IDENTIFICATION OF BIOACTIVE PEPTIDES PRODUCED IN SYNBIOTIC YOGURT HAVING ANTICANCER PROPERTIES

A thesis submitted for the degree of Doctor of Philosophy

By

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Dedicated to my parents

&

the entire SAK family

ABSTRACT

Cancer is the most widely recognized reason for human deaths globally. Conventional anticancer therapies, including chemotherapy and radiation, are very costly and induce severe side effects on the individual. The discovery of anticancer compounds including dairy-derived peptides may thus be a better alternative for cancer prevention and management. Anticancer peptides exist in the amino acid chain of milk proteins and can be generated during proteolytic activities such as gastrointestinal digestion or food processing including fermentation by lactic acid bacteria (LAB) and probiotics. However, proteolytic capacity of these bacteria is strain specific. The study was conducted to establish proteolytic activity of *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) in yogurt. Crude peptides were separated by ultra-high centrifugation and tested for antioxidant and antimutagenic activities. The degree of proteolysis highly correlated with these bioactivities, and its value (11.91 %) for samples containing all the cultures was double that of the control. Liberated peptides showed high radical scavenging activities with 1,1-diphenyl-2-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), IC_{50} 1.51 and 1.63 mg/ml respectively and strong antimutagenicity (26.35 %). These probiotics enhanced the generation of bioactive peptides in yogurt.

Pineapple waste (peel and pomace), as a low value by-product, has been reported to contain dietary fibres, sugars, proteins, and minerals, and thus, was supplemented to yogurt to enhance the growth of LAB and the subsequent release of a range of protein hydrolysates in yogurt during their growth. Oven- and freeze-dried peel and pomace were milled separately into powders and tested for prebiotic activities. The net probiotic growth (1.28 – 2.14 log cfu/g) in customized MRS broth containing the pineapple powders as a direct carbohydrate source was comparable to MRS broth containing glucose. The powders were also separately added to milk during the manufacturing of yogurt with or without probiotics. An increase (by 0.3 – 1.4 log cycles) in probiotic populations was observed in the yogurts as a consequence of pineapple powder supplementation. Crude water-soluble peptide extracts, prepared by high-speed centrifugation of the yogurts, displayed remarkable antioxidant activities assessed through *in vitro* assays, namely scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radicals (IC_{50} = 0.37 – 0.19 mg/ml) and hydroxyl

radicals (58.52 – 73.55 %). The peptide extracts also exhibited antimutagenic activities (18.60 – 32.72 %) as sodium azide inhibitor in the *Salmonella* mutagenicity test. Together, these results suggest that pineapple by-products exhibited prebiotic properties.

Furthermore, the effects of pineapple peel powder (PPP) addition on the viability and activity probiotic bacteria in refrigerated yogurts was tested. Plain and probiotic yogurts supplemented with or without PPP or inulin were prepared. The probiotic counts in supplemented yogurts at 28 days of storage ranged from 7.68 and 8.03 log cfu/g, one log cycle higher compared to non-supplemented control yogurt. Degree of proteolysis in synbiotic yogurts was significantly higher than plain yogurts and increased substantially during storage. Crude water-soluble peptide extract of the probiotic yogurt with peel possessed stronger antimutagenic and antioxidant activities (evaluated measuring reducing power and scavenging capacity of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $^{\bullet}OH$ radicals) than control yogurts and this was maintained during storage.

Search for alternative therapeutics is on the rise due to extensive rise in bacterial resistance to various conventional antibiotics or side effects of conventional cancer therapies. Thus, generation of inhibitory peptides in yogurt against pathogenic bacteria and cancer cells, during storage at 4 °C for 28 days have been conducted. Water-soluble crude peptide extracts (WSPEs) were prepared by high-speed centrifugation of plain and probiotic yogurts supplemented with or without PPP. The inhibition zone against *Escherichia coli* and *Staphylococcus aureus* by PPP fortified probiotic yogurt at 28 days storage were respectively 25.89 and 11.72 mm in diameter, significantly higher compared to non-supplemented control yogurts. Antiproliferative activity against HT29 colon cancer cells was also significantly higher in probiotic yogurt with PPP than non-supplemented probiotic yogurt. Overall, crude water-soluble peptide extract of the probiotic yogurt with peel possessed stronger inhibitory activities against bacteria and cancer cells than control and these activities were maintained during storage. However, activities were lowered substantially during *in vitro* gastrointestinal digestion.

These findings demanded further investigation to isolate and characterize these bioactive peptides from WSPEs. Thus, antioxidant peptides were purified from crude peptide extract of probiotic yogurt supplemented with PPP stored for 28 days at 4 °C by employing an ultrafiltration membrane followed by reversed-phase HPLC.

Two peptides were identified as ^{193}YQEPVLGPVRGPFPIIV^{209} (P17), ^{69}SLPQNIPPLTQTPVVVPPF^{87} (P19) derived from β-casein of milk, and their antioxidant and anticancer activities were assessed. P17 showed a high scavenging activity against 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) radicals in a dose-dependent manner, with an IC_{50} of 29.88 μg/mL, compared to P19 (IC_{50} 1.44 mg/mL). Furthermore, the proliferation of HT-29 colon cancer cell line was inhibited dose-dependently (41.49 % and 38.55 %, respectively, by P17 and P19 at 3 mg/mL) by inducing apoptosis and cell cycle arrest in G_2/M-phase. *In vitro* gastrointestinal digestion of peptides resulted in increased bioactivities. These findings indicate potentiality of utilizing both antioxidant peptides isolated from synbiotic yogurt in cancer therapy.

Fortification of PPP to yogurt has led to an improved probiotic growth resulting in enhanced release of bioactive peptides. However, this may also lead to alteration in physical, textural, and rheological properties of yogurt since gel firmness mainly depends on the strength of the three-dimensional network of milk proteins, which is in turn governed by total solids content, acidification rate, and proteolytic activity of employed cultures. Thus, the influence of adding PPP on physicochemical, textural, rheological, and microstructural attributes has been explored during refrigerated storage of probiotic yogurt for 28 days. Skim milk with or without PPP or inulin as a control was fermented by yogurt cultures with or without addition of the probiotic cultures consisting of *L. acidophilus*, *L. casei*, and *L. paracasei*. PPP supplementation at 1 % remarkably reduced fermentation time of milk co-fermented with probiotic organisms. Syneresis level in probiotic yogurt with PPP (1.16 % at day 1) was comparable with the prebiotic- inulin and increased during storage. However, firmness and storage modulus in both plain and probiotic yogurts were lowered significantly with PPP addition. The reported information on physicochemical and structural attributes of probiotic yogurts with PPP could guide the dairy industry to utilize pineapple peel discarded as processing waste to close the fiber gap by developing a new synbiotic product with comparable attributes to inulin, a commercial prebiotic. Additionally, pineapple waste, a by-product of juice production, could be proposed as a prebiotic ingredient in the manufacture of yogurts with enhanced nutrition, and functionality.

Overall, these findings support the notion that milk proteins are not only a nutritious part of a normal daily diet but also have potential for prevention and/or

management of cancer. Dairy-derived bioactive peptides show promise to serve as a functional ingredient in foods, nutraceuticals, and pharmaceuticals as well as the possibility of utilizing dairy-derived bioactive peptides in the development of a superior alternative to the current generation of antibacterial and anticancer agents. These peptides could also be exploited to improve the shelf-life of food products.

CERTIFICATE

Dr Todor Vasiljevic, PhD (*UAlberta*)
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This is to certify that the thesis entitled "**IDENTIFICATION OF BIOACTIVE PEPTIDES PRODUCED IN SYNBIOTIC YOGURT HAVING ANTICANCER PROPERTIES**" submitted by **Baidya Nath Prasad Sah** in partial fulfillment of the requirement for the award of the Doctor of Philosophy with specialization in Food Sciences 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.

[redacted]

Professor Todor Vasiljevic
(Principal supervisor)
Date: 30/06/2016

DECLARATION

"I, Baidya Nath Prasad Sah, declare that the PhD thesis by Publication entitled "IDENTIFICATION OF BIOACTIVE PEPTIDES PRODUCED IN SYNBIOTIC YOGURT HAVING ANTICANCER PROPERTIES" 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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PART A: DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	**Paper Title**	**Publication Status** (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	**Publication Title and Details** (e.g. date published, impact factor etc.)
2B	Antioxidative and antibacterial peptides derived from bovine milk proteins	Accepted for publication	Critical Reviews in Food Science and Nutrition; SJR Q1
2C	Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review	Published	Comprehensive Reviews in Food Science and Food Safety; SJR Q1
3	Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt	Published	Food Chemistry; SJR Q1
4	Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt	Published	Journal of Food Science and Technology; SJR Q2
5	Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel	Published	Journal of Dairy Science; SJR Q1
6	Antibacterial and antiproliferative peptides in synbiotic yogurt – release and stability during refrigerated storage	Published	Journal of Dairy Science; SJR Q1
7	Antioxidant peptides isolated from synbiotic yoghurt exhibit antiproliferative activities against HT-29 colon cancer cells	Published	International Dairy Journal; SJR Q1
8	Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage	Published	LWT - Food Science and Technology; SJR Q1

Declaration by (candidate name): Baidya Nath Prasad Sah **Signature**: **Date**

ORAL AND POSTER PRESENTATIONS

Oral presentations

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2014). Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. 5th International Dairy Federation (IDF) Symposium on Science and Technology of Fermented Milk, Melbourne, Australia. 06-March-2014

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Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015). Antibacterial and antiproliferative peptides in synbiotic yogurt – release and stability during refrigerated storage. Australian Institute of Food Science and Technology (AIFST) Food Science summer school 2016, Charles Sturt University, Wagga Wagga, NSW, Australia. 29-Jan-2016

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Division (ID: 113-01) at Institute of Food Technologists (IFT) 2014 Annual Meeting & Food Expo® in New Orleans, LA, USA. 23-June-2014

MEDIA UPDATE

1. Institute of Food Technologists (IFT) February 2015 Media Update on “Milk proteins show promise in prevention and treatment of cancer” featuring the article entitled “Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review” published in the Comprehensive Reviews in Food Science and Food Safety. 18-Feb-2015

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABP = Antibacterial peptide

ACP = Anticancer peptide

ABTS = 2,2'- azino- bis (3- ethyl benzothiazoline-6-sulphonic acid)

ANOVA = Analysis of variance

AOAC = Association of Official Agricultural Chemists

CA = Cluster analysis

CFU = Colony forming unit

DF = Dietary fiber

DH = Degree of hydrolysis

DMA = Davis minimal agar

DPPH = 1,1-diphenyl-2-picrylhydrazyl

GLM = General Linear Model

h = Hour

s = Second

HCA = Hierarchical cluster analysis

HMDS = 1,1,1,3,3,3-hexamethyldisilazane

HPLC = High performance liquid chromatography

HRSA = Hydroxyl radical scavenging activity

IC_{50} = Inhibitory concentration for 50 % of the measured activity

LAB = Lactic acid bacteria

MRS = de Man Rogosa and Sharpe

MW = molecular weight

OPA = O-phthaldialdehyde

PCA = Principal component analyses

pH = hydrogen ion concentration

PPP = Pineapple peel powder

RP-HPLC = Reversed-phase high performance liquid chromatography

rpm = rotation per minute

RSA = Radical scavenging activity

RSM = Reconstituted skim milk

SMP = Skim milk powder

St = *Streptococcus thermophilus*

Subsp. = subspecies

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

UV = ultraviolet

WHO = World Health Organization

WSPE = Water soluble peptide extract

mL = milliliter

mm = millimeter

mM = millimolar

μg = microgram

mV = millivolts

Pa = Pascal

× g = times gravitational force

°C = degree Celsius

v/v = volume per volume

w/w = weight per weight

Chapter 1: Introduction

1.1 Background

The rapid rate of urbanization, mechanization, and industrialization has led to dramatic changes in life cycle and dietary patterns of population, which in turn has been accompanied by an increase in the occurrence of chronic non-communicable diseases such as diabetes mellitus, cardiovascular diseases, and cancers (Collins, 2005; Sharma, 2014). Thus, demand for development of healthy foods is increasing day-by-day due to growing interest of consumers to maintain their health and well-being (Annunziata & Vecchio, 2011; Marina, Marija, & Ida, 2014). Day, Seymour, Pitts, Konczak, and Lundin (2009) defined functional foods as "foods or ingredients of foods that provide additional physiological benefit beyond their basic nutrition". Milk is considered a source of functional ingredients, such as bioactive peptides, which are encrypted in primary structure of the milk proteins and could modulate physiology of consumers only after their proteolytic release (Bhat & Bhat, 2011). There are several possible ways to obtain these bioactive peptides in order to functionalize foods. One way could be through direct release of peptides from proteins by action of proteolytic systems of bacteria commonly used in manufacturing of fermented food products (Choi, Sabikhi, Hassan, & Anand, 2012). One distinguishable feature of yogurt is the presence of lactic acid bacteria, which have well developed proteolytic systems enabling them to grow well in milk and release a large number of potentially bioactive peptides. Several lactobacillus strains displayed remarkably high proteolytic activities (Donkor, Henriksson, Vasiljevic, & Shah, 2007; Yeo & Liong, 2010). In addition, many studies have also been undertaken to confer probiotic effects by incorporating *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) into fermented food products (Ortakci & Sert, 2012; Sidira, Saxami, Dimitrellou, Santarmaki, Galanis, & Kourkoutas, 2013). For probiotic effect, consumer should aim to ingest more than 10^8 live probiotic cells per day (Hill, Guarner, Reid, Gibson, Merenstein, Pot et al., 2014; Lourens-Hattingh & Viljoen, 2001). This amount could be translated into > 10^6 CFU/g of probiotic yogurt given that 100 g is the daily serving portion. The viability of probiotic organisms is thus considered a key parameter for developing probiotic food products. The major factors for achieving and maintaining this minimal level in yogurt are – nutrients, pH, water activity, and oxygen tension of the product, the storage

conditions such as temperature, humidity, and light, the interactions with the starter cultures as well as strain types (Vasiljevic & Shah, 2008). In order to minimize their adverse effects, different approaches have been suggested, including microencapsulation of probiotics (Corona-Hernandez, Álvarez-Parrilla, Lizardi-Mendoza, Islas-Rubio, de la Rosa, & Wall-Medrano, 2013), addition of enzymes (Cruz, Castro, Faria, Bolini, Celeghini, Raices et al., 2013) and prebiotics (Al-Sheraji, Ismail, Manap, Mustafa, Yusof, & Hassan, 2013). Thus, the main goal of this project was to study the viability- and influence of selected probiotic organisms on the liberation of bioactive peptides from milk proteins in yogurt. The specific objectives were:

1. to explore the extent of proteolysis and release of bioactive peptides by selected probiotic organisms *L. acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) co-cultured with yogurt starter culture in regards to release of bioactive peptides with the antioxidant, antimutagenic, antibacterial, and anticancer activities during yogurt manufacturing,
2. to investigate pineapple waste (peel and pomace) as potential prebiotics and utilize in the production and stability of antioxidant, antimutagenic, antibacterial, and anticancer peptides in yogurts.
3. to isolate and identify antioxidant peptides from synbiotic yogurt, and evaluate anticancer activities of the isolated peptides against the HT29 colon cancer cell model.
4. to establish the impact of pineapple peel powder addition on the physicochemical, textural, rheological, and microstructural characteristics of set-type yogurt containing *L. acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) during 28 days of refrigerated storage.

1.2 Thesis outline

Chapter 1 provides background, research objectives, and outline of the thesis. Chapter 2 presents a review of current scientific knowledge on bioactive peptides, focusing on antioxidant, antimutagenic, antibacterial, and anticancer peptides released from milk proteins. Chapter 3 investigates the role of probiotic organisms including *L. acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) in the release of antioxidant and antimutagenic

peptides in yogurt during their growth. Chapter 4 presents a study on prebiotic effect of pineapple waste on probiotic organisms and the subsequent release of antioxidant and antimutagenic peptides in yogurt. Chapter 5 reports the effects of pineapple peel powder addition on viability of probiotic organisms, production and stability of antimutagenic and antioxidant peptides in yogurts throughout storage at 4 °C for 28 days. Chapter 6 presents the generation of inhibitory peptides against bacteria and HT29 human colon cancer cells in yogurts throughout storage at 4 °C for 28 days. Chapter 7 presents isolation and identification of antioxidant peptides from synbiotic yogurt, and evaluation of anticancer activities of the isolated peptides against HT29 colon cancer cells. Chapter 8 focuses on influence of adding pineapple peel powder (PPP) as a fiber source on physicochemical, textural, rheological, and microstructural attributes of probiotic yogurt during refrigerated storage for 28 days. Finally, conclusions of the overall study and future directions of the research are included in Chapter 9.

References

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., & Hassan, F. A. (2013). Prebiotics as functional foods: A review. *Journal of Functional Foods, 5*(4), 1542–1553. http://dx.doi.org/10.1016/j.jff.2013.08.009.

Annunziata, A., & Vecchio, R. (2011). Factors affecting Italian consumer attitudes toward functional foods. *AgBioForum, 14*(1), 20–32.

Bhat, Z. F., & Bhat, H. (2011). Milk and dairy products as functional foods: A review. *International Journal of Dairy Science, 6*(1), 1–12. http://dx.doi.org/10.3923/ijds.2011.1.12.

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1–12. http://dx.doi.org/10.1111/j.1471-0307.2011.00725.x.

Collins, A. R. (2005). Antioxidant intervention as a route to cancer prevention. *European Journal of Cancer, 41*(13), 1923–1930.

Corona-Hernandez, R. I., Álvarez-Parrilla, E., Lizardi-Mendoza, J., Islas-Rubio, A. R., de la Rosa, L. A., & Wall-Medrano, A. (2013). Structural stability and viability of microencapsulated probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety, 12*(6), 614–628. http://dx.doi.org/10.1111/1541-4337.12030.

Cruz, A. G., Castro, W. F., Faria, J. A. F., Bolini, H. M. A., Celeghini, R. M. S., Raices, R. S. L., Oliveira, C. A. F., Freitas, M. Q., Conte Júnior, C. A., & Mársico, E. T. (2013). Stability of probiotic yogurt added with glucose oxidase in plastic materials with different permeability oxygen rates during the refrigerated storage. *Food Research International, 51*(2), 723–728. http://dx.doi.org/10.1016/j.foodres.2013.01.028.

Day, L., Seymour, R. B., Pitts, K. F., Konczak, I., & Lundin, L. (2009). Incorporation of functional ingredients into foods. *Trends in Food Science & Technology, 20*(9), 388–395. http://dx.doi.org/10.1016/j.tifs.2008.05.002.

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait, 87*(1), 21–38. http://dx.doi.org/10.1051/lait:2006023.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology, 11*(8), 506–514. http://dx.doi.org/10.1038/nrgastro.2014.66.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1–17. http://dx.doi.org/10.1016/S0958-6946(01)00036-X.

Marina, T., Marija, C., & Ida, R. (2014). Functional Foods and the Young. *Journal of Food Products Marketing, 20*(5), 441–451. http://dx.doi.org/10.1080/10454446.2013.838535.

Ortakci, F., & Sert, S. (2012). Stability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in yogurt and in an artificial human gastric digestion system. *Journal of Dairy Science, 95*(12), 6918–6925. http://dx.doi.org/10.3168/jds.2012-5710.

Sharma, N. (2014). Free radicals, antioxidants and disease. *Biology and Medicine, 6*(3). http://dx.doi.org/10.4172/0974-8369.1000214.

Sidira, M., Saxami, G., Dimitrellou, D., Santarmaki, V., Galanis, A., & Kourkoutas, Y. (2013). Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool. *Journal of Dairy Science, 96*(5), 3369–3377. http://dx.doi.org/10.3168/jds.2012-6343.

Vasiljevic, T., & Shah, N. P. (2008). Probiotics—From Metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714–728. http://dx.doi.org/10.1016/j.idairyj.2008.03.004.

Yeo, S. K., & Liong, M. T. (2010). Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *International Journal of Food Sciences and Nutrition, 61*(2), 161–181. http://dx.doi.org/10.3109/09637480903348122.

Chapter 2: Literature review

Chapter 2 presents a comprehensive review of current scientific knowledge on bioactive peptides, focusing on antioxidant, antimutagenic, antibacterial, and anticancer peptides released from milk proteins. It has been divided into chapters 2A, 2B, and 2C.

Chapter 2A: Supplementary literature review

Chapter 2A presents a review on role of lactic acid bacteria (yogurt and probiotic cultures), and prebiotics in the generation of bioactive peptides during yogurt manufacturing.

2A.1 Yogurt

Yogurt is a fermented milk product, and its structure is a result of the casein aggregation following a pH drop due to lactic acid production during milk fermentation by starter culture, which contains certain strains of lactic acid bacteria (LAB). LAB are generally regarded as safe for human consumption and have been used in food fermentation processes, where they ferment carbohydrates, produce lactic acid and create adverse environment for the growth of food spoilage organisms and pathogens (Holzapfel & Wood, 2014). They are Gram-positive, catalase-negative, nonsporulating, and facultative anaerobic bacteria, which comprise strains of the genera *Lactobacillus* (*L.*), *Streptococcus* (*S.*), *Lactococcus*, *Leuconostoc*, and *Pediococcus* (Savijoki, Ingmer, & Varmanen, 2006).

Yogurt starter culture commonly contains non-proteolytic (Prt^-) *S. thermophilus*, and highly proteolytic *L. delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), which have symbiotic relationship (known as "proto-cooperation") involving exchange of several metabolites and/or stimulatory factors (Settachaimongkon, Nout, Antunes Fernandes, Hettinga, Vervoort, van Hooijdonk et al., 2014; Smid & Lacroix, 2013). *S. thermophilus* grows faster from its lag phase as a result. It assimilates oxygen, and reduces redox potential of the milk. Milk pH also reduces from 6.7 to 5.7 due to generation of organic acids, mainly lactic acid. Some *S. thermophilus* species produce CO_2 from milk urea owing to their urease activities (Arioli, Della Scala, Remagni, Stuknyte, Colombo, Guglielmetti et al., 2016) and from galactose via the Leloir metabolic pathway (Grossiord, Vaughan, Luesink, & De Vos, 1998; Thomas & Crow, 1984). Creation of these circumstances collectively promotes the growth of *L. bulgaricus*. In turn, short peptides and free amino acids (such as histidine, threonine, and valine) are generated from milk caseins by *L. bulgaricus* during growth. These compounds stimulate the growth of *S. thermophilus* as most *S. thermophilus* strains show few amino acid auxotrophy (Sieuwerts, De Bok, Hugenholtz, & Van Hylckama Vlieg, 2008; Tamime & Robinson, 2007). Furthermore, threonine enrichment in yogurt resulted in increased production of flavor compounds including acetaldehyde (Routray & Mishra, 2011; Settachaimongkon et al., 2014). Both *S. thermophilus* and *L. bulgaricus* are thermophilic LAB but *L. bulgaricus* is more acid tolerant than *S. thermophilus* (Sieuwerts, De Bok, Hugenholtz, & Van Hylckama Vlieg, 2008). Interestingly, other microorganisms can be combined with these strains to produce

yogurt with desirable health benefits. These beneficial microorganisms are usually termed as “probiotics”.

2A.2 Probiotics

Probiotics are “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). Several microorganisms are used as probiotics including LAB, bifidobacteria, and certain yeasts (Fijan, 2014). Most probiotic species commercially used in food belong to the genera *Bifidobacterium* and *Lactobacillus* (Vasiljevic & Shah, 2008). Among *Lactobacillus* used as probiotics, the common cultures found in (commercial) yogurt are *Lactobacillus* (*L.*) *acidophilus*, *L. casei*, and *L. paracasei*.

L. acidophilus is a species of human origin. It is a homofermentative, microaerophilic, and Gram-positive rod-shaped bacterium (Anjum, Maqsood, Masud, Ahmad, Sohail, & Momin, 2014). Like many species of the genus *Lactobacillus*, it possesses an S-layer (a part of the cell envelope), which provides protection against hostile environmental agents, resistance against low pH, and adhesion to the GI tract (Åvall-Jääskeläinen & Palva, 2005; Hynönen & Palva, 2013). Hydrogen peroxide (H_2O_2) is generated during the growth of *L. acidophilus*, and can prevent growth of other microorganisms deficient in H_2O_2-scavenging enzymes (Eschenbach, Davick, Williams, Klebanoff, Young-Smith, Critchlow et al., 1989). *L. acidophilus* (ATCC® 4356™) exhibits probiotic properties such as survival at low pH and bile acid tolerance (Campana, Federici, Ciandrini, & Baffone, 2012), cholesterol metabolism (Huang & Zheng, 2010), and antagonistic activity against *Campylobacter jejuni* pathogen (Campana, Federici, Ciandrini, & Baffone, 2012).

L. casei is a homofermentative bacterium (Holzapfel & Schillinger, 2002) and acid tolerant (Kourkoutas, Xolias, Kallis, Bezirtzoglou, & Kanellaki, 2005). *L. casei* (ATCC® 393™) has also been proposed previously for the manufacturing of probiotic fermented milk and cheese because of its excellent technological properties (Kourkoutas, Bosnea, Taboukos, Baras, Lambrou, & Kanellaki, 2006; Kourkoutas, Xolias, Kallis, Bezirtzoglou, & Kanellaki, 2005). Increased survival rates of *L. casei* cells has been reported when utilizing immobilized cells in food production compared to free cells (Kourkoutas, Bosnea, Taboukos, Baras, Lambrou, & Kanellaki, 2006; Kourkoutas, Xolias, Kallis, Bezirtzoglou, & Kanellaki, 2005). *L. casei* ATCC 393 shows probiotic properties such as survival during GI transit, modulation of intestinal

microflora (Sidira, Galanis, Ypsilantis, Karapetsas, Progaki, Simopoulos et al., 2010), and adhesion to large intestine (Saxami, Ypsilantis, Sidira, Simopoulos, Kourkoutas, & Galanis, 2012). Furthermore, sufficient survival of *L. casei* ATCC 393 to confer a probiotic effect has also been observed during refrigerated storage of yogurt for longer periods than are required by commercial sectors (Sah, Vasiljevic, McKechnie, & Donkor, 2015a; Sidira, Saxami, Dimitrellou, Santarmaki, Galanis, & Kourkoutas, 2013).

L. paracasei is a lactic acid bacterium and frequently resides on GI mucosa of healthy individuals (Ilha, Scariot, Treml, Pereira, Sant′Anna, Prudêncio et al., 2015; Molin, Jeppsson, Johansson, Ahrne, Nobaek, Stahl et al., 1993). *L. paracasei* subsp. *paracasei* strain has been widely incorporated into dairy products owing to higher survival during storage and less influence on organoleptic characteristics of the products (Buriti, Cardarelli, Filisetti, & Saad, 2007; Xie, Zhou, & Li, 2012).

Bifidobacteria also present together with lactobacilli in GI microbiota are Gram-positive bacteria. Mostly *Bifidobacterium* species are anaerobic. However, strains such as *Bifidobacterium* (*B.*) *thermophilum* and *B. animalis* subsp. *lactis* are considered as microaerophilic (Li, Chen, Ruan, Zhu, & He, 2010; von Ah, Mozzetti, Lacroix, Kheadr, Fliss, & Meile, 2007). Some *Bifidobacterium* species (*B. animalis*, *B. longum*, and *B. bifidum*) are able to survive GI transit including bile-containing environments (Ruiz, Margolles, & Sánchez, 2013) and, and exhibit health-promoting activities including treatment of diarrhea, and cholesterol-lowering activity (Zanotti, Turroni, Piemontese, Mancabelli, Milani, Viappiani et al., 2015).

The genus *Saccharomyces* comprises several yeasts including *Saccharomyces* (*Sacch.*) *cerevisiae* (used in wine, bread, and beer making), *Sacch. bayanus* (used in wine making) and *Sacch. boulardii* (used as a probiotic in medicine). Administration of *Sacch. boulardii* has resulted in positive health benefits through regulation of intestinal microbial homeostasis, and prevention of several gastrointestinal disorders including *Helicobacter pylori* infection, irritable bowel syndrome, antibiotic-associated diarrhea, and traveler's diarrhea (Kelesidis & Pothoulakis, 2012).

Consumer should aim to ingest sufficient number of viable probiotic cells (> 10^8 cells per day) for health benefits (Hill, Guarner, Reid, Gibson, Merenstein, Pot et al., 2014; Lourens-Hattingh & Viljoen, 2001). The viability of probiotic organisms is thus considered a key factor for developing foods for probiotic effects. Major parameters affecting probiotic population in yogurt are product composition and

attributes (nutrients, pH, water activity, and oxygen tension), storage conditions (such as temperature, humidity, and light), and interactions with starter cultures (Vasiljevic & Shah, 2008). In order to minimize their adverse effects, different approaches have been suggested, including microencapsulation of probiotics (Corona-Hernandez, Álvarez-Parrilla, Lizardi-Mendoza, Islas-Rubio, de la Rosa, & Wall-Medrano, 2013), addition of enzymes (Cruz, Castro, Faria, Bolini, Celeghini, Raices et al., 2013) and prebiotics (Al-Sheraji, Ismail, Manap, Mustafa, Yusof, & Hassan, 2013).

2A.3 Prebiotics

A prebiotic is a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the GI microflora that confers benefits upon host wellbeing and health" (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). Prebiotics are not digestible in the upper GI tract and reach the lower GI tract, where resident microflora utilizes prebiotics as a substrate. The amounts and types of prebiotics entering the large bowel can influence the growth of microbial populations and the production of short-chain fatty acids, thereby altering gut functionality (Macfarlane, Macfarlane, & Cummings, 2006). Prebiotic properties are exhibited by some non-digestible oligosaccharides and polysaccharides including fructooligosaccharides, and galactooligosaccharides, inulin, resistant starch, and lactulose (Thammarutwasik, Hongpattarakere, Chantachum, Kijroongrojana, Itharat, Reanmongkol et al., 2009; Watson, O'Connell Motherway, Schoterman, van Neerven, Nauta, & Van Sinderen, 2013). Pineapple peel powder has been reported to act as a prebiotic source and enhanced viability of probiotic organisms during storage of yogurt (Sah, Vasiljevic, McKechnie, & Donkor, 2015a, 2016a).

2A.3.1 Inulin

Inulin is a non-digestible carbohydrate, and occurs naturally in several plants of the Compositae and Liliaceae families such as chicory roots (15 – 20 %), garlic (4 – 12 %), onions (1 – 5 %, fresh weight basis), banana (0.2 %), artichoke, leek, asparagus, oats, wheat, and soybean (Kolida & Gibson, 2007; Schaafsma & Slavin, 2015). Chicory is mostly utilized as a raw material for industrial extraction of inulin (Boeckner, Schnepf, & Tungland, 2002). Degree of polymerization (DP) of chicory inulin varies from 2 – 60 units. A long-chain inulin, also termed inulin HP, has DP ranging between 10 and 60 units (average DP of 25 units) (Roberfroid, 2007). Partial

enzymatic hydrolysis of inulin molecules having a DP of 3 to 10 units are referred as oligofructose or fructooligosaccharides (Schaafsma & Slavin, 2015).

Inulin is a fructan consisting almost entirely of linearly beta-1,2-linked fructose units with a terminal alpha1-beta2- linked glucose molecule (Schaafsma & Slavin, 2015). It has a neutral taste, minimal effect on organoleptic properties of a product, and can be used as a fat replacer, sugar replacer, texture modifier, and prebiotic ingredient (Meyer, Bayarri, Tárrega, & Costell, 2011). Additionally, inulin affects rheological properties only slightly at low concentrations in food (Franck, 2002; Kalyani Nair, Kharb, & Thompkinson, 2010). It also exerts a protective effect on probiotic organisms resulting in improved survival and activity during storage (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007; Sah, Vasiljevic, McKechnie, & Donkor, 2015a).

Probiotic organisms possess cell-associated glycosidases, which hydrolyze oligosaccharides and subsequent utilization of prebiotics as an additional carbon and energy source (De Souza Oliveira, Perego, De Oliveira, & Converti, 2011; Rossi, Corradini, Amaretti, Nicolini, Pompei, Zanoni et al., 2005).

2A.4 Synbiotics

Incorporation of prebiotics in a probiotic product promotes survival and activity of the probiotic organisms. Combining prebiotics and probiotics in food produces a synbiotic product (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007; Sah, Vasiljevic, McKechnie, & Donkor, 2016b). Various synbiotic products have been formulated by fortifying fiber-rich fractions from herbs (Chowdhury, Chakraborty, & Raychaudhuri, 2008), cereals (Vasiljevic, Kealy, & Mishra, 2007), banana, passion, or apple processing by-products (Espírito Santo, Cartolano, Silva, Soares, Gioielli, Perego et al., 2012). Davis and Milner (2009) reported improved probiotic viability (especially bifidobacteria) in food products and in GI tract, attributable to fructooligosaccharides and galactooligosaccharides. Synbiotic yogurt can also be prepared by incorporating probiotic cultures including *L. acidophilus*, *L. casei*, and *L. paracasei* subsp. *paracasei* with yogurt culture, and supplementation of pineapple waste powder (Figure 2A.1).

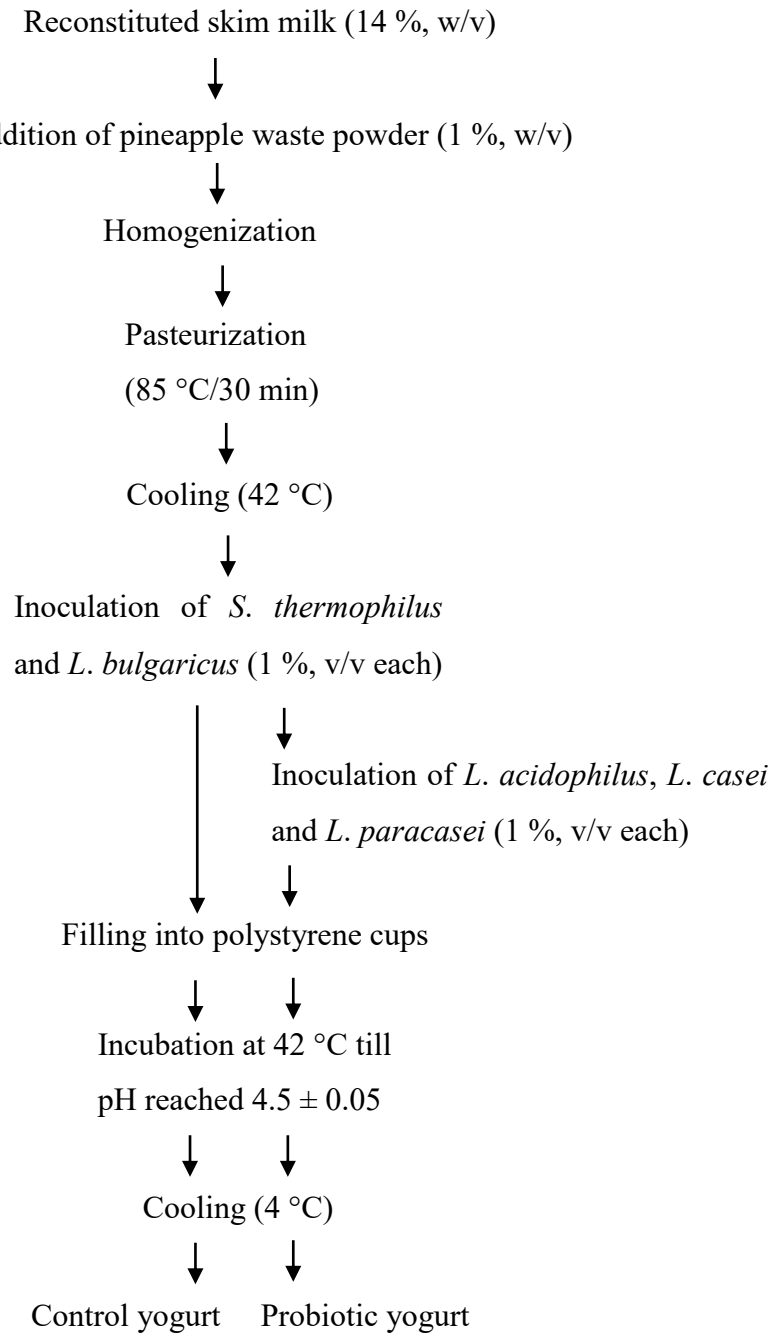

Figure 2A.1 Flow diagram for manufacturing of yogurt supplemented with pineapple waste (peel and pomace) powder

Most *S. thermophilus* strains are stimulated by the supply of 2 – 5 amino acids including Glu, Met, His, and Pro (Letort & Juillard, 2001; Neviani, Giraffa, Brizzi, & Carminati, 1995), whereas *Lactobacilli* require 3 – 14 amino acids (Hebert, Raya, & De Giori, 2000; Ledesma, de Ruiz Holgado, Oliver, De Giori, Raibaud, & Galpin, 1977; Morishita, Deguchi, Yajima, Sakurai, & Yura, 1981). Therefore, optimum

growth of LAB in milk system depends on their proteolytic system for the hydrolysis of milk caseins into short peptides, and amino acids (Thomas & Pritchard, 1987).

LAB perform many biochemical conversions of milk components during fermentation including hydrolysis of milk proteins (caseins and whey proteins) into free amino acids and peptides (Smit, Smit, & Engels, 2005). The proteolytic system of LAB consists of extracellular peptidases, peptide transport system, and intracellular peptidases. However, proteolytic enzymes found in different species of LAB exhibit different protease activities, and complex system of endo- and exopeptidases, which may differ in activity, cell location, and specificity (Kunji, Mierau, Poolman, & Konings, 1996). In general, LAB possess:

a) Proteases located in the cell envelope: Cell-wall proteinases of LAB are utilized for initial breakdown of caseins into oligopeptides.
b) Peptide transport systems: The peptides are subsequently transported by several peptide transport systems into cells; oligopeptides are transported by the oligopeptide transport system (Opp) and the di/tri-peptides are transported by a proton motive force-driven transporter DtpT or an adenosine triphosphate (ATP)-driven Dpp system (Doeven, Kok, & Poolman, 2005; Picon, García-Casado, & Nuñez, 2010).
c) Intracellular peptidases: After peptides enter cells, they are hydrolyzed to amino acids by combined actions of intracellular peptidases (such as aminopeptidases), endopeptidases, and di- and tri-peptidases (Christensen, Dudley, Pederson, & Steele, 1999; Kunji, Mierau, Poolman, & Konings, 1996; M. Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). Moreover, LAB have numerous proline peptidases, which can breakdown proline-rich proteins such as caseins (Christensen, Dudley, Pederson, & Steele, 1999).

S. thermophilus is a thermophilic lactic acid *bacterium*, and is commonly referred to as GRAS (Generally Recognized as Safe) (Delorme, 2008). It shows auxotrophy for some amino acids, which can be liberated from caseins breakdown by proteolytic enzymes of proteolytic *S. thermophilus* strains (Christensen, Dudley, Pederson, & Steele, 1999; Kunji, Mierau, Poolman, & Konings, 1996; Savijoki, Ingmer, & Varmanen, 2006). The proteolytic system of *S. thermophilus* consists of cell-wall proteinases (PrtS), ATP-binding cassette (ABC) transporters of oligopeptides, and di/tripeptide transporters, and a pool of intracellular peptidases (Fernandez-Espla, Garault, Monnet, & Rul, 2000). The peptides released by PrtS are

internalized in cell by oligopeptide and dipeptide transport systems (Garault, Le Bars, Besset, & Monnet, 2002). After their internalization, peptides are hydrolyzed by intracellular peptidases (Rul & Monnet, 1997). Similarly, *L. bulgaricus* has a cell-wall proteinase (PrtB) for the first step of milk protein hydrolysis (Gilbert, Blanc, Frot-Coutaz, Portalier, & Atlan, 1997). In *L. bulgaricus*, the peptide transport system is by both Opp and Dpp. Several studies (Choi, Sabikhi, Hassan, & Anand, 2012; Korhonen & Pihlanto, 2006; Sah, Vasiljevic, McKechnie, & Donkor, 2015b) reported a high content of peptides and free amino acids in fermented milks owing to proteolysis by LAB.

2A.5 Release of bioactive peptides in yogurt

Some *Lactobacillus* strains hydrolyze milk proteins resulting in release of bioactive peptides (Korhonen & Pihlanto, 2003a). Common biological activities exhibited by milk protein-derived bioactive peptides have been presented in Figure 2A.2.

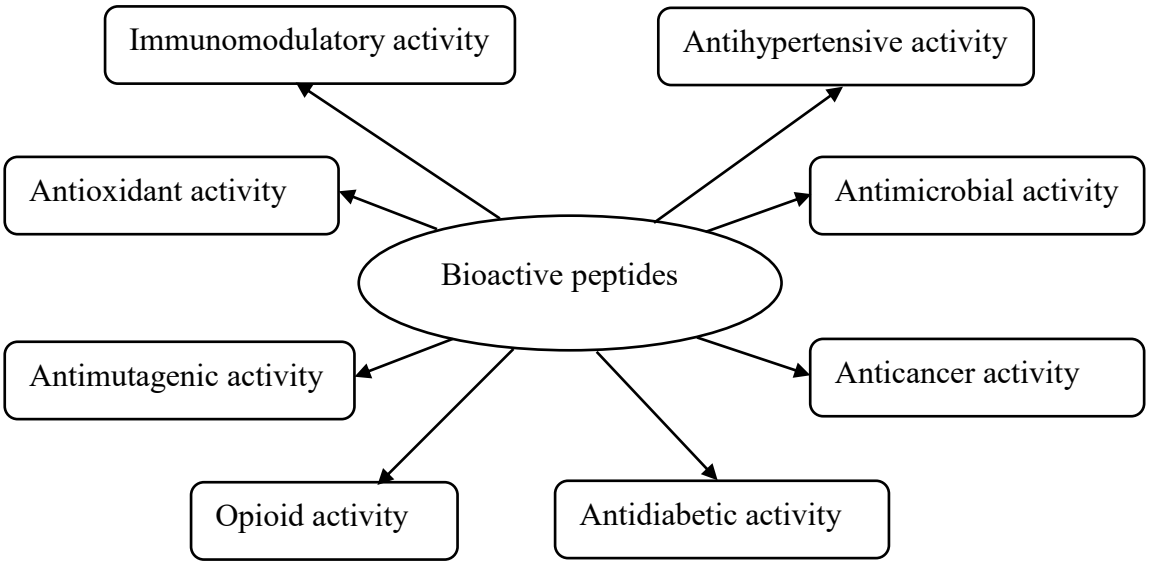

Figure 2A.2 Common bioactivities of milk protein-derived bioactive peptides

2A.6 Isolation and identification of bioactive peptides from yogurt

Application possibility of a bioactive peptide in food and pharmaceutical industries also requires optimized steps of its production, isolation, and characterization. A schematic representation of production, purification, and characterization of bioactive peptides from yogurt is presented in Figure 2A.3.

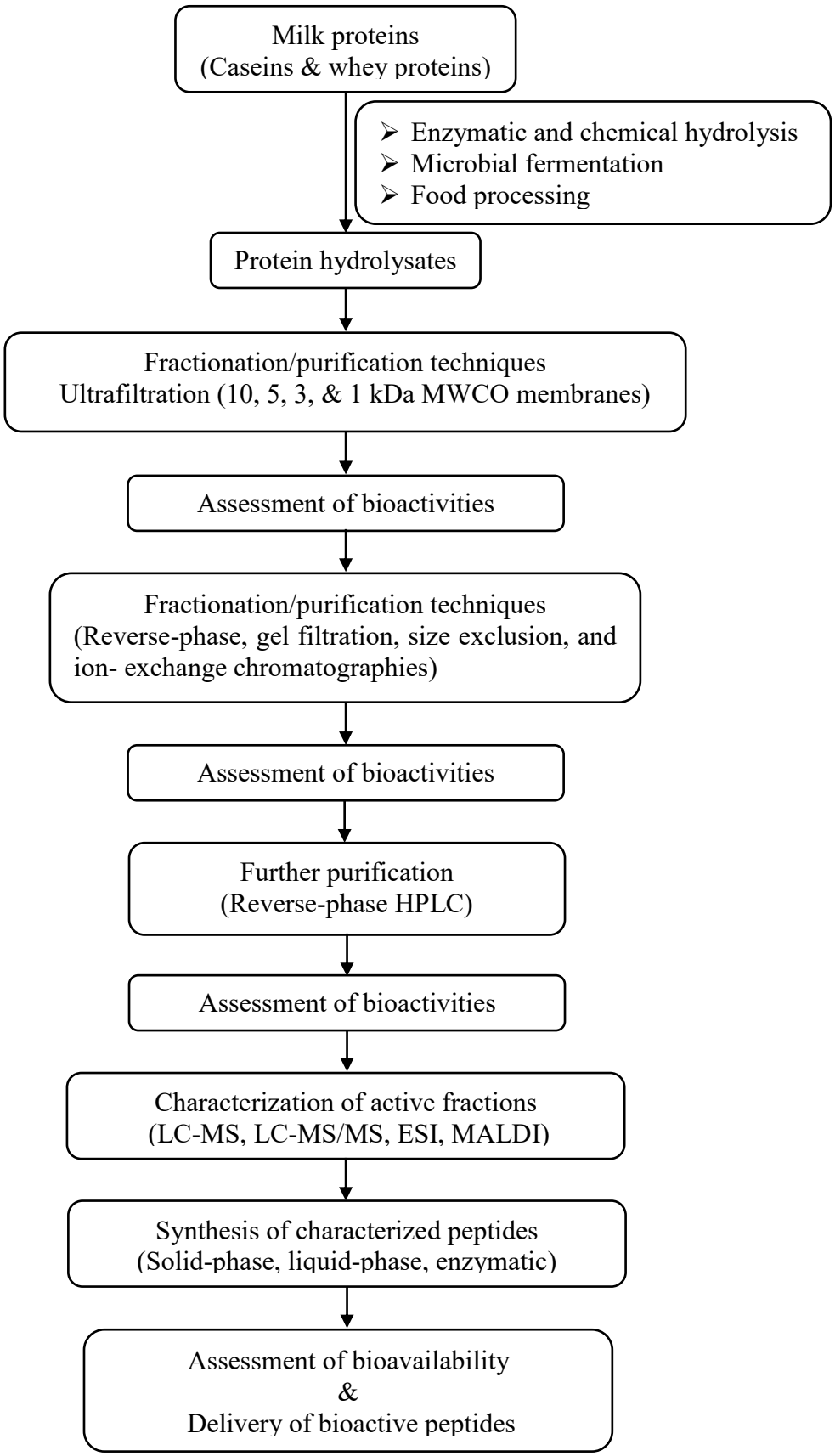

Figure 2A.3 Schematic representation of production and purification of bioactive peptides from yogurt

Solvent extraction and salt precipitation are usually utilized to prepare crude peptide extracts prior to further purification steps. In our laboratory, water-soluble peptides were extracted by high-speed centrifugation of yogurt samples followed by filtering and lyophilization of the supernatant (Sah, Vasiljevic, McKechnie, & Donkor, 2015a, 2016c). Organic solvents such as acetone can also be used to extract peptides from dairy products (Matar, Nadathur, Bakalinsky, & Goulet, 1997). Crude protein hydrolysates are purified by employing several separation techniques, and tested for relevant bioactivities. Proteins/peptides are fractionated based on differences in size during ultrafiltration, which is a simple non-denaturing membrane concentration and fractionation technique. Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) removed sugars and lactic acid present in yogurt samples using cation exchange technique and separated fractions based on different molecular sizes using ultrafiltration membranes with varying molecular weight cut off sizes of 30, 10, and 3 kDa. Hydrolysates exhibiting bioactivities were further purified by exploiting chromatographic techniques, namely ion-exchange chromatography (IEC), gel filtration chromatography (GFC), gel permeation chromatography (GPC), and high-pressure liquid chromatography (HPLC). Chromatographic techniques are considered one of the most powerful, effective and efficient approaches to isolate and purify peptides from crude extracts.

Different chromatography techniques can be employed based on properties of targeted peptides. HPLC with reversed-phase columns is a common technique for separation and detection of peptides from crude mix. When the molecular size of a peptide is of interest, size-exclusion chromatography (SEC) is used; for aqueous separation system, GFC is used whereas GPC is used for non-aqueous separation systems. When separation is based on charge properties of the peptides, IEC, capillary electrophoresis or capillary isoelectric focusing (CIEF) may be utilized for peptide separation.

Once hydrolysates are fractionated into several peptide fractions, further bioactivity assays are conducted to find the most potent fraction. The most potent fraction/s can then be purified by a combination of chromatographic techniques including reverse phase HPLC (RP-HPLC), gel filtration, ion exchange, and selective adsorption (Etzel, 2004). However, special consideration may be required during selection of the purification method because of smaller molecular mass sizes of

bioactive peptides. For instance, SEC has limited resolving power for low molecular weight compounds.

The isolation and purification techniques are very crucial during the production of bioactive peptides. Isolation of a peptide from a dairy product is a difficult task because of matrix complexity (mixture of sugars, acids, salts, and free amino acids). For this reason, classical preparative chromatography, and ultrafiltration are usually employed. With suitable columns and running conditions, HPLC gives useful data for characterization of peptides. For example, RP-HPLC can reflect the hydrophilicity of peptides; however, peptides with similar retention times are not resolved. Therefore, retention time alone cannot be used to identify peptides. Other drawbacks are sensitivity and selectivity for detection of lower mass peptides. A satisfactory chromophore, fluorophore, or electrophore is also not easily available for the separation of small peptides. Nevertheless, the innovation of mass spectrometry has offered a new choice to elucidate the mass of peptides.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to establish purity and molecular weight of a peptide. However, SDS-PAGE is effective only for relatively high molecular weight peptides because resolution with the SDS gel is usually poor for low molecular weight peptides. Size-exclusion HPLC, on the other hand, estimates peptide sizes accurately, but size-based analyses cannot provide complete information about amino acid sequence of a peptide.

Mass spectrometry (MS) techniques such as matrix-assisted laser desorption/ionization- time of flight mass spectrometry (MALDI-TOF MS) or electrospray ionization mass spectrometry (ESI-MS) can be employed to characterize the most active peptide. Furthermore, a protein sequencer and amino acid analyzer can be used to establish amino acid composition and sequence of a peptide. J. Liu, Jin, Lin, Jones, and Chen (2015) identified peptides from protein hydrolysates of egg white by employing amino acid analyzer, liquid chromatography tandem mass spectrometry (LC-MS/MS), and mid-infrared spectroscopy (MIR). Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) also used LC-MS/MS technique to characterize peptides from yogurt efficiently.

2A.7 Synthesis of antioxidant and antibacterial peptides

Structures of bioactive peptides range from a simple dipeptide to a complex peptide having a long chain of amino acids. Regardless, these peptides can be

synthesized once characterized for a large-scale production. Chemical synthesis, enzymatic synthesis, and recombinant DNA technology are three major techniques for peptide synthesis governed mainly by peptide size (Gill, López-Fandiño, Jorba, & Vulfson, 1996). Chemical synthesis is a commonly used technique for laboratory-scale production. Solid phase, homogeneous phase, and mixed phase (a combination of both solid and solution phases) syntheses are three main methods of peptide syntheses (Pichereau & Allary, 2005). Solid phase method is a small-scale production for the synthesis of intermediate size (about 10 to over 100 residues) peptides. However, the method results in high running costs. Conversely, liquid phase synthesis is usually performed to synthesize short-chain peptides and condense peptide fragments (Gill, López-Fandiño, Jorba, & Vulfson, 1996). Chemical synthesis has many drawbacks, for instance racemization during formation of peptide bonds, extra cost involved in protecting side-chain functional amino acids, tough recycling process for acyl donors and coupling reagents, and health and environmental burdens due to toxicity of some spent reagents (Gill, López-Fandiño, Jorba, & Vulfson, 1996). Recombinant DNA technology is usually preferable to synthesize comparatively large peptides (Gill, López-Fandiño, Jorba, & Vulfson, 1996; Kumar & Bhalla, 2005). This process is not usually selected for the synthesis of short peptides because of low expression efficiencies and challenges during the extraction and recovery of products (Korhonen & Pihlanto, 2003b).

Several studies have emphasized enzymatic peptide synthesis (Gill, López-Fandiño, Jorba, & Vulfson, 1996; Kumar & Bhalla, 2005). Enzymatic synthesis has been reported to produce short peptides, usually dipeptides, and tripeptides, and has been rarely employed to produce large peptides exceeding 10 amino acid residues (Kumar & Bhalla, 2005). Proteases catalyze the reactions involving peptide bond hydrolysis in normal aqueous conditions, but the reaction can be reversed at optimum reaction conditions in water-restricted reaction media such as organic solvents (Bordusa, 2002), ionic liquids (Moniruzzaman, Nakashima, Kamiya, & Goto, 2010), supercritical fluids (Noritomi, Miyata, Kato, & Nagahama, 1995), and eutectic mixtures (Maugeri, Leitner, & Domínguez De María, 2013). For example, enzymes such as boilysin from Bacillus stearothermophilus and thermolysin from Bacillus thermoproteolyticus can be exploited for dipeptide (Asp-Phe) synthesizing properties in peptide synthesis (Kühn, Dürrschmidt, Mansfeld, & Ulbrich-Hofmann, 2002). Overcoming the requirement for side-chain protection of chemical synthesis due to

reaction specificity, and utilization of nonhazardous reaction media are some of the merits of enzymatic peptide synthesis. Therefore, a combination of two or three methods, such as the chemoenzymatic approach would probably yield the best results for peptide synthesis (Yazawa & Numata, 2014). The development of cost-effective production processes for antioxidant and antibacterial peptides may promote their application in functional foods or pharmaceutical preparations.

References

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., & Hassan, F. A. (2013). Prebiotics as functional foods: A review. *Journal of Functional Foods, 5*(4), 1542–1553. http://dx.doi.org/10.1016/j.jff.2013.08.009.

Anjum, N., Maqsood, S., Masud, T., Ahmad, A., Sohail, A., & Momin, A. (2014). *Lactobacillus acidophilus*: Characterization of the Species and Application in Food Production. *Critical Reviews in Food Science and Nutrition, 54*(9), 1241–1251. http://dx.doi.org/10.1080/10408398.2011.621169.

Arioli, S., Della Scala, G., Remagni, M. C., Stuknyte, M., Colombo, S., Guglielmetti, S., De Noni, I., Ragg, E., & Mora, D. (2016). *Streptococcus thermophilus* urease activity boosts *Lactobacillus delbrueckii* subsp. *bulgaricus* homolactic fermentation. *International Journal of Food Microbiology*. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.01.006.

Åvall-Jääskeläinen, S., & Palva, A. (2005). *Lactobacillus* surface layers and their applications. *FEMS Microbiology Reviews, 29*(3 SPEC. ISS.), 511–529. http://dx.doi.org/10.1016/j.femsre.2005.04.003.

Boeckner, L. S., Schnepf, M. I., & Tungland, B. C. (2002). Inulin: A review of nutritional and health implications. *Advances in Food and Nutrition Research, 43*, 1–63. http://dx.doi.org/10.1016/S1043-4526(01)43002-6.

Bordusa, F. (2002). Proteases in Organic Synthesis. *Chemical Reviews, 102*(12), 4817–4867. http://dx.doi.org/10.1021/cr010164d.

Buriti, F. C. A., Cardarelli, H. R., Filisetti, T. M. C. C., & Saad, S. M. I. (2007). Synbiotic potential of fresh cream cheese supplemented with inulin and *Lactobacillus paracasei* in co-culture with *Streptococcus thermophilus*. *Food Chemistry, 104*(4), 1605–1610. http://dx.doi.org/10.1016/j.foodchem.2007.03.001.

Campana, R., Federici, S., Ciandrini, E., & Baffone, W. (2012). Antagonistic activity of *Lactobacillus acidophilus* ATCC 4356 on the growth and adhesion/invasion characteristics of human *Campylobacter jejuni*. *Current Microbiology, 64*(4), 371–378. http://dx.doi.org/10.1007/s00284-012-0080-0.

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1–12. http://dx.doi.org/10.1111/j.1471-0307.2011.00725.x.

Chowdhury, B. R., Chakraborty, R., & Raychaudhuri, U. (2008). Study on β-galactosidase enzymatic activity of herbal yogurt. *International Journal of Food Sciences and Nutrition, 59*(2), 116–122. http://dx.doi.org/10.1080/09637480701447787

Christensen, J. E., Dudley, E. G., Pederson, J. A., & Steele, J. L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek, 76*(1–4), 217–246. http://dx.doi.org/10.1023/A:1002001919720.

Corona-Hernandez, R. I., Álvarez-Parrilla, E., Lizardi-Mendoza, J., Islas-Rubio, A. R., de la Rosa, L. A., & Wall-Medrano, A. (2013). Structural stability and viability of microencapsulated probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety, 12*(6), 614–628. http://dx.doi.org/10.1111/1541-4337.12030.

Cruz, A. G., Castro, W. F., Faria, J. A. F., Bolini, H. M. A., Celeghini, R. M. S., Raices, R. S. L., Oliveira, C. A. F., Freitas, M. Q., Conte Júnior, C. A., & Mársico, E. T. (2013). Stability of probiotic yogurt added with glucose oxidase in plastic materials with different permeability oxygen rates during the refrigerated storage. *Food Research International, 51*(2), 723–728. http://dx.doi.org/10.1016/j.foodres.2013.01.028.

Davis, C. D., & Milner, J. A. (2009). Gastrointestinal microflora, food components and colon cancer prevention. *Journal of Nutritional Biochemistry, 20*(10), 743–752.

De Souza Oliveira, R. P., Perego, P., De Oliveira, M. N., & Converti, A. (2011). Effect of inulin as a prebiotic to improve growth and counts of a probiotic cocktail in fermented skim milk. *LWT - Food Science and Technology, 44*(2), 520–523. http://dx.doi.org/10.1016/j.lwt.2010.08.024.

Delorme, C. (2008). Safety assessment of dairy microorganisms: *Streptococcus thermophilus*. *International Journal of Food Microbiology, 126*(3), 274–277. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.08.014.

Doeven, M. K., Kok, J., & Poolman, B. (2005). Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms. *Molecular Microbiology, 57*(3), 640–649. http://dx.doi.org/10.1111/j.1365-2958.2005.04698.x.

Donkor, O. N., Nilmini, S. L. I., Stolic, P., Vasiljevic, T., & Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during

cold storage. *International Dairy Journal, 17*(6), 657–665. http://dx.doi.org/10.1016/j.idairyj.2006.08.006.

Eschenbach, D. A., Davick, P. R., Williams, B. L., Klebanoff, S. J., Young-Smith, K., Critchlow, C. M., & Holmes, K. K. (1989). Prevalence of hydrogen peroxide-producing lactobacillus species in normal women and women with bacterial vaginosis. *Journal of Clinical Microbiology, 27*(2), 251–256.

Espírito Santo, A. P., Cartolano, N. S., Silva, T. F., Soares, F. A. S. M., Gioielli, L. A., Perego, P., Converti, A., & Oliveira, M. N. (2012). Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *International Journal of Food Microbiology, 154*(3), 135–144. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.025

Etzel, M. R. (2004). Manufacture and Use of Dairy Protein Fractions. *Journal of Nutrition, 134*(4), 996S–1002S.

FAO/WHO. (2002). Guidelines for the evaluation of probiotics in food. In *Report of a Joint FAO/WHO*). London, Ontario, Canada.

Farvin, K. H. S., Baron, C. P., Nielsen, N. S., Otte, J., & Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2 – Characterisation of peptide fractions. *Food Chemistry, 123*(4), 1090–1097. http://dx.doi.org/10.1016/j.foodchem.2010.05.029.

Fernandez-Espla, M. D., Garault, P., Monnet, V., & Rul, F. (2000). *Streptococcus thermophilus* cell wall-anchored proteinase: Release, purification, and biochemical and genetic characterization. *Applied and Environmental Microbiology, 66*(11), 4772–4778. http://dx.doi.org/10.1128/AEM.66.11.4772-4778.2000.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: An overview of recent literature. *International Journal of Environmental Research and Public Health, 11*(5), 4745-4767. http://dx.doi.org/10.3390/ijerph110504745.

Franck, A. (2002). Technological functionality of inulin and oligofructose. *British Journal of Nutrition, 87*(SUPPL. 2), S287–S291.

Garault, P., Le Bars, D., Besset, C., & Monnet, V. (2002). Three oligopeptide-binding proteins are involved in the oligopeptide transport of *Streptococcus thermophilus*. *Journal of Biological Chemistry, 277*(1), 32–39. http://dx.doi.org/10.1074/jbc.M107002200.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews, 17*(2), 259–275. http://dx.doi.org/10.1079/NRR200479.

Gilbert, C., Blanc, B., Frot-Coutaz, J., Portalier, R., & Atlan, D. (1997). Comparison of cell surface proteinase activities within the *Lactobacillus* genus. *Journal of Dairy Research, 64*(4), 561–571. http://dx.doi.org/10.1017/S0022029997002355.

