School of Public Health
Nutrition, Dietetics and Food Science

Effect of yoghurt processing on beta-casomorphin 5 and beta-casomorphin 7 concentrations using novel liquid chromatography-mass spectrometry methods

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 29/06/2015

I dedicate this PhD to my father Duan and stepmother Nhung, my wife Trang and my daughters An and My.

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ABSTRACT

The main objectives of this research project were to measure the concentration of beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in milk and yoghurt and determine the effect of yoghurt processing on their concentration using novel liquid chromatography-mass spectrometry (LC-MS) methods. The thesis consists of three main parts: 1) Development of novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS) methods for simultaneous identification and quantification of BCM5 and BCM7; 2) Measurement of BCM5 and BCM7 in commercial dairy products; and 3) Effect of yoghurt processing on BCM5 and BCM7 concentrations. In this research project, commercial sandwich ELISA was also used for determination of BCM7 during yoghurt processing.

The initial study was to develop a LC-MS/MS method for identification and quantitation of BCM5 and BCM7 in yoghurt and milk. Stable isotopes labelled BCM5-d_{10} and BCM7-d_{10} were used as internal standards. Samples were centrifuged and cleaned-up by 0.45 µm membranes and then dried until 1 mL under nitrogen flow, before LC-MS/MS analysis. Linearity (R^2) observed in the range 0.01–10 ng/µL were 0.9985 and 0.9986 for BCM5 and BCM7, respectively. Limits of detection (LODs) were found to be 0.5 ng/g for BCM5 and 0.25 ng/g for BCM7. LODs improvement was achieved during sample preparation by centrifuging and cleaning-up supernatants using solid phase extraction (SPE) instead of filtering through 0.45 µm membranes and subsequently drying extracts until 0.5 mL under nitrogen flow. The concentrated extracts were injected to LC-MS/MS and LC-HRMS. LODs of LC-MS/MS method were 0.4 ng/g and 0.2 ng/g, while LODs of LC-HRMS were 0.25 ng/g and 0.1 ng/g for BCM5 and BCM7, respectively.

Measurement of BCM5 and BCM7 content in 14 samples of Australian pasteurised milk, one sample of skim milk powder and ten samples of yoghurt were performed using novel LC-MS/MS and LC-HRMS methods. Neither BCM5 nor BCM7 were found in Australian commercial yoghurts. BCM5 was also below limit of detection (LOD) in all samples of pasteurised milk and skim milk powder. BCM7 was found between 0.13 and 2.38 ng/g in ten samples of pasteurised milk and skim milk powder. However, BCM7 content in four other samples of pasteurised milk was below LOD.

Effect of fermentation and storage on the degradation of BCM5 and BCM7 was conducted by measuring the variation in BCM5 and BCM7 concentrations during these processes. Yoghurt was produced by fermentation of reconstituted milk with a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* to pH 4.8 and 4.5 and then stored for one and seven days. BCM5 was below LOD at any fermentation pH and after any period of storage. BCM7 content decreased from 1.4 ng/g to 0.29 ng/g when reconstituted milk was fermented from pH 6.5 to pH 4.8, and completely degraded as prolonged fermentation to pH 4.5. BCM7 content in yoghurt produced by milk fermentation with these yoghurt cultures to pH 4.8 decreased from 0.29 ng/g to 0.17 ng/g after one day of storage and completely degraded after seven days of storage. Yoghurt produced by fermentation of milk to pH 4.5 contained BCM7 below LOD after any time of storage.

In addition, effect of yoghurt cultures on the degradation of BCM5 and BCM7 was also conducted in this research project. Synthesised BCM5 and BCM7 were spiked (40 ng/g) into ultra-high temperature (UHT) milk to investigate the remaining of these BCMs in yoghurt. Yoghurt was produced by inoculation of UHT with *L. delbrueckii* ssp. *bulgaricus* alone, *S. thermophilus* alone or a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus,* and fermented to pH 4.5, which is a characteristic of final yoghurt, and then stored for one and seven days. Both BCMs were completely degraded in milk fermented to pH 4.5 and below LOD in yoghurt after any time of storage. The degradation of BCM5 and BCM7 in yoghurt processing is likely to be a result of X-prolyl dipeptidyl aminopeptidase (PepX) activity, which was produced by both *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. However, the degradation of BCM7 is due to the presence of yoghurt cultures, while the stability of BCM5 is affected by yoghurt cultures and pH.

The applicability of a sandwich enzyme-linked immunesorbent assay (ELISA) test for quantitation of BCM7 in yoghurt was validated using the LC-MS/MS method. A sandwich ELISA test and LC/MS/MS were used to determine the BCM7 content in the samples mentioned. Two methods showed two different results of BCM7 content. Using LC-MS/MS, BCM7 content was found to be 1.4 ng/g, while it was 3.37×10^{-3} ng/g using a sandwich ELISA test. LC-MS/MS showed that BCM7 content decreased during fermentation from 1.4 ng/g in reconstituted milk to 0.29 ng/g and below LOD in milk fermented to pH 4.8 and pH 4.5, respectively. BCM7 content in

yoghurt produced by fermentation of milk to pH 4.8 decreased to 0.17 ng/g after one day of storage and to below LOD after seven days of storage. In contrast, ELISA data showed that BCM7 content increased during fermentation from 3.37×10^{-3} ng/g in reconstituted milk to 3.52×10^{-3} ng/g and to 12.22×10^{-3} ng/g in milk fermented to pH 4.8 and then pH 4.5, respectively. In yoghurt fermented to pH 4.8, BCM7 content increased to 3.99×10^{-3} ng/g and 12.78×10^{-3} ng/g after one and seven days of storage. After one day of storage, BCM7 in yoghurt fermented to pH 4.5 decreased very slightly to 12.19×10^{-3} ng/g; however, its content dramatically decreased to 2.19×10^{-3} ng/g after seven days of storage. The results suggest that commercial sandwich ELISA test should not be used for quantitation of BCM7 in yoghurt and milk.

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ACE-I	Angiotensin-converting enzyme inhibitory
Ala	Alanine
A*m*	Area of BCM5 or BCM7 added in a yoghurt blank extract-matrix
Arb	Arbitrary
Arg	Arginine
A*s*	Area of BCM5 or BCM7 added in ultrapure water-standard solution
Asn	Asparagine
Asp	Aspartic acid
BCM	Beta-cacomorphin
BCM10	Beta-cacomorphin 10
BCM11	Beta-cacomorphin 11
BCM13	Beta-casomorphin 13
BCM4	Beta-cacomorphin 4
BCM5	Beta-cacomorphin 5
BCM5-d_{10}	Deuterated standards [^{2}H10] beta-casomorphin 5
BCM6	Beta-cacomorphin 6
BCM7	Beta-casomorphin 7
BCM7-d_{10}	Deuterated standards [^{2}H10] beta-casomorphin 7
BCM8	Beta-cacomorphin 8
BCM9	Beta-cacomorphin 9
BCMs	Beta-cacomorphins
CID	Collision-induced dissociation
Cys	Cysteine
DNA	Deoxyribonucleic acid
EC	European commission
EFSA	European Food Safety Association
ELISA	Enzyme-linked immunosorbent assay
EMC	Enzyme modified cheese
Eq.1	Equation 1
ESI	Electrospray ionisation

FAO	Food and Agriculture Organisation of the United Nations
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
Gly-pro-pNA	Glycine-proline-para-nitroaniline
His	Histidine
HPLC	High performance liquid chromatography
HPLC-API-MS	High performance liquid chromatography-atmospheric pressure ionisation mass spectrometry
HPLC-GPC	High performance liquid chromatography-gel permeation chromatography
HPLC-MRM-MS/MS	High performance liquid chromatography-multiple reaction monitoring-tandem mass spectrometry
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
HPLC-QIT-MS	High performance liquid chromatography-quadrupole ion trap-mass spectrometry
HPLC-TOF-MS	High performance liquid chromatography-time of flight mass spectrometry
HPLC-UV	High performance liquid chromatography- ultra violet
$HRMS^2$	High resolution multiple fragmentation spectra (MS/MS done in ion trap)
$HRMS^n$	High resolution multiple fragmentation spectra
HTST	High temperature short time
IDLs	Instrumental detection limits
Ile	Isoleucine
IQLs	Instrumental quantification limits
LAB	Lactic acid bacteria
LC	Liquid chromatography
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Leu	Leucine
LOD	Limit of detection

LODs	Limits of detection
LOQs	Limits of quantification
Lys	Lysine
MALDI-TOF-MS	Matrix-assisted laser desorption/ionisation time of flight mass spectrometry
ME	Matrix effect
MeOH	Methanol
Met	Methionine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple fragmentation spectra
n.a	Not available
n.d	Not detection
n° 541	Number 541
NSW	New South Wale
PepX	X-prolyl dipeptidyl aminopeptidase
Phe	Phenylalanine
PM 1, 2, 3…14	Pasteurised milk 1, 2, 3…14
pNA	Para-nitroaniline (4-nitroaniline)
Pro	Proline
pro-BCM	Pro-beta casomorphin
pro-BCMs	Pro-beta casomorphins
Q1	Quadrupole 1
Q2	Quadrupole 2 (Collision cell)
Q3	Quadrupole 3
QA	Quality assurance
QC	Quality control
QCs	Quality control sample
QIT-MS	Quadrupole ion-trap mass spectrometry
QLD	Queensland
R^2	Regression coefficient
RP-HPLC	Reversed-phase high performance liquid chromatography

RP-HPLC-UV	Reversed-phase high performance liquid chromatography-ultra violet
RSD	Relative standard deviation
S/N	Signal-to-noise
SD	Standard deviation
Ser	Serine
SMP	Skim milk powder
SPE	Solid phase extraction
SRM	Single reaction monitoring
SSC	Somatic cell count
TGase	Transglutaminase
Thr	Threonine
TOF-MS	Time-of-flight mass spectrometry
T_R	Retention time
Trp	Tryptophan
Tyr	Tyrosine
UHT	Ultra high temperature
UPLC	Ultra-performance liquid chromatography
UV	Ultra violet
UV-Vis	Ultra violet-visible
Val	Valine
Vic	Victoria
WA	Western Australia
Yg 1, 2, 3…10	Yoghurt 1, 2, 3…10
α-LA	Alpha-lactalbumin
α_{s1}-CN	Alpha s_1-casein
α_{s2}-CN	Alpha s_2-casein
β-CN	Beta-casein
β-CNf	Beta casein fragment
β-CNs	Beta-caseins
β-LG	Beta-lactoglobulin
κ-CN	Kappa-casein
%	Percent
$[M+H]^+$	Molecular ion

$[M+H]^+_{exp}$	Experimental molecular ion mass
$[M+H]^+_{teo}$	Theoretical molecular ion mass
$^{\circ}C$	Celsius (temperature)
µg/mL	Microgram/millilitre
µL	Microlitre
µm	Micrometre
C18	Octadecyl silica
Da	Dalton
g	Gram
kg	Kilogram
kPa	Kilopascal
KV	Kilovolt
L	Litre
L/h	Litre/hour
M	Molar concentration
m/z	Mass-to-charge
min	Minute
mL	Mililitre
mM	Milimolar concentration
ng/µL	Nanogram/microlitre
ng/g	Nanogram/gram
ng/mL	Nanogram/millilitre
nm	Nanometre
pg/g	Picogram/gram
ppm	Parts per million
s	Seconds
V	Volt
w/v	Weight/volume

CHAPTER 1 INTRODUCTION

Milk as a nutritional food is well known as an important source of high-quality proteins and fat, due to its essential amino acids and unsaturated fatty acids. In addition, milk also contains a number of minor constituents including enzymes, vitamins, minerals, flavour compounds and bioactive peptides. All of these constituents make important contributions to the nutritional and technological properties of milk and dairy products (Fox & McSweeney, 1998). The current demand for milk and dairy products is not only due to their valuable nutrients, but also their role in prevention of chronic health conditions including obesity, dental decay, osteoporosis and hypertension (Nagpal et al., 2011). According to the Food and Agriculture Organisation of the United Nations (FAO), global consumption of milk has increased since early 1960s, and doubled in developing countries in recent years. Nations where per capita consumption of milk is high (> 150kg/capita/year) include Australia, Argentina, Europe, Israel, North America and Pakistan, whereas Senegal, most of Central Africa, Vietnam and Southeast Asia are low milk consuming countries (<30kg/capita/year). In parallel, world milk production has risen by more than 50% in the last 30 years, between 1982 and 2012, and stood at approximately 750 million tonnes in 2012. India is the largest milk producing country with 16% of global production, followed by the United States of America, Brazil and China. A group of countries with the highest milk surplus are New Zealand, the United States of America, Germany, France and Australia

Cow milk contains approximately 80% of casein, consisting of alpha s_1-, alpha s_2-, beta- and kappa-casein (α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, respectively). Beta-casein consists of 13 variants (see detail in Chapter 2), among which β-CN A1 and A2 variants are predominant in milk. Milk containing both variants in equal amount is known as normal milk. However, milk can contain either β-CN A1 or A2 only, and these are called A1 milk or A2 milk, respectively. In recent years, Australian consumers and media have been concerned about several milk products described to contain more β-CN A2 variant than β-CN A1 variant. This concern has been triggered by epidemiological evidence that intake of milk containing more β-CN A1 variant in nations with high consumption of milk is associated with non-communicable diseases such as type 1 diabetes, heart diseases and schizophrenia (Birgisdottir, Hill, Thorsson, & Thorsdottir, 2006; Elliott, Harris, Hill, Bibby, &

Wasmuth, 1999; McLachlan, 2001). Potential factors derived from milk that may contribute to these disorders are the casein derived peptides, beta-casomorphins (BCMs).

