented culture. Therefore, some isolated bioactive peptides may exhibit less bioactivity when tested alone (Daliri et al., 2017).

2.10 The Physiological Function of Bioactive Peptides

Fermented dairy products are a source of physiologically important peptides that can affect physiological functions in the human body, in addition to supplying nutrients and energy. These potential health benefits are due to the proteolytic action of LAB on milk proteins during fermentation (Nakajima et al., 1995; Hernández et al., 2005b; Pan et al., 2005). Upon consumption of microbial fermented milk, peptides released during fermentation (Figure 2.3) may affect not only the digestive system, but also the nervous, endocrine, immune and cardiovascular systems (Korhonen, 2009a). Some identified peptides released from milk proteins have been reported to have bioactive properties, such as ACE inhibitory and immunomodulatory, antimicrobial and antithrombotic peptides (Fitzgerald & Meisel., 2003; FitzGerald et al., 2004). Antioxidant and anticarcinogenic peptides have also been purified and identified (Sah et al., 2016b, 2016c).

Bioactive peptides are usually small functional fragments of a protein that deliver efficacy, selectivity and specificity for their targets (Hummel et al., 2006; Bidwell, 2012). Peptides have several benefits for use in drug therapy. They can be manufactured at a relatively low cost, can be kept at room temperature due to their high stability, have minimal potential for interaction with the body's immune system, and have superior penetrating ability at organ or tumour sites because of their small size. Therapeutic peptides can be utilised in place of traditional drugs. The degradation products of peptides

are AAs, which minimises the risk of systemic toxicity (Loffet, 2002). Peptides derived from milk proteins may have potential anticancer effects as anticancer agents because they can be designed to target proteins of interest. Peptides can be easily manufactured and their AAs sequences can be altered using specific techniques such as chemical synthesis or molecular biology (Hruby, 2002). Although bioactive peptides have several advantages, their usefulness for cancer treatment is finite due to deficient pharmacokinetic parameters and tumour delivery (Vlieghe et al., 2010). Bioactive peptides can rapidly loss their bioactivity in circulating blood due to peptidolytic enzymes, and the impermeability of cancer cell membranes makes the utilisation of such peptides in cancer treatment challenging (Pichereau & Allary, 2005).

2.10.1 Antidiabetic Peptides

One of the prevalent clinical symptoms of diabetes is an increase in blood glucose levels in the body as a result of reduced insulin production, insulin resistance and eventual pancreatic β-cell failure (Jao et al., 2015). Statistics show that approximately 387 million people are living with diabetes mellitus worldwide and the disorder resulted in the death of around 4.9 million individuals in 2014 (Zarkogianni et al., 2015). Two gut-derived peptides (glucose-dependent insulinotropic polypeptide [GIP] and glucagon-like peptide-1 [GLP-1]), also called incretin hormones, have been found to induce insulin secretion from islet β-cells in a glucose-dependent manner. GIP and GLP-1 are secreted upon nutrient ingestion. However, these peptides can rapidly be inactivated by aminopeptidase and dipeptidyl peptidase-IV (DPP-IV) (Darmoul et al., 1994; Holst & Deacon, 1998: Drucker, 2006). Use of dipeptidyl peptidase-IV (DPP-IV) inhibitors is one of the measures for ensuring effective control of type 2 diabetes mellitus (Shi et al., 2016). Side effects such as pancreatitis, GI reaction (nausea, vomiting, diarrhoea, etc.), flu-like symptoms and skin reactions have been reported to be associated with these inhibitors,

and limit their ability to function effectively. This has prompted scientists to find a different option. Natural DPP-IV inhibitors are potent and advantageous as they lack undesirable side effects (Safavi et al., 2013).

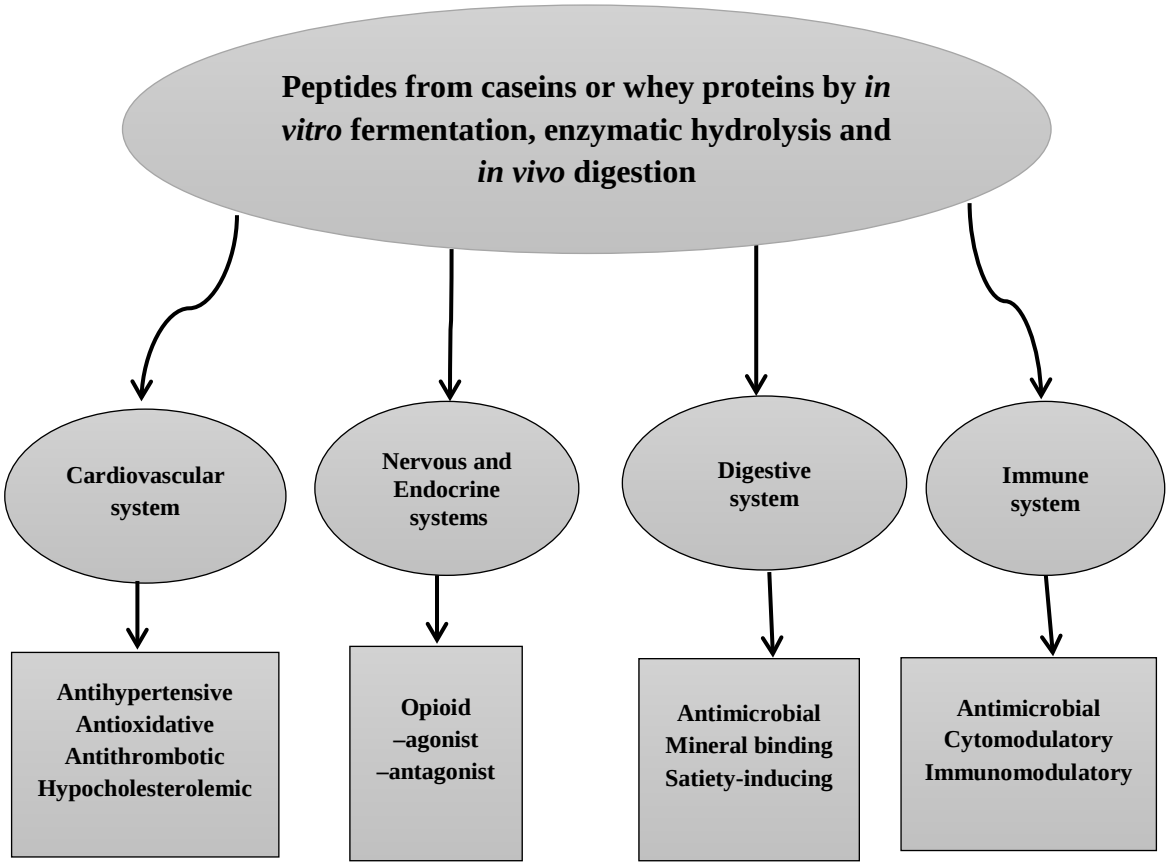

Figure 2.3 Possible pathways for the release of milk-derived bioactive peptides

Milk protein-derived peptides can serve as an alternative pharmaceutical to chemosynthetic drugs. Native AA composition and sequence largely determines the biological activity of bioactive peptides. Two peptides (Ile–Pro–Ala–Val–Phe and Ile–Pro–Ala–Val) have been isolated from whey protein concentrate (rich in β-lactoglobulin [β-Lg]) hydrolysed by pepsin and proteinase K, using a semi-preparative reverse-phase–high-performance liquid chromatography (RP–HPLC). These peptides exhibited similar

DPP-IV inhibitory activities (Tulipano et al., 2011). Further, Leu–Pro–Gln and Leu–Pro–Gln–Asn–Ile–Pro–Pro–Leu, obtained from a water-soluble extract of a Gouda-type cheese and separated by RP–HPLC, shared the common property of their derivation from ß-casein, with IC_{50} values against DPP-IV of 46 and 82 μM respectively (Uenishi et al., 2012).

2.10.2 Angiotensin-converting Enzyme-Inhibitory Peptides

ACE is a peptidyl dipeptidase enzyme that is able to cleave the C-terminal end of the substrate that may regulate the rise of blood pressure through conversion of angiotensin I to angiotensin II, an active peptide hormone and potent vaso-constrictor. This results in an increase in sodium concentration, which leads to a rise in blood pressure. Some peptides derived from milk protein are able to inhibit ACE and control hypertension (Korhonen & Pihlanto, 2007; Pihlanto et al., 2010). *L. helveticus* has proved to have high proteolytic activity among cultures in milk fermentation (Elfahri et al., 2014). The culture has been used in milk fermentation to produce VPP and IPP peptides, which are believed to have antihypertensive properties (Nakamura et al., 1995; Seppo et al., 2003). These short peptides have proline at the C-terminal end, which enables them to escape digestive enzyme degradation, cross the blood stream and exert antihypertensive activity (Yamamoto et al., 2003). Some ACE-I peptides are released from β-CN only by proteinases of *L. helveticus* (Yamamoto et al., 1993), whereas others are liberated by both proteinases and peptidases (Yamamoto et al., 1999; Yamamoto & Takano, 1999). Ueno et al. (2004) verified the production of tripeptides IPP and VPP from the *L. helveticus* endopeptidase CM4 using oligopeptides as a substrate. However, due to slight differences in proteinase specificity among *L. helveticus* strains, production of ACE-I peptides might be affected (Sadat-Mekmene et al., 2011a; Wakai et al., 2012).

2.10.3 Antimicrobial Peptides

The appearance of pathogenic bacteria resistant to chemical antimicrobial drugs has become a public health concern worldwide and has received considerable attention (Bruni et al., 2016). The search for natural compounds that may exhibit antimicrobial activity against positive and negative pathogenic bacteria is rapidly growing (Bahar & Ren, 2013). Antimicrobial peptides (AMPs) are a potential candidate for overcoming antimicrobial resistance (Bruni et al., 2016). Most reported AMPs contain 3–50 AAs (Rutherfurd-Markwick & Moughan, 2005). Milk protein-derived AMPs have been reported to reduce the growth of many pathogenic bacteria (Gram positive and Gram negative) including *Escherichia coli* MTCC82, *Salmonella typhi* MTCC3216 and *Staphylococcus aureus* MTCC 96 (Mohanty et al., 2014, 2016). Caseicidin peptide derived from casein by chymosin digestion shows antimicrobial activity against pathogenic microorganisms (*Staphylococcus* spp., *Sarcina* spp., *Bacillus subtilis*, *St. pyogenes*) (Lahov & Regelson, 1996). Further, casocidin-I, a cationic peptide released from αs2-CN, causes growth inhibition of *E. coli* and *Staphylococcus carnosus* (Zucht et al., 1995; Haque & Chand, 2008) and two peptides (f183–207 and f164–179) released from the same casein also inhibited growth of pathogens (Recio & Visser, 1999; Recio & López-Expósito, 2008). The structural properties (amphiphilic motifs and net positive charges) of AMPs are the elements essentially responsible for membranolytic activities. For example, AMPs with net positive charge expedite the interaction with negatively charged pathogenic cell surfaces. The hydrophobic properties of AMPs enable them to cross bacterial membranes (Wieprecht et al., 1997).

2.10.4 Antioxidant Peptides from Bovine Milk Proteins

Metabolic activity of body cells is essential for survival, and by-products from the process, including reactive oxygen species (ROS) and free radicals, are constantly produced (Birben et al., 2012). Moreover, the body is exposed to oxidants such as motor vehicle exhaust, tobacco smoke, oxides of nitrogen, ozone and air pollutants. Continuous interaction of body systems with these oxidants leads to an additional load of free radicals and causes increasing harm to proteins, lipids, DNA, carbohydrates and membranes, resulting in oxidative stress (Chakrabarti et al., 2014; Sah et al., 2015b, 2016c), which may lead to cancer (Sah et al., 2015b) (Figure 2.4).

Free radicals are chemical species that are composed of unpaired electrons in the exterior orbit possessed by molecules (Power et al., 2013). These unpaired reactive electrons allow the capture of electrons from other particles, resulting in free radicals that are able to destroy healthy cells. Free radicals, according to Chakrabarti et al. (2014), have the potential to attack all macromolecules, which in turn, would lead to lipid peroxidation, DNA modification and break down of cellular proteins during an oxidative event (Sah et al., 2016c). Uncontrolled formation of these free radicals has often been linked with the inception of a number of cardiovascular diseases, together with cancer and other disorders such as congestive heart failure, cardiac hypertrophy, cardiomyopathies, hypertension, ischemic heart disease and atherosclerosis (Clarkson & Thompson, 2000; Chakrabarti et al., 2014). The human body contains an endogenous antioxidant structure primarily used as a defence system that comprises a range of enzymes, particularly the superoxide dismutase and catalase, as well as other compounds with antioxidation capabilities. These antioxidants can prevent oxidation by inactivating or preventing generation of ROS (Sah et al., 2016c).

Several naturally occurring antioxidant peptides (e.g. glutathione [GSH: Glu–Cys–Gly], carnosine [β-Ala-L-His], anserine [β-Ala-3 methyl-L-His], homocarnosine [γ aminobutyryl-L-His]) with free radical scavenging activity have been identified in the human body (Marambe & Wanasundara, 2012). Figure 2.4 demonstrates the possibility for food proteins to act as biologically active peptides precursors with diverse physiological characteristics, such as antioxidant activity. Searching for potential antioxidant compounds from natural sources may provide alternative treatments to current synthetic chemicals (Sah et al., 2015a). Peptides derived from proteins originating from milk products are among those that research has linked to natural antioxidants (Sah et al., 2016c) (Table 2.11). They are involved in quenching free radicals by oxidation of AA residues. Casein proteins in bovine milk are thought to possess antioxidant peptides in their primary structure that are released upon hydrolysis. For instance, the peptide Ala–Arg–His–Pro–His–Pro–His–Leu–Ser–Phe–Met released from κ-casein upon milk fermentation with *L. delbrueckii* ssp. *bulgaricus* IFO13953 when isolated displays antioxidant activity (Kudoh et al., 2001). Further, the antioxidant activity of whey protein-derived peptides has been reported along with the antioxidant activity of whey itself (Meisel, 2005). For example, the antioxidant peptide Tyr–Tyr–Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile was released by the action of a commercial protease (corolase® PP) on β-Lg A (Hernández et al., 2005a, b). The bioactivity of antioxidative peptides is associated with AA composition (Suetsuna et al., 2000). Peptides with high amounts of histidine and some hydrophobic AAs have been reported to have more potent antioxidant activity than others (Peña-Ramos et al., 2004). For example, histidine-containing peptide is thought to be connected with hydrogen donation and lipid peroxyl radical trapping (Chan et al., 1994; Sah et al., 2016c). Further, the existence of leucine or proline with histidine–histidine dipeptides at the N-terminus may improve antioxidant activity

(Suarez-Jimenez et al., 2012). The antioxidant activities of 28 peptides were compared to that of the antioxidative peptide (Leu–Leu–Pro–His–His) by Chen et al. (1996), who found that tripeptide (Pro–His–His) exhibited the highest antioxidant activity among all the peptides assessed.

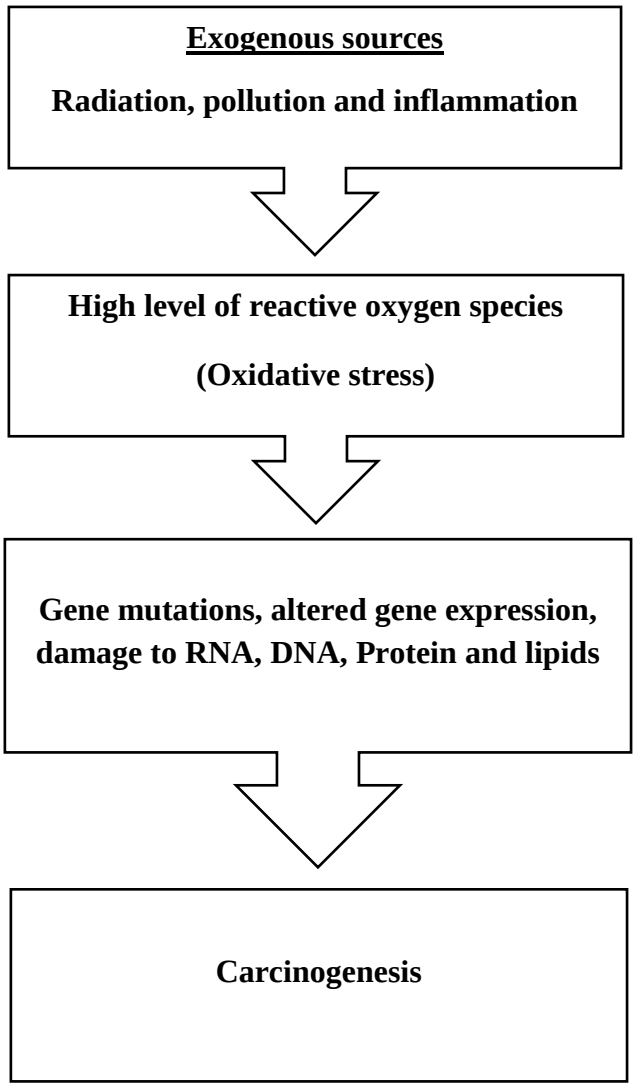

Figure 2.4 Factors that may lead to increased cancer incidence

However, deletion of the histidine C-terminal peptide has been reported to decrease antioxidant activity (Zou et al., 2016), indicating the importance of histidine in antioxidant peptides. Hydrophobicity of a peptide can also be an important determinant of its antioxidant activity and may increase accessibility to hydrophobic targets (e.g., lipophilic fatty acids) (Sah et al., 2016c).

2.10.4.1 The Structure–Activity Relationship

The correlation between antioxidant properties of peptides and their structure has not yet been described in full detail. However, some factors, such as AA composition, hydrophobicity and peptide structure, may enhance their bioactivity (Chen et al., 1998; Sah et al., 2016b). For example, antioxidant peptides that contain aromatic ring structure AAs (Trp, Phe and Tyr) can donate protons to electron-deficient radicals (Chen et al., 1998). Further, hydrophobicity has been reported to enhance antioxidant activity (Sah et al., 2016c). Hydrophobic AAs such as valine, leucine and tyrosine contained in antioxidant peptides can boost solubility in phospholipid conditions and facilitate better interaction with hydrophobic radicals (Qian et al., 2008). The hydrophobic properties of AAs at the N- and C-terminus of peptides increase antioxidant activity (Zou et al., 2016). Table 2.9 compares antioxidant peptides released from milk proteins, the majority of which have hydrophobic AAs at their N-terminus (Zou et al., 2016). Also, properties of the second AA adjacent to the C-terminal AA has been reported to enhance antioxidant activity when this AA has low hydrophobicity, strong hydrogen bonding and steric properties. This means that antioxidant activity will be increased, particularly when the second AA is basic, for example, Glu, Asp, Arg, His, Lys or hydrophilic, such as Ser or Thr (Power et al., 2013). Moreover, peptides with Cys at the N-terminal position play a key role in antioxidant activity through the R–SH group, which interacts with radicals by hydrogen donation from the SH group (Elias et al., 2008). Histidine has also been reported

to have antioxidative effects due to the imidazole group in histidine-containing peptides, which has metal chelating, hydrogen donating and lipid peroxyl trapping capabilities (Chan et al., 1994). The presence of hydrophobic residues in His-containing peptides can boost accessibility to hydrophobic radicals (Murase et al., 1993). Chymotrypsin can hydrolyse whey proteins and release antioxidant peptides through its specificity for C-terminal-cleavage bonds containing aromatic or hydrophobic residues (Pihlanto, 2006b), resulting in facilitated electron transfer from these peptides. Suetsuna et al. (2000) showed that a hydrophobic Leu-containing peptide (Tyr–Phe–Tyr–Pro–Glu–Leu) from casein hydrolysed by pepsin exhibited DPPH radical scavenging activity—Glu–Leu residues being the main factor in bioactivity.

2.10.4.2 Methods to Evaluate Antioxidant Activity

The importance of natural antioxidants with fewer side effects and lower cost than chemically synthesised drugs cannot be overemphasised. This has led to the investigation of antioxidants in dairy products by food science researchers (Sah et al., 2014; Elfahri et al., 2014, 2015). A number of detection methods has been used and developed to evaluate antioxidant capacity (Sah et al., 2015a). In terms of chemical reactions, the methods for measurement of antioxidant capacity are categorised into two groups: the first is based on hydrogen atom transfer (HAT); and the second, on electron transfer (ET) (Huang et al., 2005; Korhonen, 2009a; Sah et al., 2015a). ET-based assays measure the capacity of an antioxidant in the reduction state, and act as an indicator for monitoring the reaction endpoint (Huang et al., 2005; Dryáková et al., 2010). Ferric ion-reducing antioxidant power and DPPH radical scavenging capacity are the most common methods used. For example, ET-based assays measure the antioxidant activity of peptides during milk fermentation (Sah et al., 2015a, 2016c). However, use of these methods to investigate the *in vitro* antioxidant capacity of compounds in dairy products cannot be directly

extrapolated to their *in vivo* antioxidant capacity due to a range of factors such as bioavailability of antioxidants, reactivity, stability, concentration and storage in tissue. Thus, peptides with antioxidant properties may or may not resist proteolytic enzymes in the GIT to reach target sites in the body (Vermeirssen et al., 2002; Mills et al., 2009; Madureira et al., 2010).

2.10.5 Anticarcinogenic Peptides from Bovine Milk Proteins (Whey and Caseins Peptides)

2.10.5.1 Anticarcinogenic Peptides from Caseins

Most milk proteins belong to a group of specific acid-insoluble phosphoprotein caseins (~80% of all bovine milk proteins). These proteins were first recognised and studied in the 18th century with reports that caseins exist in milk as large particles suspended in the aqueous phase (Fox & Brodkorb, 2008). Casein proteins were usually referred to as 'calcium caseinate–calcium phosphate' particles. After 1960, the term 'casein micelle' was exclusively used (Fox & Brodkorb, 2008). Due to the significance of casein micelles in most dairy-based industries, their structure, characteristics and effects of compositional and processing factors have been extensively reported (Holt et al., 2013). The main use of casein is to supply the AAs needed for infant growth. However, the physiological functions of the casein micelle have also been reported as protecting the mammary gland from certain pathogens (Holt, 1997; Holt et al., 2013). Casein proteins, a major component of milk proteins, can be the main precursors of anticarcinogenic peptides (Table 2.12) after proteolytic enzyme attack. Hatzoglou et al. (1996) demonstrated an *in vitro* antiproliferative effect against breast cancer T47D cells in a dose-dependent manner of five casomorphins: α-casein fragments 90–95 and 90–96, β-casomorphin 7 (BCM7) fragment 60–66, β-casomorphin 5 (BCM5) fragment 60–64 and morphiceptin (β-casomorphin 4 amide).

Table 2.11 Some antioxidative peptides isolated from milk proteins

Family of proteins	Protein precursor	Peptide fragment	Isolation	Antioxidant activity	References
Caseins	β-casein	β-casein f (98–105) β-casein f (169–176) β-casein f (177–183) β-casein f (170–176)	Trypsin digestion of casein	Inhibition of enzymatic and nonenzymatic lipid peroxidation DPPH radical scavenging activity	Rival et al., 2001; Kitts & Weiler, 2003
		β-casein f (193–209) β-casein f (69–87)	Probiotic yoghurt supplemented with pineapple peel powder	2,2′-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) radical scavenging activity	Sah et al., 2016b
	αs1-casein	αs1-casein f (144–149)	Pepsin digestion of casein	DPPH radical scavenging activity	Suetsuna et al., 2000
	κ-casein	κ-casein f (96–106)	Fermentation with *Lactobacillus delbrueckii* ssp. *bulgaricus*	DPPH radical scavenging activity	Kudoh et al., 2001
Whey proteins	β-lactoglobulin	β-lactoglobulin f (19–29) β-lactoglobulin f (58–61) β-lactoglobulin f (95–101)	Corolase PP™	Oxygen radical absorbance capacity	Hernández-Ledesma et al., 2005; del Mar Contreras et al., 2011

The inhibition of cancer development and proliferation may be explained by the interaction and binding with opioid receptors and the concentration of peptides. The second explanation may be due to the AA sequence of these peptides or proteins (a high level of proline and presence of tyrosine at the N-terminus) (Teschemacher, 2003).