Gill, I., López-Fandiño, R., Jorba, X., & Vulfson, E. N. (1996). Biologically active peptides and enzymatic approaches to their production. *Enzyme and Microbial Technology, 18*(3), 163–183. http://dx.doi.org/10.1016/0141-0229(95)00097-6.

Grossiord, B., Vaughan, E. E., Luesink, E., & De Vos, W. M. (1998). Genetics of galactose utilisation via the Leloir pathway in lactic acid bacteria. *Lait, 78*(1), 77–84.

Hebert, E. M., Raya, R. R., & De Giori, G. S. (2000). Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. *Applied and Environmental Microbiology, 66*(12), 5316–5321. http://dx.doi.org/10.1128/AEM.66.12.5316-5321.2000.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology, 11*(8), 506–514. http://dx.doi.org/10.1038/nrgastro.2014.66.

Holzapfel, W. H., & Schillinger, U. (2002). Introduction to pre- and probiotics. *Food Research International, 35*(2-3), 109–116. http://dx.doi.org/10.1016/S0963-9969(01)00171-5.

Holzapfel, W. H., & Wood, B. J. B. (2014). Introduction to the LAB. In *Lactic Acid Bacteria: Biodiversity and Taxonomy*, (pp. 1–12): Wiley Blackwell. http://dx.doi.org/10.1002/9781118655252.ch1.

Huang, Y., & Zheng, Y. (2010). The probiotic *Lactobacillus acidophilus* reduces cholesterol absorption through the down-regulation of Niemann-Pick C1-like

1 in Caco-2 cells. *British Journal of Nutrition, 103*(4), 473–478. http://dx.doi.org/10.1017/S0007114509991991.

Hynönen, U., & Palva, A. (2013). *Lactobacillus* surface layer proteins: structure, function and applications. *Applied Microbiology and Biotechnology, 97*(12), 5225–5243. http://dx.doi.org/10.1007/s00253-013-4962-2.

Ilha, E. C., Scariot, M. C., Treml, D., Pereira, T. P., Sant′Anna, E. S., Prudêncio, E. S., & Arisi, A. C. M. (2015). Comparison of real-time PCR assay and plate count for *Lactobacillus paracasei* enumeration in yoghurt. *Annals of Microbiology*. http://dx.doi.org/10.1007/s13213-015-1137-7.

Kalyani Nair, K., Kharb, S., & Thompkinson, D. K. (2010). Inulin dietary fiber with functional and health attributes—A review. *Food Reviews International, 26*(2), 189–203. http://dx.doi.org/10.1080/87559121003590664.

Kelesidis, T., & Pothoulakis, C. (2012). Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. *Therapeutic Advances in Gastroenterology, 5*(2), 111–125. http://dx.doi.org/10.1177/1756283X11428502.

Kolida, S., & Gibson, G. R. (2007). Prebiotic capacity of inulin-type fructans. *Journal of Nutrition, 137*(11), 2503S–2506S.

Korhonen, H., & Pihlanto, A. (2003a). Bioactive peptides: novel applications for milk proteins. *Applied Biotechnology, Food Science and Policy, 1*(3), 133–144.

Korhonen, H., & Pihlanto, A. (2003b). Food-derived bioactive peptides – Opportunities for designing future foods. *Current Pharmaceutical Design, 9*(16), 1297–1308. http://dx.doi.org/10.2174/1381612033454892.

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

Kourkoutas, Y., Bosnea, L., Taboukos, S., Baras, C., Lambrou, D., & Kanellaki, M. (2006). Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. *Journal of Dairy Science, 89*(5), 1439–1451. http://dx.doi.org/10.3168/jds.S0022-0302(06)72212-3.

Kourkoutas, Y., Xolias, V., Kallis, M., Bezirtzoglou, E., & Kanellaki, M. (2005). *Lactobacillus casei* cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. *Process Biochemistry, 40*(1), 411–416. http://dx.doi.org/http://dx.doi.org/10.1016/j.procbio.2004.01.029.

Kühn, D., Dürrschmidt, P., Mansfeld, J., & Ulbrich-Hofmann, R. (2002). Boilysin and thermolysin in dipeptide synthesis: a comparative study. *Biotechnology and Applied Biochemistry, 36*(1), 71–76. http://dx.doi.org/10.1042/BA20020012.

Kumar, D., & Bhalla, T. C. (2005). Microbial proteases in peptide synthesis: Approaches and applications. *Applied Microbiology and Biotechnology, 68*(6), 726–736. http://dx.doi.org/10.1007/s00253-005-0094-7.

Kunji, E. R. S., Mierau, I., Poolman, B., & Konings, W. N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, 70*(2–4), 187–221.

Ledesma, O. V., de Ruiz Holgado, A. P., Oliver, G., De Giori, G. S., Raibaud, P., & Galpin, J. V. (1977). A synthetic medium for comparative nutritional studies of *Lactobacilli*. *Journal of Applied Bacteriology, 42*(1), 123–133. http://dx.doi.org/10.1111/j.1365-2672.1977.tb00676.x.

Letort, C., & Juillard, V. (2001). Development of a minimal chemically-defined medium for the exponential growth of Streptococcus thermophilus. *Journal of Applied Microbiology, 91*(6), 1023-1029. http://dx.doi.org/10.1046/j.1365-2672.2001.01469.x.

Li, Q., Chen, Q., Ruan, H., Zhu, D., & He, G. (2010). Isolation and characterisation of an oxygen, acid and bile resistant *Bifidobacterium animalis* subsp. *lactis* Qq08. *Journal of the Science of Food and Agriculture, 90*(8), 1340–1346. http://dx.doi.org/10.1002/jsfa.3942.

Liu, J., Jin, Y., Lin, S., Jones, G. S., & Chen, F. (2015). Purification and identification of novel antioxidant peptides from egg white protein and their antioxidant activities. *Food Chemistry, 175*, 258–266. http://dx.doi.org/10.1016/j.foodchem.2014.11.142.

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A., & Siezen, R. J. (2010). The proteolytic system of lactic acid bacteria revisited: A genomic comparison. *BMC Genomics, 11*(1). http://dx.doi.org/10.1186/1471-2164-11-36.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1–17. http://dx.doi.org/10.1016/S0958-6946(01)00036-X.

Macfarlane, S., Macfarlane, G. T., & Cummings, J. H. (2006). Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology and*

Therapeutics, 24(5), 701–714. http://dx.doi.org/10.1111/j.1365-2036.2006.03042.x.

Matar, C., Nadathur, S. S., Bakalinsky, A. T., & Goulet, J. (1997). Antimutagenic effects of milk fermented by *Lactobacillus helveticus* L89 and a protease-deficient derivative. *Journal of Dairy Science, 80*(9), 1965–1970. http://dx.doi.org/10.3168/jds.S0022-0302(97)76139-3.

Maugeri, Z., Leitner, W., & Domínguez De María, P. (2013). Chymotrypsin-catalyzed peptide synthesis in deep eutectic solvents. *European Journal of Organic Chemistry*(20), 4223–4228. http://dx.doi.org/10.1002/ejoc.201300448.

Meyer, D., Bayarri, S., Tárrega, A., & Costell, E. (2011). Inulin as texture modifier in dairy products. *Food Hydrocolloids, 25*(8), 1881–1890. http://dx.doi.org/10.1016/j.foodhyd.2011.04.012.

Molin, G., Jeppsson, B., Johansson, M. L., Ahrne, S., Nobaek, S., Stahl, M., & Bengmark, S. (1993). Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. *Journal of Applied Bacteriology, 74*(3), 314–323.

Moniruzzaman, M., Nakashima, K., Kamiya, N., & Goto, M. (2010). Recent advances of enzymatic reactions in ionic liquids. *Biochemical Engineering Journal, 48*(3), 295–314. http://dx.doi.org/10.1016/j.bej.2009.10.002.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., & Yura, T. (1981). Multiple nutritional requirements of Lactobacilli: Genetic lesions affecting amino acid biosynthetic pathways. *Journal of Bacteriology, 148*(1), 64–71.

Neviani, E., Giraffa, G., Brizzi, A., & Carminati, D. (1995). Amino acid requirements and peptidase activities of *Streptococcus salivarius* subsp. *thermophilus*. *Journal of Applied Bacteriology, 79*(3), 302–307. http://dx.doi.org/10.1111/j.1365-2672.1995.tb03141.x.

Noritomi, H., Miyata, M., Kato, S., & Nagahama, K. (1995). Enzymatic synthesis of peptide in acetonitrile/supercritical carbon dioxide. *Biotechnology Letters, 17*(12), 1323-1328.

Pichereau, C., & Allary, C. (2005). Therapeutic peptides under the spotlight. *EBR - European Biopharmaceutical Review*(WINTER), 88–93.

Picon, A., García-Casado, M. A., & Nuñez, M. (2010). Proteolytic activities, peptide utilization and oligopeptide transport systems of wild *Lactococcus lactis*

strains. *International Dairy Journal, 20*(3), 156–162. http://dx.doi.org/10.1016/j.idairyj.2009.10.002.

Roberfroid, M. B. (2007). Inulin-type fructans: Functional food ingredients. *The Journal of Nutrition, 137*(11), 2493S–2502S.

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., & Matteuzzi, D. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: A comparative study of pure and fecal cultures. *Applied and Environmental Microbiology, 71*(10), 6150–6158. http://dx.doi.org/10.1128/AEM.71.10.6150-6158.2005.

Routray, W., & Mishra, H. N. (2011). Scientific and technical aspects of yogurt aroma and taste: A review. *Comprehensive Reviews in Food Science and Food Safety, 10*(4), 208–220. http://dx.doi.org/10.1111/j.1541-4337.2011.00151.x.

Ruiz, L., Margolles, A., & Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Frontiers in Microbiology, 4*. http://dx.doi.org/10.3389/fmicb.2013.00396.

Rul, F., & Monnet, V. (1997). Presence of additional peptidases in *Streptococcus thermophilus* CNRZ 302 compared to *Lactococcus lactis*. *Journal of Applied Microbiology, 82*(6), 695–704.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015a). Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. *Journal of Dairy Science, 98*(9), 5905–5916. http://dx.doi.org/10.3168/jds.2015-9450.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015b). Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. *Comprehensive Reviews in Food Science and Food Safety, 14*(2), 123–138. http://dx.doi.org/10.1111/1541-4337.12126.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016a). Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt. *Journal of Food Science and Technology, 53*(3), 1698–1708. http://dx.doi.org/10.1007/s13197-015-2100-0.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016b). Physicochemical, textural and rheological properties of probiotic yogurt

fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT - Food Science and Technology, 65*, 978–986. http://dx.doi.org/10.1016/j.lwt.2015.09.027.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016c). Antibacterial and antiproliferative peptides in synbiotic yogurt — Release and stability during refrigerated storage. *Journal of Dairy Science, 99*(6), 4233–4242. http://dx.doi.org/10.3168/jds.2015-10499.

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

Saxami, G., Ypsilantis, P., Sidira, M., Simopoulos, C., Kourkoutas, Y., & Galanis, A. (2012). Distinct adhesion of probiotic strain Lactobacillus casei ATCC 393 to rat intestinal mucosa. *Anaerobe, 18*(4), 417–420. http://dx.doi.org/10.1016/j.anaerobe.2012.04.002.

Schaafsma, G., & Slavin, J. L. (2015). Significance of inulin fructans in the human diet. *Comprehensive Reviews in Food Science and Food Safety, 14*(1), 37–47. http://dx.doi.org/10.1111/1541-4337.12119.

Settachaimongkon, S., Nout, M. J. R., Antunes Fernandes, E. C., Hettinga, K. A., Vervoort, J. M., van Hooijdonk, T. C. M., Zwietering, M. H., Smid, E. J., & Van Valenberg, H. J. F. (2014). Influence of different proteolytic strains of Streptococcus thermophilus in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* on the metabolite profile of set-yoghurt. *International Journal of Food Microbiology, 177*, 29–36. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.008.

Sidira, M., Galanis, A., Ypsilantis, P., Karapetsas, A., Progaki, Z., Simopoulos, C., & Kourkoutas, Y. (2010). Effect of probiotic-fermented milk administration on gastrointestinal survival of *Lactobacillus casei* ATCC 393 and modulation of intestinal microbial flora. *Journal of Molecular Microbiology and Biotechnology, 19*(4), 224–230. http://dx.doi.org/10.1159/000321115.

Sidira, M., Saxami, G., Dimitrellou, D., Santarmaki, V., Galanis, A., & Kourkoutas, Y. (2013). Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool. *Journal of Dairy Science, 96*(5), 3369–3377. http://dx.doi.org/10.3168/jds.2012-6343.

Sieuwerts, S., De Bok, F. A. M., Hugenholtz, J., & Van Hylckama Vlieg, J. E. T. (2008). Unraveling microbial interactions in food fermentations: From classical to genomics approaches. *Applied and Environmental Microbiology, 74*(16), 4997–5007. http://dx.doi.org/10.1128/AEM.00113-08.

Smid, E. J., & Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology, 24*(2), 148–154. http://dx.doi.org/10.1016/j.copbio.2012.11.007.

Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*(3 SPEC. ISS.), 591–610. http://dx.doi.org/10.1016/j.femsre.2005.04.002.

Tamime, A. Y., & Robinson, R. K. (2007). *Tamime and Robinson's Yoghurt: Science and Technology* (3rd ed.). Cambridge, England: Woodhead Publishing Limited.

Thammarutwasik, P., Hongpattarakere, T., Chantachum, S., Kijroongrojana, K., Itharat, A., Reanmongkol, W., Tewtrakul, S., & Ooraikul, B. (2009). Prebiotics – A review. *Songklanakarin Journal of Science and Technology, 31*(4), 401–408.

Thomas, T. D., & Crow, V. L. (1984). Selection of galactose-fermenting *Streptococcus thermophilus* in lactose-limited chemostat cultures. *Applied and Environmental Microbiology, 48*(1), 186–191.

Thomas, T. D., & Pritchard, G. G. (1987). Proteolytic enzymes of dairy starter cultures. *FEMS Microbiology Letters, 46*(3), 245–268. http://dx.doi.org/10.1016/0378-1097(87)90111-X.

Vasiljevic, T., Kealy, T., & Mishra, V. K. (2007). Effects of β-glucan addition to a probiotic containing yogurt. *Journal of Food Science, 72*(7), C405–C411. http://dx.doi.org/10.1111/j.1750-3841.2007.00454.x.

Vasiljevic, T., & Shah, N. P. (2008). Probiotics—From Metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714–728. http://dx.doi.org/10.1016/j.idairyj.2008.03.004.

von Ah, U., Mozzetti, V., Lacroix, C., Kheadr, E. E., Fliss, I., & Meile, L. (2007). Classification of a moderately oxygen-tolerant isolate from baby faeces as *Bifidobacterium thermophilum*. *BMC Microbiology, 7*. http://dx.doi.org/10.1186/1471-2180-7-79.

Watson, D., O'Connell Motherway, M., Schoterman, M. H. C., van Neerven, R. J. J., Nauta, A., & Van Sinderen, D. (2013). Selective carbohydrate utilization by lactobacilli and bifidobacteria. *Journal of Applied Microbiology, 114*(4), 1132–1146. http://dx.doi.org/10.1111/jam.12105.

Xie, N., Zhou, T., & Li, B. (2012). Kefir yeasts enhance probiotic potentials of *Lactobacillus paracasei* H9: The positive effects of coaggregation between the two strains. *Food Research International, 45*(1), 394–401. http://dx.doi.org/10.1016/j.foodres.2011.10.045.

Yazawa, K., & Numata, K. (2014). Recent advances in chemoenzymatic peptide syntheses. *Molecules, 19*(9), 13755–13774. http://dx.doi.org/10.3390/molecules190913755.

Zanotti, I., Turroni, F., Piemontese, A., Mancabelli, L., Milani, C., Viappiani, A., Prevedini, G., Sanchez, B., Margolles, A., Elviri, L., Franco, B., van Sinderen, D., & Ventura, M. (2015). Evidence for cholesterol-lowering activity by *Bifidobacterium bifidum* PRL2010 through gut microbiota modulation. *Applied Microbiology and Biotechnology, 99*(16), 6813–6829. http://dx.doi.org/10.1007/s00253-015-6564-7.

Chapter 2B: Antioxidative and antibacterial peptides derived from bovine milk proteins

Chapter 2B presents a comprehensive review of current scientific knowledge on bioactive peptides, focusing on antioxidant and antibacterial peptides released from milk proteins. Efforts have also been made to discuss recent advances in production, bioavailability, and application of these peptides.

The paper entitled “Antioxidative and antibacterial peptides derived from bovine milk proteins” by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been accepted for publication in the peer-reviewed journal “Critical Reviews in Food Science and Nutrition” (2016). http://dx.doi.org/ 10.1080/10408398.2016.1217825.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** **Date:** 15-Mar-2016

Baidya Nath Prasad Sah [redacted]

Paper Title:

Antioxidative and Antibacterial Peptides Derived from Bovine Milk Proteins

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Concept development, research question, hypothesis, and manuscript preparation
Prof Todor Vasiljevic	5 %	Concept development, research question, hypothesis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Concept development, research question, hypothesis, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Concept development, research question, hypothesis, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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Antioxidative and Antibacterial Peptides Derived from Bovine Milk Proteins

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Key words: caseins, whey proteins, bioactive peptides, antioxidant and antibacterial activities, bioavailability

Running head: Antioxidative and antibacterial peptides

Abstract

The search for alternative preservatives is on the rise due to safety issues linked with the application of synthetic antioxidants and the extensive increase in bacterial resistance to several conventional antibiotics. Therefore, the quest for finding suitable alternatives including bioactive peptides has received attention. This article reports a comprehensive insight concerning antioxidative and antibacterial peptides derived from milk proteins, a prolific source of peptides having various bioactivities. Caseins and whey proteins have also been evaluated for antioxidative and antibacterial potential using the BIOPEP database. A notable number of potentially active peptides is present in milk proteins. Technological approaches are here reported for the production of these peptides. The findings of this review show potentiality of utilizing dairy-derived antioxidative and antibacterial peptides in the development of a superior alternative to the current generation of preservatives and therapeutic agents, as well as a functional ingredient in dietetic or pharmaceutical applications.

1. Introduction

Oxidative metabolism is inevitable for the survival of living cells, unfortunately with detrimental side effects due to generated free radicals (Ren et al., 2008). A free radical is defined as any chemical species containing unpaired electrons; examples are reactive oxygen and nitrogen species, which can cause oxidative and nitrosative stresses, respectively, ultimately leading to several chronic and degenerative diseases such as multiple sclerosis, cancer, cardiovascular diseases, Parkinson's disease, and Alzheimer's disease (Sharma, 2014). Pathological effects of free radicals are neutralized *in vivo* by numerous enzymatic antioxidants including catalase, glutathione peroxidase, and superoxide dismutase (Devasagayam et al., 2004) and by the consumption of foods containing antioxidants.

Many oxidation reactions also involve various food components, namely carbohydrates, proteins, and lipids. For example, lipid oxidation results in rancidity and deteriorates food quality (Pihlanto, 2006). Moreover, intermediates of lipid oxidation may react with protein molecules and further deteriorate food quality (Viljanen et al., 2004). Carbohydrates are less susceptible to oxidation compared to lipids and proteins. Lipid oxidation causes the development of undesirable dark color, off-flavors and odors, resulting in a short shelf-life of many fat containing foods (Antolovich et al., 2002). Thus, the prevention of lipid oxidation has become a challenge for the food industry.

An antioxidant is a chemical compound that can halt radical chain reactions by scavenging reactive species including reactive oxygen/nitrogen species, prevent formation of reactive oxidants (Huang et al., 2005), and inhibit auto-oxidative processes such as lipid peroxidation in foods (Mielnik et al., 2003). Synthetic compounds such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and propyl gallate (PG) have been frequently used as antioxidants in the food industry; however, safety issues linked with their application have led to limited uses or even prohibition in some nations (Jun et al., 2004). Therefore, the quest for finding suitable alternatives such as bioactive peptides is gaining momentum.

Bioactive peptides are specific protein fragments that can beneficially influence physiological functions or conditions (López-Expósito et al., 2007). Bioactive peptides are comprised of various groups of peptides including physiologically active peptides, hormonal peptides, antimicrobial peptides, neuroactive peptides, immunoactive peptides, and enzyme regulators and inhibitors

(Gill et al., 1996). Most biologically active peptides consist of 2–20 amino acids (Meisel and FitzGerald, 2003) with molecular mass less than 6 kDa (Sun et al., 2004). These peptides are latent when present in the amino acid chain of a protein molecule but can be released by hydrolysis of the protein. Thus, dietary proteins are widely recognized not only for their role as a source of amino acids but also for offering health benefits either in the intact form or as protein hydrolysates.

Milk proteins have been frequently assessed for various bioactivities due to their nutritional importance and overall applications in the food industry. These proteins comprise about 80% caseins, with the remaining portion commonly termed whey proteins. Caseins are phosphoproteins and include α_{s1}-, α_{s2}-, β-, and κ-caseins. Whey proteins are globular proteins composed of β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins (Kopf-Bolanz et al., 2012). Milk proteins can confer special support to the growth and development of neonates beyond basic nutrition as well as serve as excellent precursors of different biologically active peptides. Research into bioactive milk peptides began in the 1950s with a study by Mellander (1950), who reported improved vitamin D-independent calcification in rachitic infants owing to the ingestion of phosphorylated peptides derived from caseins. Existence of biologically active peptides in dairy foods such as yogurt, fermented milk, and cheese has been described in numerous reports (Farvin et al., 2010; Korhonen, 2009; Sah et al., 2014).

Several identified peptides derived from milk proteins display various biological roles, including antioxidative (Farvin et al., 2010; Sah et al., 2014, 2015a), antibacterial (Sah et al., 2016a), antimutagenic (Sah et al., 2014, 2016b), anticancer (Sah et al., 2015b), and antihypertensive (Korhonen, 2009) functions. These activities are governed by several factors, the major being structural properties, amino acid composition, and sequences within the peptide (Jun et al., 2004; Pihlanto-Leppälä, 2000). Dairy-derived bioactive peptides may thus offer promising avenues to prevent, control, and even treat several diseases and disorders by means of a regulated food intake. This potential has drawn attention from industrial, nutraceutical, and therapeutic sectors. Several studies (Sah et al., 2015b; Sharma, 2014) mentioned antioxidative therapy as an encouraging approach in the management of oxidative and nitrosative stress-related chronic and degenerative diseases.

The intent of this review was to assess current state of knowledge regarding dairy-derived bioactive peptides, with a particular attention to their antioxidative and

antibacterial potential. Efforts have also been made to discuss recent advances in the production, bioavailability, and application of these peptides.

2. Dairy-derived antioxidative peptides

Numerous investigations have focused on liberation of bioactive peptides from milk proteins. The molecular weight and amino acid sequence mainly govern the antioxidant activity of a peptide (Jun et al., 2004). Moreover, dairy-derived antioxidative peptides usually contain 5–11 amino acids, including hydrophobic amino acids such as proline, histidine, tyrosine, or tryptophan in the sequence (Pihlanto, 2006). Caseins are widely recognized as an important source of peptides with an antioxidative potential. Casein peptides produced during hydrolysis with alcalase, a *Bacillus licheniformis* enzyme preparation, displayed greater potential to exert bioavailability and *in vitro* antioxidant efficacy in comparison to gastrointestinal digests (Xie et al., 2013). Suetsuna et al. (2000) and López-Expósito et al. (2007) reported peptides with antioxidative properties from peptic digests of α_{s1}- and κ-casein, respectively (Table 1). Rival et al. (2001) also reported tryptic β-casein digest as source of antioxidative peptides. Several peptides liberated during the digestion of caseins and whey proteins have been reported to possess antioxidative potential. These are shown in Table 1.

The BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) was used for the scientific prediction of antioxidative peptides from caseins and whey proteins of bovine milk (Minkiewicz et al., 2008). Potential antioxidative peptides from different caseins are presented in Tables 3.1–3.6 and whey proteins in Tables 4.1–4.3. Furthermore, *in silico* determinations established that tyrosine, proline, leucine, glutamine, and histidine dominated at various positions in antioxidative peptides derived from caseins (β-casein gen. var. A1, A2, B, α_{S1}-casein gen. var. B, C and κ-casein gen. var. A precursor), and tyrosine, leucine, tryptophan, alanine, and isoleucine dominated at various positions in antioxidative peptides derived from whey proteins (α-lactalbumin gen. var. B precursor, β-lactoglobulin, gen. var. A precursor, and lactoferrin) (Figure 1A).

3. Mode of action of antioxidative peptides

Various compounds may exhibit antioxidative activity via different mechanisms; for example, interactions with free radicals or oxygen capture. Six main

reactive oxygen species are singlet oxygen ($^1O_2$), peroxyl radical ($RCOO^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) (Huang et al., 2005). A third mechanism may be through inhibition of oxidative enzymes such as lipoxygenase, resulting in prevention or reduction of oxidation reactions catalyzed by these enzymes. Given that a bioactive peptide can show antioxidative activities via multiple reaction mechanisms, several different antioxidant assays should ideally be performed to acquire comprehensive information about overall antioxidative capacity of the tested peptide. Common assays for establishing scavenging activity of free radicals include 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), 2,2'- azino- bis (3- ethyl benzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$), and hydroxyl ($^{\bullet}OH$), oxygen radical absorbance capacity (ORAC), inhibition of lipid peroxidation, iron (Fe^{2+}) chelating activity, and reducing power.

According to the mode of action, antioxidants can be grouped into primary and secondary (Antolovich et al., 2002). Primary antioxidants are capable of neutralizing free radicals, mainly via hydrogen transfer, resulting in interception of the chain reaction of lipid peroxidation and referred to chain breaking antioxidants. Ascorbic acid, BHT, BHA, and PG are examples of the primary antioxidants (Antolovich et al., 2002). They are likely effective during the induction period of lipid peroxidation. Secondary antioxidants prevent lipid oxidation by inhibiting the formation of free radicals via self-oxidation. Lecithin is an example of a secondary antioxidant (Butnariu and Grozea, 2012). Antioxidants also include metal chelators, radical chain reaction inhibitors, antioxidant enzyme cofactors, and oxidative enzyme inhibitors (Huang et al., 2005).

The antioxidative capacity of a peptide depends on various attributes including sequence, composition of amino acids, configuration, and concentration (Phanturat et al., 2010). Peptides containing amino acids residues such as aspartic acid, proline, tryptophan, tyrosine, methionine, cysteine, leucine, arginine, alanine, and histidine have shown higher antioxidative activities (Ji et al., 2014; Li et al., 2011; Sarmadi and Ismail, 2010). Hydrolysates containing proline, histidine, methionine, tyrosine, valine, lysine, cysteine, and glutamine have also displayed strong radical scavenging capacity (Rajapakse et al., 2005). A high hydroxyl radical scavenging activity by compounds possessing amino acid residues, such as aspartic acid, cysteine, glutamic acid, lysine, proline, serine, and tryptophan has similarly been reported (Furukawa et al., 2012). Non-polar aliphatic amino acid valine at the N-terminal of the sequence appears to be

needed for a peptide to exert enhanced antioxidative activity and retard lipid peroxidation (Chen et al., 1995). This may be a critical mechanism in interrupting the radical mediated chain reaction. Similarly, Elias et al. (2008) concluded that antioxidative peptides often contain hydrophobic amino acid including valine or leucine at the N-terminus of the peptides. Hydrophobic amino acid residues like valine or leucine can increase the presence of the peptides at the water–lipid interface and therefore facilitate access to scavenge free radicals generated at the lipid phase (Ranathunga et al., 2006).

4. Dairy-derived antibacterial peptides

The existence of bacterial growth inhibitory substances in milk has been known for a long time. Jones et al. (1930) reported on antibacterial factor found in milk against scarlet fever Streptococci. They named it lactenin. Antibacterial peptides (ABPs) have gained much attention worldwide since then. Most peptides possessing antimicrobial activities have low molecular mass (2000 Da on average) with considerable number of hydrophobic amino acids (Dziuba and Dziuba, 2014). Furthermore, cationic antibacterial peptides (CAPs), carrying a net positive charge, constitute the larger group, which is further categorized into 3 groups (Brogden, 2005; Vizioli and Salzet, 2002). The first group encompasses linear cationic α-helical peptides including magainin and cecropins. These peptides initially acquire linear conformation prior to interacting with the cell membrane, which changes upon contact and adopt amphipathic α-helical secondary configuration (Bechinger et al., 1993). The second cationic peptide group contains proline and arginine, which are mainly linear although some may show extended coils (Brogden, 2005). Cationic peptides containing cysteine residues that form disulfide linkages and stable β-sheets are in the third class. For instance, defensins have 6 cysteine residues and are grouped into α-, β-, and θ-defensins according to the alignment of disulfide bridges (Mehra et al., 2012).

Several dairy-derived peptides with antibacterial potential have also been obtained by enzymatic hydrolysis of the two most abundant bovine whey proteins, namely α-lactalbumin and β-lactoglobulin (Table 2). The BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) was employed for the scientific prediction of ABPs from caseins and whey proteins of bovine milk (Minkiewicz et al., 2008). Potential ABPs from different caseins are presented in Tables 3.1–3.6 and whey proteins in Tables 4.1–4.3. Furthermore, *in silico*

determinations established that proline, glutamic acid, leucine, valine, and threonine dominated at various positions in ABPs derived from caseins (β-casein gen. var. A1, A2, B, α_{S1}-casein gen. var. B, C, and κ-casein gen. var. A precursor), and arginine, lysine, alanine, cysteine, and leucine dominated at various positions in antibacterial peptides obtained from whey proteins (α-lactalbumin gen. var. B precursor, β-lactoglobulin, gen. var. A precursor, and lactoferrin) (Figure 1B). Dziuba and Dziuba (2014) also reported a higher content of Lys, Leu, Val, and Pro in the evaluated antimicrobial peptides from milk proteins. Bovine lactoferricin is a pepsin-derived lactoferrin peptide fragment, and corresponds to the 17–41 residue of bovine lactoferrin. This sequence differs from the amino acid sequence within the lactoferrin molecule responsible for iron sequestering property, which imparts bacteriostatic effect by removing iron (an essential substrate) necessary for bacterial growth. It supports a new mode of antibacterial action independent of iron-binding capacities of lactoferrin (Yamauchi et al., 1993).

5. Mode of action of antibacterial peptides

ABPs disrupt microbial cell membrane integrity via negatively charged electrostatic interactions, inhibiting synthesis of proteins, DNA and RNA, or by interacting with specific intracellular targets (Bahar and Ren, 2013). Thus, differences in membrane composition between Gram-positive and Gram-negative bacteria are important factors for antibacterial activity. Cell walls of Gram-negative bacteria are structurally and chemically more complex compared to Gram-positive bacteria, having a unique outer membrane, rich in lipopolysaccharides (LPS), phospholipids, lipoprotein, and different proteins. The net positive charge of antibacterial peptides facilitates an initial interaction with the negatively charged bacterial outer membrane (LPS) via electrostatic binding (Hancock and Rozek, 2002). ABPs may form “cracks” in the LPS layer or bind to LPS sites responsible for interactions with divalent cations (Ca^{2+} and Mg^{2+}), which are crucial for membrane integrity, resulting in distortion of the outer membrane structure and a consequent permeabilization of the membrane to peptides and other toxic molecules (da Silva Jr and Teschke, 2003). The peptide molecules, after passing through the outer membrane of the bacterial cell, bind to the negatively charged cytoplasmic membrane. For an appropriate action, peptide molecules need to adopt a conformation typical to the environment of the lipid bilayer of the cell membrane. Therefore, hydrophobicity of antimicrobial peptide appears to

facilitate important interactions with hydrophobic core of the lipid bilayer (Oren et al., 1997). Thus, increased hydrophobicity of a peptide may enhance its antimicrobial activity; this is supported by López-Expósito et al. (2006a) and López-Expósito et al. (2006b) who reported enhanced antibacterial activity of hydrophobic peptides derived from α_{S2}- and κ-caseins. However, this is yet to be confirmed as some studies have failed to establish correlation between antibacterial activity and hydrophobicity (Giangaspero et al., 2001; Pathak et al., 1995).

Net electric charge of ABPs also plays important role in antibacterial activities (Benkerroum, 2010). For example, a cationic peptide f(183–207) derived from α_{S2}-Casein, has been reported to permeabilize cell envelopes of *E. coli* ATCC 25922 and *Staphylococcus carnosus* CECT 4491T, resulting in leakage of cytoplasmic content to the extracellular medium (López-Expósito et al., 2008). Several other models (such as "carpet", "toroid pore" and "barrel-stave") have also been proposed to describe interactions between ABPs and membrane resulting in permeabilization of the membrane (Yeaman and Yount, 2003). Furthermore, secondary structures including α-helices and β-sheets of ABPs also play important roles in causing cellular damage (Giangaspero et al., 2001; Pathak et al., 1995). Basically, peptides having α-helices and β-sheets of ABPs are electrostatically attracted to the negatively charged groups (LPS layer of Gram-negative bacterial cells and lipoteichoic acids and peptidoglycan of Gram-positive bacterial cells) and accumulated on the bacterial membranes. This results in development of transient pores, channels, or extensive detergent-like disaggregation of the bilayer leading to depolarization, loss of membrane compositional specificity, and leakage of metabolites from the cell (Giangaspero et al., 2001; Pathak et al., 1995).

6. Production of antioxidative and antibacterial peptides

6.1 Generation of antioxidative and antibacterial peptides

Milk and other unfermented dairy products contain mainly full-length proteins in a native or denatured form and a few peptides or free amino acids, depending on the level of heat treatment during processing (Kopf-Bolanz et al., 2012). Bioactive peptides, engraved in their latent form in the primary protein structure, are released by hydrolysis of precursor milk proteins. The hydrolysis can be achieved by chemicals such as hydrochloric acid or proteolytic enzymes obtained from animals,

microorganisms, plants, microbial fermentation, or during food processing (Korhonen, 2009). A combination of these techniques has been proven effective and efficient in the liberation of peptides. Thus, *in silico* studies of milk proteins should be performed in order to design an appropriate approach for generating peptides of interest.

6.1.1 *In vitro* enzymatic and chemical hydrolysis of protein

Hydrolysis of a protein molecule results in a cleavage of a peptide bond, fragmentation of proteins into oligopeptides, peptides, or free amino acids, and is commonly accomplished employing enzymes or chemicals (acid/alkali). Alkali treatment may destroy protein substrates resulting in yielding potentially toxic substances such as lysinoalanine (N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine) (Faist et al., 2000; López-Fandiño et al., 2006). Furthermore, nutritional quality of released peptides and amino acids may also be reduced due to structural and chemical changes during severe acid hydrolysis (Neklyudov et al., 2000). On the other hand, enzyme-catalyzed hydrolysis appears as an effective approach that liberates biologically active peptides from the intact sequence of protein molecules with less damage under milder hydrolytic conditions (Korhonen, 2009). A type of proteolytic enzymes and protein substrates, enzyme to substrate ratio, physicochemical conditions (pH, reaction time, and temperature) influence the composition of hydrolysates and thus bioactivities. Several proteolytic enzymes, namely pepsin, pancreatin, chymotrypsin, alcalase, and thermolysin, in addition to enzymes from bacterial and fungal origins could possibly be employed to generate bioactive peptides (Korhonen and Pihlanto, 2003). Hernández-Ledesma et al. (2005) utilized corolase PP from pig pancreas glands to obtain antioxidative protein hydrolysates from α-lactalbumin and β-lactoglobulin. However, the application of animal derived proteases in food formulations may be perceived unfavorable in terms of consumers' preferences, thus plant and microbial derived proteases seem to be an appropriate alternative.

In this regard, Di Pierro et al. (2014) utilized serum prepared from latex of *Ficus carica* L. trees as a source of proteolytic enzymes to release peptides from bovine β-casein. Different ficin forms, which are cysteine proteases of the papain family, have been reported in the latex of *Ficus carica* (Azarkan et al., 2011). Hogan et al. (2009) employed three proteases of microbial-origin, validase, alkaline and neutral proteases respectively from *Aspergillus oryzae*, *Bacillus licheniformis*, and *Bacillus subtilis*, in the digestion of milk protein for production of antioxidative

hydrolysates/peptides. Several studies have been reported on antioxidant activities of hydrolysates prepared using alcalase (Peng et al., 2010). Alcalase yields comparatively shorter peptides, which usually resist digestive enzymes and reach the required sites presumably intact (Sarmadi and Ismail, 2010).

6.1.2 Microbial fermentation

Exploiting microbial fermentation for liberation of a range of bioactive peptides has drawn much attention in the dairy industry since highly proteolytic lactic acid cultures may be used in manufacturing of dairy products. The proteolytic systems of some lactic acid bacteria including *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Lactococcus lactis* have been extensively studied (Christensen et al., 1999). These systems are comprised in general of (a) cell-wall proteinases, such as P_I-type proteinase (PrtP) in *Lactococcus lactis* strains (Juillard et al., 1995), which initiate proteolytic attacks, (b) transport systems, which facilitate oligopeptides uptakes into bacterial cells, and (c) intracellular peptidases. The isolated intracellular peptidases from *Lactobacilli* and *Lactococci* include mainly aminopeptidases and endopeptidases. Small peptides liberated within the cells may be expelled outside into food products via an exchange over the cell membrane (Kunji et al., 1996), or through cell lysis. Furthermore, cell lysis may also result in release of intracellular peptidases, which can further act on large oligopeptides produced via action of the cell wall proteases but due to size, remain in the medium. The extracellular and intracellular proteolytic and peptidolytic activities of starter and probiotic cultures have been used on numerous occasions for bioactive peptide studies published in the period between 1990 and 2016 (Tables 1 and 2).

6.2 Large-scale production of antioxidative and antibacterial peptides

Incorporation of bioactive peptides in food products requires optimized steps of the production, isolation, characterization, and economic viability for industrial scale manufacturing as well as established safety. Laboratory-scale preparations are not optimized and reflect a low volume of production. The lack of viable bioprocesses, transferable to the industrial scale, is a major bottleneck for commercial existence of bioactive peptides on the market. Thus, industrial-scale manufacturing of antioxidative and antibacterial peptides is of utmost importance.

Investigations for suitable large-scale production technologies for isolation and enrichment of antioxidative and antibacterial peptides are ongoing to reduce time and cost (Agyei and Danquah, 2012; Bargeman et al., 2002). Some developments such as continuous reactors and membrane ultrafiltration processes for continuous production and separation of the peptides are encouraging (Korhonen and Pihlanto, 2006). Membrane separation techniques are one of the suitable approaches for the enrichment of peptides having a particular molecular weight range (Korhonen and Pihlanto, 2007). Ultrafiltration and nanofiltration techniques are employed industrially to concentrate antioxidative and antibacterial peptides of interest for the production of peptide ingredients. Such preparations can be supplemented in different consumer products including dairy and fruit based drinks, chewing gum, confectionery, and pastilles (Korhonen, 2009). For example, Ellegård et al. (1999) developed a process-scale method (comprised of acid precipitation, diafiltration, and anion-exchange chromatography) to produce casein phosphopeptides. Electro-membrane filtration (EMF) is another approach to isolate and enrich specific antioxidative and antibacterial peptides (Bargeman et al., 2002). This method combines membrane filtration and electrophoresis, making it more efficient for charged molecules, and less costly than chromatography. Demers-Mathieu et al. (2013) also obtained a retentate rich in anionic peptides using a pilot scale nanofiltration from ultrafiltration permeate of whey protein tryptic hydrolysate. The authors determined the antibacterial activity of these peptides derived from β-lactoglobulin against *Listeria monocytogenes* and *Staphylococcus aureus*; but further reported that improved activity could be through processing optimization. Recombined enzyme technology utilizing certain microbial strains or proteolytic enzymes can also be useful in the commercial production of a specific peptide sequence. However, lack of scalable identification and enrichment methodologies increase peptide manufacturing cost (Agyei and Danquah, 2012).

7. Application of antioxidative and antibacterial peptides

Studies have demonstrated that rapid rate of urbanization, and industrialization has resulted in immense changes in lifestyle practices leading to an increase in the risks of various diseases and disorders, such as cancer. Nutrition performs key roles in the prevention of several diseases, and optimizes health and well-being (Mills et al., 2011). Thus, consumers' interest nowadays has inclined towards food products that can impart health benefits, increase longevity, and/or reduce the risks of, or delay the

onsets of, diseases and disorders. This has contributed to the emergence of functional foods products, which provide supplementary physiological benefits in addition to basic nutrition. Thus, formulation of novel food products by incorporating antioxidative and antimicrobial peptides of milk origin as ingredients for the physiological benefits or disease prevention and control has drawn the attention of researchers, clinicians, consumers, and industrialists (Sah et al., 2016a, c). In addition, milk protein hydrolysates have a potential application as a functional ingredient in food products because of unique properties including solubility, water holding capacity, gelling activity, emulsification ability, foaming capacity, and oil absorption capacity.

7.1 Application of antioxidative peptides

Lipid oxidation results in development of off-flavors, odors, and potentially toxic reaction products, and is therefore a great concern to food industries and consumers (Sakanaka et al., 2005). Antioxidative peptides can be used to increase shelf life of foods by preventing lipid peroxidation, and for protecting living systems from oxidative damage by scavenging free reactive oxygen and nitrogen species. Increasing the oxidation stability of vegetable oils is important for industrial practice, and many antioxidant tests are based on the ability to retard or inhibit oil rancidity.

Several studies reported that the antioxidative activity of protein hydrolysates and isolated peptides prepared from milk proteins is comparable to that of natural antioxidant including α-tocopherol, and commonly used synthetic antioxidants BHA and BHT (Hernández-Ledesma et al., 2005; Qian et al., 2011; Sah et al., 2014). Protein hydrolysates and peptide fractions could be added as a functional ingredient in food systems for the reduction of oxidative changes during storage period either directly as an additive or indirectly through diffusion from the packaging material (van Aardt et al., 2004). For example, casein calcium peptides prevented lipid oxidation when added in ground beef homogenates (Sakanaka et al., 2005). Similarly, (FitzGerald, 1998) suggested a potential use of antioxidative caseinophosphopeptides, derived from tryptic digestion of casein, in breakfast cereals, breads, pastry, chocolate, juices, tea, and mayonnaise. Furthermore, some antioxidative peptides may also be added in food products together with other antioxidants such as tocopherols for synergistic effects (Chen et al., 1996; Jun et al., 2004). In agreement of this, a tripeptide (Pro–His–His) showed a synergistic action with the lipophilic antioxidants BHA and tocopherol

(Chen et al., 1996). In addition to food industry, antioxidants can also be proposed for use in oral and topical pharmaceutical and cosmetic compositions.

Common consequences of oxidative stress are increased damages to biomolecules including lipids, proteins, and DNA leading to pathogenesis of many diseases such as cancers. Reducing oxidative damages may result in prevention and/or treatment of cancer as antioxidative peptides can terminate oxidizing chain reactions and formations of free radicals including reactive oxygen species (ROS) (Choudhari et al., 2014; Tekiner-Gulbas et al., 2013). The ROS also play a critical role in the pathogenesis of neurodegenerative diseases such as amyotrophic lateral sclerosis. Cellular ROS is mostly generated at inner mitochondrial membrane by respiratory chain. Thus, cell permeable antioxidative peptides (targeted to the inner mitochondrial membrane) can be utilized to treat neuronal damage induced by oxidative stress (Calkins et al., 2012). Antioxidative peptides have been reported to ameliorate complications of diabetes, a chronic condition of high glucose levels in blood, by scavenging hyperglycemia-induced free radicals (Johansen et al., 2005; Rahimi et al., 2005). In addition, antioxidative peptides (YQEPVLGPVRGPFPIIV, SLPQNIPPLTQTPVVVPPF derived from bovine β-casein) isolated from synbiotic yogurt exhibited anticancer activity via apoptosis and cell cycle arrest in HT-29 colon cancer cells (Sah et al., 2016d). Similarly, soluble extracts of milk fermented by proteolytic strains of *Lactobacillus helveticus* demonstrated antioxidative and anti-colon cancer activities (Elfahri et al., 2016).

7.2 Application of antibacterial peptides

Dairy-derived ABPs could have the prospects of being utilized as preservative and ingredients in functional foods, nutraceuticals, cosmeceuticals, and pharmaceuticals to control and prevent various diseases. Dairy-derived peptides have been documented for antibacterial activities against several pathogens (Table 2). Moreover, the amino acid sequence of an antibacterial peptide can also be manipulated to optimize food applications of the peptide for the control of targeted food-borne pathogens. Alvarez-Ordóñez et al. (2013) studied importance of specific amino acids of α_{S2}-casein f(183–207) peptide at different pH, temperature, and salinity governing its antibacterial activity against food-borne pathogens (*Listeria monocytogenes* and *Cronobacter sakazakii*). The main finding was that environmental conditions altered antibacterial potency thus a particular antibacterial peptide can be utilized for the

preservation of a particular food product. Antimicrobial packaging, which is active food packaging delivering antimicrobial activity, deliberately interacts with the packaged food or food environment, and retards or even inhibits the growth of spoilage and pathogenic microorganisms resulting in shelf-life extension (Otoni et al., 2016). The antimicrobial agents can be incorporated directly into polymer matrix, can be coated or adsorbed onto the packaging surface, or can be immobilized to polymers by ion or covalent linkages (Appendini and Hotchkiss, 2002).

ABPs as therapeutic adjuncts offer a promising policy in the pharmaceutical area and may act as alternatives to conventional antibiotics. Advantages such as wide spectra of therapeutic actions, low levels of toxicity, absence or low levels of accumulation in body tissues and structural diversity are also associated with the utilization of ABPs (Agyei and Danquah, 2011). In addition, the use of antimicrobial peptides in the treatment of different medical conditions leads to little or no side effects (Hancock and Sahl, 2006). Because of broad spectrum of activities, ABPs can also be utilized to kill antibiotic-resistant pathogens (Rizzello et al., 2005). Apart from biological activities, other aspects including *in vivo* stability, side effects, and production costs should be considered before developing these peptides for systemic use (Nguyen et al., 2011). Water-soluble peptide extracts having antibacterial activities against Gram-positive and Gram-negative bacteria also exhibited anticancer activity against HT29 colon cancer cells (Sah et al., 2016a).

7.3 Potential challenges in application of antioxidative and antibacterial peptides

Potential challenges such as sensory perception, stability, and safety issues need to be rectified prior to exploiting antioxidative and antibacterial peptides as a food additive or drug supplement although enrichment of milk protein hydrolysates in food products has several health benefits over non-hydrolyzed protein (Hernández-Ledesma et al., 2011). Some protein hydrolysates may have negative impact on sensory perception including bitterness. This can negatively affect product quality and limit their utilization in food and pharmaceutical applications. The release of hydrophobic groups during hydrolysis of proteins may be attributed to development of bitterness. Lee et al. (1996) isolated five bitter peptides from cheddar cheese, which were rich in proline, a hydrophobic amino acid. The association of bitterness with hydrophobicity and peptide size was early reported (Ney, 1971). In general, small molecular weight peptides appear responsible for bitterness. However, there is

conflicting research on the exact molecular weight range. Ney (1971) hypothesized that small to medium size peptides can only impart bitterness and postulated different Q-values to predict bitterness based on size and hydrophobicity of peptides. Q-value is an average hydrophobicity value imparted by amino acid side-chains of a peptide. Peptides of molecular weight less than 6 kDa and Q-values greater than 1400 were described as bitter. However, there are many limitations to this assumption, as it does not consider peptide structure, which strongly influences bitterness properties of peptides (Maehashi et al., 2008).

Bitter-masking agents can be used to mask bitterness of the food products. Depending on the application, it may be necessary to remove bitterness from hydrolysates. Several approaches such as hydrophobic interaction chromatography, treatment with activated carbon, and hydrolysis with exopeptidases have been proposed previously (Saha and Hayashi, 2001). Another technique that minimizes the contact of bitter antioxidative and antibacterial peptides with taste buds is encapsulation. Microencapsulation also limits many issues associated with bioactive peptides such as hygroscopicity, reactivity, and stability under adverse environmental conditions (Saadi et al., 2015). For example, bitterness of casein hydrolysates was successfully attenuated by microencapsulation technologies, including encapsulation with soya protein isolate/gelatin (Favaro-Trindade et al., 2010), and soybean protein isolate/pectin (Mendanha et al., 2009). Barbosa et al. (2004) also reported on lessening of bitterness of casein hydrolysates through encapsulating in lipospheres.

Most allergens of food origin are proteins, which break down results in the generation of lower molecular weight fractions. Thus, a concern associated with the application of peptides as antioxidative and antibacterial agents is also a potential issue of allergenicity, especially with peptides derived from β-lactoglobulin (Rahaman et al., 2016). Possibilities of mitigating allergenic activities of the parent protein molecules during hydrolysis of peptide bonds can also be experienced. On the other hand, peptides may retain allergenic potentials of the parent protein after hydrolysis (Reddi et al., 2012), which may restrain their utilization in food and in pharmaceutical industries for human use. Pescuma et al. (2015) isolated peptides derived from the allergenic sequences of β-lactoglobulin from its hydrolysate prepared by *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454. Hence, allergenicity associated with application of antioxidative and antibacterial peptides need to be thoroughly investigated and tested.

The instability of peptide molecules is another drawback limiting their food and pharmaceutical applications. Bioavailability of peptides consumed orally mainly varied according to resistance to enzymatic breakdown and translocation across the cell membrane. Bioactive peptides must cross many barriers such as gastrointestinal (GI) and blood-circulatory systems in their active form and maintain their activity up to the target sites to exert physiological effects *in vivo* (Sienkiewicz-Szlapka et al., 2009). Although therapeutic peptides are a good alternative to commonly available antibiotics, several inherent drawbacks are also associated with them. Some have low oral bioavailability, very short half-life, rapid hepatic and renal clearance, poor capability to cross physiological barriers, high conformational flexibility (Pichereau and Allary, 2005) and high production costs (Agyei and Danquah, 2011).

GI environment with presence of digestive enzymes and very low pH in stomach could affect the structure of bioactive peptides leading to changes in bioactivity (Segura-Campos et al., 2011). Sah et al. (2016a) reported on the degradation of biologically active peptide fractions during GI digestion. Su et al. (2007) also found instability of peptides derived from α_{S1}- and α_{S2}-casein during pancreatic digestion. However, peptides containing proline and hydroxyproline residues can usually resist hydrolysis by digestive enzymes (Segura-Campos et al., 2011). In reality, GI digestion can lead to both degradation and generation of biologically active peptides. For example, Kopf-Bolanz et al. (2014) reported degradation of α_{S1}-casein leading to generation of several potential bioactive peptides during *in vitro* digestion. Chen and Li (2012) reported high susceptibility of large peptides (above 3 kDa) to gastric digestion and suggested low-molecular weight (less than 1 kDa) casein-derived antioxidative peptides as a promising antioxidant for oral administration. Casein-derived antioxidative peptides are more likely digestible in the intestine than the stomach (Chen and Li, 2012). Ao and Li (2013) reported the degradation of positively charged peptide fractions to be more significant than negatively charged peptide fractions during GI digestion resulting in lowering of antioxidative activities. Moreover, the GI tract is a well-known site for generation of various free radicals (Srigiridhar et al., 2001), which aid challenges for the efficacy of antioxidative peptides *in vivo* when ingested orally. Alternative routes of peptide administration such as parenteral, mucosal, and transdermal routes have emerged to circumvent gastric acidity, intestinal proteolytic environment, and permeability barriers (Ibraheem et al., 2014). Enzyme inhibitors to protect bioactive peptides from

proteolytic enzymes, absorption enhancers to overcome intrinsic drawbacks of peptides (charge, hydrophilicity, and molecular size) and microencapsulation are also alternative approaches for oral delivery (Lee, 2002).

8. Conclusions

Milk is considered complete food nourishment for the newborn. Research has proposed several bioactivities associated with intact milk proteins and their hydrolysates, broadening the role of milk proteins in human health. Current findings have discovered several antioxidative and antibacterial peptides obtainable from milk proteins and emphasized their potential utilization as functional food ingredients, nutraceuticals, or pharmaceuticals to promote health and reduce disease risks. These peptides could also be utilized to extend shelf life of food products by preventing lipid peroxidation and microbial growth. However, many scientific and technological issues need to be rectified prior to exploiting these peptides for human nutrition and health. Potential therapeutic applications of antioxidative and antimicrobial peptides have mostly been studied *in vitro*. Therefore, further investigations are essential to clarify the safety concerns and action modes associated with these peptides, through *in vivo* studies as well as dosage and clinical experimentations. Another challenge to food scientists and manufacturers is the lack of appropriate industrial-scale processes for commercial production of foods with physiologically significant concentrations of antioxidative and/or antibacterial peptides of milk origin.

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References

Agyei, D., and Danquah, M. K. (2011). Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnol. Adv.* **29**(3): 272–277. doi: 10.1016/j.biotechadv.2011.01.001

Agyei, D., and Danquah, M. K. (2012). Rethinking food-derived bioactive peptides for antimicrobial and immunomodulatory activities. *Trends Food Sci. Technol.* **23**(2): 62–69. doi: 10.1016/j.tifs.2011.08.010

Alvarez-Ordóñez, A., Begley, M., Clifford, T., Deasy, T., Considine, K., and Hill, C. (2013). Structure-activity relationship of synthetic variants of the milk-derived antimicrobial peptide α_{S2}-casein f(183–207). *Appl. Environ. Microbiol.* **79**(17): 5179–5185. doi: 10.1128/AEM.01394-13

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., and Robards, K. (2002). Methods for testing antioxidant activity. *Analyst*. **127**(1): 183–198. doi: 10.1039/b009171p

Ao, J., and Li, B. (2013). Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion in vitro: Charge properties of peptides affect digestive stability. *Food Res. Int.* **52**(1): 334–341. doi: 10.1016/j.foodres.2013.03.036

Appendini, P., and Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innov. Food Sci. Emerg. Technol.* **3**(2): 113–126. doi: 10.1016/S1466-8564(02)00012-7

Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J., and Baeyens-Volant, D. (2011). Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from *Ficus carica* latex. *Phytochemistry*. **72**(14-15): 1718–1731. doi: 10.1016/j.phytochem.2011.05.009

Bahar, A. A., and Ren, D. (2013). Antimicrobial Peptides. *Pharmaceuticals*. **6**(12): 1543–1575. doi: 10.3390/ph6121543

Barbosa, C. M. S., Morais, H. A., Delvivo, F. M., Mansur, H. S., De Oliveira, M. C., and Silvestre, M. P. C. (2004). Papain hydrolysates of casein: Molecular weight profile and encapsulation in lipospheres. *J. Sci. Food Agric.* **84**(14): 1891–1900. doi: 10.1002/jsfa.1855

Bargeman, G., Koops, G. H., Houwing, J., Breebaart, I., Van Der Horst, H. C., and Wessling, M. (2002). The development of electro-membrane filtration for the

isolation of bioactive peptides: The effect of membrane selection and operating parameters on the transport rate. *Desalination*. **149**(1–3): 369–374. doi: 10.1016/S0011-9164(02)00824-X

Bechinger, B., Zasloff, M., and Opella, S. J. (1993). Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. *Protein Sci.* **2**(12): 2077–2084. doi: 10.1002/pro.5560021208

Benkerroum, N. (2010). Antimicrobial peptides generated from milk proteins: A survey and prospects for application in the food industry. A review. *Int. J. Dairy Technol.* **63**(3): 320–338. doi: 10.1111/j.1471-0307.2010.00584.x

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*. **3**(3): 238–250. doi: 10.1038/nrmicro1098

Butnariu, M., and Grozea, L. (2012). Antioxidant (antiradical) compounds. *J. Bioequivalence Bioavailab.* **4**(6): xvii–xix. doi: 10.4172/jbb.10000e18

Calkins, M. J., Manczak, M., and Reddy, P. H. (2012). Mitochondria-targeted antioxidant SS31 prevents amyloid beta-induced mitochondrial abnormalities and synaptic degeneration in Alzheimer's disease. *Pharmaceuticals*. **5**(10): 1103–1119. doi: 10.3390/ph5101103

Chen, H. M., Muramoto, K., and Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-conglycinin. *J. Agric. Food. Chem.* **43**(3): 574–578. doi: 10.1021/jf00051a004

Chen, H. M., Muramoto, K., Yamauchi, F., and Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food. Chem.* **44**(9): 2619–2623.

Chen, M., and Li, B. (2012). The effect of molecular weights on the survivability of casein-derived antioxidant peptides after the simulated gastrointestinal digestion. *Innov. Food Sci. Emerg. Technol.* **16**: 341–348. doi: 10.1016/j.ifset.2012.07.009

Choudhari, S. K., Chaudhary, M., Gadbail, A. R., Sharma, A., and Tekade, S. (2014). Oxidative and antioxidative mechanisms in oral cancer and precancer: A review. *Oral Oncol.* **50**(1): 10–18. doi: 10.1016/j.oraloncology.2013.09.011

Christensen, J. E., Dudley, E. G., Pederson, J. A., and Steele, J. L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek*. **76**(1–4): 217–246. doi: 10.1023/A:1002001919720

Contreras, M. D. M., Hernández-Ledesma, B., Amigo, L., Martín-Álvarez, P. J., and Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. *LWT - Food Sci. Technol.* **44**(1): 9–15. doi: 10.1016/j.lwt.2010.06.017

da Silva Jr, A., and Teschke, O. (2003). Effects of the antimicrobial peptide PGLa on live *Escherichia coli*. *Biochim. Biophys. Acta*. **1643**(1–3): 95–103. doi: 10.1016/j.bbamcr.2003.10.001

Demers-Mathieu, V., Gauthier, S. F., Britten, M., Fliss, I., Robitaille, G., and Jean, J. (2013). Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration. *Int. Dairy J.* **28**(2): 94–101. doi: 10.1016/j.idairyj.2012.09.003

Devasagayam, T. P. A., Tilak, J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004). Free radicals and antioxidants in human health: Current status and future prospects. *J. Assoc. Physicians India*. **52**(OCT): 794–804.

Di Pierro, G., O'Keeffe, M. B., Poyarkov, A., Lomolino, G., and Fitzgerald, R. J. (2014). Antioxidant activity of bovine casein hydrolysates produced by *Ficus carica* L.-derived proteinase. *Food Chem.* **156**: 305–311. doi: 10.1016/j.foodchem.2014.01.080

Díaz, M., and Decker, E. A. (2004). Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. *J. Agric. Food. Chem.* **52**(26): 8208–8213. doi: 10.1021/jf048869e

Dionysius, D. A., and Milne, J. M. (1997). Antibacterial peptides of bovine lactoferrin: Purification and characterization. *J. Dairy Sci.* **80**(4): 667–674. doi: 10.3168/jds.S0022-0302(97)75985-X

Dziuba, B., and Dziuba, M. (2014). New milk protein-derived peptides with potential antimicrobial activity: An approach based on bioinformatic studies. *Int. J. Mol. Sci.* **15**(8): 14531–14545. doi: 10.3390/ijms150814531

Elbarbary, H. A., Abdou, A. M., Nakamura, Y., Park, E. Y., Mohamed, H. A., and Sato, K. (2012). Identification of novel antibacterial peptides isolated from a

commercially available casein hydrolysate by autofocusing technique. *BioFactors*. **38**(4): 309–315. doi: 10.1002/biof.1023

Elfahri, K. R., Vasiljevic, T., Yeager, T., and Donkor, O. N. (2016). Anti-colon cancer and antioxidant activities of bovine skim milk fermented by selected *Lactobacillus helveticus* strains. *J. Dairy Sci.* **99**(1): 31–40. doi: 10.3168/jds.2015-10160

Elias, R. J., Kellerby, S. S., and Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Crit. Rev. Food Sci. Nutr.* **48**(5): 430–441. doi: 10.1080/10408390701425615

Ellegård, K. H., Gammelgård-Larsen, C., Sørensen, E. S., and Fedosov, S. (1999). Process scale chromatographic isolation, characterization and identification of tryptic bioactive casein phosphopeptides. *Int. Dairy J.* **9**(9): 639–652. doi: 10.1016/S0958-6946(99)00135-1

Faist, V., Drusch, S., Kiesner, C., Elmadfa, I., and Erbersdobler, H. F. (2000). Determination of lysinoalanine in foods containing milk protein by high-performance chromatography after derivatisation with dansyl chloride. *Int. Dairy J.* **10**(5–6): 339–346. doi: 10.1016/S0958-6946(00)00075-3

Farvin, K. H. S., Baron, C. P., Nielsen, N. S., Otte, J., and Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2 – Characterisation of peptide fractions. *Food Chem.* **123**(4): 1090–1097. doi: 10.1016/j.foodchem.2010.05.029

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

FitzGerald, R. J. (1998). Potential uses of caseinophosphopeptides. *Int. Dairy J.* **8**(5-6): 451–457. doi: 10.1016/S0958-6946(98)00068-5

Furukawa, T., Akutagawa, T., Funatani, H., Uchida, T., Hotta, Y., Niwa, M., and Takaya, Y. (2012). Cyclic dipeptides exhibit potency for scavenging radicals. *Bioorg. Med. Chem.* **20**(6): 2002–2009. doi: 10.1016/j.bmc.2012.01.050

Giangaspero, A., Sandri, L., and Tossi, A. (2001). Amphipathic α helical antimicrobial peptides: A systematic study of the effects of structural and physical properties on biological activity. *Eur. J. Biochem.* **268**(21): 5589–5600. doi: 10.1046/j.0014-2956.2001.02494.x

Gill, I., López-Fandiño, R., Jorba, X., and Vulfson, E. N. (1996). Biologically active peptides and enzymatic approaches to their production. *Enzyme Microb. Technol.* **18**(3): 163–183. doi: 10.1016/0141-0229(95)00097-6

Hancock, R. E. W., and Rozek, A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett.* **206**(2): 143–149. doi: 10.1016/S0378-1097(01)00480-3

Hancock, R. E. W., and Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**(12): 1551–1557. doi: 10.1038/nbt1267

Hernández-Ledesma, B., Contreras, M. M., and Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* **165**(1): 23–35. doi: 10.1016/j.cis.2010.11.001

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., and Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulln. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food. Chem.* **53**(3): 588–593. doi: 10.1021/jf048626m

Hogan, S., Zhang, L., Li, J., Wang, H., and Zhou, K. (2009). Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. *Food Chem.* **117**(3): 438–443. doi: 10.1016/j.foodchem.2009.04.040

Huang, D., Boxin, O. U., and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food. Chem.* **53**(6): 1841–1856. doi: 10.1021/jf030723c

Ibraheem, D., Elaissari, A., and Fessi, H. (2014). Administration strategies for proteins and peptides. *Int. J. Pharm.* **477**(1–2): 578–589. doi: 10.1016/j.ijpharm.2014.10.059

Ji, N., Sun, C., Zhao, Y., Xiong, L., and Sun, Q. (2014). Purification and identification of antioxidant peptides from peanut protein isolate hydrolysates using UHR-Q-TOF mass spectrometer. *Food Chem.* **161**: 148–154. doi: 10.1016/j.foodchem.2014.04.010

Johansen, J. S., Harris, A. K., Rychly, D. J., and Ergul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical pratice. *Cardiovasc. Diabetol.* **4**. doi: 10.1186/1475-2840-4-5

Jones, F. S., Simms, H. S., and Maaroufi, A. (1930). The bacterial growth inhibitor (lactenin) of milk: I. The preparation in concentrated form. *J. Exp. Med.* **51**(2): 327–339.