BCMs are a group of opioid peptides with morphin-like properties. These peptides contain a sequence of 4-11 amino acids and the same amino acid sequence at the first three residues. BCMs can be released from their parent β-CN at the amino acid residues 60-70 by enzymatic action. Among these, the β-CN fragment 60-64 (β-CN f60-64) and β-CN fragment 60-66 (β-CN f60-66) are named BCM5 and BCM7 (Fig. 1), respectively and are two peptides with the most potent opioid properties. Epidemiological studies show that BCM7 is a risk factor contributing to type 1 diabetes, heart disease, autism and schizophrenia, and BCM5 is associated with apnoea in infants (Elliott et al., 1999; McLachlan, 2001; Sun et al., 2003; Wasilewska, Maczmarski, Kostyra, & Iwan, 2011).

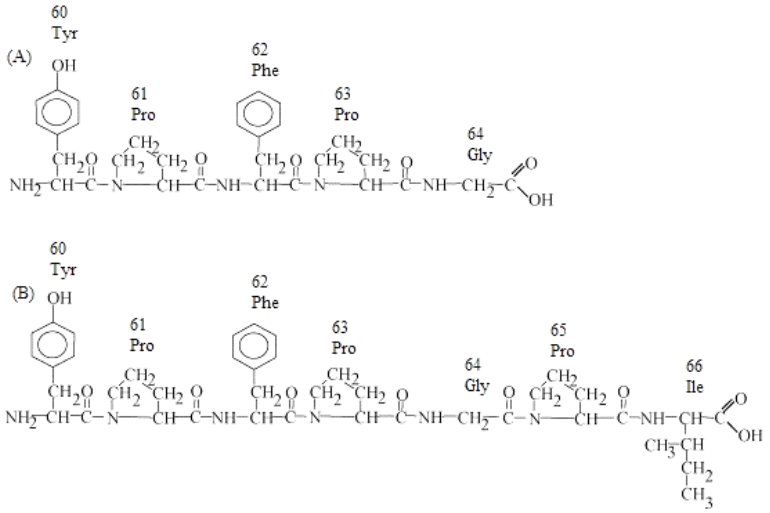

Figure 1. Structure of BCM5 (A) and BCM7 (B) (Adapted from Juan-García, Font, Juan, & Picó, 2009)

In dairy processing, bioactive peptides including BCMs are encrypted in an inactive form in parent caseins and can be released by bacteria-derived enzymes during fermentation of milk or storage of dairy products (Choi, Sabikhi, Hassan, & Anand, 2012). In fermented milk, the amount and profile of bioactive peptides depends on the strains of microorganisms used for fermentation or the conditions and time of maturation and storage. Many cheeses have been reported to contain BCM5 and BCM7; while other cheeses contain neither BCM5 nor BCM7 (De Noni & Cattaneo, 2010; Sienkiewicz-Szłapka et al., 2009). BCM7 is a factor causing bitter taste of

cheese (Shinoda, Tada, Okai, & Fukui, 1986). However, the presence of these peptides in pasteurised milk, UHT milk, milk powder and yoghurt are still equivocal.

Yoghurt is a milk product widely used cross the world. It is made by fermentation of milk with two common lactic acid bacteria (LAB), *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and is defined as a lactic acid dairy product. In addition, yoghurt is also considered as a health beneficial food, rich in calcium, B group vitamins, essential fatty acids and bioactive peptides. These peptides are mainly formed by LAB during fermentation or storage of the yoghurt. A large number of bioactive peptides have been reported as being generated in yoghurt manufacture, including antimutagenic, antimicrobial, anticarcinogenic, antioxidant, angiotensin I-converting enzyme inhibitory (ACE-I) peptides as well as opioid peptides (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007; Papadimitriou et al., 2007; Poyrazoglu Coban, Biyik, & Yavuz, 2012; Sabeena Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010). However, some bioactive peptides, (for instance, those with antimicrobial and hypertensive activity) can be degraded by LAB during yoghurt fermentation or storage.

The formation and/or degradation of BCM5 and BCM7 during manufacture of yoghurt has received a little attention in the scientific literature. There have been various studies attempting to measure these peptides in commercial yoghurt by different analytical methods. However, findings of the identification and quantification of BCM5 and BCM7 in yoghurt have provided contradictory results. This may be the result of differences in the source of milk, yoghurt starter cultures, storage conditions or sensitivity of the method used.

In recent years, reversed-phase high performance liquid chromatography (RP-HPLC) and ELISA are methods of choice for detection and quantification of BCM5 and BCM7 (Muehlenkamp & Warthesen, 1996; Sienkiewicz-Szłapka et al., 2009). RP-HPLC coupled to ultra violet-visible (UV-Vis) detection can be limited by interference of other peptides with similar physical-chemical properties, which can co-elute with target BCMs, increasing the absorption values and resulting in overestimated results. Furthermore, UV-Vis absorption may lack the sensitivity required to quantify BCM5 and BCM7 at low levels (Cass et al., 2008; Muehlenkamp & Warthesen, 1996). For ELISA, the quality of antibodies play important role for detection of target BCMs and it can influence the effectiveness of

analysis (Lutter, Parisod, & Weymuth, 2011). In addition, heat treatment of milk during yoghurt manufacture may modify the conformation of BCM7 by the interaction between lactose and amino acid residues, leading to a reduction in the binding affinity of the modified BCM7 to the antibody and resulting in underestimated results.

Currently, liquid chromatography-mass spectrometry (LC-MS) is a powerful method for identification and quantification of peptides in complex matrices such as milk and yoghurt. Different mass spectrometry techniques such as tandem mass spectrometry (MS/MS), quadrupole ion-trap mass spectrometry (QIT-MS) have been applied to determine BCM5 and BCM7 in yoghurt and milk (De Noni & Cattaneo, 2010; Juan-García et al., 2009). The choice of the calibration technique plays a critical role in LC-MS quantitative analysis. External, internal, as well as matrix matched calibration standards can be used for this purpose. The effects of matrix components present in complex samples such as dairy products are known to be responsible for suppressing and, less frequently, for enhancing the absolute response of analytes. The use of external calibration standards is generally not recommended as it can result in erroneous quantitative results. The use of deuterated homologues is by far the most effective way of correcting matrix effects, as the analytes of interest and the deuterated homologues behave identically in the ionisation interface and therefore, are subjected to identical matrix effects. Alternatively, matrix matched calibration standards can be used to compensate fully for matrix effects. However, this approach is generally time consuming as it requires using the matrix (i.e. sample/ sample extract) to build the calibration curves (Jessome & Volmer, 2006).

Therefore, the main objectives of this research are the:

1. Development of LC-MS/MS using stable isotope-labelled compounds as internal standards for simultaneous quantification of BCM5 and BCM7 in milk and yoghurt.

2. Identification and quantitation of BCM5 and BCM7 in pasteurised milk, skim milk powder and commercial yoghurt .

3. Investigation of the formation and/or degradation of BCM5 and BCM7 during fermentation and storage of yoghurt.

4. Investigation of the effect of yoghurt cultures, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* used for fermentating individually on the formation and/or degradation of BCM5 and BCM7.

CHAPTER 2 LITERATURE REVIEW

2.1 Bovine protein

Milk is an important source of nutrients for not only newborn humans but also for adults. It contains minerals, vitamins, essential fatty acids and high quality proteins. The major milk proteins consist of caseins and whey proteins, amounting to approximately 80% and 20% of milk protein, respectively (Kopf-Bolanz et al., 2012). Casein exists in milk in form of micelles (Fig. 2), consisting of four fractions: α_{s1}, α_{s2}, β and κ-CN. The α_{s1}-, α_{s2}- and β-CN are located in the core region of the micelle, whereas κ-CN is on micelle surface (Fig. 2) (Considine, Patel, Anema, Singh, & Creamer, 2007).

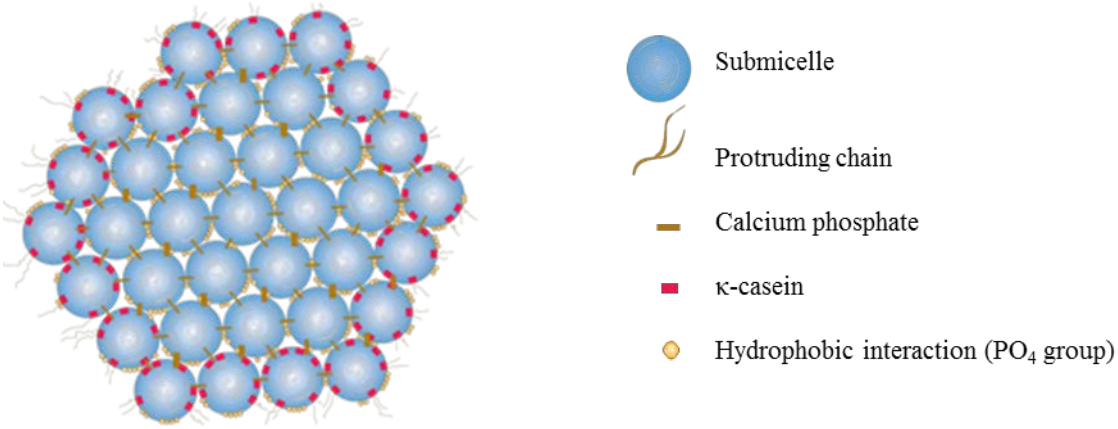

Figure 2. Model of the casein micelle (Adapted from Gosta, 1995)

β-CNs have a chain length of 209 amino acids (Fig. 3) and have been found as 13 variants in cow milk, namely β-CN A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and H (Kamiński, Cieślińska, & Kostyra, 2007). Milk from most cows contains both β-CN A1 and β-CN A2 variants. Originally, when first domesticated, cows produce only pure β-CN A2 variant, however many herds in European countries produced mainly β-CN A1 variant (http://www.a2milk.co.uk). Holstein-Friesian cows for instance, produce milk containing predominantly β-CN A1 variant (Tailford, Berry, Thomas, & Campbell, 2003), while Guernsey and Jersey breeds produce more β-CN A2 than β-CN A1 variant (Bell, Grochoski, & Clarke, 2006). The difference between β-CN A1 and A2 variant is only a single amino acid at position 67 at which the residue is histidine on β-CN A1 variant and proline on β-CN A2 variant (Fig. 3) (http://www.betacasein.org; Kamiński et al., 2007), leading to a difference in milk functionality between β-CN A1/A1 variant containing milk and β-CN A2/A2 variant

containing milk (Raynes, Day, Augustin, & Carver, 2015). More importantly, although both β-CN variants have sequence homology at residues 60-66, β-CN A1 variant can be hydrolysed between residues 66-67 by proteases to give the opioid peptide known as BCM7, whereas β-CN A2 variant is resistant to enzymatic hydrolysis at these residues (Thiri et al., 2012; Jinsmaa & Yoshikawa, 1999).

Epidemiological studies have reported that there are strong correlations between consumption of high β-CN A1 variant and type 1 diabetes and ischemic heart diseases (Elliott et al., 1999; McLachlan, 2001). However, there is insufficient data for demonstration of the relationship between BCM7 and other related BCMs and non-communicable diseases (EFSA, 2009; Swinburn, 2004; Truswell, 2005).