Other bioactive peptides are caseinphosphopeptides (CPPs) derived from casein during digestion. These peptides have been reported to have an *in vitro* antiproliferative effect on human colon carcinoma cell line HT-29 based on calcium uptake and binding with extracellular calcium (Cosentino et al., 2010). Perego et al. (2012) reported that CPPs protected differentiated intestinal cells from the toxic effect of high concentrations of extracellular calcium, frustrated apoptosis and supported proliferation. Simultaneously, they promoted apoptosis in undifferentiated tumour cells. The authors further suggested that this effect was due to binding of CPPs with extracellular calcium in a specific dose response, resulting in reduction of cell proliferation rates and apoptosis. Recently, Sah et al. (2016b) isolated and identified two peptides (^{193}YQEPVLGPVRGPFPIIV^{209} and ^{69}SLPQNIPPLTQTPVVVPPF^{87}) derived from β-CN with *in vitro* antiproliferative properties against the HT-29 colon cancer cell line by inducing apoptosis and cell cycle arrest in G2/M.

2.10.5.2 Anticarcinogenic Peptides from Whey Proteins

Some whey proteins have been reported to have an antiproliferative effect on different cancer cell lines: for example, α-lactalbumin with colon adenocarcinoma cell lines (Caco-2 or HT-29 monolayers); β-Lg in an animal model with colon cancer progression; and bovine serum albumin (BSA) with the MCF-7 human breast cancer cell line (Pepe et al., 2013). Bovine lactoferricin (B-Lfcin) is a peptide released from bovine lactoferrin (B-Lf) by acid pepsin enzyme that has been shown to have an *in vitro* cytotoxic effect on many types of cancer cell lines (including leukaemia, fibrosarcoma, various carcinoma and neuroblastoma cells) (Bellamy et al., 1992; Eliassen et al., 2006). B-Lfcin must be used at a concentration that does not affect the viability of healthy cells (Mader et al., 2005; Furlong et al., 2006; Furlong et al., 2010). The cytotoxic effect results from a strong cationic property that permits B-Lfcin to target cancer cells, which are negatively

charged; healthy cells are not affected due to their net neutral charge (Burdick et al., 1997). Although there is a net negative charge associated with the outer membrane leaflet of many cancer cells, certain cancer cells may not be affected by the cytotoxic effect of B-Lfcin due to inadequate net negative charge that promotes a strong electrostatic interaction with cationic B-Lfcin (Eliassen et al., 2003; Gifford et al., 2005).

B-Lfcin kills human cancer cells such as T-cell leukaemia and breast cancer, by inducing apoptosis via caspase-3 (a member of the cysteine–aspartic acid protease family) activation (mitochondrial pathway). Yoo et al. (1997) found that using B-Lfcin at 100 μg/mL and an incubation time of up to 10 h induced cell death in THP-1 cells (a human monocytic cell line derived from an acute monocytic leukaemia patient) via the apoptotic pathway. However, there was no cell death induction with the treatment of lactoferrin protein even at high concentrations.

2.11 Apoptosis Induction in Cancer Cells

Apoptosis, according to Nikoletopoulou et al. (2013), is a systematic procedure of cell death that occurs in multicellular organisms. The process is also known as the programmed death of cells, referring to the controlled nature of the process (Chang & Yang, 2000; McIlwain et al., 2015). The word apoptosis originates from a Greek word meaning ‘dropping off’ that has been used since the 1970s in biology to describe the situation in which a cell receives certain stimuli following which it actively pursues a course towards death (Lawen, 2003). This is a systemic programme controlling the cellular balance in body systems. Apoptosis and the process known as necrosis are fundamental forms of cell death. Nikoletopoulou et al. (2013) defined necrosis as an unintentional process that leads to premature disruption of cellular membranes as a result of external perturbation such as hypoxia or mechanical trauma.

Table 2.12 Selected anticarcinogenic peptides from bovine milk proteins

Family of proteins	Protein precursor	Peptide fragment	References
Caseins	β-casein	β-casomorphins 5 f (60–64)	Pepe et al., 2013
		β-casomorphin 7 f (60–66)	Pepe et al., 2013
		Morphiceptin f (60–63)-NH2	Pepe et al., 2013
		β-casomorphin-7, also known as β-casein f (1–25)4P	Hata et al., 1998 Sah et al, 2016b
		β-casein f (193–209)	
		β-casein f (69–87)	
	α_{s1}-casein	α_{s1}-casein f (90–95)	Pepe et al., 2013
		α_{s1}-casein f (90–96)	Pepe et al., 2013
		α_{s1}-casomorphin f (158–162)	Pepe et al., 2013
		αs1-casein f (1–3)	Otani & Suzuki, 2003
		αs1-casein f (101–103)	Otani & Suzuki, 2003
		αs1-casein f (104–105)	Otani & Suzuki, 2003
	κ-casein	κ-casein f (17–21), also known as κ-casecidin	Matin & Otani, 2002
Whey proteins	Lactoferrin	lactoferricin (LfcinB)	Pepe et al., 2013
		β-lactoferrin f (17–38)	Roy et al., 2002
		β-lactoferrin f (1–16)	Roy et al., 2002
		β-lactoferrin f (45–48)	Roy et al., 2002

Although apoptosis is a physiological process that necessitates the activation of energy-requiring intracellular machinery, it has a critical role in development and defence in multicellular organisms. Apoptosis has an effect on single cells asynchronously and usually occurs in the absence of inflammation (Nikoletopoulou et al., 2013). It also has a significant role in the morphogenesis of embryonic tissues and the delicate balance of organ and tissue cell number in adults. Further, apoptosis takes place in cells exposed to danger such as viral infection or cells with damaged DNA (McIlwain et al., 2015). The

main physiognomies of apoptotic cells are fragmentation, nuclear chromatin condensation, membrane blebbing and cellular shrinkage. The final step in the apoptotic process is forming apoptotic bodies. During this stage, the cell breaks into membrane-bound fragments and is engulfed by macrophages and dendritic cells (Nikoletopoulou et al., 2013).

In the case of cancer, the complication is an imbalance between cell division and death. Due to the absence of a cell death signal, cancer cells do not have the ability to die and thus continue to grow (McIlwain et al., 2015). For example, the down-regulation of the tumour suppressor gene *p53* leads to a reduction in apoptosis and thus cancer cells can continuously grow and progress (Nikoletopoulou et al., 2013). It is important to understand that the concentrations and the degree of apoptotic stimulants can change the type of cell death. Using small doses of anticancer medications can lead to the induction of apoptosis, but a high dose of the same stimulus can result in necrosis. Apoptosis, on the other hand, can be a double-edged sword and may be part of both the problem and the treatment (Zhang & Yu, 2013; Kim et al., 2015). Also, many biological studies are currently examining antiproliferative agents from natural sources as potential anticancer drugs targeting apoptosis with no/low side effects on normal cells (Kannan et al., 2010; Perego et al., 2012; Sah et al., 2016a, b).

2.12 The Mechanisms of Apoptosis and Caspase Activation

Understanding the mechanisms of apoptosis is crucial for the development of anticancer drugs that target one of the many apoptotic inducers or its respective pathway. The induction and inhibition of apoptosis are multiple biochemical reactions including receptors, enzymes and signalling molecules such as in the caspase–cascade system (Fabian et al., 2005). Two main pathways of apoptotic mechanisms (Figure 2.5) have

been reported: the extrinsic, occurring in the death receptor path and the intrinsic that transpires within the mitochondrial pathway (Andón & Fadeel, 2012). An additional pathway has also been reported called the perforin/granzyme pathway (Elmore, 2007; Iwasaki et al., 2009). All of these pathways terminate with the same execution pathway steps that include caspase-3's cleavage, leading to the fragmentation of DNA (Wong, 2011; Bell & Megeney, 2017), degradation of nuclear proteins and cytoskeleton, the formation of apoptotic figures and finally the engulfment of these apoptotic bodies by phagocytic cells.

Caspases are a family of interleukin-1β-converting enzyme proteases (Fan et al., 2005; Li & Yuan, 2008). Fourteen intracellular caspases have been identified that share some characteristics: all are aspartate-specific cysteine proteases, with seven of them having apoptosis-related activity (Fan et al., 2005; Wang et al., 2005). Caspases exist intracellularly as inactive 'proenzymes' (procaspase) that can be stimulated by other caspases at their aspartic acid residues (Thornberry & Lazebnik, 1998). Apoptosis passes through many stages, involving some caspases (long prodomain) that are used as initiator caspases (upstream signal transducers) and others called downstream caspases that are used as proteolytically active effector caspases (short prodomain) (Jin & El-Deiry, 2005). The initiator caspases, that is caspase-10 and -8, possess a death effector domain within their prodomain, which is important in binding with adaptor proteins. Caspase-2 and -9 contain a caspase activation and recruitment domain that is involved in the activation of effector caspases, relevant for binding to adaptor molecules (Pop & Salvesen, 2009). Effector caspases react with cellular proteins by cleaving, leading to death by apoptosis.

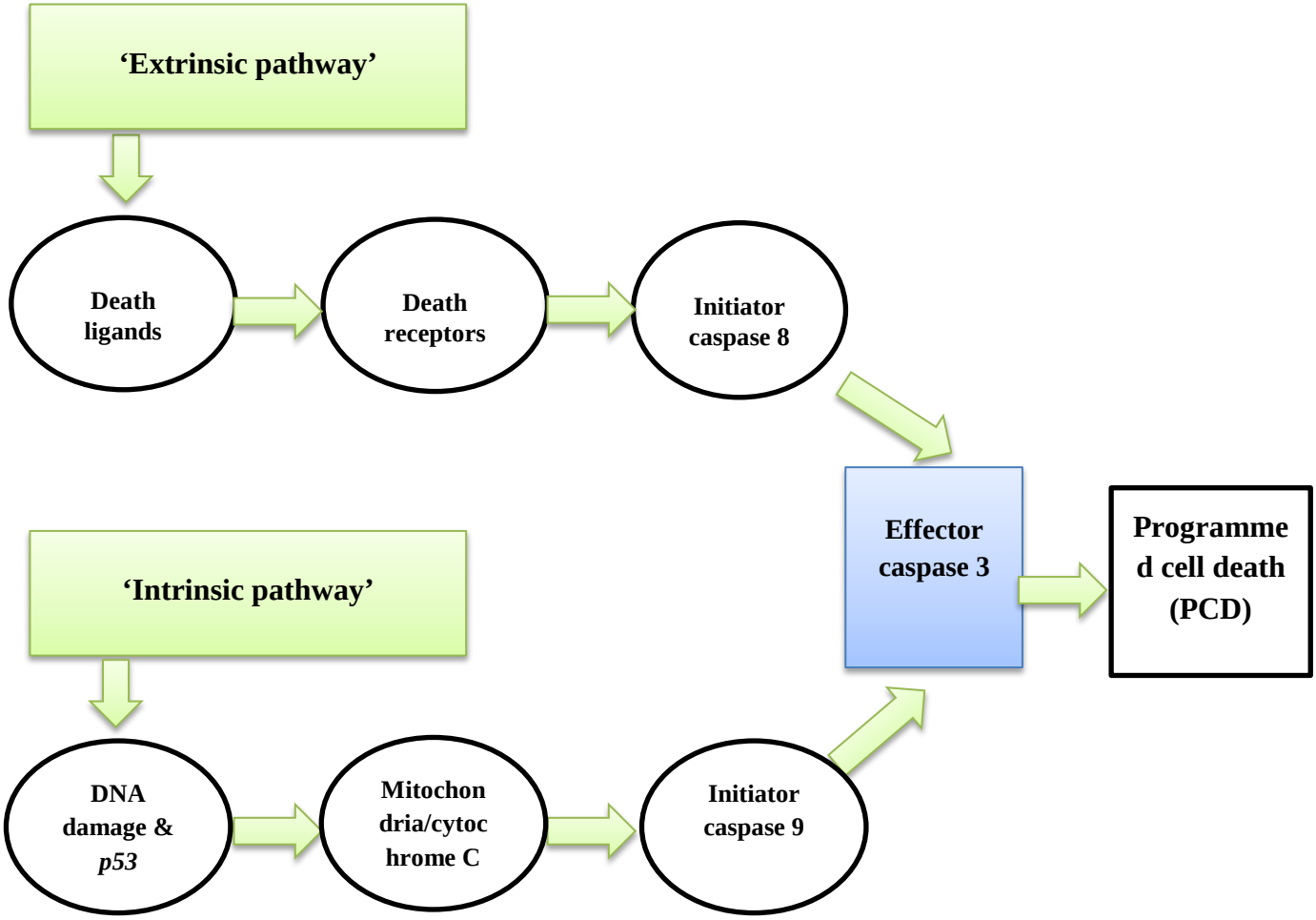

Figure 2.5 Intrinsic and extrinsic pathways of apoptosis

2.13 Apoptosis Induction by Peptides and Potential Pathways of Anti-Colon Cancer Peptides

Many efforts have been made within the last decade to explore natural compounds able directly or indirectly to activate proapoptotic caspases such as caspase-3 for use in cancer therapy. These compounds should cause little or no harm to normal cells (Kasibhatla & Tseng, 2003; Sah et al., 2015b). A range of possible pathways has been proposed to prevent or cure cancers (including colon cancer) by utilising peptides (Thundimadathil, 2012). These include drug carriers (tumour-targeting agents that carry cytotoxic drugs), hormones, radionuclides (targeted chemotherapy and radiation therapy), drugs (e.g., as

angiogenesis inhibitors) and vaccines (Figure 2.6). The antiproliferative effect of peptides on colon cancer is most likely attributed to mechanisms including inhibition of some pathways such as angiogenesis, proteins, enzymes, protein–protein interactions and gene expression (Rosca et al., 2011; Karagiannis & Popel, 2008; Thundimadathil, 2012). Another mechanism is that peptides may act as antagonists, which can attach to a specific receptor (Cornelio et al., 2007; Sotomayor et al., 2010; Sah et al., 2015b). Peptides may also act as proapoptotic peptides causing induction of apoptosis in cancer cells (Smolarczyk et al., 2005; Sah et al., 2016b).

Based on the target site, therapeutic peptides have been categorised into three pathway groups: signal transduction, cell cycle regulation and cell death (Raucher et al., 2009). The first group of peptides is designed to inhibit mitogen-activated protein kinases (MAPKs) that have a significant function in cellular signal transduction cascades and prompt intracellular interactions in response to any cellular changes caused by external growth factors, hormones, nutrient status or stress (Hughes et al., 2016). These alterations or signals affect the nucleus, influencing gene expression. Changes in MAPKs have been observed in some cancers including colon cancer (Hoshino et al., 1999). Induction or inhibition of apoptosis is controlled by B-cell lymphoma-2 (BCL-2) family protein members (Table 2.13). For example, cell survival is promoted by anti-apoptotic proteins such as BCL-2 and BCL-X large (BCL-XL) (Burlacu, 2003; Dai et al., 2016), whereas inhibition of anti-apoptotic proteins and existing proapoptotic proteins such as BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK) promotes cell suicide (Dai et al., 2016). In the case of colon cancer treated with peptides, apoptotic signals initiated by B-cell lymphoma-2 homology (BH3), a proapoptotic member of the BCL-2 protein family, which activates BAX and BAK in a direct way by binding to them or to

anti-apoptotic proteins, or uses an indirect pathway to activate these proteins (Dai et al., 2016).

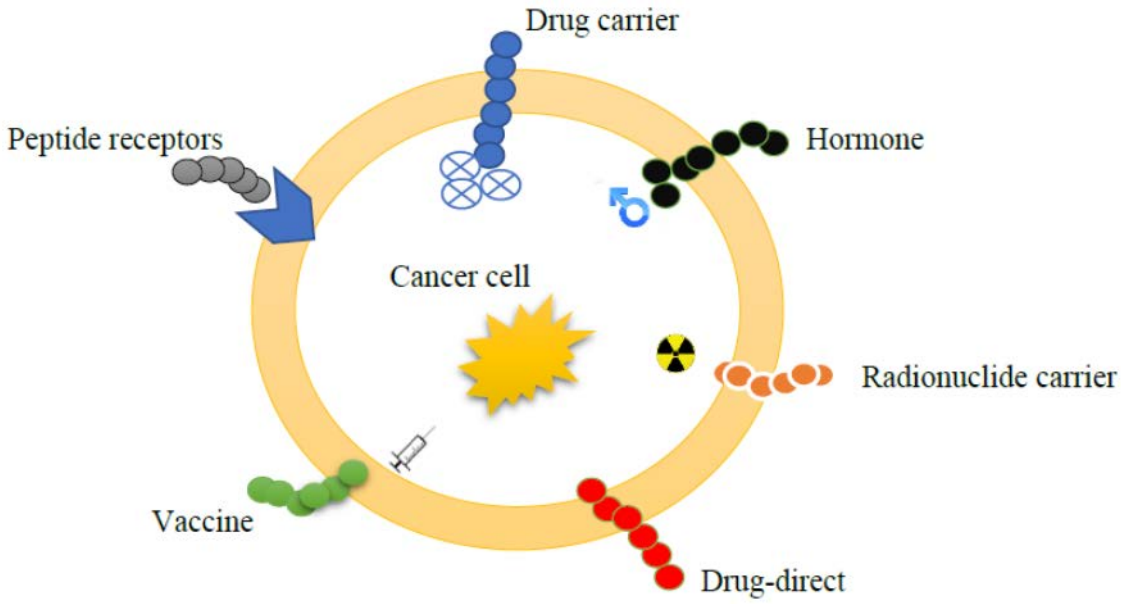

Figure 2.6 Potential treatment options for anticarcinogenic peptides

The activated BAX and BAK result in the forming of pores in the outer mitochondrial membrane and release cytochrome c into the cytosol. The activated cytochrome c binds to apoptotic protease-activating factor and procaspase-9 to form an apoptosome (Youle & Strasser, 2008; Marqus et al., 2017). Once the apoptosome is formed, it leads to caspase-9 (initiator caspase) activation, which in turn triggers caspase-7 and caspase-3 (apoptotic caspase) and promotes apoptosis (Riedl & Salvesen, 2007; McIlwain et al., 2015). Therefore, uncontrollable cell growth is due to overexpression of anti-apoptotic proteins leading to deregulation of apoptosis as a result of the disturbance in the balance of anti-apoptotic and proapoptotic proteins, as is observed in many types of cancer (Pistritto et al., 2016).

Table 2.13 B-cell lymphoma-2 (BCL-2) family proteins and their regulation in cancer. Data adopted from Dai et al. (2016)

BCL-2 family member	Abbreviation	Category
BCL-2 antagonist/killer	BAK	Apoptotic factor
BCL-2 ‑associated X protein	BAX	Apoptotic factor
BCL-2 -related ovarian killer	BOK	Apoptotic factor
BCL-2	BCL-2	Anti-apoptotic factor
BCL‑X large	BCLXL	Anti-apoptotic factor
Myeloid cell leukaemia 1	MCL1	Anti-apoptotic factor
BCL-2-like protein 2	BCLW	Anti-apoptotic factor
BCL-2-related protein A1	BFL1	Anti-apoptotic factor
BCL-2-like protein 10	BCLB	Anti-apoptotic factor

2.14 Isolation, Purification and Characterisation of Peptides

The separation of bioactive peptides such as antioxidant and anticarcinogenic peptides from crude extracts has been carried out using a number of techniques including ultrafiltration, acid and isoelectric precipitation and chromatography (Sah et al., 2016c). It is a difficult process due to the presence of these compounds as a complex blend of acids, sugars, salts and free AAs (Donkor et al., 2007; Sah et al., 2016c). A schematic representation of milk fermentation and protein hydrolysis, followed by separation, purification and characterisation of dairy-derived bioactive peptides is presented in Figure 2.7. Using HPLC with suitable C_{18} peptide columns and appropriate running conditions, bioactive peptides with potential physiological properties have been isolated (Donkor et al., 2007; Sah et al., 2016c). For instance, injecting soluble peptide extracts (SPEs) into a RP–HPLC fitted with a peptide column (C_{18} column) can reveal the hydrophilicity of peptides; however, some peptides could have almost identical retention times (Sah et al., 2016c). In addition, HPLC cannot provide sufficient information about the structure of an isolated peptide, such as its AA sequence. Mass spectrometry (MS) is

the most common system used to characterise bioactive peptides. Further, a protein sequencer and AA analyser can be run to establish the AA composition and sequence of a peptide. Liu et al. (2015) used an AA analyser, LC–MS/MS and mid-infrared spectroscopy techniques for purification and identification of antioxidant peptides from white egg protein. LC–MS/MS has also been used to characterise antioxidant peptides derived from yoghurt (Farvin et al., 2010).

2.15 Bioavailability of Bioactive Peptides

Human GI digestion of proteins involves a complex group of mechanical, physiochemical and physiological processes. Many factors affect the secretion and digestion of proteins, including fast or feed state, individual variation, pH and proteolytic enzyme secretions (Ulleberg et al., 2011; Delgado et al., 2015). In the case of protein and peptide digestion, although gastric juice consists of many materials, the most significant components for the digestion of proteins are hydrochloric acid (HCl) and protease pepsin (Sah et al., 2016c). HCl is released by parietal cells located in the gastric mucosa. It is usually secreted in three stages: the cephalic stage, in response to smell; the gastric stage when food is present in the stomach; and the final stage, called the intestinal stage, where gastric digestion moves to the duodenum (Guyton & Hall, 2006; Isackson & Ashley, 2014). The low pH of ~2 in the stomach assists with protein digestion in two ways: it denatures the tertiary structure of consumed proteins, which makes them more enzymatically accessible and digestible (Renukuntla et al., 2013); and provides the acidic environment necessary for proteolytic enzyme (pepsin) activation. Pepsin is produced as *pepsinogen* (zymogenic form) from gastric mucosa in an inactive form (Renukuntla et al., 2013). When pepsinogen diffuses into the stomach, self-digestion is enabled by the low pH, eliminating inhibitory fragments and thus converting pepsinogen into the active pepsin form. The function of pepsin is to hydrolyse proteins by breaking peptide bonds between

hydrophobic side chains of AAs in the middle of polypeptides, releasing shorter polypeptides.