Juillard, V., Laan, H., Kunji, E. R. S., Jeronimus-Stratingh, C. M., Bruins, A. P., and Konings, W. N. (1995). The extracellular P_I-type proteinase of *Lactococcus lactis* hydrolyzes β-casein into more than one hundred different oligopeptides. *J. Bacteriol.* **177**(12): 3472–3478.

Jun, S. Y., Park, P. J., Jung, W. K., and Kim, S. K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *Eur. Food Res. Technol.* **219**(1): 20–26. doi: 10.1007/s00217-004-0882-9

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergéres, G., Portmann, R., and Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *J. Nutr.* **142**(2): 245–250. doi: 10.3945/jn.111.148635

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., and Egger, L. (2014). Impact of milk processing on the generation of peptides during digestion. *Int. Dairy J.* **35**(2): 130–138. doi: 10.1016/j.idairyj.2013.10.012

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *J. Funct. Foods*. **1**(2): 177–187. doi: 10.1016/j.jff.2009.01.007

Korhonen, H., and Pihlanto, A. (2003). Food-derived bioactive peptides – Opportunities for designing future foods. *Curr. Pharm. Des.* **9**(16): 1297–1308. doi: 10.2174/1381612033454892

Korhonen, H., and Pihlanto, A. (2006). Bioactive peptides: production and functionality. *Int. Dairy J.* **16**(9): 945–960. doi: 10.1016/j.idairyj.2005.10.012

Korhonen, H., and Pihlanto, A. (2007). Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Curr. Pharm. Des.* **13**(8): 829–843. doi: 10.2174/138161207780363112

Kudoh, Y., Matsuda, S., Igoshi, K., and Oki, T. (2001). Antioxidative peptide from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO13953. *J JPN SOC FOOD SCI*. **48**(1): 44–50.

Kunji, E. R. S., Mierau, I., Poolman, B., and Konings, W. N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. **70**(2–4): 187–221.

Lahov, E., and Regelson, W. (1996). Antibacterial and immunostimulating casein-derived substances from milk: Casecidin, isracidin peptides. *Food Chem. Toxicol.* **34**(1): 131–145. doi: 10.1016/0278-6915(95)00097-6

Lee, H. J. (2002). Protein drug oral delivery: The recent progress. *Arch. Pharmacal Res.* **25**(5): 572–584. doi: 10.1007/BF02976925

Lee, K. D., Lo, C. G., and Warthesen, J. J. (1996). Removal of bitterness from the bitter peptides extracted from Cheddar cheese with peptidases from *Lactococcus lactis* ssp. *cremoris* SK11. *J. Dairy Sci.* **79**(9): 1521–1528. doi: 10.3168/jds.S0022-0302(96)76512-8

Li, Y. W., Li, B., He, J., and Qian, P. (2011). Quantitative structure–activity relationship study of antioxidative peptide by using different sets of amino acids descriptors. *J. Mol. Struct.* **998**(1–3): 53–61. doi: 10.1016/j.molstruc.2011.05.011

López-Expósito, I., Amigo, L., and Recio, I. (2008). Identification of the initial binding sites of α_{S2}-casein f(183–207) and effect on bacterial membranes and cell morphology. *Biochim. Biophys. Acta Biomembr.* **1778**(10): 2444–2449. doi: 10.1016/j.bbamem.2008.06.018

López-Expósito, I., Gómez-Ruiz, J. A., Amigo, L., and Recio, I. (2006a). Identification of antibacterial peptides from ovine α_{S2}-casein. *Int. Dairy J.* **16**(9): 1072–1080. doi: 10.1016/j.idairyj.2005.10.006

López-Expósito, I., Minervini, F., Amigo, L., and Recio, I. (2006b). Identification of antibacterial peptides from bovine κ-casein. *J. Food Prot.* **69**(12): 2992–2997.

López-Expósito, I., Quirós, A., Amigo, L., and Recio, I. (2007). Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. *Lait*. **87**(4–5): 241–249. doi: 10.1051/lait:2007019

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

Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., and Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. *Biochem. Biophys. Res. Commun.* **365**(4): 851–855. doi: 10.1016/j.bbrc.2007.11.070

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., and Reynolds, E. C. (2001). Kappacin, a novel antibacterial

peptide from bovine milk. *Antimicrob. Agents Chemother.* **45**(8): 2309–2315. doi: 10.1128/AAC.45.8.2309-2315.2001

McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H., and Coventry, M. J. (2005). Isolation and characterisation of antibacterial peptides derived from the f(164–207) region of bovine α_{S2}-casein. *Int. Dairy J.* **15**(2): 133–143. doi: 10.1016/j.idairyj.2004.06.008

Mehra, T., Köberle, M., Braunsdorf, C., Mailänder-Sanchez, D., Borelli, C., and Schaller, M. (2012). Alternative approaches to antifungal therapies. *Exp. Dermatol.* **21**(10): 778–782. doi: 10.1111/exd.12004

Meisel, H., and FitzGerald, R. J. (2003). Biofunctional Peptides from Milk Proteins: Mineral Binding and Cytomodulatory Effects. *Curr. Pharm. Des.* **9**(16): 1289–1295.

Mellander, O. (1950). The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. *Acta Soc. Med. Ups.* **55**(5-6): 247–255.

Mendanha, D. V., Ortiz, S. E. M., Favaro-Trindade, C. S., Mauri, A., Monterrey-Quintero, E. S., and Thomazini, M. (2009). Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin. *Food Res. Int.* **42**(8): 1099–1104. doi: 10.1016/j.foodres.2009.05.007

Mielnik, M. B., Aaby, K., and Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. *Meat Sci.* **65**(3): 1147–1155. doi: 10.1016/S0309-1740(02)00345-5

Mills, S., Ross, R. P., Hill, C., Fitzgerald, G. F., and Stanton, C. (2011). Milk intelligence: Mining milk for bioactive substances associated with human health. *Int. Dairy J.* **21**(6): 377–401. doi: 10.1016/j.idairyj.2010.12.011

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., and Darewicz, M. (2008). BIOPEP database and other programs for processing bioactive peptide sequences. *J. AOAC Int.* **91**(4): 965–980.

Neklyudov, A. D., Ivankin, A. N., and Berdutina, A. V. (2000). Production and purification of protein hydrolysates (review). *Appl. Biochem. Microbiol.* **36**(4): 317-324. doi: 10.1007/BF02738038

Ney, K. H. (1971). Prediction of bitterness of peptides from their amino acid composition. *Z. Lebensm. Unters. Forsch.* **147**(2): 64-68. doi: 10.1007/BF01879606

Nguyen, L. T., Haney, E. F., and Vogel, H. J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29**(9): 464–472. doi: 10.1016/j.tibtech.2011.05.001

Oren, Z., Hong, J., and Shai, Y. (1997). A repertoire of novel antibacterial diastereomeric peptides with selective cytolytic activity. *J. Biol. Chem.* **272**(23): 14643–14649. doi: 10.1074/jbc.272.23.14643

Otoni, C. G., Espitia, P. J. P., Avena-Bustillos, R. J., and McHugh, T. H. (2016). Trends in antimicrobial food packaging systems: Emitting sachets and absorbent pads. *Food Res. Int.* **83**: 60–73. doi: 10.1016/j.foodres.2016.02.018

Pathak, N., Salas-Auvert, R., Ruche, G., Janna, M. H., McCarthy, D., and Harrison, R. G. (1995). Comparison of the effects of hydrophobicity, amphiphilicity, and α-helicity on the activities of antimicrobial peptides. *Proteins: Structure, Function and Genetics*. **22**(2): 182–186. doi: 10.1002/prot.340220210

Pellegrini, A., Dettling, C., Thomas, U., and Hunziker, P. (2001). Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochim. Biophys. Acta, Gen. Subj.* **1526**(2): 131–140. doi: 10.1016/S0304-4165(01)00116-7

Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., and Von Fellenberg, R. (1999). Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochim. Biophys. Acta Gen. Subj.* **1426**(3): 439–448. doi: 10.1016/S0304-4165(98)00165-2

Peng, X., Kong, B., Xia, X., and Liu, Q. (2010). Reducing and radical-scavenging activities of whey protein hydrolysates prepared with Alcalase. *Int. Dairy J.* **20**(5): 360–365. doi: 10.1016/j.idairyj.2009.11.019

Peng, X., Xiong, Y. L., and Kong, B. (2009). Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. *Food Chem.* **113**(1): 196–201. doi: 10.1016/j.foodchem.2008.07.068

Pescuma, M., Hébert, E. M., Haertlé, T., Chobert, J. M., Mozzi, F., and Font De Valdez, G. (2015). *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454 cleaves allergenic peptides of β-lactoglobulin. *Food Chem.* **170**: 407–414. doi: 10.1016/j.foodchem.2014.08.086

Phanturat, P., Benjakul, S., Visessanguan, W., and Roytrakul, S. (2010). Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the

production of gelatin hydrolysate with antioxidative activity. *LWT - Food Sci. Technol.* **43**(1): 86–97. doi: 10.1016/j.lwt.2009.06.010

Pichereau, C., and Allary, C. (2005). Therapeutic peptides under the spotlight. *EBR Eur. Biopharm. Rev.*(WINTER): 88–93.

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* **11**(9–10): 347–356. doi: 10.1016/S0924-2244(01)00003-6

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *Int. Dairy J.* **16**(11): 1306–1314. doi: 10.1016/j.idairyj.2006.06.005

Qian, B., Xing, M., Cui, L., Deng, Y., Xu, Y., Huang, M., and Zhang, S. (2011). Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340. *J. Dairy Res.* **78**(1): 72–79.

Rahaman, T., Vasiljevic, T., and Ramchandran, L. (2016). Effect of processing on conformational changes of food proteins related to allergenicity. *Trends Food Sci. Technol.* **49**: 24–34. doi: 10.1016/j.tifs.2016.01.001

Rahimi, R., Nikfar, S., Larijani, B., and Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomed. Pharmacother.* **59**(7): 365–373. doi: 10.1016/j.biopha.2005.07.002

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., and Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.* **38**(2): 175–182. doi: 10.1016/j.foodres.2004.10.002

Ranathunga, S., Rajapakse, N., and Kim, S. K. (2006). Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*). *Eur. Food Res. Technol.* **222**(3–4): 310–315. doi: 10.1007/s00217-005-0079-x

Recio, I., and Visser, S. (1999a). Identification of two distinct antibacterial domains within the sequence of bovine α_{s2}-casein. *Biochim. Biophys. Acta Gen. Subj.* **1428**(2–3): 314–326. doi: 10.1016/S0304-4165(99)00079-3

Recio, I., and Visser, S. (1999b). Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin. *In situ* enzymatic hydrolysis on an ion-exchange membrane. *J. Chromatogr. A*. **831**(2): 191–201. doi: 10.1016/S0021-9673(98)00950-9

Reddi, S., Kapila, R., Dang, A. K., and Kapila, S. (2012). Evaluation of allergenic response of milk bioactive peptides using mouse mast cell. *Milchwissenschaft*. **67**(2): 189–191.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., and Xue, S. J. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chem.* **108**(2): 727–736. doi: 10.1016/j.foodchem.2007.11.010

Rival, S. G., Boeriu, C. G., and Wichers, H. J. (2001). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J. Agric. Food. Chem.* **49**(1): 295–302. doi: 10.1021/jf0003911

Rizzello, C. G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M. D., and Zambonin, P. G. (2005). Antibacterial activities of peptides from the water-soluble extracts of Italian cheese varieties. *J. Dairy Sci.* **88**(7): 2348–2360.

Saadi, S., Saari, N., Anwar, F., Hamid, A. A., and Ghazali, H. M. (2015). Recent advances in food biopeptides: Production, biological functionalities and therapeutic applications. *Biotechnol. Adv.* **33**(1): 80–116. doi: 10.1016/j.biotechadv.2014.12.003

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2014). Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. *Food Chem.* **156**: 264–270. doi: 10.1016/j.foodchem.2014.01.105

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2015a). Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. *J. Dairy Sci.* **98**(9): 5905–5916. doi: 10.3168/jds.2015-9450

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2015b). Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. *Compr. Rev. Food Sci. Food Saf.* **14**(2): 123–138. doi: 10.1111/1541-4337.12126

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2016a). Antibacterial and antiproliferative peptides in synbiotic yogurt — Release and stability during refrigerated storage. *J. Dairy Sci.* **99**(6): 4233–4242. doi: 10.3168/jds.2015-10499

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2016b). Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt. *J Food Sci Technol*. **53**(3): 1698–1708. doi: 10.1007/s13197-015-2100-0

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2016c). Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT - Food Sci. Technol.* **65**: 978–986. doi: 10.1016/j.lwt.2015.09.027

Sah, B. N. P., Vasiljevic, T., McKechnie, S., and Donkor, O. N. (2016d). Antioxidant peptides isolated from synbiotic yoghurt exhibit antiproliferative activities against HT-29 colon cancer cells. *Int. Dairy J.* **63**: 99–106. doi: 10.1016/j.idairyj.2016.08.003

Saha, B. C., and Hayashi, K. (2001). Debittering of protein hydrolyzates. *Biotechnol. Adv.* **19**(5): 355–370. doi: 10.1016/S0734-9750(01)00070-2

Sakanaka, S., Tachibana, Y., Ishihara, N., and Juneja, L. R. (2005). Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates. *J. Agric. Food. Chem.* **53**(2): 464–468.

Sarmadi, B. H., and Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*. **31**(10): 1949–1956. doi: 10.1016/j.peptides.2010.06.020

Sedaghati, M., Ezzatpanah, H., Boojar, M. M. A., Ebrahimi, M. T., and Aminafshar, M. (2014). Plasmin digest of κ-casein as a source of antibacterial peptides. *J. Dairy Res.* **81**(2): 245–251. doi: 10.1017/S0022029914000120

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., and Hernandez-Escalante, V. M. (2011). Bioavailability of bioactive peptides. *Food Rev. Int.* **27**(3): 213–226. doi: 10.1080/87559129.2011.563395

Sharma, N. (2014). Free radicals, antioxidants and disease. *Biol. Med.* **6**(3). doi: 10.4172/0974-8369.1000214

Sienkiewicz-Szlapka, E., Jarmolowska, B., Krawczuk, S., Kostyra, E., Kostyra, H., and Bielikowicz, K. (2009). Transport of bovine milk-derived opioid peptides across a Caco-2 monolayer. *Int. Dairy J.* **19**(4): 252–257. doi: 10.1016/j.idairyj.2008.10.007

Srigiridhar, K., Nair, K. M., Subramanian, R., and Singotamu, L. (2001). Oral repletion of iron induces free radical mediated alterations in the gastrointestinal

tract of rat. *Mol. Cell. Biochem.* **219**(1–2): 91–98. doi: 10.1023/A:1011023111048

Su, R., Qi, W., He, Z., Yuan, S., and Zhang, Y. (2007). Pancreatic hydrolysis of bovine casein: Identification and release kinetics of phosphopeptides. *Food Chem.* **104**(1): 276–286. doi: 10.1016/j.foodchem.2006.11.039

Suetsuna, K., Ukeda, H., and Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.* **11**(3): 128–131. doi: 10.1016/S0955-2863(99)00083-2

Sun, J., He, H., and Bi, J. X. (2004). Novel antioxidant peptides from fermented mushroom *Ganoderma lucidum*. *J. Agric. Food. Chem.* **52**(21): 6646–6652. doi: 10.1021/jf0495136

Tang, W., Yuan, H., Zhang, H., Wang, L., Qian, H., and Qi, X. (2015). An antimicrobial peptide screened from casein hydrolyzate by *Saccharomyces cerevisiae* cell membrane affinity method. *Food Control*. **50**: 413–422. doi: 10.1016/j.foodcont.2014.09.030

Tekiner-Gulbas, B., Westwell, A. D., and Suzen, S. (2013). Oxidative stress in carcinogenesis: New synthetic compounds with dual effects upon free radicals and cancer. *Curr. Med. Chem.* **20**(36): 4451–4459. doi: 10.2174/09298673113203690142

van Aardt, M., Duncan, S. E., Long, T. E., O'Keefe, S. F., Marcy, J. E., and Sims, S. R. (2004). Effect of antioxidants on oxidative stability of edible fats and oils: Thermogravimetric analysis. *J. Agric. Food. Chem.* **52**(3): 587–591. doi: 10.1021/jf030304f

Viljanen, K., Kivikari, R., and Heinonen, M. (2004). Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *J. Agric. Food. Chem.* **52**(5): 1104–1111. doi: 10.1021/jf034785e

Vizioli, J., and Salzet, M. (2002). Antimicrobial peptides from animals: focus on invertebrates. *Trends Pharmacol. Sci.* **23**(11): 494–495. doi: 10.1016/S0165-6147(02)02105-3

Xie, N., Wang, C., Ao, J., and Li, B. (2013). Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its *in vitro* gastrointestinal digest. *Food Res. Int.* **51**(1): 114–122. doi: 10.1016/j.foodres.2012.12.001

Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison III, R. T. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.* **61**(2): 719–728.

Yeaman, M. R., and Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **55**(1): 27–55. doi: 10.1124/pr.55.1.2

Zucht, H. D., Raida, M., Adermann, K., Mägert, H. J., and Forssmann, W. G. (1995). Casocidin-I: a casein-α_{s2} derived peptide exhibits antibacterial activity. *FEBS Lett.* **372**(2–3): 185–188. doi: 10.1016/0014-5793(95)00974-E

Figure captions

Figure 1. Occurrence frequency of amino acids in the predicted antioxidative peptides (**A**), and antibacterial peptides (**B**) from caseins (▧ ; β-casein gen. var. A1, A2, B, α_{S1}-casein gen. var. B, C and κ-casein gen. var. A precursor) and whey proteins (▩ ; α-lactalbumin gen. var. B precursor, β-lactoglobulin, gen. var. A precursor, and lactoferrin) using the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Table 1. Antioxidative peptides derived from milk protein

Substrate	Generation approach	Amino acid sequence[§]	Antioxidative activity	References
β-lactoglobulin A	hydrolysis using Corolase PP	W_{19}YSLAMAASDI_{29}	Oxygen radical absorbance capacity (ORAC)	(Hernández-Ledesma et al., 2005)
Casein	hydrolysis using pepsin	YFYPEL	Scavenging activity of free radicals (superoxide anion and DPPH)	(Suetsuna et al., 2000)
κ-casein	fermentation of skim milk using *Lactobacillus delbrueckii* ssp. *bulgaricus* IFO13953	A_{96}RHPHPHLSFM_{106}	Scavenging activity of DPPH radicals	(Kudoh et al., 2001)
κ-casein	hydrolysis using porcine pepsin A	-	ORAC	(López-Expósito et al., 2007)
β-casein	hydrolysis using corolase PP, trypsin	-	Inhibitory activity to lipoxygenase-catalyzed oxidation reactions & Antiradical activity against DPPH	(Rival et al., 2001)
β-lactoglobulin	hydrolysis using thermolysin, < 3 kDa fraction	L_{58}QKW_{61}, L_{95}DTDYKK_{101}	Oxygen radical absorbance capacity	(Contreras et al., 2011)
Milk protein	fermentation of skim milk using starter and probiotic cultures	-	Scavenging activity of DPPH and ABTS radicals	(Sah et al., 2014)
Casein	hydrolysis using trypsin, < 10 kDa	-	ORAC, inhibition of thiobarbituric acid reactive substances (TBARS)	(Díaz and Decker, 2004)

Milk protein isolate	hydrolysis using microbial proteases	-	ORAC, and DPPH radical scavenging activity	(Hogan et al., 2009)
Whey protein isolate	hydrolysis using alcalase, 0.1 - 2.8 kDa fraction	-	Scavenging activity DPPH, hydroxyl, and superoxide radicals	(Peng et al., 2009)

[§]Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 2. Antimicrobial peptides derived from milk proteins

Substrate	Generation approach	Amino acid sequence[§]	Strains tested	References
α_{s1}-casein	hydrolysis using pepsin	L_{99}RLKKYKVPQL$_{109}$	*Salmonella enterica* serovar *Typhimurium* ATCC 14028, *Staphylococcus aureus* *Escherichia coli* NBRC 3301, *Bacillus subtilis* NBRC 3134	(Tang et al., 2015)
α_{s2}-casein	commercial bovine casein hydrolysate prepared by a mixture of proteases	K_{165}KISQRYQKFALPQYLKTVYQHQK$_{188}$		(Elbarbary et al., 2012)
α_{s1}-casein		I_6KHQGLPQEV$_{15}$		
κ-casein		T_{136}EAVESTVATL$_{146}$		
α_{s2}-casein	Digestion using chymosin	K_{181}TVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$, L_{180}KTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$, A_{175}LPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$, L_{164}KKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$, Q_{172}KFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$	Several Gram-positive and Gram-negative bacteria	(McCann et al., 2005)
α_{s2}-casein	Digestion using pepsin	L_{164}KKISQRYQKFALPQY$_{179}$, V_{183}YQHQKAMKPWIQPKTKVIPYVRYL$_{207}$	*Escherichia coli* ATCC 25952, *Micrococcus flavus* DSM 1790	(Recio and Visser, 1999a)
κ-casein	digestion using plasmin	M_1MK$_3$, F_{17}FSDK$_{21}$, I_{22}AK$_{24}$	*Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus casei*, *Lactobacillus acidophilus*	(Sedaghati et al., 2014)

κ-casein	digestion with chymosin	f(106-169)	*Streptococcus mutans*, *Porphyromonas gingivalis*, *Escherichia coli*	(Malkoski et al., 2001)
α_{s1}-casein	chymosin digestion	R_1PKHPIKHQGLPQEVLNENLLRF$_{23}$	*Staphylococci*, *Sarcina*, *Bacillus subtilis*, *Diplococcus pneumoniae*, *Streptococcus pyogenes*	(Lahov and Regelson, 1996)
Peptide extract	prepared by high-speed centrifugation of yogurt	–	*Escherichia coli (ATCC CRM-8739)*, *Staphylococcus aureus* subsp. *aureus* (ATCC 25923)	(Sah et al., 2016a)
Lactoferrin	digestion with porcine pepsin A	A_1PRKNVRW**C***TISQPEW$_{16}$ **C**$^*_{45}$IRA$_{48}$ [f(1-16)S-S(45-48)], A_1PRKNVRW**C***TI$_{11}$ F$_{17}$K**C**‡RRWQWRMKKLGAPS IT**C**‡VRRAFALE**C***IR$_{47}$ [f(1-11)S-S(17-47)]	*Micrococcus flavus* DSM 1790	(Recio and Visser, 1999b)
Lactoferrin	digestion with pepsin	A_1PRKNVRW**C***TISQPEWFK**C**‡RRWQWRMKKL GAPSIT**C**‡VRRAFA$_{42}$ L$_{43}$E**C***IRA$_{48}$ [f(1-42)S-S(43-48)]	*Escherichia coli*	(Dionysius and Milne, 1997)
Lactoferrin	digestion with pepsin	A_1PRKNVRW**C***TISQPEW$_{16}$ L$_{43}$E**C***IRA$_{48}$ [f(1-16)S-S(43-48)]	many Gram-positive and Gram-negative bacteria	(Dionysius and Milne, 1997)
β-Lactoglobulin	Digestion with trypsin	V$_{15}$AGTWY$_{20}$, A$_{25}$ASDISLLDAQSAPLR$_{40}$, I$_{78}$PAVFK$_{83}$, V$_{92}$LVLDTDYK$_{100}$	many Gram-positive bacteria	(Pellegrini et al., 2001)
α-Lactalbumin	Digestion with chymotrypsin	**C***KDDQNPH IS**C***DKF [f(61-68)S-S(75-80)]	many Gram-positive bacteria	(Pellegrini et al., 1999)

α-Lactalbumin	Digestion with trypsin	E_1QLTK_5, G_{17}YGGVSLPEWV**C***TTF_{31} A_{109}L**C***SEK_{114} [f(17-31)S-S(109-114)]	many Gram-positive bacteria	(Pellegrini et al., 1999)
α_{s2}-casein	Proteolytic cleavage using trypsin, pronase or endoproteinase Glu-C	K_{165}TKLLTEEEKNRLNFLKKISQRYQKFALPQ YLKTVYQHQK_{203}	*Escherichia coli*, *Staphylococcus carnosus*	(Zucht et al., 1995)

[§]Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

[*], [‡]Cysteine residues (**C**) with the same superscript are linked by a disulfide bridge (S-S).

Table 3.1 Potential antioxidative and antibacterial peptides of β-casein gen. var. A1 of *Bos taurus* (amino acid residues = 209; formula weight = 23622.4 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence

1 RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY

61 PFPGPIHNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVQPFT

121 ESQSLTLTDV ENLHLPPLLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPEKAVPY

181 PQRDMPIQAF LLYQQPVLGP VRGPFPIIV

Potential antioxidative peptides	Potential antibacterial peptides
f(133-134), f(134-135), f(98-105), f(192-193), f(177-183), f(2-3), f(5-6), f(44-45), f(179-182), f(133-135), f(147-149), f(59-60), f(178-182), f(199-208), f(183-188).	ND

*The amino acid sequence of β-casein gen. var. A1 of *Bos taurus* (ID = 1097) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). ND = Not detected.

Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 3.2 Potential antioxidative and antibacterial peptides of β-casein gen. var. A2 of *Bos taurus* (amino acid residues = 209; formula weight = 23582.4 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence

1 RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY

61 PFPGPI**P**NSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVQPFT

121 ESQSLTLTDV ENLHLPPLLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPEKAVPY

181 PQRDMPIQAF LLYQQPVLGP VRGPFPIIV

Potential antioxidative peptides	Potential antibacterial peptides
f(133-134), f(134-135), f(98-105), f(192-193), f(177-183), f(2-3), f(5-6), f(44-45), f(179-182), f(133-135), f(147-149), f(59-60), f(178-182), f(199-208), f(183-188).	ND

*The amino acid sequence of β-casein gen. var. A2 of *Bos taurus* (ID = 1098) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). ND = Not detected.
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 3.3 Potential antioxidative and antibacterial peptides of β-casein gen. var. B of *Bos taurus* (amino acid residues = 209; formula weight = 23652.5 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence	
1 RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY 61 PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVEPFT 121 ERQSLTLTDV ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY 181 PQRDMPIQAF LLYQEPVLGP VRGPFPIIV	
Potential antioxidative peptides	Potential antibacterial peptides
f(133-134), f(134-135), f(98-105), f(192-193), f(177-183), f(169-176), f(170-176), f(193-196), f(193-200), f(2-3), f(5-6), f(44-45), f(179-182), f(133-135), f(147-149), f(59-60), f(178-182), f(199-208), f(183-188).	ND

*The amino acid sequence of β-casein gen. var. B of *Bos taurus* (ID = 1100) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). ND = Not detected.
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 3.4 Potential antioxidative and antibacterial peptides of α_{S1}-casein gen. var. B of *Bos taurus* (amino acid residues = 199; formula weight = 22973.9 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence

1 RPKHPIKHQG LPQEVLNENL LRFFVAPFPQ VFGKEKVNEL SKDIGSESTE DQAMEDIKEM

61 EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR LKKYKVPQLE IVPNSAEERL

121 HSMKQGIHAQ QKEPMIGVNQ ELAYFYPELF RQFYQLDAYP SGAWYYVPLG TQYTDAPSFS

181 DIPNPIGSEN SEKTTMPLW

Potential antioxidative peptides	Potential antibacterial peptides
f(120-121), f(143-144), f(158-159), f(144-149), f(39-40), f(141-142), f(148-149), f(164-165), f(165-167), f(164-166), f(144-146), f(120-122), f(42-43), f(147-149), f(146-149), f(145-149), f(101-102), f(163-164), f(198-199), f(170-173), f(158-161), f(154-157), f(93-97), f(146-150), f(100-104), f(20-22), f(98-100), f(154-156), f(153-156).	f(1-23), f(10-14), f(1-7), f(6-14), f(15-22), f(180-193).

*The amino acid sequence of α_{S1}-casein gen. var. B of *Bos taurus* (ID = 1087) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 3.5 Potential antioxidative and antibacterial peptides of α_{S1}-casein gen. var. C of *Bos taurus* (amino acid residues = 199; formula weight = 22901.9 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence

1 RPKHPIKHQG LPQEVLNENL LRFFVAPFPQ VFGKEKVNEL SKDIGSESTE DQAMEDIKEM

61 EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR LKKYKVPQLE IVPNSAEERL

121 HSMKQGIHAQ QKEPMIGVNQ ELAYFYPELF RQFYQLDAYP SGAWYYVPLG TQYTDAPSFS

181 DIPNPIGSEN SGKTTMPLW

Potential antioxidative peptides	Potential antibacterial peptides
f(120-121), f(143-144), f(158-159), f(144-149), f(39-40), f(141-142), f(148-149), f(164-165), f(165-167), f(164-166), f(144-146), f(120-122), f(42-43), f(147-149), f(146-149), f(145-149), f(101-102), f(163-164), f(198-199), f(170-173), f(158-161), f(154-157), f(93-97), f(146-150), f(100-104), f(20-22), f(98-100), f(154-156), f(153-156).	f(1-23), f(10-14), f(1-7), f(6-14), f(15-22).

*The amino acid sequence of α_{S1}-casein gen. var. C of *Bos taurus* (ID = 1088) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 3.6 Potential antioxidative and antibacterial peptides of κ-casein gen. var. A, precursor of *Bos taurus* (amino acid residues = 190; formula weight = 21303.1 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence

1 MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPIQ YVLSRYPSYG
61 LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL SNTVPAKSCQ AQPTTMARHP
121 HPHLSFMAIP PKKNQDKTEI PTINTIASGE PTSTPTTEAV ESTVATLEDS PEVIESPPEI
181 NTVQVTSTAV

Potential antioxidative peptides	Potential antibacterial peptides
f(121-124), f(80-82), f(119-121), f(121-123), f(123-124), f(117-127), f(81-83), f(63-65), f(62-64), f(122-124), f(34-35), f(49-51), f(51-53), f(30-31), f(67-68), f(84-85), f(186-189), f(47-53), f(82-86), f(117-122), f(118-122), f(55-58), f(54-58), f(82-85), f(52-58), f(145-151), f(59-62).	f(127-190), f(63-70), f(183-190), f(162-167), f(39-45), f(49-51), f(51-53), f(139-142), f(160-167), f(85-96), f(159-179).

*The amino acid sequence of κ-casein gen. var. A, precursor of *Bos taurus* (ID = 1117) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 4.1 Potential antioxidative and antibacterial peptides of α-lactalbumin gen. var. B, precursor of *Bos taurus* (amino acid residues = 142; formula weight= 16381.5 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence				
1	MMSFVSLLLV	GILFHATQAE	QLTKCEVFRE	LKDLKGYGGV SLPEWVCTTF
61	HTSGYDTQAI	VQNNDSTEYG	LFQINNKIWC	KDDQNPHSSN ICNISCDKFL
121	DDDLTDDIMC	VKKILDKVGI	NYWLAHKALC	SEKLDQWLCE KL

Potential antioxidative peptides	Potential antibacterial peptides
f(125-127), f(125-126), f(30-31), f(86-88), f(32-33), f(81-82), f(31-32), f(34-35).	f(20-24), f(36-50), f(128-133), f(80-87), f(94-99).

*The amino acid sequence of α-lactalbumin gen. var. B, precursor of *Bos taurus* (ID = 1115) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 4.2 Potential antioxidative and antibacterial peptides of β-lactoglobulin, gen. var. A, precursor of *Bos taurus* (amino acid residues = 178; formula weight = 20059.4 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

*Amino acid sequence	
1 MKCLLLALAL TCGAQALIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE	
61 ELKPTPEGDL EILLQKWEND ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL	
121 FCMENSAEPE QSLACQCLVR TPEVDDEALE KFDKALKALP MHIRLSFNPT QLEEQCHI	
Potential antioxidative peptides	Potential antibacterial peptides
f(61-62), f(35-45), f(161-165), f(58-62), f(35-36), f(35-37), f(35-38), f(35-39), f(35-40), f(35-41), f(163-164), f(62-64), f(62-63), f(156-157), f(63-64), f(57-58), f(113-115), f(34-35).	f(31-36), f(94-99), f(41-56), f(108-116).

*The amino acid sequence of β-lactoglobulin, gen. var. A, precursor of *Bos taurus* (ID = 1116) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Table 4.3 Potential antioxidative and antibacterial peptides of lactoferrin of *Bos taurus* (amino acid residues = 689; formula weight = 72637.3 Da) predicted using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) accessed on 17 June, 2016.

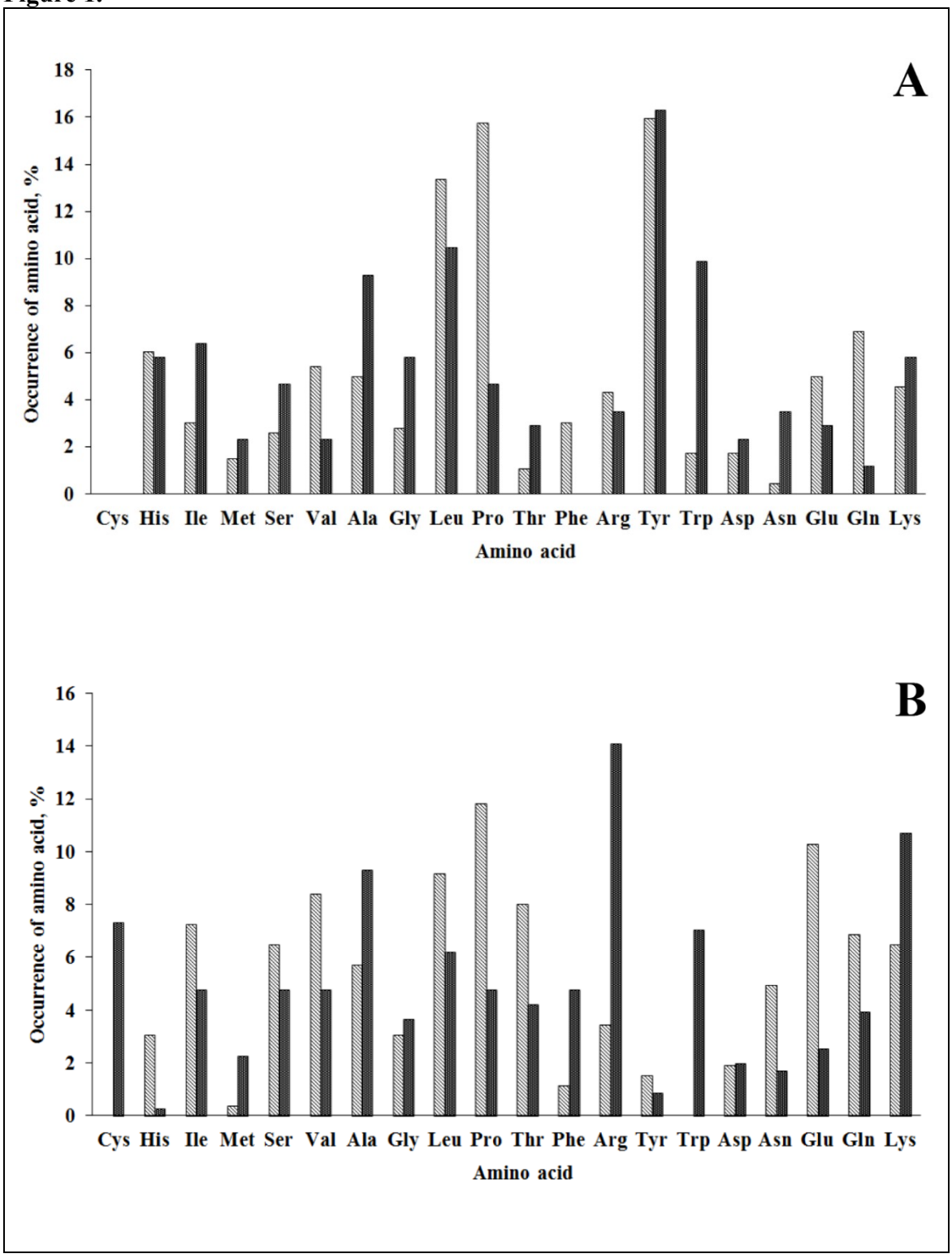
*Amino acid sequence

1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD
61 GGMVFEAGRD PYKLRPVAAE IYGTKESPQT HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG
121 RSAGWIIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ
181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD
241 AFKECHLAQV PSHAVVARSV DGKEDLIWKL LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF
301 KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ
361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKSSKH
421 SSLDCVLRPT EGYLAVAVVK KANEGLTWNS LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS
481 CAFDEFFSQS CAPGADPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV
541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR
601 SDRAAHVKQV LLHQQALFGK NGKNCPDKFC LFKSETKNLL FNDNTECLAK LGGRPTYEEY
661 LGTEYVTAIA NLKKCSTSPL LEACAFLTR

Potential antioxidative peptides	Potential antibacterial peptides
f(612-613), f(246-247), f(588-589), f(165-166), f(318-319), f(81-82), f(399-400), f(605-606), f(228-229), f(91-93), f(522-524), f(92-94), f(522-524), f(92-94), f(523-525), f(398-400), f(524-526), f(612-614), f(21-23), f(301-302), f(452-453), f(7-8), f(21-22), f(46-47), f(328-329), f(385-386), f(451-452), f(672-673), f(579-580), f(656-657), f(465-470), f(465-469), f(467-470), f(466-470), f(466-469), f(524-529), f(594-597), f(447-448), f(346-347), f(548-549), f(652-655).	f(17-41), f(268-284), f(1-8), f(17-42), f(17-43), f(1-16), f(45-48), f(17-47), f(1-11), f(19-37), f(17-30), f(43-48), f(1-42), f(17-48).

*The amino acid sequence of lactoferrin of *Bos taurus* (ID = 1212) was obtained from the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).
Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

Figure 1.


A
Occurrence of amino acid, %
0
2
4
6
8
10
12
14
16
18
Cys His Ile Met Ser Val Ala Gly Leu Pro Thr Phe Arg Tyr Trp Asp Asn Glu Gln Lys
Amino acid
B
Occurrence of amino acid, %
0
2
4
6
8
10
12
14
16
Cys His Ile Met Ser Val Ala Gly Leu Pro Thr Phe Arg Tyr Trp Asp Asn Glu Gln Lys
Amino acid

Chapter 2C: Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review

Chapter 2C presents a scientific prediction of anticancer peptides that could be potentially acquired from milk proteins and explores related work with milk and milk products.

The paper entitled "Identification of Anticancer Peptides from Bovine Milk Proteins and Their Potential Roles in Management of Cancer: A Critical Review" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*Comprehensive Reviews in Food Science and Food Safety*" (2015), 14(2): 123–138. http://dx.doi.org/10.1111/1541-4337.12126.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** **Date:** 15-Mar-2016

Baidya Nath Prasad Sah

[redacted]

Paper Title:

Identification of Anticancer Peptides from Bovine Milk Proteins and Their Potential Roles in Management of Cancer: A Critical Review

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Concept development, research question, hypothesis, and manuscript preparation
Prof Todor Vasiljevic	5 %	Concept development, research question, hypothesis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Concept development, research question, hypothesis, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Concept development, research question, hypothesis, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s):
College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne, VIC, Australia

and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	15/03/2016
Signature 2		15/03/2016
Signature 3		15/3/16
Signature 4		16/3/16

Sah, B.N.P., Vasiljevic, T., McKechnie, S. and Donkor, O.N. (2015), Identification of Anticancer Peptides from Bovine Milk Proteins and Their Potential Roles in Management of Cancer: A Critical Review. COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY, 14: 123-138. doi:10.1111/1541-4337.12126

Chapter 3: Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt

Chapter 3 describes the role of probiotic organisms including *Lactobacillus* (*L*.) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) in the release of antioxidant and antimutagenic peptides in yogurt during their growth. The performance of these strains (individually or in a combination) co-culturing with yogurt starter culture has been studied in regards to release of bioactive peptides with the antioxidant (free radical scavenging activity) and antimutagenic activities during yogurt manufacturing.

The paper entitled "Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*Food Chemistry*" (2014), 156: 264–270. http://dx.doi.org/10.1016/j.foodchem.2014.01.105.

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PART B:

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** [redacted] **Date:** 15-Mar-2016

Baidya Nath Prasad Sah

Paper Title:

Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	15/03/2016
Signature 2		15/03/2016
Signature 3		15/03/16
Signature 4		16/03/16

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Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt

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ABSTRACT

Search for bioactive peptides is intensifying because of the risks associated with the use of synthetic therapeutics, thus peptide liberation by lactic acid bacteria and probiotics has received a great focus. However, proteolytic capacity of these bacteria is strain specific. The study was conducted to establish proteolytic activity of *Lactobacillus acidophilus* (ATCC® 4356™), *Lactobacillus casei* (ATCC® 393™) and *Lactobacillus paracasei* subsp. *paracasei* (ATCC® BAA52™) in yogurt. Crude peptides were separated by high-speed centrifugation and tested for antioxidant and antimutagenic activities. The degree of proteolysis highly correlated with these bioactivities, and its value (11.91%) for samples containing all the cultures was double that of the control. Liberated peptides showed high radical scavenging activities with 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), IC_{50} 1.51 and 1.63 mg/ml, respectively and strong antimutagenicity (26.35%). These probiotics enhanced the generation of bioactive peptides and could possibly be commercially applied in new products, or production of novel anticancer peptides.

1. Introduction

Chronic diseases and ageing phenomena are relevant to the imbalance of free radical levels in the body. An excess of free radicals can cause lethal cellular damage through oxidising cellular proteins, enzymes, membrane lipids, and DNA; thus arresting cellular respiration (Urso & Clarkson, 2003). When the damage cannot be repaired diseases, such as cancer, cardiovascular diseases and diabetes, can arise (Collins, 2005). Their presence in foods, in addition to lipolytic and peroxidative changes, can cause sensory and nutritional value deterioration (Spitzer, Doucet, & Buettner, 2010). Therefore, it becomes essential to inhibit the peroxidation of lipids and the generation of free radicals in the living cells and foodstuffs.

Although artificial antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, and n-propyl gallate, display strong activity against various oxidation systems, the use of these antioxidants in food is prohibited in some countries due to the potential risks to human health (Kahl & Kappus, 1993). This leads to a growing interest towards natural antioxidants for chemotherapeutic and preservation properties. In addition to the functional and physiological properties, antioxidants from protein hydrolysates may confer nutritional value (Pownall, Udenigwe, & Aluko, 2010). Consequently, the search for natural antioxidants becomes a subject of interest to replace artificial antioxidants.

Kudoh, Matsuda, Igoshi, and Oki (2001) identified a κ-casein derived peptide, which displayed 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, from the milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus*. Sabeena Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) also found antioxidant peptides comprised of several fragments from β-casein and a few N-terminal fragments of α_{s1}-, α_{s2}- and κ-casein from yogurt. Korhonen and Pihlanto (2006) concluded that most of the peptides are encrypted within the native protein, and can be released during several food processes, or during gastrointestinal activities. Moreover, Gupta, Mann, Kumar, and Sangwan (2009) found that degree of proteolysis, which depends on the strain used in cheese making, is directly related to antioxidant activity. Donkor, Henriksson, Vasiljevic, and Shah (2007) reported that a large number of oligopeptides are generated by extracellular proteinases, and further breakdown into peptides by intracellular peptidases.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 1,1-diphenyl-2-picrylhydrazyl; WSPE, Water soluble peptide extract; HCA, Hierarchical cluster analysis.

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http://dx.doi.org/10.1016/j.foodchem.2014.01.105
0308-8146/

Nowadays, yogurt consumption has expanded rapidly because of the fact that it fulfils many current nutritional requirements. One distinguishable feature of yogurt is the presence of lactic acid bacteria, which have well developed proteolytic systems enabling them to grow well in milk and release a large number of potentially bioactive peptides. Several studies have been undertaken to confer probiotic effects by incorporating *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) into fermented food products (Ortakci & Sert, 2012; Sidira et al., 2013). Additionally, these strains displayed remarkably high proteolytic activities (Donkor et al., 2007; Yeo & Liong, 2010). However, the potential of these strains to release peptides with a particular physiological activity has not been assessed to-date. Thus, the aim of this study was to establish the performance of these strains (individually or in a combination) by co-culturing with yogurt starter culture, in regards to release of bioactive peptides with the antioxidant (free radical scavenging activity) and antimutagenic activities during yogurt manufacturing.

2. Materials and methods

2.1. Substrates and chemicals

Most of the chemicals were supplied by Sigma Chemical Company (St Louis, MO, USA) and used without further purification. Milli-Q water (18.2 MΩ cm) was used to prepare all aqueous solutions. M17 media, de Man Rogosa and Sharpe (MRS) media, and bacteriological agar were purchased from Oxoid (West Heidleberg, Australia). Clindamycin, vancomycin, o-phthaldialdehyde (OPA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA) and serine were purchased from Sigma Chemical Company (St Louis, MO, USA). Instant skimmed milk powder was purchased from a local store (Woolworths Limited, Australia). Acetonitrile was purchased from Merck (Darmstadt, Germany).

2.2. Propagation of cultures

Streptococcus thermophilus 1275 (*S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 (*L. bulgaricus*) were obtained from the Victoria University Culture Collection (Werribee, Australia). *Lactobacillus acidophilus* ATCC 4356 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*) and *Lactobacillus paracasei* subsp. *paracasei* ATCC BAA52 (*L. paracasei*) were purchased from Cell Biosciences Pty Ltd (Heidelberg, Victoria, Australia). The strains were stored at −80 °C in 40% glycerol in MRS broth. The strains were activated by triplicate sequential transfer, by inoculating 1% (v/v) of each strain in MRS broth (except *S. thermophilus*, which was inoculated in M17 broth) and incubating for 18 h at 42 °C for *L. bulgaricus*, 37 °C for *S. thermophilus*, *L. acidophilus*, *L. casei* and 30 °C for *L. paracasei*. The activated strains were inoculated at 1% (v/v) into 10 ml of sterile reconstituted skimmed milk (RSM, 14%, w/v) containing glucose (2%) and yeast extract (1%). Finally, the cultures were inoculated at 1% (v/v) into sterile RSM and incubated at 37 °C for 20 h to obtain approximately 10^8 colony-forming units (CFU) per millilitre. The final step was performed so that yeast extract was not carried over during yogurt manufacturing, which might interfere with results (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007).

2.3. Yogurt preparation

Yogurt was prepared as described by Donkor et al. (2007). Briefly, yogurt mix was prepared by heat treating reconstituted skimmed milk (140 g/l) at 85 °C for 30 min followed by cooling to 45 °C, and aseptically inoculating with 1% (v/v) of each of *L. bulgaricus* and *S. thermophilus*. The inoculated milk was divided into eight equal portions; one portion was used as a control, while the other portions were further inoculated with 1% (v/v) of each probiotic cultures (*L. acidophilus*, *L. casei* and *L. paracasei*) separately, two in- combination and all three in- combination as shown in Table 1. The mixes were poured into polystyrene cups aseptically and incubated at 42 °C until the required pH of 4.5 ± 0.1 was reached. Cooling to 4 °C was done to halt further acidification. The pH of the heat treated RSM was adjusted to 4.5 ± 0.1 by using aqueous 1 M HCl.

2.4. Selective enumeration of probiotic and yogurt strains

Cell populations of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. casei* and *L. paracasei* were determined using the pour plate technique as described by Donkor et al. (2007) with a few modifications. Briefly, the samples (10 g) weighed aseptically into sterile stomacher bags were diluted with sterile (0.15%, w/v) peptone (Oxoid) water to 100 g and homogenised using a Stomacher (Stomacher400, John Morris Scientific Pty Limited). The resulting diluted sample (10^{-1}) was serially diluted in sterile peptone water, and 1 ml of appropriate dilutions were used for enumeration by the pour plate technique. *S. thermophilus* was selectively enumerated using M17 medium, supplemented with lactose under aerobic incubation at 45 °C for 24 h; *L. bulgaricus* using MRS agar (pH adjusted to 5.2 using 1 M HCl) at 45 °C for 72 h anaerobically and *L. acidophilus* using MRS-clindamycin (pH 6.2; 0.5 ppm clindamycin) at 37 °C for 72 h anaerobically (Casteele et al., 2006). Both *L. casei*/*L. paracasei* together or alone were selectively enumerated from other probiotic and yogurt cultures using MRS-vancomycin agar (pH 6.2; 1 ppm vancomycin) at 37 °C for 72 h anaerobically (Sakai et al., 2010). Plates containing 25–250 colonies were considered for enumeration and the results were reported as log CFU/g.

2.5. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was analysed using the OPA method described by Nielsen, Petersen, and Dambmann (2001) and Donkor et al. (2007) with a few modifications. The OPA reagent was prepared daily by combining 25 ml of sodium tetraborate buffer (100 mM; pH 9.3), 2.5 ml of sodium dodecyl sulphate (20%, w/w), 40 mg of OPA (dissolved in 1 ml of methanol), and 100 µl of β-mercaptoethanol and diluting to 50 ml with water. The serine standard (0.9516 meqv/l) was prepared by dissolving 50 mg serine (Sigma Chemical Company) in 500 ml deionized water. Three millilitres of 0.75 M TCA were added to 3 g aliquots of yogurt samples; and incubated for 10 min at room temperature. The mixture was centrifuged at 4000×*g* for 30 min at 4 °C. The supernatant was passed through a 0.45 µm syringe filter and stored at −20 °C until assayed.

Small aliquots (400 µl) of the samples were added to a test tube containing 3 ml of the OPA reagent and mixed gently for 5 s. The absorbance (A_{sample}) was measured at 340 nm using a Nova-Spec®-II Spectrophotometer (Pharmacia, England, UK) after exactly two minutes of incubation at room temperature. The experimental steps were repeated with Milli-Q water as a blank (A_{blank}); and with the serine solution as standard ($A_{standard}$). Degree of hydrolysis was determined by using Eq. (1).

$$DH(\%) = \frac{h}{h_{tot}} \times 100 \quad (1)$$

where, h_{tot} was the total number of peptide bonds per protein equivalent; for casein, the h_{tot} value was 8.2 mEq/g protein (Nielsen

Table 1
Culture combinations in yogurt preparation.

Yogurt types	Code	Combination of strains (1% v/v)
Plain yogurt	1	*S. thermophilus* + *L. bulgaricus*
Probiotic yogurt	2	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus*
	3	*S. thermophilus* + *L. bulgaricus* + *L. casei*
	4	*S. thermophilus* + *L. bulgaricus* + *L. paracasei*
	5	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei*
	6	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. paracasei*
	7	*S. thermophilus* + *L. bulgaricus* + *L. casei* + *L. paracasei*
	8	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*

et al., 2001), and h was the number of hydrolysed bonds, which was determined by using Eq. (2).

$$h = \frac{(\text{Serine}\,NH_2 - \beta)}{\alpha} \tag{2}$$

For casein, $\alpha = 1.039$; $\beta = 0.383$ mEq/g protein (Nielsen et al., 2001) and the value of Serine-NH_2 was determined using the Eq. (3).

$$\text{Serine} - NH_{2\,(\text{mEq/g protein})} = \left[\frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{standard}} - A_{\text{blank}})}\right] \times \text{Conc}^{n} \text{ of Serine Std (mEq/l)} \times V \times \frac{100}{X} \times P \tag{3}$$

where, V = final volume make-up of the sample, litre; X = weight of yogurt sample, g; P = protein% (w/w) in yogurt sample

2.6. Preparation of water-soluble peptide extracts (WSPE)

Yogurt samples (pH 4.5 ± 0.1) and heat-treated RSM (pH adjusted to 4.5 ± 0.1) were centrifuged at 15,000×g (J2-HS rotor, Beckman Instruments Inc., USA) at 4 °C for 30 min. The filtrate obtained by filtering the supernatant using 0.45 μm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) was freeze-dried in a Dynavac FD 300 freeze dryer (Airvac Engineering Pty. Ltd, Rowville, Australia) at −30 °C for 40 h (primary drying) and −10 °C for 24 h (secondary drying). All the lyophilised samples were kept at −80 °C for further analysis. The protein content (mg/ml) of the samples was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

2.7. Determination of antioxidant activity

The radical scavenging activity by peptide extract of the yogurt samples was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sabeena Farvin, Baron, Nielsen, & Jacobsen, 2010) and ABTS (Şanlidere Aloğlu & Öner, 2011) radicals with a few modifications. Briefly, 1.5 ml of DPPH solution (0.2 mM in buffered methanol prepared by mixing 40 ml of 0.1 M acetate buffer, pH 5.5 with 60 ml methanol) was added to 1.5 ml of properly diluted crude peptide extracts in Milli-Q water; followed by incubation for 30 min in dark and the absorbance was measured at 517 nm using a Pharmacia UV-Vis spectrophotometer. Similarly, 1.5 ml of Milli-Q water was used instead of the sample for the blank. Radical-scavenging activity (RSA) was calculated using Eq. (4).

Similarly, the working solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical ($ABTS^{\bullet+}$) was prepared by mixing stock solutions of 7.4 mM ABTS (MW = 548.68) aqueous solution and 2.6 mM potassium persulphate (MW = 270.32) aqueous solution in equal quantities (molar ratio = 1:0.35) and allowing them to react for 12–16 h at room temperature in the dark. This 1 ml of $ABTS^{\bullet+}$ solution was then diluted by mixing with 50–60 ml of the buffered methanol in order to obtain an absorbance of 0.70 ± 0.02 at 734 nm after equilibration at 30 °C. The reagent was prepared daily. Twenty microlitres of properly diluted crude peptide extracts in Milli-Q water was added to 2 ml reagent, and incubated at 30 °C for 6 min. The absorbance of the mix was measured by using a Pharmacia UV-Vis spectrophotometer at 734 nm. Similarly, 20 μl of Milli-Q water was used instead of the sample for the blank. Radical-scavenging activity was calculated using Eq. (4).

$$\text{RSA},\% = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100 \tag{4}$$

The % RSA was plotted against the different peptide concentrations and a linear regression curve was established in order to calculate the IC_{50} value. IC_{50} is the concentration of peptides needed to inhibit 50% of the radicals.

2.8. Determination of antimutagenic activity by the Ames test

A direct-acting mutagen, sodium azide (Sigma–Aldrich Pty. Ltd.) dissolved in Milli-Q water at a concentration of 0.01 μg/ml, was used for the antimutagenic tests of the crude WSPE from yogurt, using a base pair substitution type mutated strain- *Salmonella enterica* subsp. *enterica* serovar *typhimurium* (ATCC® 29629™) (Cell Biosciences Pty Ltd, Australia) according to the preincubation protocol as described by Espeche Turbay, De Moreno de LeBlanc, Perdigón, Savoy de Giori, and Hebert (2012) with a few modifications. Briefly, *S. typhimurium* was activated at 37 °C for 48 h on a plate containing Davis minimal agar (BD Australia) supplemented with biotin (3 μM) and histidine (260 μM). Before each test, the strain was grown in nutrient broth No. 2 (Oxoid Ltd, England) at 37 °C overnight with agitation (120 rpm). The strain check for the spontaneous mutation rate and genetic integrity was also conducted with the overnight cultures. For preliminary toxicity test, aliquots of 100 μl of the crude WSPE (10, 50, 100 μg crude peptide per plate) were combined with 100 μl of the overnight culture (approximately 10^8 CFU). Similarly, aliquots of 100 μl of the sodium azide (0.1, 1, 5 μg/plate) were combined with 100 μl of the same culture. The mix was pre-incubated at 37 °C for 20 min with agitation (120 rpm). Serial dilutions were performed with sterile 100 mM phosphate buffered saline (pH 7.4), and then 100 μl of the aliquot was spread onto previously prepared petri plates containing 25 ml of Davis minimal agar supplemented with biotin (3 μM) and histidine (260 μM), and incubated at 37 °C for 48 h. After this period, the number of revertant colonies were counted and a toxicity effect on the strain was confirmed if the viable count of the test sample was found significantly lower than that of the negative control.

Exactly 100 μl of sodium azide aqueous solution (1 μg), 100 μl test solution (50 ± 1 μg peptide) was delivered to 500 μl of 100 mM sodium phosphate buffer (pH 7.4) in sterile 13 × 100 mm capped culture tubes. After adding 100 μl of the tester strain, the tube was vortexed gently and incubated at 37 °C for 20 min on a rotary shaker (120 rpm). 2 ml of sterile molten top agar (0.05 mM L-histidine, 0.05 mM D-biotin, 0.5% NaCl, and 0.6% agar; 45 °C) was added to the tube. The mixture was poured over the surface of a Davis minimal agar plate. Positive and negative controls were also included in each assay. Revertant colonies were counted after the incubation of plates at 37 °C for 48 h. The percentage of inhibition was calculated using Eq. (5).

$$\%\ \text{Inhibition} = \frac{(M - S_1)}{(M - S_0)} \times 100 \tag{5}$$

where, S_1 was number of revertant colonies/plate induced by the extract plus the mutagen; M was number of revertant colonies/plate induced by mutagen alone (positive control); S_0 was number of spontaneous revertant colonies (negative control).

2.9. RP-HPLC analysis of water-soluble peptides extract

The peptide profile of crude WSPE of control yogurt, probiotic yogurts and RSM samples were examined according to the method used by Donkor et al. (2007) with few modifications, on a reversed-phase HPLC (Varian Analytical Instruments, Walnut Creek, USA) equipped with C-18 monomeric column (5 mm, 300 Å, 250 mm × 4.6 mm; Grace Vydac, Hesperia CA, USA). All samples were filtered through a 0.45 μm Phenex syringe filter (Phenomenex, Inc. Australia) and loaded using a 20 μl injection loop. The peptides were eluted at a flow rate of 0.75 ml/min by a linear gradient from 100% to 0% solvent A (0.1%, v/v trifluoroacetic acid (TFA) in deionised water) in solvent B (0.1%, v/v TFA in acetonitrile) over 90 min. The eluted peptides were detected at 215 nm using a Varian 9065 Polychrom UV/Vis detector. Separations were conducted at room temperature (~22 °C).

2.10. Statistical analysis

The experiments, including yogurt manufacturing, were performed in triplicate. Six values for each samples were averaged ($n = 6$) and reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with cultures as the main factor, and Tukey Honestly Significant Difference (HSD) post hoc tests were conducted to determine significant differences ($P < 0.05$) between the means. The analyses were carried out using the SPSS package (SPSS 21.0 for windows, SPSS Inc., Chicago, USA). A two-tailed Pearson's correlation test was conducted to assess the correlations among means. Hierarchical cluster analysis Hierarchical cluster analysis (HCA) was performed to make clusters on the basis of similarity index among plain yogurt and probiotic yogurts with different combinations of strains using the Ward's algorithm method (Pennington & Fisher, 2009).