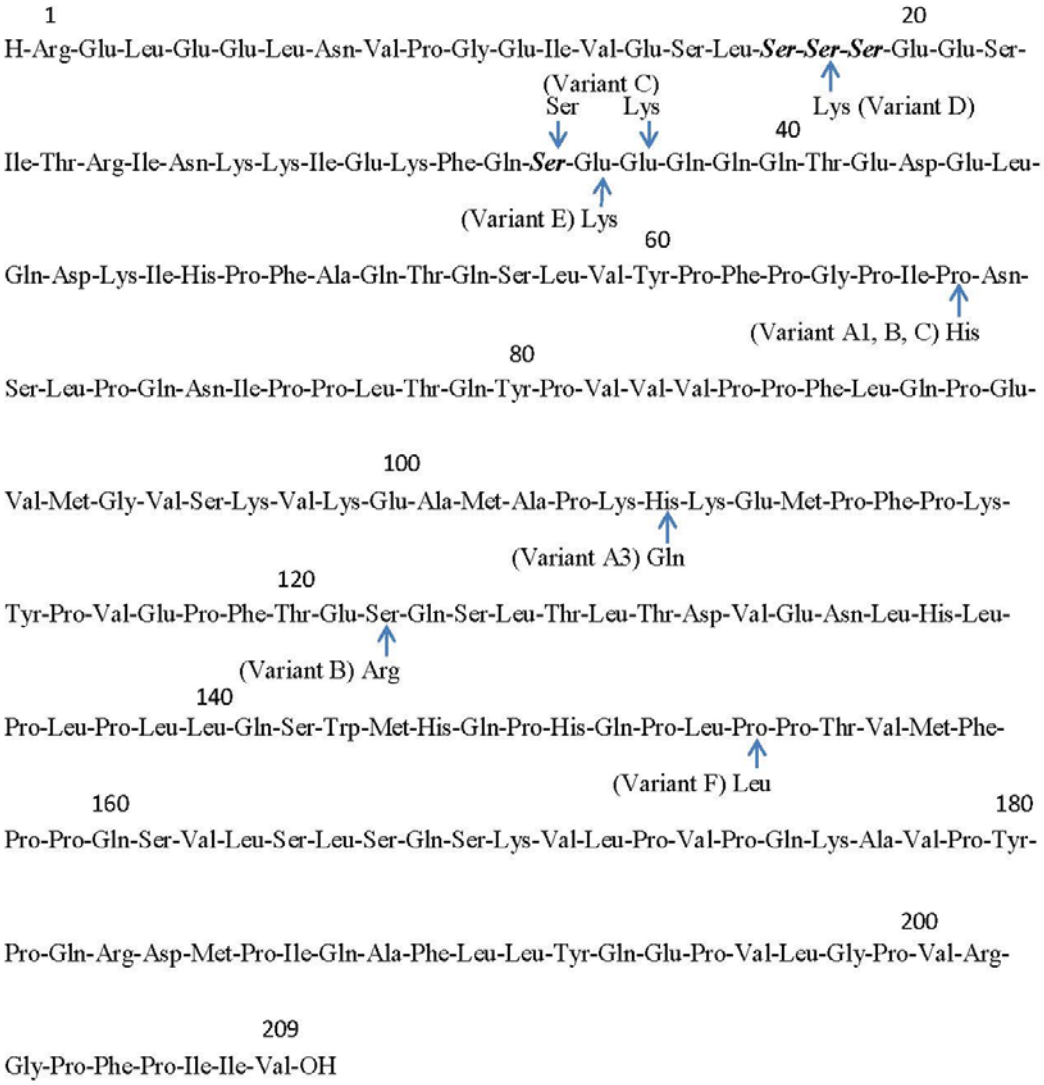

Figure 3. The primary structure of β-CN A2 variant indicating amino acid substitutions of the other variants (Adapted from Swaigood, 2003).

2.2 Source of beta-casomorphins

Beta-casomorphins (BCMs) are a group of opioid peptides released from enzymatic hydrolysis of bovine β-CN (De Noni & Cattaneo, 2010) at amino acid positions 60-70 (Boutrou et al., 2013). These peptides have a sequence of 4-11 amino acids (Kamiński et al., 2007) and the same sequence at the first three residues (-Tyr-Pro-Phe-) (Muehlenkamp & Warthesen, 1996) (Table 1). Among these peptides, BCM5 and BCM7 are peptides with the highest opioid activity (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981; Kálmán, Cserháti, Valkó, & Neubert, 1992) and have been studied more extensively than the other BCMs.

BCM7 is cleaved from β-CN at amino acid positions 60-66 (Fig. 4) during *in vitro* enzymatic hydrolysis (Brantl, Teschemacher, Henschen, & Lottspeich, 1979; De Noni, 2008; Jinsmaa & Yoshikawa, 1999) and during *in vivo* human gastrointestinal digestion (Boutrou et al., 2013). BCM7 was hydrolysed only from β-CN A1 variant by pepsin *in vitro* (De Noni, 2008; Jinsmaa & Yoshikawa, 1999). Other BCMs such as BCM9 was generated from enzymatic hydrolysis of both β-CN A1 and β-CN A2 variant *in vitro* (Jinsmaa & Yoshikawa, 1999).

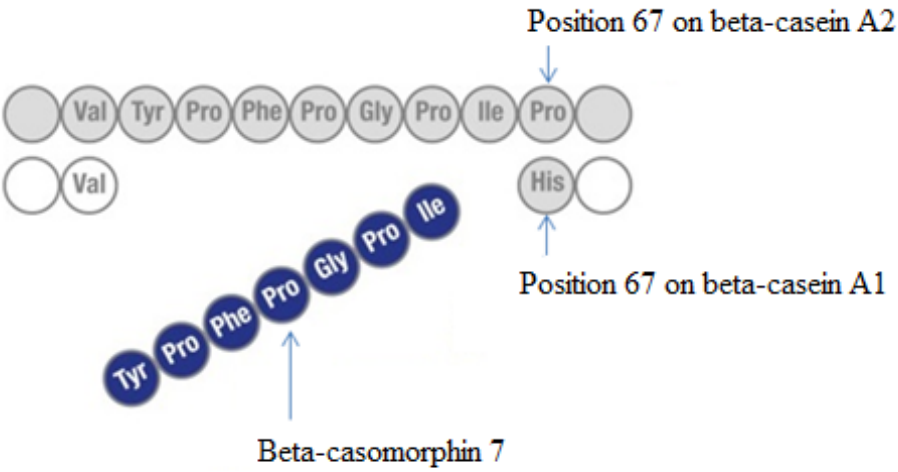

Figure 4. The release of BCM7 (Adapted from Woodford, 2009)

Table 1. Sequence of bovine BCMs

Beta casomorphins	Fragments	Structure	References
Beta-casomorphin 4 (BCM4)	β-CNf(60-63)	Tyr-Pro-Phe-Pro	Kamiński et al. (2007), Matar and Goulet (1996), Brantl et al. (1981)
Beta-casomorphin 5 (BCM5)	β-CNf(60-64)	Tyr-Pro-Phe-Pro-Gly	Kamiński et al. (2007), Juan-García et al. (2009), De Noni (2008), Brantl et al. (1981)
Beta-casomorphin 6 (BCM6)	β-CNf(60-65)	Tyr-Pro-Phe-Pro-Gly-Pro	Kamiński et al. (2007)
Beta-casomorphin 7 (BCM7)	β-CNf(60-66)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	De Noni (2008), Kamiński et al. (2007) Juan-García et al. (2009), Jinsmaa and Yoshikawa (1999), Brantl et al. (1981)
Beta-casomorphin 8 (BCM8)	β-CNf(60-67)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	Kamiński et al. (2007)
Beta-casomorphin 9 (BCM9)	β-CNf(60-68)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn	Saito, Nakamura, Kitazawa, Kawai, and Itoh (2000), Jinsmaa and Yoshikawa (1999)
Beta-casomorphin 10 (BCM10)	β-CNf(60-69)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser	Toelstede and Hofmann (2008)
Beta-casomorphin 11 (BCM11)	β-CNf(60-70)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	Kamiński et al. (2007)

2.3 Beta-casomorphins in dairy products

2.3.1 Raw and heat-treated milk

Bovine milk is not only a source of essential fatty acids, calcium and phosphate, and vitamins, but also provides high quality of protein that can generate bioactive peptides. These peptides are in an inactive form in the parent proteins and are released during fermentation of milk or during gastric digestion. Milk peptides with health beneficial effects may play an important role in prevention of hypertension (Jauhiainen & Korpela, 2007) and cardiovascular disease (Elwood, 2005). However, several bioactive peptides with opioid property released from bovine β-CN may increase risk of chronic disease (Cieślińska, Kaminski, Kostyyra, & Sienkiewicz-Szłapka, 2007; Cieślińska et al., 2012).

Bovine milk often contains somatic cells, native milk enzymes or bacteria contaminated from external environment. These are three factors that can contribute to the hydrolysis of proteins to peptides. Somatic cell count (SSC) in milk is an important factor for assessment of the severity of mastitis which indicates the inflammation of cow udders. Milk is considered as coming from a mastitis infected cow if somatic cells exceed 500,000 per millilitre (Ginn, Packard, Mochrie, Kelley, & Schultz, 1985). Many studies have measured BCM7 in milk collected from cows with different severities of mastitis. This peptide was found in fresh cow milk, in which somatic cells were not characterised (Cieślińska et al., 2007). Simlarly, Cieślińska et al. (2012) detected BCM7 in cow milk collected from healthy cows that did not indicate any sub-clinical symptoms of mastitis. In contrast, Napoli et al. (2007) have reported that BCM7 was undetectable at any level in milk collected from cows with mastitis. Consequently, somatic cells are not a factor associating with the presence of BCM7 in fresh milk.

Endogenous proteases often present in milk during lactation consist of plasmin, cathepsins and elastase. According to Weinstein and Doolittle (1972), plasmin specifically cleaves the peptide bonds between lysine and arginine residue. As shown in Fig. 3, there is no lysine or arginine amino acid residue at positions from 59-70 on β-CN chain. In addition, plasmin hydrolyses peptide bonds on β-CN at amino acid residues Lys_{28} and Lys_{29}, Lys_{105} and His_{106}, and Lys_{107} and Glu_{108} (Fig. 3) (Aslam & Hurley, 1998). Meanwhile, cathepsins and elastase cleaved β-CNs to pro-beta casomorphins (pro-BCMs) such as β-CNf(54-68), β-CNf(55-68), β-CNf(57-68) and

β-CNf(55-65), but no BCMs (Gaucher, Mollé, Gagnaire, & Gaucheron, 2008). More interestingly, cathepsin B cleaves β-CNs to BCM10 (β-CN f60-69) and an undefined peptide (β-CN f60-) (Considine, Healy, Kelly, & McSweeney, 2004). However, Meltretter, Schmidt, Humeny, Becker, and Pischetsrieder (2008) detected unknown peptides with mass-to-charge (*m/z*) from 1900 to 4500 in raw milk by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS). None of these *m/z* values matched those of BCMs suggesting the absence of BCMs in the raw milk.

The contamination of bacteria from the external environment into milk always occurs during milking. Enzymes produced by these bacteria may hydrolyse β-CNs into BCM7 and other BCMs. Hamel, Kielwein, and Teschemacher (1985) have detected BCM7, BCM6, BCM5 and BCM4 immunoreactive material in fresh milk inoculated with bacteria frequently found in contaminated milk.

Investigations of the occurrence of BCM7 and other BCMs in human milk may also assist in our understanding of the origin of formation of these peptides in bovine milk. Jarmołowska et al. (2007) have showed that BCM5 and BCM7 were detected in human milk that was collected from healthy women and stored at -70 °C immediately after expression. Obviously, in these conditions of collection and storage of milk samples, the hydrolysis of proteins by somatic cell or bacteria-derived enzymes was very unlikely. Therefore, BCM5 and BCM7 were probably released from β-CNs in the mammary glands by endogenous proteinases before the milk was expressed (Koch et al., 1988). However, the identity of specific endogenous enzymes in human milk and their mechanism of action are unknown.

The presence of β-CN variants and their ratio in milk plays an important role in the formation of BCM7 and other BCMs. As previously described, *in vitro* studies demonstrated that BCM7 is only released from β-CN A1 variant, but other BCMs can be released from both β-CN A1 and β-CN A2 variant (Jinsmaa & Yoshikawa, 1999). Surprisingly however, BCM7 has been found in milk containing not only the pure β-CN A1 variant, but also β-CN A2 and β-CN A1/A2 (Cieślińska et al., 2012). The level of BCM7 is the highest in milk containing only β-CN A1 variant, followed by in milk containing β-CN A1/A2 variant and then β-CN A2 variant. In this regard, BCM7 may be formed from β-CN A2 variant at very low rate (Korhonen & Marnila, 2013). In contrast, De Noni and Cattaneo (2010) did detect neither BCM5 nor BCM7

in unprocessed milk, although this milk contains both β-CN A1 and β-CN A2 variant.