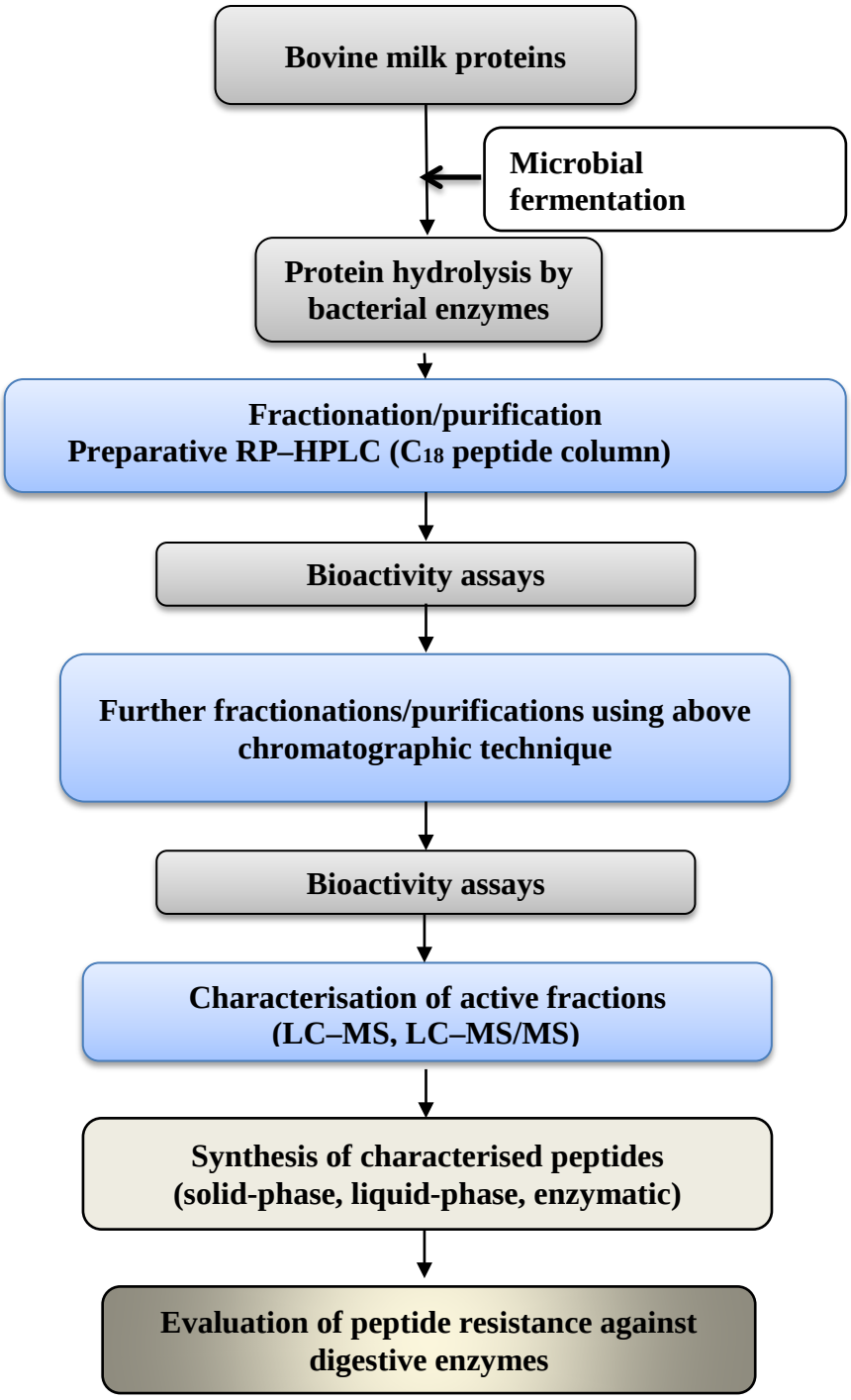

Figure 2.7 Schematic representation of the generation, isolation, purification and characterisation of fermented milk-derived bioactive peptides

Although this proteolytic activity occurs in the stomach, most protein hydrolysis occurs in the small intestine, where other proteolytic enzymes are secreted from the intestinal mucosa and pancreas. Pancreatic juice has a number of different proteases, mostly secreted in inactive forms that are stimulated by enzymes in brush border cells. Once activated, proteases begin digesting polypeptides into short peptides and AAs, which are taken up by the intestinal epithelium (Renukuntla et al., 2013; Abumrad et al., 2016). Activation of pepsin in the stomach and of pancreatic zymogens by brush border enzymes in the small intestine phosphorylate the protease zymogen trypsinogen, changing it to trypsin, which is its active form (Abumrad et al., 2016). After digestion of proteins by proteolytic enzymes *in vitro* or in the stomach, some small peptides and oligopeptides are formed that may be subjected to further hydrolysis by membrane peptidases (Molhoek et al., 2011; Renukuntla et al., 2013).

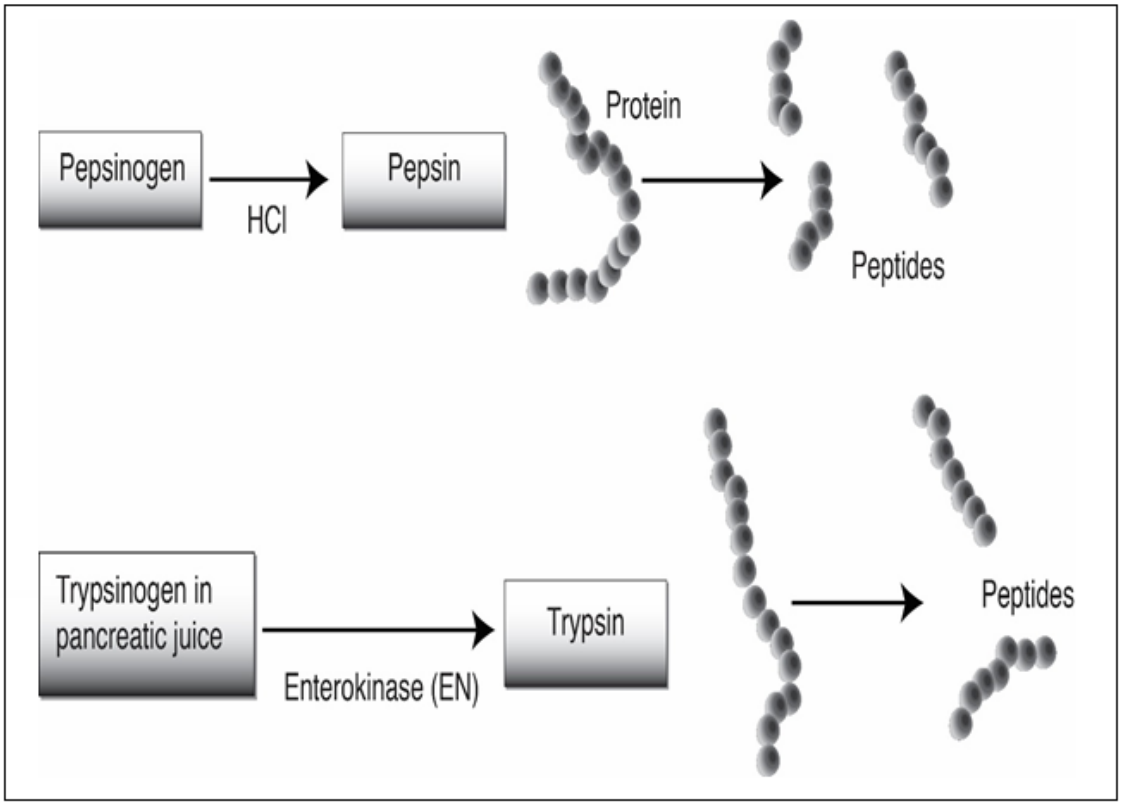

Figure 2.8 Activation of pepsin and trypsin and their hydrolysis of proteins in the gastrointestinal tract

GI enzymes may cleave some bioactive peptides formed *in vitro* and thus alter their physiological effects. To activate peptides, they should be resistant against proteolytic enzymes and move to their target sites in the body (Meisel & Schlimme, 1996; Roufik et al., 2006; Seo et al., 2012). Although it has been reported that some small peptides and oligopeptides can pass into blood circulation to reach physiologically significant levels, some *in vitro* bioactive peptides cannot cross into the blood stream due to the loss of bioactivity *in vivo* as a result of GIT proteolytic enzymes (Ohsawa et al., 2008). Vermeirssen et al. (2002) found that a peptide fragment (Ala–Leu–Pro–Met–His–Ile–Arg) derived from β-Lg is an ACE-I peptide able to cross Caco-2 cell monolayers. Other studies revealed that this peptide was hydrolysed and was not resistant to GIT and serum proteolytic enzymes, such that it lost its bioactivity (Walsh et al., 2004; Roufik et al., 2006). Further, a study found that a bioactive peptide fragment (142–148) derived from whey β-Lg protein was not affected by pepsin activity, but was almost completely broken down by chymotrypsin (Roufik et al., 2006). Thus, some *in vitro* bioactive peptides extracted from fermented milk proteins should be able to avoid the action of digestive enzymes (*in vivo* and *in vitro*) and reach a target site at the desired concentration (Sørensen & Petersen, 1993).

Low concentrations of bioactive peptides can be up taken by the intestinal barrier and convey physiological effects at cellular levels based on the peptide transporter route. The ability of bioactive peptides to cross the intestinal layer and reach the lymphatic system is affected by many factors including permeability, lipid solubility, escaping hepatic metabolism and hydrophobicity, all of which can affect the main transport systems for peptides (Deak & Csaky, 1984). The molecular weight and AA sequence can affect the absorption of peptides. Some bioactive peptides with 2–6 AAs (Grimble, 1994), and even 2–3 or up to 51 AAs are capable of crossing the intestinal layer (Roberts et al., 1999).

The presence of proline in peptides can make them more resistant to digestive enzymes and tripeptides; Pro–Pro at the C-terminus has been reported to be resistant to proline-specific peptidases (FitzGerald & Meisel, 2000). It can be deduced that uptake of *in vitro* bioactive peptides after oral ingestion might exert little or no activity *in vivo* (Erdmann et al., 2008). The increase of this bioactivity may be due to further breakdown of these peptides by GIT enzymes (Sah et al., 2016b).

Chapter 3: Anti-Colon Cancer and Antioxidant Activities of Bovine Skim Milk Fermented by Selected *Lactobacillus helveticus* Strains

3.1 Introduction

Environmental and dietary changes have been identified as the main causes of noninfectious diseases globally, including aging-related diseases and cancers (Itano et al., 2012; Johannesdottir et al., 2012; Mogili et al., 2012; Sah et al., 2015b). More than 100 types of cancers have been identified that are a common cause of death (Hoskin & Ramamoorthy, 2008). The rate of cancer mortality diagnosed worldwide in 2008 increased by 7 million from 12.7 million in the same year (Jemal et al., 2011). Although some anticancer treatments, such as surgery, chemotherapy and radiotherapy, have been used with varying success in many types of cancer patients (Perez-Tomas, 2006), these treatments are expensive (O'Leary et al., 2004) and have deleterious side effects (Chau & Cunningham, 2002) including cardiotoxicities (Curigliano et al., 2010), diarrhoea, narrowing of the bowel and inability of nutrients to be absorbed efficiently (Chau & Cunningham, 2002). The most common side effects include damage to healthy cells and induction of drug resistance in cancer cells (Kakde et al., 2011). These complications have led to increased interest in approaches using anticancer compounds from natural foods as a treatment alternative to chemotherapy drugs, which can reduce the cost and side effects.

Recently, milk proteins and peptides have drawn attention as potential anticarcinogenic drugs through a therapeutic or prophylactic effect of carcinogenesis (Sah et al., 2015a). These compounds may have an inhibitory effect on cancer cells, mainly by disrupting cell membrane by pore formation or micellisation and caspase-related apoptosis

activation (Sah et al., 2015), without a major cytotoxic effect on healthy cells. Bioactive peptides are inactive in milk proteins and are only released by certain proteolytic enzymes such as digestive enzyme or proteases extracted from plants or microbial sources, including proteolytic enzymes of LAB, which cleave milk proteins during fermentation (Korhonen & Pihlanto, 2006). Peptides derived from bovine milk proteins are associated with significant biological activity, such as anticancer and antioxidant activity (Sah et al., 2015).

Under oxidative stress, ROS such as superoxide ($^{\bullet}O2^{-}$, $^{\bullet}OOH$), hydroxyl ($^{\bullet}OH$) and peroxyl ($ROO^{\bullet}$) radicals are formed (Benbrook, 2005). In the presence of insufficient amounts of antioxidants in the human body, free radicals can lead to degenerative or pathological processes such as aging and cancer through cellular damage, including oxidation of cellular membrane lipids, proteins, enzymes and DNA (Benbrook, 2005; Sah et al., 2015). Peptides derived from milk proteins are able to scavenge free radicals *in vitro*, which may mean that they can enhance endogenous antioxidants in the human body to prevent tissue damage through neutralising free radicals. Increasing evidence has shown that milk protein-derived cytomodulatory peptides may act as specific signals that can trigger apoptosis of cancer cells while not affecting normal cells (Phelan et al., 2009). The antiproliferative effect of casein-derived peptides on cancer cell lines suggests that they might have a role in the prevention of cancer by blocking hyper-proliferation of the epithelium and promoting apoptosis (MacDonald et al., 1994; Ganjam et al., 1997). For instance, a peptide derived from B-Lf (fragment 17–38) exhibits an antiproliferative effect against human leukaemia cell line HL-60 via apoptosis induction (Roy et al., 2002). Further, B-Lfcin, a peptide derived from lactoferrin by pepsin digestion, has an antiproliferative effect on human neuroblastoma cell lines (Kelly, SK-N-DZ, IMR-32) through the induction of cleavage of caspase-6, -7 and -9 followed by cell death (Eliassen

et al., 2006). Therefore, milk fermented by LAB may contain peptides with antioxidant and antiproliferative properties.

The proteolytic enzymes of LAB and their specificity on milk proteins play a crucial role in bioactive peptide production (Korhonen, 2009a). This mechanism varies among fermented milk microorganisms, which hydrolyse milk proteins at different levels and release different bioactive peptides depending on strain selection (Donkor et al., 2007; Gupta et al., 2009). Many strains of *L. helveticus* have been extensively used in dairy studies due to their rapid growth in milk, ability to grow under acid stress and high proteolytic activity accompanied by release of bioactive peptides from milk proteins (Elfahri et al., 2014). Selected *L. helveticus* strains are capable of releasing a variety of bioactive peptides with potentially different physiological functions including inhibition of ACE and immunomodulation (Elfahri et al., 2014). However, the release of antioxidant and anti-colon cancer components by these strains during growth in milk has not been fully detailed. Therefore, understanding the role of different strains of *L. helveticus* in relation to the kinetics of potential antioxidant and anti-colon cancer peptides released from milk proteins, and their stability during milk fermentation, is imperative for appropriate strain selection. In the present study, four highly proteolytic strains of *L. helveticus* (ASCC 953, ASCC 474, ASCC 1188 and ASCC 1315) were studied for their ability to release antioxidative and anti-colon cancer compounds from skim milk during fermentation for up to 24 h at 37°C.

3.2 Materials and Methods

3.2.1 Culture Propagation

L. helveticus ASCC953, *L. helveticus* ASCC474, *L. helveticus* ASCC1188 and *L. helveticus* ASCC1315 were obtained from the Australian Starter Culture Collection

(Dairy Innovation Australia, Werribee, Australia). The strains were stored at −80°C. Each strain was propagated as reported previously (Elfahri et al., 2014). Briefly, 10-mL aliquots of aseptic MRS broth (Merck KGaA, Darmstadt, Germany) were prepared and inoculated separately with 100 μL of each strain, followed by incubation at 37°C for 24 h. After two consecutive transmissions of 24-h incubations, the preinocula cultures were prepared by transferring 1% (vol/vol) of activated culture (10^8–10^9 cfu/mL) to 10-mL aliquots of sterile reconstituted skim milk (RSM) containing 12% (wt/wt) total milk solids (Fonterra, Auckland, New Zealand) to reach the initial concentration of 106–107 cfu/mL in medium. The last step was to produce food-grade strains in RSM before transferring the culture into milk.

3.2.2 Fermented Milk Sample Preparation

One L of RSM (12% TS) was prepared by adding an appropriate amount of skim milk powder (Fonterra) to Milli-Q water, followed by stirring for full dispersion and heat treatment at 85°C for 30 min. The heat-treated milk was then cooled to approximately 40°C. The sterile RSM was aseptically inoculated with 1% (vol/vol) of each activated strain (approximately 10^7 cells /mL) and incubated at 37°C for 24 h for optimal growth. Samples were taken for analysis at 0-, 4-, 8-, 12- and 24-h intervals during incubation. The change in pH during incubation was measured with a pH meter (model 8417; Hanna Instruments, Singapore, Singapore) at the given time intervals. The cell population of each *L. helveticus* strain was assessed using sterile bacteriological peptone (vol/vol) and the pour plate technique (Elfahri et al., 2014). Assessment of samples were performed in triplicate and 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 per millilitre of the samples were calculated.

3.2.3 Determination of Proteolytic Activity

The extent of proteolytic activity in milk at 0, 4, 8, 12 and 24 h of fermentation was measured by assessing the release of free amino groups using the *o*-phthaldialdehyde (OPA) method (Church et al., 1983) and following the procedure reported previously (Elfahri et al., 2014). Briefly, 10 mL of 1% (wt/vol) trichloracetic acid (TCA) was added to 10 mL of each sample to precipitate large proteins. The mixture was centrifuged (Sorvall RT7 centrifuge, DuPont, Newtown, CT) at 4,000 × *g* for 30 min at 4°C and the supernatant was vacuum-filtered using a 0.45-μm filter tip (Schleicher & Schuell GmbH, Dassel, Germany). The collected suspension (150 μL) was then mixed with 3 mL of OPA reagent and left at room temperature (~20°C) for 2 min. The absorbance of each mixture was measured at 340 nm with a NovaSpec-II Spectrophotometer (Pharmacia, Cambridge, UK) and proteolytic activity was expressed as the absorbance of OPA derivatives at 340 nm. The relative degree of proteolytic activity of all samples was compared with that of control milk at the start of fermentation.

3.2.4 Preparation of Soluble Extracts

Each sample of fermented and untreated RSM (200 mL) had its pH adjusted to 4.6 by addition of 2 M HCl. The samples were then centrifuged (J2-HS rotor, Beckman Instruments Inc., Palo Alto, CA) at 14,000 × *g* at 4°C for 30 min. The supernatant was filtered separately through a 0.22-μm membrane filter (Schleicher & Schuell GmbH) to avoid microbial contamination. The filtrate was then freeze-dried (Dynavac freeze drier; Dynavac Eng. Pty. Ltd., Melbourne, Australia) and stored at –80°C for further analysis.

3.2.5 Determination of Radical Scavenging Activity

The DPPH radical was used to determine the free radical scavenging ability of extracted samples (Elfahri et al., 2014). Briefly, 800 μL of 0.1 mM DPPH dissolved in 95% methanol were added to 0.2 mL of each soluble extract in glass test tubes. The solutions were shaken vigorously, sealed with parafilm and incubated in the dark at room temperature for 30 min. Methanolic DPPH was used as a blank and methanol was used for the baseline correction. After incubation, the reduction of absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Biochrom Ltd., Cambridge, UK). All experiments were carried out in triplicate. The radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Radical scavenging activity (%) = [1 − (absorbance of sample/absorbance of blank)] × 100.

3.2.6 Cell Thawing and Maintenance

Human colonic epithelial carcinoma cell line HT-29 (colorectal adenocarcinoma) was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and human primary colon cell line T4056 was obtained from Applied Biological Material (ABM Inc., Richmond, Canada). To propagate and activate each culture, 1 mL of HT-29 or T4056 (10^6/mL concentration) was quickly thawed and rapidly diluted in 20-mL aliquots of RPMI-1640 growth medium containing 10% foetal bovine serum (FBS) (Invitrogen, Waltham, MA), and the cell cultures were pelleted by centrifugation at 200 × *g* for 5 min at 20°C. The pelleted cells were resuspended in 20 mL of complete growth medium supplemented with 1% of penicillin–streptomycin and incubated in 75-cm^2 cell culture flasks for 1 week. The medium was replaced with fresh growth medium every 2 d. Following this step, the aggregated colon cancer and primary colon cells were

dissociated by washing with phosphate-buffered saline (PBS) and then replacement of the medium with 5 mL of trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C for 5 min. The trypsin solution was inactivated by adding 15 mL of complete growth medium. The trypsinised cell suspension was removed by centrifugation (200 × *g* for 5 min at 20°C) and cells were resuspended in complete medium in a 50-mL falcon tube. The cells were stained using trypan blue at a 1:1 ratio for 3 min before counting via a haemocytometer (Sigma-Aldrich, St Louis, MO). Cells were counted on the four outer quadrants of the haemocytometer using a light microscope (Olympus CH-2, model CHT, Olympus Optical Co. Ltd., Tokyo, Japan) at 400× magnification. The diluted cells were divided into two portions: one was for passaging into a new flask for continuing propagation (1.0×10^6/mL) and the other (1.0×10^3/100 μL) was used in the antiproliferative assay.

3.2.7 MTS Proliferation Assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI) was performed as described in the manufacturer's instructions. Briefly, cells (HT-29 or T4056) were initially passaged and counted in a haemocytometer for the purpose of calculating the appropriate seeding. Cells were plated at a concentration of 1.0×10^3 cells per well and the inhibition of cell proliferation was measured using the MTS assay. Briefly, 20 μL of soluble extract sample were added to the cells (1.0×10^3 in 80 μL of complete medium). After 72 h of exposure of the sample to the cells, MTS reagent (20 μL/100 μL of medium) was added and incubated for an additional 4 h under the same conditions. The absorbance of formazan was measured at 495 nm and unstimulated cells were used as a control. All assays were performed in triplicate and the results expressed as mean values ± standard error. The

percentage of cell inhibition of colon cancer and normal colon cells was calculated (Kim et al., 2000) from a ratio of treatment values compared with controls as follows:

$$\text{Proliferative inhibition (\%)} = (\text{Treatment A495}/\text{Control A495}) \times 100$$

where A495 = absorbance at 495 nm.

3.3 Statistical Analysis

All the experiments were carried out in triplicate for each bacterial culture. Results obtained were analysed as a blocked split plot in time design with two main factors: strains and replications as the main plot and time as a subplot. The replications served as a block. Statistical evaluation of the data was performed using the General Linear Model of Statistical Analysis System software (SAS) (v. 6.11; SAS Institute Inc., Cary, NC). 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 $P < 0.05$.

3.4 Results and Discussion

The four *L. helveticus* strains (953, 474, 1188 and 1315) selected for our study have been shown to be highly proteolytic, mainly due to cell wall proteases (Virtanen et al., 2007; Nielsen et al., 2009; Elfahri et al., 2014), and to have metabolic activities leading to the release of several different peptides. The culture performance was assessed by the production of organic acids as the primary metabolites, measured by decline in pH (Table 3.1). The growth of the selected strains was evaluated by determining viable cell counts (Figure 3.1), which increased significantly after 12 h of fermentation. The bacterial population levelled after 12 h, except for *L. helveticus* 953, which showed slightly

decreased counts towards the end of fermentation. Although the growth of *L. helveticus* 1315 was consistent during the first 4 h of fermentation, it grew to significantly higher cell densities than *L. helveticus* 953, was similar to other strains at the end of fermentation, and was able to continue proliferating under highly acidic conditions, which indicates strain dependence and acid resistance (Elfahri et al., 2014). For appropriate growth performance in milk, a starter culture requires highly developed proteolytic and glycolytic systems capable of providing essential compounds for the culture growth (AA and glucose; Kunji et al., 1996). Although glucose is primarily required for meeting energy requirements, provision of AAs is needed to support an adequate growth rate. These AAs are obtained through a complex proteolytic apparatus that starts with a cell wall-bound protease (Elfahri et al., 2014). This protease liberates oligopeptides that are taken across the cell wall into the cytoplasm, where they are further degraded to simpler peptides and, subsequently, AAs. It is not clear which enzymes are responsible for liberation of bioactive peptides, but even oligopeptides appear to have very potent physiological activity (Ashar & Chand, 2004). Apparent bioactivity in water extracts is most likely time dependent, indicating that some of these peptides may be further degraded, resulting in loss or gain of potency (Donkor et al., 2007). In the current study, proteolytic activity of selected *L. helveticus* strains in pasteurised skim milk was first assessed using the ability of OPA to react with primary amines to form fluorescent moieties. As indicated in Table 3.1, all strains showed significantly higher proteolytic activity than the control. The control sample also contained simpler proteinaceous forms that reacted with OPA, but these levels remained fairly constant, unlike in the fermented samples.