3. Results and discussion

S. thermophilus, *L. bulgaricus*, *L. acidophilus*, *L. casei* and *L. paracasei* were enumerated selectively and tabulated in Table 2. The counts for *S. thermophilus* and *L. bulgaricus* are in line with previous studies with probiotic yogurts (Beheshtipour, Mortazavian, Haratian, & Darani, 2012; Elizaquível et al., 2011). *L. bulgaricus* presented a lower viable count as compared to *S. thermophilus*, probably due to its greater oxygen sensitivity (Beshkova, Simova, Frengova, Simov, & Spasov, 2002). For probiotic effect, the consumer should aim to ingest more than 10^8 live probiotic cells per day (Lourens-Hattingh & Viljoen, 2001). This amount could be translated into $\geqslant 10^6$ CFU/g of probiotic yogurt given that 100 g is the daily serving portion. Therefore, the counts for *L. acidophilus*, *L. casei* and *L. paracasei* as indicated, in Table 2, were sufficient for the probiotic effect.

The degree of hydrolysis measures the extent of peptide bonds cleaved in the substrate by a proteolytic agent (lactic acid bacteria strains, in this case): the higher the value, the higher amount of amino groups released. The proteolytic activities of yogurt culture with or without probiotic cultures, were measured and recorded as percentage of the bonds cleaved (Table 3). Degree of hydrolysis for yogurt 8, containing all cultures (11.91%), was double in comparison with that of yogurt 1, containing only starter cultures (5.38%). This shows that probiotics enhanced the utilisation of the protein source as substrate for hydrolysis to yogurt culture.

To confirm the hydrolysis of milk protein, the crude WSPE were profiled using a reversed-phase HPLC (Figs. 1 and 2). The chromatograms of the control (yogurt 1) and seven probiotic yogurts (2–8) showed higher degrees of liberated peptides as compared with that of the RSM. The peptide profile of probiotic yogurts also varied in comparison to that of the control yogurt, which supported the variations in the proteolytic capacities of the cultures; moreover, indicating selected probiotic cultures were highly proteolytic. The peaks in the region of solvent A (0.1% TFA in Milli-Q water) represented the hydrophilic peptides while those in the region of solvent B (0.1% TFA in acetonitrile) represented hydrophobic peptides at the wavelength of 215 nm. This observation also supports that the hydrophobic regions of milk proteins are more accessible for proteolysis; this result is in accordance with the findings that the hydrophobic region (especially the C-terminal region) of casein, was more accessible to the cell envelope proteinase (CEP) of *S. thermophilus* (Miclo et al., 2012).

In the present study, the antioxidant activity was assessed using two methods-DPPH and ABTS. The radical scavenging capacity of antioxidants for DPPH and ABTS radicals could be different owing to the difference in their solubility and diffusivity in the reaction medium. Even though the DPPH method is a popular method for determination of radical scavenging activity of natural products, it has significant drawbacks if the radical scavenger is hydrophilic in nature, because it is only soluble in organic media (especially in alcoholic media) and not in aqueous media. Additionally, DPPH acts both as an oxidising substrate and as the reaction indicator; and this may easily result into the problem of spectral interference. In contrast, the ABTS is soluble in both aqueous and organic media; and the radical scavenging activities of both hydrophilic and lipophilic antioxidants can be assessed using this method (Tang et al., 2010).

In order to confirm that the measured antioxidant activity was generated during the fermentation, the absorbance of crude extract from heat treated skimmed milk (reduced to pH 4.5 ± 0.1 with 1 M HCl) was subtracted from that of the aqueous extracts from yogurts. The DPPH radical scavenging activity indicates the capacity of the antioxidants to donate hydrogen or electrons, resulting into a more stable species (Prior, Wu, & Schaich, 2005). Yogurt 8, containing all organisms, was the most potent scavenger of free DPPH

Table 2
Cell concentration of yogurt and probiotic cultures in yogurt samples (pH of 4.5 ± 0.1) prepared by fermenting at 42 °C.

Yogurt types	Count (log CFU/g)					
	S. thermophilus	*L. bulgaricus*	*L. acidophilus*	*L. casei*	*L. paracasei*	*L. casei & L. paracasei*
1	9.32 ± 0.14^{a}	8.00 ± 0.15^{a}	–	–	–	–
2	9.21 ± 0.08^{a}	$8.17 \pm 0.07^{a,b,c}$	7.58 ± 0.292^{a}	–	–	–
3	9.18 ± 0.14^{a}	$8.23 \pm 0.15^{b,c}$	–	8.22 ± 0.20^{a}	–	–
4	9.23 ± 0.16^{a}	$8.08 \pm 0.09^{a,b}$	–	–	7.38 ± 0.34^{a}	–
5	9.17 ± 0.23^{a}	$8.17 \pm 0.15^{a,b,c}$	7.44 ± 0.27^{a}	8.20 ± 0.22^{a}	–	–
6	9.13 ± 0.06^{a}	$8.23 \pm 0.12^{b,c}$	7.28 ± 0.24^{a}	–	7.33 ± 0.17^{a}	–
7	9.14 ± 0.10^{a}	8.35 ± 0.06^{c}	–	–	–	7.78 ± 0.10^{a}
8	9.20 ± 0.14^{a}	$8.28 \pm 0.11^{b,c}$	7.34 ± 0.10^{a}	–	–	7.83 ± 0.10^{a}

^{a,b,c}Different superscripts within a column depict statistical difference between means for yogurt types ($P < 0.05$). Results were expressed as means ± standard deviation ($n = 6$).

Table 3
Degree of hydrolysis of milk proteins, antioxidant activity, antimutagenic activity of peptides in yogurts (with and without probiotics).

Yogurt types	DH,%	Antioxidant activity (IC_{50}, mg/ml)		Antimutagenic activity (% inhibition)
		DPPH	ABTS	
1	5.38 ± 0.32[a]	2.23 ± 0.03[a]	2.43 ± 0.01[a]	15.87 ± 2.16[a]
2	9.11 ± 0.42[b]	2.05 ± 0.05[b]	2.28 ± 0.03[b]	18.35 ± 2.40[a,b]
3	9.94 ± 0.57[b,c]	1.83 ± 0.02[c]	1.91 ± 0.02[c]	18.83 ± 2.52[a,b]
4	10.19 ± 0.54[c]	1.82 ± 0.02[c,d]	1.98 ± 0.04[d]	18.48 ± 0.70[a,b]
5	10.03 ± 0.62[b,c]	1.80 ± 0.01[c,d]	1.73 ± 0.03[e]	20.25 ± 3.21[b,c]
6	10.02 ± 0.59[b,c]	1.77 ± 0.01[d]	1.80 ± 0.02[f]	23.06 ± 2.39[c,d]
7	10.38 ± 0.62[c]	1.71 ± 0.02[e]	1.72 ± 0.03[e]	23.23 ± 1.84[c,d]
8	11.91 ± 0.68[d]	1.51 ± 0.04[f]	1.63 ± 0.03[g]	26.35 ± 1.63[d]

[a,b,c,d,e,f,g] Different superscripts within a column were significantly different ($p < 0.05$). Results were expressed as means ± standard deviation ($n = 6$).

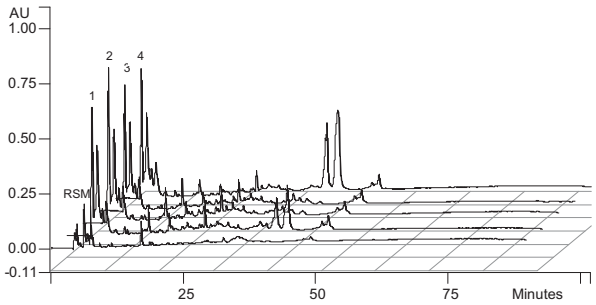

Fig. 1. Reversed-phase HPLC profile of the water-soluble fraction of yogurt types: 1, 2, 3, 4 compared with RSM as control using a linear gradient from 100% to 0% solvent A (0.1% TFA in Milli Q water) in solvent B (0.1% TFA in acetonitrile) over 90 min at a flow rate of 0.75 ml/min. The eluted peptides were detected by UV absorption at 215 nm.

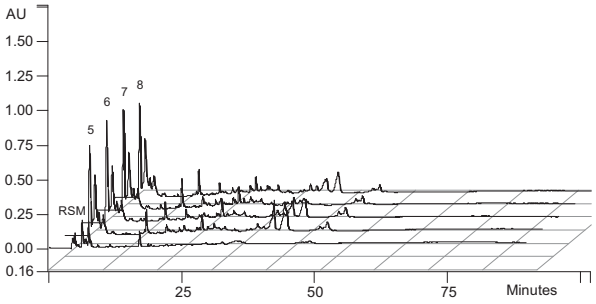

Fig. 2. Reversed-phase HPLC profile of the water-soluble fraction of yogurt types: 5, 6, 7, 8 compared with RSM as control using a linear gradient from 100% to 0% solvent A (0.1% TFA in Milli Q water) in solvent B (0.1% TFA in acetonitrile) over 90 min at a flow rate of 0.75 ml/min. The eluted peptides were detected by UV absorption at 215 nm.

radicals, as shown by the lowest IC_{50} value of 1.51 mg/ml. In contrast, yogurt 1, containing starter cultures had the highest IC_{50} value of 2.23 mg/ml and was the least potent (Table 3). The IC_{50} of yogurt 8 was comparable with the standard-L-ascorbic acid whose IC_{50} was 0.192 mg/ml. Similarly, the decolourisation of the ABTS radical cation ($ABTS^{\bullet+}$) was used to assess the antioxidant activity of yogurt samples, and was expressed in terms of IC_{50} value. Yogurt 8 had the lowest IC_{50} value of 1.63 mg/ml while yogurt 1 with the highest IC_{50} value of 2.43 mg/ml (Table 3). The IC_{50} of yogurt 8 was also comparable to the standard, trolox, whose IC_{50} was found to be 0.23 mg/ml. These results are in agreement with Virtanen, Pihlanto, Akkanen, and Korhonen (2007), who found that fermented milk produced using mixed cultures of lactic acid bacteria, showed a higher radical scavenging activity than milks fermented using a single strain. According to Foh, Amadou, Foh, Kamara, and Xia (2010), the bioactivities of protein hydrolysates are dependent on a number of factors, such as amino acid sequence, size and structure of the peptides. The antioxidant capacity of hydrolysates for the same substrate, depends on the types of enzyme from the lactic acid bacteria, as specific proteases are involved in the hydrolysis of specific peptide bonds. Peptides consisting of methionine, glutamine, tyrosine, lysine, histidine, cysteine, valine, and proline possess strong antioxidant activity (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Kudoh et al. (2001) also isolated a DPPH radical scavenging peptide having an amino acid sequence of Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met derived from κ-casein in fermented milk using *Lactobacillus delbrueckii* ssp. *bulgaricus*. In another study, Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005) also investigated an antioxidative peptide (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) obtained from β-lactoglobulin after hydrolysis using corolase PP. Similar results were observed in a previous study by Dryáková, Pihlanto, Marnila, Čurda, and Korhonen (2010), who evaluated the effect of hydrolysis time on the development of the antioxidant activity, and by Li et al. (2013), who isolated five antioxidant oligopeptides (Val-Tyr-Pro-Phe, Phe-Gly-Gly-Met-Ala-His, Phe-Pro-Tyr-Cys-Ala-Pro, Tyr-Val-Pro-Glu-Pro-Phe, and Tyr-Pro-Pro-Tyr-Glu-Thr-Tyr) from goat milk casein by using a combination of neutral and alkaline proteases. These results are also in agreement with Kim, Jang, and Kim (2007), who found the radical scavenging effects of casein hydrolysates, and proved that low molecular weight fraction of the casein hydrolysates had greater antioxidant activity. Indeed, both ABTS and DPPH are nitrogen radicals that can be neutralised either by radical quenching through hydrogen atom transfer, or by direct reduction through electron transfer, and display similar stoichiometry with standard antioxidants such as trolox and ascorbic acid (Prior et al., 2005). However, the DPPH- and $ABTS^{\bullet+}$-radical scavenging activities of aqueous extracts were distinct quantitatively, supporting the differences in the reaction kinetics of both radicals with antioxidants (Tang et al., 2010). Co-culturing probiotics with yogurt cultures resulted in enhanced antioxidant activity (Table 3). The degree of hydrolysis and the IC_{50} value using the DPPH method highly correlated ($P < 0.01$, $r = -0.897$). Similarly, the degree of hydrolysis and the IC_{50} value using the ABTS method was also found highly correlated ($P < 0.01$, $r = -0.827$). The results were in accordance with Virtanen et al. (2007) who reported the increment of the antioxidative activity during fermentation and Igoshi, Kondo, Kobayashi, Kabata, and Kawakami (2008) who found significant correlation between the antioxidative activity, and the amount of peptides generated during cheese ripening.

The toxicity test results showed that the survival of *S. typhimurium* in the presence of crude peptide extract was close to that of the controls (data not shown), indicating that these fractions were not toxic toward the test strains. The inhibitory effect of crude

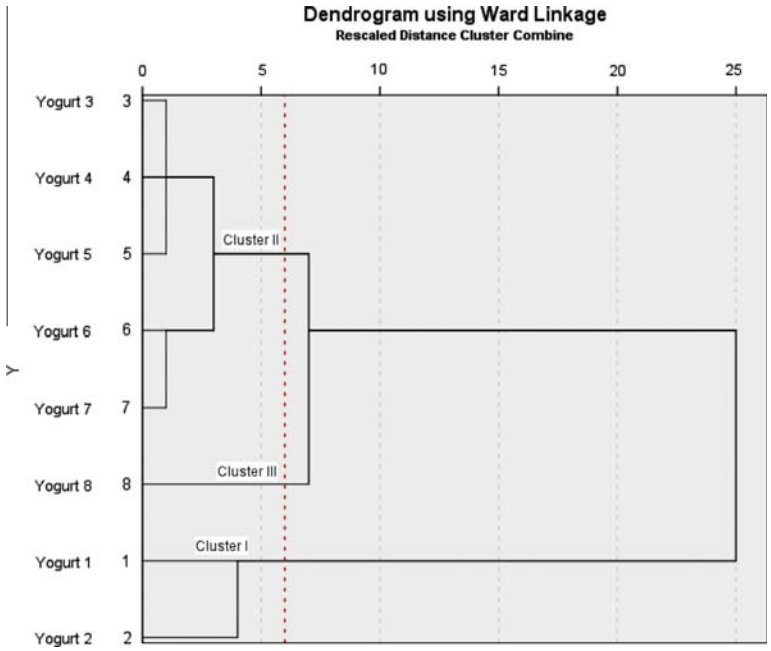

Fig. 3. Dendrogram visualising the clustering of eight yogurt types in the study based on similarities of the parameters (degree of hydrolysis, antioxidant and antimutagenic activities).

peptide against mutagenesis on *S. typhimurium* induced by sodium azide is shown in Table 3, which is in agreement with Espeche Turbay et al. (2012) and Bakalinsky, Nadathur, Carney, and Gould (1996). Similarly, Van Boekel, Weerens, Holstra, Scheidtweiler, and Alink (1993) found that the hydrolysis of casein by pepsin enhanced the inhibitory activity against several mutagens; related to the released peptides. Degree of hydrolysis and antimutagenic activity (% inhibition) using Ames test was also found highly correlated ($P < 0.01$, $r = 0.640$), therefore supporting previous findings.

Using the hierarchical clustering method with Ward's linkage, three statistically significant clusters were identified based on similarities in their degree of hydrolysis, antioxidant capacity, and antimutagenic activity; the results obtained are shown as a dendrogram (Fig. 3). This indicates that the peptides produced have different behaviour depending on the type of *Lactobacillus* strains. Yogurts 1 and 2 are arranged in the first cluster characterised by less degree of hydrolysis, weak antioxidant and antimutagenic activity; yogurts 3, 4, 5, 6 and 7 belong to the second cluster with moderate degree of hydrolysis, antioxidant capacity, and antimutagenic activity, whereas yogurt 8 belongs to the third cluster with a high in degree of hydrolysis, antioxidant and antimutagenic power. It can also be seen that within each cluster the difference between each yogurt is clear, since all observations under each time form a group which is different from another within the same type of strains. This grouping gives evidence that probiotics in each group have different characteristics. Clusters I and II were well separated showing remarkably different proteolytic, antioxidant and antimutagenic activities.

4. Conclusion

Our results revealed that probiotic organisms had a statistically significant effect on the proteolytic activity, and enhanced the generation of peptides with potential antioxidant and antimutagenic properties with good correlation between proteolytic and antioxidant or antimutagenic activities. Probiotic yogurt containing all three probiotic strains (*Lactobacillus acidophilus* ATCC 4356, *L. casei* ATCC 393 and *L. paracasei* subsp. *paracasei* ATCC BAA52) had the highest value of degree of hydrolysis, and the strongest antioxidant and antimutagenic activities. The activities displayed by the milk protein hydrolysates could have resulted from the synergistic effects of various peptides in the mixture. The peptides generated by milk fermentation with *Lactobacillus* strains may contribute with a variety of bioactive compounds to a positive effect on human health, specifically antimutagenic and antioxidant properties. Moreover, these hydrolysates could also be considered for the development of functional foods and simultaneously enhance the shelf-life of the food products. The powerful proteolytic system of these strains opens up future opportunities to develop novel functional foods with potential health promoting properties.

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References

Bakalinsky, A. T., Nadathur, S. R., Carney, J. R., & Gould, S. J. (1996). Antimutagenicity of yogurt. *Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis, 350*(1), 199–200.

Beheshtipour, H., Mortazavian, A. M., Haratian, P., & Darani, K. K. (2012). Effects of Chlorella vulgaris and Arthrospira platensis addition on viability of probiotic bacteria in yogurt and its biochemical properties. *European Food Research and Technology, 235*(4), 719–728.

Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., & Spasov, Z. N. (2002). Effect of oxygen on batch yogurt cultures. *World Journal of Microbiology and Biotechnology, 18*(4), 361–365.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Casteele, S. V. D., Vanheuverzwijn, T., Ruyssen, T., Assche, P. V., Swings, J., & Huys, G. (2006). Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. *International Dairy Journal, 16*(12), 1470–1476.

Collins, A. R. (2005). Antioxidant intervention as a route to cancer prevention. *European Journal of Cancer, 41*(13), 1923–1930.
Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., & Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal, 17*(11), 1321–1331.
Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait, 87*(1), 21–38.
Dryáková, A., Pihlanto, A., Marnila, P., Čurda, L., & Korhonen, H. J. T. (2010). Antioxidant properties of whey protein hydrolysates as measured by three methods. *European Food Research and Technology, 230*(6), 865–874.
Elizaquível, P., Sánchez, G., Salvador, A., Fiszman, S., Dueñas, M. T., López, P., et al. (2011). Evaluation of yogurt and various beverages as carriers of lactic acid bacteria producing 2-branched (1,3)-β-d-glucan. *Journal of Dairy Science, 94*(7), 3271–3278.
Espeche Turbay, M. B., Moreno, De., de LeBlanc, A., Perdigón, G., Savoy de Giori, G., et al. (2012). B-Casein hydrolysate generated by the cell envelope-associated proteinase of Lactobacillus delbrueckii ssp. lactis CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. *Journal of Dairy Science, 95*(3), 1108–1118.
Foh, M. B. K., Amadou, I., Foh, B. M., Kamara, M. T., & Xia, W. (2010). Functionality and antioxidant properties of tilapia (Oreochromis niloticus) as influenced by the degree of hydrolysis. *International Journal of Molecular Sciences, 11*(4), 1851–1869.
Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3), 339–347.
Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulln. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588–593.
Igoshi, K., Kondo, Y., Kobayashi, H., Kabata, K., & Kawakami, H. (2008). Antioxidative activity of cheese. *Milchwissenschaft, 63*(4), 424–427.
Kahl, R., & Kappus, H. (1993). Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E. *Toxikologie der synthetischen Antioxidantien BHA und BHT im Vergleich mit dem natürlichen Antioxidans Vitamin E, 196*(4), 329–338.
Kim, G. N., Jang, H. D., & Kim, C. I. (2007). Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase. *Food Chemistry, 104*(4), 1359–1365.
Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945–960.
Kudoh, Y., Matsuda, S., Igoshi, K., & Oki, T. (2001). Antioxidative peptide from milk fermented with Lactobacillus delbrueckii subsp. Bulgaricus IFO13953. *Nippon Shokuhin Kagaku Kogaku Kaishi, 48*(1), 44–50.
Li, Z., Jiang, A., Yue, T., Wang, J., Wang, Y., & Su, J. (2013). Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. *Journal of Dairy Science, 96*(7), 4242–4251.
Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1–17.
Miclo, L., Roux, E., Genay, M., Brusseaux, E., Poirson, C., Jameh, N., et al. (2012). Variability of hydrolysis of β-, αs1-, and αs2-caseins by 10 strains of streptococcus thermophilus and resulting bioactive peptides. *Journal of Agricultural and Food Chemistry, 60*(2), 554–565.
Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science, 66*(5), 642–646.
Ortakci, F., & Sert, S. (2012). Stability of free and encapsulated Lactobacillus acidophilus ATCC 4356 in yogurt and in an artificial human gastric digestion system. *Journal of Dairy Science, 95*(12), 6918–6925.
Pennington, J. A. T., & Fisher, R. A. (2009). Classification of fruits and vegetables. *Journal of Food Composition and Analysis, 22*(SUPPL.), S23–S31.
Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino acid composition and antioxidant properties of pea seed (Pisum sativum L.) Enzymatic protein hydrolysate fractions. *Journal of Agricultural and Food Chemistry, 58*(8), 4712–4718.
Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*(10), 4290–4302.
Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., & Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International, 38*(2), 175–182.
Sabeena Farvin, K. H., Baron, C. P., Nielsen, N. S., & Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 1 – In vitro assays and evaluation in omega-3 enriched milk. *Food Chemistry, 123*(4), 1081–1089.
Sabeena Farvin, K. H., Baron, C. P., Nielsen, N. S., Otte, J., & Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2 – Characterisation of peptide fractions. *Food Chemistry, 123*(4), 1090–1097.
Sakai, T., Oishi, K., Asahara, T., Takada, T., Yuki, N., Matsumoto, K., et al. (2010). M-RTLV agar, a novel selective medium to distinguish Lactobacillus casei and Lactobacillus paracasei from Lactobacillus rhamnosus. *International Journal of Food Microbiology, 139*(3), 154–160.
Şanlidere Aloğlu, H., & Öner, Z. (2011). Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. *Journal of Dairy Science, 94*(11), 5305–5314.
Sidira, M., Saxami, G., Dimitrellou, D., Santarmaki, V., Galanis, A., & Kourkoutas, Y. (2013). Monitoring survival of Lactobacillus casei ATCC 393 in probiotic yogurts using an efficient molecular tool. *Journal of Dairy Science, 96*(5), 3369–3377.
Spitzer, J., Doucet, S., & Buettner, A. (2010). The influence of storage conditions on flavour changes in human milk. *Food Quality and Preference, 21*(8), 998–1007.
Tang, X., He, Z., Dai, Y., Xiong, Y. L., Xie, M., & Chen, J. (2010). Peptide fractionation and free radical scavenging activity of zein hydrolysate. *Journal of Agricultural and Food Chemistry, 58*(1), 587–593.
Urso, M. L., & Clarkson, P. M. (2003). Oxidative stress, exercise, and antioxidant supplementation. *Toxicology, 189*(1–2), 41–54.
Van Boekel, M. A. J. S., Weerens, C. N. J. M., Holstra, A., Scheidtweiler, C. E., & Alink, G. M. (1993). Antimutagenic effects of casein and its digestion products. *Food and Chemical Toxicology, 31*(10), 731–737.
Virtanen, T., Pihlanto, A., Akkanen, S., & Korhonen, H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of Applied Microbiology, 102*(1), 106–115.
Yeo, S. K., & Liong, M. T. (2010). Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *International Journal of Food Sciences and Nutrition, 61*(2), 161–181.

Chapter 4: Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt

Chapter 4 describes a study on prebiotic potential of a fibre-rich fraction from pineapple waste (peel and pomace) to enhance the metabolic performance of the selected probiotic organisms- *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) leading the augmented liberation of antioxidant and antimutagenic peptides encrypted into milk protein primary structures during manufacture of yogurt.

The paper entitled "Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*Journal of Food Science and Technology*" (2016), 53:1698–708, http://dx.doi.org/10.1007/s13197-015-2100-0, and included in thesis "With permission of Springer".

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** **Date:** 15-Mar-2016

Baidya Nath Prasad Sah

[redacted]

Paper Title:

Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
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ORIGINAL ARTICLE

Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt

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Abstract Although many fruit by-products are good sources of nutrients, little is known about their prebiotic potential. This research was aimed at establishing the prebiotic effect of pineapple wastes on probiotics including *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* spp. *paracasei* (ATCC® BAA52™) and the subsequent release of antioxidant and antimutagenic peptides in yogurt during their growth. Oven- and freeze- dried peel and pomace were milled separately into powders and tested for prebiotic activities. The net probiotic growth (1.28–2.14 log cfu/g) in customized MRS broth containing the pineapple powders as a direct carbohydrate source was comparable to MRS broth containing glucose. The powders were also separately added to milk during the manufacturing of yogurt with or without probiotics. An increase (by 0.3–1.4 log cycle) in probiotic populations was observed in the yogurts as a consequence of pineapple powder supplementation. Crude water-soluble peptide extracts, prepared by high-speed centrifugation of the yogurts, displayed remarkable antioxidant activities assessed through in vitro assays, namely scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radicals (IC_{50} = 0.37–0.19 mg/ml) and hydroxyl radicals (58.52–73.55 %). The peptide extracts also exhibited antimutagenic activities (18.60–32.72 %) as sodium azide inhibitor in the *Salmonella* mutagenicity test. Together, these results suggest that pineapple by-products exhibited prebiotic properties and could possibly be commercially applied in new functional food formulations.

Keywords Pineapple by-products · Probiotics · Peptide · Antioxidant activity · Antimutagenic activity

Abbreviations

DPPH	1,1-diphenyl-2-picrylhydrazyl
WSPE	Water soluble peptide extract
CA	Cluster analysis
PCA	Principal component analysis

Highlights
- Pineapple by-products are a potential source of bacterial growth factors.
- Pineapple fiber increased probiotic growth and production of organic acids.
- Pineapple fiber addition resulted in enhanced antioxidative activity of peptide extract.
- Pineapple fiber addition resulted in enhanced antimutagenic property of peptide extract.

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Introduction

Yogurt is a fermented dairy product, which has long been considered a prolific source for nutritious and therapeutic constituents. Some of these are attributable to metabolic activity of starter cultures and can be further enhanced by addition of probiotic organisms. According to FAO/WHO (2002), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Despite the importance of minimum level of these bacteria to achieve probiotic effects, many studies showed poor viability of these bacteria in yogurt (Lourens-Hattingh and Viljoen

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2001). Several strategies have been employed to enhance probiotic growth in milk medium. Fortunately, some legislations authorize addition of total solids (thickeners, stabilizers, emulsifiers, or gelling agents) up to 2 % (Canadian Legal Legislation Institute 2014). This opens an opportunity of supplementation to yogurt for the enhancement of probiotic growth and prebiotic supplementation could be an approach. A prebiotic is a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health" (Gibson et al. 2004). Non-digestible oligosaccharides and polysaccharides usually exhibited prebiotic properties, such as fructooligosaccharides, and galactooligosaccharides, inulin, resistant starch, and lactulose derived from various sources including fruits and vegetables (Thammarutwasik et al. 2009). Some of them including fructooligosaccharides, galactooligosaccharides, and inulin are established prebiotics and are available commercially. Combining probiotics and prebiotics into a single product creates a synbiotic (Donkor et al. 2007a; Sah et al. 2016). Industry and scientific community are very keen to explore and introduce new prebiotic ingredients with added functionalities such as fibre-rich fraction from fruits, vegetables, and cereals due to several reasons including commercial importance, sustainability, and health benefits. Several studies have sought to enhance the growth of probiotic organisms by fortifying the fibre-rich fractions from herbs (Chowdhury et al. 2008), cereals (Vasiljevic et al. 2007), and banana, passion, or apple processing by-products (Espírito Santo et al. 2012).

Pineapple waste contains mainly peel and pomace, presenting more than one third of the whole fruit mass (Huang et al. 2011) and is only partially utilized as feed or composting for fertilization but mainly discarded, creating environmental issues such as pollution and climate change and consequently economic loss. However, these by-products have been reported to contain dietary fibres, sugars, proteins, and minerals (Huang et al. 2011), and availability of these nutrients greatly depends on the recovery processes such as drying techniques employed in the preparation of powder. The drying techniques, namely oven drying and freeze drying have significantly different effects on the functional properties like, solubility, flow behaviours, water- and oil-holding capacity, and foaming capacity of the dried powder (Mirhosseini and Amid 2013). Sogi et al. (2013) also suggested that freeze-drying has advantages over conventional drying techniques in solubility, preservation of nutrients etc. These nutrients could serve as a prominent source of growth factors such as prebiotic factors for lactic acid bacteria (LAB) and may enhance protease production and activity leading to liberation of a range of protein hydrolysates, when incorporated in yogurt.

There is a growing interest in protein hydrolysates as they may provide enhanced nutritional value (Pownall et al. 2010) in addition to chemotherapeutic potentialities in the treatment and management of many diseases and disorders such as cancer (Sah et al. 2015a). Mota et al. (2006); Sah et al. (2015b) reported that bioactivity of protein hydrolysates depends on the protein source, enzyme profile and activity, and hydrolysis time.

In this context, the aim of the present research was to establish a prebiotic potential of a fibre-rich fraction from pineapple wastes to enhance the metabolic performance of the selected probiotic organisms– *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* spp. *paracasei* (ATCC® BAA52™). This would consequently lead to augmented liberation of antioxidant and antimutagenic peptides encrypted into milk protein primary structures during manufacture of yogurt.

Materials and methods

Substrates and chemicals

Trifluoroacetic acid, vancomycin, clindamycin, 1,1-diphenyl-2-picrylhydrazyl, o-phthaldialdehyde (OPA), serine, salicylic acid and sodium azide were purchased from Sigma-Aldrich Corporation (St Louis, Missouri, USA). De Man Rogosa and Sharpe (MRS) and M17 media were supplied by Oxoid Australia (West Heidelberg, Victoria, Australia) while Becton Dickinson Pty Ltd. (BD) (Sydney, NSW, Australia) supplied Davis minimal agar (DMA). Skim milk powder was procured from a local supermarket (Woolworths Limited, Australia). All aqueous solutions were prepared using Milli-Q water (18.2 MΩcm).

Propagation of cultures

Streptococcus thermophilus ASCC 1275 (*S. thermophilus*) and *L. delbrueckii* spp. *bulgaricus* Lb1466 (*L. bulgaricus*) were collected from the Victoria University Culture Collection (Werribee, Australia). Cell Biosciences Pty Ltd. (Heidelberg, Victoria, Australia) supplied *L. acidophilus* ATCC 4356 (*L. acidophilus*), *L. casei* ATCC 393 (*L. casei*) and *L. paracasei* spp. *paracasei* ATCC BAA52 (*L. paracasei*). All strains were preserved at −80 °C in MRS broth containing 40 % (*v*/*v*) glycerol. The strains were resuscitated and starter cultures were prepared as described by Sah et al. (2014).

Preparation and analysis of pineapple waste powder (PWP)

Whole pineapples (*Ananas comosus* [L.] Merrill) without crown were purchased from a local supermarket (Woolworths Limited, Australia). Peel and pomace powder

were prepared as described by Espírito Santo et al. (2012) with some modifications. Briefly, crushed peel and pomace were dipped in hot water (90 °C) for 30 min to inactivate potential pathogens and enzymes, dried separately using an oven dryer (Memmert GmbH+ Co. KG., Schwabach, Germany) at 60 °C for 24 h and a Dynavac FD 300 freeze dryer (Airvac Engineering Pty. Ltd., Rowville, Australia), and milled to fine powders. Particle size of the powders was standardized to less than 180 μm using sieves (Mesh Series S410/1986; Endecotts Ltd., London, UK) and then sterilized in UV irradiation for 30 min.

Proximate analysis of PWPs was performed by measuring moisture (AOAC official method 934.06), crude protein (AOAC official method 920.152), crude fat (AOAC official method 963.15), total ash (AOAC official method 940.26), and total dietary fibre (AOAC official method 985.29) (Horwitz and Latimer 2006). Total available carbohydrates were calculated by subtracting % moisture, % crude protein, % crude fat, % total ash, and % total dietary fibre from 100. Trace metals were also estimated by using an ICPE-9000 Multitype Inductively Coupled Plasma Atomic Emission Spectrometer (Shimadzu Corporation, Kyoto, Japan) according to AOAC official method 985.01 (Horwitz and Latimer 2006).

Assessment of prebiotic activity of PWPs

The prebiotic activity of the PWPs was assessed through the growth of *L. acidophilus*, *L. casei* and *L. paracasei* according to Moreno-Vilet et al. (2014) with some modifications. The customized culture media were prepared by substituting glucose with the pineapple powders in the same formulations of MRS broth (CM0359, Oxoid). Customized MRS medium without glucose, hereafter referred to as cMRS broth, was prepared by mixing different ingredients in Milli-Q water, i.e. 10 g/l of bacteriological peptone, 8 g/l Lab Lemco powder, 4 g/l yeast extract, 1 g/l of Tween 80, 2 g/l of di-potassium hydrogen phosphate, 5 g/l of sodium acetate trihydrate, 2 g/l of ammonium citrate tribasic, 0.2 g/l of magnesium sulphate heptahydrate, and 0.05 g/l of manganese sulphate tetrahydrate. The pH was adjusted to 6.2 ± 0.2 at 25 °C. The PWPs, whose amounts were quantified on the basis of total soluble carbohydrates content determined by phenol-sulphuric acid method (Dubois et al. 1956), were mixed separately at the rate of 20 g/l to the cMRS and autoclaved at 121 °C for 15 mins. The broths (at room temperature) were aseptically inoculated with 1 % (*v*/*v*) of each probiotic cultures (1–2 × 10^9 cfu/ml) separately and incubated at 37 °C for 24 h with agitation (120 rpm). The bacterial colonies were enumerated on MRS agar after anaerobic incubation at 37 °C for 48 h. The net growth of probiotic organisms was determined by subtracting count as log cfu/g at 0 h to that at 24 h. The experiment was repeated using cMRS supplemented with glucose as the positive control and cMRS only as the negative control.

Lactic and acetic acids of the samples collected at 24 h were also determined as described by Donkor et al. (2007a) employing a Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) fitted with an Aminex HPX-87 H (300 × 7.8 mm) ion-exchange column (Biorad Life Science Group, Hercules, CA, USA) maintained at 65 °C. Flow rate of the mobile phase (4.5 mM H_2SO_4) was 0.6 ml/min and peaks were detected at 210 nm.

Preparation of yogurt supplemented with PWPs

Three replicate experiments were performed by making set-type yogurt as described by Sah et al. (2014) with some modifications. Briefly, the quantity of PWP corresponding to 1 % (*w*/*v*) of the yogurt mix was dissolved separately in a small quantity of Milli-Q water, pH adjusted to 6.7, blanched to inactivate bromelain and other proteolytic enzymes, and supplemented separately to four lots of milk bases prepared by reconstituting skim milk powder in Milli-Q water at 14 % (*w*/*v*). The fortified milk bases were homogenized, and heated to 85 °C for 30 min, cooled to 45 °C and inoculated aseptically with 1 % (*v*/*v*) of each *S. thermophilus* and *L. bulgaricus* monocultures. They were divided into two equal portions; one portion was further inoculated with each of *L. acidophilus*, *L. casei* and *L. paracasei* monocultures at 1 % (*v*/*v*) (Table 1). The final mixes were poured into polystyrene cups, incubated at 42 °C until pH of 4.5 ± 0.05 was achieved.

Enumeration of yogurt and probiotic cultures

S. thermophilus, *L. bulgaricus*, *L. acidophilus*, *L. casei* and *L. paracasei* in yogurt samples were enumerated by spreading 0.1 ml of the appropriate dilutions of the sample in sterile peptone salt solution (bacteriological peptone 0.1 % *w*/*v*; sodium chloride 0.85 % *w*/*v*, pH 7.0 ± 0.2) onto selective agar plates and expressed as log cfu/g. The selective agar plates and incubation conditions were M17 medium supplemented with lactose and 45 °C for 24 h aerobically for *S. thermophilus*, acidified MRS agar (pH 5.2) and 45 °C for 72 h anaerobically for *L. bulgaricus*, MRS-clindamycin agar (pH 6.2; 0.5 ppm clindamycin) and 37 °C for 72 h anaerobically for *L. acidophilus*, and MRS-vancomycin agar (pH 6.2; 1 ppm vancomycin) and 37 °C for 72 h anaerobically for both *L. casei*/*L. paracasei* together (Sah et al. 2014).

Determination of pH and titratable acidity of yogurt samples

The pH of yogurt samples was measured using pH 720 precision pH meter (WTW inoLab®, Weilheim, Germany) and titratable acidity was estimated according to the AOAC

Table 1 Experimental design and the coding used in the study to evaluate the effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt

Pineapple waste powder (1 % *w/v*)	Code	Combination of cultures (1 % *v/v* each)
Oven dried peel powder	1	*S. thermophilus* + *L. bulgaricus*
	2	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Freeze dried peel powder	3	*S. thermophilus* + *L. bulgaricus*
	4	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Oven dried pomace powder	5	*S. thermophilus* + *L. bulgaricus*
	6	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Freeze dried pomace powder	7	*S. thermophilus* + *L. bulgaricus*
	8	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*

Yogurt culture = *S. thermophilus* + *L. bulgaricus*; Probiotic cultures = *L. acidophilus* + *L. casei* + *L. paracasei*

official method 947.05 and expressed as % lactic acid (Horwitz and Latimer 2006).

Determination of proteolysis in yogurt samples

Proteolysis was assessed using the OPA method as described by Sah et al. (2014) and expressed as percentage degree of hydrolysis (DH) with some modifications in the sample preparation. Briefly, 1 ml Milli-Q water was added to a 5 g aliquot of yogurt sample and the final volume adjusted to 10 ml with TCA solution (0.75 M). The mix was filtered using a 0.45 μm Phenex syringe filter (Phenomenex Inc., Lane Cove, Australia) after centrifugation at 2684 × *g* for 30 mins at 4 °C and analyzed using a Biochrom Libra S12 UV/Vis spectrophotometer (Biochrom Ltd., Cambridge, UK).

Preparation and profiling of water-soluble peptide extract (WSPE)

The WSPEs from yogurts and heat treated reconstituted skim milk (RSM; pH adjusted to 4.5 ± 0.05) were prepared and profiled using a HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, and a 9065 Polychrom UV/Vis detector (Varian Inc., Palo Alto, California, USA) as described by Sah et al. (2014).

Determination of antioxidant activities

DPPH radical scavenging activity

The radical scavenging activity (RSA) of WSPEs was measured against DPPH$^{\bullet}$ radical as described by Siow and Gan (2013) with some modifications. 1.0 ml of DPPH reagent (0.075 mM in ethanol) was added to 0.1 ml of aqueous WSPE of different protein concentrations and left to stand in dark at room temperature for 30 mins. The mix was clarified by centrifuging at 16,000× *g* (5415C microcentrifuge, Eppendorf, Hamburg, Germany) for 5 mins at room temperature and subjected for absorbance measurement at 517 nm. Milli-Q water was used for blank and % RSA was calculated using Eq. (1).

$$RSA, \% = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100 \quad (1)$$

IC_{50} value, which is the concentration that scavenges 50 % of DPPH radicals, was calculated from a linear regression curve of protein content of the WSPE (mg/ml) versus the % RSA.

Hydroxyl radical ($^{\bullet}$OH) scavenging activity (HRSA)

The HRSA of WSPEs was assayed as described by Zheng et al. (2015) with some modifications. Briefly, 500 μl aqueous $FeSO_4 \cdot 7H_2O$ (2 mM) and 100 μl aqueous H_2O_2 (2 mM) were added to 20 μl of sample (0.1 mg protein/ml), allowed to stand for 10 min at room temperature and 500 μl of aqueous salicylic acid (2.5 mM) was then added. Absorbance of the reaction mixture was measured at 510 nm after incubation at 37 °C for 30 min. Milli-Q water was used for the blank (instead of salicylic acid solution) and the control (instead of sample) in the reaction mixture. The HRSA was calculated using Eq. (2), where A_s, A_b, and A_c represented absorbance for sample, blank, and control respectively.

$$\text{HRSA}(\%) = \left[1 - \frac{A_s - A_b}{A_c}\right] \times 100 \quad (2)$$

Determination of antimutagenic activity by the Ames test

The antimutagenicity of WSPEs was conducted using *Salmonella enterica* spp. *enterica* serovar *typhimurium* (ATCC® 29629™) (genotype: *his*, *rfa*, *uvrB-bio*) through the preincubation protocol of the Ames test as described by Sah et al. (2014). Briefly, 0.1 ml of test sample (50 ± 1 μg peptide), 0.1 ml sodium azide solution (1 μg) were mixed to

0.5 ml of sodium phosphate buffer (0.1 M; pH 7.4). After inoculating 0.1 ml culture, the mix was preincubated for 20 min at 37 °C. The mix was then poured over a Davis minimal agar plate with 2 ml molten top agar. The plate was incubated aerobically for 48 h at 37 °C and revertant colonies were enumerated. The antimutagenic activity as percentage of inhibition was calculated using Eq. (3).

$$\% \text{ Inhibition} = \frac{(M - S_1)}{(M - S_0)} \times 100 \quad (3)$$

Where, S_1 = revertant counts/plate induced by mutagen in the presence of peptide extract; M = revertant counts/plate induced by mutagen alone; S_0 = spontaneous revertant counts/plate.

Statistical analysis

Experiments were conducted in triplicate and results were averaged and expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was conducted to explore the influence of pineapple fibres as the main effect, followed by Tukey Honestly Significant Difference post hoc test to evaluate significant differences between the means at $P < 0.05$. Two-way ANOVA was carried out to examine the interaction effect of yogurt types and different pineapple fibres on the measured variables. A two-tailed Pearson's correlation test was carried out to measure the correlations between proteolysis and antioxidant activity or antimutagenic activity. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were conducted to show correlation among the measured variables and their relationship with different yogurt samples. Statistical analyses were performed using the SPSS 22.0 for windows (SPSS Inc., Chicago, Illinois, USA).

Results and discussion

Chemical composition and prebiotic effect of PWPs

The powders were prepared from pineapple peel and pomace and subjected to proximate and elemental analyses; the results are presented in Table 2. Although significant ($P < 0.05$) differences in the composition between peel and pomace were noted, oven and freeze-drying methods employed in the preparation of powders had no pronounced ($P > 0.05$) effect. The high dietary fibre content (44.90–58.48 g/100 g of dry matter), comparable in the pineapple peel and pomace powders, reflected those fibre-rich fractions in wine grape pomace (61.32 g/100 g of dry matter) (Tseng and Zhao 2013) and passion fruit seed fibres (64.8 g/100 g of dry matter) (Chau and Huang 2004). Crude protein, crude fat, and total ash were found in low concentrations: 3.82–6.89 g/100 g of dry matter, 0.34–1.35 g/100 g of dry matter, and 2.52–4.76 g/100 g of dry matter, respectively. Of the minerals tested, potassium was found at the highest level (5967.28–10,144.32 mg/kg of dry matter), followed by calcium (893.03–2206.75 mg/kg of dry matter) and sodium (610.69–1033.57 mg/kg of dry matter). Other trace elements such as copper, zinc, and cobalt were available at very low concentrations. Chromium and iron were only detected in peel powders and manganese was only detected in oven- dried peel. Overall, the pineapple powders, due to their high dietary fibre and essential minerals contents, can be considered an excellent source of growth factors including prebiotic components for probiotic bacteria.

The principal characteristic of prebiotics is their ability to enhance the proliferation of beneficial microbes or probiotics that are part of human colonic microbiota (Gibson et al. 2004). However, this synbiotic association is strain specific. The net probiotic growth (in 24 h) of *L. acidophilus*, *L. casei* and *L. paracasei* in customized MRS broth containing freeze dried pineapple peel and pomace powders as a direct carbohydrate source were not substantially different ($P \geq 0.05$) from those of customized MRS broth supplemented with glucose (Fig. 1a). In all the pineapple powders tested, bacterial growth was more than one log higher compared to the negative control (customized MRS broth without carbohydrate). Both pineapple peel and pomace powders have been reported to be rich in dietary fibre, mainly cellulose, hemicellulose, lignin, fructans, pectin, and pectic substances (Chitturi et al. 2013; Huang et al. 2011). Furthermore, several studies have reported prebiotic properties of pectin-derived oligosaccharides (Gullón et al. 2013). Thus, the enhanced growth of tested probiotic organisms in our study was likely due to prebiotic components and other growth factors present in the fibre-rich fractions of pineapple powders.

LAB metabolize carbohydrate substrates for growth and energy and the predominant fermentation products are short chain fatty acids, mainly lactic and acetic acids (Gibson 1999). Thus, the production of lactic and acetic acids after 24 h of fermentation was also investigated to assess the growth of probiotic species in terms of release of metabolites (Fig. 1b). Espírito Santo et al. (2012) also reported similar results, where the total dietary fibre from by-products of banana, apple or passion processing increased the probiotic viability in yogurt. The peel and pomace powders in our study supported the growth of the probiotic organisms, in a manner similar to that of complex medium, as evidenced by the equivalent production of organic acids.

These observations showed the in vitro synbiotic potential of pineapple powders upon the probiotic organisms tested, which were similar to the previous reports with dietary fibres from various fruit processing by-products on probiotics (Espírito Santo et al. 2012). These synbiotic combinations were therefore applied in yogurt manufacturing for enhancing liberation of peptides through improved growth.

Table 2 Proximate and elemental analysis of the powders prepared by milling oven- and - freeze dried pineapple peel and pomace

Parameters	Pineapple waste powder			
	Oven-dried peel	Freeze-dried peel	Oven-dried pomace	Freeze-dried pomace
Moisture†	4.32 ± 0.09^{c}	5.25 ± 0.14^{a}	4.77 ± 0.11^{b}	4.55 ± 0.06^{bc}
Crude protein§	6.89 ± 0.13^{a}	6.89 ± 0.04^{a}	3.91 ± 0.09^{b}	3.82 ± 0.13^{b}
Crude fat§	1.19 ± 0.06^{b}	1.35 ± 0.06^{a}	0.47 ± 0.02^{c}	0.34 ± 0.03^{d}
Total ash§	4.56 ± 0.16^{a}	4.76 ± 0.05^{a}	2.69 ± 0.15^{b}	2.52 ± 0.03^{b}
Total dietary fiber§	57.76 ± 1.33^{a}	58.48 ± 0.49^{a}	46.19 ± 1.41^{b}	44.90 ± 0.71^{b}
Total available carbohydrates§	29.60 ± 1.18^{b}	28.52 ± 0.53^{b}	46.73 ± 1.42^{a}	48.43 ± 0.59^{a}
Total soluble carbohydrates§	26.27 ± 0.87^{b}	27.12 ± 0.82^{b}	37.88 ± 1.64^{a}	38.29 ± 1.13^{a}
Calcium*	1997.97 ± 10.13^{b}	2206.75 ± 20.41^{a}	893.03 ± 3.06^{c}	911.34 ± 7.66^{c}
Magnesium*	883.36 ± 3.92^{b}	1031.70 ± 7.44^{a}	713.47 ± 2.75^{d}	846.16 ± 3.14^{c}
Sodium*	967.97 ± 4.89^{b}	1033.57 ± 6.34^{a}	610.69 ± 4.57^{d}	915.73 ± 18.87^{c}
Potassium*	8574.78 ± 8.31^{b}	10,144.32 ± 51.62^{a}	5967.28 ± 11.28^{d}	6395.15 ± 58.55^{c}
Cobalt*	5.21 ± 0.16^{a}	3.25 ± 0.12^{b}	1.21 ± 0.12^{c}	1.16 ± 0.03^{c}
Manganese*	1.22 ± 0.07^{a}	ND	ND	ND
Iron*	9.90 ± 0.98^{b}	14.82 ± 0.62^{a}	ND	ND
Zinc*	62.66 ± 0.08^{b}	85.46 ± 0.69^{a}	38.04 ± 0.08^{d}	40.00 ± 0.37^{c}
Copper*	285.47 ± 6.83^{b}	400.44 ± 2.69^{a}	17.83 ± 0.02^{c}	20.47 ± 0.13^{c}
Chromium*	36.96 ± 0.08^{b}	57.68 ± 0.68^{a}	ND	ND

† Results were expressed as g/100 g of pineapple peel/pomace powder; § Results were expressed as g/100 g of dry matter; *Results were expressed as mg/kg of dry matter; *ND* Not Detected, ^{abcd} Results were expressed as mean ± standard deviation ($n = 3$) and values with different superscripts within a row were significantly different ($P < 0.05$)

Synbiotic study in yogurt

The viable count as log cfu/g of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, and *L. casei*, *L. paracasei* in yogurt samples supplemented with PWPs were performed selectively and reported in Table 3. The viable counts of *S. thermophilus* and *L. bulgaricus* in the yogurts supplemented with pineapple powders (Table 3) showed similar value in comparison to those in yogurts without supplementation (Sah et al. 2014). On the other hand, probiotic yogurts with PWPs (yogurts 2, 4, 6, 8) experienced enhanced growth of the probiotic species- high counts of *L. acidophilus* (8.13–8.71 log cfu/g; Table 3) and *L. casei*, *L. paracasei* (8.12–8.72 log cfu/g; Table 3) than non-supplemented probiotic yogurts (7.34 log cfu/g and 7.83 log cfu/g, respectively) (Sah et al. 2014). Moreover, the probiotic counts were very high compared to the minimum therapeutic count of 6 log cfu/g, if 100 g yogurt is considered as a daily serving dose (Lourens-Hattingh and Viljoen 2001).

The pH and titratable acidity of all yogurt samples were within 4.51–4.48 and 1.01 % – 1.07 %, respectively (Table 3), within 5 h 45 min. These results were in agreement with the various works on addition of fibres from fruit by-products to yogurt (Espírito Santo et al. 2012; García-Pérez et al. 2005). The pineapple powders contained dietary fibres, proteins, and minerals as well as fats (Table 2), which could have served as growth factors for probiotics and therefore improved growth. Similarly, improved growth would have resulted in high metabolic activities and hence proteolysis (Donkor et al. 2007a).

Enhancement of milk proteolysis by supplementing PWPs

The proteolytic enzymes of LAB used in our study cleaved peptide bonds of milk proteins and the extent of proteolysis determined was expressed as percentage degree of hydrolysis as presented in Table 4. There were significant effects of probiotic addition and type of pineapple fibre supplementation on degree of proteolysis in yogurt (Table 5). The probiotic yogurts (numbers 2, 4, 6, 8) with pineapple powder supplementation producing a significantly high degree of hydrolysis (13.07–14.30 %; Table 4) compared with control yogurts (numbers 1, 3, 5, 7) with pineapple powder supplementation (6.47–7.13 %; Table 4) or without the supplementation (5.38 %) (Sah et al. 2014). For probiotic yogurts, supplementation of freeze-dried peel and pomace led to higher degree of proteolysis than that of oven-dried peel and pomace (Tables 4 and 5). Freeze-drying has advantages such as nutrients preservation, solubility etc. over conventional drying techniques (Sogi et al. 2013). The principal milk proteins, caseins, are broken down first by extracellular proteinases to oligopeptides and then by intracellular peptidases into peptides (Donkor

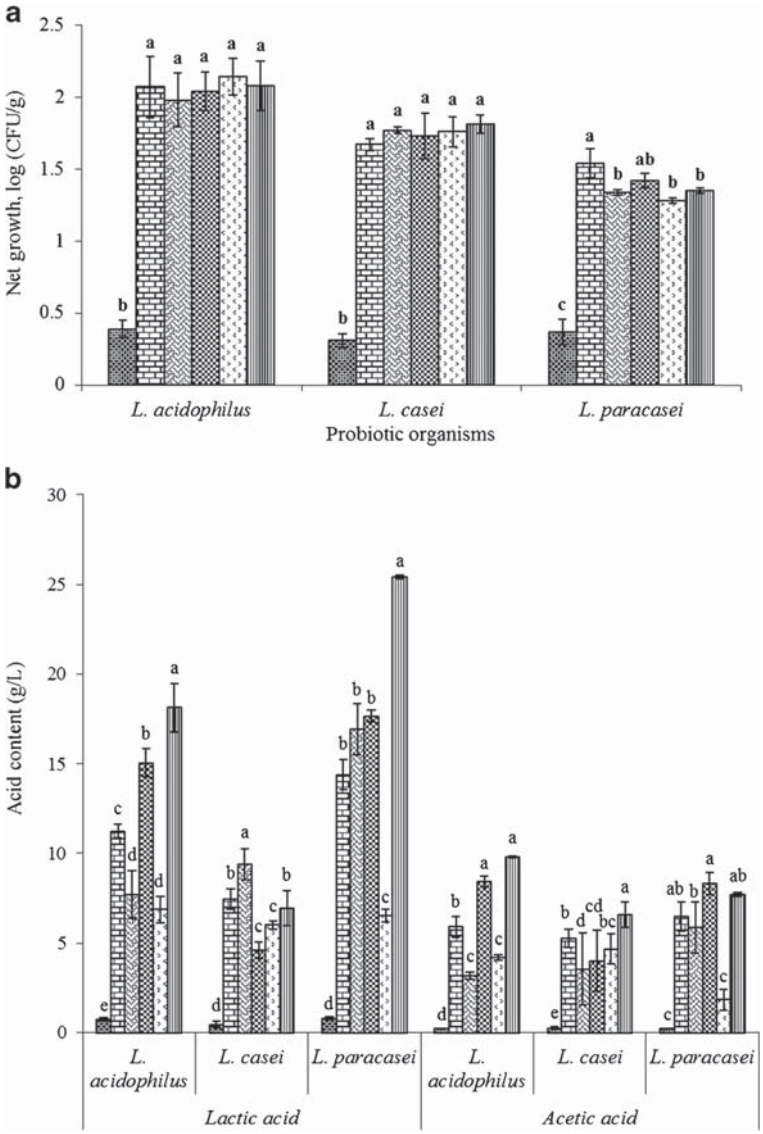

Fig. 1 **a** and **b** display net growth of probiotic organisms (*L. acidophilus*, *L. casei*, *L. paracasei*), and concentration of organic acids (lactic and acetic) respectively in customized MRS broth without glucose (cMRS) separately containing freeze dried pineapple pomace powder (▥), oven dried pineapple pomace powder (▩), freeze dried pineapple peel powder (▦), oven dried pineapple peel powder (▧) as a carbohydrate source compared to glucose as positive control (▤) and without carbohydrate as negative control (▨) at 37 °C for 24 h. The monocultures were aseptically inoculated at 1 % (*v*/*v*) separately. [a,b,c,d,e]Results were expressed as means ± standard deviation ($n = 3$), values with different letters within each organisms **a** and each acids **b** were significantly different ($P < 0.05$)

et al. 2007b). Additionally, the activity of proteolytic enzymes could have been markedly enhanced because of the increased level of divalent ions such as Ca^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+} in the yogurts due to the supplementation with pineapple powders (Table 2). Divalent metal ions have been reported to enhance production and activity of proteases in microorganisms (Llorente-Bousquets et al. 2008).

The proteolytic activities of the enzymes produced by LAB were evidenced through chromatographic profiling of water-soluble extracts from yogurt samples using a reversed-phase HPLC (Figs 2a and 2b). Control yogurts (numbers 1, 3, 5, 7) with PWPs addition and probiotic yogurts (numbers 2, 4, 6, 8) with PWPs addition displayed more peaks (peptides) than that of the reconstituted skim milk (RSM) sample only. The WSPE containing these peptides were further tested for their antioxidant and antimutagenic activities.

Enhancement of antioxidant activities by supplementation with PWPs

In this study, the antioxidant activity was assessed by measuring scavenging capacity of WSPE for DPPH$^{\bullet}$ and $^{\bullet}$OH radicals. The DPPH$^{\bullet}$ scavenging capacity of WSPE was expressed as IC_{50} value and presented in Table 4. Probiotic yogurts (numbers 2, 4, 6, 8) with PWPs supplementation displayed strong scavenging capacity (IC_{50} values of 0.27–0.19 mg/ml; Table 4) compared to the control yogurts (numbers 1, 3, 5, 7) with PWPs supplementation (IC_{50} values of 0.37–0.23 mg/ml;

Table 3 Viable counts, pH, titratable acidity of control and probiotic yogurts supplemented with pineapple peel and pomace powder at ultimate pH (4.5 ± 0.05) prepared by fermenting aseptically inoculated yogurt mix with 1 % (*v*/*v*) of *S. thermophilus*, *L. bulgaricus* cultures with or without *L. acidophilus*, *L. casei* and *L. paracasei* cultures at 42 °C

Yogurt types		Count (log cfu/g)				pH	Titratable acidity (% lactic acid)
Cultures	Pineapple powders	*S. thermophilus*	*L. bulgaricus*	*L. acidophilus*	*L. casei* & *paracasei*		
SC	Oven-dried peel powder	9.32 ± 0.14^{a}	8.00 ± 0.15^{c}	–	–	4.51 ± 0.02^{a}	1.03 ± 0.02^{ab}
SC + PC		9.21 ± 0.08^{a}	8.17 ± 0.07^{abc}	8.25 ± 0.02^{c}	8.11 ± 0.03^{d}	4.49 ± 0.02^{a}	1.04 ± 0.01^{ab}
SC	Freeze-dried peel powder	9.25 ± 0.15^{a}	8.17 ± 0.14^{abc}	–	–	4.50 ± 0.02^{a}	1.02 ± 0.04^{b}
SC + PC		9.13 ± 0.06^{a}	8.23 ± 0.13^{ab}	8.71 ± 0.08^{a}	8.73 ± 0.08^{a}	4.49 ± 0.01^{a}	1.01 ± 0.01^{b}
SC	Oven-dried pomace powder	9.24 ± 0.03^{a}	8.22 ± 0.16^{ab}	–	–	4.49 ± 0.02^{a}	1.05 ± 0.04^{ab}
SC + PC		9.23 ± 0.16^{a}	8.08 ± 0.09^{bc}	8.13 ± 0.03^{d}	8.37 ± 0.05^{c}	4.48 ± 0.02^{a}	1.04 ± 0.03^{ab}
SC	Freeze-dried pomace powder	9.14 ± 0.10^{a}	8.35 ± 0.06^{a}	–	–	4.48 ± 0.02^{a}	1.05 ± 0.03^{ab}
SC + PC		9.20 ± 0.14^{a}	8.28 ± 0.11^{ab}	8.50 ± 0.10^{b}	8.50 ± 0.10^{b}	4.49 ± 0.02^{a}	1.07 ± 0.01^{a}

SC Starter culture (*S. thermophilus* + *L. bulgaricus*); PC: Probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*)

^{abcd} Results were expressed as means ± standard deviation (n = 6), values with different superscripts within a column were significantly different ($P < 0.05$)

Table 4) or without the supplementation (IC_{50} value of 2.23 mg/ml) (Sah et al. 2014). The significant interaction (Table 5) indicates that the effect of different pineapple powders on the antioxidant activity was different in probiotic yogurts compared to control yogurts. Specifically, the probiotic yogurts supplemented with freeze-dried peel or pomace powders exhibited the most potent radical scavenging activities as compared to the other yogurts. Several studies (Kudoh et al. 2001; Sah et al. 2014) also reported the generation of potent antioxidant peptides from milk proteins. It can be inferred that the generated peptides had acted as hydrogen or electron donors and could have reacted with free DPPH radicals resulting into stable products (Prior et al. 2005).

In current study, $^{\bullet}$OH scavenging capacity of WSPEs was determined and presented in Table 4. Probiotic yogurts (numbers 2, 4, 6, 8) with PWPs supplementation displayed strong scavenging capacity (67.63–73.55 %) compared to the control yogurts (numbers 1, 3, 5, 7). The significant interaction (Table 5) indicates that the effect of different pineapple powders on the $^{\bullet}$OH scavenging activity was different in probiotic yogurts compared to control yogurts. Specifically, the probiotic yogurts supplemented with freeze-dried peel powder exhibited the most potent hydroxyl radical scavenging activities as compared to the other yogurts. These results suggest that WSPEs could have the potential to safeguard human against $^{\bullet}$OH-induced damage or prevent food spoilage.