Heat-treated liquid milk is a product wildly consumed across the world. It consists of pasteurised milk (75 °C/15s), UHT milk (UHT-149 °C/2s) and sterilised milk (110 °C/10min). In addition to destroying bacteria, heat treatment of milk also changes its physio-chemical properties; for instance, increasing viscosity, browning of colour, changes in protein conformation and release of peptides. BCM7 has been found in commercial UHT milk (Juan-García et al., 2009; Thiri, Ardeshir, Andrew, and Xiaomin, 2012) and in pasteurised and sterilised milk (Cieślińska et al., 2012) (Table 2). In contrast, De Noni and Cattaneo (2010) did not identify BCM5 and BCM7 in similar milk products. Another study showed five new peptides identified in cow milk that was heated at 72, 85 and 120 °C for 10, 20 and 30 min respectively, but neither contained BCM7 nor other BCMs (Meltretter et al., 2008). These BCMs were also not identified in commercial UHT and pasteurised milk (Gaucher et al., 2008; Meltretter et al., 2008).

Overall, while some peptides in milk and heated milk released from β-CNs by endogenous proteases or heat treatment have been known, the presence of BCM5 and BCM7 in these milk products has been contradicted. This may be due to analytical methods used for identification and quantification of BCM5 and BCM7 (see detail in Section 2.6 and Chapter 4) or different origins of raw milk and heated milk used for measurement of these BCMs.

Table 2. BCMs in milk and milk products

Milk and milk products		Beta-casomorphins	Levels	References
Milk				
	Fresh milk		Identified	Cieślińska et al.(2007)
			1.1-2.1[a]	Cieślińska et al. (2012)
	Pasteurised milk		3.3-6.1[a]	Cieślińska et al. (2012)
	UHT milk	BCM7	1.4[a]	Juan-García et al. (2009)
			0.2[a]	Thiri et al. (2012)
	Sterilised milk		2.9-7.4[a]	Cieślińska et al. (2012)
Cheese				
	Brie		5 – 15[b]	Jarmolowska et al. (1999)
		BCM7	0.15[b]	De Noni and Cattaneo (2010)
			6.48[b]	Sienkiewicz-Szłapka et al. (2009)
		BCM5	3.14[b]	Sienkiewicz-Szłapka et al. (2009)
	Cheddar	BCM7	0.11-1.0[b]	De Noni and Cattaneo (2010); Norris, Coker, Boland, and Hill (2003)
		BCM6	Identified	Norris et al. (2003)
		BCM9	Identified	Singh et al. (1997)
	EMC	BCM7	Identified	Haileselassie, Lee, and Gibbs (1999)
	Gorgonzola	BCM7	0.01[b]	De Noni and Cattaneo (2010)
	Fontina	BCM7	0.04[b]	De Noni and Cattaneo (2010)

Gouda		0.06[b]	Sienkiewicz-Szłapka et al. (2009)
	BCM7	0.1[b]	De Noni and Cattaneo (2010)
		0.066[b]	Norris et al. (2003)
	BCM5	0.05[b]	Sienkiewicz-Szłapka et al. (2009)
	BCM9	identified	Saito et al.(2000), Toelstede and Hofmann (2008)
	BCM10	identified	Toelstede and Hofmann (2008)
Rokpol	BCM7	1.66[b]	Sienkiewicz-Szłapka et al. (2009)
	BCM5	2.57[b]	Sienkiewicz-Szłapka et al. (2009)
Edamski	BCM7	1[b]	Sienkiewicz-Szłapka et al. (2009)
	BCM5	0.46[b]	Sienkiewicz-Szłapka et al. (2009)
Kasztelan	BCM7	0.04[b]	Sienkiewicz-Szłapka et al. (2009)
	BCM5	0.14[b]	Sienkiewicz-Szłapka et al. (2009)
Swiss	BCM7	0.22[b]	Norris et al. (2003)
	BCM6	identified	Norris et al. (2003)
Elsberg	BCM7	0.11[b]	Norris et al. (2003)
	BCM6	identified	Norris et al. (2003)
Feta	BCM7	0.15[b]	Norris et al. (2003)
	BCM6	identified	Norris et al. (2003)
Blue	BCM7	23.2[b]	Norris et al. (2003)
	BCM6	identified	Norris et al. (2003)
Milk powder and formula			

	Milk powder	BCM7	0.8-2.7[c]	Cieślińska et al. (2012)
	Infant formula	BCM5	0.39[d]	Jarmołowska et al.(2007)
	High protein formula	BCM5	0.004[b]	Wocior (2008)
Yoghurt				
	Probiotic yoghurt	BCM7	46[e]	Jarmolowska (2012)
	Traditional yoghurt	BCM7	54[e]	Jarmolowska (2012)
	Kefir	BCM7	29[e]	Jarmolowska (2012)

[a] ng/mL of milk

[b] ng/mg of cheese or high protein formula

[c] ng/mL of reconstituted milk

[d] ng/µL of reconstituted infant formula

[e] ng/mg of protein in yoghurt

ECM – enzyme modified cheese (Cheddar) treated with Neutrase protease produced from *Bacillus subtilis*

2.3.2 Cheese

Cheese is a dairy product produced by coagulation of casein protein in cow, buffalo, ewe or goat milk. During cheese production, milk is heated, acidified by inoculation with stater culture and the addition of the enzyme rennet to coagulate caseins to give curd and whey. Further processes are: to separate the whey, press the curd into a mount and ripening of the curd into the final cheese. Moreover, for many varieties of cheese, specific moulds are also added during ripening.

Milk used for cheese making is commonly pasteurised at 72 $^{\circ}$C for 15s (Hayaloglu, Deegan, & McSweeney, 2010). However, some varieties of cheeses are produced from milk without heat treatment or alternately milk heated at higher temperature than pasteurisation (>72 $^{\circ}$C). Heat treatment of milk causes denaturation of the whey proteins, beta-lactoglobulin (β-LG) and alpha-lactalbumin (α-LA), leading to interaction between caseins and denatured whey proteins (Fig. 5). This interaction not only leads to increase in the yield of cheese, but also significantly affects hydrolysis of proteins during ripening, impacting the flavour and texture of final products (Benfeldt & Sørensen, 2001). In addition, using different starter cultures for fermentation or moulds for ripening also contributes to variations in the peptide profile in the ripened cheese. In general, protease systems cleave proteins into a number of larger peptides during ripening, which are subsequently further hydrolysed into smaller peptides (Benfeldt & Sørensen, 2001). The peptide, β-CNf58-72 called pro-beta casomorphin (pro-BCM), was found in Crescenza cheese after 8-10 day ripening (Smacchi & Gobbetti, 1998) and in Cheddar and Jarlsberg cheese (Stepaniak, Fox, Sorhaug, & Grabska, 1995). This peptide may be cleaved by peptidases into beta-casomorphin 13 (BCM13, β-CNf60-72) as reported by Jinsmaa and Yoshikawa (1999), or into other smaller BCMs if the cheese is ripened for a longer period.

In recent years, the presence of BCMs in cheese, especially BCM5 and BCM7, has been investigated (De Noni & Cattaneo, 2010; Sienkiewicz-Szłapka et al., 2009). The levels of BCMs in some cheeses are showed in Table 2. BCM7 has been found in varieties such as Brie, Cheddar, Gorgonzola, Fontina, Gouda, Rokpol, Edamski, Kasztelan, Swiss, Elsberg, Feta and Blue (De Noni & Cattaneo, 2010; Norris et al., 2003; Sienkiewicz-Szłapka et al., 2009). Some cheeses have also been reported to contain BCM5, for example Brie, Rokpol, Edamski, Gouda and Kasztelan

(Sienkiewicz-Szłapka et al., 2009), BCM6 in Blue, Cheddar, Feta, Swiss, Gouda and Elsberg (Norris et al., 2003). BCM9 has been reported in Cheddar and Gouda (Saito et al., 2000; Singh et al., 1997; Toelstede & Hofmann, 2008), BCM10 in Gouda (Toelstede & Hofmann, 2008), and BCM11 in the Italian goat cheese Caprino del Piemonte (Rizzello et al., 2005). In contrast, other studies did not detect BCM7 or other BCMs in Brie and Cheddar (Alli, Okoniewska, Gibbs, & Konishi, 1998; Muehlenkamp & Warthesen, 1996). These findings may indicate that BCM7 is (a) not probably formed from β-CNs, (b) may be degraded during cheese ripening or (c) present at a level lower than LOD of the analytical method used.

SCC in raw milk is one of important factors for assessment of the suitability of milk for production of cheese (Siobhan, Denise, Patrick, & Alan, 2000). Increasing SCC in milk is significantly associated with higher endogenous protease activity, from enzymes such as cathepsin B and D and elastase. This enzymatic activity plays a major role in the hydrolysis of caseins, with potential for reducing yields due to loss of proteose-peptones in whey (Albenzio et al., 2009). Endogenous proteases significantly contribute to peptide profiles in cheese during ripening, however; neither BCM nor other BCMs were identified (Gagnaire et al., 2011).

Plasmin is a main endogenous protease originating from the cow's blood (Considine, Healy, Kelly, & McSweeney, 1999) and its activity is therefore, not associated with SCC (Siobhan et al., 2000). Furthermore, its activity is unaffected by pasteurisation of milk at 72 °C/60 s or at 80 °C/15s (Benfeldt, Sørensen, Ellegård, & Petersen, 1997). This enzyme has been thought to play a role in aging of Cheddar, Swiss-type cheese and Gouda (Farkye & Fox, 1992; McSweeney, Pochet, Fox, & Healy, 1994). Some peptides released from β-CNs by plasmin have been isolated from Cheddar cheese, but no BCM7 or other BCMs were reported (Farkye & Fox, 1992; McSweeney et al., 1994). According to McSweeney (2004), rennet enzymes (chymosin) also cleaved β-CNs in a similar manner to plasmin, elastase and cathepsin. Therefore, any single endogenous protease or chymosin is incapable of releasing BCM7 or other BCMs during cheese ripening. However, a combination of these enzymes and others derived from LAB or mould may play a role in formation of BCMs and the BCM-releasing activities, however, this needs further investigation.

LAB is often used as a starter culture for fermentation of milk during cheese making. In addition to producing lactic acid to coagulate milk protein, LAB also hydrolyses

milk proteins into peptides, including BCM7 and other BCMs, during fermentation and cheese ripening. The formation or degradation of BCM7 and other BCMs may depend on the strains of LAB culture and cheese making conditions. The BCM7 and other related BCMs in cheese could not originate from the milk, because if so, these peptides would be removed in the whey fraction during washing of the curd (Jarmolowska et al., 1999). De Noni and Cattaneo (2010) found BCM7 at 0.11 ng/mg in Cheddar cheese. In the manufacture of Cheddar, starter cultures that are commonly used for fermentation include a mixture of *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* (Robinson, 1995). Muehlenkamp and Warthesen (1996) showed that BCM7 level decreased by 50% after 6-15 weeks of storage when it was incubated with *Lactococcus lactis* ssp. *cremoris* at pH 5.0 and 1.5% NaCl. These conditions of incubation and storage are similar to those used in production of Cheddar cheese. According to Haileselassie et al. (1999), BCM7 was detected in emulsified Cheddar cheese with added Neutrase, an enzyme produced from *Bacillus subtilis*, after an eight hour incubation. However, the produced BCM7 was degraded by peptidases from *L. casei*, *Lactococcus lactis* and *Aspergillus oryzae* after a 72 hour incubation. Therefore, BCM7 may be generated from hydrolysis of β-CNs during fermentation, followed by degradation into smaller peptides by LAB in ripening.

Non-starter microflora plays a role similar to starter cultures in cheese making. Non-starter microflora is microflora that has contaminated the raw milk from cow udders, equipment and pipes during milking and transportation. In addition to lactic acid fermentation, these microorganisms hydrolyse caseins into peptides, and further into amino acids, leading to production of unique desired flavours of these cheeses. Therefore, many types of cheese are made from milk without heat treatment in order to benefit from the flavour profiles generated by these non-starter microorganisms. In addition, in cheese making, pasteurisation of milk does not kill all non-stater microflora, therefore, those that do survive can still hydrolyse milk proteins into peptides during fermentation or ripening. Hayaloglu et al. (2010) showed that content of peptides in Malatya cheese made from raw milk without addition of starter culture is higher than that in cheese made from pasteurised milk inoculated with *Lactococcus lactis* ssp. *lactis* and *S. thermophilus*. The authors suggested that the resultant peptides were generated from activity of non-starter culture derived

enzymes, not from those derived from *Lactococcus lactis* ssp. *lactis* and *S. thermophilus* starter culture. However, BCM7 and other BCMs in this cheese were not identified.