Table 3.1 Decline of pH and extent of proteolysis as measured using the *o*-phthaldialdehyde (OPA) method during growth of *Lactobacillus helveticus* strains in sterile reconstituted skim milk for 24 h at 37°C

Incubation time (h)	Strain code	pH	OPA[1] at 340 nm
0	Control	6.61 ±0.01^{A}	0.31 ±0.01^{B}
	L1188	6.42 ±0.03^{B}	0.52 ±0.01^{A}
	L1315	6.41 ±0.02^{B}	0.48 ±0.01^{A}
	L474	6.44 ±0.04^{B}	0.50 ±0.02^{A}
	L953	6.48 ±0.06^{B}	0.33 ±0.02^{B}
4	Control	6.61 ±0.01^{A}	0.33 ±0.01^{E}
	L1188	6.14 ±0.05^{B}	0.55 ±0.01^{C}
	L1315	6.06 ±0.15^{B}	0.71 ±0.01^{A}
	L474	6.07 ±0.05^{B}	0.58 ±0.01^{B}
	L953	6.29 ±0.03^{B}	0.37 ±0.01^{D}
8	Control	6.58 ±0.01^{A}	0.34 ±0.01^{D}
	L1188	4.92 ±0.18^{B}	0.65 ±0.01^{C}
	L1315	4.66 ±0.36^{B}	0.97 ±0.0^{A}
	L474	4.50 ±0.09^{B}	0.93 ±0.02^{A}
	L953	4.91± 0.26^{B}	0.83 ±0.02^{B}
12	Control	6.54 ±0.01^{A}	0.35 ±0.03^{C}
	L1188	3.85 ±0.03^{C}	0.90 ±0.01^{B}
	L1315	3.77 ±0.03^{C}	1.20 ±0.01^{A}
	L474	3.75 ±0.03^{C}	1.21 ±0.01^{A}
	L953	3.99 ±0.05^{B}	1.21 ±0.02^{A}
24	Control	6.53 ±0.07^{A}	0.35 ±0.02^{C}
	L1188	3.48 ±0.14^{B}	1.30 ±0.01^{B}
	L1315	3.34 ±0.06^{B}	1.73 ±0.02^{A}
	L474	3.38 ±0.06^{B}	1.51 ±0.01^{BA}
	L953	3.46 ±0.10^{B}	1.52 ±0.16^{BA}

$^{A–C}$Means in the same column at particular incubation time with different uppercase letters are significantly different ($P < 0.05$)

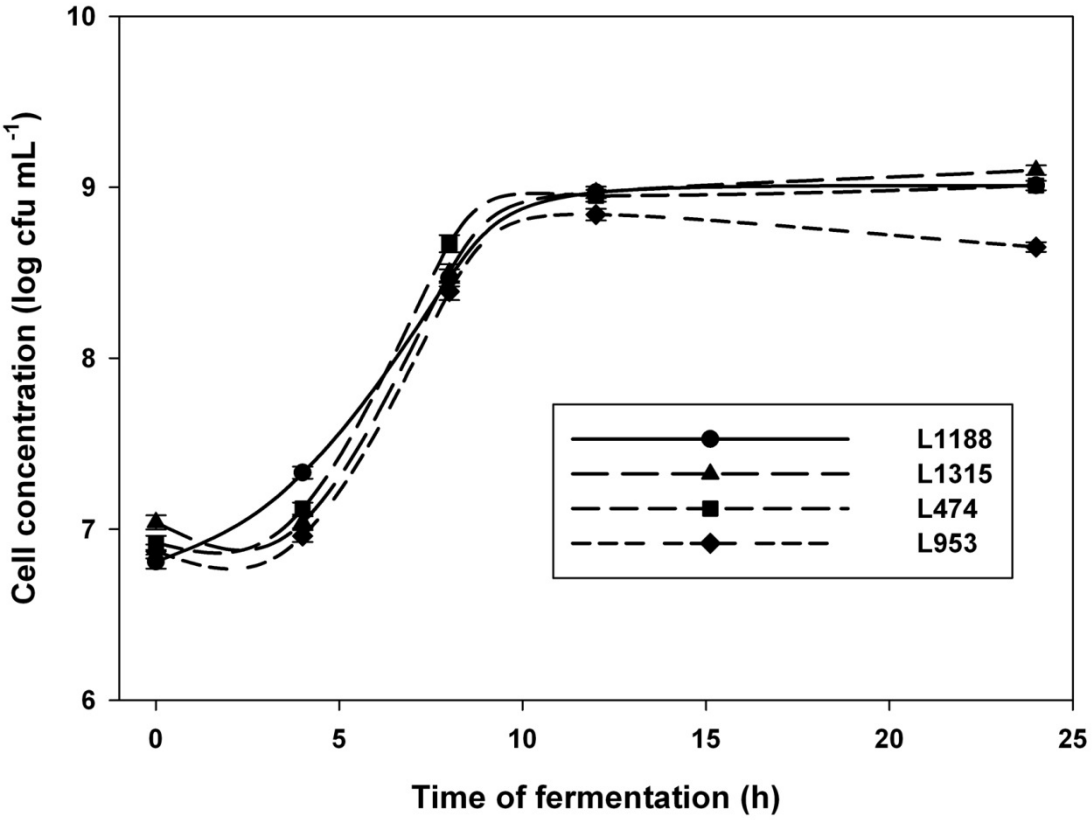

Figure 3.1 Growth performance of selected *Lactobacillus helveticus* strains cultivated in 12% reconstituted skim milk for 24 h at 37°C. Error bars represent pooled SEM ($P < 0.05$)

The proteolytic activity was significantly highly strain dependent, with *L. helveticus* 1315 appearing to be the most proteolytic ($P < 0.05$) at the end of fermentation; this could be related to viability and activity of this strain under the fermentation conditions. In general, this increase indicates formation of peptides based on cultural proteolytic enzyme specificity, with some peptides likely showing different bioactivities (Elfahri et al., 2014). Milk protein-derived bioactive peptides, such as antioxidant and antiproliferation peptides, have been reported to be a potential natural alternative to synthetic drugs and complementary to traditional dietary intake (Kannan et al., 2012); however, the mechanism of action is yet to be established.

The antioxidant activity of crude peptide extracts was evaluated by measuring the reduction of DPPH radical concentration when it encountered radical scavengers. The free radical scavenging activity in the samples changed significantly between 0 and 12 h compared with the control (Figure 3.2). This change varied among selected *L. helveticus* strains, which might be due to the fact that antioxidant capacity is not usually connected with continuing protein hydrolysis or bacteria growth but is likely related to strain selection and proteolytic enzyme specificity (Virtanen et al., 2007). In the current study, peak antioxidant activity was recorded at 12 h but declined significantly after 24 h of fermentation (Figure 3.2). However, antioxidative peptides have been reported to be hydrolysed from milk proteins by LAB fermentation after 24 h (Virtanen et al., 2007; Osuntoki et al., 2010). Sample extracts from RSM fermented with *L. helveticus* 474 showed the highest ($P < 0.05$) antioxidant activity (from 6.54% at 0 h to 48.01% at 12 h). Similar trends were observed for *L. helveticus* 1315 and 1188 (42.98 and 41.34%, respectively) at 12 h (Figure 3.2). These results indicate that antioxidants of crude peptide extracts released during fermentation may have the ability to neutralise free DPPH radicals either by hydrogen atoms or by direct reduction through ET (Sah et al., 2014). These compounds might be peptides formed during fermentation by the action of *L. helveticus* proteolytic enzymes. Although many studies have measured antioxidant activity from fermented milk in different models and fermented microorganisms (Qian et al., 2011; Sah et al., 2014), confirmation of the type of enzyme responsible for this activity is still not available (Kilpi et al., 2007). Further, the size and composition of peptides released are connected with certain bioactivity. Histidine-rich peptides isolated from milk fermented by *L. delbrueckii* ssp. *bulgaricus* IFO13953 show DPPH radical scavenging activity (Kudoh et al., 2001). Ala-, Phe-, Gly- and Pro-rich peptides have also been reported (Hernández et al., 2005b; Dryáková et al., 2010; Li et al., 2013); therefore, *L.*

helveticus stains used in the current study might be able to produce antioxidant peptides from milk proteins rich in the previously mentioned AAs. The strain-dependent variations of these activities may also be attributed to the production of different bioactive compounds such as peptides, which exhibit different antioxidant properties (Qian et al., 2011). Further hydrolysis of some potential antioxidative components by *L. helveticus* enzymes could be the reason for the reduction of antioxidant activity after 24 h of incubation. Conversely, a continuing increase in DPPH radical scavenging activity during 28 days of probiotic and symbiotic yoghurt storage at 4°C has been reported (Madhu et al., 2012). Thus, antioxidant activity in fermented milk might be based on metabolic activity of LAB—which is different among species and even varies between strains of the same species—and the ability of a fermented culture to grow under low pH (Virtanen et al., 2007). DPPH radical scavenging activity might also be attributed to fermentation processes (37°C, final pH of 3.7; Nishino et al. (2000)).

An imbalance between free radical formation and antioxidant presence can lead to DNA damage and mutation, which can increase cancer incidence. Thus, antioxidants may play a role in prevention or treatment of cancers; they may affect cancer incidence by minimising reactions that affect cell proliferation induction, not only *in vitro* (Kim et al., 2000), but also *in vivo* in rats and in human clinical trials (Tsuda et al., 2002; Kozu et al., 2009). The current study evaluated the inhibition effect of crude peptide extracts during fermentation on colon cancer cell (HT-29) proliferation (Figure 3.3A) and a healthy colon cell line (T4056) (Figure 3.3B).

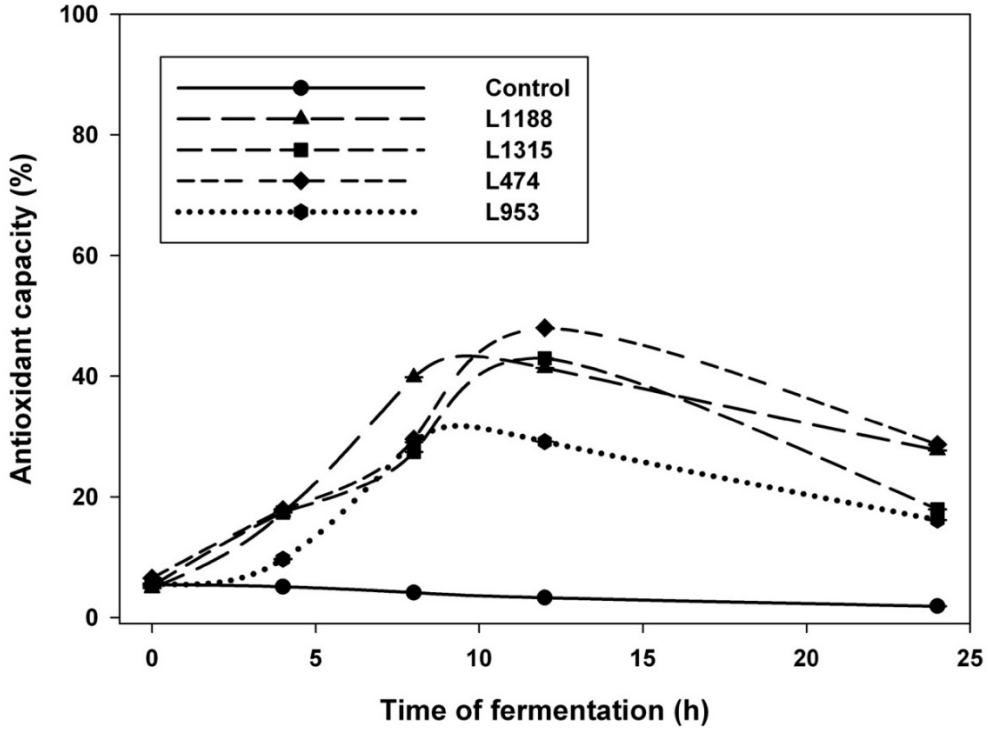

Figure 3.2 Free radical scavenging activity of peptides in the soluble extract released from skim milk proteins fermented by selected *Lactobacillus helveticus* strains during 24 h of incubation at 37°C. Error bars represent pooled SEM ($P < 0.05$)

A cell viability (MTS) assay was used to evaluate *in vitro* the inhibition of both cell lines (HT-29 and T4056) after treatment with crude peptide extract. Figure 3.3A shows the effect on the proliferation of HT-29 colon cancer cells of milk fermented with *L. helveticus* strains. All samples exhibited antiproliferative effects at various levels compared with the control. The most significant reduction in HT-29 proliferation was observed for samples obtained after 12 h of fermentation. The effect was substantially reduced when samples obtained at the end of fermentation (24 h) were applied. The percentage of proliferative inhibition after 12 h of milk fermented by *L. helveticus* 1315 increased significantly and was higher than other strains. No significant inhibition of all soluble extracts was detected with normal T4056 cells (Figure 3.3B). It is possible that the reduction in proliferation of the HT-29 cell line was due to the increase in

concentration of potential bioactive compounds in the medium, in which certain cell death mechanisms were activated, such as apoptosis (programmed cell death). It has been previously reported that different concentrations (25, 50, 100 or 200 μg/mL) of lactoferricin—a peptide derived from whey lactoferrin protein—have a cytotoxic effect on colon carcinoma cell lines (COLO-35 and HT-29) in a dose-dependent manner (Mader et al., 2006). Further, the duration of cell stimulation may also play a role in the decrease in proliferation of cancer cells. Long-term (5 weeks) treatment of Caco-2 human colon cancer cells with lactoferricin resulted in a 35% reduction of cell numbers compared with the control (unstimulated cells; Freiburghaus et al. (2009)). Thus, long-term exposure of cancer cells to bioactive compounds may play a role in delaying or reducing cancer progression.

The mechanism of this activity is mainly related to cytoplasmic membrane disruption and apoptosis induction (Sah et al., 2015b). The hydrophobicity and net charge of peptides with bioactivity may also have a crucial role in inducing apoptosis and reducing cancer cell proliferation (Hoskin & Ramamoorthy, 2008; Huang et al., 2011).

It can be deduced that these *L. helveticus* strains had a statistically significant effect on the release of potential antioxidant and anti-colon cancer compounds during fermentation. Therefore, purification of these bioactive compounds from fermented milk is important to confirm specific bioactivities, as is the establishment of the relationship between fermentation time and concentrations of these potential bioactive compounds. In general, bioactive compounds released in this study will likely have multiple physiological properties manifested at different time points during fermentation (Meisel & FitzGerald, 2003).

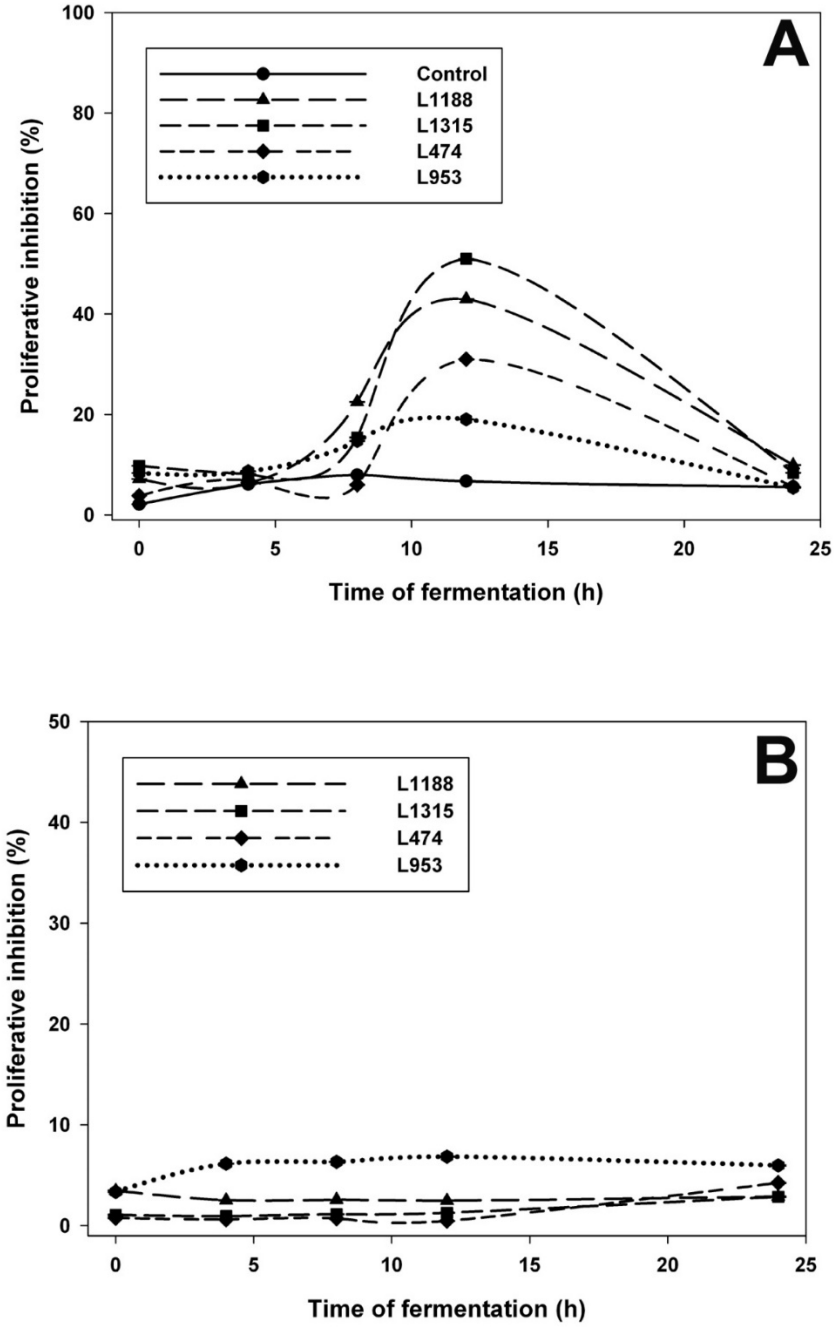

Figure 3.3 Proliferative inhibition of colon cell lines treated with peptides contained in the soluble extract obtained during fermentation of reconstituted skim milk by *Lactobacillus helveticus* 1188, 1315, 474 and 953. A, colon cancer cells (HT-29); B, normal colon cells (T4056); error bars represent pooled SEM ($P < 0.05$)

For example, soluble extracts with antioxidative and anti-colon cancer ability appear to be generated continuously, and accumulated in the fermented milk in the first 12 h of incubation at 37°C. However, antioxidative and anti-colon cancer compounds produced during culture growth or enzyme activity may not exhibit bioactivity *in vivo* due to the action of GI enzymes (Walsh et al., 2004; Roufik et al., 2006). However, ACE-I peptides derived from whey β-Lg are able to pass through GI cell monolayers to reach target sites (Vermeirssen et al., 2002).

3.5 Conclusions

This study reported the use of four proteolytic strains of *L. helveticus* (953, 474, 1188 and 1315) as potential functional dairy starter cultures capable of releasing a wide range of bioactive compounds from milk. *L. helveticus* strains grew well in skim milk under acidic conditions. Their growth apparently resulted in the release of antioxidative and anti-colon cancer compounds from milk. However, the level of bioactivity appeared to be strain specific and dependent on fermentation time, temperature, pH changes and the concentration of released bioactive compounds in fermented milk. *L. helveticus* strains have the potential to produce bioactive compounds with antioxidative and anticancerogenic activities.

Chapter 4: Release of Bioactive Peptides with Antioxidative Properties During Cold storage of Milk Fermented with *Lactobacillus helveticus* 1315

4.1 Introduction

Diet and lifestyle interventions are justifiably targeted as preventative options for many diseases. As such, it is pertinent to investigate endogenous physiological systems that can be manipulated through the use of nutraceutical agents, food or food products that reportedly provide health or medical benefits, to safely advance the prevention of diseases (Nyström & Mutanen, 2009; Tsuda et al., 2010). In the human body, oxygen is a necessary factor in normal metabolism; it is involved in creating ROS such as superoxide anions (O_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (-$OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) through final electron acceptors in electron transport (Apak et al., 2016). Under normal metabolic conditions, these radicals are not only important during respiration, but also essential for the normal germicidal activity of macrophages (Apak et al., 2016). However, excessive generation of free radicals (reactive oxygen and nitrogen species) may result in mortal damage to cells, which may lead to the development of chronic diseases such as cardiovascular disease, diabetes and cancer (Collins, 2005; Sharma, 2014; Sah et al., 2016c).

Due to failure of the defensive system to synthesise enzymatic antioxidants (catalase, superoxide dismutase and glutathione peroxidase) to neutralise the excessive release of free radicals, patients are usually treated with extra antioxidative compounds. Synthetic antioxidants such as butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), and propyl gallate (PG) can boost free radical scavengers in the human body's defence system (Sah et al., 2016c). However, some countries restrict or prohibit the use

of these chemical antioxidants because of safety concerns (Jun et al., 2004). Therefore, searching for natural antioxidants as an alternative to synthetic medicines has increased momentum in terms of their prospective functional activities and food quality.

Milk proteins have been recognised as important sources of bioactive peptides (Elfahri et al., 2015; Sah et al., 2015b); some of these peptides have potential antioxidative capacity (Elfahri et al., 2014). Antioxidative peptides, mainly derived from caseins in milk by the action of LAB, are bioactive compounds that show reducing power and radical scavenging capacity towards DPPH, 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) and hydroxyl radicals (Suetsuna et al., 2000; Sah et al., 2015b). For example, a strong free radical scavenging peptide (Ala–Arg–His–Pro–His–Pro–His–Leu–Ser–Phe–Met) was isolated from κ-casein of milk fermented with *L. delbrueckii* subsp. *bulgaricus* IFO13953 (Kudoh et al., 2001). Farvin et al. (2010) reported other free radical peptide scavengers liberated from β-, αs1-, αs2- and κ-caseins in yoghurt. Released antioxidant peptides derived from milk proteins have been found to be strain dependent and partly linked with levels of protein hydrolysis (Gupta et al., 2009). Cell wall proteases and peptidases of various LAB play a crucial role in releasing peptides from milk proteins (Elfahri et al., 2015). *L. helveticus* strains in particular exhibit strong protease and peptidase activities compared to other LAB (LeBlanc et al., 2002; Elfahri et al., 2014), and are capable of generating peptides with ACE-I and antioxidative properties (Hernández et al., 2005b; Elfahri et al., 2014).

It is apparent from previous studies that storage of starter cultures may change the bioactivity of peptide extracts (Donkor et al., 2007; Sah et al., 2016a). However, very little attention has been given to the isolation during storage and the fate of peptides during GI passage. Therefore, the aim of this study was to investigate the effect of 28-day cold storage (4^{o}C) on the liberation of bioactive peptides extracted from milk

fermented with proteolytic strain *L.* 1315, and stability of the peptides under *in vitro* GI digestion. Special emphasis was given to peptides with elevated antioxidative capacity.