Additionally, degree of protein hydrolyses were correlated with the IC_{50} values of DPPH$^{\bullet}$ scavenging activities ($P < 0.05$, $r = -0.676$) and the percentage of $^{\bullet}$OH scavenging activities ($P < 0.05$, $r = 0.942$). These results were in agreement with

Table 4 Degree of hydrolysis (DH) of milk proteins, antioxidant activity (evaluated by measuring scavenging activities of DPPH$^{\bullet}$ and $^{\bullet}$OH radicals), and antimutagenic activity of water-soluble peptide extracts from yogurts supplemented with pineapple peel and pomace powder with or without probiotic addition at ultimate pH (4.5 ± 0.05)

Yogurt types		DH, %	Antioxidant activity		Antimutagenic activity (% Inhibition)
Cultures	Pineapple powders		DPPH (IC_{50}, mg/ml)	HRSA (%)	
SC	Oven-dried peel powder	6.47 ± 0.30^{e}	0.37 ± 0.01^{a}	58.99 ± 0.72^{e}	22.04 ± 1.65^{c}
SC + PC		13.07 ± 0.23^{c}	0.23 ± 0.02^{d}	70.21 ± 0.54^{b}	27.39 ± 0.89^{b}
SC	Freeze-dried peel powder	6.99 ± 0.45^{de}	0.23 ± 0.01^{d}	60.59 ± 0.60^{d}	25.42 ± 1.24^{b}
SC + PC		13.79 ± 0.21^{ab}	0.19 ± 0.01^{e}	73.55 ± 0.68^{a}	32.72 ± 1.98^{a}
SC	Oven-dried pomace powder	7.02 ± 0.09^{de}	0.30 ± 0.02^{b}	58.52 ± 0.32^{e}	18.60 ± 1.44^{d}
SC + PC		13.43 ± 0.42^{bc}	0.27 ± 0.01^{c}	67.63 ± 0.54^{c}	26.27 ± 1.73^{b}
SC	Freeze-dried pomace powder	7.13 ± 0.09^{d}	0.26 ± 0.01^{c}	58.79 ± 0.37^{e}	20.15 ± 0.98^{cd}
SC + PC		14.30 ± 0.51^{a}	0.20 ± 0.00^{e}	68.06 ± 0.48^{c}	27.85 ± 1.60^{b}

SC: Starter culture (*S. thermophilus* + *L. bulgaricus*); PC: Probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*)

^{abcde} Results were expressed as means ± standard deviation (n = 6), values with different superscripts within a column were significantly different ($P < 0.05$)

Table 5 Analysis of variance (ANOVA) depicting the significance (at $P < 0.05$) of types of yogurt (control and probiotic yogurts), pineapple powder (oven dried and freeze dried - peel and - pomace powder) and their effect on degree of protein hydrolysis, antioxidant (evaluated by measuring scavenging activities of DPPH$^{\bullet}$ and $^{\bullet}$OH radicals) and antimutagenic activities

Source of variation	*P*- value			
	Degree of proteolysis	Antioxidant activity		Antimutagenic activity
		DPPH	HRSA	
Yogurt type	< 0.000	< 0.000	< 0.000	< 0.000
Pineapple powder type	< 0.000	< 0.000	< 0.000	< 0.000
Yogurt type × Pineapple powder type	0.038	< 0.000	< 0.000	0.180

Igoshi et al. (2008), who reported significant correlation between generated peptides in cheese ripening and antioxidative activity. Thus, the more proteins degraded during yogurt manufacturing, because of enhanced proteolysis, the more potent peptides with antioxidant activity are generated.

Enhancement of antimutagenic activities by supplementing PWPs

The antimutagenic activity of the WSPEs was determined by evaluating inhibitory activity of peptides against mutagenicity

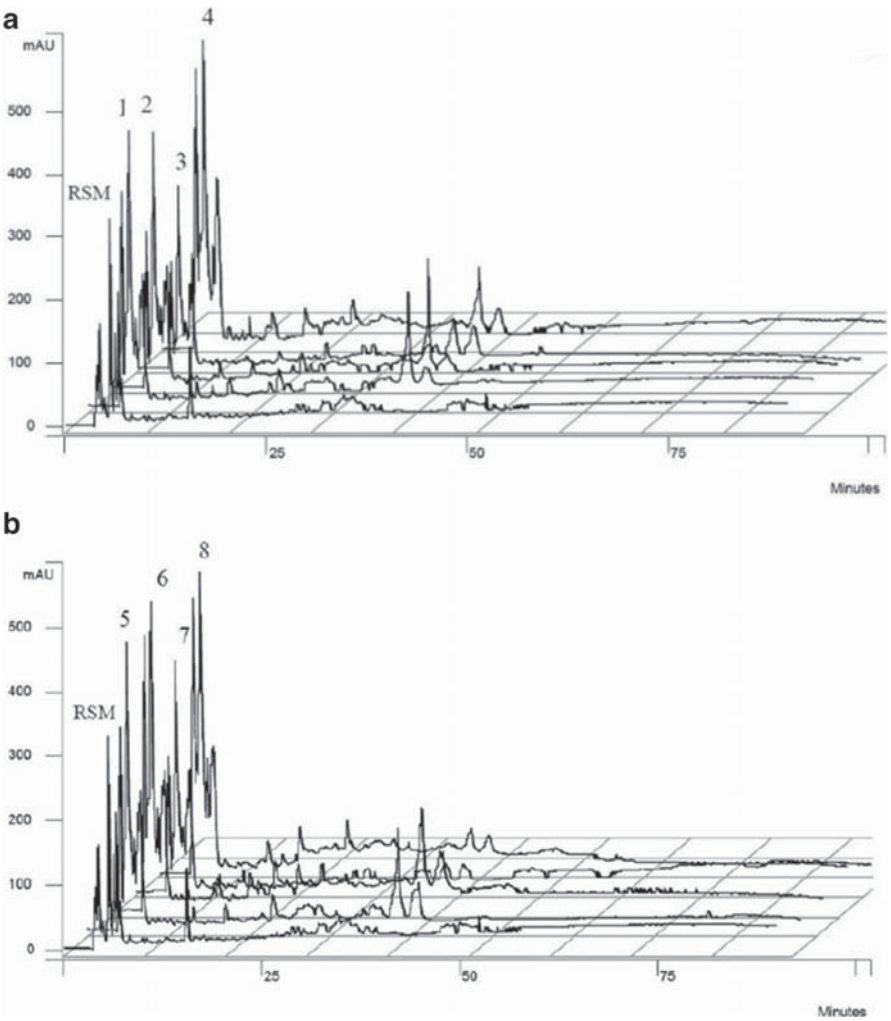

Fig. 2 RP-HPLC profile of the WSPEs of yogurts: a) 1, 2, 3, 4; and b) 5, 6, 7, 8, compared with reconstituted skim milk (RSM). Yogurt samples denoted 1, 3, 5 and 7 were fermented with the starter culture only and supplemented respectively with oven-dried peel, freeze-dried peel, oven-dried pomace, and freeze-dried pomace. Yogurt samples denoted 2, 4, 6 and 8 were fermented with the starter culture and probiotics supplemented with oven-dried peel, freeze-dried peel, oven-dried pomace, and freeze-dried pomace, respectively. The peptides were eluted at a flow rate of 0.75 ml/min and detected by UV absorption at 215 nm. The flow of solvent A (0.1 % TFA in Milli-Q water) was at a linear gradient from 100 % to 0 % in solvent B (0.1 % TFA in acetonitrile) over 90 mins

effect of sodium azide on *S. typhimurium* and reported in Table 4. The effect of PWPs addition on antimutagenic activity of liberated peptides was not significantly different for both control and probiotic yogurts (Table 5). However, the probiotic yogurts (numbers 2, 4, 6, 8) with PWPs supplementation showed high mutagen inhibitory activity (26.27–32.72 %; Table 4) compared to the control yogurts (numbers 1, 3, 5, 7) with PWPs supplementation (18.60–25.42 %; Table 4) or without the supplementation (15.87 %) (Sah et al. 2014). Moreover, the probiotic yogurt supplemented with freeze-dried peel (yogurt 4) exhibited a significantly higher antimutagenic activity compared to other yogurts. These results were in line with others (Espeche Turbay et al. 2012) and indicated that the probiotic yogurts containing pineapple peel may be a contributor to the dietary prevention of cancer.

Antimutagenic activities and degree of protein hydrolysis were also highly correlated ($P < 0.05$, $r = 0.817$). This result supported the outcome of Matar et al. (1997) who concluded a linkage between proteolytic activity of *Lactobacillus helveticus* L89 and antimutagenicity of the fermented milk. Enhanced proteolysis increases peptide production, which in turn enhances antimutagenic activity of the probiotic yogurt.

Effect of drying techniques employed during preparation of PWPs on overall properties of yogurts

Cluster analysis (CA) was carried out using the hierarchical clustering method with Ward's linkage and identified two clusters based on similarities in the pH, titratable acidity, degree of protein hydrolysis, antioxidant, and antimutagenic activities (Fig. 3a). Control yogurts with PWPs supplementation (numbers 1, 3, 5, 7) arranged in the first cluster and probiotic yogurts with PWPs supplementation (numbers 2, 4, 6, 8) were arranged in the second cluster. The yogurts were clustered according to yogurt types rather than peel and pomace powder supplementation.

Principal component analysis was also carried out and two interpretable components were chosen based on the Kaiser's criterion of eigenvalues greater than 1.0. Score plot (Fig. 3b) displayed the relationships among all yogurts in two main components. Samples were grouped in four distinct groups: the yogurts enriched with pomace powder at the bottom left and right quadrants, while yogurts enriched with peel powder at top left and right. Distributions of yogurts enriched with freeze- dried and oven- dried powders in each quadrants conferred that types of drying techniques had no pronounced effects on probiotic bioactivities.

Conclusions

The pineapple peel and pomace powders were rich in nutrients such as dietary fibre, protein, and divalent cations and

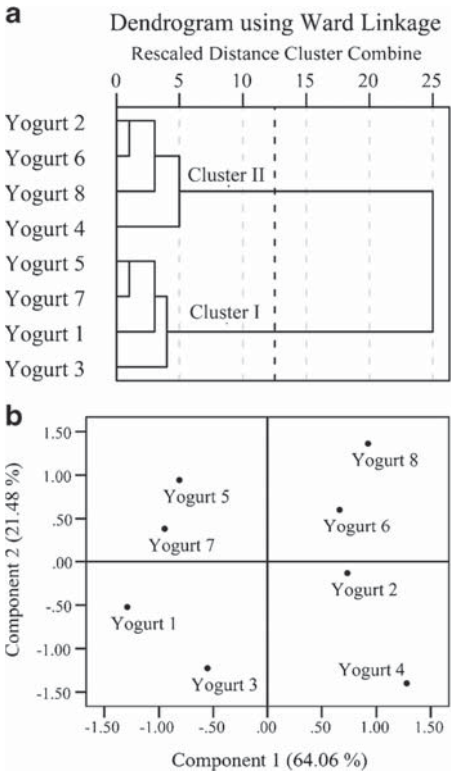

Fig. 3 **a** and **b.** Dendrogram **a** displays the clustering of yogurts according to similarities amongst measured variables. **b** shows score plot of principal components of measured variables in yogurts. The measured variables were pH, titratable acidity, degree of protein hydrolysis, antioxidant (evaluated by measuring scavenging activities of DPPH˙ and ˙OH radicals) and antimutagenic activities

exhibited prebiotic effects on *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* spp. *paracasei* (ATCC® BAA52™). Simultaneously, the proteolytic activities of starter and probiotic cultures were increased substantially in the presence of PWPs. PWPs addition into yogurts also resulted into increased antioxidant and antimutagenic activities compared to the non-supplemented yogurts. Moreover, the WSPE of probiotic yogurt with freeze-dried pineapple peel powder displayed greater antioxidant and antimutagenic activities. Interestingly, both oven and freeze-drying techniques employed in the preparation of powders displayed similar outcomes. Overall, PWPs inclusion in yogurt comprises an interesting approach for enhancement of probiotic growth and improvement of health benefits, and an ecological alternative to reduce fruit-processing waste. However, further works need to be performed to isolate and characterize these bioactive peptides from the WSPEs.

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References

Canadian Legal Legislation Institute (2014). Regulation respecting food, CQLR c P-29, r 1. from http://www.canlii.org/en/qc/laws/regu/cqlr-c-p-29-r-1/latest/cqlr-c-p-29-r-1.html

Chau CF, Huang YL (2004) Characterization of passion fruit seed fibres —a potential fibre source. Food Chem 85(2):189–194

Chitturi S, Talatam VG, Vuppu S (2013) Studies on protein content, protease activity, antioxidants potential, melanin composition, glucosinolate and pectin constitution with brief statistical analysis in some medicinally significant fruit peels. Der Pharm Lett 5(1):13–23

Chowdhury BR, Chakraborty R, Raychaudhuri U (2008) Study on β-galactosidase enzymatic activity of herbal yogurt. Int J Food Sci Nutr 59(2):116–122

Donkor ON, Nilmini SLI, Stolic P, Vasiljevic T, Shah NP (2007a) Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int Dairy J 17(6):657–665

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

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28(3):350–356

Espeche Turbay MB, de Moreno de LeBlanc A, Perdigón G, Savoy de Giori G, Hebert EM (2012) β-casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. J Dairy Sci 95(3):1108–1118

Espírito Santo AP, Cartolano NS, Silva TF, Soares FASM, Gioielli LA, Perego P, Converti A, Oliveira MN (2012) Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. Int J Food Microbiol 154 (3):135–144

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO, London, Ontario, Canada

García-Pérez FJ, Lario Y, Fernández-López J, Sayas E, Pérez-Alvarez JA, Sendra E (2005) Effect of orange fiber addition on yogurt color during fermentation and cold storage. Color Res Appl 30(6):457–463

Gibson GR (1999) Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. J Nutr 129(7 SUPPL.): 1438S–1441S

Gibson GR, Probert HM, Van Loo J, Rastall RA, Roberfroid MB (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17(2):259–275

Gullón B, Gómez B, Martínez-Sabajanes M, Yáñez R, Parajó JC, Alonso JL (2013) Pectic oligosaccharides: manufacture and functional properties. Trends Food Sci Technol 30(2):153–161

Horwitz W, Latimer Jr GW (2006) Official methods of analysis of AOAC international. AOAC International, AOAC International, Gaithersburg, Md

Huang YL, Chow CJ, Fang YJ (2011) Preparation and physicochemical properties of fiber-rich fraction from pineapple peels as a potential ingredient. J Food Drug Anal 19(3):318–323

Igoshi K, Kondo Y, Kobayashi H, Kabata K, Kawakami H (2008) Antioxidative activity of cheese. Milchwissenschaft 63(4):424–427

Kudoh Y, Matsuda S, Igoshi K, Oki T (2001) Antioxidative peptide from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO13953. J Jpn Soc Food Sci 48(1):44–50

Llorente-Bousquets A, Pérez-Munguía S, Farrés A (2008) Novel extracellular proteolytic activity in *Pediococcus acidilactici* ATCC 8042. Can J Microbiol 54(8):694–699

Lourens-Hattingh A, Viljoen BC (2001) Yogurt as probiotic carrier food. Int Dairy J 11(1–2):1–17

Matar C, Nadathur SS, Bakalinsky AT, Goulet J (1997) Antimutagenic effects of milk fermented by *Lactobacillus helveticus* L89 and a protease-deficient derivative. J Dairy Sci 80(9):1965–1970

Mirhosseini H, Amid BT (2013) Effect of different drying techniques on flowability characteristics and chemical properties of natural carbohydrate-protein gum from durian fruit seed. Chem Cent J 7(1)

Moreno-Vilet L, Garcia-Hernandez MH, Delgado-Portales RE, Corral-Fernandez NE, Cortez-Espinosa N, Ruiz-Cabrera MA, Portales-Perez DP (2014) In vitro assessment of agave fructans (*Agave salmiana*) as prebiotics and immune system activators. Int J Biol Macromol 63:181–187

Mota MVT, Ferreira IMPLVO, Oliveira MBP, Rocha C, Teixeira JA, Torres D, Gonçalves MP (2006) Trypsin hydrolysis of whey protein concentrates: characterization using multivariate data analysis. Food Chem 94(2):278–286

Pownall TL, Udenigwe CC, Aluko RE (2010) Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. J Agric Food Chem 58(8):4712–4718

Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53(10):4290–4302

Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2014) Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. Food Chem 156:264–270

Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2015a) Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: a critical review. Compr Rev Food Sci Food Saf 14(2):123–138

Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2015b) Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. J Dairy Sci 98(9):5905–5916

Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2016) Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. LWT Food Sci Technol 65:978–986

Siow HL, Gan CY (2013) Extraction of antioxidative and antihypertensive bioactive peptides from *Parkia speciosa* seeds. Food Chem 141 (4):3435–3442

Sogi DS, Siddiq M, Greiby I, Dolan KD (2013) Total phenolics, antioxidant activity, and functional properties of 'Tommy Atkins' mango peel and kernel as affected by drying methods. Food Chem 141(3): 2649–2655

Thammarutwasik P, Hongpattarakere T, Chantachum S, Kijroongrojana K, Itharat A, Reanmongkol W, Tewtrakul S, Ooraikul B (2009) Prebiotics – a review. Songklanakarin J Sci Technol 31(4):401–408

Tseng A, Zhao Y (2013) Wine grape pomace as antioxidant dietary fibre for enhancing nutritional value and improving storability of yogurt and salad dressing. Food Chem 138(1):356–365

Vasiljevic T, Kealy T, Mishra VK (2007) Effects of β-glucan addition to a probiotic containing yogurt. J Food Sci 72(7):C405–C411

Zheng XQ, Wang JT, Liu XL, Sun Y, Zheng YJ, Wang XJ, Liu Y (2015) Effect of hydrolysis time on the physicochemical and functional properties of corn glutelin by Protamex hydrolysis. Food Chem 172:407–415

Chapter 5: Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel

Chapter 5 reports the effect of pineapple peel powder addition on viability and performance of *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) in regard to the liberation of bioactive peptides with antioxidant and antimutagenic potential in yogurts during 28 days of storage at 4 °C.

The paper entitled "Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*Journal of Dairy Science*" (2015), 98(9): 5905–5916. http://dx.doi.org/10.3168/jds.2015-9450.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** **Date:** 15-Mar-2016

Baidya Nath Prasad Sah [redacted]

Paper Title:

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel

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ABSTRACT

Fruit by-products are good resources of carbohydrates, proteins, vitamins, and minerals, which may function as growth nutrients for probiotic bacteria. This research aimed at evaluating effects of pineapple peel powder addition on the viability and activity of *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus casei* (ATCC 393), and *Lactobacillus paracasei* ssp. *paracasei* (ATCC BAA52) in yogurts throughout storage at 4°C for 28 d. Plain and probiotic yogurts supplemented with or without pineapple peel powder or inulin were prepared. The probiotic counts in supplemented yogurts at 28 d of storage ranged from 7.68 and 8.03 log cfu/g, one log cycle higher compared with nonsupplemented control yogurt. Degree of proteolysis in synbiotic yogurts was significantly higher than plain yogurts and increased substantially during storage. Crude water-soluble peptide extract of the probiotic yogurt with peel possessed stronger antimutagenic and antioxidant activities [evaluated measuring reducing power and scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl; 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid), and hydroxyl radicals] than control and maintained during storage. Pineapple peel, a by-product of juice production, could be proposed as a prebiotic ingredient in the manufacture of yogurts with enhanced nutrition, and functionality.
Key words: pineapple, probiotic, peptide, antioxidative activity, and antimutagenic activity

INTRODUCTION

Demand for development of healthy foods is increasing rapidly due to growing interest of consumers to maintain their health and well-being. Day et al. (2009) defined functional foods as "foods or ingredients of foods that provide additional physiological benefit beyond their basic nutrition." Milk is considered a source of functional ingredients, such as bioactive peptides, which are encrypted in the primary structure of milk proteins and could modulate physiology of consumers only after their proteolytic release (Bhat and Bhat, 2011). Several possible ways exist to obtain these bioactive peptides to functionalize foods. One way could be through direct release of peptides from proteins by action of proteolytic systems of bacteria commonly used in manufacturing of fermented food products (Choi et al., 2012). Therefore, yogurt appears to be a suitable matrix for production of such functional ingredients.

Yogurt is an excellent vehicle to deliver probiotics to consumers; however, to be beneficial for health, the product should contain the suggested minimum number of 10^6 cfu/g at the time of consumption (Shiby and Mishra, 2013; Mani-López et al., 2014). The viability of probiotic organisms is thus considered a key parameter for developing probiotic food products. The major factors for achieving and maintaining this minimal level in yogurt include nutrients, pH, water activity, oxygen tension of the product, storage conditions (e.g., temperature, humidity, and light), the interactions with the starter cultures, as well as strain types (Vasiljevic and Shah, 2008). To minimize their adverse effects, different approaches have been suggested, including microencapsulation of probiotics (Corona-Hernandez et al., 2013), addition of enzymes (Cruz et al., 2013), and prebiotics (Al-Sheraji et al., 2013).

A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al., 2004). Common prebiotics are inulin, fructooligosaccharides, galactooligosaccharides, and other oligosaccharides, such as resistant starch and lactulose (Thammarutwasik et al., 2009). Inulin represents a group of plant polysaccharides having linear fructans

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with β-(2←1) fructosyl-fructose glycosidic linkages and usually prepared by aqueous extraction of chicory roots. However, human digestive enzymes are specific for the hydrolysis of α-glycosidic bonds. Consequently, they are indigestible and only fermented by colonic microflora (Roberfroid, 2007). A high-performance type of inulin is a long-chain inulin with degree of polymerization of 10 to 60, average being 25. In addition to inulin, pineapple peel powder appeared a good source of dietary fiber and has been reported to show prebiotic potential (Diaz-Vela et al., 2013).

Several investigations (Donkor et al., 2007a; Al-Sheraji et al., 2012) have focused on probiotic viability in yogurt containing prebiotic supplements and during refrigerated storage. Whereas prebiotic supplementations may result into several functional benefits for probiotic organisms and ultimately consumers, this approach may influence the bioactivity of yogurt, as bacterial proteolytic enzymes may further hydrolyze milk proteins as well as bioactive peptides during storage (Donkor et al., 2007b). Notably, milk proteins emerge as a prolific source of peptides with anticarcinogenic potentials (Sah et al., 2015). However, studies are lacking regarding the effects of prebiotic addition on antimutagenic and antioxidant activities of the liberated peptides in yogurt during storage. Thus, our study aimed to assess the effect of pineapple peel powder (**PPP**) addition on viability and performance of *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus casei* (ATCC 393), and *Lactobacillus paracasei* ssp. *paracasei* (ATCC BAA52) in regard to the liberation of bioactive peptides with antioxidant and antimutagenic potentials in yogurts during 28 d of refrigerated storage.

MATERIALS AND METHODS

Substrates and Chemicals

Trichloroacetic acid, o-phthaldialdehyde (**OPA**), trifluoroacetic acid, 1,1-diphenyl-2-picrylhydrazyl (**DPPH**), 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (**ABTS**), salicylic acid, vancomycin, clindamycin, sodium azide, and serine were purchased from Sigma Chemical Company (St. Louis, MO), whereas acetonitrile was from Merck (Darmstadt, Germany). Hydrogen peroxide, ferrous sulfate, and potassium ferricyanide were obtained from Ajax Finechem (Seven Hills, NSW, Australia). Bacteriological agar, M17 medium, de Man Rogosa and Sharpe (**MRS**) medium, and peptone were supplied by Oxoid Australia (West Heidelberg, Victoria, Australia), whereas Davis minimal agar was purchased from Becton Dickinson Pty Ltd. (Sydney, NSW, Australia). Skim milk powder was procured from a local store (Woolworths Limited, Melbourne, Australia). Aqueous solutions were prepared in Milli-Q water (18.2 MΩ·cm) obtained from a Millipore water-purification system (Millipore, North Ryde, Australia). Whole pineapples were purchased from a local supermarket (Woolworths Limited).

Propagation of Cultures

Pure cultures of *Streptococcus thermophilus* ASCC 1275 and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 were obtained from the Victoria University Culture Collection (Werribee, Australia). *Lactobacillus acidophilus* ATCC 4356, *L. casei* ATCC 393, and *L. paracasei* spp. *paracasei* ATCC BAA52 were procured from Cell Biosciences Pty Ltd. (Heidelberg, Victoria, Australia). All organisms were stored at −80°C in MRS broth containing 40% (vol/vol) glycerol. The resuscitated strains after 3 successive transfers were employed to prepare starters as described by Sah et al. (2014).

Preparation of PPP

Pineapple peel powder was prepared from the peel of pineapples [*Ananas comosus* (L.) Merrill], as described by do Espírito Santo et al. (2012) with some modifications. Briefly, crushed peel (~2 × 2 cm sizes) was washed by dipping in hot water (90°C) for 30 min to inactivate potential pathogens and proteolytic enzymes (Jutamongkon and Charoenrein, 2010). The peel was then freeze-dried using an Alpha 1–4 LSC Christ freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The dried peel was milled to fine powder, standardized particle size less than 180 μm using wire mesh sieves (Endecotts Ltd., London, UK; Mesh Series BS410/1986) and sterilized with UV irradiation for 30 min (Coman et al., 2013).

Preparation of Yogurts Supplemented with Prebiotics

Set-type plain and probiotic yogurts with inulin or PPP supplementation or without supplementation (control) were prepared as described by Sah et al. (2014) with some modifications. Briefly, 3 batches of milk base were prepared by reconstituting skim milk powder in Milli-Q water at 140 g/L; 2 batches were separately supplemented with 1.0% (wt/vol) of commercial inulin Orafti HP (Beneo-Orafti Ltd., Tienen, Belgium) and PPP. All milk bases were heated for 30 min at 85°C, cooled to 45°C, and then inoculated with 1% (vol/vol) of each *S. thermophilus* and *L. bulgaricus* monocultures aseptically. The mixes were divided into 2 equal portions; one portion was further inoculated with 1% (vol/vol) of each probiotic monocultures. The final

mixes were poured into polystyrene cups and incubated at 42°C until pH of 4.5 ± 0.05 was achieved. Thereafter, the yogurts were immediately cooled to 4°C and stored for 28 d at the same temperature.

Enumeration of Starter and Probiotic Cultures

Cell populations of starter (*S. thermophilus* and *L. bulgaricus*) and probiotic (*L. acidophilus*, *L. casei*, and *L. paracasei*) cultures in freshly inoculated yogurt mixes (0 h) and in yogurts during storage were counted by spread plate technique and expressed as the log of colony-forming units per gram. The selective agar plates and incubation conditions for the cultures were as follows: M17 medium supplemented with lactose and aerobic incubation of 24 h at 45°C for *S. thermophilus*; acidified MRS agar (pH 5.2) and anaerobic incubation of 72 h at 45°C for *L. bulgaricus*. *Lactobacillus acidophilus* and both *L. casei* and *L. paracasei* together were respectively enumerated using MRS-clindamycin agar (pH 6.2; 0.5 mg/L clindamycin), MRS-vancomycin agar (pH 6.2; 1 mg/L vancomycin), and anaerobic incubation of 72 h at 37°C (Sah et al., 2014).

Determination of Titratable Acidity

Titratable acidity (TA), as percent lactic acid, of yogurt samples was determined according to AOAC official method 947.05 (Horwitz and Latimer, 2006) using equation [1]:

$$\%\ \text{TA (wt/wt), as lactic acid} = \frac{V \times N \times 90.08}{W \times 10}, \quad [1]$$

where V = volume of NaOH consumed, mL; N = normality of the NaOH; and W = mass of sample, g.

Determination of Proteolysis

Degree of protein hydrolysis was measured using the OPA method as described by Sah et al. (2014) with some modifications. Briefly, 1 mL of Milli-Q water was added to a 5-g yogurt sample and the final volume adjusted to 10 mL with trichloroacetic acid solution (0.75 *M*). The supernatant collected after centrifugation of the sample at 2,684 × *g* for 30 min at 4°C was filtered using a 0.45-μm Phenex syringe filter (Phenomenex Inc., Lane Cove, Australia). An aliquot of clarified sample (400 μL) was mixed with 3 mL of freshly prepared OPA reagent and left to stand for 2 min. Absorbance of the sample (A_{sample}) was then measured using a Biochrom Libra S12 UV/Vis spectrophotometer (Biochrom Ltd., Cambridge, UK) at 340 nm. In place of sample, Milli-Q water was used for the blank (A_{blank}) and serine solution (0.9516 mEqv/L) for the standard ($A_{standard}$). Degree of hydrolysis (DH) was estimated using equation [2],

$$\text{DH}\ (\%) = \frac{\text{h}}{\text{h}_{\text{tot}}} \times 100, \quad [2]$$

where h_{tot} was total number of peptide bonds per protein equivalent (8.2 mEq/g for casein), and h was number of hydrolyzed bonds, determined by using equation [3]:

$$\text{h} = \frac{(\text{Serine}\,\text{NH}_2 - \beta)}{\alpha}. \quad [3]$$

For casein, $\alpha = 1.039$, $\beta = 0.383$ mEq/g of protein, and Serine NH_2 (mEq/g of protein) was determined using equation [4],

$$\text{Serine NH}_2 = \left[\frac{(\text{A}_{\text{sample}} - \text{A}_{\text{blank}})}{(\text{A}_{\text{standard}} - \text{A}_{\text{blank}})}\right] \times S \times V \times \frac{100}{X} \times P, \quad [4]$$

where S = strength of serine standard, mEq/L; V = final volume make-up of the sample, L; X = weight of yogurt sample, g; and P = protein content of yogurt sample, % (wt/wt).

Preparation and Profiling of Water-Soluble Peptide Extracts

The water-soluble peptide extracts (**WSPE**) from yogurts and heat-treated reconstituted skim milk (pH-adjusted to 4.5 ± 0.05) were prepared according to Sah et al. (2014) with a few modifications. Briefly, samples were centrifuged at 22,680 × *g* using JLA-16.250 rotor in Avanti J-26S XPI High-Performance Centrifuge (Beckman Coulter Inc., Brea, CA) for 30 min at 4°C. The supernatant was filtered using a sintered glass crucible to remove coagulated proteins, debris, and cells. The filtrate was freeze-dried using an Alpha 1–4 LSC Christ freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH) at 0.1 mbar for 24 to 36 h (main drying) and 0.08 mbar for 12 h (final drying) and stored at −80°C until further analysis. The protein content (mg/mL) of the WSPE was estimated according to Bradford (1976) using BSA (0.1–1.4 mg/mL) as standards.

The WSPE were also profiled using a reversed-phase HPLC system as described by Sah et al. (2014). Briefly, the samples were loaded using a 20-μL injection loop to a Varian HPLC system (Varian Inc., Palo Alto, CA) equipped with a C-18 monomeric column (5 μm, 300 Å, 250 × 4.6 mm; Grace Vydac, Hesperia, CA) and detected eluted peptides at 215 nm.

Determination of Antimutagenic Activity by the Ames Test

The antimutagenicity of crude WSPE was assessed in a bacterial reverse mutation assay through the preincubation protocol of Ames test as described by Sah et al. (2014) and conducted using *Salmonella enterica* ssp. *enterica* Typhimurium (ATCC 29629; genotype: *his*, *rfa*, *uvrB-bio*) with aqueous sodium azide (1 μg/plate) as a direct mutagen. Briefly, 0.1 mL of WSPE solution (50 ± 1 μg of peptide) and 0.1 mL of sodium azide solution (1 μg) were mixed with 0.5 mL of sodium phosphate buffer (0.1 *M*; pH 7.4) in a culture tube. An aliquot (0.1 mL) of 14- to 16-h-old culture was inoculated and preincubated for 20 min at 37°C. The mix was poured over a Davis minimal agar plate after mixing with 2 mL of molten top agar at 45°C. Revertant colonies were enumerated after incubating aerobically at 37°C for 48 h. The antimutagenic activity was determined using equation [5]:

$$\% \text{ Inhibition} = \frac{M - S_1}{M - S_0} \times 100, \qquad [5]$$

where S_1 = revertant colonies per plate induced by mutagen in the presence of peptide extract; M = revertant colonies per plate induced by mutagen alone; and S_0 = spontaneous revertant colonies per plate.

Determination of Antioxidant Activity

Assay of Reducing Power. Reducing power of the crude WSPE was evaluated by assessing reduction of $Fe^{3+}(CN^-)^6$ to $Fe^{2+}(CN^-)^6$, as described by Jiang et al. (2014) with some modifications. Briefly, 50 μL of aqueous WSPE (at 0.5 mg of protein/mL) was mixed with 0.5 mL of phosphate buffer (0.2 *M*, pH 6.6) and 0.5 mL of aqueous potassium ferricyanide solution (1%, wt/vol). After incubation at 50°C for 20 min, 0.5 mL of aqueous trichloroacetic acid solution (10%, wt/vol) was added to terminate the reaction. The reaction mixture was centrifuged at 16,000 × *g* for 10 min at room temperature. Finally, 0.5 mL of the supernatant solution was mixed with 0.5 mL of Milli-Q water, then 0.1 mL of aqueous ferric chloride solution (0.1%, wt/vol) was added. After 10 min of standing at room temperature, the absorbance of resulting Prussian blue solution was measured using a Biochrom Libra S12 UV/Vis spectrophotometer (Biochrom Ltd.) at 700 nm. The experimental steps were repeated with Milli-Q water as a blank. The reducing power of the sample was reported as absorbance at 700 nm after subtracting the absorbance value of the blank. A higher absorbance value indicated greater reducing power.

Assay of DPPH Radical Scavenging Activity. The radical scavenging activity (RSA) of WSPE was measured against DPPH radical as described by Siow and Gan (2013) with some modifications. Briefly, 20 μL of aqueous WSPE (at 0.5 mg of protein/mL) was mixed with 1.0 mL of DPPH reagent (50 μ*M* in ethanol). The mix was vortexed and left to stand for 30 min in dark. Then, the mix was clarified by centrifuging at 16,000 × *g* (5415C microcentrifuge, Eppendorf, Hamburg, Germany) for 5 min at room temperature and subjected for absorbance measurement at 517 nm. For the blank, Milli-Q water was used instead of sample. Antioxidant activity of WSPE was calculated using equation [6]:

$$\text{RSA, } \% = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100. \qquad [6]$$

Assay of ABTS Radical Scavenging Activity. The $ABTS^{\bullet+}$ scavenging activity of WSPE was assayed according to the method described by Ozgen et al. (2006) with some modifications. The working solution of $ABTS^{\bullet+}$ was prepared by mixing stock solutions of 7.4 m*M* ABTS (molecular = 548.68) in sodium acetate buffer (20 m*M*, pH 4.5) and 2.6 m*M* potassium persulphate (molecular = 270.32) aqueous solution in equal quantities and allowing them to react for 12 to 16 h at room temperature in the dark. One milliliter of $ABTS^{\bullet+}$ solution was then diluted by mixing with 50 to 60 mL of sodium acetate buffer (20 m*M*, pH 4.5) to obtain an absorbance of 0.70 ± 0.02 at 734 nm after equilibration at 30°C. The $ABTS^{\bullet+}$ reagent was prepared daily.

Exactly 10 μL of aqueous WSPE (at 0.5 mg of protein/mL) was added to 1 mL of the $ABTS^{\bullet+}$ reagent and incubated at 30°C for 6 min after vortexing. The absorbance of the mix was measured at 734 nm. Similarly, 10 μL of Milli-Q water was used instead of the sample for the blank. Radical scavenging activity was calculated using equation [6].

Assay of Hydroxyl Radical Scavenging Activity. The scavenging capacity of WSPE for hydroxyl radical was assayed according to the method described by Zheng et al. (2015) with some modifications. Briefly, 500 μL of aqueous ferrous sulfate (2 m*M*) and 100 μL of aqueous hydrogen peroxide (2 m*M*) were mixed to 20 μL of sample (0.1 mg of protein/mL). The reaction mixture was left for 10 min and then 500 μL of aqueous salicylic acid (2.5 m*M*) was added. The mixture was incubated for 30 min at 37°C and subjected for absorbance measurement at 510 nm. Milli-Q water was used for the blank (in place of salicylic acid) and the

control (in place of sample) in the reaction. Hydroxyl radical scavenging activity (HRSA) of the WSPE was calculated using equation [7]

$$\text{HRSA}\ (\%) = \left[1 - \frac{A_s - A_b}{A_c}\right] \times 100, \qquad [7]$$

where A_s, A_b, and A_c were absorbance for sample, blank, and control, respectively.

Statistical Analyses

Experiments were conducted as a randomized split-plot blocked design in time with types of yogurt as the main plot, prebiotic addition and time as subplots. The design was replicated independently on 3 different occasions with subsequent subsampling giving at least 6 observations (n $\geq$6). Results were analyzed using GLM procedure of the SAS System (SAS, 1996) to explore the effects of probiotic and potential prebiotic, PPP, and inulin addition on properties of different yogurt types over time at 3 levels (d 1, 14, and 28) of storage. Significance level was considered at $P < 0.05$. Chemometric methods such as hierarchical cluster analysis (**HCA**) and principal component analysis (**PCA**) were employed to discriminate yogurts with different culture and prebiotic combinations (Matera et al., 2014). The HCA categorized different yogurts into clusters based on their similarities by applying the squared Euclidean distance and Ward linkage methods to the standardized data set (z-scores). The eigenvalues were extracted from the correlation matrix; varimax rotation was used in the PCA to produce orthogonal transformations to the reduced factors to identify the high and low correlations. Pearson correlation, HCA, and PCA were performed on d 1 results using SPSS 22.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Viability of Starter and Probiotic Cultures in Yogurt During Refrigerated Storage

Prebiotics are usually supplemented to fermented milk and milk products for stimulation of the growth of probiotic strains, which are a part of the human colonic microbiota. Because of widened attention on lactobacilli, the effect of addition of PPP as a potential prebiotic on the viable count of probiotic (*L. acidophilus*, *L. casei*, and *L. paracasei*) and starter (*S. thermophilus*, *L. bulgaricus*) cultures in yogurt was investigated during 28 d of refrigerated storage and reported in Tables 1 and 2.

Supplementation of milk with selected probiotic organisms and PPP resulted in a substantially faster lowering of pH (data not shown), suggesting a higher rate of acid production compared with control yogurt. The viability of *S. thermophilus* and *L. bulgaricus* reduced significantly during the 28-d storage period by half of a log cycle in all types of yogurt (Table 1). Table 2 shows the viable count of probiotic organisms before fermentation and during refrigerated storage for yogurt containing PPP, yogurt with inulin, and control yogurt. Overall, the presence of PPP in yogurts effectively improved probiotic growth, comparable to that of inulin and one log cycle higher than the nonsupplemented control sample. Stability of the probiotic organisms in

Table 1. Viability of starter culture in yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C; 0 h counts represent count of starter culture in yogurt mix taken immediately after culture addition[1]

Yogurt type[2]		Viable count (log cfu/g)							
		Streptococcus thermophilus				*Lactobacillus bulgaricus*			
Culture	Prebiotic	0 h	d 1	d 14	d 28	0 h	d 1	d 14	d 28
SC	None	$7.41^{a,D}$	$9.18^{c,A}$	$8.98^{d,B}$	$8.58^{d,C}$	$7.33^{a,D}$	$8.13^{c,A}$	$8.04^{d,B}$	$7.51^{f,C}$
SC	Inulin	$7.40^{a,D}$	$9.23^{bc,A}$	$9.03^{cd,B}$	$8.62^{cd,C}$	$7.31^{a,D}$	$8.27^{a,A}$	$8.20^{a,B}$	$7.90^{a,C}$
SC	PPP	$7.41^{a,D}$	$9.27^{ab,A}$	$9.10^{ab,B}$	$8.72^{b,C}$	$7.33^{a,D}$	$8.19^{b,A}$	$8.13^{bc,B}$	$7.70^{d,C}$
SC + PC	None	$7.40^{a,D}$	$9.22^{bc,A}$	$9.05^{bc,B}$	$8.66^{c,C}$	$7.33^{a,D}$	$8.21^{b,A}$	$8.09^{c,B}$	$7.63^{e,C}$
SC + PC	Inulin	$7.40^{a,D}$	$9.27^{ab,A}$	$9.11^{a,B}$	$8.82^{a,C}$	$7.33^{a,D}$	$8.28^{a,A}$	$8.19^{ab,B}$	$7.84^{b,C}$
SC + PC	PPP	$7.41^{a,D}$	$9.31^{a,A}$	$9.15^{a,B}$	$8.86^{a,C}$	$7.33^{a,D}$	$8.25^{ab,A}$	$8.11^{c,B}$	$7.77^{c,C}$
SEM[3]		0.02				0.02			

[a–f]Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($P < 0.05$).

[A–D]Different uppercase superscripts in the same row depict the significant difference between means for yogurts with the same culture and prebiotic combination at d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*S. thermophilus* + *L. bulgaricus*); PC = probiotic culture (*Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*).

[3]Pooled standard error of the mean for predetermined $P < 0.05$.

Table 2. Viability of probiotic cultures in yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C; 0 h counts represent probiotic count in yogurt mix taken immediately after culture addition[1]

Yogurt type[2]		Viable count (log cfu/g)							
		Lactobacillus acidophilus				*Lactobacillus casei* and *Lactobacillus paracasei*			
Culture	Prebiotics	0 h	d 1	d 14	d 28	0 h	d 1	d 14	d 28
SC + PC	None	$7.35^{a,D}$	$7.66^{b,C}$	$7.07^{b,A}$	$6.72^{c,B}$	$7.52^{a,B}$	$7.94^{b,A}$	$7.05^{b,C}$	$6.74^{b,D}$
SC + PC	Inulin	$7.35^{a,D}$	$8.45^{a,A}$	$8.25^{a,B}$	$7.86^{b,C}$	$7.54^{a,D}$	$8.68^{a,A}$	$8.25^{a,B}$	$7.83^{a,C}$
SC + PC	PPP	$7.35^{a,D}$	$8.54^{a,A}$	$8.28^{a,B}$	$8.03^{a,C}$	$7.54^{a,C}$	$8.70^{a,A}$	$8.30^{a,B}$	$7.68^{a,C}$
SEM[3]			0.04				0.06		

[a–c]Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($P < 0.05$).

[A–D]Different uppercase superscripts in the same row depict the significant difference between means for yogurts with the same culture and prebiotic combination at d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*Streptococcus thermophilus* + *Lactobacillus bulgaricus*); PC = probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*).

[3]Pooled standard error of the mean for predetermined $P < 0.05$.

PPP enriched medium might be due to buffering capacity of the prebiotics, which would aid in maintaining the viability of live bacterial cells (Kailasapathy et al., 2008). It has been reported that PPP may serve as a source of growth factors for improved growth of probiotic bacteria in yogurt (Diaz-Vela et al., 2013). Iyer and Kailasapathy (2005) also reported enhanced survival of probiotic organisms under in vitro acidic and bile salt conditions when the organisms were coencapsulated with prebiotics such as Hi-maize (Starch Australasia Ltd., Lane Cove, Australia). Thus, addition of PPP resulted in improved survival and metabolic activity during storage at 4°C. Conversely, factors such as postfermentation acidification and dissolved oxygen may adversely influence the viability of probiotic strains in fermented milk (Vasiljevic and Shah, 2008). The reported loss of probiotic viability in yogurts during storage is likely due to acid injury, as titratable acidity increases significantly during storage (Table 3; Donkor et al., 2006). Nonetheless, counts of the probiotic organisms during 28 d of cold storage were sufficient to exhibit probiotic effect on the consumer as the reported minimum therapeutic count of 10^6 cfu/g (Lourens-Hattingh and Viljoen, 2001) is below that obtained in the current study. These observations point out an in vitro symbiotic effect of PPP on selected probiotic organisms similar to previous reports (Sendra et al., 2008; do Espírito Santo et al., 2012), where fibers from various fruit processing by-products stimulated the growth of probiotic strains.

Titratable Acidity of Yogurt During Refrigerated Storage

In all yogurt samples, the titratable acidity increased significantly ($P < 0.05$) during 28 d of storage (Table 3), suggesting continued production of organic acids during storage. Supplementation with PPP or inulin increased the acidifying ability of starter and probiotic cultures during refrigerated storage. Similar acidification in yogurt-type products was observed during refrigerated storage in various studies (Gilliland et al., 2002; Donkor et al., 2006).

Degree of Proteolysis in Yogurt During Refrigerated Storage

Lactic acid bacteria produce proteolytic enzymes during yogurt manufacturing which cleave peptide bonds of milk proteins leading to generation of peptides and free AA (Donkor et al., 2007c). The extent of protein hydrolysis in yogurt samples during storage was estimated by determining free amino groups and results reported as degree of hydrolysis in Table 3. In all samples, the degree of proteolysis increased significantly ($P < 0.05$) over a storage period of 28 d, suggesting continuation of proteolytic activities during storage. Additionally, a significant ($P < 0.05$) increase in proteolytic activity was observed in probiotic yogurt compared with plain yogurt. Furthermore, proteolytic activities increased significantly ($P < 0.05$) in PPP- or inulin-supplemented yogurt compared with the nonsupplemented control yogurt. Interactions among yogurt types, prebiotic (inulin or PPP) additions, and storage time (Table 4) indicated that the prebiotics increased the generation of new peptides in probiotic yogurts compared with plain yogurts during storage. Pineapple powders contain dietary fibers, proteins, and minerals, including divalent cations, and serve as growth factors or growth promoters for probiotics in the yogurts (Diaz-Vela et al., 2013). For better growth, bacterial cells require free AA for protein synthesis; therefore, bacterial extracellular

Table 3. Titratable acidity, degree of protein hydrolysis, and antimutagenic activity of plain and probiotic yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C[1]

Yogurt type[2]		Titratable acidity (as % lactic acid)			Degree of protein hydrolysis (%)			Antimutagenic activity (% inhibition)		
Culture	Prebiotic	d 1	d 14	d 28	d 1	d 14	d 28	d 1	d 14	d 28
SC	None	$1.01^{a,C}$	$1.05^{e,B}$	$1.09^{e,A}$	$6.15^{e,B}$	$6.49^{e,A}$	$6.65^{e,A}$	$21.28^{f,C}$	$22.74^{d,B}$	$23.60^{e,A}$
SC	Inulin	$1.02^{a,C}$	$1.06^{d,B}$	$1.11^{d,A}$	$6.90^{d,C}$	$7.38^{d,B}$	$7.69^{d,A}$	$24.74^{d,B}$	$25.03^{c,B}$	$27.11^{c,A}$
SC	PPP	$1.02^{a,C}$	$1.08^{c,B}$	$1.12^{d,A}$	$7.06^{d,B}$	$7.35^{d,A}$	$7.52^{d,A}$	$22.47^{e,C}$	$24.79^{c,B}$	$25.54^{d,A}$
SC + PC	None	$1.02^{a,C}$	$1.10^{b,B}$	$1.14^{c,A}$	$11.29^{c,B}$	$11.24^{c,B}$	$12.60^{c,A}$	$31.74^{c,C}$	$33.31^{b,B}$	$34.25^{b,A}$
SC + PC	Inulin	$1.01^{a,C}$	$1.12^{a,B}$	$1.15^{b,A}$	$12.42^{b,C}$	$12.79^{b,B}$	$13.64^{b,A}$	$37.76^{a,B}$	$37.68^{a,B}$	$38.77^{a,A}$
SC + PC	PPP	$1.02^{a,C}$	$1.12^{a,B}$	$1.17^{a,A}$	$13.26^{a,C}$	$13.67^{a,B}$	$13.92^{a,A}$	$36.19^{b,C}$	$37.65^{a,B}$	$39.04^{a,A}$
SEM[3]			0.005			0.07			0.26	

[a–e]Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($P < 0.05$).

[A–C]Different uppercase superscripts in the same row depict the significant difference between means for yogurts with the same culture and prebiotic combination at d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*Streptococcus thermophilus* + *Lactobacillus bulgaricus*); PC = probiotic culture (*Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*).

[3]Pooled standard error of the mean for predetermined $P < 0.05$.

proteinases hydrolyze milk proteins into oligopeptides and further hydrolysis by intracellular peptidases of peptides into AA (Donkor et al., 2007c). The mineral-rich PPP likely stimulated bacterial enzymes, resulting in higher proteolytic activities.

The generation of peptides from milk proteins during yogurt manufacturing was confirmed by profiling WSPE from yogurts using a reversed-phase HPLC, as presented in Figure 1. Probiotic yogurts supplemented with inulin or PPP (yogurts 5 and 6) exhibited more peaks (peptides) compared with nonsupplemented plain yogurt (yogurt 1) and reconstituted skim milk. The peptide profiles obtained are in line with previous study (Miclo et al., 2012), who reported casein break down by cell envelope proteinases of *S. thermophilus*. Several peptides may be generated during storage and may display different biological activities. A study by van Boekel et al. (1993), found that the generated peptides from pepsin hydrolysis of casein were more potent in antimutagenic activity than intact casein.

Antimutagenic Activity of Yogurt During Refrigerated Storage

Mutagen inhibitory activity of WSPE was evaluated using Ames test by assessing inhibitory activity of peptides against sodium azide on *S. typhimurium* and presented in Table 3. In all yogurt samples, the antimutagenic activities of the WSPE at the end of storage increased significantly ($P < 0.05$) compared with the first day of storage, indicating increased generation of these peptides during storage. In addition, enhanced antimutagenic activity was also observed in the probiotic yogurts compared with control. The an-

Table 4. Analysis of variance depicting the significance ($P < 0.05$) of types of yogurt (plain and probiotic yogurts), prebiotics (inulin and pineapple peel powder), storage time at 3 levels (d 1, 14, and 28) and their effects on degree of protein hydrolysis, antimutagenic and antioxidant activities

	P-value					
			Antioxidant activities			
				Radical scavenging activity[1]		
Source of variation	Degree of protein hydrolysis	Mutagen inhibitory activity	Reducing power	DPPH$^{\bullet}$	ABTS$^{\bullet+}$	$^{\bullet}$OH
Yogurt type	**	**	**	**	**	**
Prebiotic	**	**	**	**	**	**
Time	**	**	**	**	**	**
Yogurt type × Prebiotic	**	**	**	**	**	**
Yogurt type × Time	**	NS	**	**	**	**
Prebiotic × Time	**	**	**	**	**	**
Yogurt type × Prebiotic × Time	**	NS	**	**	**	**

[1]DPPH = 1,1-diphenyl-2-picrylhydrazyl; ABTS = 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid); OH = hydroxyl.

**$P < 0.05$, NS = nonsignificant at $P > 0.05$.

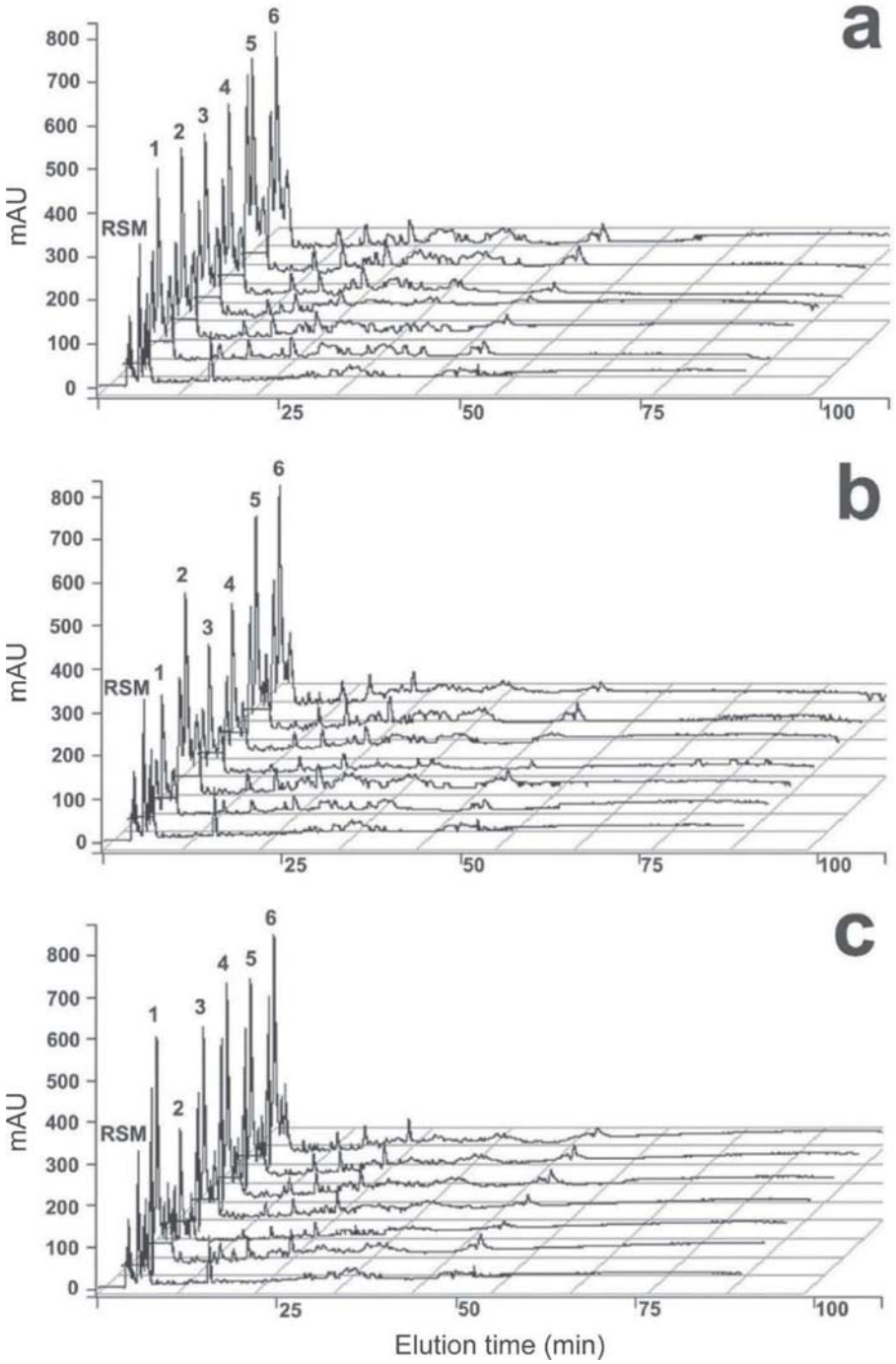

Figure 1. Reversed-phase HPLC profile of crude water-soluble peptide extract of yogurt types 1, 2, 3, 4, 5, and 6 during 28 d of refrigerated storage at (a) d 1, (b) 14, and (c) 28 compared with reconstituted skim milk (RSM). Yogurts denoted 1, 2, and 3 were plain yogurts with or without prebiotic supplementation; yogurt 1 was without supplementation, whereas yogurts 2 and 3 were supplemented with inulin and pineapple peel powder (PPP), respectively. Yogurts denoted 4, 5, and 6 were probiotic yogurts without prebiotic, with inulin, and PPP supplementation, respectively. The peptides were eluted using a linear gradient from 100 to 0% solvent A (0.1% trifluoroacetic acid in Milli-Q water from a Millipore water-purification system; Millipore, North Ryde, Australia) in solvent B (0.1% trifluoroacetic acid in acetonitrile) over 90 min at a flow rate of 0.75 mL/min and detected by UV absorption at 215 nm.

timutagenic activity in yogurt supplemented with PPP was similar to that of inulin-supplemented yogurts and both showed significantly higher activity compared with nonsupplemented control yogurts. The insignificant interactions among yogurt types, prebiotic (inulin or PPP) addition, and storage time (Table 4) indicated that increment pattern of antimutagenic activities for both plain and probiotic yogurts during storage was similar because of prebiotic supplementation. Pearson correlation suggested a direct relationship between degree of proteolysis and antimutagenic activity ($P < 0.01$, r = 0.96); Bakalinsky et al. (1996) also reported of similar results.

In Vitro Antioxidant Activity of Yogurt During Refrigerated Storage

An antioxidant compound can protect the human body by scavenging free radicals, such as reactive oxygen species, and increase shelf life of foodstuffs by retarding the process of lipid peroxidation through hydrogen atom or electron transfer. For instance, donation of hydrogen ions involves interrupting free radical chain reactions, which is a basis for the assessment of reducing power. Additionally, the reaction mechanisms involving mainly a hydrogen atom transfer is basis for DPPH-based methods and both hydrogen atom transfer and single electron transfer for ABTS-based methods (Gülçin, 2012). Due to the involvement of a cascade of reaction steps in an oxidation process, protein hydrolyzates can exhibit antioxidant activities through multiple reaction mechanisms (Chen et al., 2003). Thus, several different assays must be performed to provide a comprehensive information about total antioxidant capacity of the compound tested. This justifies the current study of evaluation for the antioxidant potential of WSPE by conducting 4 different assays. Reducing power and scavenging capacities for DPPH, ABTS, and hydroxyl radicals of WSPE were measured during 28 d of refrigerated storage and presented in Table 5. All yogurt samples exhibited varying degrees of reducing power, scavenging capacities for DPPH, ABTS, and hydroxyl radicals, indicating differences in generated WSPE of the yogurts. The activities increased significantly ($P < 0.05$) during storage compared with the first day. Furthermore, these activities were stronger in PPP- or inulin-supplemented yogurt compared with the nonsupplemented control yogurt. This implied that that the generated peptides in WSPE acted as potent antioxidant compounds. The interactions among yogurt types, prebiotic (inulin or PPP) additions, and storage time (Table 4) showed that prebiotics influenced differently the antioxidant capacities of WSPE in plain and probiotic yogurts during storage.

A positive correlation between degree of proteolysis with reducing power ($P < 0.01$, r = 0.91), scavenging capacities of DPPH ($P < 0.01$, r = 0.76), ABTS ($P < 0.01$, r = 0.89), and hydroxyl ($P < 0.01$, r = 0.98) radicals were observed. Several potent antioxidant peptides liberated from milk proteins have been reported, such as Tyr-Val-Pro-Glu-Pro-Phe, Phe-Pro-Tyr-Cys-Ala-Pro, Phe-Gly-Gly-Met-Ala-His, Val-Tyr-Pro-Phe, and Tyr-Pro-Pro-Tyr-Glu-Thr-Tyr from casein hydrolyzates of goat milk using a combination of alkaline and neutral proteases (Li et al., 2013); Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile from β-lactoglobulin hydrolyzates using corolase PPP (Hernández-Ledesma et al., 2005); and Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met from fermented milk by using *Lactobacillus delbrueckii* ssp. *bulgaricus* (Kudoh et al., 2001). Furthermore, low-molecular weight casein hydrolyzates displayed strong radical scavenging activities (Kim et al., 2007). The antioxidative capacity of a peptide depends on various attributes including sequence, composition of AA, configuration, and concentration of the peptide (Phanturat et al., 2010). Again, the type of enzymes involved in the hydrolysis of protein is also a determining factor (Foh et al., 2010). In addition, Rajapakse et al. (2005) reported that hydrolyzates containing Pro, His, Met, Tyr, Val, Lys, Cys, and Gln displayed strong radical scavenging capacity.

Effect of PPP and Probiotic Addition on Overall Characteristics of Yogurts

Cluster analysis was performed using hierarchical clustering method with Ward's linkage and revealed 2 clusters based on similarities in titratable acidity, degree of protein hydrolysis, and antimutagenic and antioxidant activities (evaluated measuring reducing power and scavenging capacity for $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $^{\bullet}OH$ radicals) during the first day of storage at 4°C (Figure 2A). Plain and probiotic yogurts were arranged in 2 separate clusters. Inclusion of probiotic cultures resulted in pronounced effects on the measured variables compared with the supplementation of inulin or PPP.

Principal component analysis was also conducted and 2 components were identified based on Kaiser's criterion of eigenvalues greater than 1.0 (Figure 2B). The first component explains higher variance (76.95%) than the second component (17.60%). Samples are distributed in all 4 quadrants of the score plot. Yogurts prepared with starter culture only are at the left quadrants, whereas yogurts prepared with both starter and probiotic cultures are at the right quadrants. Yogurt 6, designated in a separate quadrant, shows higher antioxidant and antimutagenic activities, indicating differences in properties compared with the other studied yogurts.