In addition to starter culture, mould is also used for ripening in some cheeses such as Brie, Camembert or Gorgonzola. This microorganism strongly influences the appearance, flavour and texture of these cheeses (Wilhelm, 1999). According to Takafuji (1993), mould-derived proteases play a role in degradation of β-CNs into large peptides, once the level of activity the enzymes increased after 20 day ripening. Subsequently, large peptides were hydrolysed into smaller ones by LAB derived enzymes. However, the role of LAB or mould in formation of BCM5 and BCM7 in these cheeses remains poorly understood. The level of BCM7 and BCM5 in Brie cheese and Rokpol cheese ripened with both mould and LAB was considerably higher than that in other cheeses ripened with only LAB (De Noni & Cattaneo, 2010; Sienkiewicz-Szłapka et al., 2009). Similarly, other authors have reported high levels of BCM7 in Brie (Jarmolowska et al., 1999) and in Blue cheese (Norris et al., 2003).

As previoulsy described in Section 2.2, BCM7 is generated from digestion of β-CN A1 variant by gastric enzymes. However, the link between β-CN A1 or β-CN A2 variants in the formation of BCM7 in milk products or in cheese currently has beeen received little attention. Almost all studies that have investigated BCM7 and other related BCMs in commercial cheeses did not characterise the type of β-CN variant of the raw milk or the resulting cheese. However, according to De Noni and Cattaneo (2010), both β-CN A1 and β-CN A2 variants were identified in cheeses where BCM7 was detected. Therefore, it will now be necessary to elucidate which variant is the source of BCM7 during cheese making by future investigations on the occurrence of BCM7 and other related BCMs in cheese produced from milk of a single β-CN variant.

2.3.3 Milk powder and milk formulas

Milk powder is a dairy product mainly produced from bovine milk by dehydration, so that the product contains 3-5% water. It is one of main ingredients to be blended into formulas. In general, the main process in the manufacture of milk powder is heat treatment (including pasteurisation), concentration under vacuum and spray drying. In recent years, several studies have reported the occurrence of BCM7 and other related BCMs in the milk powder. Cieślińska et al. (2012) indicated that the level of

BCM7 of milk reconstituted from powder containing β-CN A1 and β-CN A2 variants ranged between 0.8 and 2.7 ng/ml (Table 2). Wocior (2008) and Jarmołowska et al. (2007) found BCM5 in milk powder-based high protein formulas and infant formulas. In contrast, De Noni and Cattaneo (2010) in their study on products containing both β-CN A1 and β-CN A2 variants, detected neither BCM7 nor BCM5 in commercial skim milk powder or in other products such as calcium caseinate, milk protein concentrate or milk-powder based infant formulas. Therefore, the presence of BCM7 and BCM5 milk powder and formulas involves pre-existence of these peptides in the raw milk before it was processed into milk powder.

2.3.4 Yoghurt

Yoghurt is a dairy product mainly produced from bovine milk by fermentation. Yoghurt fermented with LAB, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (Tamime & Robinson, 1999) is known as traditional yoghurt. In recent years, probiotic yoghurt also has been produced by incubation of milk with probiotic bacteria such as *Bifidobacterium, L. acidophilus* or *L. casei*. Yoghurt is regarded as a nutritious milk product with health beneficial properties since it is rich in calcium, B-group vitamins, essential fatty acids and bioactive peptides. Peptides with bioactive properties such as ACE-I, antioxidant, antimicrobial or opioid peptides have been found in yoghurt (Donkor et al., 2007; Jarmolowska, 2012; Kunda et al., 2012; Papadimitriou et al., 2007; Poyrazoglu Coban et al., 2012; Sabeena Farvin et al., 2010).

Recently, BCM5 and BCM7 have been investigated in commercial yoghurt or fermented milk. According to De Noni and Cattaneo (2010), yoghurt and other fermented milk products contained neither BCM5 nor BCM7. In contrast, the later was found in traditional and probiotic yoghurt (Jarmolowska, 2012). A number of different peptides are also formed from β-CNs during traditional and probiotic yoghurt production (Donkor et al., 2007; Kunda et al., 2012; Schieber & Brückner, 2000). Various identified peptides are pro-BCMs, namely β-CNf57-68 and β-CNf57-72 (Plaisancié et al., 2013; Schieber & Brückner, 2000); β-CNf58-72, β-CNf59-68, β-CNf59-70 (Kunda et al., 2012), and β-CNf59-72 (Plaisancié et al., 2013). In principle, these pro-BCMs may be further degraded by peptidase system of yoghurt starter cultures into BCMs during storage. To date, the peptide, β-CNf60-72

called BCM13 (Kunda et al., 2012) and BCM9 (Sabeena Farvin et al., 2010) have been found in commercial yoghurt.

According to Kunda et al. (2012), two di-peptides, probably β-CNf60-61 and β-CNf62-63, cleaved from both β-CN A1 and β-CN A2 variants were identified in commercial yoghurt. As illustrated in Fig. 3, residues at position 61 and 63 on the chain of parent β-CNs are proline residues. The peptide bonds between proline and other amino acids are hydrolysed by the enzyme PepX that is released from LAB (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002). Therefore, the finding of Kunda et al. (2012) indicates that BCM7 and other BCMs are likely to be digested into smaller peptide fragments during yoghurt fermentation or storage. However, Kunda et al. (2012) did not isolate and characterise bacteria, a key factor influencing generation and degradation of the BCMs, present in the investigated yoghurt.

L. delbrueckii ssp. *bulgaricus* and *S. thermophilus* grow symbiotically (proto-cooperation), during yoghurt fermentation. In this symbiosis, metabolites released by each organism stimulate the growth of the other (Tamime & Robinson, 1999). In summary, *L. delbrueckii* ssp. *bulgaricus* produces peptides or amino acids that stimulate the growth of *S. thermophilus* (Courtin, Monnet, & Rul, 2002). In turn, *S. thermophilus* releases pyruvic acid, formic acid, folic acid, fatty acids and CO_2 that stimulate the growth of *L. delbrueckii* ssp. *bulgaricus* (Settachaimongkon et al., 2014). However, the symbiosis between these two yoghurt bacteria is dependent on the strain of *S. thermophilus* used for co-fermentation (Settachaimongkon et al., 2014). Therefore, in yoghurt fermentation there may formation of BCM7 and other BCMs by *L. delbrueckii* ssp. *bulgaricus* with their subsequent degradation by *S. thermophilus*, and vice versa. It is unknown if, and when BCMs are released or degraded in yoghurt processing as using starter cultures *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. Therefore, this interaction needs further investigation.

2.4 Effect of processing on formation and degradation of beta-casomorphins and other peptides

2.4.1 Effect of heat treatment

Thermal treatment plays an important role in dairy processing in terms of food safety and quality. In addition to killing unwanted bacteria, thermal treatment causes change in composition and properties of milk (Hattem, Manal, Hanaa, & Elham, 2011), especially resulting in the denaturation of whey proteins. Heating of milk above 70 °C (the temperature above which most milk products are exposed to during manufacture), causes significant denaturation of two major whey proteins, β-LG and α-LA (Considine et al., 2007). The denatured whey proteins can interact with casein micelles, particularly the κ-CN on the micelle surface (Fig. 2). This forms a complex of κ-CN~β-LG (Fig.5), which affects the physical and chemical properties of milk products (Krasaekoopt, 2003). In addition, heat treatment can induce protein fragmentation to form lower molecular weight peptides (Gaucheron, Mollé, Briard, & Léonil, 1999).

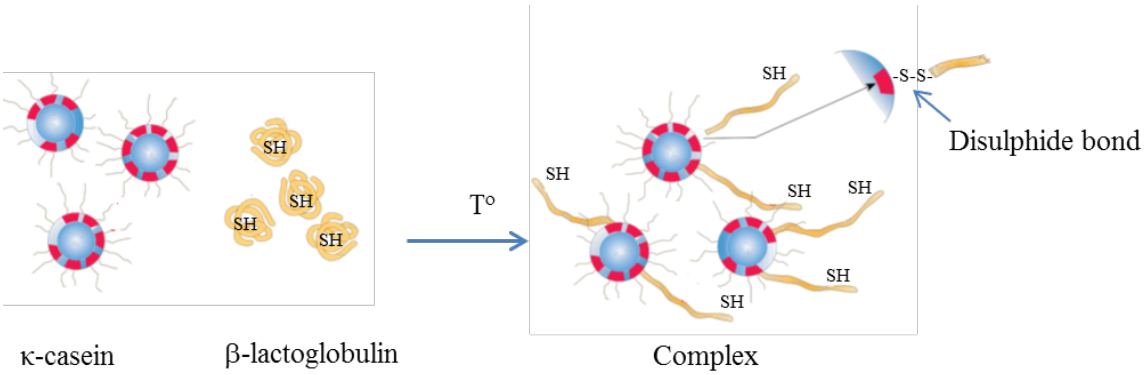

Figure 5. Formation of κ-CN~β-LG complex during heat treatment of milk (Adapted from Gosta, 1995)

According to Meltretter et al. (2008), five new peptides were generated after heat treatment of milk. Peptides of unknown structure with mass-to-charge (*m/z*) ratios of 974.4, 2218.7, 3730.1, 4297.8 and 4436.8 were generated from caseins, none of which were reported as BCM7 nor other BCMs. In contrast, in another study, the level of BCM7 was reported to significantly increase on heating milk (Cieślińska et al., 2012). Compared to the raw milk, after pasteurisation (90 °C/20 min) or

sterilisation (117 °C/ 5 min), the BCM7 content increased approximately 3 or 2 fold, respectively (Table 2) (Cieślińska et al., 2012).

In principle, the formation of new peptides or BCM7 can be due to heat treatment, enzymes or acid hydrolysis. The hydrolysis of proteins by enzymes or acid during heat treatment of milk can be excluded because enzymes are inactivated and pH of heated milk changes a little compared to that of raw milk. However, thermal treatment results in a Maillard reaction that may form radicals attacking the peptide bonds to cleave the bonds releasing peptides (Meltretter et al., 2008).

The strength of peptide bonds is an important factor for fragmentation of protein by heat treatment. Gaucheron et al. (1999) have reported that ten peptides were generated in raw skim milk heated at 120 °C for 30 min. Two of which were liberated from β-CNs, including β-CNf1-14 and β-CNf1-16, but neither BCMs nor pro-BCMs were found. The peptides were liberated after heating due to cleavage at bonds containing aspartic acid (Asp), asparagine (Asn) or glutamic acid (Glu). The peptide bonds involving these amino acids are thus more susceptible to cleavage by heat treatment (Hustinx, Singh, & Fox, 1997). Gaucher et al. (2008) identified pro-BCMs in UHT milk, namely β-CNf54-68, β-CNf54-69, β-CNf55-65, β-CNf55-68 and β-CNf57-68, and suggested that these peptides derived from elastase and cathepsin enzymatic hydrolysis. Three peptides (β-CNf54-68, β-CNf55-68 and β-CNf57-68) resulted from cleavage of peptide bonds involving the Asn residue at position 68. Therefore, this finding may indicate that peptide bonds containing Asn are more susceptible to hydrolysis by enzymes if milk is heated. Several studies have showed that the peptides β-CNf57-68 and β-CNf59-68 both contain Asn residue at position 68, are also found in yoghurt (Kunda et al., 2012; Plaisancié et al., 2013; Schieber & Brückner, 2000). The formation of these two peptides is due to the heat treatment of milk rather than at the later fermentation and storage stages.

The pasteurisation of milk prior to manufacture of dairy products plays an important role in subsequent proteolysis during the fermentation and storage stages (El-Zahar, Chobert, Dalgalarrondo, Sitohy, & Haertle', 2004). Although heating is widely used for killing non-starter microflora in the milk used for cheese production, though as previously described, some cheeses are still made from raw milk because of their distinctive flavours (Atasoy, Yetişmeyen, Türkoğlu, & Özer, 2008). Hayaloglu et al. (2010) showed that pasteurisation of milk (72 °C/ 30 s) resulted in a decrease in

peptide content of the resulting cheese compared to that in cheese made from raw milk; however, the identity of the peptides was not reported. According to Hayaloglu et al. (2010), heat treatment of milk at 72 °C/ 30 s can kill non-starter microflora that may result in less peptides in cheese made from pasteurised compared to raw milk. In contrast, Mendia et al. (2000) reported that pasteurisation resulted in increase in proteolysis of β-CNs in cheese. This phenomenon may be linked to heat-induced activation of plasmin, one of the native enzymes in milk is quite heat stable. According to Benfeldt and Sørensen (2001) and Mendia et al. (2000), when milk is pasteurised, plasmin is not inactivated and β-CN protein is dissociated from the casein micelles (Considine et al., 2007), which allows plasmin or other still active proteases hydrolyse the β-CNs more easily.

2.4.2 Effect of fermentation and storage

Fermentation is an extremely important process in the production of milk products such as yoghurt and cheese. In addition to conversion of lactose into lactic acid and breakdown milk fat into fatty acids, bacteria also hydrolyse proteins into peptides and amino acids during fermentation (Smit, Smit, & Engels, 2005). The extent and kinetics of these reactions depends on the strains of bacteria used for fermentation, temperature and time of incubation, and period of storage.