4.2 Materials and Methods

4.2.1 Culture Performance of *Lactobacillus helveticus* During Cultivation in Milk

L. helveticus 1315 (*L.* 1315) strain was obtained from the Australian Starter Culture Collection (Dairy Innovation, Werribee, Australia) and stored as a stock culture (10^9 cfu/mL) at –80°C. Propagation of the culture was performed as described by Elfahri et al. (2015). The fermentation trial was also conducted as described by Elfahri et al. (2014) but with some modifications. Briefly, 5 L of RSM was prepared by dissolving 12% total solids of skim milk powder (Fonterra, Palmerston North, New Zealand) in Milli-Q water, heat treated at 90°C for 10 min and cooled to 40°C. The milk was inoculated with 1% (v/v) of *L.* 1315 strain and incubated at 37°C until pH ~4.5 was reached. All fermented milk samples were then stored at 4°C and sampled at 0 h, 1, 7, 14, 21 and 28 days. Each sample was divided into two portions: one portion (10 mL) was immediately assessed for cell counts, while the remaining portion was stored at –80°C for further analysis. Cell populations were assessed as previously reported (Elfahri et al., 2014) using the pour plate technique on MRS–sorbitol agar under anaerobic incubation using anaerobic kits (Oxoid limited, Basingstoke, Hampshire, England) at 37°C for 72 h. Proteolytic activity of the culture during fermentation and cold storage was also measured according to Elfahri et al. (2014) by assessing released free amino groups using the OPA method.

4.2.2 Simulated Gastrointestinal Digestion

The simulated GI digestion of fermented milk protein was carried out according to Quirós et al. (2009) with some modifications. Approximately 100 mL were taken from each

sample previously stored at –80°C and thawed overnight at 4°C. The thawed sample was then aseptically homogenised and mixed with pepsin (Sigma) (enzyme:substrate ratio of 1:100 w/w) and incubated for 60 min at 37°C (pH 2–3). Pancreatin at the same enzyme:substrate ratio as pepsin was added to each resulting mixture at pH 7–8 and further incubated at 37°C for 60 min. Hydrolysis was carried out in a thermally controlled incubator under constant stirring (120 rpm). Protein digestion was stopped by heating at 80°C for 20 min, followed by cooling to 4°C. Each digested enzyme sample (designated as DEs) was centrifuged at 14,000 × g for 30 min at 4°C; supernatant was filtered through a 0.22-μm filter (Phenomenex Inc., Lane Cove, Australia), freeze-dried using an Alpha 1–4 LSC Christ freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH) and stored at –80°C for further analysis. Fermented milk with nondigested enzyme, and unfermented milk samples, were used as positive and negative controls, respectively.

4.2.3 Preparation of Soluble Peptide Extract and Chromatographic Determination

SPEs for each sample were prepared as described by Elfahri et al. (2015). The SPEs of fermented milk were designated as NDEs, while the SPEs of fermented milk digested with GI enzymes were labelled as DEs. The protein content (mg/mL) of the NDEs and DEs was estimated according to the Bradford method using BSA as a standard. The freeze-dried extract of each sample (300 mg/mL) was resuspended in 1 mL of 0.1% trifluoroacetic acid (TFA), filtered with 0.22 μm into a vial and analysed using RP–HPLC on an LC-2010 system (Shimadzu, Kyoto, Japan). A linear gradient was used from 100% to 0% solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%, acetonitrile in water) over 40 min at a flow rate of 0.75 mL/min. All samples were injected onto a C_{18} column (Jupiter, 300A Column 250 × 4.6 mm, Phenomenex) respectively at room temperature and eluted peaks were detected with a Varian 9050 UV/VIS detector (Varian Analytical Instruments) at 214 nm.

4.2.4 Assessment of Bioactivity

4.2.4.1 Angiotensin-converting Enzyme-Inhibitory Activity

ACE-I activity was determined according to the method of Elfahri et al. (2014) by measuring the release of hippuric acid at 228 nm.

4.2.4.2 Antibacterial Activity

The antibacterial activities of NDE and DE samples (at 0.2 mg protein/mL of PBS, pH 7.4) against negative and positive target pathogenic strains [*E. coli* (ATCC CRM-8739) and *Staphylococcus aureus* ssp. *aureus* (ATCC 25923) respectively] were assessed using the agar disc diffusion method as described by Sah et al. (2016a). Briefly, a bacterial suspension was spread on a nutrient agar plate. Sterilised filter paper discs (6 mm) were soaked with each sample separately and left to dry for 30 min. These discs were then aseptically placed in the centre of each plate, sealed and incubated aerobically at 37°C for 12–24 h. The inhibition zones were measured in millimetres including the discs. Ampicillin (1mg/mL of PBS) was used as a control.

4.2.4.3 Antioxidative Activity

4.2.4.3.1 DPPH Free Radical Scavenging Activity

The DPPH radical (Sigma-Aldrich Pty. Ltd. Australia) was used to determine the free radical scavenging ability of extracted samples (Elfahri et al., 2015). Samples (NDEs or DEs—0.2 mg protein/mL) were mixed with 0.1 mM DPPH dissolved in 95% methanol and vigorously shaken, sealed with parafilm and incubated in the dark at room temperature for 30 min. The methanolic DPPH was used as a blank and methanol was used for the baseline correction. After incubation, the reduction in absorbance was

measured at 517 nm using a UV/VIS spectrophotometer (Biochrom Ltd, Cambridge, England).

4.2.4.3.2 ABTS Radical Scavenging Activity

The $ABTS^{\bullet+}$ scavenging activity of NDEs and DEs was evaluated according to the method described by Sah et al. (2015a) with some modifications. Briefly, $ABTS^{\bullet+}$ reagent (Sigma-Aldrich Pty. Ltd. Australia) was prepared fresh. To measure scavenging activity, precise aliquots of 10 µL of NDE or DE (at 0.2 mg of protein/mL) were added to 990 µL of the $ABTS^{\bullet+}$ reagent, vortexed for 10 s and then incubated at 30°C for 20 min. $ABTS^{\bullet+}$ scavenging activity was measured at an absorbance of 734 nm. Milli-Q water was used as a blank.

4.2.4.3.3 Reducing Power

Reducing power of SPEs was measured by assessing reduction of $Fe^{3+}(CN^{-})^{6}$ to $Fe^{2+}(CN^{-})^{6}$, as described by Sah et al. (2015a), with some modifications. Briefly, 50 µL of NDE or DE (0.2 mg of protein/mL) was mixed with 500 µL of 0.2 M phosphate buffer (pH 6.6) and 500 µL of aqueous potassium ferricyanide solution (1%, wt/vol). To start the reaction, the mixture was incubated at 50°C for 20 min, and then 500 µL of 10% of TCA solution (w/v) was added to stop the reaction. The resulting mixture was centrifuged at 10,000 × *g* for 15 min at room temperature. Finally, 500 µL of the top layer formed was mixed with 500 µL of Milli-Q and 100 µL of 0.1% of ferric chloride solution (w/v in Milli-Q water). After 10 min at ambient temperature, the absorbance of the resulting solution was measured at 700 nm using a Biochrom Libra S12 UV/VIS spectrophotometer (Biochrom Ltd.). A high absorbance indicates high reducing power.

4.2.4.3.4 Hydroxyl Radical Scavenging Activity

Nine isolated peptides were additionally assessed for their hydroxyl radical scavenging activity (HRSA) following a method described by Sah et al. (2015a) with some modifications. Briefly, a ferrous sulphate solution (2 mM in Milli-Q water) and hydrogen peroxide (2 mM in Milli-Q water) were mixed with 20 µL of each peptide (concentration 100 or 200 µg/mL). The resulting mixture was vortexed and incubated at room temperature for 10 min. Salicylic acid (2.5 mM) was added to the reaction mixture and incubated for 30 min at 37°C. The absorbance of the mixture was measured at 510 nm. Milli-Q water was used instead of salicylic acid as a blank, while control (in place of sample) in the reaction.

4.2.4.4 Alpha Glucosidase Inhibition Assay

α-Glucosidase inhibition of isolated peptides as a marker of antidiabetic properties was assayed following a method described by Apostolidis et al. (2007) by mixing 50 µL of each purified peptide at a concentration of 100 or 200 µg/mL with 100 µL of α-glucosidase (Sigma-Aldrich) solution containing 1.0 unit/mL in phosphate buffer (0.1 M; pH 6.9) and incubated in a 96-well plate at 25°C for 10 min. After pre-incubation, a precise aliquot of 50 µL of *p*-nitrophenyl-α-D-glucopyranoside (5 mM in 0.1 M phosphate buffer; pH 6.9) was added to each well at timed intervals. The resulting mixtures were incubated at 25°C for 5 min and the absorbance readings at 405 nm were recorded before and after incubation using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA). Similarly, the buffer solution was used as control. The α-glucosidase inhibitory activity was expressed as percentage inhibition.

4.2.5 Fractionation and Peptide Purification

Semi-preparative RP–HPLC (Varian Analytical Instruments, Santa Clara, CA) was carried out according to the method of López-Expósito et al. (2006) with some modifications. The freeze-dried water SPE of fermented milk not subjected to simulated digestion was dissolved in 0.1% TFA at 0.3 g/mL. The solution was centrifuged at 14,000 × g (Beckman Coulter, Inc., Brea, CA, USA) for 30 min at room temperature. The supernatants were collected and filtered (0.22-mm membrane filter) into 50 mL falcon tubes for peptide analysis. All samples were eluted at a flow rate of 16 mL/min, with a linear gradient of solvent B (water:acetonitrile:TFA of 100:900:1.0) in solvent A (water:TFA of 1000:1.0) increasing from 0% to 100 % B in 40 min. Before each chromatographic run, a C_{18} column (Phenomenex Jupiter 10u Proteo 90A, AXIA Packed; 250 × 21.2 mm; 7.8 μm; 90 A) fitted with a security guard cartridge (C_{18}; 21.2mm, Phenominex) was equilibrated for 10 min with 100% solvent A. The fractions were collected manually at 5-min intervals, snap-frozen and freeze-dried. The powders were redissolved in 15 mL Milli-Q water and assayed for antioxidative activity as described above only. The fraction with the greatest antioxidative activities was selected for further steps of purification and peptide analysis. At each stage of purification, antioxidative capacity was confirmed, yielding the most potent fraction. Peptides of the potent fraction were identified using LC–MS/MS (Sah et al., 2016b) employing a quadrupole time-of-flight mass spectrometer (micrOTOF-Q; Bruker Daltonik GmbH, Bremen, Germany) coupled online to a nano-HPLC (Ultimate 3000; Dionex Corporation, SunnyBrook, CA, USA). The data were compared with Swiss-Prot databases using the MASCOT search engine (v. 2.4, Matrix Science Inc., London, UK). Nine peptides were identified, and synthesised by Mimotopes Pty Ltd (Clayton, VIC, Australia) at purity >95%. Their potency was assayed following the methods described above.

4.3 Statistical Analysis

All experiments were carried out in triplicate. Results obtained were analysed as a blocked split plot in time design with digestion as the main factor (two levels: digested and nondigested) and the replications as the block with the cold storage period as a subplot. Statistical evaluation of the data was performed using the General Linear Model (SAS, 1996). Significant differences between treatments were tested by ANOVA followed by a comparison between treatments performed by Fisher's LSD method, with a level of significance of $P < 0.05$.

4.4 Results and Discussion

4.4.1 Growth Performance of *L.* 1315 During Refrigerated Storage

L. 1315 strain has previously been shown to have high proteolytic activity, resulting in release of free AAs and peptides during growth in skim milk (Elfahri et al., 2015, 2014). As this activity appears growth related, growth of *L.* 1315 was assessed in a previous study (Elfahri et al., 2015). The culture continued to grow during the first week of storage (>8 log cfu/mL) but declined significantly after 7 days, likely due to the reduction in pH. Continuous metabolic activity during cold storage resulted in the production of organic acids, which compromised culture growth over time (Damin et al., 2008). As shown in the previous study of Elfahri et al. (2015), appreciable proteolytic activity was also detected in all fermented milk samples determined using the OPA method. In general, the amount of free AA groups in fermented milk samples increased during storage in comparison with fermented samples before storage. Proteolysis increased significantly after 14 days and the trend continued until the end of 28 days storage at 4°C, possibly due to increased intracellular enzymes as a result of microbial cell lysis (Otte et al., 2011).

This may indicate increased proteolytic activity resulting in better *L.* 1315 growth and thus the release of bioactive compounds in milk during fermentation and storage (Elfahri et al., 2014; Sah et al., 2015a). Bacterial cells in milk require free AAs for growth; thus, bacterial enzymes (extracellular proteinases and peptidases) liberate oligopeptides, small peptides and AAs from milk proteins during fermentation (Donkor et al., 2007; Korhonen, 2009a). Although liberated peptides usually constitute ~1–2 % of total milk proteins (Matar et al., 1996)**,** a number of distinct peptides with different biological activities might be released during fermentation and cold storage (Sah et al., 2015a).

4.4.2 Bioactivity of Soluble Peptide Extracts Obtained During Cold Storage of Fermented Milk

4.4.2.1 Angiotensin-converting Enzyme-Inhibitory and Antimicrobial Activity of Soluble Extracts

A spectrophotometry assay is commonly used in dairy research for the determination of ACE-I activity (Donkor et al., 2007; Elfahri et al., 2014). This method is based on the presence of hippuric acid and histidyl–leucine, which results from the hydrolytic action of ACE on the specific substrate (hippuryl–histidyl–leucine). Figure 4.1 displays the *in vitro* ACE-I activity of NDEs from the milk fermented with *L.* 1315 at weekly intervals during refrigerated storage. ACE-I activity in NDEs increased significantly during cold storage, from 8% at 0 h to 65% after 14 days. Similar to a previous report (Donkor et al., 2007), the activity was almost halved after 21 days followed by a significant increase after 28 days. Apparently, further activity of proteolytic enzymes continued to cleave liberated peptides, releasing peptides with bioactive properties during storage (Sah et al., 2016a). All samples (NDEs and DEs) were further assessed for antimicrobial activity; no such activity was observed against negative and positive target pathogenic strains.

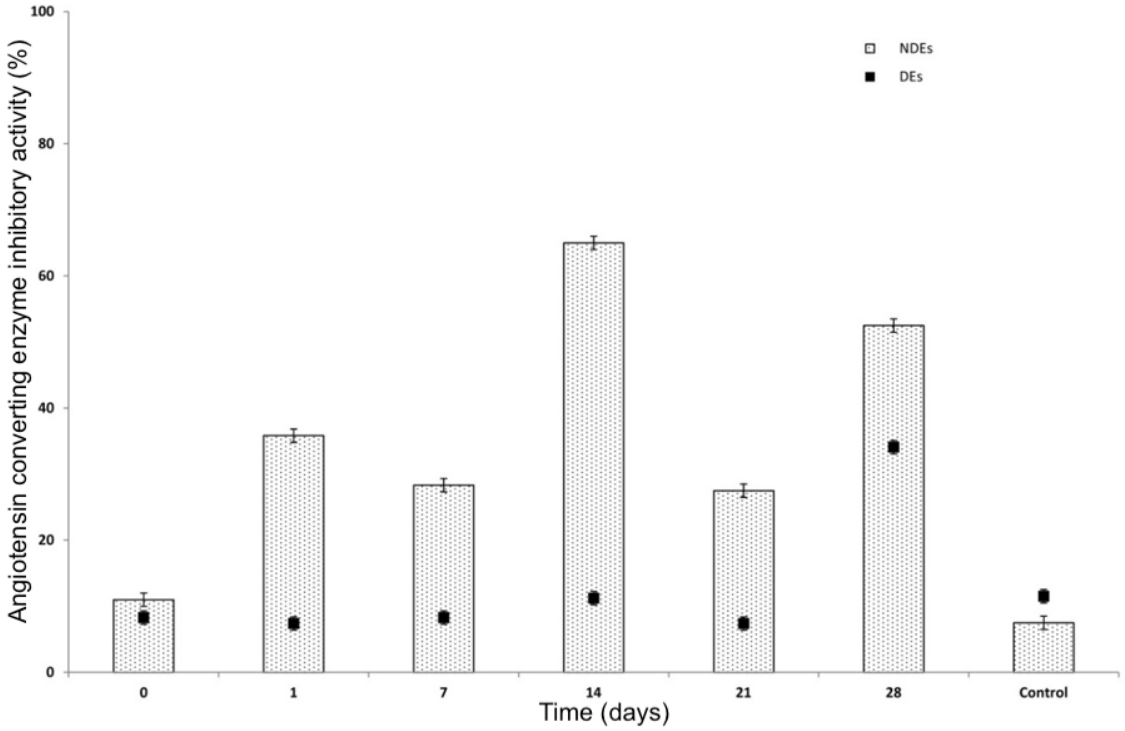

Figure 4.1. Angiotensin-converting enzyme-inhibitory activity of milk fermented by *L.* 1315 during fermentation until pH ~ 4.5 and cold storage at 4ºC for 4 weeks. Samples were water extracted and assessed as is (non-digestion, NDE) or subjected to simulated digestion (DE); error bars represent pooled SEM ($P < 0.05$)

4.4.2.2 Antioxidative Activity of Soluble Extracts

Due to the contribution of a series of reaction steps in an oxidisation process, food protein hydrolysates can neutralise free radicals through multiple reaction mechanisms (Samaranayaka & Li-Chan, 2011). Thus, as a confirmatory test and to provide sufficient information about the total antioxidant capacity of a compound, more than two different assays may be used. In this study, DPPH, ABTS–based methods and reducing power were used separately to assess the production of antioxidative compounds released by bacterial fermentation in milk, as exhibited in Figure 4.2A, B and C. The free radical scavenging activity measured for NDE samples was significantly higher with each assay after 14 days of storage compared with samples at the beginning or end of storage. Although *L.* 1315 growth decreased after 14 days of storage, the antioxidative activity of the extracts

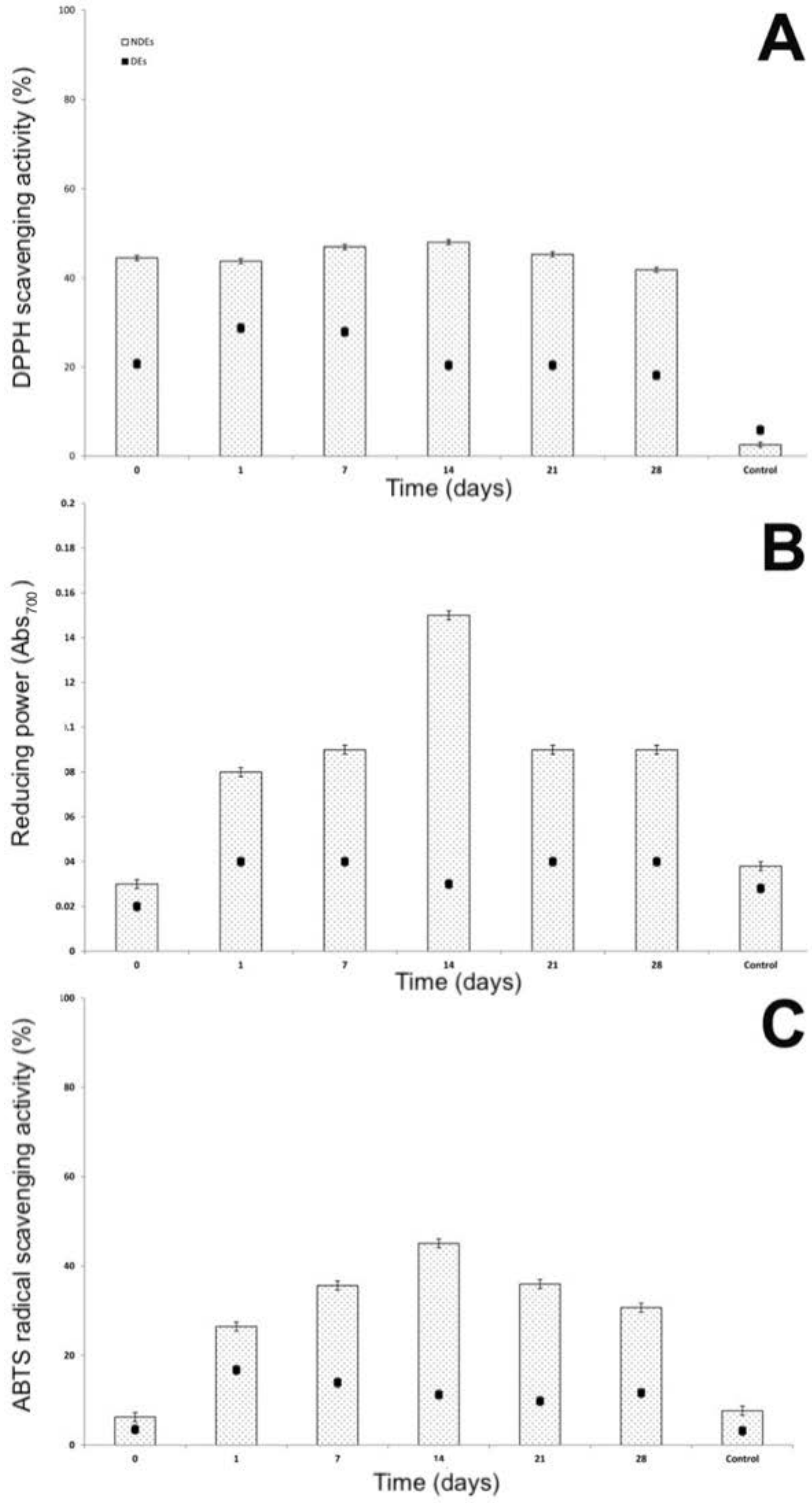

Figure 4.2. Antioxidative capacity, as assessed using the DPPH (A), ABTS (B) and reducing power (C) methods, of water-soluble peptide extracts obtained from milk fermented by *Lactobacillus helveticus* 1315 upon fermentation until pH ~ 4.5 and cold storage at 4ºC for 4 weeks. Samples were assessed as is (NDEs) or were

subjected to simulated gastrointestinal digestion (DEs); error bars represent pooled SEM ($P < 0.05$)

increased significantly after the same storage time, possibly due to enhanced activity of intracellular enzymes released due to cell lysis (Otte et al., 2011). In DPPH analysis, the reaction mechanism involves mainly HAT whereas in ABTS analysis, both single ET and HAT take place (Gülçin, 2012). Interruption of free radical chain reactions by donation of hydrogen ions is involved in the assessment of reducing power for antioxidant capacity determination (Sah et al., 2015a). A strong positive correlation between proteolytic activity and antioxidative capacity of released peptides has been previously reported (Sah et al., 2015a). Type of proteolytic enzyme (Foh et al., 2010), AA sequence and concentration of released peptides are some of the factors governing antioxidative properties of peptides (Hernández et al., 2005b). For example, peptides with large amounts of histidine and some hydrophobic AAs such as proline, histidine, tyrosine or tryptophan in the sequence have been reported to have more potent antioxidative activity than others (Peña-Ramos et al., 2004). Chen et al. (1996) evaluated the antioxidant activity of 28 peptides and compared this to the antioxidative peptide Leu–Leu–Pro–His–His, showing that the tripeptide Pro–His–His exhibited the highest antioxidant activity among all peptides assessed, and further reported that deletion of histidine at the C-terminus decreased antioxidant activity.