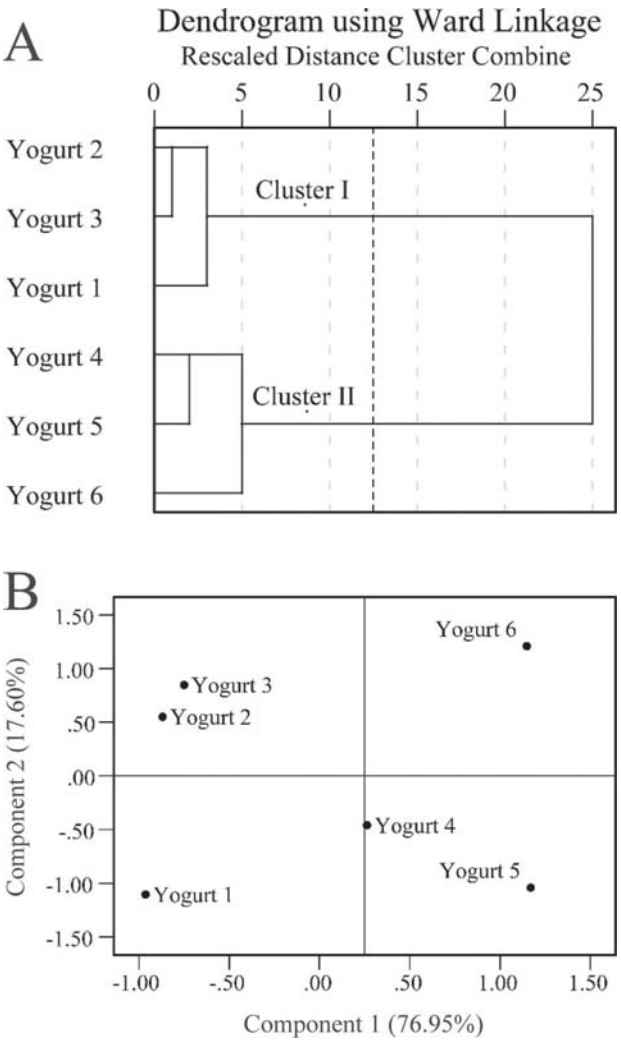

Figure 2. Dendrogram (A) exhibits the clustering of yogurts according to similarities among measured variables. Score plot (B) is of principal components of measured variables in yogurts stored at 4°C in d 1. The measured variables were titratable acidity, degree of protein hydrolysis, antimutagenic and antioxidant activities. Yogurts denoted 1, 2, and 3 were fermented using starter culture only, whereas yogurts denoted 4, 5, and 6 were fermented using both starter and probiotic cultures. Yogurts 2 and 5 were supplemented with inulin, yogurts 3 and 6 were supplemented with pineapple peel powder, and yogurts 1 and 4 were nonsupplemented control yogurts.

CONCLUSIONS

Improved growth and retention of viability of *L. acidophilus* (ATCC 4356), *L. casei* (ATCC 393), and *L. paracasei* spp. *paracasei* (ATCC BAA52) was observed during refrigerated storage at 4°C for 28 d in synbiotic yogurt formulations with added PPP. The proteolytic activities of cultures were enhanced substantially in

Table 5. Antioxidant activities (evaluated by assessing reducing power and scavenging of DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and $^{\bullet}$OH radicals) of plain and probiotic yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C[1]

Yogurt[2]		Reducing power (A_{700})			Radical scavenging activity[3] (%)							
					DPPH$^{\bullet}$			ABTS$^{\bullet+}$			$^{\bullet}$OH	
Culture	Prebiotic	d 1	d 14	d 28	d 1	d 14	d 28	d 1	d 14	d 28	d 1	d 14	d 28
SC	None	$0.36^{d,C}$	$0.47^{b,B}$	$0.48^{c,A}$	$34.35^{d,B}$	$33.61^{e,B}$	$44.25^{e,A}$	$35.92^{d,C}$	$38.31^{f,B}$	$46.76^{f,A}$	$61.12^{e,C}$	$63.38^{d,B}$	$66.79^{e,A}$
SC	Inulin	$0.36^{d,C}$	$0.45^{c,B}$	$0.53^{b,A}$	$36.80^{c,B}$	$37.83^{d,B}$	$46.14^{d,A}$	$38.13^{c,C}$	$43.83^{e,B}$	$53.41^{e,A}$	$61.70^{de,C}$	$66.56^{c,B}$	$70.98^{d,A}$
SC	PPP	$0.39^{c,B}$	$0.40^{d,B}$	$0.58^{c,A}$	$34.91^{d,C}$	$39.69^{c,B}$	$50.54^{c,A}$	$45.93^{b,B}$	$46.12^{d,B}$	$54.67^{d,A}$	$62.15^{d,C}$	$67.41^{c,B}$	$72.13^{c,A}$
SC + PC	None	$0.40^{b,C}$	$0.47^{b,B}$	$0.59^{bc,A}$	$42.18^{b,B}$	$51.88^{c,A}$	$46.06^{b,C}$	$50.06^{c,B}$	$59.12^{c,A}$	$68.08^{c,C}$	$69.08^{b,B}$	$75.54^{b,A}$
SC + PC	Inulin	$0.48^{a,C}$	$0.51^{a,B}$	$0.59^{b,A}$	$38.98^{b,C}$	$40.61^{c,B}$	$55.98^{b,A}$	$52.94^{a,C}$	$51.92^{b,B}$	$61.91^{b,A}$	$70.70^{b,C}$	$74.07^{a,B}$	$78.83^{a,A}$
SC + PC	PPP	$0.47^{a,C}$	$0.50^{a,B}$	$0.64^{a,A}$	$43.90^{a,B}$	$44.35^{a,B}$	$58.03^{a,A}$	$53.06^{a,C}$	$55.44^{a,B}$	$62.79^{a,A}$	$73.13^{a,C}$	$74.45^{a,B}$	$79.65^{a,A}$
SEM[4]		0.005			0.55			0.29			0.33		

[a–f]Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($P < 0.05$).

[A–C]Different uppercase superscripts in the same row depict the significant difference between means for yogurts with the same culture and prebiotic combination at d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*Streptococcus thermophilus* + *Lactobacillus bulgaricus*); PC = probiotic culture (*Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*).

[3]DDPH = 1,1-diphenyl-2-picrylhydrazyl; ABTS = 2,2′-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid); OH = hydroxyl.

[4]Pooled standard error of the mean for predetermined $P < 0.05$.

the presence of prebiotic during storage. All WSPE prepared from yogurt samples possessed antimutagenic activity, which increased during storage. In addition, the WSPE exhibited excellent antioxidant properties measured through in vitro assays employing $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $^{\bullet}OH$ free radicals. Interestingly, WSPE of the probiotic yogurt with PPP exhibited the most potent antimutagenic and antioxidant activities. In addition, incorporation of PPP and probiotics in food products would provide new opportunities in the design and preparation of novel functional foods. Establishing the stability of bioactive peptides during refrigerated storage would add in expanding the functional food market as well. However, these findings demand further investigations to identify and purify these bioactive peptides from WSPE and to understand the possible health benefits, as well as their effect on sensory attributes of food products enriched with protein hydrolyzates.

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REFERENCES

Al-Sheraji, S. H., A. Ismail, M. Y. Manap, S. Mustafa, and R. M. Yusof. 2012. Viability and activity of bifidobacteria during refrigerated storage of yoghurt containing *Mangifera pajang* fibrous polysaccharides. J. Food Sci. 77:M624–M630. http://dx.doi.org/10.1111/j.1750-3841.2012.02955.x.

Al-Sheraji, S. H., A. Ismail, M. Y. Manap, S. Mustafa, R. M. Yusof, and F. A. Hassan. 2013. Prebiotics as functional foods: A review. J. Funct. Foods 5:1542–1553. http://dx.doi.org/10.1016/j.jff.2013.08.009.

Bakalinsky, A. T., S. R. Nadathur, J. R. Carney, and S. J. Gould. 1996. Antimutagenicity of yogurt. Mutat. Res. 350:199–200. http://dx.doi.org/10.1016/0027-5107(95)00113-1.

Bhat, Z. F., and H. Bhat. 2011. Milk and dairy products as functional foods: A review. Int. J. Dairy Sci. 6:1–12. http://dx.doi.org/10.3923/ijds.2011.1.12.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248–254.

Chen, J., H. Lindmark-Månsson, L. Gorton, and B. Åkesson. 2003. Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods. Int. Dairy J. 13:927–935. http://dx.doi.org/10.1016/S0958-6946(03)00139-0.

Choi, J., L. Sabikhi, A. Hassan, and S. Anand. 2012. Bioactive peptides in dairy products. Int. J. Dairy Technol. 65:1–12. http://dx.doi.org/10.1111/j.1471-0307.2011.00725.x.

Coman, M. M., M. C. Verdenelli, C. Cecchini, S. Silvi, A. Vasile, G. E. Bahrim, C. Orpianesi, and A. Cresci. 2013. Effect of buckwheat flour and oat bran on growth and cell viability of the probiotic strains *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and their combination SYNBIO®, in synbiotic fermented milk. Int. J. Food Microbiol. 167:261–268. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.09.015.

Corona-Hernandez, R. I., E. Álvarez-Parrilla, J. Lizardi-Mendoza, A. R. Islas-Rubio, L. A. de la Rosa, and A. Wall-Medrano. 2013. Structural stability and viability of microencapsulated probiotic bacteria: A review. Compr. Rev. Food Sci. Food Saf. 12:614–628. http://dx.doi.org/10.1111/1541-4337.12030.

Cruz, A. G., W. F. Castro, J. A. F. Faria, H. M. A. Bolini, R. M. S. Celeghini, R. S. L. Raices, C. A. F. Oliveira, M. Q. Freitas, C. A. Conte Júnior, and E. T. Mársico. 2013. Stability of probiotic yogurt added with glucose oxidase in plastic materials with different permeability oxygen rates during the refrigerated storage. Food Res. Int. 51:723–728. http://dx.doi.org/10.1016/j.foodres.2013.01.028.

Day, L., R. B. Seymour, K. F. Pitts, I. Konczak, and L. Lundin. 2009. Incorporation of functional ingredients into foods. Trends Food Sci. Technol. 20:388–395. http://dx.doi.org/10.1016/j.tifs.2008.05.002.

Diaz-Vela, J., A. Totosaus, A. E. Cruz-Guerrero, and M. De Lourdes Pérez-Chabela. 2013. In vitro evaluation of the fermentation of added-value agroindustrial by-products: Cactus pear (*Opuntia ficus-indica* L.) peel and pineapple (*Ananas comosus*) peel as functional ingredients. Int. J. Food Sci. Technol. 48:1460–1467. http://dx.doi.org/10.1111/ijfs.12113.

do Espírito Santo, A. P., N. S. Cartolano, T. F. Silva, F. A. S. M. Soares, L. A. Gioielli, P. Perego, A. Converti, and M. N. Oliveira. 2012. Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. Int. J. Food Microbiol. 154:135–144. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.025.

Donkor, O. N., A. Henriksson, T. K. Singh, T. Vasiljevic, and N. P. Shah. 2007b. ACE-inhibitory activity of probiotic yoghurt. Int. Dairy J. 17:1321–1331. http://dx.doi.org/10.1016/j.idairyj.2007.02.009.

Donkor, O. N., A. Henriksson, T. Vasiljevic, and N. P. Shah. 2006. Effect of acidification on the activity of probiotics in yoghurt during cold storage. Int. Dairy J. 16:1181–1189. http://dx.doi.org/10.1016/j.idairyj.2005.10.008.

Donkor, O. N., A. Henriksson, T. Vasiljevic, and N. P. Shah. 2007c. Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. Lait 87:21–38. http://dx.doi.org/10.1051/lait:2006023.

Donkor, O. N., S. L. I. Nilmini, P. Stolic, T. Vasiljevic, and N. P. Shah. 2007a. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int. Dairy J. 17:657–665. http://dx.doi.org/10.1016/j.idairyj.2006.08.006.

Foh, M. B. K., I. Amadou, B. M. Foh, M. T. Kamara, and W. Xia. 2010. Functionality and antioxidant properties of tilapia (*Oreochromis niloticus*) as influenced by the degree of hydrolysis. Int. J. Mol. Sci. 11:1851–1869. http://dx.doi.org/10.3390/ijms11041851.

Gibson, G. R., H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutr. Res. Rev. 17:259–275. http://dx.doi.org/10.1079/NRR200479.

Gilliland, S. E., S. S. Reilly, G. B. Kim, and H. S. Kim. 2002. Viability during storage of selected probiotic lactobacilli and bifidobacteria in a yogurt-like product. J. Food Sci. 67:3091–3095. http://dx.doi.org/10.1111/j.1365-2621.2002.tb08864.x.

Gülçin, I. 2012. Antioxidant activity of food constituents: An overview. Arch. Toxicol. 86:345–391. http://dx.doi.org/10.1007/s00204-011-0774-2.

Hernández-Ledesma, B., A. Dávalos, B. Bartolomé, and L. Amigo. 2005. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulln. Identification of active peptides by HPLC-MS/MS. J. Agric. Food Chem. 53:588–593. http://dx.doi.org/10.1021/jf048626m.

Horwitz, W., and G. W. Latimer Jr. 2006. Official Methods of Analysis of AOAC International. 18th ed. AOAC International, Gaithersburg, Md.

Iyer, C., and K. Kailasapathy. 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt condi-

tions and in yogurt. J. Food Sci. 70:M18–M23. http://dx.doi.org/10.1111/j.1365-2621.2005.tb09041.x.

Jiang, H., T. Tong, J. Sun, Y. Xu, Z. Zhao, and D. Liao. 2014. Purification and characterization of antioxidative peptides from round scad (*Decapterus maruadsi*) muscle protein hydrolysate. Food Chem. 154:158–163. http://dx.doi.org/10.1016/j.foodchem.2013.12.074.

Jutamongkon, R., and S. Charoenrein. 2010. Effect of temperature on the stability of fruit bromelain from smooth cayenne pineapple. Kasetsart J. (Nat. Sci.) 44:943–948.

Kailasapathy, K., I. Harmstorf, and M. Phillips. 2008. Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred fruit yogurts. LWT Food Sci. Technol. (Campinas.) 41:1317–1322. http://dx.doi.org/10.1016/j.lwt.2007.08.009.

Kim, G. N., H. D. Jang, and C. I. Kim. 2007. Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase. Food Chem. 104:1359–1365. http://dx.doi.org/10.1016/j.foodchem.2007.01.065.

Kudoh, Y., S. Matsuda, K. Igoshi, and T. Oki. 2001. Antioxidative Peptide from Milk Fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* IFO13953. J. Jpn. Soc. Food Sci. 48:44–50.

Li, Z., A. Jiang, T. Yue, J. Wang, Y. Wang, and J. Su. 2013. Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. J. Dairy Sci. 96:4242–4251. http://dx.doi.org/10.3168/jds.2012-6511.

Lourens-Hattingh, A., and B. C. Viljoen. 2001. Yogurt as probiotic carrier food. Int. Dairy J. 11:1–17. http://dx.doi.org/10.1016/S0958-6946(01)00036-X.

Mani-López, E., E. Palou, and A. López-Malo. 2014. Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. J. Dairy Sci. 97:2578–2590. http://dx.doi.org/10.3168/jds.2013-7551.

Matera, J. A., A. G. Cruz, R. S. L. Raices, M. C. Silva, L. C. Nogueira, S. L. Quitério, R. N. Cavalcanti, M. Q. Freiras, and C. A. Conte Júnior. 2014. Discrimination of Brazilian artisanal and inspected pork sausages: Application of unsupervised, linear and non-linear supervised chemometric methods. Food Res. Int. 64:380–386. http://dx.doi.org/10.1016/j.foodres.2014.07.003.

Miclo, L., E. Roux, M. Genay, E. Brusseaux, C. Poirson, N. Jameh, C. Perrin, and A. Dary. 2012. Variability of hydrolysis of β-, α_{s1}-, and α_{s2}-caseins by 10 strains of *Streptococcus thermophilus* and resulting bioactive peptides. J. Agric. Food Chem. 60:554–565. http://dx.doi.org/10.1021/jf202176d.

Ozgen, M., R. N. Reese, A. Z. Tulio Jr., J. C. Scheerens, and A. R. Miller. 2006. Modified 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) methods. J. Agric. Food Chem. 54:1151–1157. http://dx.doi.org/10.1021/jf051960d.

Phanturat, P., S. Benjakul, W. Visessanguan, and S. Roytrakul. 2010. Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. LWT - . Food Sci. Technol. (Campinas.) 43:86–97. http://dx.doi.org/10.1016/j.lwt.2009.06.010.

Rajapakse, N., E. Mendis, W. K. Jung, J. Y. Je, and S. K. Kim. 2005. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Res. Int. 38:175–182. http://dx.doi.org/10.1016/j.foodres.2004.10.002.

Roberfroid, M. 2007. Prebiotics: The concept revisited. J. Nutr. 137(Suppl 2):830S–837S.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2014. Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. Food Chem. 156:264–270. http://dx.doi.org/10.1016/j.foodchem.2014.01.105.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2015. Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. Compr. Rev. Food Sci. Food Saf. 14:123–138. http://dx.doi.org/10.1111/1541-4337.12126.

SAS. 1996. SAS/STAT Softwares: Changes and Enhancements Through Release 6.11. SAS Institute Inc., Cary, NC.

Sendra, E., P. Fayos, Y. Lario, J. Fernández-López, E. Sayas-Barberá, and J. Pérez-Alvarez. 2008. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. Food Microbiol. 25:13–21. http://dx.doi.org/10.1016/j.fm.2007.09.003.

Shiby, V. K., and H. N. Mishra. 2013. Fermented Milks and milk products as functional foods—A review. Crit. Rev. Food Sci. Nutr. 53:482–496. http://dx.doi.org/10.1080/10408398.2010.547398.

Siow, H. L., and C. Y. Gan. 2013. Extraction of antioxidative and antihypertensive bioactive peptides from *Parkia speciosa* seeds. Food Chem. 141:3435–3442. http://dx.doi.org/10.1016/j.foodchem.2013.06.030.

Thammarutwasik, P., T. Hongpattarakere, S. Chantachum, K. Kijroongrojana, A. Itharat, W. Reanmongkol, S. Tewtrakul, and B. Ooraikul. 2009. Prebiotics—A review. Songklanakarin J. Sci. Technol. 31:401–408.

van Boekel, M. A. J. S., C. N. J. M. Weerens, A. Holstra, C. E. Scheidtweiler, and G. M. Alink. 1993. Antimutagenic effects of casein and its digestion products. Food Chem. Toxicol. 31:731–737. http://dx.doi.org/10.1016/0278-6915(93)90144-N.

Vasiljevic, T., and N. P. Shah. 2008. Probiotics—From Metchnikoff to bioactives. Int. Dairy J. 18:714–728. http://dx.doi.org/10.1016/j.idairyj.2008.03.004.

Zheng, X. Q., J. T. Wang, X. L. Liu, Y. Sun, Y. J. Zheng, X. J. Wang, and Y. Liu. 2015. Effect of hydrolysis time on the physicochemical and functional properties of corn glutelin by Protamex hydrolysis. Food Chem. 172:407–415. http://dx.doi.org/10.1016/j.foodchem.2014.09.080.

Chapter 6: Antibacterial and antiproliferative peptides in synbiotic yogurt – release and stability during refrigerated storage

Chapter 6 deals with the effect of pineapple peel powder addition on performance of *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) in regard to the generation of inhibitory peptides against bacteria and HT-29 human colon cancer cells in yogurts during 28 days of refrigerated storage at 4 °C.

The paper entitled "Antibacterial and antiproliferative peptides in synbiotic yogurt – release and stability during refrigerated storage" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*Journal of Dairy Science*" (2016), 99(6): 4233–4242. http://dx.doi.org/10.3168/jds.2015-10499. It has also been selected as an "Editor's Choice" article for the June 2016 issue, and featured on the journal's homepage.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** [redacted] **Date:** 15-Mar-2016

Baidya Nath Prasad Sah

Paper Title:

Antibacterial and antiproliferative peptides in synbiotic yogurt – release and stability during refrigerated storage

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Antibacterial and antiproliferative peptides in synbiotic yogurt—Release and stability during refrigerated storage

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ABSTRACT

The search for alternative therapeutics is on the rise due to the extensive increase in bacterial resistance to various conventional antibiotics and side effects of conventional cancer therapies. Bioactive peptides released from natural sources such as dairy foods by lactic acid bacteria have received attention as a potential source of biotherapeutic peptides. However, liberation of peptides in yogurt depends on proteolytic activities of the cultures used. Thus, this research was conducted to establish generation of inhibitory peptides in yogurt against pathogenic bacteria and cancer cells during storage at 4°C for 28 d. Water-soluble crude peptide extracts were prepared by high-speed centrifugation of plain and probiotic yogurts supplemented with or without pineapple peel powder (PPP). The inhibition zones against *Escherichia coli* and *Staphylococcus aureus* by PPP-fortified probiotic yogurt at 28 d of storage were, respectively, 25.89 and 11.72 mm in diameter, significantly higher than that of nonsupplemented control yogurts. Antiproliferative activity against HT29 colon cancer cells was also significantly higher in probiotic yogurt with PPP than in nonsupplemented probiotic yogurt. Overall, crude water-soluble peptide extracts of the probiotic yogurt with PPP possessed stronger inhibitory activities against bacteria and cancer cells than controls, and these activities were maintained during storage. However, activities were lowered substantially during in vitro gastrointestinal digestion. These findings support the possibility of utilizing dairy-derived bioactive peptides in the development of a superior alternative to the current generation of antibacterial and anticancer agents, as well as a functional ingredient in foods, nutraceuticals, and pharmaceuticals.

Key words: pineapple, probiotics, peptides, antibacterial activity, anticancer activity

INTRODUCTION

Rapid industrialization and urbanization has resulted in immense changes to lifestyle practices, leading to increased risks of various diseases and disorders, such as cancer. Cancer, an uncontrolled growth and spreading of abnormal cells, has become a major health burden in the United States and many other parts of the world (Siegel et al., 2012). Colorectal cancer is a widespread cancer, the fourth most common in men and third in women in Latin America (Goss et al., 2013). Side effects such as alopecia (hair loss), fatigue, nausea, and vomiting are associated with conventional cancer therapies, such as chemotherapy and radiotherapy, because they adversely affect healthy cells as they destroy malignant cells. In addition, there is increasing resistance against conventional chemotherapy. Consequently, there is an urgent demand for natural anticancer compounds, including bioactive peptides, as an alternative treatment to chemotherapy drugs, which could eliminate some drawbacks of chemotherapy.

Some bioactive peptides exhibit interesting cytotoxic activities against both malignant and microbial cells (Hoskin and Ramamoorthy, 2008). Positively charged antimicrobial peptides (**AMP**) can bind with negatively charged components of bacterial and cancer cells electrostatically, which may play a critical role for the disruption of bacterial and cancer cell membranes (Yeaman and Yount, 2003; Hoskin and Ramamoorthy, 2008). Most AMP are relatively small (6 to 100 AA), cationic, amphipathic, and α-helical peptides and demonstrate broad-spectrum antibacterial and antifungal activities, usually by lysing cell membranes (Giuliani et al., 2007; Yeung et al., 2011). The widespread increase in bacterial resistance to several common antibiotics has inspired scientists to focus on exploring new groups of antibiotics with new target sites and action modes.

Consequently, interest is growing in food-derived peptides as drug candidates, mainly due to several specific key merits over common chemotherapeutics. Notably, milk proteins emerge as a prolific source of biologically active peptides, which are encrypted in the

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primary structure of the proteins and could modulate the physiology of consumers following the proteolytic release of peptides with anticarcinogenic potential (Bhat and Bhat, 2011; Sah et al., 2015a). One way to obtain these bioactive peptides is by direct release from proteins by proteolytic actions of bacteria commonly used in manufacturing fermented foods (Choi et al., 2012). Therefore, yogurt appears to be an appropriate matrix for production of such functional ingredients.

Several investigations have been conducted to increase the functionality of yogurt such as probiotic inclusion in culture and prebiotic supplementation (Donkor et al., 2007a; Al-Sheraji et al., 2012; Sah et al., 2015b, 2016). A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al., 2004). Common prebiotics are inulin, fructooligosaccharides, galactooligosaccharides, and other oligosaccharides, such as resistant starch and lactulose (Thammarutwasik et al., 2009). Inulin represents a group of plant polysaccharides having linear fructans with β-(2←1) fructosyl-fructose glycosidic linkages, and "inulin HP" is a long-chain inulin with a degree of polymerization of 10 to 60, the average being 25 (Roberfroid, 2007). Besides inulin, pineapple peel powder (**PPP**) appears to be a good source of dietary fiber, protein, and minerals, with apparent prebiotic potential (Sah et al., 2015c).

Although prebiotic supplementations may result in several functional benefits for probiotic organisms and ultimately consumers, the approach may influence the bioactivity of yogurt because bacterial proteolytic enzymes further hydrolyze milk proteins and peptides during storage (Donkor et al., 2007b). However, studies are still largely limited regarding the effects of prebiotic addition on inhibitory activities against bacteria and HT29 human colon cancer cells of the released peptides in yogurt during storage. This work thus aimed to assess the effect of PPP addition on performance of *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus casei* (ATCC 393), and *Lactobacillus paracasei* ssp. *paracasei* (ATCC BAA52) in regard to the liberation of bioactive peptides with antibacterial and anticancer potential in yogurts during 28 d of refrigerated storage at 4°C.

MATERIALS AND METHODS

Substrates and Chemicals

McCoy's 5A (Modified) medium and trypsin-EDTA (0.25%) were procured from Life Technologies (Carlsbad, CA). Bovogen Biologicals Pty Ltd. (Melbourne, Australia) supplied fetal bovine serum (**FBS**). CellTiter 96 AQ_{ueous} One Solution reagent containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; **MTS**] and an electron-coupling reagent (phenazine ethosulfate) was purchased from Promega Corp. (Madison, WI) for the cell proliferation assay. Antibiotic/antimycotic solution (100×) and staurosporine solution (from *Streptomyces* sp.) were obtained from Sigma Chemical Co. (St. Louis, MO). Pepsin (cat. no. P7000; pepsin A; EC 3.4.23.1, 570 U/mg solid, from porcine gastric mucosa), pancreatin [cat. no. 1494057; pancreatin, amylase, and protease United States Pharmacopeia reference standard; each mg contains 344 USP units of amylase activity and 358 USP units of protease activity], and bile (catalog number B3883; bile bovine) were also purchased from Sigma Chemical Co. Cellstar T25 and T75 flasks, 96-well flat-bottomed microplate (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany) were obtained from Interpath Services Pty. Ltd. (Heidelberg West, VIC, Australia). Ampicillin sodium salt was purchased from Progen Industries Ltd. (Darra, QLD, Australia). Thermo Fisher Scientific Australia Pty Ltd. (Scoresby, VIC, Australia) supplied nutrient agar no. 1 (CM0003; Oxoid, Basingstoke, UK). Aqueous solutions were prepared in Milli-Q water (18.2 MΩ·cm) obtained from a Millipore water purification system (Millipore Australia Pty Ltd., North Ryde, NSW, Australia). Skim milk powder and whole pineapples were bought from a local supermarket (Woolworths Limited, Werribee, Australia). Pineapple peel powder was prepared from the peel of pineapple (*Ananas comosus* [L.] Merrill) as described by Sah et al. (2015b).

Propagation of Cultures and Preparation of Yogurts Supplemented with Prebiotics

Pure cultures of *Streptococcus thermophilus* ASCC 1275 and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 (*L. bulgaricus*) were obtained from the Victoria University Culture Collection (Werribee, Australia). *Lactobacillus acidophilus* ATCC 4356, *L. casei* ATCC 393, and *L. paracasei* ssp. *paracasei* ATCC BAA52 (*L. paracasei*) were procured from Cell Biosciences Pty Ltd. (Heidelberg, VIC, Australia). All organisms were stored at −80°C in de Man, Rogosa, and Sharpe broth containing 40% (vol/vol) glycerol. The strains resuscitated after 3 successive transfers were used to prepare starters as described by Sah et al. (2014).

Set-type plain and probiotic yogurts with inulin or PPP supplementation or without supplementation (control) were prepared as described by Sah et al.

Table 1. Experimental design to evaluate production and stability of antibacterial and antiproliferative peptides in yogurt during refrigerated storage[1]

Prebiotic (1% wt/vol)	Combination of cultures (1% vol/vol each)
None (Control 1)	*Streptococcus thermophilus* + *Lactobacillus bulgaricus*
None (Control 2)	*S. thermophilus* + *L. bulgaricus* + *Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*
Inulin (Orafti HP[2])	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Pineapple peel powder	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*

[1]Yogurt culture = *S. thermophilus* + *L. bulgaricus*; probiotic cultures = *L. acidophilus* + *L. casei* + *L. paracasei*.
[2]Beneo-Orafti Ltd. (Tienen, Belgium).

(2014). Briefly, 4 batches of milk base were prepared by reconstituting skim milk powder in Milli-Q water at 140 g/L; 2 batches were separately supplemented with 1.0% (wt/vol) of commercial inulin Orafti HP (Beneo-Orafti Ltd., Tienen, Belgium) or PPP. All milk bases were heated for 30 min at 85°C, cooled to 45°C, and then inoculated with 1.0% (vol/vol) of *S. thermophilus* and *L. bulgaricus* monocultures aseptically. Three mixes (2 supplemented mixes, and 1 nonsupplemented control) were further inoculated with 1% (vol/vol) of each probiotic monoculture (Table 1). The final mixes were aliquoted into polystyrene cups, and incubated at 42°C until pH of 4.5 ± 0.05 was achieved. Thereafter, the yogurts were immediately cooled to 4°C and stored for 28 d.

Preparation of Water-Soluble Peptide Extracts

Water-soluble peptide extracts (**WSPE**) were prepared by high-speed centrifugation of yogurt samples as described by Sah et al. (2014). Briefly, samples were centrifuged at 22,680 × *g* using a JLA-16.250 rotor in an Avanti J-26S XPI High-Performance Centrifuge (Beckman Coulter Inc., Brea, CA) at 4°C for 30 min. The supernatant was collected and freeze-dried using an Alpha 1-4 LSC Christ freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and stored at −80°C until further analysis. The protein content (mg/mL) of the WSPE was estimated according to Bradford (1976) using BSA (0.1–1.4 mg/mL) as standard.

Determination of Antibacterial Activity

An agar well diffusion assay was performed to assess inhibitory activity of WSPE against target strains [gram-negative: *Escherichia coli* (ATCC CRM-8739) and gram-positive: *Staphylococcus aureus* ssp. *aureus* (ATCC 25923)] as described by Vieira et al. (2014) with some modifications. Briefly, 100 μL of a serially diluted overnight culture of the test organism (1 to 5 × 10^5 cells/mL) was spread on nutrient agar plates. Wells (6 mm in diameter) were made in agar using a sterilized stainless steel borer. Each well was filled with 100 μL of sterilized WSPE in PBS (NaCl = 8.475 g/L, Na_2HPO_4 = 1.093 g/L, and NaH_2PO_4 = 0.276 g/L; pH 7.4; 500 μg of protein/mL). The plates were left at 4°C for 4 h to allow peptide diffusion in the medium, and then incubated aerobically at 37°C for 16 to 18 h. Subsequently, the diameter of inhibition zones in mm (including the well) was measured. Ampicillin (500 μg/mL) was used as a positive control and PBS was used as a negative control.

The morphological changes induced by the WSPE on *E. coli* ATCC 8739 and *S. aureus* ATCC 25923 were studied using scanning electron microscopy as described by Zhao et al. (2015) with some modifications. Briefly, 300 μL of suspension of log-phase tested bacteria in Nutrient broth No. 1 (optical density at 600 nm of ∼0.1) was treated with 600 μL of sterilized WSPE sample (at 500 μg of protein/mL in PBS) in a sterile 1.5-mL Eppendorf tube and incubated for 6 h at 37°C. The WSPE was prepared from probiotic yogurt with PPP stored at 4°C for 28 d. After incubation, cells were washed twice with sterile PBS, pelleted (16,000 × *g*, 2 min). Then, 200 μL of fixative (2.5% glutaraldehyde solution in PBS) was slowly added and gently mixed. After 10 min, the cells were pelleted; the spent fixative was replaced with the fresh, and further fixing was allowed overnight at 4°C. The pellet was washed thrice with sterile Milli-Q water, dehydrated rapidly with ascending concentrations of aqueous ethanol series (25, 50, 75, and 90%, and 3 times with 100% for 10 min each), and dried further using 1,1,1,3,3,3-hexamethyldisilazane (**HMDS**) at 1:2 HMDS:ethanol, 2:1 HMDS:ethanol, and 100% HMDS for 10 min each. Finally, the cell pellet was directly mounted on an aluminum scanning electron microscopy stubs, air-dried overnight at room temperature in a biosafety cabinet, and sputtered with gold (∼18 nm) using a Jeol NeoCoater (model MP-19020NCTR). Fields of the specimen were examined under a high-vacuum NeoScope JCM-5000 benchtop SEM (Jeol Ltd., Tokyo, Japan) and micrographs were recorded.

Cell Culture and Assessment of Antiproliferative Activity Against HT-29 Cells

A human colorectal cancer cell line, HT29 (ATCC HTB38), was obtained from the American Type Culture Collection (Manassas, VA). The HT-29 cells were maintained in McCoy's 5A (Modified) complete growth medium containing 10% FBS and 1% antibiotic-antimycotic solution and incubated at 37°C in a CO_2 incubator (Shanghai Lishen Scientific Equipment Co. Ltd., Shanghai, China) in a humidified air atmosphere containing 5% CO_2. The cells grew as monolayers in 75-cm^2 tissue culture flasks, where the cell culture medium was changed every 48 to 72 h and cells passaged at 80 to 90% confluency using 0.25% trypsin-EDTA (1×) to detach cell lines. Viable cells were counted according to the trypan blue dye exclusion method using a hemocytometer.

The antiproliferative effect of WSPE on HT29 cells was assessed through MTS assay as described by Yan et al. (2013) with some modifications. Briefly, 100 μL of a logarithmically growing cell suspension in the McCoy's 5A complete growth medium (~2.0×10^4 cells/mL) was dispensed in a well of a 96-well flat-bottomed plate, and the plate was preincubated at 37°C for 24 h in a CO_2 incubator to allow cells to adhere. The medium was replaced with 100 μL of fresh McCoy's 5A complete medium containing WSPE at a protein concentration of 250 μg/mL prepared by dissolving WSPE in complete growth medium, adjusting pH to 7.3 ± 0.1, and filter-sterilized using a sterile cellulose acetate syringe filter (0.20 μm; Advantec MFS Inc., Dublin, CA). The microplate was further incubated at 37°C for 24 h in the CO_2 incubator. Then, 20 μL of CellTiter 96 AQ_{ueous} One Solution reagent was added to each well, incubated at 37°C for 4 h in the CO_2 incubator, and subjected to absorbance measurement at 490 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA). The plate included blank wells containing the same volume of complete McCoy's 5A medium instead of WSPE sample. Staurosporine (500 ng/mL) was used as a positive control in the assay. Antiproliferative activity of the WSPE was calculated as follows:

$$Antiproliferative\ acitivity\ (\%) = \left[\frac{A_b - A_s}{A_b - A_c}\right] \times 100,$$

where A_s is the absorbance of sample; A_b is the absorbance of blank, using the same volume of culture medium instead of the sample; and A_c is the absorbance of the control, using the same volume of culture medium without cells and samples.

In Vitro Gastrointestinal Digestion

In vitro gastrointestinal (**GI**) digestion of WSPE was performed as described by Minekus et al. (2014) with some modifications. Briefly, 10 mL of aqueous WSPE (at 200 mg/mL) from 28-d-stored probiotic yogurt with PPP was mixed with 7.5 mL of simulated gastric fluid electrolyte stock solution [6.9 mL of KCl (0.5 *M*), 0.9 mL of KH_2PO_4 (0.5 *M*), 12.5 mL of $NaHCO_3$ (1 *M*), 11.8 mL of NaCl (2 *M*), 0.4 mL of $MgCl_2{\cdot}6H_2O$ (0.15 *M*), 0.5 mL $(NH_4)_2CO_3$ (0.5 *M*); volume made up to 400 mL with Milli-Q water; pH 3.0], 1.6 mL of pepsin stock solution (25,000 U/mL in simulated gastric fluid electrolyte stock solution), and 5 μL of $CaCl_2$ (0.3 *M*). The pH of the mixture was adjusted to 3.0, the volume made up to 20 mL with Milli-Q water, and digested for 2 h in a shaking waterbath (model SWB20; Ratek Instruments Pty Ltd., Boronia, VIC, Australia) at 37°C with shaking (100 horizontal strokes/min). Subsequently, 20 mL of gastric chyme was mixed with 11 mL of simulated intestinal fluid electrolyte stock solution [6.8 mL of KCl (0.5 *M*), 0.8 mL of KH_2PO_4 (0.5 *M*), 42.5 mL of $NaHCO_3$ (1 *M*), 9.6 mL of NaCl (2 *M*), 1.1 mL of $MgCl_2{\cdot}6H_2O$ (0.15 *M*); volume made up to 400 mL with Milli-Q water; pH 7.0], 5.0 mL of a pancreatin stock solution (800 U/mL in simulated intestinal fluid electrolyte stock solution), 2.5 mL of bile (160 m*M*), and 40 μL of $CaCl_2$ (0.3 *M*). The pH of mixture was adjusted to 7.0, and the volume made up to 40 mL with Milli-Q water, and digested for 2 h in the water bath at 37°C with shaking (100 horizontal strokes/min). The digestate was immediately heated at 95°C for 15 min to inactivate the enzymes, and then cooled to room temperature, frozen, and freeze-dried.

Statistical Analyses

Experiments were conducted as a randomized split-plot blocked design in time with type of yogurt as the main plot and prebiotic addition and time as subplots; the results obtained were analyzed using the general linear model (GLM) procedure. The design was replicated in triplicate with simultaneous subsampling of the samples, resulting in at least 6 observations ($n \geq 6$). A paired samples *t*-test was also carried out to explore the effects of gastrointestinal digestion on stability of bioactive peptides using the PROC TTEST procedure. These analyses were performed using SAS software at a significance level of $P < 0.05$ (SAS Institute, 1996). In addition, hierarchical cluster analysis was performed to categorize yogurt samples with different culture and prebiotic combinations based on their similarities by applying the squared Euclidean distance and Ward linkage methods to the standardized data set (z-scores)

of d-28 results using SPSS 22.0 (SPSS Inc., Chicago, IL), with results graphically displayed as a dendrogram.

RESULTS AND DISCUSSION

Lactic acid bacteria produce proteolytic enzymes during yogurt manufacturing, which cleave peptide bonds of milk proteins, leading to generation of peptides and free AA (Donkor et al., 2007c). In our previous study (Sah et al., 2015b), the viability of probiotic (*L. acidophilus*, *L. casei*, and *L. paracasei* spp. *paracasei*) and starter (*S. thermophilus* and *L. bulgaricus*) cultures in yogurt was improved during 28 d of refrigerated storage due to supplementation with PPP or inulin, and subsequently the extent of protein hydrolysis increased in yogurts during storage. This resulted in generation of several peptides, which may display antibacterial and anticancer activities.

Antibacterial Activity of Yogurts During Refrigerated Storage

Despite the large numbers of antibiotics available currently, the growing bacterial resistance against many conventional antibiotics in recent decades has directed the investigation of alternative compounds. In addition, the use of natural antimicrobial compounds has received great attention due to consumer demands for minimally processed food. Thus, inhibitory activities of WSPE were evaluated against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria, and the findings are presented in Figure 1 and Table 2. All samples displayed antibacterial activity against both *E. coli* and *S. aureus*. Moreover, the inhibition zones induced by the WSPE increased significantly ($P < 0.05$) at the end of storage compared with d 1 of storage, indicating increased generation of the peptides. Overall, enhanced antibacterial activity was observed in the probiotic yogurts supplemented with PPP compared with the nonsupplemented probiotic yogurt, and similar activities were observed for inulin-supplemented yogurts. Furthermore, growth inhibition of WSPE against *E. coli* was comparable to that of ampicillin at 500 μg/mL (19.94 ± 1.27 mm). However, the extent of inhibition against *S. aureus* was significantly less than that of ampicillin at 500 μg/mL (41.72 ± 1.61 mm). Several potent antibacterial peptides (**ABP**) liberated from milk proteins have been reported, such as Leu-Arg-Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu ($f_{99–109}$ of α_{S1}-CN) from the pepsin hydrolysate of bovine casein (Tang et al., 2015). Additionally, Sedaghati et al. (2014) also reported 3 ABP [Met-Met-Lys ($f_{1–3}$), Phe-Phe-Ser-Asp-Lys ($f_{17–21}$), Ile-Ala-Lys ($f_{22–24}$)] from bovine κ-CN digested using plasmin. McCann et al. (2005) isolated many ABP derived from the f(164–207) region of bovine α_{S2}-CN from the digested bovine milk proteins by chymosin. Furthermore, the antibacterial activity and selectivity of a peptide depends on various attributes, including peptide charges, amphipathicity, and the size of hydrophobic or hydrophilic domain (Zelezetsky and Tossi, 2006).

Bacterial morphology was determined after treatment with WSPE using scanning electron microscopy. The observations demonstrated that the peptides possessed membrane-lytic activities against microbial cells (Figure 2). Fibrous material, likely due to leakage of

Table 2. Inhibition zones of plain and probiotic yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C against *Escherichia coli* (ATCC CRM-8739; gram-negative) and *Staphylococcus aureus* ssp. *aureus* (ATCC 25923; gram-positive)[1]

Yogurt type[2]		Inhibition zone (mm)					
		E. coli			*S. aureus*		
Culture	Prebiotic	d 1	d 14	d 28	d 1	d 14	d 28
SC	None	$23.83^{b,A}$	$23.50^{b,AB}$	$23.00^{c,B}$	$10.06^{b,A}$	$9.44^{c,B}$	$10.50^{c,A}$
SC + PC	None	$22.83^{c,B}$	$23.28^{b,AB}$	$23.67^{c,A}$	$10.17^{ab,B}$	$10.28^{b,B}$	$11.00^{bc,A}$
SC + PC	Inulin	$23.17^{bc,B}$	$23.44^{b,B}$	$24.56^{b,A}$	$10.33^{ab,B}$	$10.44^{b,B}$	$11.22^{ab,A}$
SC + PC	PPP	$24.61^{a,B}$	$24.89^{a,B}$	$25.89^{a,A}$	$10.67^{a,B}$	$11.06^{a,B}$	$11.72^{a,A}$
SEM[3]			0.24			0.19	

[a–c]Different lowercase superscripts in the same column depict significant differences between means for yogurt types ($P < 0.05$).

[A,B]Different uppercase superscripts in the same row depict significant differences between means for yogurts with the same culture and prebiotic combination on d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*Streptococcus thermophilus* + *Lactobacillus bulgaricus*); PC = probiotic culture (*Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*).

[3]SEM = pooled standard error of the mean for predetermined $P < 0.05$.

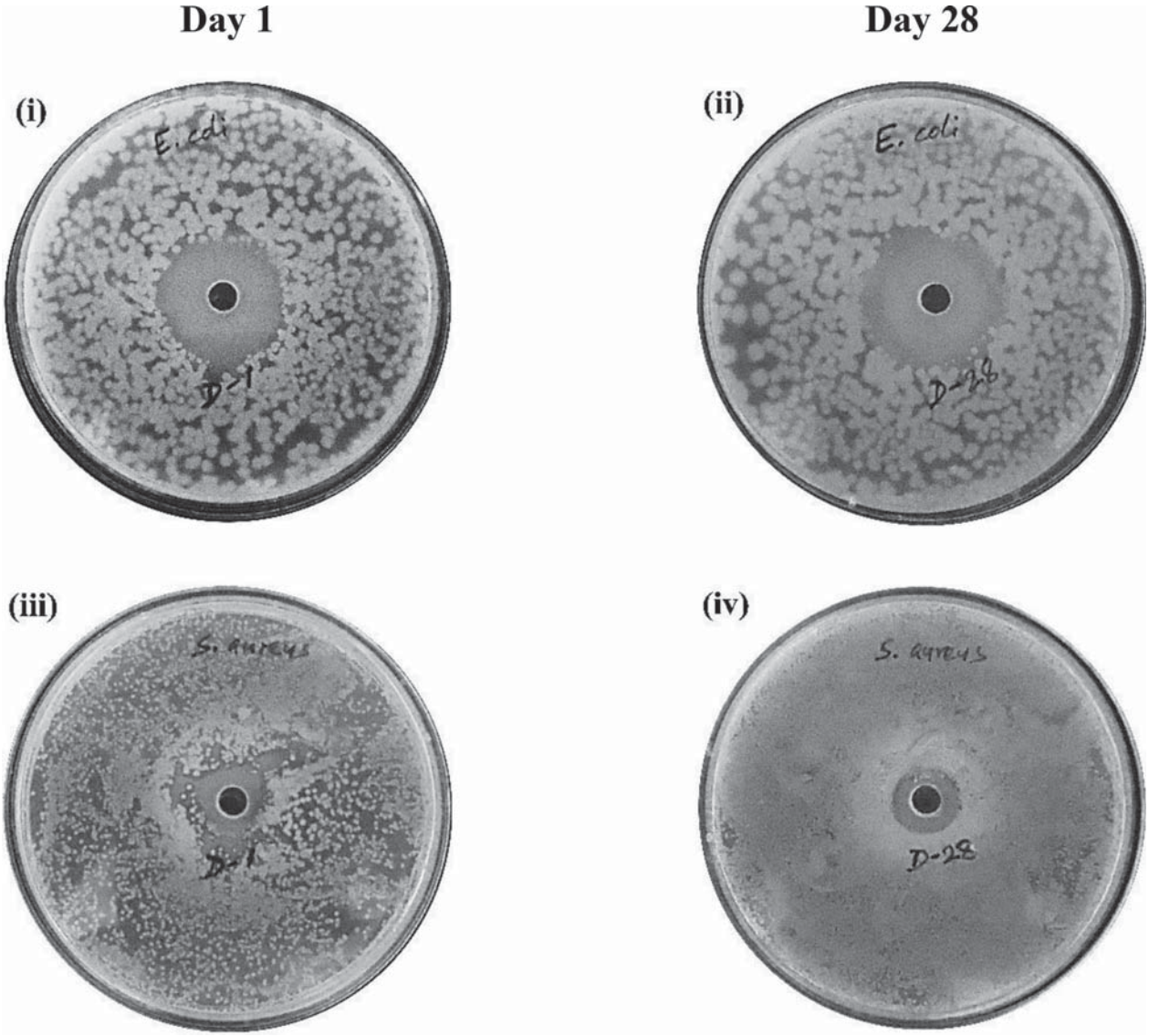

Figure 1. Probiotic yogurts supplemented with pineapple peel powder stored at 4°C for 28 d against test microorganisms: *Escherichia coli* (i, ii), and *Staphylococcus aureus* (iii, iv), showing inhibitory zones for d 1 (i and iii) and d 28 (ii and iv).

cell content, and cell debris were seen scattered around the cells (Figures 2B and 2D). Some cationic peptides are believed to interact with gram-negative bacteria first by binding to the anionic lipopolysaccharides of cell membrane (Yeaman and Yount, 2003; Hoskin and Ramamoorthy, 2008). These peptides can also displace divalent cations such as Ca^{2+} and Mg^{2+}, causing distortion of the outer membrane bilayer because the ions are essential for integrity of the outer membrane (Peterson et al., 1987). Consequently, the membrane lyses, resulting in cell death.

Antiproliferative Activity of Yogurts Against HT-29 Cells During Storage

Antiproliferative activity of WSPE against cancer cells was investigated by assessing their potency to inhibit the growth of HT-29 colon cancer cells, and the results are presented in Figure 3 and Table 3. All samples reduced proliferation of HT-29 cells to varying degrees, indicating differences in generated WSPE of the yogurts. The antiproliferative activities were stronger in PPP-supplemented probiotic yogurt

(56.36%) compared with the nonsupplemented control probiotic yogurt (40.52%) and plain yogurt (35.71%) after 28 d of refrigerated storage. Moreover, activities in probiotic yogurt with PPP increased significantly ($P < 0.05$) during storage compared with on d 1 (40.10 vs. 56.36%). The effectiveness of WSPE was comparable to that of staurosporine at 500 ng/mL (36.28 ± 2.80%). Several potent anticancer peptides liberated from milk proteins have been reported, such as Phe-Phe-Ser-Asp-Lys (κ-casecidin; $f_{17–21}$ of bovine κ-CN) against human leukemic cells lines (Matin and Otani, 2002), and Ile-Asn-Lys-Lys-Ile ($f_{41–45}$ of β-CN) against B16F10 melanoma cells (Azevedo et al., 2012). Furthermore, a partially purified peptide subfraction from buffalo cheese acid whey, called f3, reduced the proliferation of human epithelial colon cancer (Caco-2) cells by modulating the cell cycle (De Simone et al., 2009). The peptide Pro-Gly-Pro-Ile-Pro-Asn ($f_{63–68}$ of β-CN) inhibited proliferation of SKOV3 human ovarian cancer cells partly by promoting apoptosis by hindering BCL2 pathway (Wang et al., 2013). α-Casecidins [Arg-Pro-Lys ($f_{1–3}$), Leu-Lys-Lys ($f_{101–103}$), and Tyr-Lys ($f_{104–105}$) derived from α_{S1}-CN] caused necrosis in leukemic T and B cell lines (Otani and Suzuki, 2003). Bovine lactoferrin reduced the proliferation of MCF-7 breast cancer cells dose-dependently by inducing apoptosis (Zhang et al., 2015). The antiproliferative activity observed in this study requires further investigation to elucidate mechanisms of cell death or suppression.

Synbiotic Effect of Prebiotic and Probiotic on the Overall Antibacterial and Antiproliferative Activities in Yogurts

Cluster analysis was conducted using hierarchical clustering method with Ward's linkage and revealed 2 clusters based on similarities in measured inhibitory activities against *E. coli*, *S. aureus*, and HT29 colon cancer cells during 28 d of storage at 4°C (Figure 4). These findings implied that the liberated peptides behaved differently according to sample types. Overall, nonsupplemented probiotic yogurt (denoted yogurt 2) and probiotic yogurt supplemented with inulin (denoted yogurt 3) displayed similar bioactivities. Plain

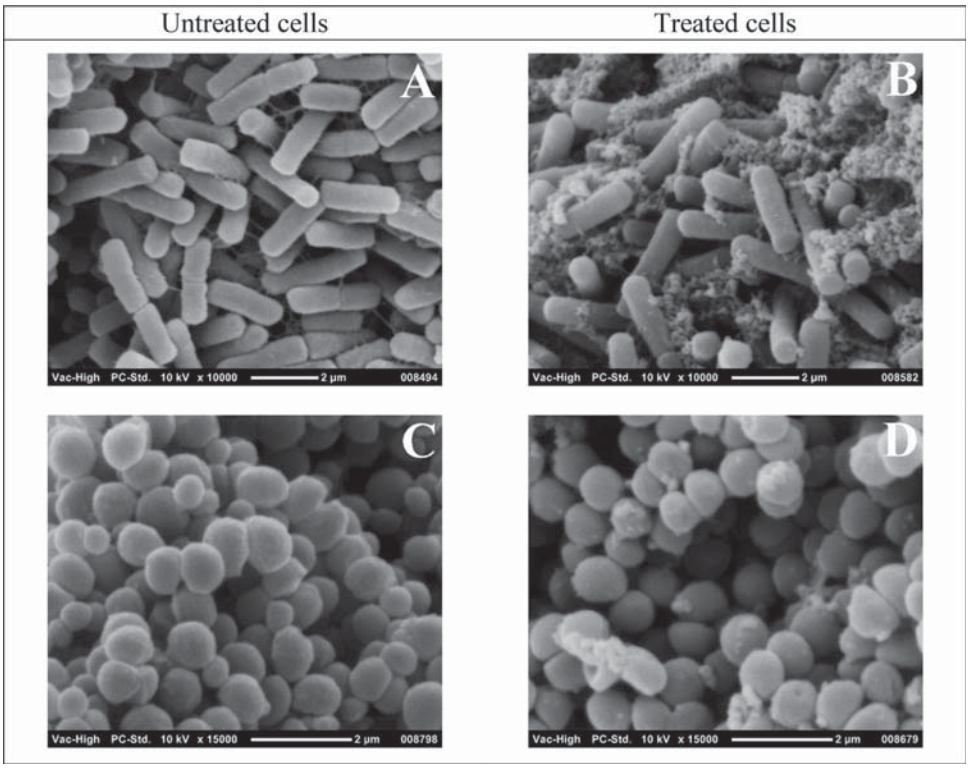

Figure 2. Scanning electron micrographs showing morphological changes of *Escherichia coli* (A = untreated control cells; B = treated cells), and *Staphylococcus aureus* (C = untreated control cells; D = treated cells) induced by treating for 6 h at 37°C with water-soluble peptide extract (WSPE) probiotic yogurts supplemented with pineapple peel powder and stored for 28 d.

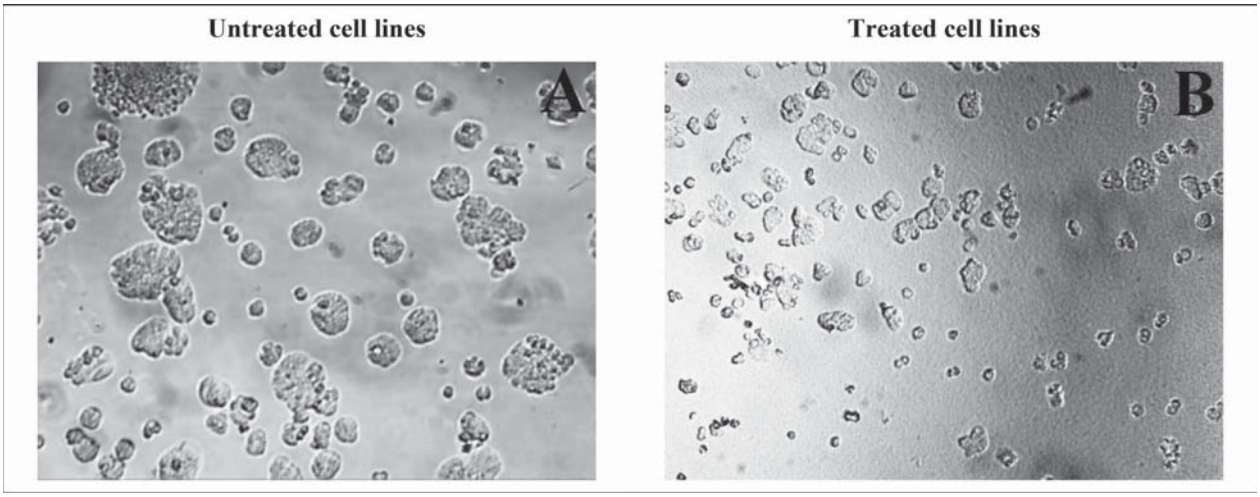

Figure 3. Images examined under phase-contrast microscopy showing morphological changes in HT29 human colon cancer cells: (A) untreated control, and (B) treated for 24 h at 37°C with water-soluble peptide extract (WSPE) probiotic yogurts supplemented with pineapple peel powder and stored for 28 d.

yogurt was arranged in a separate cluster characterized by weak antibacterial and antiproliferative activities, whereas probiotic yogurt with PPP was in a separate cluster showing strong inhibitory activities against bacterial and HT 29 colon cancer cells. Thus, the PPP-fortified probiotic yogurt stored for 28 d was selected to study the stability of measured bioactivities during in vitro GI digestion.

Table 3. Antiproliferative activity of plain and probiotic yogurts supplemented with or without pineapple peel powder (PPP) or inulin during 28 d of storage at 4°C against a human colorectal cancer cell line, HT29 (ATCC HTB38)[1]

Yogurt type[2]		Antiproliferative activity (%)		
Culture	Prebiotic	d 1	d 14	d 28
SC	None	$44.73^{a,A}$	$39.44^{a,AB}$	$35.71^{b,B}$
SC + PC	None	$21.81^{b,B}$	$29.87^{b,B}$	$40.52^{b,A}$
SC + PC	Inulin	$46.78^{a,A}$	$35.66^{ab,B}$	$40.36^{b,AB}$
SC + PC	PPP	$40.10^{a,B}$	$40.94^{a,B}$	$56.36^{a,A}$
SEM[3]			2.82	

[a,b]Different lowercase superscripts in the same column depict significant differences between means for yogurt types ($P < 0.05$).

[A,B]Different uppercase superscripts in the same row depict significant differences between means for yogurts with the same culture and prebiotic combination on d 1, 14, and 28 of refrigerated storage ($P < 0.05$).

[1]Results are expressed as mean of 3 trials.

[2]SC = starter culture (*Streptococcus thermophilus* + *Lactobacillus bulgaricus*); PC = probiotic culture (*Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus paracasei*).

[3]SEM = pooled standard error of the mean for predetermined $P < 0.05$.

Stability of Bioactivities During In Vitro GI Digestion

One of the greatest challenges of in vivo efficacy of bioactive peptides is the ability to reach target organs after oral administration because of hydrolysis

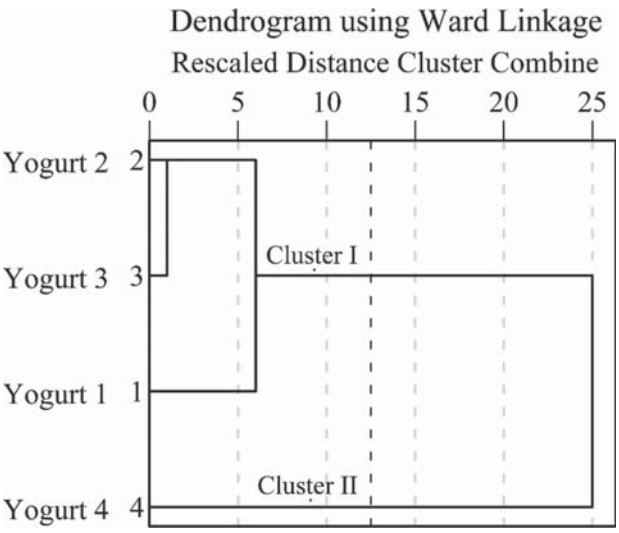

Figure 4. Dendrogram exhibiting the clustering of yogurts according to similarities among measured variables in yogurts stored at 4°C in d 28. The measured variables were inhibitory zones against *Escherichia coli* and *Staphylococcus aureus*, and antiproliferative activity against HT29 colon cancer cell lines. Yogurt 1 = fermented using starter culture only; yogurts 2, 3, and 4 = fermented using both starter and probiotic cultures. Yogurts 3 and 4 were supplemented with inulin and pineapple peel powder, respectively, whereas yogurts 1 and 2 were nonsupplemented control yogurts.

by digestive enzymes and low pH in stomach. Results after GI tract simulation with WSPE sample showed insignificant changes ($P > 0.05$) in inhibitory activities against *S. aureus* (10.94 ± 0.49 mm) compared with undigested sample (11.72 ± 0.49 mm). However, GI tract simulation resulted in significantly lower ($P < 0.05$) inhibitory activities against *E. coli* and HT29 colon cancer cells (17.28 ± 0.77 mm and 27.56 ± 3.74%, respectively) compared with undigested sample (25.89 ± 0.40 mm and 56.36 ± 3.73%, respectively). This decrease could have resulted from the breakdown of bioactive peptides of the WSPE due to hydrolysis by the GI tract enzymes. Ao and Li (2013) also reported the degradation of peptide fractions during GI digestion. Consistent with these findings, Su et al. (2007) showed that multi-phosphorylated α_{S1}- and α_{S2}-CN peptides were not stable during pancreatic digestion. However, peptides containing proline and hydroxyproline residues can usually resist breakdown by digestive enzymes (Segura-Campos et al., 2011). In fact, GI digestion can result in both formation and degradation of bioactive peptides, as reported by Kopf-Bolanz et al. (2014). Therefore, parenteral administration may be the preferable delivery mode for the purified peptides compared with the consumption of bioactive peptides in probiotic yogurts fortified with PPP. Encapsulation of active peptides may be another approach to minimize possible hydrolysis by the GIT system.

CONCLUSIONS

All WSPE prepared from yogurt samples possessed antibacterial activities against gram-positive and gram-negative bacteria, and activities increased during storage. The WSPE exhibited stronger inhibitory activity against gram-negative compared with gram-positive bacteria. In addition, the WSPE inhibited proliferation of HT29 human colon cancer cells. Generation of inhibitory peptides against bacteria and HT29 human colon cancer cells improved with PPP supplementation of yogurt. However, activities reduced substantially after GI tract digestion. Taken together, the incorporation of PPP and probiotics in yogurts offers new opportunities in the development of novel functional foods, and this approach could lead to the development of novel bioactive peptides having antibacterial and anticancer activity. These findings demand further investigation to isolate and characterize these inhibitory peptides from WSPE.

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REFERENCES

Al-Sheraji, S. H., A. Ismail, M. Y. Manap, S. Mustafa, and R. M. Yusof. 2012. Viability and activity of bifidobacteria during refrigerated storage of yoghurt containing *Mangifera pajang* fibrous polysaccharides. J. Food Sci. 77:M624–M630. http://dx.doi.org/10.1111/j.1750-3841.2012.02955.x.

Ao, J., and B. Li. 2013. Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion in vitro: Charge properties of peptides affect digestive stability. Food Res. Int. 52:334–341. http://dx.doi.org/10.1016/j.foodres.2013.03.036.

Azevedo, R. A., A. K. Ferreira, A. V. V. Auada, K. F. M. Pasqualoto, R. Marques-Porto, D. A. Maria, and I. Lebrun. 2012. Antitumor effect of cationic INKKI peptide from bovine β-casein on melanoma B16F10. J. Cancer Ther. 3:237–244. http://dx.doi.org/10.4236/jct.2012.34034.