The choice of strains of culture plays a crucial role in determining the properties of fermented milk including the peptides profile in the final products (Jarmolowska, 2012). Gobbetti et al. (2000) have identified some peptides, namely β-CNf6-14, βCN f7-14, β-CNf73-82, β-CNf74-82 and β-CNf75-82, in yoghurt inoculated with a single strain of *L. delbruekii* ssp. *bulgaricus* SS1. Papadimitriou et al. (2007) have identified β-CNf176-180 and β-CNf1-8 in yoghurt fermented with only *L. delbruekii* ssp. *bulgaricus* ϒ 10.13.To date, many studies have reported the finding of BCM7 and other BCMs in some types of cheese (Table 2), however, the bacteria strains responsible have not been characterised. Some types of bacterial strains that have been reported as possibly capable of releasing BCM7 and other BCMs in cheese or yoghurt are summarised in Table 3.

L. delbrueckii ssp. *bulgaricus* and *S. thermophilus* are often used for co-fermentation in traditional yoghurt and have been characterised in commercial yoghurt (De Noni & Cattaneo, 2010; Jarmolowska, 2012; Schieber & Brückner, 2000). De Noni and

Cattaneo (2010) showed that yoghurt stored for 30 days at cold temperature before analysis did not contain BCM5 or BCM7. The same finding was reported by Schieber & Brückner (2000) investigating yoghurt stored for 21 days at 4 °C. Thus, these results suggest that the mixture of starter culture *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is not capable of forming BCMs, or they can generate BCMs which are subsequently degraded during storage. This suggestion is also supported by the analysis of a 35-day stored Taleggio cheese fermented using *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* as the starter culture, in which no BCM7 nor BCM5 were detected (De Noni & Cattaneo, 2010). Donkor et al. (2007) have identified several peptides originated from β-CNs in probiotic yoghurt fermented with *L. delbrueckii* ssp. *bulgaricus* LB1466 and *S. thermophilus* St1342, and probiotic bacteria *L. casei* L26, *L. acidophilus* L10 and *Bifidobacterium lactis* B94, but the presence of neither BCM7 nor other BCMs has been reported. In contrast, Jarmolowska (2012) quantified BCM7 in commercial traditional and in probiotic yoghurt and showed that its level decreased during storage.

As described previously, BCM7 is a peptide rich in proline (Fig. 4) and therefore is highly susceptible to LAB derived PepX that preferentially cleaves peptide bonds involving this amino acid. Atlan et al. (1990) demonstrated that PepX is located in the cytoplasm and thus is released into the surrounding medium when there is lysis of bacteria cells. In the low pH and cold conditions during yoghurt storage, bacterial cells become unstable and more susceptible to lysis to potentially release more PepX (Otte, Lenhard, Flambard, & Sørensen, 2011). As a result, the decrease in BCM7 during storage demonstrated by Jarmolowska (2012) and the absence of BCM7 or BCM5 in yoghurt and cheese stored for 30 or 35 days reported by De Noni and Cattaneo (2010) may be associated with increase in PepX activity. On the other hand, during fermentation, proteases associated to the cell wall of bacteria can hydrolyse caseins into large peptides which subsequently are transported into the bacteria cell and hydrolysed into di and tri-peptides (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009). Consequently, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* used as co-culture in yoghurt production may cleave β-CNs into BCM7 and other related BCMs during fermentation and subsequently degrade them into even smaller peptides during storage.

The pH at which the fermentation is halted is a key factor affecting subsequent proteolysis during cold storage of fermented milk. Fermentation process is often stopped at pH 3.5-4.6 prior to cold storage. According to Nielsen et al. (2009), fermentation by *Lactococcus lactis* and *L. helviticus* strains to pH 4.6 or 4.3 resulted in release of large peptides from β-CNs after 7-day cold storage. However, there was no change in peptide profile during storage when milk had been fermented with *S. thermophilus* to these same pH values. Antimicrobial and hypotensive peptides are degraded very slightly after a four hour incubation with strains of *S. thermophilus* at pH 4.5 (Paul & Somkuti, 2009). Thus, these findings suggest that *S. thermophilus* derived proteases or peptidases may not be active during cold storage at these pH values.

Unlike fermentation with *S. thermophilus,* fermentation with *L. delbrueckii* ssp. *bulgaricus* results in significant hydrolyses of antimicrobial and hypotensive peptides after a four hour incubation at pH 4.5 (Paul & Somkuti, 2009). This study indicated that any BCMs formed during yoghurt fermentation or pre-existing in the milk before yoghurt making may be degraded during storage at pH 4.5. Currently, there is very little research on the effect of final fermentation pH value on the changes in level (e.g., by release of degradation) of BCMs during cold storage of yoghurt fermented with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*.

Table 3. Presence of BCMs in milk products fermented with different bacteria

Beta-casomorphins	Milk products	Starter culture	Origin	References
BCM4	Fermented milk	*L. helveticus* (mutant)	Bovine milk	Matar and Goulet (1996)
BCM5	Cheese	*Lactococcus lactis* ssp. *lactis* and *S. thermophilus*[a]	Bovine milk	Sienkiewicz-Szłapka et al. (2009)
BCM7	Cheese	*S. salivarius* ssp. *thermophilus*, *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*[b]	Bovine milk	De Noni and Cattaneo (2010), Sienkiewicz-Szłapka et al. (2009), Jarmolowska et al. (1999)
	Traditional yoghurt	*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*	Bovine milk	Jarmolowska (2012)
	Probiotic yoghurt	*L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus* and *Bifidobacterium*	Bovine milk	Jarmolowska (2012)
BCM9	Cheese	*Lactococcus lactis* spp. *lactis* and *Leuconostoc mesenteroides* ssp. *dextranicum*	Ovine milk	Gómez-Ruiz, Ramos, and Recio (2004)

[a] stater culture used for Gouda cheese making (Messens, Estepar-Garcia, Dewettinck, & Huyghebaert, 1999)

[b] stater culture used for Brie cheese making (Lefier, Lamprell, & Mazerolles., 2000)

2.4.3 Effect of cross-linking of protein

It is well-known that heat treatment of milk forms a κ-CN~β-LG complex (Fig.5) resulting from di-sulphide linkage. There are many studies reporting the effect of heat treatment of milk on the interaction between κ-CN and β-LG and leading to changes in properties of milk products. For β-CNs alone, the formation of inter/intra-molecular di-sulphide bonds by heat treatment of milk is very unlikely due to the lack of cysteine residues. However, the sequence of β-CNs contains four tyrosines, eleven lysines and twenty glutamines, so its higher level structure can be modified by formation of covalent cross-linking between tyrosine-tyrosine or lysine-glutamine by treatment of milk with the enzymes tyrosinase and transglutaminase (TGase), respectively (Monogioudi et al., 2009).

TGase (EC.2.3.2.13) catalyses the formation of covalent cross-linking between lysine and glutamine (Domagała, Wszołek, Tamime, & Kupiec-Teahan, 2013), leading to modification of the water holding capacity, viscosity or foaming properties of food products. Recently, application of TGase to modify proteolysis and texture of yoghurt has been reported (Domagała et al., 2013; SanlI, Sezgin, Deveci, Senel, & Benli, 2011; Yuksel & Erdem, 2010). Treatment of milk with TGase causes increase in viscosity and firmness, and lowers syneresis of yoghurt (Domagała et al., 2013). Treatment of milk with TGase has been reported to result in a significant decrease in peptide content in yoghurt, but the peptide profile do not change (Yuksel & Erdem, 2010). Yuksel and Erdem (2010) showed that peptide content significantly decreased when TGase is still active during fermentation. To date, there is little research reporting the effect of this enzyme on formation of BCM7 and other BCMs. However, it is presumed that a major impact of cross-linking by TGase on formation of BCMs is unlikely, due to the lack of lysine and glutamine in the BCM sequences (Table 1).

Tyrosinase (EC 1.14.18.1) catalyses oxidation of the phenolic ring of tyrosine to diquinones, which further react chemically with each other or free sulfhydryl to form covalent cross-linking (Ercili Cura et al., 2010; Monogioudi et al., 2009). Therefore, tyrosinase has been applied to increase the firmness of raw milk gels (Ercili Cura et al., 2010). To date, there has been no study investigating the effect of tyrosinase on the physio-chemical properties of yoghurt. As described in Table 1, the sequences of BCMs contain a tyrosine residue that could potentially form cross-linking catalysed

by tyrosinase. Therefore, treatment of milk with this enzyme may reduce the formation of BCMs and is thus, worthy of future investigation.

2.5 Analytical methods for identification and quantitation of beta-casomorphin 5 and beta-casomorphin 7

Milk and dairy products are complex matrices, containing proteins, lactose, lipids and peptides all of which can interfere with separation and detection of target peptides such as BCM5 and BCM7, leading to erroneous quantitative and qualitative results. Therefore, sample preparation and choice of an analytical method play a crucial role in generating accurate and reproducible results (Table 4). Reversed-phase high performance liquid chromatography (RP-HPLC) has been used widely in separation of peptides and amino acids in food products. RP-HPLC coupled with ultra violet (UV) detector has been applied for separation and quantification of BCM5 and BCM7 in milk and dairy products (Cieślińska et al., 2007; Latha & Mathur, 2001; Muehlenkamp & Warthesen, 1996; Wocior, 2008). In addition, HPLC-UV and ion-exchange chromatography have been also used for the analysis of BCM7 human milk or in cheese (Jarmolowska et al., 1999; Jarmołowska et al., 2007). In analysis of peptides, reversed-phase high performance liquid chromatography-ultra violet (RP-HPLC-UV) may generate chromatographic peaks that are in fact, constituted from the co-elution of multiple peptides with similar physical-chemical and spectrophotometric absorption properties to the analytes of interest (Muehlenkamp & Warthesen, 1996). This phenomenon can lead to a quantitative overestimation of target peptides as demonstrated by Cass et al. (2008). In addition, RP-HPLC-UV has low sensitivity to quantify levels of BCM7 found in dairy products. Muehlenkamp and Warthesen (1996) indicated that using RP-HPLC-UV is not possible to quantify the level of BCM7 below 2 µg/ml of cheese extract. Despite these limitations, RP-HPLC-UV is a well-established, relatively cheap, simple and "user friendly" analytical technique, thus, its wide popularity.

In recent years, ELISA has been used for determination of BCM5 and BCM7 in milk and fermented milk (Cieślińska et al., 2012; Jarmolowska, 2012; Sienkiewicz-Szłapka et al., 2009). This analytical technique can quantify the levels of BCM5 and BCM7 in cheese at 0.05 and 0.04 ng/mg, respectively. However, the heat treatment commonly applied to milk during processing may modify the conformation of BCM7 and other BCMs, leading to reduction in the binding affinity of the modified BCM7

to the antibody and a potential underestimation of the BCM7 level when milk is heated at high temperatures (Cieślińska et al., 2012).

Currently, HPLC coupled with mass spectrometry (MS) represents "the-state-of-the-art" analytical technique for identification and quantification of peptides in food matrices. There are three commonly used MS techniques namely tandem mass spectrometry (MS/MS), quadrupole ion-trap mass spectrometry (QIT-MS) and time-of-flight mass spectrometry (TOF-MS) that have been applied successfully to analyse BCM7 and other BCMs in milk and dairy products (Table 4).

The selectivity and sensitivity of MS/MS, especially when operated in multiple reaction monitoring (MRM) mode, allows accurate quantification of BCM5 and BCM7 at low levels (sub-ng/mg) in samples containing co-eluting peptides. Using this method, Haileselassie et al. (1999) detected BCM7 from an enzyme-modified cheese (EMC). More recently, HPLC-MS/MS has been used for quantitative determination of BCM5 and BCM7 in cheese, milk, fermented milk, yoghurt, milk-based infant formulas and extracts of these products after *in vitro* simulated gastro-intestinal digestion. BCM7 was quantified to a level as low as 0.01 ng/mg (De Noni, 2008; De Noni & Cattaneo, 2010). In other studies, BCM9 was detected in the water-soluble extracts of an Italian goat cheese (Rizzello et al., 2005).

QIT-MS mass spectrometry can perform multiple fragmentation spectra (MS^n), allowing to some extent, structural elucidation of unknown peptides. Similar to MS/MS, QIT-MS operated in single reaction monitoring (SRM) mode can be also used for quantification of BCM5 and BCM7. Juan-Garcia et al. (2009) examined BCMs in cheese and milk using a nano electrospray QIT-MS providing a fast and easy means of analysis of these compounds in different matrices. The main fragmentation pathways for BCMs and structures for the major fragment ions recorded in the MS^n spectra were also reported. Most recently, Thiri et al. (2012) used a hybrid triple quadrupole linear ion trap mass spectrometer operated in MRM mode to quantify BCM7 in milk at a level of 0.2 ng/ml.