4.4.3 The Effect of Gastrointestinal Digestion on Bioactivity in Fermented Milk

Bioactive peptides that resist GI enzyme hydrolysis are able to reach target sites in the body (Vermeirssen et al., 2002; Roufik et al., 2006; Mills et al., 2009; Madureira et al., 2010). To establish the stability of released crude bioactive extracts, all fermented milk samples were exposed to a two-step hydrolysis process that simulates GI digestion conditions. After exposure to digestive enzymes, ACE-I and antioxidative activities

decreased significantly in all SPEs samples compared with the ACE-I activity of the NDE samples (Figure 4.1 and Figure 4.2A, B and C). The highest ($P< 0.05$) decrease in activity was determined for the sample obtained after 14 days cold. This reduction in ACE-I and antioxidant activity was likely due to specificity of digestive enzymes hydrolysing ACE-I and antioxidative peptides (Roufik et al., 2006; Virtanen et al., 2007; Nielsen et al., 2009). Many factors affect the stability and absorption of bioactive peptides when exposed to GI digestion. For instance, the physiological environment of the GIT, including the presence of proteinases and peptidases, and low pH, could affect the structure of bioactive peptides, leading to a change in bioactivity in the organism. Therefore, digestion of peptides in the GIT may or may not result in formation of bioactive peptides. Molecular weight, AA sequence and charge can also affect absorption of peptides through the intestinal epithelium layer (Sah et al., 2016c). However, the presence of proline in peptides may provide resistance to digestive enzymes, for example tripeptides with Pro–Pro at the C-terminus have been reported to resist proline-specific peptidases (FitzGerald & Meisel, 2000). On the other hand, peptides formed during fermentation may be cleaved, resulting in altered physiological effects (Sah et al., 2016c). Thus, it is not certain that uptake of *in vitro* bioactive peptides after oral ingestion will lead to their physiological activity *in vivo* (Li et al., 2004; Erdmann et al., 2008). Vermeirssen et al. (2002) found that an ACE-I peptide fragment derived from β-Lg f (Ala–Leu–Pro–Met–His–Ile–Arg), was able to cross Caco-2 cell monolayers. However, other studies suggested that the same peptide was hydrolysed by digestive and serum proteolytic enzymes, thus losing its bioactivity (Walsh et al., 2004; Roufik et al., 2006).

4.4.4 Isolation and Identification of Antioxidant Peptides

NDEs after 14 days of cold storage exhibited higher antioxidative activity than all other samples tested; they were therefore selected for fractionation (Figure 4.3A) to identify

potential antioxidative peptides. Figure 4.3A and Table 4.1 show seven peptide fractions (from F1 to F7) that exhibited various levels of antioxidative activity. The DPPH free radical scavenging effects of soluble fractions F1 and F2 were significantly higher than those of the other fractions. As the antioxidant activity of F2 was slightly higher than F1, F2 (Figure 4.3B) was selected for further fractionation. Sub-fractions 2–3 of F2 showed significantly higher antioxidant activity (Table 4.1), likely due to a synergistic effect of the peptides (Kwak et al., 2009).

Table 4.1. Antioxidative properties expressed as DPPH scavenging activity of water-soluble peptide extracts obtained from milk fermented with *Lactobacillus helveticus* 1315 after 14 days of cold storage collected by semi-preparative RP–HPLC

	Fraction						
Bioactivity	F1	F2	F3	F4	F5	F6	F7
DPPH %	32.2±0.50^{A}	34±1.27^{A}	16.6±0.79^{CB}	5.9±0.88^{D}	14±0.95^{C}	3.8±1.02^{D}	17.90±1.19^{B}
F2 sub-fraction							
		F2-1	F2-2	F2-3	F2-4	F2-5	
DPPH %		15.3±1.22^{C}	26.5±1.70^{B}	38.5±1.29^{A}	14.3±2.23^{CD}	9.9±2.76^{D}	

Different superscripts in the same row indicate a significant difference ($P < 0.05$)

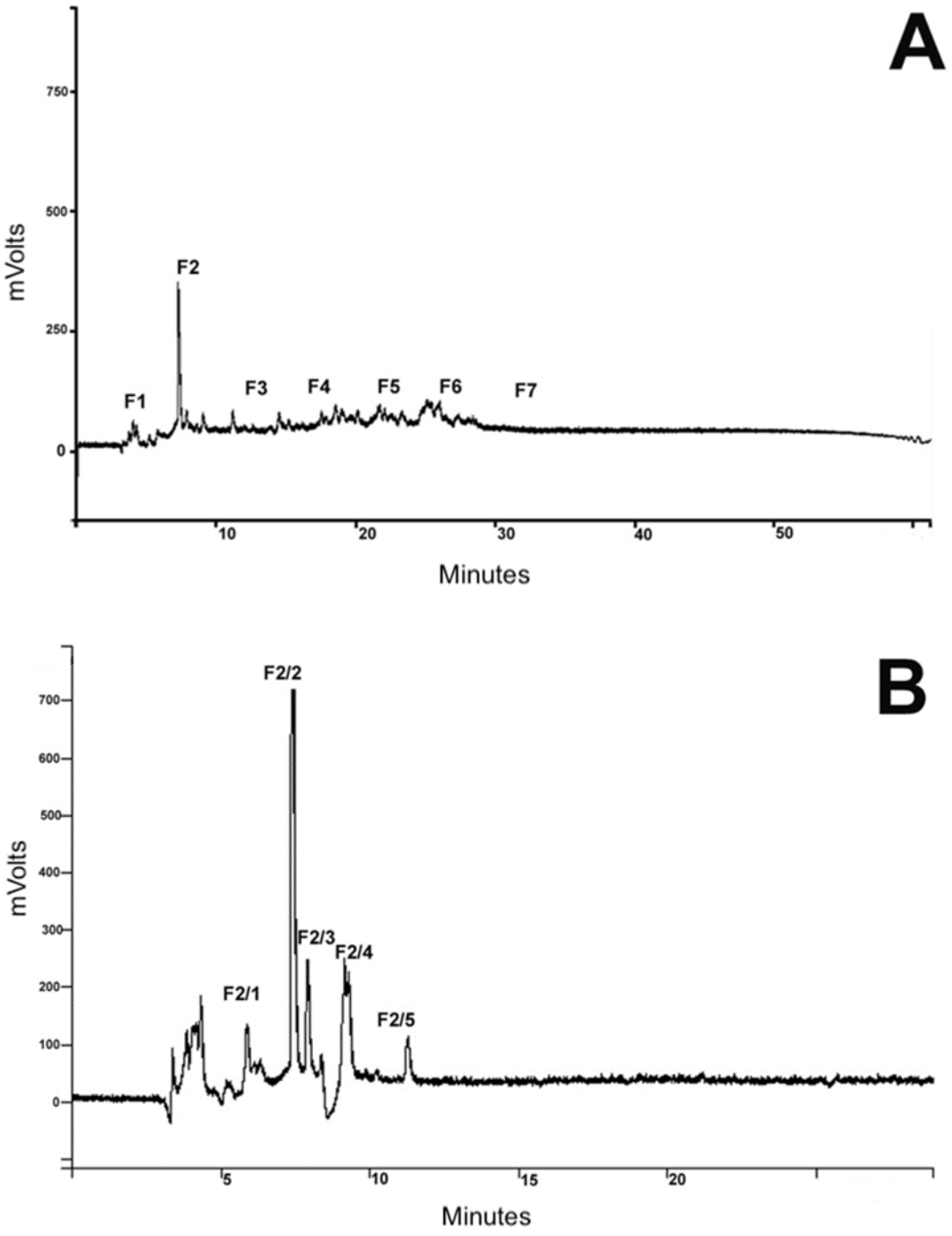

Figure 4.3. Representative semi-preparative RP–HPLC chromatograms of a whole water-soluble peptide extract (A) and the fraction (F2) with greatest antioxidative capacity (B) established by the DPPH method obtained after 14 days of cold storage of milk fermented by *Lactobacillus helveticus* 1315

Overall, nine peptides were obtained from sub-fractions 2–3 after further analysis (SWMHQPHQPLPP, SWMHQPHQPLPPT, LQSWMHQPHQPLPP, SLSQSKVLPVPQ, RDMPIQAF, LQSWMHQPHQPLPPT, FPGPIPN,

KVLPVPQKAVPYPQ and SQSKVLPVPQKAVPYPQ). The peptides designated PP1– to PP9 were found to have been released from β-CN (Table 4.2). The identified peptides were synthesised and reassessed for ACE-I, antidiabetic and antioxidative activities at concentrations of 100 and 200 μg/mL, respectively.

Table 4.2 Structure and amino acid sequence of peptides isolated from sub-fraction 2–3 of milk fermented by *Lactobacillus helveticus*1315 after 14 days of cold storage

Peptide number	Structure and amino acid sequence
PP1	SWMHQPHQPLPP
PP2	SWMHQPHQPLPPT
PP3	LQSWMHQPHQPLPP
PP4	SLSQSKVLPVPQ
PP5	RDMPIQAF
PP6	LQSWMHQPHQPLPPT
PP7	FPGPIPN
PP8	KVLPVPQKAVPYPQ
PP9	SQSKVLPVPQKAVPYPQ

4.4.4.1 Angiotensin-converting Enzyme-Inhibitory and Antidiabetic Activity of Synthesised Peptides

ACE-I peptides mainly have a hypotensive effect (FitzGerald et al., 2004). The results in Table 4.3 show that peptides PP1, PP2, PP3 and PP5 demonstrated ACE-I activity in a dose-dependent manner. PP1 exhibited the highest (*P* <ependent maI (29.81%) followed by PP3 (22.80%), PP2 (21.04%), PP5 (12.27%) and PP7 (5.26%) at 200 μg/mL; equivalent values were 17.53%, 12.27%, 7.01%, 5.26% and 0% at 100 μg/mL. However, these ACE-I peptides were significantly less potent than the positive control (captopril) at the same concentrations (Table 4.3). Several antihypertensive peptides derived from

milk proteins have been isolated and identified (Korhonen, 2009a). For instance, peptides containing Val–Pro–Pro and Ile–Pro–Pro released from casein proteins have been isolated from some fermented dairy products, such as 'Calpis' sour milk (Calpis Co., Japan) and 'Evolus' sour milk (Valio Ltd., Finland) (Korhonen, 2009a). Further, peptide structure plays a key role in ACE-I activity. The existence of aromatics such as phenylalanine, tryptophan and tyrosine, or the position of the hydrophobic AA proline at the C-terminus of peptides has been reported to have a positive correlation with ACE-I activity (Pripp et al., 2004). In addition, the presence of a Pro–Pro (P–P) bond and tryptophan (W) at the C-terminal region in the peptide sequence of PP1, PP2 and PP3 likely contributed to their ACE-I activity in the current study. The presence of the hydrophobic AA phenylalanine (F) at the C-terminus and methionine (M) in PP5 could also have contributed to their observed ACE-I activity. In conclusion, all ACE-I peptides in this study contain a hydrophobic AA at the C-terminus and that proline is the dominant AA in the sequences. All nine peptides were also assessed for the antidiabetic activity using the α-glucosidase inhibition assay but none showed any observable inhibition.

4.4.4.2 Antioxidative Activity of Synthesised Peptides

In the current study, the antioxidative activity of peptides was determined according to the scavenging capacity of radicals DPPH, $ABTS^{\bullet+}$ and HRSA (Table 4.3). PP8 and 9 exhibited significantly higher DPPH, $ABTS^{\bullet+}$ and $^{\bullet}OH$ radical scavenging capacities (at concentrations of 100 and 200 μg/mL) than did peptides PP1–7 but these were significantly lower than the positive control (L-ascorbic acid) at the same concentrations (Table 4.3). The mode of action by which these peptides exhibit antioxidative activity is not fully understood or explained; however free radical scavenging, metal ion chelation and singulet oxygen quenching have been reported as the main properties of potential antioxidative peptides (Erdmann et al., 2008). Free radical scavenging by peptides

depends mainly on AA composition, sequence, molecular weight and concentration (Zou et al., 2016). Such milk-derived antioxidant peptide activities have been reported in several studies (Sarmadi & Ismail, 2010; Li et al., 2011; Ji et al., 2014; Sah et al., 2016c).

Table 4.3. Angiotensin-converting enzyme-inhibitory (ACE-I), antidiabetic and antioxidative properties of nine peptides (Table 4.2) isolated from the most potent antioxidative fraction of milk fermented by *Lactobacillus helveticus* 1315

Peptide number	ACE-I %		Antidiabetic %		DPPH %		ABTS$^{\bullet+}$ %		HRSA %	
	100 µg	200 µg	100 µg	200 µg	100 µg	200 µg	100 µg	200 µg	100 µg	200 µg
PP1	17.53^{B}	29.81^{B}	ND	ND	0.40^{C}	1.90^{D}	1.95^{C}	3.24^{D}	0.73^{D}	1.26^{D}
PP2	7.01^{C}	21.04^{C}	ND	ND	0.56^{C}	0.56^{D}	2.09^{C}	2.66^{D}	0.62^{D}	0.94^{E}
PP3	12.27^{C}	22.80^{C}	ND	ND	0.51^{C}	1.12^{D}	2.33^{C}	2.66^{D}	ND	0.10^{E}
PP4	ND	ND	ND	ND	0.71^{C}	1.33^{D}	2.23^{C}	3.43^{D}	0.10^{D}	0.52^{E}
PP5	5.26^{C}	12.27^{D}	ND	ND	0.56^{C}	1.02^{D}	2.62^{C}	3.62^{D}	0.41^{D}	0.52^{E}
PP6	ND	ND	ND	ND	0.50^{C}	1.02^{D}	1.76^{C}	3.10^{D}	0.20^{D}	0.38^{E}
PP7	ND	5.26^{E}	ND	ND	0.71^{C}	1.02^{D}	1.76^{C}	1.81^{D}	0.52^{D}	1.89^{D}
PP8	ND	ND	ND	ND	13.77^{B}	26.36^{B}	13.74^{B}	25.40^{C}	6.85^{C}	13.81^{C}
PP9	ND	ND	ND	ND	13.82^{B}	23.16^{C}	13.51^{B}	27.88^{B}	11.38^{B}	26.15^{B}
Captopril	33.33^{A}	85.96^{A}								
Ascorbic acid					42.92^{A}	81.93^{A}	25.44^{A}	43.55^{A}	41.03^{A}	63.49^{A}
SEM	1.46				0.43		0.34		0.48	

Different capital superscripts in the same column were significantly different ($P < 0.05$); SEM, standard error of the mean; ND, not detected

They usually contain 5–11 AAs and hydrophobic AAs such as proline, tyrosine or tryptophan (Pihlanto, 2006a). Further, strong hydroxyl radical scavenging by peptides containing AA residues such as lysine, proline, and serine has also been reported (Ji et al., 2014). For example, hydrophobicity of a peptide can be an important factor in antioxidative activity and may increase accessibility to hydrophobic targets (e.g.,

lipophilic fatty acids) (Chen et al., 1998). Therefore, the high antioxidative activity of PP8 and PP9 might be due to the presence of hydrophobic AA residues such as Leu or Val. Such peptides have been reported at the water–lipid interface to facilitate access to scavenge free radicals generated in the lipid phase (Ranathunga et al., 2006). Further, the aromatic AA tyrosine (Y) in the third position of PP8 and PP9, and phenylalanine (F) in PP5, may be active sites on antioxidants for radical exchange (Chen et al., 1998). This study has shown that PP8 and PP9 contain Tyr–Pro–Glu at the C-terminus of the peptides, which is likely to have been a factor in inducing the release of a hydrogen atom of phenolic hydroxyl in tyrosine, to enhance the antioxidative activity (Sah et al., 2016b, c). In contrast, the indole ring in the tryptophan-containing PP1–3 and 6 and the pyrrolidine ring in the proline-containing PP1–7 may serve as hydrogen donors through hydroxyl groups, acting as anti-hydroxyl radicals (Zou et al., 2016).

A number of *in vitro* antioxidant peptides isolated from milk proteins has been reported with different structures and AA sequences (Sah et al., 2016b, c). A hexapeptide (Tyr–Phe–Tyr–Pro–Glu–Leu) derived from digested casein (αs_1-CN) showed strong superoxide anion scavenging activity (Suetsuna et al., 2000). However, deletion of the last three AAs from the N-terminus of the hexapeptide resulted in a loss of antioxidant activity, indicating the importance of tyrosine and phenylalanine in antioxidant peptides. Further, whey protein (β-Lg) digested by Corolase PP® produced an antioxidant peptide with AA sequence Trp–Tyr–Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile, and Trp, Tyr and Met were the main contributors to the measured antioxidative activity. The current results show that cleaving the Ser–Lys bond from PP9 resulted in decrease antioxidant activity, suggesting that in some instances a peptide bond or structural conformation of a peptide can enhance or decrease antioxidative activity (Pihlanto, 2006a; Ha et al., 2015). On the other hand, the differing levels of antioxidative activity among the nine peptides

examined could be due to primary composition and location of AAs in the peptide sequence. For example, hydrophobic AAs (Val or Leu at the N-terminus) and Pro, His, or Tyr in the AA sequences of peptides may lead to linoleic acid peroxidation (Chen et al., 1995). Therefore, some AAs, such as Tyr, Leu, Val and Pro in PP8 and PP9 may have a key role in scavenging of free radicals through electron and/or hydrogen donation, and thus represent promising candidates among emerging functional foods with special health.

4.5 Conclusions

This study showed that cold storage of milk fermented by *L*. 1315 may provide more than a simple process for preservation of milk and extension of its shelf life. Via multiplex reactions during culture growth, fermented milk is enriched with different bioactive compounds, having various bioactivities and potential antioxidative properties that may maintain oxidation balance in the human body. *L*. 1315 was able to release bioactive peptides from bovine milk proteins that are associated with strain growth during fermentation and cold storage. However, digestive enzymes greatly decreased the bioactivities of the peptides. The nine peptides (PP1–PP9) derived from bovine β-CN identified were (^{142}SWMHQPHQPLPP^{153}, ^{142}SWMHQPHQPLPPT^{154}, ^{140}LQSWMHQPHQPLPP^{153}, ^{164}SLSQSKVLPVPQ^{175}, ^{183}RDMPIQAF^{190}, ^{140}LQSWMHQPHQPLPPT^{154}, ^{62}FPGPIPN^{68}, ^{169}KVLPVPQKAVPYPQ^{182} and ^{166}SQSKVLPVPQKAVPYPQ^{182}). PP8 and 9 showed free radical scavenging activity in a dose-dependent manner. Understanding of the stability of these antioxidant peptides against *in vitro* GI enzymes and their derivatives requires further investigation.

Chapter 5: Identification of Colon Cancer Inhibitory Peptides from Bovine Beta Casein in Fermented Milk

5.1 Introduction

Cancer, a disease characterised by uncontrolled cell growth, is a key public health concern worldwide (Siegel et al., 2013; Torre et al., 2016). It is a leading cause of death, with more than 14.1 million new cancer cases and around 8.2 million mortality cases reported in 2012 (Torre et al., 2016). Among cancers, colon cancer (also known as bowel cancer) has been reported as one of the most commonly diagnosed cancers in both males and females (Jemal et al., 2011). According to 2012 data (Ferlay et al., 2015), there were more than half a million deaths caused by colon cancer from 1.4 million new colorectal cancer cases in that year. A number of different treatment regimens are employed for patients suffering from colon cancer including surgery, monoclonal antibodies, radiotherapy and chemotherapy (Woude & Klein, 2009). The main application of anticarcinogenic agents is to induce cell death via different mechanisms including altering the tubulin–microtubule balance (Stanton et al., 2011), inhibiting angiogenesis (Bracci et al., 2014) and inducing apoptosis (programmed cell death) (Sah et al., 2015b). The induction of apoptosis is a key element of chemotherapeutic drug treatment (Ziegler & Kung, 2008). A number of targeted therapeutics have been proposed for the prevention and treatment of colon cancer. These include MMP inhibitors (Otero-Estévez et al., 2015), VEGF inhibitors (Ferrara & Adamis, 2016), histone deacetylase inhibitors (Mottamal et al., 2015), cyclooxygenase-2 inhibitors (Tietz et al., 2016), farnesyltransferase inhibitors (Knickelbein & Zhang, 2015), S100P/RAGE interaction inhibitors (Moravkova et al., 2016), *p53*-MDM2 inhibitors (Pereira et al., 2016), Hsp90 inhibitors (Wang et al., 2016), proteasome inhibitors (Krętowski et al., 2015), TKIs (Kircher et al., 2016) and

antioxidants (Sah et al., 2015b). However, side effects from the use of these anticancer drugs, such as alopecia (hair loss), fatigue, nausea and vomiting, are serious drawbacks (Jaehde et al., 2008). In addition, long-term treatment with chemotherapy drugs can lead to cancer cell resistance to these treatments (Housman et al., 2014). These significant side effects, in addition to high treatment cost, have prompted research into natural anticancer compounds including bioactive peptides from milk proteins, which may provide alternative treatment strategies to maximise the efficiency of cancer treatment with reduced toxicity and expense.

Antioxidant peptides present one class of anticarcinogenic peptides. Antioxidants are chemical compounds that suppress or delay oxidative stress by inhibiting oxidative processes (Srinivasan, 2014). Deleterious effects of ROS such as hydroxyl radicals, superoxide anion radicals and hydrogen peroxide molecules, along with reactive nitrogen species including peroxynitrites and nitrogen oxides, result in oxidative DNA damage. This damage is predominantly linked with the initiation process of carcinogenesis. Minimising oxidative damage would represent a significant advance in the prevention or treatment of cancer; antioxidants are able to reduce free radical formation and prevent oxidising chain reactions (Sah et al., 2016b). This correlation has generated great interest in the development of antioxidant-based anticancer drugs (Tekiner-Gulbas et al., 2013; Mut-Salud et al., 2015).

Milk proteins have emerged as a prolific source of biologically active peptides, which are part of the primary structure of proteins (mainly caseins) and could modulate the physiology of consumers following proteolytic release of peptides with anticarcinogenic potential (Sah et al., 2016b). One way to obtain these bioactive peptides is by direct release from casein proteins by proteolytic actions of LAB commonly used in the dairy industry (Akalin, 2014; Sah et al., 2015b). Among LAB, *L. helveticus* strains are

considered to be highly proteolytic cultures for applications in milk fermentations (Elfahri et al., 2014). Extracellular proteinases followed by intracellular peptidases of *L. helveticus* strains have the potential to release bioactive peptides such as ACE, immunomodulatory and antioxidant peptides from milk proteins during fermentation (Elfahri et al., 2014). Among *L. helveticus* strains examined in a previous study (Elfahri et al., 2015), crude peptide extracts from milk fermented by *L. helveticus* 1315 caused greater growth inhibition in human colorectal cancer cell line HT-29 than did other *L. helveticus* strains, with no significant cytotoxic effect on human primary colon cell line T4056. However, the peptide(s) that may be responsible for such bioactivity has not been not investigated. The objective of this study was to further isolate and identify potential antiproliferative peptides from milk fermented by *L. helveticus* 1315 on colon cancer cell line HT-29, and to evaluate the antioxidant and anti-colon cancer activities of these peptides after *in vitro* GI digestion. The mechanism of anti-colon cancer activity (apoptotic activity, caspace-3 and cell cycle arrest) was also examined.

5.2 Materials and Methods

5.2.1 Chemicals and Substrates

Antibiotic/antimycotic solution (100 mL), staurosporine solution (from *Streptomyces* sp.), ABTS, DPPH, pepsin from porcine gastric mucosa and pancreatin were purchased from Sigma Chemical Company (St Louis, MO, USA). RPMI-1640 growth medium and trypsin-EDTA (0.25%) were obtained from Life Technologies (Carlsbad, CA, USA). FBS was purchased from Bovogen Biologicals Pty Ltd. (Melbourne, Australia). CellTiter 96® AQueous One Solution reagent containing the tetrazolium compound MTS was purchased from Promega Corp. (Madison, WI, USA). CELLSTAR T75 flasks, 24- and 96-well flat-bottomed microplates (CELLSTAR®, Greiner Bio-One GmbH,

Frickenhausen, Germany) were procured from Interpath Services Pty., Ltd. (Heidelberg West, VIC, Australia).