Bhat, Z. F., and H. Bhat. 2011. Milk and dairy products as functional foods: A review. Int. J. Dairy Sci. 6:1–12. http://dx.doi.org/10.3923/ijds.2011.1.12.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248–254.

Choi, J., L. Sabikhi, A. Hassan, and S. Anand. 2012. Bioactive peptides in dairy products. Int. J. Dairy Technol. 65:1–12. http://dx.doi.org/10.1111/j.1471-0307.2011.00725.x.

De Simone, C., G. Picariello, G. Mamone, P. Stiuso, A. Dicitore, D. Vanacore, L. Chianese, F. Addeo, and P. Ferranti. 2009. Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. J. Pept. Sci. 15:251–258. http://dx.doi.org/10.1002/psc.1093.

Donkor, O. N., A. Henriksson, T. K. Singh, T. Vasiljevic, and N. P. Shah. 2007b. ACE-inhibitory activity of probiotic yoghurt. Int. Dairy J. 17:1321–1331. http://dx.doi.org/10.1016/j.idairyj.2007.02.009.

Donkor, O. N., A. Henriksson, T. Vasiljevic, and N. P. Shah. 2007c. Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. Lait 87:21–38. http://dx.doi.org/10.1051/lait:2006023.

Donkor, O. N., S. L. I. Nilmini, P. Stolic, T. Vasiljevic, and N. P. Shah. 2007a. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int. Dairy J. 17:657–665. http://dx.doi.org/10.1016/j.idairyj.2006.08.006.

Gibson, G. R., H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutr. Res. Rev. 17:259–275. http://dx.doi.org/10.1079/NRR200479.

Giuliani, A., G. Pirri, and S. F. Nicoletto. 2007. Antimicrobial peptides: An overview of a promising class of therapeutics. Cent. Eur. J. Biol. 2:1–33. http://dx.doi.org/10.2478/s11535-007-0010-5.

Goss, P. E., B. L. Lee, T. Badovinac-Crnjevic, K. Strasser-Weippl, Y. Chavarri-Guerra, J. S. Louis, C. Villarreal-Garza, K. Unger-Saldaña, M. Ferreyra, M. Debiasi, P. E. R. Liedke, D. Touya, G. Werutsky, M. Higgins, L. Fan, C. Vasconcelos, E. Cazap, C. Vallejos, A. Mohar, F. Knaul, H. Arreola, R. Batura, S. Luciani, R. Sullivan, D. Finkelstein, S. Simon, C. Barrios, R. Kightlinger, A. Gelrud, V. Bychkovsky, G. Lopes, S. Stefani, M. Blaya, F. H. Souza, F. S. Santos, A. Kaemmerer, E. de Azambuja, A. F. C. Zorilla, R. Murillo, J. Jeronimo, V. Tsu, A. Carvalho, C. F. Gil, C. Sternberg, A. Dueñas-Gonzalez, D. Sgroi, M. Cuello, R. Fresco, R. M. Reis, G. Masera, R. Gabús, R. Ribeiro, R. Knust, G. Ismael, E. Rosenblatt, B. Roth, L. Villa, A. L. Solares, M. X. Leon, I. Torres-Vigil, A. Covarrubias-Gomez, A. Hernández, M. Bertolino, G. Schwartsmann, S. Santillana, F. Esteva, L. Fein, M. Mano, H. Gomez, M. Hurlbert, A. Durstine, and G. Azenha. 2013. Planning cancer control in Latin America and the Caribbean. Lancet Oncol. 14:391–436. http://dx.doi.org/10.1016/S1470-2045(13)70048-2.

Hoskin, D. W., and A. Ramamoorthy. 2008. Studies on anticancer activities of antimicrobial peptides. Biochim. Biophys. Acta Biomembr. 1778:357–375. http://dx.doi.org/10.1016/j.bbamem.2007.11.008.

Kopf-Bolanz, K. A., F. Schwander, M. Gijs, G. Vergères, R. Portmann, and L. Egger. 2014. Impact of milk processing on the generation of peptides during digestion. Int. Dairy J. 35:130–138. http://dx.doi.org/10.1016/j.idairyj.2013.10.012.

Matin, A., and H. Otani. 2002. Cytotoxic and antibacterial activities of chemically synthesized kappa-casecidin and its partial peptide fragments. J. Dairy Res. 69:329–334. http://dx.doi.org/10.1017/s0022029902005435.

McCann, K. B., B. J. Shiell, W. P. Michalski, A. Lee, J. Wan, H. Roginski, and M. J. Coventry. 2005. Isolation and characterisation of antibacterial peptides derived from the f(164–207) region of bovine α_{s2}-casein. Int. Dairy J. 15:133–143. http://dx.doi.org/10.1016/j.idairyj.2004.06.008.

Minekus, M., M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. MacIerzanka, A. MacKie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies, and A. Brodkorb. 2014. A standardised static *in vitro* digestion method suitable for food—An international consensus. Food Funct. 5:1113–1124. http://dx.doi.org/10.1039/c3fo60702j.

Otani, H., and H. Suzuki. 2003. Isolation and characterization of cytotoxic small peptides, α-casecidins, from bovine α_{s1}-casein digested with bovine trypsin. Anim. Sci. J. 74:427–435. http://dx.doi.org/10.1046/j.1344-3941.2003.00135.x.

Peterson, A. A., S. W. Fesik, and E. J. McGroarty. 1987. Decreased binding of antibiotics to lipopolysaccharide from polymyxin-resistant strains of *Escherichia coli* and *Salmonella typhimurium*. Antimicrob. Agents Chemother. 31:230–237. http://dx.doi.org/10.1128/AAC.31.2.230.

Roberfroid, M. B. 2007. Inulin-type fructans: Functional food ingredients. J. Nutr. 137:2493S–2502S.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2014. Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. Food Chem. 156:264–270. http://dx.doi.org/10.1016/j.foodchem.2014.01.105.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2015a. Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. Compr. Rev. Food Sci. Food Safety 14:123–138. http://dx.doi.org/10.1111/1541-4337.12126.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2015b. Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. J. Dairy Sci. 98:5905–5916. http://dx.doi.org/10.3168/jds.2015-9450.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2015c. Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt. J. Food Sci. Technol. http://dx.doi.org/10.1007/s13197-015-2100-0.

Sah, B. N. P., T. Vasiljevic, S. McKechnie, and O. N. Donkor. 2016. Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. Food Sci. Technol. (Campinas.) 65:978–986. http://dx.doi.org/10.1016/j.lwt.2015.09.027.

SAS Institute. 1996. SAS/STAT Software: Changes and Enhancements through Release 6.11. SAS Institute Inc., Cary, NC.

Sedaghati, M., H. Ezzatpanah, M. M. A. Boojar, M. T. Ebrahimi, and M. Aminafshar. 2014. Plasmin digest of κ-casein as a source of antibacterial peptides. J. Dairy Res. 81:245–251. http://dx.doi.org/10.1017/S0022029914000120.

Segura-Campos, M., L. Chel-Guerrero, D. Betancur-Ancona, and V. M. Hernandez-Escalante. 2011. Bioavailability of bioactive peptides. Food Rev. Int. 27:213–226. http://dx.doi.org/10.1080/87559129.2011.563395.

Siegel, R., D. Naishadham, and A. Jemal. 2012. Cancer statistics, 2012. CA Cancer J. Clin. 62:10–29. http://dx.doi.org/10.3322/caac.20138.

Su, R., W. Qi, Z. He, S. Yuan, and Y. Zhang. 2007. Pancreatic hydrolysis of bovine casein: Identification and release kinetics of phosphopeptides. Food Chem. 104:276–286. http://dx.doi.org/10.1016/j.foodchem.2006.11.039.

Tang, W., H. Yuan, H. Zhang, L. Wang, H. Qian, and X. Qi. 2015. An antimicrobial peptide screened from casein hydrolyzate by *Saccharomyces cerevisiae* cell membrane affinity method. Food Contr. 50: http://dx.doi.org/10.1016/j.foodcont.2014.09.030.

Thammarutwasik, P., T. Hongpattarakere, S. Chantachum, K. Kijroongrojana, A. Itharat, W. Reanmongkol, S. Tewtrakul, and B. Ooraikul. 2009. Prebiotics—A review. Songklanakarin J. Sci. Technol. 31:401–408.

Vieira, M., C. Pinheiro, R. Fernandes, J. P. Noronha, and C. Prudêncio. 2014. Antimicrobial activity of quinoxaline 1,4-dioxide with 2- and 3-substituted derivatives. Microbiol. Res. 169:287–293. http://dx.doi.org/10.1016/j.micres.2013.06.015.

Wang, W., F. Gu, C. Wei, Y. Tang, X. Zheng, M. Ren, and Y. Qin. 2013. PGPIPN, a therapeutic hexapeptide, suppressed human ovarian cancer growth by targeting BCL2. PLoS ONE 8: http://dx.doi.org/10.1371/journal.pone.0060701.

Yan, Z., Z. Zhu, J. Wang, J. Sun, Y. Chen, G. Yang, W. Chen, and Y. Deng. 2013. Synthesis, characterization, and evaluation of a novel inhibitor of WNT/β-catenin signaling pathway. Mol. Cancer 12: http://dx.doi.org/10.1186/1476-4598-12-116.

Yeaman, M. R., and N. Y. Yount. 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. 55:27–55. http://dx.doi.org/10.1124/pr.55.1.2.

Yeung, A. T. Y., S. L. Gellatly, and R. E. W. Hancock. 2011. Multifunctional cationic host defence peptides and their clinical applications. Cell. Mol. Life Sci. 68:2161–2176. http://dx.doi.org/10.1007/s00018-011-0710-x.

Zelezetsky, I., and A. Tossi. 2006. Alpha-helical antimicrobial peptides: Using a sequence template to guide structure-activity relationship studies. Biochim. Biophys. Acta Biomembr. 1758:1436–1449. http://dx.doi.org/10.1016/j.bbamem.2006.03.021.

Zhang, Y., C. F. Lima, and L. R. Rodrigues. 2015. In vitro evaluation of bovine lactoferrin potential as an anticancer agent. Int. Dairy J. 40:6–15. http://dx.doi.org/10.1016/j.idairyj.2014.08.016.

Zhao, L., H. Zhang, T. Hao, and S. Li. 2015. *In vitro* antibacterial activities and mechanism of sugar fatty acid esters against five food-related bacteria. Food Chem. 187:370–377. http://dx.doi.org/10.1016/j.foodchem.2015.04.108.

Chapter 7: Antioxidant peptides isolated from synbiotic yogurt exhibit antiproliferative activities against HT-29 colon cancer cells

Chapter 7 focuses on isolation of antioxidant peptides from crude peptide extract of probiotic yogurt supplemented with pineapple peel powder stored for 28 days at 4 °C and anticancer activities were also assessed against HT-29 human colon cancer cells.

The paper entitled "Antioxidant peptides isolated from synbiotic yoghurt exhibit antiproliferative activities against HT-29 colon cancer cells" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*International Dairy Journal*" (2016), 63, 99–106. http://dx.doi.org/10.1016/j.idairyj.2016.08.003.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** **Date:** 15-Mar-2016

Baidya Nath Prasad Sah

[redacted]

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Antioxidant peptides isolated from synbiotic yoghurt exhibit antiproliferative activities against HT-29 colon cancer cells

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ABSTRACT

Milk proteins are considered a reservoir of peptides possessing various bioactivities. Using ultrafiltration followed by reversed phase-high performance liquid chromatography, antioxidant peptides were purified from crude peptide extract of probiotic yoghurt supplemented with pineapple peel powder stored for 28 days at 4 °C. Two β-casein-derived peptides, ^{193}YQEPVLGPVRGPFPIIV^{209} and ^{69}SLPQNIPPLTQTPVVVPPF^{87} (designated P17 and P19, respectively), were identified and their antioxidant and anticancer activities assessed. P17 showed high scavenging activity against 2,2′-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) radicals, with an IC_{50} of 29.88 μg mL^{-1}, compared with P19 (IC_{50} 1.44 mg mL^{-1}). Furthermore, the proliferation of HT-29 colon cancer cell line was inhibited (41.49% and 38.55%, respectively, by P17 and P19 at 3 mg mL^{-1}) via inducing apoptosis and cell cycle arrest in G_2/M-phase. In vitro gastrointestinal digestion of peptides resulted in increased bioactivities. These findings indicate a potential for utilising both of these peptides to manage oxidative stress mediated diseases and disorders including cancers.

1. Introduction

Oxidative metabolism is a principal pathway for the biosynthesis of adenosine triphosphate (ATP), which provides energy to cellular processes (Ren et al., 2008). However, generation of free radicals such as reactive oxygen and nitrogen species during oxidation can cause lethal damage to cells, leading to the development of several chronic and degenerative diseases such as multiple sclerosis, cardiovascular disease, diabetes, and cancer (Collins, 2005; Sharma, 2014). Cancer, an uncontrolled growth and spreading of abnormal cells, has become a major health burden worldwide (Siegel, Naishadham, & Jemal, 2012). Colorectal cancer is one of the widespread cancers, the third most commonly diagnosed cancer in males and the second in females in 2008, with the highest incidence rates in Australia, New Zealand, Europe, and North America (Jemal et al., 2011). Current therapeutic agents employed in chemotherapy and radiotherapy indiscriminately target both healthy and malignant cells, resulting in many side effects including nausea, vomiting, fatigue, and alopecia (hair loss). Many limitations such as lack of aqueous solubility, lack of selectivity, and multidrug resistance restrict the use of conventional chemotherapy (Moorthi, Manavalan, & Kathiresan, 2011). Thus, research on more effective and less toxic medications are needed; the heightened research interest on isolation and identification of novel anticancer compounds including bioactive peptides as alternative treatment to chemotherapy drugs is on the rise.

Dairy-derived peptides have exhibited various health benefits including recognition as a source for cancer therapy (Sah, Vasiljevic, McKechnie, & Donkor, 2015a). Kudoh, Matsuda, Igoshi, and Oki (2001) isolated a κ-casein derived antioxidative peptide (Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met) from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO13953. Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) also reported antioxidant peptides derived from β-, α_{S1}-, α_{S2}- and κ-caseins from yoghurt. Korhonen and Pihlanto (2006) concluded that most of the peptides are encrypted within the amino acid sequence of protein molecule, and can be liberated during several food processes or gastrointestinal activities. Moreover, Gupta, Mann, Kumar, and Sangwan (2009) stated a direct correlation of antioxidant activity with the extent of protein hydrolysis, which was found dependent on the

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lactic acid bacterial strain used in cheese making. Extracellular proteinases of lactic acid bacteria involve in the release of several oligopeptides from proteins and further hydrolysis by intracellular peptidases results into peptides of low molecular weight in fermented milk products including yoghurt (Donkor, Henriksson, Vasiljevic, & Shah, 2007).

Yoghurt consumption is associated with health benefits due to the presence of active constituents including biologically active peptides. The availability of bioactive peptides can be increased when prebiotics are incorporated in yoghurt (Sah, Vasiljevic, McKechnie, & Donkor, 2015b, 2016a). Pineapple peel powder (PPP) is a good source of dietary fibre and has been reported to show prebiotic potential (Sah, Vasiljevic, McKechnie, & Donkor, 2016b), and its supplementation in probiotic yoghurt resulted in increased biological activities of crude peptide extract from the yoghurt (Sah, Vasiljevic, McKechnie, & Donkor, 2016c; Sah et al., 2015b). This directed further investigation to isolate and identify bioactive peptides having anticancer potential. In this context, the objective of this study was to isolate antioxidative peptides from peptide extract of the probiotic yoghurt containing PPP as the prebiotic, and evaluate antiproliferative activities of the isolated peptides against HT29 colon cancer cells. Stability of the isolated peptides was also assessed during in vitro gastrointestinal digestion.

2. Material and methods

2.1. Substrates and chemicals

Salicylic acid, and 2,2′- azino-bis (3- ethyl benzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma Chemical Company (St Louis, MO, USA). McCoy's 5A (Modified) medium and trypsin-EDTA (0.25%) were obtained from Life Technologies (Carlsbad, CA, USA). Antibiotic/antimycotic solution (100×), and staurosporine solution (from *Streptomyces* sp.) were purchased from Sigma Chemical Company. Bovogen Biologicals Pty Ltd, Melbourne, Australia supplied foetal bovine serum (FBS). CellTiter 96® AQ_{ueous} One Solution reagent containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulphate; PES) was purchased from Promega Corp. (Madison, WI, USA). CELLSTAR T75 flasks, and 96-well flat-bottomed microplate (CELLSTAR®, Greiner Bio-One GmbH, Frickenhausen, Germany) were procured from Interpath Services Pty., Ltd. (Heidelberg West, VIC, Australia). Pepsin (catalogue number P7000; pepsin A; EC 3.4.23.1, 570 Units mg^{-1} solid, from porcine gastric mucosa), bile (catalogue number B3883; bile bovine), and pancreatin [catalogue number 1494057; pancreatin, amylase and protease United States Pharmacopeia (USP) Reference Standard; each mg contains 344 USP units of amylase activity and 358 USP units of protease activity] were also purchased from Sigma Chemical Company. Skim milk powder and whole pineapple (*Ananas comosus* [L.] Merrill) were procured from a local store (Woolworths Limited, Melbourne, VIC, Australia). Peel was separated from whole pineapple, and used to prepare PPP as described by Sah et al. (2016b). Aqueous solutions were prepared in Milli-Q water (18.2 MΩ cm) obtained from a Millipore water purification system (Millipore Australia Pty Ltd., North Ryde, NSW, Australia).

2.2. Propagation of cultures

Streptococcus thermophilus ASCC 1275 (*S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 (*L. bulgaricus*) were collected from Victoria University Culture Collection (Werribee, VIC, Australia). *Lactobacillus acidophilus* ATCC 4356 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*) and *Lactobacillus paracasei* spp. *paracasei* ATCC BAA52 (*L. paracasei*) were purchased from Cell Biosciences Pty Ltd (Heidelberg, VIC, Australia). All strains were stored at −80 °C in MRS broth containing 40% (v/v) glycerol. Three successive transfers in M17 broth for *S. thermophilus* and in MRS broth for *L. bulgaricus*, *L. acidophilus*, *L. casei*, and *L. paracasei* were performed to resuscitate the strains. These strains were then used in the preparation of starters (~10^8 cfu mL^{-1}) as described by Sah, Vasiljevic, McKechnie, and Donkor (2014).

2.3. Preparation of water-soluble peptide extract from probiotic yoghurt with pineapple peel powder

L. acidophilus, *L. casei* and *L. paracasei* have been incorporated previously into several fermented food products to confer probiotic properties (Ortakci & Sert, 2012; Sidira et al., 2013), and hence used to prepare set-type probiotic yoghurt supplemented with PPP as described by Sah et al. (2015b). Briefly, milk base was prepared by reconstituting skim milk powder in Milli-Q water at 140 g L^{-1} and supplemented with 1% (w/v) of PPP. The milk base was heated for 30 min at 85 °C, cooled to 45 °C and then aseptically inoculated with 1% (v/v) of each *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. casei* and *L. paracasei* monocultures. The final mix was poured into polystyrene cups, incubated at 42 °C until pH of 4.5 ± 0.05 was accomplished. Thereafter, the yoghurt was immediately cooled, and stored at 4 °C for 28 days, and used to prepare a water-soluble peptide extract (WSPE) according to Sah et al. (2015b). Briefly, yoghurt samples were centrifuged at 22,680 × *g* using a JLA-16.250 rotor in Avanti J-26S XPI High-Performance Centrifuge (Beckman Coulter, Inc., Brea, CA, USA) for 30 min at 4 °C. The supernatant was collected, freeze-dried using a Dynavac FD 300 freeze dryer (Airvac Engineering Pty. Ltd, Rowville, VIC, Australia).

2.4. Purification and identification of antioxidant peptides from crude WSPE, and synthesis of identified peptides

The WSPE was fractionated based on molecular mass employing an ultrafiltration membrane, cut-off 3 kDa (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane; Merck Millipore Corporation, Darmstadt, Germany), at 2684 × *g* for 120 min at 4 °C. Two fractions (retentate, >3 kDa, and permeate, <3 kDa, of filtration) were collected separately, concentrated, and assayed for ABTS radical ($ABTS^{\bullet+}$) scavenging activity.

The ultra-filtered fraction (i.e., <3 kDa fraction) was further fractionated by injecting 1000 μL sample onto a reversed phase-high performance liquid chromatography (RP-HPLC) system (Varian Inc., Palo Alto, CA, USA) equipped with a C-18 monomeric column (10 μm, 300 Å, 22 mm × 250 mm; Grace Vydac, Hesperia, CA, USA), peptides being eluted at a flow rate of 17 mL min^{-1}, and detected at 215 nm. The fractions were collected, concentrated, and screened for antioxidant activity. Peptides of the potent fraction was identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) employing a quadrupole time-of-flight (TOF) mass spectrometer (micrOTOF-Q; Bruker Daltonik GmbH, Bremen, Germany) coupled online to a nano HPLC (Ultimate 3000; Dionex Corporation, SunnyBrook, CA, USA). Data from LC-MS/MS were exported in Mascot generic file format (*.mgf) and searched against the Swiss-Prot databases using the MASCOT search engine (version 2.4, Matrix Science Inc., London, UK). Two peptides, YQEPVLGPVRGPFPIIV (P17) and SLPQNIPPLTQTPVVVPPF (P19), derived from β-casein were identified, and synthesised by Mimotopes Pty Ltd (Clayton, VIC, Australia) at purity > 95%.

2.5. Measurement of antioxidant activity

2.5.1. Assay of ABTS radical scavenging activity of peptide fractions from WSPE

The $ABTS^{\bullet+}$ radical scavenging activity (ARSA) of peptide fractions from WSPE was assayed according to the method described by Sah et al. (2015b) with some modifications. Briefly, 7 mM ABTS (MW = 548.68) in 2.45 mM aqueous potassium persulphate (MW = 270.32) was prepared and left for 12–16 h at room temperature in dark to generate ABTS radical cation ($ABTS^{\bullet+}$). Prior to each measurement, the $ABTS^{\bullet+}$ stock solution was diluted with 10 mM phosphate buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette after equilibration at 30 °C. Exactly 40 μL of peptide fraction was mixed with 960 μL of the $ABTS^{\bullet+}$ reagent, and incubated in a hot water bath at 30 °C for 30 min. The protein content of ultra-filtered fractions [retentate (>3 kDa) and permeate (<3 kDa)] of the WSPE of probiotic yoghurt was 1500 μg mL^{-1}, and peptide fractions of the ultra-filtered permeate (<3 kDa) using preparative RP-HPLC was at 250 μg mL^{-1}. The absorbance of the mix was measured at 734 nm. Similarly, 40 μL of Milli-Q water was used instead of the sample for the blank. Radical-scavenging activity (RSA) was calculated using Eq. (1).

$$\text{RSA}\ (\%) = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100 \quad (1)$$

2.5.2. Assay of ABTS radical scavenging activity of synthetic peptides

The $ABTS^{\bullet+}$ radical scavenging activity of peptides P17 and P19 was assayed according to procedure described above for peptide fractions, with some modifications. Briefly, 200 μL sample (concentration range: 15–40 μg mL^{-1} for P17; 0.5–2.0 mg mL^{-1} for P19) was added to 800 μL of the $ABTS^{\bullet+}$ reagent and incubated in a hot water bath at 30 °C for 30 min after vortexing. The absorbance of the mix was measured at 734 nm. Similarly, 200 μL of Milli-Q water was used instead of the sample for the blank. RSA (%) was calculated using Eq. (1). IC_{50}, concentration of peptide to inhibit 50% of the radicals, was calculated from the linear regression curve prepared by plotting RSA (%) versus different peptide concentrations. Similarly, IC_{50} value of ascorbic acid was estimated for comparison.

2.5.3. Assay of hydroxyl radical scavenging activity of synthetic peptides

The scavenging capacity of peptides (P17 and P19) for hydroxyl radical ($^{\bullet}OH$) was assayed according to the method described by Sah et al. (2015b). Briefly, 500 μL of aqueous ferrous sulphate (2 mM) and 100 μL of aqueous hydrogen peroxide (2 mM) were mixed with 50 μL aqueous peptide sample (5 mg mL^{-1}). The reaction mixture was left for 10 min and then 500 μL of aqueous salicylic acid (2.5 mM) was added. The mixture was incubated at 37 °C for 30 min, and subjected for absorbance measurement at 510 nm. Milli-Q water was used for the blank (in place of salicylic acid), the control (in place of sample) in the reaction, and the hydroxyl radical scavenging activity (HRSA) was calculated using Eq. (2):

$$\text{HRSA}\ (\%) = \left[1 - \frac{A_s - A_b}{A_c}\right] \times 100 \quad (2)$$

where A_s, A_b, and A_c were absorbance for sample, blank, and control respectively.

Similarly, HRSA of ascorbic acid (1 mg mL^{-1}) was also estimated for comparison.

2.6. Cell culture and assay of antiproliferative activity of peptides against HT-29 cells

A human colorectal cancer cell line, HT-29 (ATCC® HTB38™), was obtained from the American Type Culture Collection (Manassas, VA, USA). The HT-29 cells were maintained in McCoy's 5A (Modified) complete growth medium containing 10% FBS and 1% antibiotic-antimycotic solution and incubated at 37 °C in a CO_2 incubator (New Brunswick™ Galaxy® 170 R; New Brunswick Scientific Co Inc., Edison, NJ, USA) having humidified air atmosphere containing 5% CO_2. The cells grew as monolayers in 75-cm^2 T flasks, where the cell culture medium was changed every 48–72 h and cells passaged at 80–90% confluency using 0.25% trypsin-EDTA (1×) to detach cell lines. Viable cells were counted using Scepter™ 2.0 Handheld Automated Cell Counter (Merck KGaA, Darmstadt, Germany).

The antiproliferative effect of peptides P17 and P19 on HT-29 cells was assessed by employing MTS assay according to Sah et al. (2016c) with some modifications. Briefly, 100 μL of a logarithmically growing cell suspension in the McCoy's 5A complete growth medium (~2.0 × 10^4 cells mL^{-1}) was dispensed in a well of a 96-well flat-bottomed plate, and the plate was pre-incubated at 37 °C for 24 h in the CO_2 incubator for cell adherence. The medium was replaced with 100 μL fresh McCoy's 5A complete medium containing peptides at a concentration of 1, 3, and 5 mg mL^{-1}. The microplate was further incubated at 37 °C for 72 h in the CO_2 incubator. Then, 20 μL of CellTiter 96® AQ_{ueous} One Solution Reagent was added to each well, incubated at 37 °C for 2 h in the CO_2 incubator, and subjected for absorbance measurement at 490 nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA). The plate also included blank wells containing same volume of complete McCoy's 5A medium instead of WSPE sample. Staurosporine (50 ng mL^{-1}) was used as a positive control in the assay. Antiproliferative activity of the WSPEs was calculated using Eq. (3).

$$\text{Antiproliferative acitivity}\ (\%) = \left[\frac{A_b - A_s}{A_b - A_c}\right] \times 100 \quad (3)$$

where A_s was the absorbance of sample; A_b was the absorbance of blank, using the same volume of culture medium instead of the sample; A_c was the absorbance of the control, using the same volume of culture medium without cells and samples.

Apoptotic activity of peptides was assessed using Muse Annexin V & Dead Cell Reagent (Merck Millipore Corporation) according to the manufacturer's instructions. Briefly, 500 μL of a logarithmically growing cell suspension in the McCoy's 5A complete growth medium (~2.0 × 10^4 cells mL^{-1}) was dispensed in 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 for cell adherence. The medium was replaced with 500 μL McCoy's 5A complete medium containing peptides at a concentration of 5 mg mL^{-1}. The plate was further incubated at 37 °C for 72 h. Treated cells (both floating and attached) were harvested, washed twice with cold PBS, and resuspended in McCoy's 5A (Modified) complete growth medium (containing 10% FBS and 1% antibiotic-antimycotic solution) to a cell density of ~1.5 × 10^5 cells mL^{-1}. Exactly, 100 μL of Annexin V & Dead Cell Reagent was added to 100 μL of a single cell suspension for staining, and left to stand at room temperature for 20 min in dark. Cells were then analysed using Muse Cell Analyzer (Merck Millipore Corporation).

Multicaspase activity was also measured using Muse Multi-Caspase Kit according to the manufacture's instruction. Briefly, 5 μL of Muse™ MultiCaspase working solution was added to 50 μL of cell suspension in caspase buffer (~5 × 10^5 cells mL^{-1}), capped the tubes loosely, and incubated at 37 °C in the CO_2 incubator for 30 min. Then, 150 μL of 7-AAD (7-Aminoactinomycin D) working solution was added, and subjected for analysis using the Muse Cell Analyzer.

Cell cycle analyses were performed using a Muse Cell Cycle Reagent (Merck Millipore Corporation) according to the manufacturer's instructions. Briefly, 2 mL of a logarithmically growing cell suspension in the McCoy's 5A complete growth medium (~2.0 × 10^4 cells mL^{-1}) was dispensed in a well of a 6-well flat-bottomed plate, and the plate was pre-incubated at 37 °C for 24 h in the CO_2 incubator for cell adherence. The medium was replaced with 2 mL fresh McCoy's 5A medium containing only 0.1% FBS and incubated at 37 °C for further 48 h to synchronise cells in G_0 phase. The medium was then replaced with McCoy's 5A complete medium (10% FBS and antibacterial-antimycotic solution) containing peptides at a concentration of 5 mg mL^{-1}. The medium of negative control well was replaced with McCoy's 5A complete medium (10% FBS and antibacterial-antimycotic solution). The medium of positive control well was replaced with McCoy's 5A complete medium (10% FBS and antibacterial-antimycotic solution) containing staurosporine at a concentration of 50 ng mL^{-1}. The plate was further incubated at 37 °C for 24 h. Treated cells were harvested, washed twice with cold PBS, and fixed in ice-cold 70% ethanol for overnight at −20 °C. The fixed cells were stained with 200 μL of Muse™ Cell Cycle Reagent at room temperature for 30 min in dark, and the percentages of cells in G_0/G_1, S and G_2/M phases were determined using the Muse Cell Analyzer.

2.7. In vitro gastro-intestinal digestion

In vitro gastro-intestinal (GI) digestion of the peptides P17 and P19 was performed as described by Minekus et al. (2014) with some modifications. Briefly, 455 μL aqueous peptide (10 mg) was mixed with 455 μL of simulated gastric fluid (SGF), pH adjusted to 3.0, and digested for 2 h at 37 °C in a shaking incubator. Subsequently, pH of the gastric chyme was adjusted to 7.0, mixed with the same volume of simulated intestinal fluid (SIF), and digested at 37 °C for 2 h in the shaking incubator. The digestate was immediately heated at 95 °C for 15 min to inactivate the enzymes, followed by cooling to room temperature, frozen, and freeze-dried.

The SGF was prepared by mixing 7.5 mL SGF electrolyte stock solution [1.725 mL KCl (0.5 M), 0.225 mL KH_2PO_4 (0.5 M), 3.125 mL $NaHCO_3$ (1 M), 2.950 mL NaCl (2 M), 0.1 mL $MgCl_2 \cdot 6H_2O$ (0.15 M), 0.125 mL $(NH_4)_2CO_3$ (0.5 M); volume made up to 100 mL with Milli-Q water; pH 3.0], 1.6 mL pepsin stock solution (25,000 Units mL^{-1} in SGF electrolyte stock solution), and 5 μL $CaCl_2$ (0.3 M). The pH of mixture was adjusted to 3.0, volume made up to 10 mL with Milli-Q water.

The SIF was prepared by mixing 5.5 mL SIF electrolyte stock solution [1.7 mL KCl (0.5 M), 0.2 mL KH_2PO_4 (0.5 M), 10.625 mL $NaHCO_3$ (1 M), 2.4 mL NaCl (2 M), 0.275 mL $MgCl_2 \cdot 6H_2O$ (0.15 M); volume made up to 100 mL with Milli-Q water; pH 7.0], 2.5 mL pancreatin stock solution (800 Units mL^{-1} in SIF electrolyte stock solution), 1.25 mL bile (160 mM), and 20 μL $CaCl_2$ (0.3 M). The pH of mixture was adjusted to 7.0, volume made up to10 mL with Milli-Q water.

2.8. Statistical analysis

The experiments were performed in triplicate (n = 3), and the data are reported as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was conducted to determine the significance of the main effects. Paired samples t-test was also carried out to explore the effects of gastrointestinal digestion on stability of bioactive peptides employing the PROC TTEST procedure. These analyses were performed using Statistical Analysis System at a significance level of $P < 0.05$ (SAS, 1996).

3. Results and discussion

Antioxidants are chemical compounds that can convert reactive species into stable products and significantly decrease the adverse effects on normal physiological functions (Huang, Boxin, & Prior, 2005), and in auto-oxidative processes including lipid peroxidation in food systems (Mielnik, Aaby, & Skrede, 2003). Artificial compounds like butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), and propyl gallate (PG) can display remarkable antioxidant activities. However, safety concerns associated with their utilisation have resulted in restrictions of their use or prohibition in some countries (Jun, Park, Jung, & Kim, 2004). Therefore, the quest for alternatives such as bioactive peptides is gaining momentum. Water soluble peptide extract of probiotic yoghurt supplemented with PPP after 28 days of storage showed high antioxidant activities in our previous study (Sah et al., 2015b), which lent itself for further investigation.

3.1. Isolation and identification of antioxidant peptides

Ultrafiltration, a separating technique based on molecular size, was employed to fractionate the WSPE from probiotic yoghurt supplemented with PPP stored for 28 days at 4 °C. Two fractions, retentate (>3 kDa fraction) and permeate (<3 kDa fraction) were obtained and evaluated for antioxidant activity by measuring $ABTS^{\bullet+}$ radical scavenging assay; this assay is widely used in the screening of protein hydrolysates, their fractions, and purified peptides for antioxidant activities. The permeate exhibited a high $ABTS^{\bullet+}$ radical scavenging activity (50.48 ± 1.77%), which was significantly higher than the retentate (23.40 ± 1.15%) and was, therefore, chosen for further fractionation using a preparative RP-HPLC. Fractions from preparative RP-HPLC showed $ABTS^{\bullet+}$ radical scavenging activities ranged from 2.61 ± 0.95% to 71.31 ± 1.13% and the most potent fraction contained the peptides YQEPVLGPVRGPFPIIV and SLPQNIPPLTQTPVVVPPF, which are present in the primary structure of bovine β-casein, i.e., f (193–209) and f (69–87), respectively. These peptides designated P17 and P19, respectively, and were synthesised by Mimotopes Pty Ltd (Clayton, VIC, Australia) at purity > 95% and subjected for reassessment of their antioxidant activities.

The antioxidant activity of P17 and P19 was evaluated by assessing scavenging capacity of the radicals: $ABTS^{\bullet+}$ and $^{\bullet}OH$. The $ABTS^{\bullet+}$ radicals are scavenged by antioxidants via an electron and/or hydrogen donation (Gülçin, 2012). A positive reaction changes the reaction solution from blue to colourless, which is detected as a decrease in absorbance at 734 nm. As highlighted in Fig. 1A, P17 demonstrated strong $ABTS^{\bullet+}$ scavenging capacities in a dose-dependent manner with IC_{50} of 29.88 μg mL^{-1}, comparable with that of L-ascorbic acid (IC_{50} of 12.77 μg mL^{-1}). The P19 showed less potency for scavenging $ABTS^{\bullet+}$ radicals (IC_{50} of 1.44 mg mL^{-1}; Fig. 1B). Therefore, P17 might be a potent $ABTS^{\bullet+}$ inhibitor by donating electrons and/or hydrogen atoms, to prevent the occurrence of free radical damage in the human body. Additionally, P17 demonstrated $^{\bullet}OH$ radical scavenging activities (34.97 ± 1.61%), significantly less than that of L-ascorbic acid at 1 mg mL^{-1} (73.12 ± 1.37%). However, P19 did not scavenge hydroxyl radicals (Fig. 2A).

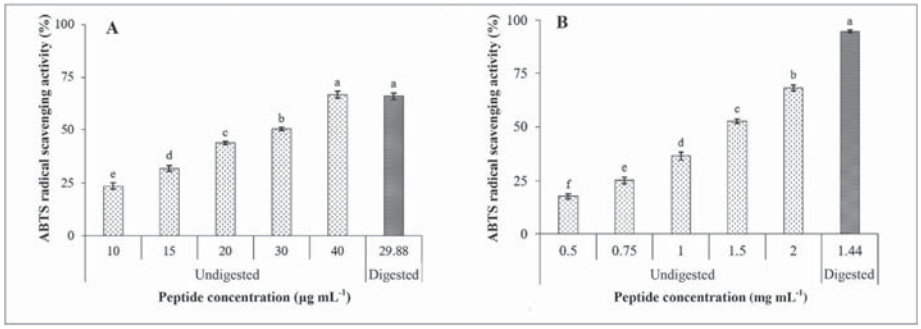

Fig. 1. ABTS$^{\bullet+}$ radical scavenging activities of peptides P17 (A: YQEPVLGPVRGPFPIIV), P19 (B: SLPQNIPPLTQTPVVVPPF), and their digestates from in vitro gastrointestinal digestion. Results are expressed as means ± standard deviation (n = 3); values within a data set (peptides P17 or P19) with different letters are significantly different ($P < 0.05$).

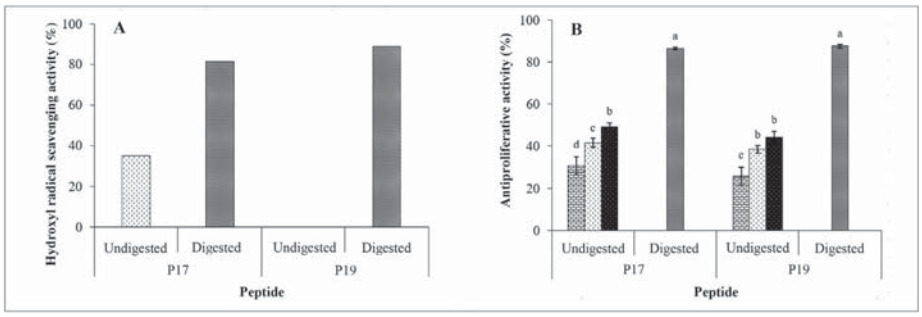

Fig. 2. Hydroxyl radical ($^{\bullet}$OH) scavenging activities (A) of peptides P17 (YQEPVLGPVRGPFPIIV), P19 (SLPQNIPPLTQTPVVVPPF) and their digestates (all at 5 mg mL^{-1}) from in vitro gastrointestinal digestion and antiproliferative activities (B) of P17 and P19 (▨, 1 mg mL^{-1}; □; 3 mg mL^{-1}; ■, 5 mg mL^{-1}), and their digestates (3 mg mL^{-1}) from in vitro gastrointestinal digestion against HT-29 human colorectal cancer cells. Results are expressed as means ± standard deviation (n = 3); values within a data set (peptides P17 or P19) with different letters are significantly different ($P < 0.05$).

Antioxidative activity of a peptide depends mainly on amino acid sequence, concentration, and configuration (Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010). Particular amino acid residues such as Asp, Pro, Trp, Tyr, Met, Cys, Leu, Arg, Ala, and His possessing peptides, displayed high antioxidant capacities in various studies (Ji, Sun, Zhao, Xiong, & Sun, 2014; Li, Li, He, & Qian, 2011; Sarmadi & Ismail, 2010). In addition, Furukawa et al. (2012) reported a strong radical scavenging capacity for hydroxyl radicals by peptides containing amino acid residues such as Asp, Cys, Glu, Lys, Pro, Ser, and Trp. Moreover, hydrophobic amino acid residues like Val or Leu can increase the presence of the peptides at the water–lipid interface and therefore facilitate access to scavenge free radicals generated at the lipid phase (Ranathunga, Rajapakse, & Kim, 2006). Chen, Muramoto, and Yamauchi (1995) also reported retardation of linoleic acid peroxidation by peptides containing hydrophobic amino acids (Val or Leu) at the N-terminal positions, and Pro, His, or Tyr in the amino acid sequences. Both P17 and P19 from probiotic yoghurt supplemented with PPP contained one or more of these amino acids, which likely have made these peptides exhibit radical scavenging activities. Antioxidant peptides may provide enhanced nutritional value in addition to chemotherapeutic promise in the management and treatment of various oxidation related diseases and disorders including cancer (Sah et al., 2015a; Sosa et al., 2013).

3.2. Antiproliferative activity of peptides against HT-29 colon cancer cells

In this study, anticancer activity of the peptides has been investigated by assessing their potential to inhibit the proliferation of HT-29 colon cancer cells (Figs. 2B and 3). Both peptides reduced proliferation of HT-29 cells at varying degrees in a dose-dependent manner. As reported in Fig. 2B, P17 and P19 demonstrated antiproliferative activities by 41.49 ± 2.18% and 38.55 ± 1.75% at 3 mg mL^{-1} respectively, comparable with staurosporine at 50 ng mL^{-1} (51.27 ± 3.64%). Both peptides induced early and late apoptosis in HT-29 cells (Fig. 4). The population of total apoptotic cells increased significantly from 13.37 ± 1.00% in blank (negative control) to 28.58 ± 1.42% and 23.53 ± 1.06% in cells treated with P17 and P19, respectively (Fig. 4E), indicating death of HT-29 cells via apoptotic mechanism following exposure to the peptide. Similarly, Wang et al. (2013) reported induction of cell apoptosis in SKOV3 human ovarian cancer cells after exposure to a hexapeptide (Pro-Gly-Pro-Ile-Pro-Asn) derived from bovine β-casein. Moreover, apoptotic pathways activate a cascade of caspases, which involve cleavage of aspartic acid-containing motifs leading to apoptosis induction (Boatright & Salvesen, 2003). Results of multicaspase activity assessed have been presented in Fig. 5. The total caspase activity increased significantly from 13.68 ± 0.74% in negative control (negative control) to 31.33 ± 3.24% and 23.30 ± 1.60% in cells treated with P17 and P19, respectively (Fig. 5E). These results revealed apoptosis induction by the tested peptides via multicaspase activation.

Besides apoptosis, cell cycle arrest (retardation of cell division) is another crucial pathway to inhibit the proliferation of a cancer cell. The examined effect of P17 and P19 on cell cycle progression of HT-29 cells and the proportions of cells in G_0/G_1, S, and G_2/M phases have been presented in Fig. 6. The population of G_2/M increased significantly from 19.85 ± 0.63% in blank (negative control) to 25.18 ± 0.66% and 28.38 ± 1.72% in cells treated with P17 and P19,

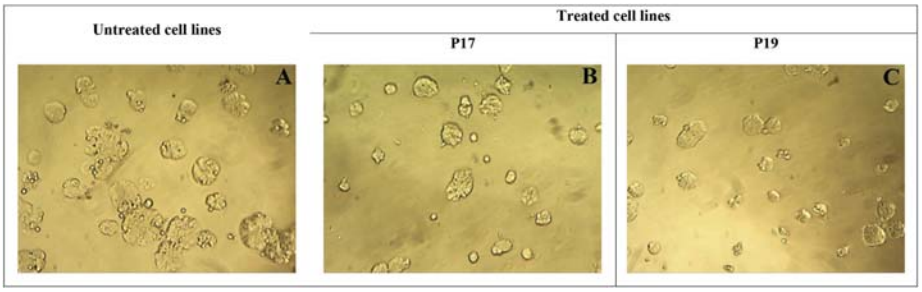

Fig. 3. Images examined under phase-contrast microscopy showing morphological changes in HT-29 human colon cancer cells: (A) untreated control; (B) and (C) treated with P17 and P19, respectively, for 72 h at 37 °C.

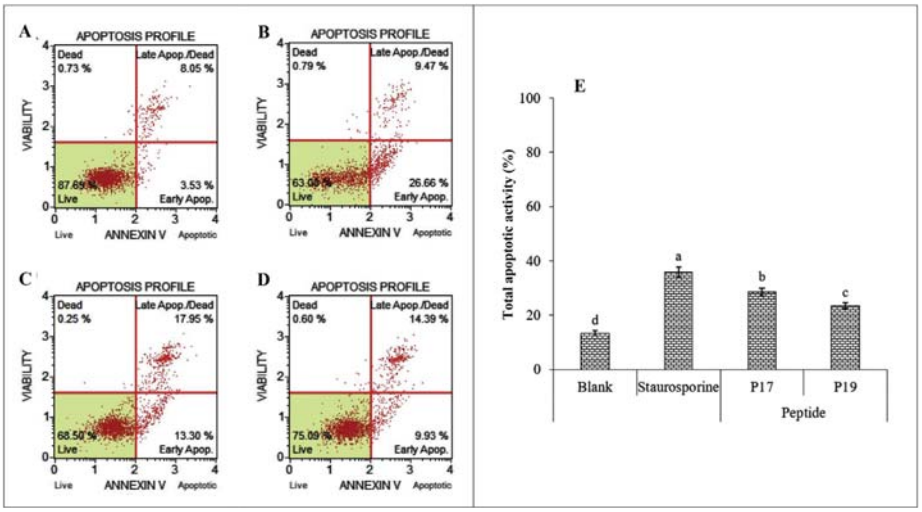

Fig. 4. Peptides P17 and P19 effectively induced apoptosis in HT-29 cells: untreated (A) or treated with (B) staurosporine, (C) peptide P17, and (D) peptide P19. Annexin V and Dead Cell analysis of cells distinguishes cells into four groups: lower left panel for viable cells [Annexin V (−) and 7-AAD (−)]; lower right panel for early apoptotic cells [Annexin V (+) and 7-AAD (−)]; upper right panel for late apoptotic cells [Annexin V (+) and 7-AAD (+)]; and upper left panel for necrotic cells [Annexin V (−) and 7-AAD (+)]. Panel E shows total apoptotic activity induced in HT-29 cells: results are expressed as means ± standard deviation (n = 3); values with different letters are significantly different ($P < 0.05$).

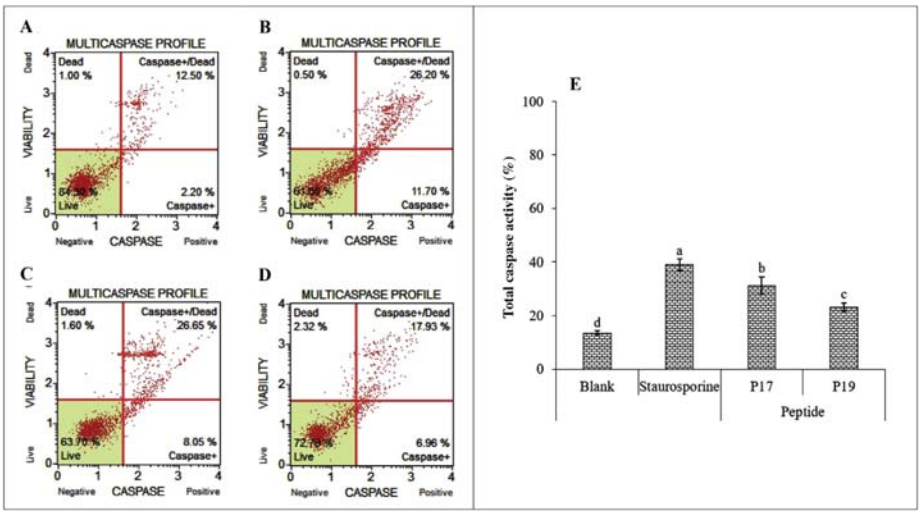

Fig. 5. Peptides P17 and P19 effectively exhibited pan caspase activity in HT-29 cells: untreated (A) or treated with (B) staurosporine, (C) peptide P17, and (D) peptide P19. Multicaspase analysis of cells can distinguish cells into four groups: lower left panel for live cells [Caspase (−) and 7-AAD (−)]; lower right panel for caspase (+) cells exhibiting pan caspase activity [Caspase (+) and 7-AAD (−)]; upper right panel for late stage of caspase activity cells [Caspase (+) and 7-AAD (+)]; and upper left panel for necrotic cells [Caspase V (−) and 7-AAD (+)]. Panel E shows total caspase activity induced in HT-29 cells: results are expressed as means ± standard deviation (n = 3); values with different letters are significantly different ($P < 0.05$).

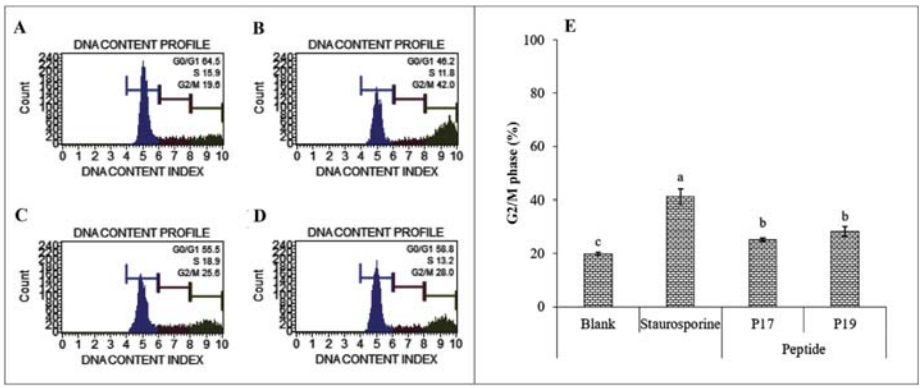

Fig. 6. The cell-cycle distribution (% of cells in G_0/G_1, S, and G_2/M phases) of HT-29 cells: untreated (A), treated with staurosporine (B), peptide P17 (C), and peptide P19 (D). (E) displays cell population at G2/M phase of HT-29 cells. ^{a,b,c}Results were expressed as means ± standard deviation ($n = 3$), values with different letters were significantly different ($P < 0.05$).

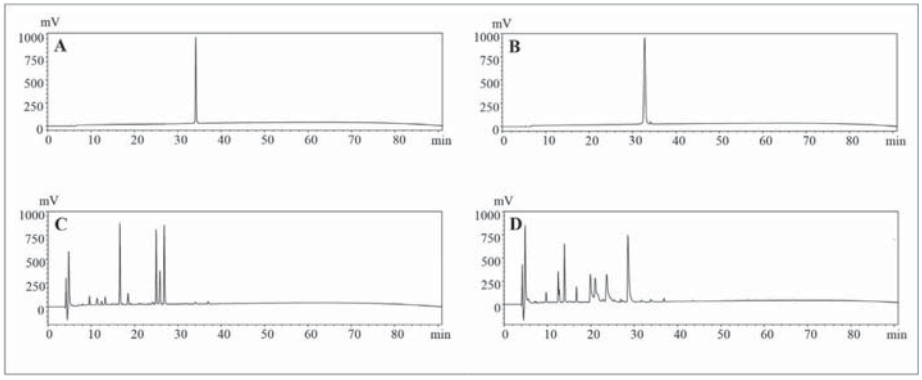

Fig. 7. Peptide profiles of in vitro gastrointestinal digestion: (A) peptide P17; (B) peptide P19; (C) digestate of peptide P17; (D) digestate of peptide P19. Profiles were generated using a reversed phase-high performance liquid chromatography system as described by Sah et al. (2014).

respectively (Fig. 6E), indicating that peptide-treated HT-29 cells were arrested at G_2/M cell cycle transition. Staurosporine similarly showed cell arrest of HT-29 at G_2/M cell cycle transition (Fig. 6). However, De Simone et al. (2009) reported reduction of proliferation of Caco-2 cells caused by peptide extract from waste whey modulating cell cycle at G_1 phase. Collectively, these results indicate a considerable potential of the isolated peptides for use in cancer therapy.

3.3. Stability of peptides during in vitro GI digestion

Peptides must cross the GI barrier, reach circulation and target sites intact to exert their biological activities (Vermeirssen et al., 2002). Proteolytic enzymes of the digestive system might hydrolyse these peptides before being transferred to the basolateral side of the intestinal enterocytes. Features such as molecular weight (Aito-Inoue, Lackeyram, Fan, Sato, & Mine, 2007), hydrophobicity (Tateoka et al., 2001) and charge (Ao & Li, 2013) of a peptide determine its resistance possibility to GI enzymes. Digested P17 exhibited significantly higher ($P < 0.05$) $ABTS^{\bullet+}$ scavenging activity (66.00 ± 1.61%) at 29.88 μg peptide mL^{-1} (Fig. 1). Similarly, digested P19 demonstrated significantly higher ($P < 0.05$) $ABTS^{\bullet+}$ scavenging activity (94.77 ± 0.66%) at 1.44 mg mL^{-1} (Fig. 1). Digested P17 showed significantly higher ($P < 0.05$) $^{\bullet}OH$ scavenging activity (81.64 ± 1.62%) at peptide concentration of 5 mg mL^{-1} compared with undigested P17 (Fig. 2B). Although undigested P19 did not scavenge hydroxyl radicals, when digested showed a high $^{\bullet}OH$ scavenging activity (88.87 ± 1.53%) at 5 mg mL^{-1} (Fig. 2B). The GIT simulation resulted in significantly higher ($P < 0.05$) antiproliferative activities against HT-29 colon cancer cells, 86.63 ± 0.57% by digestate of P17 (at 3 mg mL^{-1}) and 87.60 ± 0.89% by digestate of P19 (at 3 mg mL^{-1}), respectively, compared with their respective undigested samples (Fig. 2B). The increased antioxidant and antiproliferative activities resulted from generation of potent bioactive peptides during further hydrolysis by digestive enzymes (Fig. 7). Peptides identified in the digestate of P17 were YQEPVLGPV, YQEPVLGPVR, YQEPVLGPVRGPFPI, EPVLGPV, LGPVRGPFPI, GPVRGPF, GPVRGPFPI, RGPFPIIV, and GPFPI. Similarly, peptides identified in the digestate of P19 were LPQNIPPLTQT, NIPPL, NIPPLT, NIPPLTQT, NIPPLTQTP, NIPPLTQTPV, NIPPLTQTPVVVPP, NIPPLTQTPVVVPPF, PVVVPP, and PVVVPPF. Several studies also reported both formation and degradation of bioactive peptides during GI digestion (Ao & Li, 2013; Kopf-Bolanz et al., 2014). These findings demand further investigation to isolate and assess bioactivities of each peptide from the digestates of P17 and P19.

4. Conclusions

Peptides derived from bovine β-casein were identified with amino acid sequences ^{193}YQEPVLGPVRGPFPIIV^{209} and ^{69}SLPQNIPPLTQTPVVVPPF^{87}; designated P17 and P19, respectively.

P17 showed high scavenging activity against $ABTS^{\bullet+}$ radicals in a dose-dependent manner with IC_{50} value of 29.88 μg mL^{-1}, compared with P19 (IC_{50} value of 1.44 mg mL^{-1}). Moreover, both peptides also inhibited proliferation of HT-29 human colon cancer cells. In addition, both antioxidant and antiproliferative activities increased significantly during in vitro gastrointestinal digestion, directing to isolate and measure biological activities of individual peptides from the digestates of P17 and P19. The peptides identified could be utilised as a new ingredient in the development of functional foods, nutraceuticals, and pharmaceuticals with the aim of reducing oxidative stress mediated diseases and disorders including cancers. However, in vivo animal trials and human clinical trials are still needed to confirm the full potential of these peptides to exert anticancer activity as observed in vitro in this study.

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References

Aito-Inoue, M., Lackeyram, D., Fan, M. Z., Sato, K., & Mine, Y. (2007). Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane. *Journal of Peptide Science, 13*, 468–474.

Ao, J., & Li, B. (2013). Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion in vitro: Charge properties of peptides affect digestive stability. *Food Research International, 52*, 334–341.

Boatright, K. M., & Salvesen, G. S. (2003). Mechanisms of caspase activation. *Current Opinion in Cell Biology, 15*, 725–731.

Chen, H. M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-conglycinin. *Journal of Agricultural and Food Chemistry, 43*, 574–578.

Collins, A. R. (2005). Antioxidant intervention as a route to cancer prevention. *European Journal of Cancer, 41*, 1923–1930.

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., Vanacore, D., et al. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251–258.

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

Farvin, K. H. S., Baron, C. P., Nielsen, N. S., Otte, J., & Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2 – Characterisation of peptide fractions. *Food Chemistry, 123*, 1090–1097.

Furukawa, T., Akutagawa, T., Funatani, H., Uchida, T., Hotta, Y., Niwa, M., et al. (2012). Cyclic dipeptides exhibit potency for scavenging radicals. *Bioorganic and Medicinal Chemistry, 20*, 2002–2009.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Archives of Toxicology, 86*, 345–391.

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*, 339–347.

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians, 61*, 69–90.

Ji, N., Sun, C., Zhao, Y., Xiong, L., & Sun, Q. (2014). Purification and identification of antioxidant peptides from peanut protein isolate hydrolysates using UHR-Q-TOF mass spectrometer. *Food Chemistry, 161*, 148–154.

Jun, S. Y., Park, P. J., Jung, W. K., & Kim, S. K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology, 219*, 20–26.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2014). Impact of milk processing on the generation of peptides during digestion. *International Dairy Journal, 35*, 130–138.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945–960.

Kudoh, Y., Matsuda, S., Igoshi, K., & Oki, T. (2001). Antioxidative peptide from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO13953. *Journal of the Japanese Society for Food Science and Technology – Nippon Shokuhin Kagaku Kogaku Kaishi, 48*, 44–50.

Li, Y. W., Li, B., He, J., & Qian, P. (2011). Quantitative structure–activity relationship study of antioxidative peptide by using different sets of amino acids descriptors. *Journal of Molecular Structure, 998*, 53–61.

Mielnik, M. B., Aaby, K., & Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned Turkey meat. *Meat Science, 65*, 1147–1155.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food – An international consensus. *Food and Function, 5*, 1113–1124.

Moorthi, C., Manavalan, R., & Kathiresan, K. (2011). Nanotherapeutics to overcome conventional cancer chemotherapy limitations. *Journal of Pharmacy and Pharmaceutical Sciences, 14*, 67–77.

Ortakci, F., & Sert, S. (2012). Stability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in yogurt and in an artificial human gastric digestion system. *Journal of Dairy Science, 95*, 6918–6925.

Phanturat, P., Benjakul, S., Visessanguan, W., & Roytrakul, S. (2010). Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. *LWT – Food Science and Technology, 43*, 86–97.

Ranathunga, S., Rajapakse, N., & Kim, S. K. (2006). Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*). *European Food Research and Technology, 222*, 310–315.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., et al. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry, 108*, 727–736.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2014). Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. *Food Chemistry, 156*, 264–270.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015a). Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: A critical review. *Comprehensive Reviews in Food Science and Food Safety, 14*, 123–138.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015b). Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. *Journal of Dairy Science, 98*, 5905–5916.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016a). Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT - Food Science and Technology, 65*, 978–986.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016b). Effect of pineapple waste powder on probiotic growth, antioxidant and antimutagenic activities of yogurt. *Journal of Food Science and Technology, 53*, 1698–1708.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2016c). Antibacterial and antiproliferative peptides in synbiotic yogurt — Release and stability during refrigerated storage. *Journal of Dairy Science, 99*, 4233–4242.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31*, 1949–1956.

SAS. (1996). *SAS/STAT softwares: Changes and enhancements through release 6.11*. Cary, NC, USA: SAS Institute Inc.

Sharma, N. (2014). Free radicals, antioxidants and disease. *Biology and Medicine, 6*. Article 214.

Sidira, M., Saxami, G., Dimitrellou, D., Santarmaki, V., Galanis, A., & Kourkoutas, Y. (2013). Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool. *Journal of Dairy Science, 96*, 3369–3377.

Siegel, R., Naishadham, D., & Jemal, A. (2012). Cancer statistics, 2012. *CA: A Cancer Journal for Clinicians, 62*, 10–29.

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., & Lleonart, M. E. (2013). Oxidative stress and cancer: An overview. *Ageing Research Reviews, 12*, 376–390.

Tateoka, R., Abe, H., Miyauchi, S., Shuto, S., Matsuda, A., Kobayashi, M., et al. (2001). Significance of substrate hydrophobicity for recognition by an oligopeptide transporter (PEPT1). *Bioconjugate Chemistry, 12*, 485–492.

Vermeirssen, V., Deplancke, B., Tappenden, K. A., van Camp, J., Gaskins, H. R., & Verstraete, W. (2002). Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *Journal of Peptide Science, 8*, 95–100.

Wang, W., Gu, F., Wei, C., Tang, Y., Zheng, X., Ren, M., et al. (2013). PGPIPN, a therapeutic hexapeptide, suppressed human ovarian cancer growth by targeting BCL2. *PloS one, 8*, e60701.

Chapter 8: Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage

Chapter 8 focuses on the influence of pineapple peel powder as a fiber source on the physicochemical, textural, rheological, and microstructural characteristics of set-type yogurt containing *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) during 28 days of refrigerated storage.