TOF-MS is a high accuracy and high resolving power mass spectrometry technique. Using this technique can unequivocally identify unknown compounds in complex matrices. This is usually achieved by combining elemental formula information (i.e. $C_xH_yO_zN_j$…) from experiments of high mass accuracy with structural information from fragmentation experiments. Using HPLC-TOF-MS, Toelstede and Hofmann

(2008) identified BCM9 and BCM10 in the water-soluble extract of Gouda cheese. TOF-MS also allowed Cass et al (2008) to demonstrate that a peak previously analysed by HPLC-UV was in fact erroneously identified as BCM7.

In conclusion, RP-HPLC coupled with different mass analysers is a suitable method for quantifying BCM7 and other BCMs in milk and dairy products at low levels (sub-ng/g or ng/mL).

Table 4. Methods used for identification and quantification of BCMs

Beta-casomorphins	Matrix	Sample preparation	Analytical technique	Limits of detection	References
BCM7	Cow milk	Extraction chloroform/methanol, centrifugation, lyophilisation and purification by solid-phase extraction (SPE) using C_{18} T cartridge	ELISA	Not reported	Cieślińska et al. (2012)
BCM5 and BCM7	Cheese	Extraction chloroform/ methanol, centrifugation, fat removal, and lyophilisation and purification by SPE using C_{18}T cartridge	ELISA	11.10^{-3} µg/mL BCM5 13.10^{-3} µg/mL BCM7	Sienkiewicz-Sz1apka et al. (2009)
BCM7	Yoghurt, other fermented milk	Centrifugation and ultrafiltration	ELISA	Not reported	Jarmolowska (2012)
BCM7	Cheese	Extraction chloroform/methanol, gel filtration	Ion exchange chromatography	Not reported	Jarmołowska et al., (1999)
BCM3, BCM4,	Cheese	Extraction chloroform/	HPLC-UV	2 µg/mL	Muehlenkamp and

BCM5, BCM6 and BCM7		methanol, centrifugation, fat removal, and lyophilisation	(220, 254, 280 nm)	(BCMs)	Warthesen (1996)
BCM5 and BCM7	Human colostrum, milk	Extraction methanol/chloroform followed by shaking, centrifugation, lyophilisation and purification on C_{18} SPE cartridge	HPLC-UV (280 nm)	0.8 µg/mL BCM5 0.6 µg/mL BCM7	Jarmołowska et al., (2007)
BCM7, BCM9 and BCM13	Cow milk	Enzymatic digestion Chromatographic fractionation/purification on SPE cartridge	HPLC-UV (280 nm)	Not reported	Jinsmaa and Yoshikawa (1999)
BCM7	Cow milk	Extraction chloroform/ methanol followed by centrifugation, lyophilisation and purification on SPE cartridge	HPLC-UV	Not reported	Cieślińska et al.(2007)
BCM5	High protein formulas	Extraction chloroform/ methanol followed by centrifugation, lyophilisation and purification on SPE cartridge	HPLC-UV	Not reported	Wocior (2008)

BCM4	Fermented milk	Extraction chloroform/ methanol, absorption/desorption on charcoal/ evaporation	HPLC-UV (220 nm) LC-MS MS scan (200-800 *m/z*) HPLC-MS/MS MRM transitions (*m/z*)	Not reported	Matar and Goulet (1996)
BCM5 and BCM7	Milk-based infant formulas	milk coagulation, centrifugation, filtration and lyophilisation	BCM5 580.3→408.2; 286.0 BCM7 790.3→381.1; 530.2	Not reported	De Noni (2008)
BCM5 and BCM7	Cheese, milk, yoghurt, infant formulas, dried milk	Centrifugation and ultrafiltration	HPLC-MS/MS MRM transitions (*m/z*) BCM5 580.3→408.2; 286.0 BCM7	Not reported	De Noni and Cattaneo (2010)

BCM7	Pasteurised milk	Centrifugation followed by filtration with molecular weight cut off filter	790.3→381.1; 530.2 UPLC-hybrid triple quadruple linear ion trap MS, MRM transition	0.2ng/ml	Thiri et al. (2012)
BCM5, BCM7, BCM10 and BCM11	Cheese and milk	Extraction, fat removal, purification on polymeric SPE cartridge	HPLC-QIT-MS Q-IT in full scan and MS^n	Not reported	Juan-Garcia et al. (2009)
BCM7	Enzyme modified cheese	Centrifuge, methylene chloride and water, concentrated by Speed Vac	HPLC-API-MS	Not reported	Haileselassie et al. (1999)
BCM9	Cheese	Centrifugation, filtration through Whatman paper and 0.22 µm membrane.	HPLC-QIT-MS operated in MS scan and MS/MS full scan	Not reported	Rizzello et al. (2005)
BCM9	Cheese	Homogenisation with water following by centrifuge, dialysation, lyophilisation	HPLC-GPC Fast atom bombardment-MS	Not reported	Saito et al. (2000)

BCM7	Cheese	Coker (2002)	HPLC-MRM-MS/MS	Not reported	Norris et al. (2003)
BCM9 and BCM10	Cheese	Extraction with deionised water, centrifugation, paper filtration, lyophilisation	HPLC-TOF-MS LC-MS/MS	Not reported	Toelstede and Hofmann (2008)

2.6 Papers

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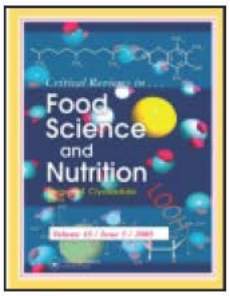

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Formation and Degradation of Beta-casomorphins in Dairy Processing

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Formation and Degradation of Beta-casomorphins in Dairy Processing

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Milk proteins including casein are sources of peptides with bioactivity. One of these peptides is beta-casomorphin (BCM) which belongs to a group of opioid peptides formed from β-casein variants. Beta-casomorphin 7 (BCM7) has been demonstrated to be enzymatically released from the A1 or B β-casein variant. Epidemiological evidence suggests the peptide BCM 7 is a risk factor for development of human diseases, including increased risk of type 1 diabetes and cardiovascular diseases but this has not been thoroughly substantiated by research studies. High performance liquid chromatography coupled to UV-Vis and mass spectrometry detection as well as enzyme–linked immunosorbent assay (ELISA) has been used to analyze BCMs in dairy products. BCMs have been detected in raw cow's milk and human milk and a variety of commercial cheeses, but their presence has yet to be confirmed in commercial yoghurts. The finding that BCMs are present in cheese suggests they could also form in yoghurt, but be degraded during yoghurt processing. Whether BCMs do form in yoghurt and the amount of BCM forming or degrading at different processing steps needs further investigation and possibly will depend on the heat treatment and fermentation process used, but it remains an intriguing unknown.

Keywords Beta-casomorphin, cheese, yoghurt, heat treatment, fermentation, ripening

INTRODUCTION

Milk protein is a source of peptides that are bioactive (Rizzello et al., 2005) including: angiotensin-converting enzyme inhibitory (ACE-I) activity (Minervini et al., 2003; Donkor et al., 2007); antihypertensive activity (Muguerza et al., 2006), antibacterial activity (Minervini et al., 2003), and opioid activity (De Noni and Cattaneo, 2010). The term "opioid" is used for substances having morphine-like activity that act by binding to opioid receptors. Opioid substances are found in the center nervous system and the gastrointestinal tract. These peptides play a crucial role in the response to pain and stress (Kamiński et al., 2007).

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Beta-casomorphins (BCMs) are a group of peptides with opioid properties and are formed from proteolytic digestion of β-casein (De Noni and Cattaneo, 2010). Among these peptides, only beta-casomorphin 7 (BCM7) has been widely studied. The release of BCM7 during hydrolysis of β-casein depends on the β-casein variant containing a histidine residue at position 67. In recent years, epidemiological evidence has indicated that consumption of milk containing the A1 variant is linked to increased risk of type 1 diabetes and heart disease (Elliott et al., 1999; McLachlan, 2001; Laugesen and Elliott, 2003). The European Food Safety Authority (EFSA) concluded there was insufficient data to determine a causal relationship between BCM7 exposure and non-communicable diseases (EFSA, 2009). Nevertheless the presence of these peptides in dairy products needs further research due to their putative link to elevated chronic disease risk.

The BCM7 peptide has been identified in raw milk (Cieślińska et al., 2007; Cieślińska et al., 2012) and some cheeses (Jarmolowska et al., 1999; De Noni and Cattaneo, 2010), but was not found in commercial yoghurt (Kahala et al., 1993; Schieber and Brückner, 2000; De Noni and Cattaneo, 2010). Whether BCMs do form and the amount of BCM forming at different processing steps needs further

investigation and possibly will depend on the heat treatment and fermentation process but remains an intriguing unknown.

Analytical trends in the analysis of BCMs indicate reverse phase high performance liquid chromatography (RP-HPLC) as the preferred choice for separation of these polar, nonvolatile peptides in a variety of dairy products. For detection, UV-Vis absorbance has been widely used in a number of analytical laboratories around the world (Muehlenkamp and Warthesen, 1996). Nowadays, at the forefront of detection techniques, is mass spectrometry coupled to electrospray ionization (ESI) which is by far the most reliable detection technique for small peptides (De Noni and Cattaneo, 2010). Alternatively, enzyme–linked immunosorbent assay (ELISA) has been applied to detect and quantify BCMs in dairy products (Siencikiewicz-Szłapka et al., 2009; Cieślińska et al., 2012).

This review aims to bring together the current knowledge on BCMs, analyzing their possible sources and presence in dairy products such as milk, cheese, and yogurt. The impacts of processing conditions such as thermal treatment, fermentation, and cross link on formation and degradation of BCMs in dairy products are also discussed. Finally, the advantages and limitations of each analytical technique currently in use to identify and quantify BCMs are also reported along with some examples of applications taken from the current scientific literature.

SOURCES OF BETA-CASOMORPHINS

Protein in bovine milk contains four caseins α_{s1}, α_{s2}, β, and κ-casein and two major whey proteins, β-lactoglobulin and α-lactoalbumin (Kamiński et al., 2007). In recent years, the 209 amino acid residues in the chains of β-casein (Fig. 1) (Swaigood, 2003; Truswell, 2005) have been studied extensively. It was found that there are 13 variants present in bovine milk including A1, A2, A3, A4, B, C, D, E, F, H1, H2, I, and H (Kamiński et al., 2007), of which A1 and A2 are the most common variants. The primary structure of some variants is showed at Figure 1. Milk from European breeds such as Holstein–Friesian mainly contains A1 β-casein (Tailford et al., 2003), while A2 β-casein is commonly detected in milk from Guernsey and Jersey breeds (Bell et al., 2006; Merriman, 2009). The difference between A1 and A2 variant is a single amino acid at position 67 on the chain; being histidine in A1 and proline in A2 (Truswell, 2005; Kamiński et al., 2007).

Interestingly, this structural difference leads to different properties when the two β-casein variants are hydrolyzed in the intestine. According to Jinsmaa and Yoshikawa (1999), the peptide bond between proline and isoleucine in the A2 variant has more enzymatic resistance than that between histidine and isoleucine in the A1 variant. So A1 β-casein is more easily hydrolyzed at this residue by gastrointestinal enzymes, resulting in the release of BCM7 (Jinsmaa and Yoshikawa, 1999). Consequently, BCM7 is liberated in the digestive tract from β-caseins containing histidine residue at 67 position, including A1 as well as B β-casein (Figure 2).

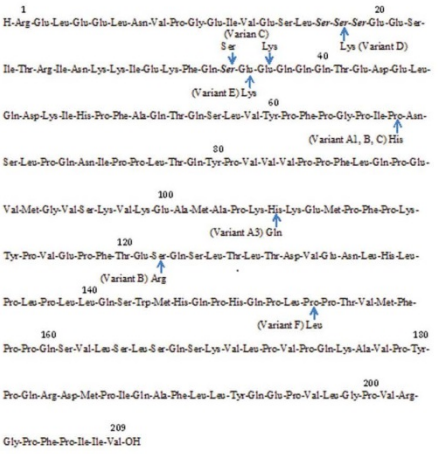

Figure 1. The primary structure of β-casein A2 indicating amino acid substitution of the variants (adapted from Swaigood, 2003).

BCMs are a group of structurally similar peptides (Table 1) with a sequence of 4–11 amino acids (Kamiński et al., 2007). All BCMs have the same sequence for the first three amino acids, tyrosine, proline, and phenylalanine (Muehlenkamp and Warthesen, 1996), and have been identified in and isolated from bovine milk (Kamiński et al., 2007; EFSA, 2009). These peptides are released from β-casein at position 60 which is the tyrosine residue and the other residues are released from other positions (table 1) if the conditions are right.