5.2.2 Propagation of Cultures and Sample Preparation

Fermented milk was prepared as described by Elfahri et al. (2015) with some modifications. Briefly, 5 L of RSM (120 g/L) was heated to 85°C for 30 min followed by cooling to 40°C. The heated RSM was inoculated with 1% (v/v) of *L. helveticus strain* 1315 and incubated at 37°C until pH ~4.6 and then stored at 4°C for 14 days. SPEs were centrifuged at 16,000 × *g* using an Avanti J-26S XPI high-performance centrifuge (Beckman Coulter Inc., Brea, CA) at 4°C for 30 min. The supernatant was filtered through a 0.22-μm filter, and then collected and freeze-dried (Dynavac freeze drier; Dynavac Eng. Pty. Ltd., Melbourne, Australia) and stored at −80°C until further analysis. The protein content (mg/mL) of the SPEs was estimated according to Bradford (1976) using BSA (0.1–1.4 mg/mL) as a standard.

5.2.3 Preparation and Synthesis of Identified Peptides Released from Milk Fermented by *L.* 1315

After 14 days of cold storage, the water-soluble peptides that showed the highest anti-colon cancer activity were fractionated by injecting 1mL sample onto a preparative RP–HPLC system (Varian Inc., Palo Alto, CA, USA) equipped with a C_{18} monomeric column (10 mm, 300 Å, 22–250 mm; Grace Vydac, Hesperia, CA, USA). The peptides were eluted at a flow rate of 16 mL/min and detected at 214 nm. A crude peptide fraction with the highest inhibition against colon cancer HT-29 cell growth was collected and concentrated. Peptides of the potent fraction were identified using LC–MS/MS employing a quadrupole time-of-flight mass spectrometer (micrOTOF-Q; Bruker Daltonik GmbH, Bremen, Germany) coupled online to a nano-HPLC (Ultimate 3000;

Dionex Corporation, SunnyBrook, CA, USA). The data were compared against the Swiss-Prot databases using the MASCOT search engine (v. 2.4, Matrix Science Inc., London, UK). Nine peptides, SWMHQPHQPLPP, SWMHQPHQPLPPT, LQSWMHQPHQPLPP, SLSQSKVLPVPQ, RDMPIQAF, LQSWMHQPHQPLPPT, FPGPIPN, KVLPVPQKAVPYPQ and SQSKVLPVPQKAVPYPQ (designated PP1–PP9, respectively) derived from β-CN were identified, and synthesised by Mimotopes Pty Ltd (Clayton, VIC, Australia) at purity >95%. The identified peptides were further assessed against the HT-29 colon cancer cell line to measure growth inhibition as described by Elfahri et al. (2015). The two peptides that showed the highest antiproliferative activity were selected and exposed to *in vitro* GI digestion, and their hydrolysates were further assessed against DPPH and $ABTS^{\bullet+}$ free radicals and HT-29 colon cancer growth. The peptides derived from PP8 and PP9 digestion were identified using the above technique.

5.2.4 *In Vitro* Gastrointestinal Digestion of Peptides

The fate of PP8 and PP9 during *in vitro* GI digestion (Sah et al., 2016a; harmonised INFOGEST *in vitro* digestion method) was investigated to determine the effect of GI enzymes on the peptides and bioactivities after digestion. Briefly, 10 mL of each peptide (3 mg/mL Milli-Q water) was mixed with 7.5 mL of a freshly prepared simulated gastric fluid electrolyte stock solution and pH adjusted to pH 3.0. Pepsin was added and the volume was topped up to 20 mL with Milli-Q water. Digestion of peptide was performed at 37°C for 2 h in a shaking incubator at 120 rpm. Subsequently, 20 mL of gastric chyme was mixed with 11 mL of another simulated intestinal fluid electrolyte stock solution, followed by pH adjustment to 7.0. The volume was adjusted to 40 mL with Milli-Q water and the whole mixture was incubated at 37°C for an additional 2 h at the same speed for further digestion with pancreatin. The digestive enzyme activity was stopped by plunging

the solution into an ice bath for 10 min, followed by centrifugation for 30 minutes at 4,000 × g. The supernatant was syringe filtered (0.22 μm) and frozen at –80°C before lyophilisation (Dynavac FD-300, Dynavac Engineering Pty Ltd, Melbourne, Australia). Samples were stored at –80°C until required for further analysis against free radicals and colon cancer growth. Similarly, digestive enzyme solution alone was used for comparison.

5.2.5 Antioxidative Activity

5.2.5.1 DPPH Free Radical Scavenging Activity

The DPPH radical was used to determine the free radical scavenging activities as described by Elfahri et al. (2015) with some modifications. Briefly, each digested peptide (0.1, 0.2 and 0.4 mg protein/mL) was mixed with 0.1 mM DPPH dissolved in 95% methanol and vortexed for 30 seconds in the dark, and then sealed with parafilm sheet and incubated in the dark at room temperature for 30 min. The undigested peptide was used as control. Methanolic DPPH was used as a blank and methanol was used for the baseline correction. Ascorbic acid was used as a positive control for comparison. After incubation, the reduction of absorbance was measured at 517 nm using a UV/VIS spectrophotometer (Biochrom Ltd, Cambridge, England).

5.2.5.2 ABTS Radical Scavenging Activity

The $ABTS^{\bullet+}$ scavenging activity of undigested and digested peptides was evaluated according to the method described by Sah et al. (2015b) with some modifications. Briefly, $ABTS^{\bullet+}$ reagent was prepared fresh. To measure scavenging activity, precise aliquots of 10 μL of peptidase or DPP (at 0.1, 0.2 and 0.4 mg protein/mL) was added to 990 μL of the $ABTS^{\bullet+}$ reagent, vortexed for 30 seconds and then incubated at 30°C for 20 minutes

in the dark. Ascorbic acid was used as a positive control for comparison. $ABTS^{\bullet+}$ scavenging activity was measured at absorbance of 734 nm. Milli-Q water was used as a blank.

5.2.6 Assessment of Percentage Inhibition and IC_{50}

Radical scavenging activity of peptidase and DPP for PP8 and PP9, and positive control samples were expressed in terms of percentage inhibition of free radical. This was calculated using the formula ((A–B) / A) × 100, where A is the absorbance of the blank, and B is the absorbance of sample. The IC_{50} value was calculated according to Thenmozhi and Rajan (2015): $IC_{50\ was}$ defined as the concentration (in mg/mL) of a sample that produced a 50% antioxidant effect.

5.2.7 Cell Culture and Assay for Antiproliferative Activity of Peptides Against the HT-29 Human Colon Cancer Cell Line

The HT-29 cell line was obtained from the ATCC (Rockville, MD). Cells were grown in RPMI-1640 growth medium supplemented with 10% FBS (Invitrogen, Waltham, MA). The cells were incubated at 37°C in a humidified CO_2 incubator (New Brunswick™ Galaxy® 170 R; New Brunswick Scientific Co Inc., Edison, NJ, USA). The antiproliferative effect of peptides (PP1–PP9) or DPP8 and DPP9 on HT-29 cells were assessed by employing the MTS assay according to Sah et al. (2016a).

5.2.8 Apoptotic Activity of Undigested and Digested Peptides

Apoptotic activity of nondigestive and digestive peptides was assessed using a Muse Annexin V & Dead Cell Reagent (Merck Millipore Corporation) according to the manufacturer's instructions. Briefly, 350 µL of a logarithmically growing cell suspension in complete growth medium (~1.0×10^4 cells/mL) was dispensed into a well of a 24-well

flat-bottomed plate, and the plate was pre-incubated at 37°C for 24 h in the CO_2 incubator to allow cell adherence. The medium was replaced with 500 µL RPMI-1640 complete medium containing peptides at a concentration of 0.4 mg/mL. The treated cells were further incubated at 37°C for 48 h. Both floating and attached treated cells were harvested, washed with cold PBS, and resuspended in complete growth medium to a cell density of ~1.0×10^5 cells/mL. Precise aliquots of 100 µL of Annexin V & Dead Cell Reagent were mixed with the same volume of cell suspension for staining, and incubated in the dark at room temperature for 20 min. Cells were then analysed for apoptotic activity using a Muse Cell Analyser (Merck Millipore Corporation). Untreated cells were used as a negative control (blank) and cells treated with staurosporine were used as a positive control.

5.2.9 Measurement of the Caspase-3 Activity of Undigested and Digested Peptides

Proapoptotic caspase-3 activity was evaluated using a Colorimetric Caspase-3 Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. This assay is based on the release of a coloured p-nitroaniline molecule from the peptide substrate (Ac-DEVD-pNA) when cleaved by active caspase-3. HT-29 cancer cells were seeded into 24-well plates (5×10^4 cells/well) and incubated for 24 h to allow adherence. Following cellular adhesion, cells were treated with nondigested or digested peptides (0.4 mg/mL concentration), staurosporine (10 µg/mL concentration) as a positive control and untreated cells (uninduced cells) as a negative control, and then further incubated in a CO_2 incubator for 24 h. At the end of the incubation period, cells were harvested by centrifugation at 600 × g for 5 min at 4°C, washed in ice-cold PBS, lysed in lysis buffer (50 mM HEPES (pH 7.4), 5 mM CHAPS, and 5 mM dithiothreitol). The lysed cells were incubated for 20 min on ice and centrifuged at 16.000 × g for 15 min at 4°C. The supernatants were removed to new tubes. To start the caspace-3 reaction, cell lysates were

added to caspase-3 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (final concentration, 2 mM) in assay buffer. Similarly, a control reaction mixture contained cell lysates and specific caspase-3 inhibitor acetyl-DEVD-CHO (final concentration, 200 μM) in assay buffer. Each mixture was gently mixed before incubation for 2 h at 37°C and the absorbance was read at 405 nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.10 Cell Cycle Analysis

The effect of undigested and digested peptides on the cell cycle distribution of HT-29 cells was determined using Muse™ Cell Cycle Reagent (Merck Millipore Corporation) according to the manufacturer's instructions. Briefly, 1.5 mL of HT-29 cells growing in RPMI-1640 complete growth medium (cell density ~5.0×10^4 cells/mL) was placed into each well (6-well flat-bottomed tissue culture plate) and incubated at 37°C for 24 h in a 5% CO_2 atmosphere incubator to allow adherence of cells to the base of wells. The medium after this first incubation was replaced with growth medium (containing 0.1% FBS) and plates were further incubated for 48 h at 37°C. This step was performed to synchronise cells in the G_0 phase. The medium was then replaced with 2 mL of RPMI-1640 growth medium containing 10% FBS, 1% antibacterial–antimycotic solution and peptides at a concentration of 2 mg/mL. Similarly, in untreated-cell wells (as negative control), the medium was substituted with complete growth medium (10% FBS and antibacterial–antimycotic solution); while in positive control wells, the medium was replaced with complete growth medium containing staurosporine (10 μg/mL). The plate was covered and further incubated under 5% CO_2 atmosphere at 37°C for 24 h. Treated and untreated cancer cells were harvested by trypsinisation and resuspended in 300 μL of cold PBS. The cells were then fixed by treatment with 800 μL of ice-cold 70% ethanol overnight at –20°C. The fixed cells were collected by centrifugation (300 × g for 5 min

at room temperature) and washed with PBS. The washed cells were suspended in 200 µL of Muse™ Cell Cycle Reagent and incubated for 30 min in the dark at room temperature. After staining, the percentages of cells in the G_0/G_1, S and G_2/M phases were determined using the Muse Cell Analyser.

5.3 Statistical Analysis

Values are expressed as the means ± SEM. Significant differences were calculated using an ANOVA. These analyses were performed using SAS (1996) and $P < 0.05$ was used to determine significance.

5.4 Results and Discussion

5.4.1 Antiproliferative Activity of Isolated Peptides Against the HT-29 Colon Cancer Cell Line

Nine peptides isolated from the highest antioxidant activity fraction were assessed separately for their DPPH, $ABTS^{\bullet+}$ and hydroxyl radical scavenging activities. These peptides exhibited differing antioxidant activities (Chapter 4) in a dose-dependent manner. Therefore, the isolated peptides were further investigated for their potential antiproliferative effect against HT-29 cell growth using the MTS assay. All nine peptides reduced proliferation of HT-29 cells to varying degrees in a dose-dependent manner, with the exception of PP3 where no activity was determined, probably due to peptide structure or AA composition. As reported in Tables 5.1 and 5.4, PP8 and PP9 showed significantly higher antiproliferative activity at 0.4 mg/mL (24.82 ± 0.60% and 26.91 ± 0.60%, respectively) than did the other fractions, although they were not as potent as the positive control (staurosporine) at 0.1 mg/mL (81.7 ± 0.60%). Consequently, PP8 and PP9 were selected for further investigation to determine the potential mechanism of HT-29 growth

inhibition of these peptides. Apparently PP8 and PP9 induced apoptosis in the HT-29 cell line, which was not seen in untreated cancer cells (Figure 5.1A, C and F).

Table 5.1 The inhibitory effect of nine purified peptides (PP1–PP9) at concentrations of 0.1, 0.2 and 0.4 mg/mL on the growth of HT-29 cancer cells using the MTS assay; staurosporine (concentration 0.025, 0.05 and 0.1 mg/mL) was used as a positive control

Inhibition of HT-29 cancer cells (%)											
	Peptide										
Concentration mg/mL	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	Staurosporine	Concentration of staurosporine (mg/mL)
0.1	0	0	0	0	0	0.05	0.08	10.08	6.33	42.13	0.025
0.2	0	0.02	0	0.28	0	2.97	0	19.19	17.31	55.52	0.05
0.4	0.78	0.09	0	5.33	0.16	5.48	1.5	24.82	26.91	81.72	0.1
SEM ±							0.60				

Both selected peptides induced early and late apoptosis, and the total apoptotic percentage of PP8 and PP9 was significantly higher (21.28% and 18.22%, respectively) than for untreated cancer cells (12.96%) (Figure 5.2). These results indicate that these two peptides have an antiproliferative effect on HT-29 cells via apoptosis induction. Similarly, a study conducted by Sah et al. (2016b) showed induction of cell apoptosis in the HT-29 cell line after exposure to two peptides (YQEPVLGPVRGPFPIIV and SLPQNIPPLTQTPVVVPPF) derived from bovine β-CN. Moreover, the activation of caspases (pro-caspases) in cancer cells is central for the occurrence of apoptosis. Caspase-3 (cysteine–aspartic acid protease caspase family), as one of the cysteine proteases, plays a vital role in the execution phase of apoptosis.

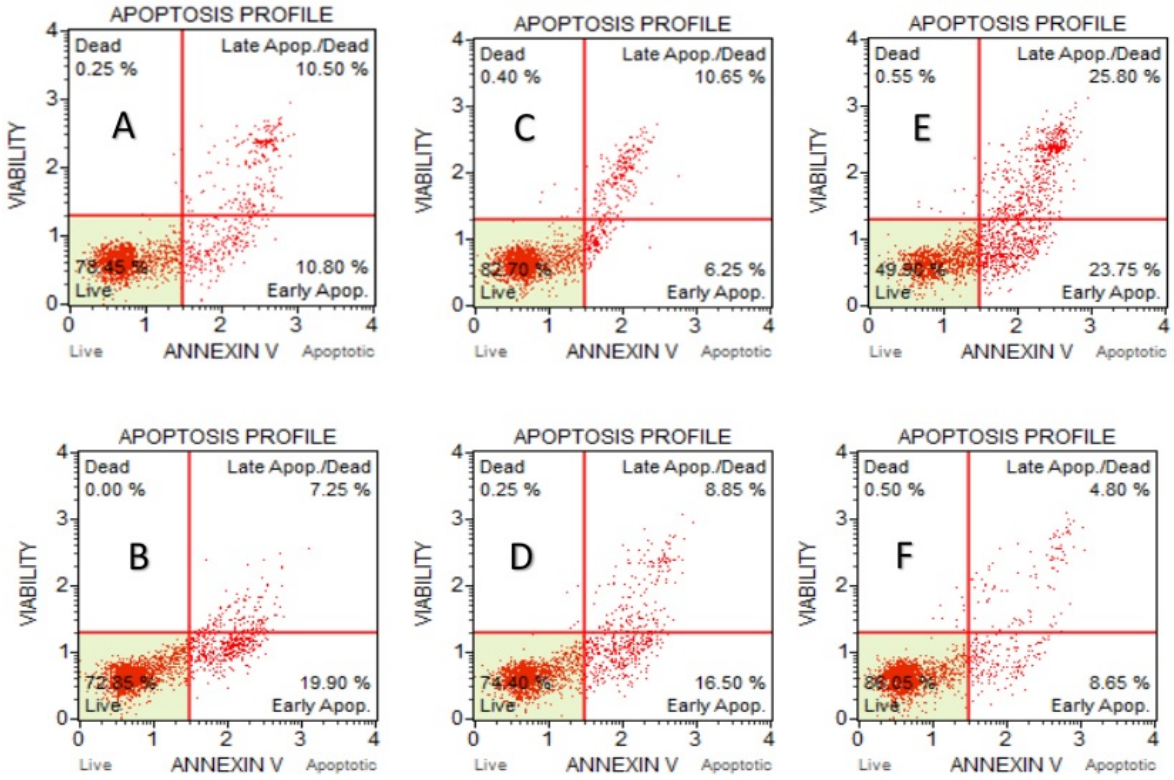

Figure 5.1 Apoptotic profile peptides PP8 (A), PP9 (C) and their digestates DPP8 (B) and DPP9 (D) effectively induced apoptosis in HT-29 cancer cells. Cells untreated (F) or treated with staurosporine (E) are the negative and positive controls, respectively; the lower left value of each panel is for viable cells; the lower right value is for early apoptotic cells; the upper right panel is for late apoptotic cells; and the upper left panel is for necrotic cells

The main two apoptotic pathways—intrinsic (mitochondrial) and extrinsic (death receptor)—activate a cascade of caspases, including caspase-3, that involve cleavage of aspartic acid-containing motifs, leading to induction of apoptosis (McIlwain et al., 2015). The results of a caspase-3 activity assay were assessed using a caspase-3 colourimetric assay and are presented in Figure 5.3. The mean level of caspase-3 activity increased significantly in HT-29 cells treated with 0.4 mg/mL of PP8 and PP9, in comparison with untreated cells (Figure 5.3).

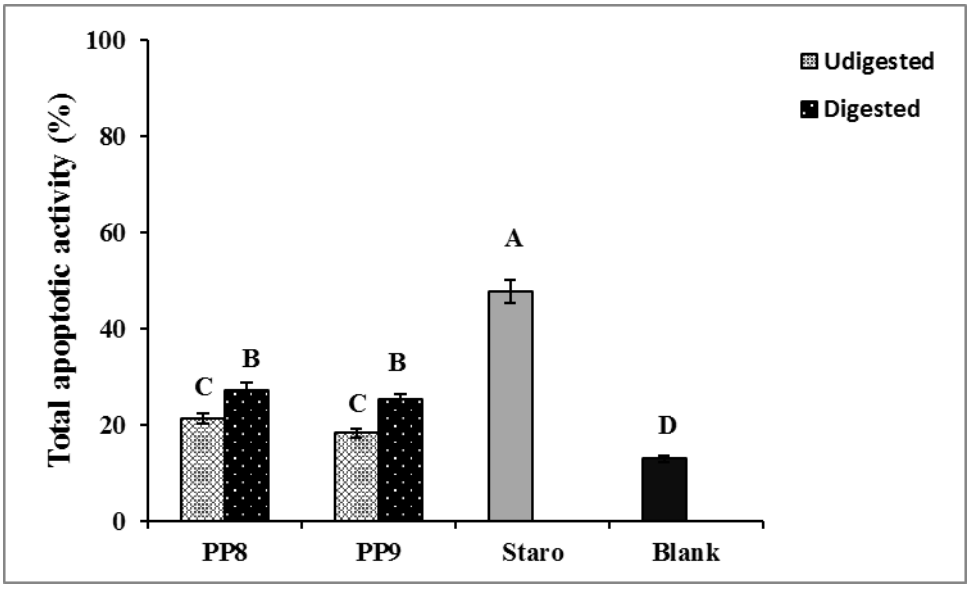

Figure 5.2 Total apoptotic activity induced by peptides (PP8, PP9, DPP8 and DPP9) in HT-29 cancer cells. Results are expressed as means ± standard deviation (*n* = 3); values with different letters are significantly different (*P* < 0.05)

The contribution of caspase-3 to the isolated peptide-induced apoptosis in HT-29 cells was confirmed by adding a caspase-3 inhibitor (Ac-DEVD-HO), which blocked caspase-3 activity in treated cells, indicating that cell death was caspase-3-dependent. Aside from proapoptotic caspase-3 and apoptotic activity, cell cycle arrest (retardation of cell division) is another crucial pathway to inhibit the proliferation of cancer cells.

Apoptosis and cell cycle arrest are the major controlling mechanisms for cell growth; this means that apoptosis may be induced when a disturbance in the cell cycle progression occurs at certain checkpoints (Orren et al., 1997). A number of effective chemotherapeutic drugs against cancer cause cell cycle arrest through microtubule damage (Shapiro & Harper, 1999). The effects of the cell cycle distribution (G0/G1, S and G2/M phases) were analysed using the Muse Cell Analyser to determine the mechanism associated with the growth inhibitory effect of PP8 and PP9 on HT-29 cancer cells (Figure 5.4A and C). Cells treated with each peptide showed a significant increase in the population of G2/M phase cells (Figure 5.5) from 17.74% in untreated cells

(negative control; Figure 5.4E) to 23.3% and 23.26% in cells treated with PP8 and PP9, respectively (Figure 5.4A and C). However, these percentages were significantly lower than for the positive control (0.025 mg/mL)-treated cells, indicating that peptides arrested HT-29 cells during G2/M cell cycle transition (Figure 5.4F and 5.5). These results are in line with those of Sah et al. (2016b), who found that two peptides from β-CN reduced HT-29 cell proliferation through induction of apoptosis and cell cycle arrest at the G2/M phase. On the other hand, De Simone et al. (2009) found a significant reduction in Caco-2 proliferation after exposure to a different set of peptides isolated from Mozzarella di Bufala waste whey, which modulated the cell cycle at G1 phase.