The paper entitled "Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage" by B. N. P. Sah, T. Vasiljevic, S. McKechnie, and O. N. Donkor has been published in the peer-reviewed journal "*LWT - Food Science and Technology*" (2016), 65: 978–986. http://dx.doi.org/10.1016/j.lwt.2015.09.027.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: **Signature:** [redacted] **Date:** 15-Mar-2016

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Baidya Nath Prasad Sah	85 %	Design and perform experiment, perform sample analysis, evaluate analytical data, perform statistical analysis, and prepare manuscript
Prof Todor Vasiljevic	5 %	Design experiment, perform statistical analysis, and contribute in writing manuscript
Dr Sandra McKechnie	5 %	Design experiment, and contribute in writing manuscript
Dr Osaana N. Donkor	5 %	Design experiment, contribute in writing manuscript, and submission to journals

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage

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ABSTRACT

Health benefits associated with dietary fibre consumption have shifted its applications towards inclusions into food products. This work aimed at exploring the influence of adding pineapple peel powder (PPP) as a fibre source on physicochemical, textural, rheological, and microstructural attributes of probiotic yogurt during refrigerated storage for 28 days. Skim milk with or without PPP or inulin as a control was fermented by yogurt cultures with or without addition of the probiotic cultures consisting of *Lactobacillus acidophilus* (ATCC® 4356™), *Lactobacillus casei* (ATCC® 393™) and *Lactobacillus paracasei* spp. *paracasei* (ATCC® BAA52™). PPP supplementation at 1% remarkably reduced fermentation time of milk co-fermented with probiotic organisms. Syneresis level in probiotic yogurt with PPP (1.16% at day 1) was comparable with the prebiotic-inulin and increased during storage. However, firmness and storage modulus in both plain and probiotic yogurts were lowered significantly with PPP addition. The reported information on physicochemical and structural attributes of probiotic yogurts with PPP could guide the dairy industry to utilize pineapple peel discarded as processing waste to close the fibre gap by developing a new synbiotic product with comparable attributes to inulin, a commercial prebiotic.

1. Introduction

Most by-products from fruit processing industries are underutilized, which usually leads to economic and environmental issues. Some fibres prepared from these by-products may find applications as functional ingredients since they exhibit functional attributes including swelling, water-holding, gel forming, and cation-exchange abilities (Lamsal & Faubion, 2009). DeVries et al. (2001) defined dietary fibre (DF) as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine". Many epidemiological studies have reported a direct association between consumption of high-fibre diets and a decreased risk of various chronic diseases (Lim, Ferguson, & Tannock, 2005). Regardless of the fact that a deficit of fibre in the dietary plan could also be the reason behind several diet-related diseases, the average consumption continues to be significantly lower than the adequate intake (AI) of 38 and 25 g dietary fibre for men and women respectively, as reported by Institute of Medicine (IOM), the National Academies of Sciences, Engineering, and Medicine, Washington, DC, USA, cited in Gajewska, Bawa, Harton, and Myszkowska-Ryciak (2013). This fact has prompted the consideration that dietary fibre should be included as an ingredient into various food formulations. However, consequences of a fibre addition need to be established since it may induce changes in water-holding capacity, textural, or structural properties of food products like yogurts, which ordinarily have a smooth body and texture (Espírito Santo, Perego, Converti, & Oliveira, 2012; Vasiljevic, Kealy, & Mishra, 2007).

Gel formation is among the basic phenomena occurring during yogurt manufacturing. Yogurt structure is a result of the casein aggregation following a drop in pH due to acid release during fermentation of milk by the starter cultures composed of

Abbreviations: PPP, pineapple peel powder; DF, dietary fibre; CA, cluster analysis; PCA, principal component analysis.

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Streptococcus thermophilus and *Lactobacillus delbrueckii* spp. *bulgaricus*, and reinforced by disulphide crosslinks between denatured whey proteins and κ-casein molecules. Yogurt gel firmness mainly depends on the strength of the three-dimensional network of milk proteins, which is in turn governed by total solids content, acidification rate and proteolytic activity of employed cultures (Lee & Lucey, 2004, 2010; Liu et al., 2014). Furthermore, the yogurt culture may be co-cultured with probiotic bacteria to enhance physiological functionalities of yogurt (Kailasapathy, Harmstorf, & Phillips, 2008). Of the various probiotic strains, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus paracasei* ssp. *paracasei* have been studied extensively and can also be utilized in the generation of bioactive peptides possessing antioxidant, antimutagenic, and anticancer potentials from milk proteins (Sah, Vasiljevic, McKechnie, & Donkor, 2014, 2015a). However, food products sold with any claim of probiotic benefits should meet the criteria of advised minimum number $\geq 10^6$ cfu/g at the time of consumption (Vasiljevic & Shah, 2008). Unfortunately, the growth of some probiotic organisms (e.g. bifidobacteria) is not rapid in milk due to inadequate amount of growth promoting nutrients (Roy, 2005). Consequently, many studies have been conducted to enrich milk with supplements in order to improve the growth of probiotic organisms, which successively offer a chance for innovation. These ingredients, commonly termed prebiotics, improve probiotic growth, which may also lead to alteration in physical, textural, and rheological properties of yogurt (Gustaw, Kordowska-Wiater, & Kozioł, 2011). Prebiotic fortification by adding fibre-rich sources like fruits, grains, and nut presents an excellent model in this regard (Espírito-Santo et al., 2013; Vasiljevic et al., 2007).

Several studies reported pineapple peel powder (PPP) as a good source of dietary fibre, protein, and minerals, capable of accelerating growth and/or activity of the tested *Lactobacilli* (Diaz-Vela, Totosaus, Cruz-Guerrero, & De Lourdes Pérez-Chabela, 2013; Sah, Vasiljevic, McKechnie, & Donkor, 2015b). A further study reported the utilization of pineapple segments containing immobilized *Lactococcus lactis* in prepared yogurt to enhance the probiotic viability during 28 days storage (Phuapaiboon, Leenanon, & Levin, 2013). Whereas the addition of PPP displayed functional merits for probiotic organisms and thereafter for consumers, this effort could influence physicochemical, textural, rheological, and microstructural attributes of yogurt. These attributes play critical roles towards the overall acceptability and sensory perception of the product (Lucey, 2002). Little information is available on effects of pineapple fibre-rich fraction fortification on textural, rheological, and microstructural attributes of set-type probiotic yogurt during storage.

Taking the above into consideration, this study aimed to establish the impact of PPP addition on the physicochemical, textural, rheological, and microstructural characteristics of set-type yogurt containing *Lactobacillus (L.) acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* ssp. *paracasei* (ATCC® BAA52™) during 28 days of refrigerated storage.

2. Materials and methods

2.1. Materials

Streptococcus thermophilus ASCC 1275 (*S. thermophilus*) and *L. delbrueckii* ssp. *bulgaricus* Lb1466 (*L. bulgaricus*) were collected from the Victoria University Culture Collection (Werribee, Australia) whereas *L. acidophilus* ATCC 4356 (*L. acidophilus*), *L. casei* ATCC 393 (*L. casei*) and *L. paracasei* spp. *paracasei* ATCC BAA52 (*L. paracasei*) were purchased from Cell Biosciences Pty Ltd (Heidelberg, Victoria, Australia). Strains were stocked at −80 °C in MRS broth containing 40% glycerol and used to prepare starters according to Sah et al. (2014).

Whole pineapples [*Ananas comosus* (L.) Merrill] were purchased from a local supermarket (Woolworths Limited, Werribee, Australia) and utilized for preparation of pineapple peel powder (PPP) according to (Sah et al., 2015b). Briefly, crushed peel was immersed for 30 min in hot water (90 °C) to inactivate proteolytic enzymes and potential pathogens, freeze-dried, and ground to fine powder. The particle size of powder was standardized to less than 180 μm using sieves (Mesh Series S410/1986; Endecotts Ltd, London, UK) and then sterilized in UV irradiation for 30 min.

2.2. Preparation of yogurts supplemented with prebiotics

Set-type yogurts were prepared as described by Sah et al. (2014). Briefly, three lots of milk bases were prepared by reconstituting skim milk powder procured from a local store (Woolworths Limited, Werribee, Australia) in warm Milli-Q water at 14% (w/v); two lots were further fortified separately with 1% (w/v) inulin Orafti®HP (Beneo-Orafti Ltd., Tienen, Belgium) or PPP. Control (without prebiotic supplementation), inulin and PPP fortified milk bases were homogenized, heated to 85 °C for 30 min, cooled to 45 °C and aseptically inoculated with 1% (v/v) of each *S. thermophilus* and *L. bulgaricus* monocultures. These samples were then divided into two equal portions; one portion was further inoculated with 1% (v/v) of each of *L. acidophilus*, *L. casei* and *L. paracasei* monocultures (Table 1). The final mixes were poured into polystyrene cups, incubated at 42 °C in an incubator until pH of 4.5 ± 0.05 was achieved. Thereafter, the yogurts were immediately cooled and stored for 28 days at 4 °C.

2.3. Determination of pH of yogurt samples

The pH of yogurt samples was recorded at 1st, 14th, and 28th day during storage at 4 °C using WTW inoLab® pH 720 precision pH meter (Weilheim, Germany).

Table 1
Experimental design to evaluate physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage.

Prebiotic and culture combinations of yogurts	
Prebiotic (1% w/v)	Combination of cultures (1% v/v each)
None (Control 1)	*S. thermophilus* + *L. bulgaricus*
Inulin (Orafti®HP)	*S. thermophilus* + *L. bulgaricus*
Pineapple peel powder	*S. thermophilus* + *L. bulgaricus*
None (Control 2)	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Inulin (Orafti®HP)	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*
Pineapple peel powder	*S. thermophilus* + *L. bulgaricus* + *L. acidophilus* + *L. casei* + *L. paracasei*

Prebiotics = pineapple peel powder and inulin; Yogurt culture = *S. thermophilus* + *L. bulgaricus*; Probiotic cultures = *L. acidophilus* + *L. casei* + *L. paracasei*.

2.4. Determination of spontaneous whey separation from yogurt

The level of spontaneous whey separation from undisturbed set yogurt was measured according to the siphon method as explained by Prasanna, Grandison, and Charalampopoulos (2013) applying Eq. (1), where W_i is initial weight (g) of the cup with yogurt and W_f is final weight (g) of the cup with yogurt after whey removal and W_c is weight (g) of the empty cup.

$$\%\ Spontaneous\ whey\ separation = \left(\frac{W_i - W_f}{W_i - W_c}\right) \times 100 \quad (1)$$

2.5. Measurement of yogurt colour

The colour parameters (L*, a*, and b*) of yogurt samples were measured using a chromameter (Model CR-400, Konica Minolta Sensing Inc., Osaka, Japan) as described by Dissanayake, Kelly, and Vasiljevic (2010). The chroma (C*, brightness) is calculated using Eq. (2) and hue angle using Eq. (3).

$$C^* = \left(a^{*^2} + b^{*^2}\right)^{1/2} \quad (2)$$

$$h = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (3)$$

Different values of hue angle represent various colours: 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue.

2.6. Measurement of rheological properties of yogurt during storage at 4 °C

The rheological properties of yogurt supplemented with PPP were characterized as described by Vasiljevic et al. (2007) using a controlled-stress rheometer (Physica MCR 301; Anton Paar, GmbH, Ostfildern, Germany) equipped with a cone and plate geometry (CP50-1, 50 mm diameter, 1° angle, and 49 μm gap) and maintained at 5 ± 1 °C. An aliquot of homogeneously mixed sample was loaded on inset plate, pre-sheared for 30 s at a shear rate of 500 s^{-1}, and followed by equilibration for 300 s to allow for a structural rebuilding of a sample (Purwandari, Shah, & Vasiljevic, 2007). Firstly, frequency sweep test was carried out by performing a frequency ramp from 0.1 to 10 Hz (in log progression with 6 points per decade) at constant strain amplitude of 0.5% (predetermined from amplitude sweep at 10 rad s^{-1}, within linear viscoelastic region). The storage modulus (G′) and loss tangent (G″/G′) at frequency of 1 Hz were reported. Then, the flow behaviour of yogurt samples was evaluated by subjecting a shear rate sweep to the same sample after 10 s of equilibration. Shear rate was first increased from 0.1 to 100 s^{-1} (in log progression with 10 points per decade for upward curve) in 900 s and then decreased from 100 to 0.1 s^{-1} (in log progression with 10 points per decade for downward curve) in another 900 s. Herschel–Bulkley model (Eq. (4)) was fitted to the upward flow curve.

$$Herschel - Bulkley\ model: \quad \sigma = \sigma_0 + \kappa \cdot \dot{\gamma}^n \quad (4)$$

where σ_0, κ and $\dot{\gamma}$ represent yield stress (Pa), consistency index ($Pa \cdot s^n$) and shear rate (s^{-1}), respectively; and n is a dimensionless number known as flow behaviour index ($n = 1$ for Newtonian fluids, $n < 1$ for pseudoplastic fluids, and $n > 1$ for dilatant fluids).

2.7. Determination of firmness of yogurt gel

The firmness of set-type yogurt samples was determined as described by Domagała (2009) using a TA.XTplus texture analyser (Stable Micro Systems, Godalming, UK) with a 25 kg load cell and flat-ended aluminium cylinder probe (SMS P/20). The probe moved down at a pre-test speed of 1 mm/s until a surface trigger force of 5 g and then at a test speed of 1 mm/s into the yogurt sample for a penetration depth of 10 mm and moved up a post-test speed of 10 mm/s. The firmness, which is peak force of compression, was recorded from the force vs time graph and expressed as gram (g).

2.8. Microstructure of yogurt gel

Microstructure of yogurt gel was studied using a scanning electron microscope (SEM) as described by Espírito-Santo et al. (2013) with some modifications. Briefly, yogurt samples stored at 4 °C for 1 day and 28 days were lyophilized using an ALPHA 1–4 LSC CHRIST freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), mounted on an aluminium SEM stub with a double-sided adhesive carbon tape and sputtered with gold (upto 15 nm) using a JEOL NeoCoater (model MP-19020NCTR). Fields of the specimen were examined under a high-vacuum Neo-Scope JCM-5000 benchtop SEM (JEOL Ltd., Tokyo, Japan) and micrographs were recorded.

2.9. Statistical analysis

Experiments were conducted as a randomized split-plot blocked design in time with the types of yogurt as the main plot, and prebiotic supplementation and time as subplots. The design was independently replicated on three occasions with subsequent subsampling resulting in 6 observations. General Linear Model (GLM) was applied to analyse the results for exploration of the effects of probiotic and potential prebiotic (inulin and PPP) addition on attributes of yogurts over time at three levels (1st, 14th and 28th day of storage), and significance was considered at $p < .05$. Correlations among variables were determined by measuring Pearson's correlation coefficients employing the PROC CORR procedure using Statistical Analysis System (SAS, 1996). Hierarchical cluster and principal component analyses were carried out using SPSS 22.0 (SPSS Inc., Chicago, Illinois, USA) for multifactorial comparison.

3. Results and discussion

3.1. Acidification during yogurt production and refrigerated storage

Semisolid texture of yogurt gel is a consequence of the development of a three-dimensional network of milk proteins brought about by reduction of pH. Dissolution of colloidal calcium phosphate and reduction of net negative charge on the casein micelles enhance protein attractions and aggregation. Casein micelles first create strong, covalent bonds with denatured whey proteins, which concomitantly with the pH decline, leads into chain formation and clustering through hydrophobic and electrostatic bonds during fermentation and finally creation of the structure of yogurt (Lee & Lucey, 2004). Rate of acidification is one of the important detriments of yogurt making as it is important to achieve a balance between strength of the gel as well as commercially feasible fermentation time.

The fermentation time needed for the pH of the milk to attain 4.5 in the production of yogurt ranged from 5.67 to 7.75 h (Table 2). Furthermore, the shortest fermentation time was achieved upon addition of PPP (Table 2) likely due to presence of dietary fibre in the powder that provided an additional source of carbohydrates for

Table 2
Yogurt-making time, pH, syneresis, and firmness of yogurt samples manufactured using starter and probiotic cultures in absence or presence of inulin or pineapple peel powder (PPP) during storage at 4 °C for 28 days.

Yogurt types		Fermentation time (h)[§]	pH			Syneresis (%)			Firmness (g)		
Culture	Prebiotics		Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
SC	Control	7.75 ± 0.25a	$4.49^{a,A}$	$4.40^{a,B}$	$4.29^{a,C}$	$1.09^{a,C}$	$1.32^{d,B}$	$2.05^{d,A}$	$86.60^{a,B}$	$96.78^{a,A}$	$95.47^{a,A}$
SC	Inulin	7.50 ± 0.25ab	$4.49^{a,A}$	$4.39^{a,B}$	$4.29^{a,C}$	$1.07^{a,C}$	$1.40^{c,B}$	$2.15^{bc,A}$	$84.79^{ac,A}$	$86.73^{bc,A}$	$86.78^{bc,A}$
SC	PPP	5.83 ± 0.14c	$4.49^{a,A}$	$4.35^{c,B}$	$4.25^{bc,C}$	$1.12^{ab,C}$	$1.63^{b,B}$	$2.31^{a,A}$	$76.55^{bd,A}$	$78.67^{d,A}$	$78.04^{d,A}$
SC + PC	Control	7.17 ± 0.52ab	$4.50^{a,A}$	$4.37^{b,B}$	$4.26^{b,C}$	$1.18^{a,C}$	$1.46^{c,B}$	$2.13^{c,A}$	$85.82^{ac,B}$	$92.22^{ab,A}$	$92.69^{ab,A}$
SC + PC	Inulin	6.75 ± 0.25b	$4.49^{a,A}$	$4.36^{bc,B}$	$4.24^{c,C}$	$1.16^{ab,C}$	$1.65^{b,B}$	$2.20^{b,A}$	$80.56^{bc,A}$	$81.14^{cd,A}$	$82.01^{cd,A}$
SC + PC	PPP	5.67 ± 0.14c	$4.48^{a,A}$	$4.32^{d,B}$	$4.23^{d,C}$	$1.16^{ab,C}$	$1.82^{a,B}$	$2.34^{a,A}$	$73.36^{d,A}$	$71.46^{e,A}$	$76.99^{d,A}$
SEM		–		0.01			0.02			2.14	

SC: Starter culture (*S. thermophilus* + *L. bulgaricus*); PC: Probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*).
Results are expressed as mean of 3 trials.
[§]Fermentation times (means ± SD) represents the time required to reach pH 4.5 during manufacture of plain and probiotic yogurts with or without addition of inulin/pineapple peel powder.
^{a,b,c}Different lowercase superscripts in the same column depict significant differences between means of fermentation times for yogurt types ($p < .05$).
^{a,b,c,d}Different lowercase superscripts in the same column depict the significant difference between means of pH, syneresis and firmness for yogurt types ($p < .05$).
^{A,B,C}Different uppercase superscripts in the same row depict the significant difference between means of pH, syneresis and firmness for same type of yogurt sample at 1st, 14th, and 28th day of refrigerated storage ($p < .05$).
SEM: Pooled standard error of the mean for predetermined $p < .05$.

the probiotic bacteria. Others who used fibre from passion fruit peel (Espírito Santo et al., 2012) and orange fibre García-Pérez et al. (2005) reported similar fermentation times (5–6 h). In all samples, pH reduced significantly ($p < .05$) over the storage period of 28 days at 4 °C and a higher pH decline was observed in PPP fortified yogurt. Prasanna et al. (2013) also observed similar pH changes during refrigerated storage of yogurt-type products. Reduction in fermentation time would be beneficial in yogurt manufacture to reduce the production cost. However, lower yogurt-making time can adversely affect the physicochemical and sensory attributes of set-type yogurts. An accelerated release of colloidal calcium phosphates from casein micelles due to a faster acidification rate induces early release of individual caseins from the micelles facilitating early development of the casein network (Lee & Lucey, 2010). This leads to fast protein aggregation resulting in the formation of a small number of protein–protein bonds and extensive rearrangement of the particles/clusters and consequently a weak gel with large pores and greater whey separation (Lee & Lucey, 2003, 2004, 2010).

3.2. Spontaneous whey separation from yogurt with PPP during storage

Spontaneous syneresis is a major visible defect, appearing as an accumulation of whey on the surface of yogurt gels, and can adversely affect the consumer acceptability of product (Purwandari et al., 2007). All yogurt samples exhibited a varying degree of syneresis (Table 2), with probiotic inclusion enhancing syneresis. Furthermore, PPP or inulin supplemented yogurts displayed greater whey separation compared to unsupplemented control yogurt throughout the storage period, likely due to thermodynamic incompatibility between polysaccharide of PPP/inulin and milk proteins (Grinberg & Tolstoguzov, 1997; Vasiljevic et al., 2007), and/or unbalanced osmotic potential due to depletion flocculation of the casein micelles in the presence of non-adsorbing polymers such as dietary fibre from PPP (Repin, Scanlon, & Gary Fulcher, 2012). A significant ($p < .05$) increase in syneresis was observed during cold storage, which was more pronounced in yogurt with PPP. Lowering of pH from 4.5 to 4.23–4.29 during storage likely resulted in contraction of the casein network and consequently greater whey expulsion owing to the reduction in net negative charge of the casein micelles leading to a decrease in electrostatic repulsion between the charged molecules and increased casein–casein attractions due to enhanced hydrophobic interactions (Lee & Lucey, 2010). Moreover, the magnitude of positive charge on casein molecules increased below its isoelectric pH during storage whereas the magnitude of negative charge on pectin molecules present in the PPP notably decreased below pH 4.5 (Liu, Nakamura, & Corredig, 2006). The negative pectin molecules may bind electrostatically to the positive casein surface, and lead to a uniformly

Table 3
Storage modulus (G′) and loss tangent at frequency of 1 Hz, apparent viscosity at shear rate of 20 s^{-1} ($\eta_{a,20}$), and yield stress (σ_0) calculated using Herschel–Bulkley model of yogurts manufactured using starter and probiotic cultures in absence or presence of inulin or pineapple peel powder (PPP) during storage at 4 °C for 28 days.

Yogurt types		Rheological characteristics											
		G′ (Pa)			Loss tangent			$\eta_{a,20}$ (Pa s)			σ_0 (Pa)		
Culture	Prebiotics	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
SC	Control	$119.72^{a,B}$	$125.33^{ab,B}$	$138.83^{a,A}$	$0.296^{ab,A}$	$0.286^{a,B}$	$0.282^{a,B}$	$0.92^{a,A}$	$0.93^{a,A}$	$0.96^{a,A}$	$4.30^{a,B}$	$4.48^{a,B}$	$5.11^{a,A}$
SC	Inulin	$118.88^{a,B}$	$127.58^{a,B}$	$141.75^{a,A}$	$0.298^{a,A}$	$0.285^{ab,B}$	$0.281^{ab,C}$	$0.89^{ab,B}$	$0.89^{ab,B}$	$0.98^{a,A}$	$4.18^{ab,C}$	$4.78^{a,B}$	$5.77^{a,A}$
SC	PPP	$87.50^{c,B}$	$101.96^{c,A}$	$114.08^{b,A}$	$0.291^{c,A}$	$0.282b^{c,B}$	$0.279^{ab,B}$	$0.72^{c,C}$	$0.82^{b,B}$	$0.93^{a,A}$	$2.95^{d,B}$	$3.13^{c,B}$	$3.66^{c,A}$
SC + PC	Control	$115.49^{ab,B}$	$124.83^{ab,AB}$	$132.17^{a,A}$	$0.293^{bc,A}$	$0.286^{a,B}$	$0.281^{ab,C}$	$0.89^{ab,A}$	$0.88^{ab,A}$	$0.94^{a,A}$	$3.88^{bc,B}$	$4.04^{b,B}$	$4.47^{b,A}$
SC + PC	Inulin	$103.64^{b,B}$	$114.83^{b,B}$	$129.33^{a,A}$	$0.293^{bc,A}$	$0.284^{ab,B}$	$0.277^{b,C}$	$0.82^{b,B}$	$0.85^{b,B}$	$0.94^{a,A}$	$3.80^{c,B}$	$4.06^{b,B}$	$4.79^{b,A}$
SC + PC	PPP	$79.83^{c,B}$	$88.19^{d,AB}$	$105.81^{b,A}$	$0.291^{c,A}$	$0.279^{c,B}$	$0.277^{b,B}$	$0.65^{c,B}$	$0.70^{c,B}$	$0.80^{b,A}$	$2.47^{e,B}$	$2.62^{d,B}$	$3.39^{c,A}$
SEM			4.50			0.001			0.03			0.12	

SC: Starter culture (*S. thermophilus* + *L. bulgaricus*); PC: Probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*).
Results are expressed as mean of 3 trials.
^{a,b,c,d}Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($p < .05$).
^{A,B,C}Different uppercase superscripts in the same row depict the significant difference between means for same type of yogurt sample at 1st, 14th, and 28th day of refrigerated storage ($p < .05$).
SEM: Pooled standard error of the mean for predetermined $p < .05$.

distributed negative charge, which can keep casein molecules apart by electrostatic repulsion. Additionally, many sites of pectin molecules would not interact with the casein micelles. Instead, the electrostatic repulsion and hydrophobic attractions between these parts can disperse casein systems in yogurt leading to a higher whey separation in PPP enriched yogurts during storage. The findings were similar to that reported by García-Pérez et al. (2005) who observed increased syneresis of set-yogurt upon orange fibre addition below 1% and Pimentel, Garcia, and Prudêncio (2012) who also noticed increased syneresis in yogurt supplemented with inulin. Syneresis usually takes place due to loss of yogurt gel ability to entrap water phase owing to weakening of gel structure (Lucey, 2002).

3.3. Firmness of yogurt with PPP during refrigerated storage

Texture is an important attribute of yogurt quality. Yogurt gel structure is the result of casein aggregation by pH reduction and disulphide bonding between κ-caseins and denatured whey proteins. In the current study, the firmness values of yogurt during 28 days storage at 4 °C are presented in Table 2. In general, firmness significantly increased during cold storage. Specifically, PPP incorporation resulted in lower firmness of yogurt, reflecting weak gel attributable to incompatibility between milk proteins and polysaccharides of PPP (Corredig, Sharafbafi, & Kristo, 2011; Grinberg & Tolstoguzov, 1997), and likely enhanced proteolytic activities of probiotic strains owing to PPP supplementation (Sah et al., 2015b). Our observations were in concert with those reported by Tudorica, Jones, Kuri, and Brennan (2004) who fortified milk curd with β-glucans.

A rise in firmness during storage could be related to further pH reduction that likely caused gel structure to shrink, with a consequent elevation of gel strength. The semi-solid yogurt gel resulting from elevated gel strength, may display relatively dynamic network properties that are susceptible to structural rearrangement. These results are in agreement with others (Domagała, 2009). A higher firmness of yogurts has also been related to a longer fermentation time (Damin, Minowa, AlcÂntara, & Oliveira, 2008). In our study, yogurts with longer fermentation time (plain yogurt – control) also displayed greater firmness compared to yogurts with shorter fermentation time (probiotic yogurt with PPP). Pearson's correlation showed a positive but weak correlation between firmness and yield stress ($r = 0.31$, $p < .01$). Similar relation between yield stress and firmness has also been previously noted by Kealy (2006).

3.4. Rheological properties of yogurt with PPP during refrigerated storage

Small amplitude oscillatory shear tests were performed to establish viscoelastic properties of yogurt samples stored at 4 °C for 28 days. All yogurt samples exhibited characteristics typical of weak gels (Table 3). The G′ values ranged from 79.83 to 119.72 Pa at day 1 storage, with lower values for PPP fortified yogurts (Fig. 1A), reflecting formation of a weaker gel with a reduced elastic behaviour, compared to the control. The G′ values increased with storage time for all yogurts. The values for loss tangent ranged from 0.291 to 0.298 at day 1 storage, with again lower values for PPP fortified yogurts compared to the control. Loss tangent of this sample decreased with the storage time, indicating enhanced network elasticity supporting the fact that rearrangement of the gel structures shifted towards a more solid-like behaviour during storage at 4 °C (Table 3).

All yogurts demonstrated non-Newtonian behaviour ($n < 1$; Fig. 1B) and the values of consistency index ranged from 1.49 to 1.81 Pa·s^n at day 1 storage, with lower values for PPP fortified

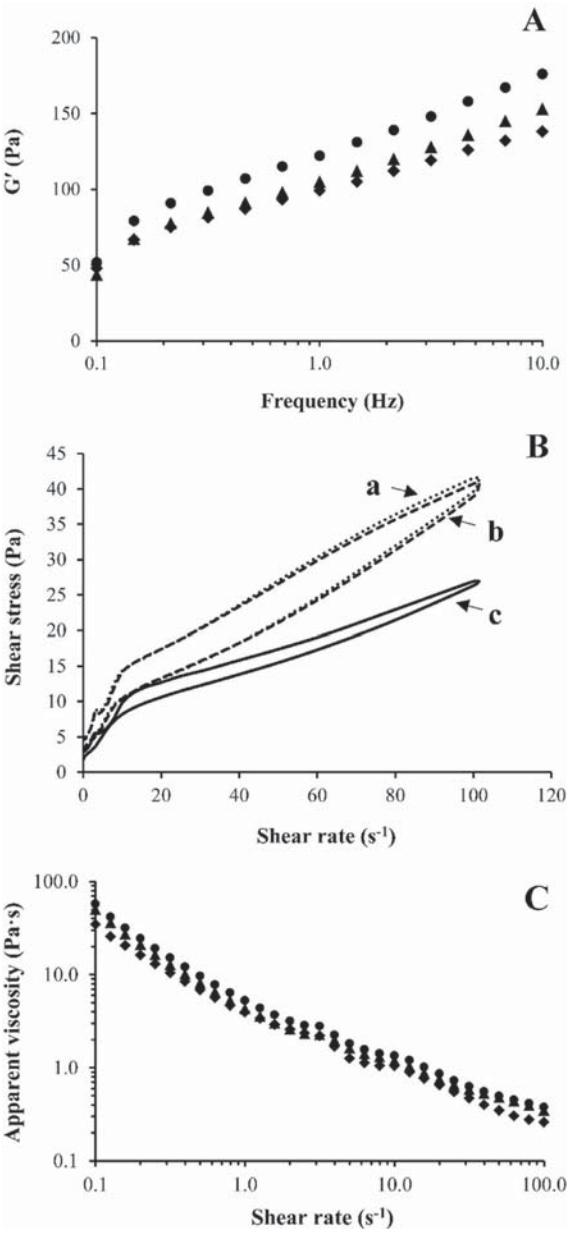

Fig. 1. (A) Storage modulus (G′) values for probiotic yogurts without prebiotic supplementation (●) or with supplementation of inulin (▲) or pineapple peel powder (◆) obtained from small strain oscillation frequency sweep of yogurt samples stored at 4 °C during 1 day. (B) Flow curves (shear stress vs. shear rate) for probiotic yogurts without prebiotic supplementation (a) or with supplementation of inulin (b) or pineapple peel powder (c) stored at 4 °C during 1 day. (C) Apparent viscosity as a function of shear rate for probiotic yogurts without prebiotic supplementation (●) or with supplementation of inulin (▲) or pineapple peel powder (◆) stored at 4 °C during 1 day.

yogurts compared to the control, suggesting a less viscous nature of the fortified product (Bourne, 2002). In addition, the apparent viscosities of yogurt decreased with increasing shear rate during shearing (Fig. 1C) and increased substantially during storage (Table 3). Furthermore, PPP supplemented yogurts displayed significantly lower apparent viscosity values compared to control yogurt without supplementation throughout the storage period. Paseephol, Small, and Sherkat (2008) previously observed similar

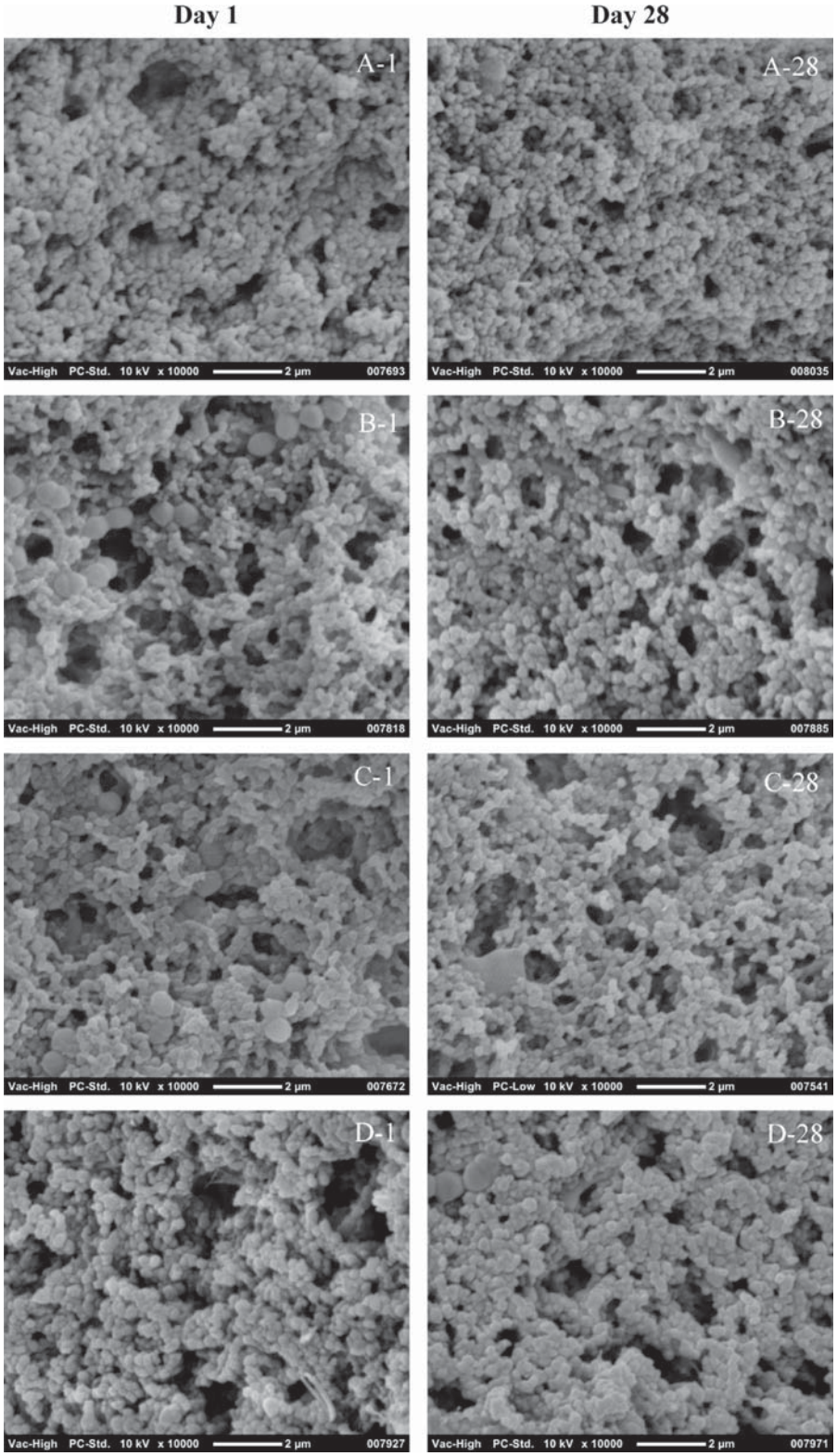

Fig. 2. Scanning electron micrographs of yogurt stored at 4 °C for 28 days. Micrographs A-1, A-28 = plain and B-1, B-28 = probiotic, yogurts at days 1 and 28, both sample sets are without prebiotic supplementation; micrographs C-1, C-28 and D-1, D-28 represent probiotic yogurts supplemented with inulin and pineapple peel powder at days 1 and 28 respectively.

results in a study in which yogurts supplemented with inulin displayed lower values of apparent viscosity compared to non-supplemented control yogurts. The apparent viscosity in our study was positively correlated with yield stress ($r = 0.77$, $p < .01$). Lower values of yield stress for yogurt with PPP or inulin compared to the unsupplemented control (Table 3) also denote a weak network of the yogurt gel (Lee & Lucey, 2010; Lucey, 2001). This is in line with Paseephol et al. (2008) who also reported lower yield stress values of yogurts supplemented with inulin compared to non-supplemented control yogurts.

3.5. Microstructure of yogurt with PPP during refrigerated storage

Micrographs were recorded to visualize changes in the microstructure of yogurt gel and explain modifications in its physical and structural properties because of PPP fortification and refrigerated storage. The micrographs show differences in gel structures such as compactness of the three-dimensional network of casein micelles and pore sizes (Fig. 2A–D). Three-dimensional network of casein micelle aggregation show globular shapes interspaced by void zones of the original serum. Remarkable differences were noticed between the microstructures of non-supplemented and PPP supplemented yogurts. Moreover, presence of the PPP particles were also observed in the micrographs of PPP supplemented yogurt (Fig. 2D). Espírito-Santo et al. (2013) have reported similar results in yogurts enriched with passion fruit fibre. However, a more open network with larger pores has been observed in PPP supplemented probiotic yogurts (Fig. 2D) than the control non-supplemented plain yogurt (Fig. 2A), possibly owing to thermodynamic incompatibility between polysaccharide of PPP and milk proteins (Corredig et al., 2011; Grinberg & Tolstoguzov, 1997). These observations are in line with Lee and Lucey (2003) who reported greater whey separation from a weak yogurt gel, which also had relatively larger pores in the gel network and Tudorica et al. (2004) who also reported enlarged pore sizes and development of a more open network attributable to increasing levels of β-glucans in milk curds. Additionally, the larger pores and reduced cross-linking between casein micelles might explain the reduced firmness and yield stress of structures formed in probiotic yogurt with PPP (Tables 1 and 2). More densely packed casein networks in the micrographs of stored yogurts were visible in day 28 yogurt samples compared to day 1, supporting structural rearrangements during storage resulting in higher values of firmness, shear stress, and storage modulus at the end of storage period.

3.6. Colour of yogurt with PPP during refrigerated storage

Colour is an important attribute in food; it is the first characteristic perceived by the consumers and thus often influences the consumer's preference. Lightness (L*), chroma (C*) and hue angle (h°) of plain and probiotic yogurts fortified with or without inulin/PPP stored at 4 °C for 28 days are presented in Table 4. The colour parameters of yogurts differed with addition of PPP due to its pigmentation and remained unchanged during storage. The L* values of non-supplemented control yogurts were significantly higher than that of PPP supplemented yogurts throughout the storage period ($p < .05$). Colour intensity (C*) increased with colour perception (h°) shifting towards yellow owing to PPP fortification. On the other hand, fortification of yogurt by inulin produced no substantial effect as observed by L*, C* and hue values because inulin has no colour/white and blends in with yogurt colour.

3.7. Effect of PPP addition on overall characteristics of probiotic yogurts

Cluster analysis (CA) was carried out using the hierarchical clustering method with Ward's linkage and two clusters were identified based on similarities in the syneresis, firmness, storage modulus, loss tangent, yield stress, consistency index, flow behaviour index and apparent viscosity of stored yogurts at day 1 of the storage period (Fig. 3A). Plain and probiotic yogurts with PPP (numbers 3, 6) arranged in a separate cluster, reflected remarkable influences of PPP on overall characteristics of yogurts.

Principal component analysis was also conducted and two interpretable components were chosen based on the Kaiser's criterion of eigenvalues greater than 1.0. The scores plot (Fig. 3B) displayed the relationships among overall observations for yogurts in two principal components; the first component showed higher percentage variance (60.74%) than the second component (27.55%). Samples were grouped in four distinct groups: yogurts fermented using starter culture only, at the top left and right quadrants while yogurts fermented using both starter and probiotic cultures, at the bottom left and right. Distributions of yogurts supplemented with PPP only at the top and bottom left quadrants confirmed the influence of PPP fortification on yogurt characteristics.

4. Conclusions

The influence of PPP addition to milk formulations on physicochemical, textural, rheological, and microstructural characteristics

Table 4
Colour-space parameters of yogurts manufactured using starter and probiotic cultures in absence or presence of inulin or pineapple peel powder (PPP) during storage at 4 °C for 28 days.

Yogurt types		Colour-space parameters								
		L*			Chroma (C*)			Hue angle (h°)		
Culture	Prebiotics	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
SC	Control	$89.88^{a,B}$	$91.54^{ab,A}$	$90.79^{ab,A}$	$11.93^{b,A}$	$11.76^{b,A}$	$11.47^{c,A}$	$110.47^{a,A}$	$110.89^{a,A}$	$110.37^{a,A}$
SC	Inulin	$90.09^{a,A}$	$90.93^{ab,A}$	$89.92^{b,A}$	$12.46^{b,A}$	$11.75^{b,AB}$	$11.36^{c,B}$	$110.39^{a,A}$	$111.58^{a,A}$	$111.35^{a,A}$
SC	PPP	$84.53^{b,A}$	$84.90^{c,A}$	$84.84^{d,A}$	$19.16^{a,A}$	$19.10^{a,A}$	$18.57^{a,A}$	$100.31^{b,A}$	$100.46^{b,A}$	$100.91^{c,A}$
SC + PC	Control	$90.43^{a,B}$	$91.82^{a,A}$	$91.54^{a,AB}$	$12.30^{b,A}$	$12.20^{b,A}$	$11.94^{bc,A}$	$108.93^{a,AB}$	$109.65^{a,A}$	$107.53^{b,B}$
SC + PC	Inulin	$90.31^{a,A}$	$90.52^{b,A}$	$89.71^{b,A}$	$12.15^{b,A}$	$12.23^{b,A}$	$12.43^{b,A}$	$109.02^{a,A}$	$110.12^{a,A}$	$110.02^{a,A}$
SC + PC	PPP	$85.01^{b,A}$	$85.41^{c,A}$	$86.20^{c,A}$	$19.02^{a,A}$	$18.54^{a,A}$	$18.64^{a,A}$	$100.59^{b,A}$	$100.80^{b,A}$	$100.95^{c,A}$
SEM			0.46			0.26			0.72	

SC: Starter culture (*S. thermophilus* + *L. bulgaricus*); PC: Probiotic culture (*L. acidophilus* + *L. casei* + *L. paracasei*).
Results are expressed as mean of 3 trials.
^{a,b,c,d}Different lowercase superscripts in the same column depict the significant difference between means for yogurt types ($p < .05$).
^{A,B,C}Different uppercase superscripts in the same row depict the significant difference between means for same type of yogurt sample at 1st, 14th, and 28th day of refrigerated storage ($p < .05$).
SEM: Pooled standard error of the mean for predetermined $p < .05$.

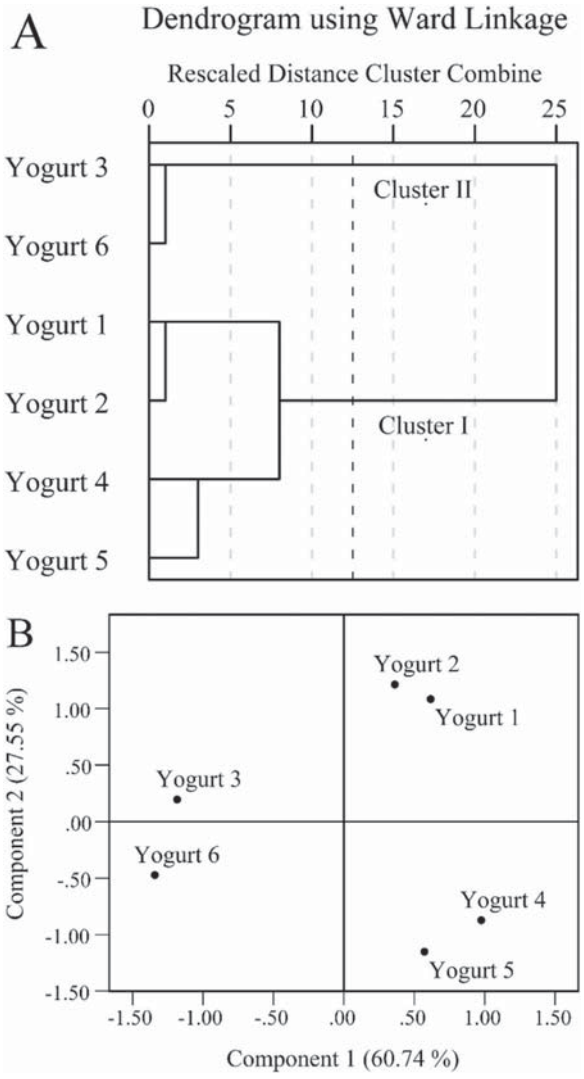

Fig. 3. Dendrogram (A) showing the clustering of six yogurt types based on similarities of measured attributes, and score plot (B) of the first two principal components of the measured attributes of yogurts stored at 4 °C during 1 day. The measured attributes were syneresis (%), firmness (g), storage modulus (Pa), loss tangent, yield stress (Pa), consistency index ($Pa \cdot s^n$), flow behaviour index and apparent viscosity (Pa·s). Yogurts denoted 1, 2 and 3 were fermented with the starter culture only; yogurt 1 without supplementation while yogurts 2 and 3 were supplemented with inulin, and pineapple peel powder (PPP), respectively. Yogurts denoted 4, 5 and 6 were fermented with both starter and probiotic cultures; yogurt 4 without supplementation while yogurts 5 and 6 with supplementation of inulin and PPP, respectively.

of set-type yogurt was investigated. Addition of PPP and probiotic organism substantially reduced yogurt-making time, which could be beneficial for manufacturing to cut cost of production. A significant reduction in pH of yogurts was also observed during storage and more pronounced in probiotic yogurts with PPP. PPP fortification apparently affected textural and rheological properties of the yogurt, similar to that of inulin, an established prebiotic. Firmness and storage modulus of yogurts were significantly increased during cold storage. These results were in agreement with the microstructural observations. Colour intensity increased with colour perception shifting towards a yellow colour, attributable to PPP addition. Both fibre and probiotic organisms are well known for their health advantages, and together they could boost the functional attributes of yogurts with industrial applications. However, these findings demand further studies to evaluate consumer acceptance of yogurt supplemented with PPP.

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References

Bourne, M. C. (2002). Chapter 3-physics and texture. In M. C. Bourne (Ed.), *Food texture and viscosity* (2nd ed., pp. 59–106). London: Academic Press.
Corredig, M., Sharafbafi, N., & Kristo, E. (2011). Polysaccharide-protein interactions in dairy matrices, control and design of structures. *Food Hydrocolloids, 25*(8), 1833–1841. http://dx.doi.org/10.1016/j.foodhyd.2011.05.014.
Damin, M. R., Minowa, E., AlcÂntara, M. R., & Oliveira, M. N. (2008). Effect of cold storage on culture viability and some rheological properties of fermented milk prepared with yogurt and probiotic bacteria. *Journal of Texture Studies, 39*(1), 40–55. http://dx.doi.org/10.1111/j.1745-4603.2007.00129.x.
DeVries, J. W., Camire, M. E., Cho, S., Craig, S., Gordon, D., Jones, J. M., et al. (2001). *The definition of dietary fiber* (Vol. 46, pp. 112–126). Cereal Foods World.
Diaz-Vela, J., Totosaus, A., Cruz-Guerrero, A. E., & De Lourdes Pérez-Chabela, M. (2013). *In vitro* evaluation of the fermentation of added-value agroindustrial by-products: Cactus pear (*Opuntia ficus-indica* L.) peel and pineapple (*Ananas comosus*) peel as functional ingredients. *International Journal of Food Science and Technology, 48*(7), 1460–1467. http://dx.doi.org/10.1111/ijfs.12113.
Dissanayake, M., Kelly, A. L., & Vasiljevic, T. (2010). Gelling properties of micro-particulated whey proteins. *Journal of Agricultural and Food Chemistry, 58*(11), 6825–6832. http://dx.doi.org/10.1021/jf1009796.
Domagała, J. (2009). Instrumental texture, syneresis and microstructure of yoghurts prepared from goat, cow and sheep milk. *International Journal of Food Properties, 12*(3), 605–615. http://dx.doi.org/10.1080/10942910801992934.
Espírito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2012). Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yoghurts. *LWT – Food Science and Technology, 47*(2), 393–399. http://dx.doi.org/10.1016/j.lwt.2012.01.038.
Espírito-Santo, A. P., Lagazzo, A., Sousa, A. L. O. P., Perego, P., Converti, A., & Oliveira, M. N. (2013). Rheology, spontaneous whey separation, microstructure and sensorial characteristics of probiotic yoghurts enriched with passion fruit fiber. *Food Research International, 50*(1), 224–231. http://dx.doi.org/10.1016/j.foodres.2012.09.012.
Gajewska, D., Bawa, S., Harton, A., & Myszkowska-Ryciak, J. (2013). Dietary fibre intake among different groups of patients as compared to recommendations. *Agro Food Industry Hi-Tech, 24*(6), 26–29.
García-Pérez, F. J., Lario, Y., Fernández-López, J., Sayas, E., Pérez-Alvarez, J. A., & Sendra, E. (2005). Effect of orange fiber addition on yogurt color during fermentation and cold storage. *Color Research and Application, 30*(6), 457–463. http://dx.doi.org/10.1002/col.20158.
Grinberg, V. Y., & Tolstoguzov, V. B. (1997). Thermodynamic incompatibility of proteins and polysaccharides in solutions. *Food Hydrocolloids, 11*(2), 145–158. http://dx.doi.org/10.1016/S0268-005X(97)80022-7.
Gustaw, W., Kordowska-Wiater, M., & Kozioł, J. (2011). The influence of selected prebiotics on the growth of lactic acid bacteria for bio-yoghurt production. *Acta Scientiarum Polonorum, Technologia Alimentaria, 10*(4), 455–466.
Kailasapathy, K., Harmstorf, I., & Phillips, M. (2008). Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred fruit yogurts. *LWT – Food Science and Technology, 41*(7), 1317–1322. http://dx.doi.org/10.1016/j.lwt.2007.08.009.
Kealy, T. (2006). Application of liquid and solid rheological technologies to the textural characterisation of semi-solid foods. *Food Research International, 39*(3), 265–276. http://dx.doi.org/10.1016/j.foodres.2005.07.016.
Lamsal, B. P., & Faubion, J. M. (2009). The beneficial use of cereal and cereal components in probiotic foods. *Food Reviews International, 25*(2), 103–114. http://dx.doi.org/10.1080/87559120802682573.
Lee, W. J., & Lucey, J. A. (2003). Rheological properties, whey separation, and microstructure in set-style yogurt: effects of heating temperature and incubation temperature. *Journal of Texture Studies, 34*(5–6), 515–536. http://dx.doi.org/10.1111/j.1745-4603.2003.tb01079.x.
Lee, W. J., & Lucey, J. A. (2004). Structure and physical properties of Yogurt gels: effect of inoculation rate and incubation temperature. *Journal of Dairy Science, 87*(10), 3153–3164. http://dx.doi.org/10.3168/jds.S0022-0302(04)73450-5.
Lee, W. J., & Lucey, J. A. (2010). Formation and physical properties of yogurt. *Asian-Australasian Journal of Animal Sciences, 23*(9), 1127–1136. http://dx.doi.org/10.5713/ajas.2010.r.05.
Lim, C. C., Ferguson, L. R., & Tannock, G. W. (2005). Dietary fibres as "prebiotics": implications for colorectal cancer. *Molecular Nutrition and Food Research, 49*(6), 609–619. http://dx.doi.org/10.1002/mnfr.200500015.
Liu, J., Nakamura, A., & Corredig, M. (2006). Addition of pectin and soy soluble polysaccharide affects the particle size distribution of casein suspensions

prepared from acidified skim milk. *Journal of Agricultural and Food Chemistry, 54*(17), 6241–6246. http://dx.doi.org/10.1021/jf060113n.
Liu, X. T., Zhang, H., Wang, F., Luo, J., Guo, H. Y., & Ren, F. Z. (2014). Rheological and structural properties of differently acidified and renneted milk gels. *Journal of Dairy Science, 97*(6), 3292–3299. http://dx.doi.org/10.3168/jds.2013-7568.
Lucey, J. A. (2001). The relationship between rheological parameters and whey separation in milk gels. *Food Hydrocolloids, 15*(4–6), 603–608. http://dx.doi.org/10.1016/S0268-005X(01)00043-1.
Lucey, J. A. (2002). Formation and physical properties of milk protein gels. *Journal of Dairy Science, 85*(2), 281–294. http://dx.doi.org/10.3168/jds.S0022-0302(02)74078-2.
Paseephol, T., Small, D. M., & Sherkat, F. (2008). Rheology and texture of set yogurt as affected by inulin addition. *Journal of Texture Studies, 39*(6), 617–634. http://dx.doi.org/10.1111/j.1745-4603.2008.00161.x.
Phuapaiboon, P., Leenanon, B., & Levin, R. E. (2013). Effect of lactococcus lactis immobilized within pineapple and yam bean segments, and Jerusalem Artichoke powder on its viability and quality of Yogurt. *Food and Bioprocess Technology, 6*(10), 2751–2762. http://dx.doi.org/10.1007/s11947-012-0940-4.
Pimentel, T. C., Garcia, S., & Prudêncio, S. H. (2012). Effect of long-chain inulin on the texture profile and survival of *Lactobacillus paracasei* ssp. *paracasei* in set yoghurts during refrigerated storage. *International Journal of Dairy Technology, 65*(1), 104–110. http://dx.doi.org/10.1111/j.1471-0307.2011.00739.x.
Prasanna, P. H. P., Grandison, A. S., & Charalampopoulos, D. (2013). Microbiological, chemical and rheological properties of low fat set yoghurt produced with exopolysaccharide (EPS) producing *Bifidobacterium* strains. *Food Research International, 51*(1), 15–22. http://dx.doi.org/10.1016/j.foodres.2012.11.016.
Purwandari, U., Shah, N. P., & Vasiljevic, T. (2007). Effects of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological and rheological properties of set-type yoghurt. *International Dairy Journal, 17*(11), 1344–1352. http://dx.doi.org/10.1016/j.idairyj.2007.01.018.
Repin, N., Scanlon, M. G., & Gary Fulcher, R. (2012). Phase behaviour of casein micelles and barley beta-glucan polymer molecules in dietary fibre-enriched dairy systems. *Journal of Colloid and Interface Science, 377*(1), 7–12. http://dx.doi.org/10.1016/j.jcis.2012.02.021.
Roy, D. (2005). Technological aspects related to the use of *Bifidobacteria* in dairy products. *Lait, 85*(1–2), 39–56. http://dx.doi.org/10.1051/lait:2004026.
Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2014). Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. *Food Chemistry, 156*, 264–270. http://dx.doi.org/10.1016/j.foodchem.2014.01.105.
Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015a). Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: a critical review. *Comprehensive Reviews in Food Science and Food Safety, 14*(2), 123–138. http://dx.doi.org/10.1111/1541-4337.12126.
Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2015b). Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. *Journal of Dairy Science, 98*(9), 5905–5916. http://dx.doi.org/10.3168/jds.2015-9450.
SAS. (1996). *SAS/STAT softwares: Changes and enhancements through release 6.11.* Cary, NC: SAS Institute Inc.
Tudorica, C. M., Jones, T. E. R., Kuri, V., & Brennan, C. S. (2004). The effects of refined barley β-glucan on the physico-structural properties of low-fat dairy products: curd yield, microstructure, texture and rheology. *Journal of the Science of Food and Agriculture, 84*(10), 1159–1169. http://dx.doi.org/10.1002/jsfa.1789.
Vasiljevic, T., Kealy, T., & Mishra, V. K. (2007). Effects of β-glucan addition to a probiotic containing yogurt. *Journal of Food Science, 72*(7), C405–C411. http://dx.doi.org/10.1111/j.1750-3841.2007.00454.x.
Vasiljevic, T., & Shah, N. P. (2008). Probiotics- from Metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714–728. http://dx.doi.org/10.1016/j.idairyj.2008.03.004.

Chapter 9: Conclusions and future directions

9.1 Overall conclusions

Examined probiotic organisms exhibited a statistically greater proteolytic activity in yogurt. The organisms enhanced the generation of peptides with potential antioxidant and antimutagenic properties which showed good correlation between proteolytic and antioxidant or antimutagenic activities. Probiotic yogurt containing all three bacteria strains [*Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™), and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™)] produced the highest degree of protein hydrolysis, and exhibited the strongest antioxidant and antimutagenic activities.

The net probiotic growth in customized MRS broth containing pineapple peel or pomace powders as a direct carbohydrate source were comparable to MRS broth containing glucose, indicating prebiotic properties of pineapple waste. The addition of pineapple powders into yogurt also resulted in increased antioxidant and antimutagenic activities compared to the non-supplemented yogurt, suggesting the possibility of commercial application of pineapple waste in functional food formulations.

Improved growth and retention of viability of *L. acidophilus*, *L. casei*, and *L. paracasei* was observed during refrigerated storage at 4 °C for 28 days in synbiotic yogurt formulations with added pineapple peel powder (PPP). The degree of proteolysis in synbiotic yogurts was significantly higher than plain yogurts and increased substantially during storage. All water-soluble peptide extracts (WSPEs) prepared from yogurt samples possessed antimutagenic activity, which increased during storage. In addition, the WSPEs exhibited excellent antioxidant properties measured through *in vitro* assays employing $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $^{\bullet}OH$ free radicals. However, WSPE of the probiotic yogurt with PPP exhibited the most potent antimutagenic and antioxidant activities.

WSPEs prepared from yogurt samples possessed antibacterial activities against Gram-positive and Gram-negative bacteria, which increased during storage. Moreover, the inhibitory activity was greater against Gram-negative compared to Gram-positive bacteria. In addition, the WSPEs also showed potential for inhibition of the proliferation of HT29 human colon cancer cells. Generation of inhibitory peptides against bacterial and cancer cells was improved owing to PPP supplementation in

yogurt. Remarkably, WSPE of the probiotic yogurt with PPP exhibited the most potent antibacterial and anti-colon cancer activities than control and these activities were maintained during storage. However, the activities lowered substantially during *in vitro* GI digestion suggesting degradation of bioactive peptides of the WSPE due to hydrolysis by the GIT enzymes.

Two peptides with potent $ABTS^{\bullet+}$ radical scavenging capacity were purified from the WSPE of stored probiotic yogurt with PPP for 28 days at 4 °C by employing ultrafiltration and chromatographic techniques. The amino acid sequences identified were ^{193}YQEPVLGPVRGPFPIIV^{209} (P17), ^{69}SLPQNIPPLTQTPVVVPPF^{87} (P19) derived from bovine β-casein. P17 showed high scavenging activity against $ABTS^{\bullet+}$ radicals in a dose-dependent manner with IC_{50} value of 29.88 μg/mL, compared to P19 (IC_{50} value of 1.44 mg/mL). Moreover, both peptides also inhibited proliferation of HT-29 human colon cancer cells by inducing apoptosis and arresting cell cycle in G_2/M phase. In addition, both antioxidant and antiproliferative activities increased significantly during *in vitro* GI digestion, directing to isolate and measure biological activities of individual peptides from the digestates of P17 and P19.

Addition of PPP and probiotic organism substantially reduced yogurt-making time, which could be beneficial for manufacturing by cutting production cost. A significant reduction in pH of yogurts was also observed during storage and more pronounced in probiotic yogurts with PPP. PPP fortification apparently affected textural and rheological properties of the yogurt, similar to that of inulin, an established prebiotic. Firmness and storage modulus of yogurts were significantly increased during cold storage. These results were in agreement with the microstructural observations. Color intensity increased with color perception shifting towards a yellow color, attributable to PPP addition.

Pineapple peel, a by-product of juice production, could be proposed as a prebiotic ingredient in the manufacture of yogurts with enhanced nutrition, and functionality. Identified peptides could be utilized as a new ingredient in the development of functional foods, nutraceuticals, and pharmaceuticals with the aim of reducing oxidative stress mediated diseases and disorders including cancer.

9.2 Future directions

Cancer is the most widely recognized reason for human deaths globally. Conventional anticancer therapies, including chemotherapy and radiation, are very costly and induce severe side effects on the individual. The discovery of natural anticancer compounds like peptides may thus be a better alternative for cancer prevention and management. Milk proteins are considered a natural reservoir of peptides possessing bioactivities, with the number of identified dairy-derived peptides increasing. Thus, selected probiotic organisms: *Lactobacillus acidophilus* (ATCC® 4356™), *Lactobacillus casei* (ATCC® 393™) and *Lactobacillus paracasei* subsp. *paracasei* (ATCC® BAA52™) with powerful proteolytic systems may open future opportunities to developing functional foods with potential health promoting properties.

Antibacterial and anticancer peptides should also be explored as potential alternatives for fighting infectious diseases and in cancer therapy either alone or in combination with other conventional compounds. In addition, both fiber, and probiotic organisms are well known for their health advantages, and together they could boost the functional attributes of yogurts with industrial applications. However, these findings demand further studies to evaluate consumer acceptance of yogurt supplemented with PPP. Establishing the stability of bioactive peptides during refrigerated storage would add to the expanding functional food market as well. Furthermore, clinical trials with animal and human subjects are still essential to confirm the anticancer effects of the isolated peptides.