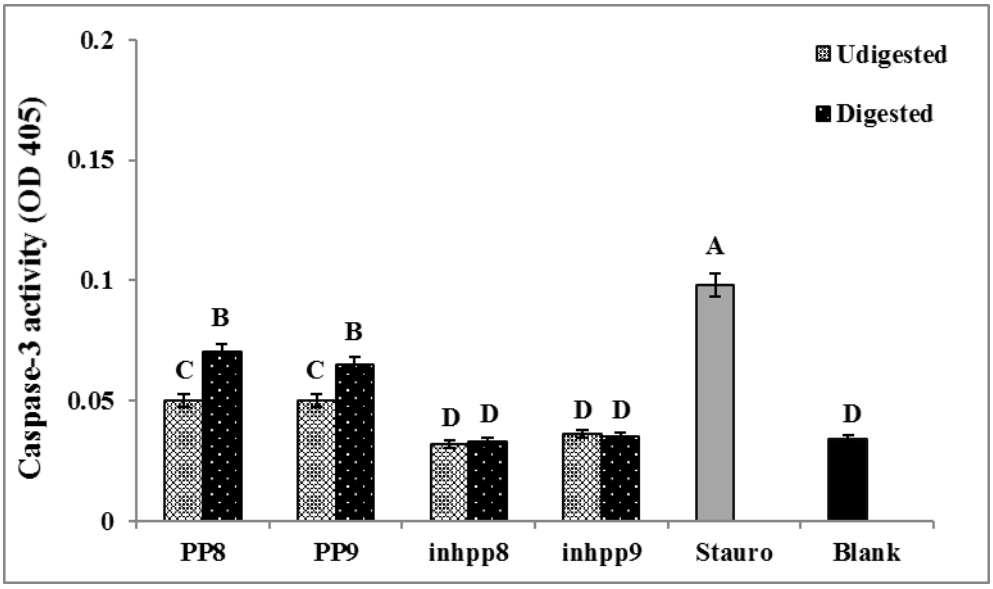

Figure 5.3 Colourimetric assay of caspase-3 activity; HT-29 cancer cells treated with peptides (PP8, PP9, DPP8 and DPP9) or staurosporine as a positive control, and untreated cells (blank); inhPP8 and inhPP9 are cells treated by adding caspase-3 inhibitor (Ac-DEVD-HO). Results are expressed as means ± standard deviation (*n* = 3); values with different letters are significantly different ($P < 0.05$)

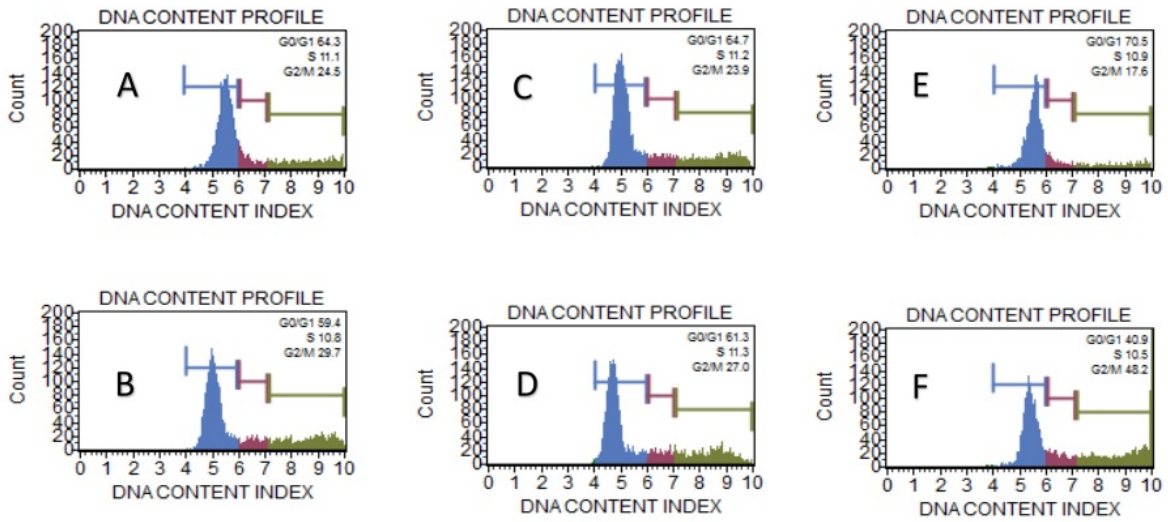

Figure 5.4 The cell cycle distribution (% cells in the G0/G1, S and G2/M phases) of HT-29 cancer cells induced by peptides PP8 (A), PP9 (C) and their digestates DPP8 (B) and DPP9 (D), in comparison with cells untreated (E) or treated with staurosporine (F)

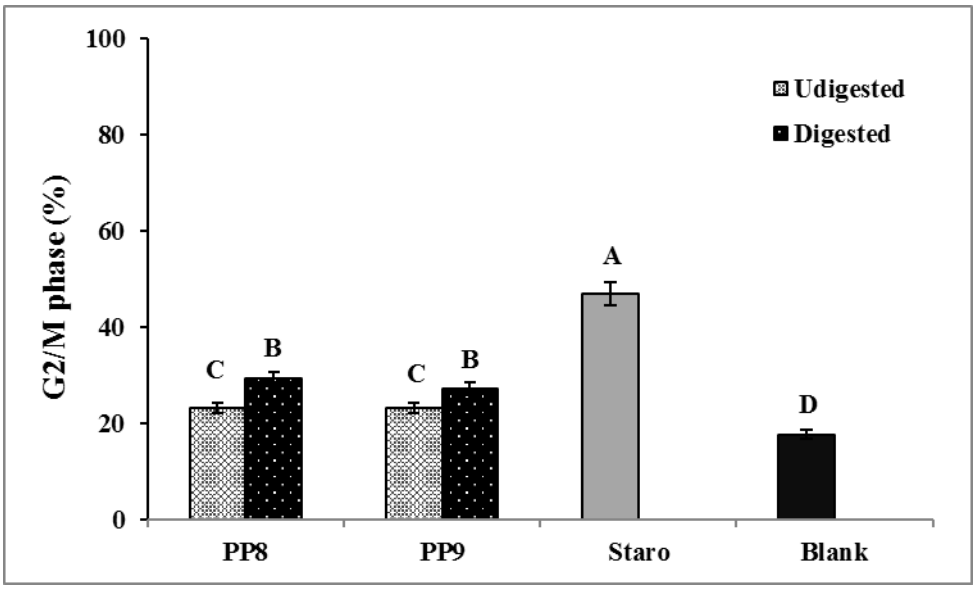

Figure 5.5 The cell population at the G2/M phase of HT-29 cancer cells treated with or without peptides (PP8, PP9, DPP8 and DPP9) compared with untreated cells (blank) and cells treated with staurosporine (Staro) as a positive control. Results are expressed as means ± standard deviation (n = 3); values with different letters are significantly different ($P < 0.05$)

5.4.2 Stability of Peptides During *in Vitro* Gastrointestinal Digestion

One of the greatest challenges in relation to the therapeutic use of isolated antiproliferative peptides is their *in vivo* efficacy after oral administration (Thanki et al., 2013). Proteolytic enzymes in the GIT under certain pH conditions may influence the structure and bioactivity of these peptides before they are transported across the plasma membrane of the epithelial cells of the intestinal enterocytes (Segura-Campos et al., 2011). The digestion of both isolated PP8 and PP9 resulted in cleavage of these peptides due to pepsin and/or pancreatin activities (Table. 5.5). Apparently, many peptide bonds were cleaved and new short peptides were formed. However, it is not clear whether one or both digestive enzymes were responsible for the release of peptides from PP8 and PP9. When a peptide is exposed to pepsin enzyme, aromatic and hydrophobic AAs at the C-terminus are preferred sites for pepsin activity. However, pancreatin has numerous cleavage sites in milk proteins and thus may hydrolyse isolated peptides more randomly.

5.4.3 Antioxidant Activity of Digested Peptides

The two digested peptide samples, DPP8 and DPP9, assessed for antioxidant activity by measuring DPPH and $ABTS^{\bullet+}$ radical scavenging assays, showed promising results. No antioxidant activity was observed with digestive enzyme alone.

As shown in Table 5.2, each digested peptide sample (DPP8 and DPP9) exhibited significant DPPH radical scavenging activity (64.18 ± 0.88% and 50.70 ± 0.88% respectively), with IC_{50} values of 0.31 ± 0.009 mg/mL for DPP8 and 0.39 ± 0.009 mg/mL for DPP9, compared with IC_{50} values for undigested PP8 and PP9 of 0.62 ± 0.009 mg/mL and 0.78 ± 0.009 mg/mL respectively). Similarly, ascorbic acid (the positive control) at a concentration of 0.1 mg/mL showed high activity against DPPH free radicals, indicating that digestion of isolated peptides enhanced DPPH free radical scavenging activity

(Table. 5.2). Further, $ABTS^{\bullet+}$ radical scavenging capacity with DPP8 and DPP9 showed that antioxidants were scavenged through transfer of an electron and/or hydrogen atom to radical molecules (Sah et al., 2016b, c) and exhibited significant $ABTS^{\bullet+}$ scavenging capacities (Table 5.3).

Table 5.2 Antioxidative capacity as measured using the DPPH method for PP8, PP9 and their digestates (DPP8 and DPP9) at concentrations of 0.1, 0.2 and 0.4 mg/mL; ascorbic acid was used as a positive control at concentrations of 0.025, 0.05 and 0.1 mg/mL

DPPH radical scavenging activity (%)						
Peptide →	PP8		PP9		Ascorbic acid	Ascorbic acid concentration
Peptide concentration (mg/mL) ↓	Undigested	Digested	Undigested	Digested		(mg/mL) ↓
0.1	14.19	32.99	12.15	37.25	20.66	0.025
0.2	28.65	49.14	23.48	49.37	53.36	0.050
0.4	32.21	64.18	25.37	50.70	81.93	0.100
SEM ±	0.88					
IC_{50} (mg/mL)	0.62	0.31	0.78	0.39	0.06	
SEM ±	0.009					

These activities were observed in a dose-dependent manner with IC_{50} values of 0.32 ± 0.005 and 0.28 ± 0.005 mg/mL for DPP8 and DPP9, respectively, in comparison with that for L-ascorbic acid (IC_{50} of 0.06 mg/mL). The results indicate that both hydrolysed peptides might be potent radical scavengers by providing electrons and/or hydrogen

atoms to excess unpaired oxidants, and thus prevent damage caused by radicals in the body. Antioxidative properties of peptides depend mainly on factors such as peptide structure, AA composition, C- and N-terminus, length and weight of the peptide sequence, concentration, and hydrophobicity and charge of the AAs (Li & Yu, 2015). For example, peptides containing AAs such as Leu, Pro, Trp, Met, Cys, Arg, Ala and Tyr have been shown to display strong antioxidant activity (Sah et al., 2016b; Zou et al., 2016).

Table 5.3 Antioxidative capacity, as assessed using the ABTS$^{\bullet+}$ method, of PP8, PP9 and their digestates (DPP8 and DPP9) at concentrations of 0.1, 0.2 and 0.4 mg/mL; ascorbic acid was used as positive control at concentrations of 0.025, 0.05 and 0.1 mg/mL

ABTS$^{\bullet+}$ radical scavenging activity (%)

Peptide →	PP8		PP9		Ascorbic acid	Ascorbic acid concentration
Peptide concentration (mg/mL) ↓	Undigested	Digested	Undigested	Digested		(mg/mL) ↓
0.1	12.85	31.42	12.80	34.96	18.45	0.025
0.2	23.78	46.36	25.14	44.41	48.19	0.050
0.4	27.14	60.74	28.67	69.68	77.55	0.100
SEM ±	0.93					
IC_{50} (mg/mL)	0.73	0.32	0.69	0.28	0.06	
SEM ±	0.005					

In addition, when hydrophobic AAs such as Val or Leu are included in a peptide sequence, they may play a crucial role in increasing antioxidant activity by enabling greater access by peptides to free radicals generated at the lipid phase, consequently neutralising their detrimental effects (Liu et al., 2016). Therefore, both digested peptides may contain one or more such AAs, which may contribute to free radical scavenging

activity. These may provide chemotherapeutic potential in the management and treatment of various diseases caused by oxidation, including cancer (Sosa et al., 2013; Sah et al., 2015b).

5.4.4 Antiproliferative Activity of Digested Peptides Against HT-29 Colon Cancer Cells

In this study, *in vitro* antiproliferative activity against colon cancer cells (HT-29) by the digested peptides DPP8 and DPP9 was investigated using the MTS assay (Table 5.4). Both digested peptides inhibited the proliferation of HT-29 cells to varying degrees in a dose-dependent manner. This activity was not observed in digestive enzymes alone. DPP8 and DPP9 significantly reduced the proliferation of cancer cells, by 60.21 ± 1.18% (IC_{50} = 0.33 mg/mL) and 50.70 ± 1.18% (IC_{50} = 0.39 mg/mL), respectively, in comparison with PP8 and PP9 and staurosporine (Table 5.4). There was no antiproliferative activity observed with digestive enzyme alone. Moreover, both digested peptides induced HT-29 cells apoptosis in early and late stages (Figure 5.1B and D). The total apoptotic cells increased significantly from 12.96 ± 0.56% in untreated cells (negative control) to 27.26% and 25.17% in cells treated with DPP8 and DPP9, respectively, compared with staurosporine (Figure 5.1E). These results indicate that death of HT-29 cells was caused by peptide interactions with the cells through apoptotic mechanism, similar to Wang et al. (2013) and Sah et al. (2016c) who reported the induction of apoptosis in SKOV3 human ovarian cancer and HT-29 colon cancer cells, respectively, after treatment with peptides derived from bovine β-CN.

Table 5.4 The inhibitory effect of PP8, PP9 and their digestates (DPP8 and DPP9) at concentrations of 0.1, 0.2 and 0.4 mg/mL on the growth of HT-29 cancer cells using the MTS assay; staurosporine (at concentrations of 0.025, 0.05 and 0.1 mg/mL) was used as a positive control

Inhibition of HT-29 cancer cells (%)

Peptide →	PP8		PP9		Staurosporine	Staurosporine concentration (mg/mL) ↓
Peptide concentration (mg/mL) ↓	Undigested	Digested	Undigested	Digested		
0.1	10.08	29.59	6.33	27.36	42.13	0.025
0.2	19.19	50.18	17.31	44.15	55.52	0.050
0.4	24.82	60.21	26.91	50.70	81.72	0.100
SEM ±	1.18					
IC_{50} (mg/mL)	0.8	0.33	0.74	0.39	0.06	
SEM ±	0.03					

5.4.5 Caspase-3 and Cell Cycle Arrest Activities

Caspase-3 activity was assessed using a calorimetric assay measuring the formation of *p*-nitroanilide at 405 nm using a microplate spectrophotometer (Figure 5.3). Caspase-3 activity increased significantly from 0.03 in the blank sample (untreated cells and after adding caspase-3 inhibitor to a cell sample treated with peptides) to 0.07 and 0.06 in HT-29 cancer cells treated with DPP8 and DPP9, respectively (Figure 5.3). These results reveal that digestion of isolated PP8 and PP9 enhanced growth inhibition in HT-29 cancer cells via induction of apoptosis resulting from caspase-3 activation. Further, Figure 5.5 illustrated that the population of G2/M was significantly higher in cancer cells treated with DPP8 (29.34%) and DPP9 (27.17%) than with undigested PP8 and PP9 (23.30% and 23.26%, respectively). When cell cycle arrest in treated cells (Figure 5.4B and D) was compared with that in untreated cells (Figure 5.4E) and cells treated with

staurosporine (Figure 5.4F), it suggested that digested peptides may have affected the cell cycle of HT-29 cancer cells by arresting cells at the G2/M transition (Sah et al., 2016b). However, not all peptides can arrest cancer cells at the same cell cycle checkpoint (De Simone et al., 2011; Sah et al., 2016b). The different cytotoxic effects of peptides on cancer cells is dependent on many features, such as AA sequence, composition and concentration of peptides. Further, the type of cancer cells, growth medium, sample preparation and laboratory conditions may also affect the results. De Simone et al. (2011) found that the cytotoxic effect of a sub-fraction (f3) peptide extract derived from buffalo cheese acid whey on H-Caco-2 cells was active at the G1/G0 phase of the cell cycle. Therefore, the digested peptides may have potential advantages in the development of nutraceuticals and cancer therapy.

5.4.6 Identification of Antioxidant and Antiproliferative Peptides from DPP8 and DPP9

The increased antioxidant and antiproliferative activities of identified peptides (DPP8 and DPP9) as a result of further hydrolysis on PP8 and PP9 are presented in Tables 5.2, 5.3 and 5.4. The digestion of PP8 resulted in eight peptides, while 13 peptides arose from PP9 digestion (Table 5.5). As the findings revealed higher free radical scavenging activities against DPPH by DPP8 and $ABTS^{\bullet+}$ by DPP9, and higher inhibition against HT-29 cell proliferation by DPP8, it is difficult to determine which peptide is responsible for antioxidation and/or anti-HT-29 cell proliferation; further investigation assessing each peptide individually for their bioactivity is thus necessary. Nevertheless, some digests of PP8 and PP9, such as the VLPVPQK and SKVLPVPQK peptides, may provide these bioactivities (Ha et al., 2015); proline (P) and valine (V) AAs are dominant in these peptide sequences, and most likely are the key factors in their bioactivity (Sah et al., 2016b). Further, the formation of some of these peptides has been reported as a result of

GI digestion (Ha et al., 2015; Shanmugam et al., 2015). These findings require further investigation to assess the bioactivity of each peptide from DPP8 and DPP9.

Table 5.5 Identified peptides of PP8 and PP9 after *in vitro* digestion

Digested peptide	Identified peptides after digestion
PP8 (KVLPVPQKAVPYPQ)	VLPVPQK, KAVPYPQ, VLPVPQKA, KVLPVPQK, LPVPQKAVPYP, VLPVPQKAVPYP, LPVPQKAVPYPQ, VLPVPQKAVPYPQ
PP9 (SQSKVLPVPQKAVPYPQ)	VLPVPQK, KAVPYPQ, VLPVPQKA, KVLPVPQK, SKVLPVPQK, SQSKVLPVPQ, LPVPQKAVPYP, SQSKVLPVPQK, VLPVPQKAVPYP, LPVPQKAVPYPQ, VLPVPQKAVPYPQ, KVLPVPQKAVPYPQ, SKVLPVPQKAVPYPQ

5.5 Conclusions

This study has shown that *L. helveticus* 1315 is able to release bioactive peptides from bovine milk proteins during milk fermentation. Two peptides derived from β-CN, among nine peptides examined, exhibited moderate antiproliferative activity against HT-29 human colon cancer cells in a dose-dependent manner. However, after digestion of PP8 and PP9, anti-colon cancer activity increased significantly, with IC_{50} values of 0.33 and 0.39 mg/mL for DPP8, respectively. The peptides identified during milk fermented by *L. helveticus* 1315 followed by *in vitro* GI digestion could be used as potential anticarcinogenic drugs and/or to reduce oxidative stress-mediated diseases and disorders including cancers. Further studies are still required to investigate which proteinase and/or peptidase is responsible for releasing those peptides before GI digestion to avoid further hydrolysis of the peptides by other bacterial peptidase and to minimise the cost and time for such peptide release. Animal and clinical trials are also needed to confirm these bioactivities.

Chapter 6: General Conclusions and Recommendations for Further Studies

6.1 Introduction

L. helveticus strains are widely used as a starter culture in some dairy products including cheeses, yoghurt and fermented milk (Hassan & Frank, 2001; Gatti et al., 2004). Moreover, many bioactive peptides including antihypertensive and immunomodulatory peptides have been identified and linked to their release by several *L. helveticus* strains during their growth in milk due to their high proteolytic specificity on milk proteins, mainly caseins (Laffineur et al., 1996; LeBlanc et al., 2002; Gobbetti et al., 2010). The proteolytic pattern of these strains is commonly associated with milk as the growth medium. Milk proteins (mainly caseins) hydrolysed by *L. helveticus* strains usually result in the release of bioactive components, including bioactive peptides, as a result of metabolic activities (Daliri et al., 2017). Proteolytic enzymes, mainly proteinases and peptidases, are an important factor enabling *L. helveticus* to grow well in milk. Besides, these enzymes are able to release bioactive peptides during milk fermentation, as a function of strain and fermentation time (Wakai et al., 2012; Elfahri et al., 2015), reflecting differences in proteolytic systems. The differences are probably due to proteinase and/or peptidase specificity, which is subject to factors including pH, temperature, incubation and storage time (Elfahri et al., 2015). ACE-I peptides such as IPP and VPP are the most common bioactive peptides, and are associated with *L. helveticus* proteolytic enzyme activity on caseins (Beltrán-Barrientos et al., 2016). However, a few studies have attempted to investigate the effects of *L. helveticus* strains on the release of antioxidative and anti-colon cancer peptides from milk proteins during milk fermentation and storage, with low cytotoxicity to healthy colon calls.

The proteolytic activity of *L. helveticus* strains has been found to be higher than some other LAB (Elfahri et al., 2014). This provides more opportunities to release more peptides with *in vitro* bioactive properties. In contrast, some bioactive peptides such as antioxidative and anti-colon cancer peptides have different peptide structures (AA sequence and composition) although they exhibit the same bioactivity. This makes it difficult to determine the interaction between bioactive peptide and the target site *in vivo*. Therefore, *in vitro* bioactivities can be enhanced or decreased, or inactivated *in vivo* due to hydrolysis by digestive proteolytic enzymes; thus, *in vitro* bioactive peptides must be resistant to GIT digestion, and absorbed, to reach the target organ at the desired concentrations.

6.2 General Conclusions

Four strains of *L. helveticus* (ASCC953, ASCC474, ASCC1188 and ASCC1315) were able to grow under low acidic conditions in bovine skimmed milk; all released antioxidative and anticarcinogenic compounds at different levels during fermentation *in vitro*, but no such bioactivity was observed in unfermented milk. The differences between these bioactivities indicate strain specificity. The inhibition of HT-29 colon cancer cell growth in milk fermented by *L. helveticus* 1315 was the highest among the examined strains. More importantly, this soluble extract of this fermented milk had no significant inhibition on primary colon cells T4056. These findings indicate that *L. helveticus* strains in milk have a large effect on the release of bioactive compounds with multifunctional properties, and these activities appear highly strain and fermentation time dependent. With further investigations, the highest proteolytic *L.* 1315 strain released bioactive peptides from casein, indicating that proteolytic pattern, strain growth during

fermentation and cold storage are the main factors in production of such bioactivity. However, digestive enzymes were most likely hydrolysed these bioactive peptides.

Nine peptides (PP1–PP9) were released and identified from bovine β-CN (fragments: 142–153, 142–154, 140–153, 164–175, 183–190, 140–154, 62–68, 169–182 and 166–182). PP8 and PP9 showed higher antioxidant activity than other purified peptides, in a dose-dependent manner. PP8 and PP9 were also assessed for their anticancer activity. Both peptides exhibited moderate anticarcinogenic activity against HT-29 human colon cancer cells in a dose-dependent manner. However, when PP8 and PP9 were exposed to GI digestion, their anti-DPPH and $ABTS^{\bullet+}$ free radical activity and HT-29 cancer cell growth inhibition activity increased significantly, also in a dose-dependent manner, with IC_{50} values of 0.31 for DPP8, 0.28 for DPP9 and 0.33 mg/mL for DPP8 respectively, indicating that the bioactivities of PP8 and PP9 contained in milk fermented by *L. helveticus* 1315 may increase after GI digestion. Therefore, these peptides may increase antioxidant activity, and antiproliferative activity of HT-29 cancer cells *in vivo* through induction of apoptosis resulting in G2/M cell cycle arrest. This might help in preventing development or reducing the severity of oxidative stress-mediated diseases and disorders including cancer, with consisting dosing.

6.3 The Outcomes of this Study

The outcomes of this investigation will enable these bioactive peptides at desired concentrations to enhance immunity against free radical damage for cancer patients while they undergo cancer drug treatment. This study has revealed that it is possible to support and assist the body's immunity with non-synthetic antioxidative and anticancer peptides, which enables replacement of natural substances that are depleted during treatment. In

addition, natural bioactive peptides most likely have fewer side effects and lower cost than synthetic substances, radiation and chemotherapy.

6.4 Further Studies

This research found that milk fermented with *L. helveticus* 1315 released two peptides from β-CN with *in vitro* antioxidant and anti-colon cancer activity. Therefore, further research into the mechanism for how these peptides are released and which proteinase(s) or/and peptidase(s) are responsible, is recommended. Also, more research would be required in *in vivo* and clinical studies to confirm these findings. Treatment of peptides derived from milk fermented by *L. helveticus* strains on colon cancer cell lines resulted in an inhibitory effect reducing the proliferation rate of treated cancer cells compared to untreated controls. Significantly, simulated GI-digested antioxidant and anti-colon cancer peptides exhibited greater free radical scavenging activity and inhibitory response on colon cancer cells than did undigested peptides. Further studies are required to determine the molecular mechanisms behind these effects. Also, it is suggested that future research should seek to clarify this relationship in both colon cancer cells and normal colon cells. This represents a milestone towards inhibition of colon cancer using alternative therapies.

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