PRESENCE OF BETA-CASOMORPHINS IN DAIRY PRODUCTS

Milk

Bovine milk is a crucial food for humans as a source not only of high quality protein, fat, minerals, and vitamins (Bell et al., 2006; Kamiński et al., 2007; Nagpal et al., 2011), but also of bioactive peptides that are linked to positive health effects on the cardiovascular, nervous, and gastrointestinal systems (Choi et al., 2012). Consumption of milk and dairy products may play an important role for prevention of a number of diseases such as hypertension (Jauhiainen and Korpela, 2007), osteoporosis (Uenishi, 2006), obesity (Jaffiol, 2008), dental decay (Shimazaki et al., 2008) and cardiovascular disease (Elwood et al., 2004a, 2004b; Elwood, 2005; Elwood et al., 2005). In addition, bovine milk also contains peptides with opioid properties, for instance BCM7 that may increase risk of chronic disease (Cieślińska et al., 2007; Cieślińska

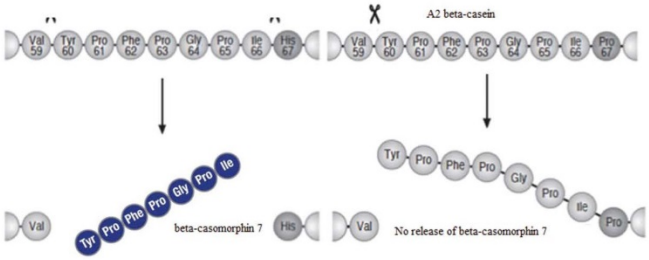

Figure 2. Release of BCM7 β-casein variant (adapted from Woodford, 2009).

et al., 2012). Epidemiologically, however, intake of milk containing A1 β-casein strongly correlates with ischemic heart disease (McLachlan, 2001; Laugesen and Elliott, 2003) and type 1 diabetes (Elliott et al., 1999; McLachlan, 2001; Birgisdottir et al., 2006). This implies that BCM7 released from A1-β casein may be a significant contributor to the risk of cardiovascular disease and type 1 diabetes.

Bovine milk naturally contains somatic cell, endogenous proteases, and unwanted bacteria, which may hydrolyze β-casein into peptides, including BCMs. Cieślińska et al. (2007) detected BCM7 in unprocessed cow milk but this study did not characterize whether somatic cells were present. Therefore, the presence of BCM7 in bovine milk may have been a result of microbial or somatic cell-derived enzyme activity (EFSA, 2009) rather than BCM formation in the mammary gland. However, Napoli et al. (2007) showed that BCM7 was not detected in milk from cows with mastitis, where concentration of somatic cells exceeds 500,000 per milliliter (Ginn et al., 1985). So high levels of somatic cell may degrade BCM7; but this needs further investigation. Similarly, Cieślińska et al. (2012) investigated the presence of BCM7 in bovine milk collected from healthy cows which did not indicate any subclinical symptom of mastitis and showed that BCM7 was detected in all milk samples with the highest amount in A1 milk, followed by A1A2 milk and A2 milk. Surprisingly, BCM7 was found in the milk containing the A1 variant but also in A2 variant-containing milk. However, the authors did not characterize the number of bacterial cells, the conditions and duration of storage of samples, or the presence of plasmin, an endogenous protease present in milk. Consequently, it is possible that the release of BCM7 may involve hydrolysis by bacterial-derived enzymes or endogenous enzymes of A1 β-casein to form BCM7. This research, however, still contradicts other studies that have not found BCM7 in milk of the A2 β-casein variant.

Findings in human milk may assist in our understanding of the origin and fate of BCMs in bovine milk. For instance, Jarmołowska et al. (2007) reported that beta-casomorphin 5 (BCM5) and BCM7 were found in human colostrums and mature milk; the BCM content of the former being much higher than that of the later. According to the authors, milk samples were selected from healthy women and frozen immediately after expression and stored at -70°C; excluding the possibility of formation of BCMs by somatic cell and bacterial enzyme action. These findings indicate that BCMs may be released from β-casein in the mammary gland by endogenous enzyme activity (Koch et al., 1988). Endogenous enzyme activity may also result in peptide formation during the manufacture of cheese and other fermented dairy products as will be described in the following sections.

Cheese

Cheese is a dairy product that is a commonly consumed part of the diet in Western countries, and is commonly produced from bovine milk. Major steps in cheese manufacture include

Table 1 BCMs released from bovine milk

Beta-casomorphins	Structure	References
BCM 4, β-CN f(60–63)	Tyr-Pro-Phe-Pro	EFSA (2009), Kamiński et al. (2007)
BCM 5, β-CN f(60–64)	Tyr-Pro-Phe-Pro-Gly	EFSA (2009), Kamiński et al. (2007)
BCM 6, β-CNf(60–65)	Tyr-Pro-Phe-Pro-Gly-Pro	Kamiński et al. (2007)
BCM 7, β-CN f(60–66)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	EFSA (2009), Kamiński et al. (2007)
BCM 8, β-CN f(60–67)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	EFSA (2009), Kamiński et al. (2007)
BCM 9, β-CN f(60–68)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn	Saito et al. (2000)
BCM 10, β-CN f(60–69)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser	Toelstede and Hofmann (2008)
BCM 11, β-CN f(60–70)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	Kamiński et al. (2007)

heat treatment of milk, coagulation with rennet and starter culture, separation of curd from whey, and ripening of curd to produce the final cheese. Heat treatment of milk is commonly done by pasteurization at 72°C for 15 seconds (Hayaloglu et al., 2010). Cheese studies that used milk heated at above 72°C involving peptide profiles (Hougaard et al., 2010) showed denaturation of whey protein, leading to the interaction between whey protein and casein. High temperature pretreatment may significantly affect proteolysis of cheese during ripening as well as its final flavor and texture (Benfeldt and Sørensen, 2001). Protelolysis during ripening may involve the formation of large peptides, some of which are subsequently degraded into smaller peptides that may include pro-BCMs and BCMs as well as free amino acids and aroma compounds (Benfeldt and Sørensen, 2001).

Currently, only a few studies have investigated the formation of BCMs in cheese varieties (Sienkiewicz-Szłapka et al., 2009; De Noni and Cattaneo, 2010). In Brie cheese, BCM7 was found in the range 5 to 15 μg/g following treatments of different ripening times by Jarmołowska et al. (1999), whereas De Noni and Cattaneo (2010) detected only 0.15 μg/g and Sienkiewicz-Szłapka et al. (2009) found 6.48 μg/g. BCM7 was found 0.01 to 0.11 μg/g in Gorgonzola, Gouda, Fontina, and Cheddar (De Noni and Cattaneo, 2010). Similarly, Sienkiewicz-Szłapka et al. (2009) quantified BCM7 in some locally produced Polish mould cheeses (Brie and Rokpol) and determined that levels were much higher than that of the semi-hard cheeses they analyzed (Edamski, Gouda and Kasztelan). The levels of BCM7 and other related BCMs reported in some cheeses are shown in Table 2. In addition to BCM7, BCM5 was found in Brie, Rokpol, Edamski, Gouda, and Kasztelan (Sienkiewicz-Szłapka et al., 2009), BCM9 (Saito et al., 2000; Toelstede and Hofmann, 2008) and BCM10 in Gouda (Toelstede and Hofmann, 2008) and BCM 11 in Caprino del Piemonte, an Italian goat cheese (Rizzello et al., 2005). In contrast, Muehlenkamp and Warthesen (1996) reported that BCM7 were not detected in Brie and Cheddar. According to the authors, the absence of BCM7 in these products may be due to (a) nonformation of BCMs from β-casein, (b) degradation of BCM7 during ripening, or (c) smaller amounts of BCM7 than the minimum detectable level by RP-HPLC (<2 μg/mL of cheese extract).

The formation of BCM7 in some cheeses is due to the digestion of β-casein with starter culture derived enzymes. By using a HPLC-MS/MS method, De Noni and Cattaneo (2010) found 0.11 μg/g of BCM7 in Cheddar cheese. According to Jarmolowska et al. (1999), the presence of BCM7 in cheese does not originate from that originally present in milk, because milk-derived peptides would be removed from the curd during the drainage of whey. Cheddar cheese making commonly use starter cultures which are a mixture of *Lactococcus. lactic* ssp. *cremoris* and *L. lactic* ssp. *lactic* (Robinson, 1995). Muehlenkamp and Warthesen (1996) showed that enzymes derived from *L. lactic* ssp. *cremoris* are capable of reducing the amount of BCM7 found by 50% at pH 5.0 and 1.5% NaCl after 6–15 weeks; conditions which are similar to those in Cheddar cheese making. Thus, the concentration of BCM7 in Cheddar cheese at the beginning period of ripening may be higher than

Table 2 BCMs in cheeses

Cheese	β-casomorphins	Content (μg/g)	References
Brie	BCM7	5–15	Jarmolowska et al. (1999)
		0.15	De Noni and Cattaneo (2010)
		6.48	Sienkiewicz-Szłapka et al. (2009)
	BCM5	3.14	Sienkiewicz-Szłapka et al. (2009)
Cheddar	BCM7	0.11	De Noni and Cattaneo (2010)
	BCM5	Not reported	De Noni and Cattaneo (2010)
	BCM9	Identified	Singh et al. (1997)
Gorgonzola	BCM7	0.01	De Noni and Cattaneo (2010)
	BCM5	Not reported	De Noni and Cattaneo (2010)
Fontina	BCM7	0.04	De Noni and Cattaneo (2010)
	BCM5	Not reported	De Noni and Cattaneo (2010)
Gouda	BCM7	0.06	Sienkiewicz-Szłapka et al. (2009)
		0.1	De Noni and Cattaneo (2010)
	BCM5	0.05	Sienkiewicz-Szłapka et al. (2009)
	BCM9	Identified	Saito et al. (2000); Toelstede and Hofmann (2008)
	BCM10	Identified	Toelstede and Hofmann (2008)
Rokpol	BCM7	1.66	Sienkiewicz-Szłapka et al. (2009)
	BCM5	2.57	Sienkiewicz-Szłapka et al. (2009)
Edamski	BCM7	1	Sienkiewicz-Szłapka et al. (2009)
	BCM5	0.46	Sienkiewicz-Szłapka et al. (2009)
Kasztelan	BCM7	0.04	Sienkiewicz-Szłapka et al. (2009)
	BCM5	0.14	Sienkiewicz-Szłapka et al. (2009)
Taleggio, Caprino and Grana Padano	BCM7	No detected	De Noni and Cattaneo (2010)
	BCM5	Not reported	De Noni and Cattaneo (2010)
	BCM5	Not reported	De Noni and Cattaneo (2010)

that in the ripened cheese reported by De Noni and Cattaneo (2010), since BCM7 may have been formed from β-casein on the addition of starter culture, but degraded into even smaller peptides during ripening.

As well as through starter cultures, there is potential for bioactive peptides including BCMs to be formed by hydrolysis of β-casein by endogenous proteases and nonstarter culture bacterial proteases from contaminated udder, pipes, and equipment (EFSA, 2009). However, the endogenous protease plasmin has been shown not to release BCMs (Gaucher et al., 2008) and similarly the rennet enzymes used to coagulate milk protein also do not liberate bioactive peptides (McSweeney, 2004); excluding these enzymes activities as sources of BCMs. Hayaloglu et al. (2010) investigated the peptide profile of Malatya cheese made from raw milk without addition of starter culture. The authors demonstrated considerably higher concentration of peptides but did not measure BCMs, although they may have been present in this cheese. Hayaloglu et al. (2010) compared to raw milk cheese to cheese made from pasteurized milk with added *L. lactis* ssp. *lactis* and *S. Thermophilus* and suggested that the resultant peptides were as a result of nonstarter culture derived enzyme-activity. However, to date, there is no published paper investigating formation of BCMs by nonstarter culture-derived proteases.

The presence of BCM7 in cheese may also be due to the activity of enzymes derived from mould during ripening. The level of BCM7 in Brie (Sienkiewicz-Szłapka et al., 2009; De Noni and Cattaneo, 2010) and Rokpol ripened with fungi and lactic acid bacteria (LAB) (Sienkiewicz-Szłapka et al., 2009) was considerably higher than that in cheese ripened with only LAB. Similarly, Jarmolowska et al. (1999) also observed a high level of BCM7 in Brie. Consequently, the formation of BCM7 in Brie and Rokpol cheese may be due to proteolytic digestion of protein by both enzymes derived from fungi (De Noni and Cattaneo, 2010) and LAB (Jarmolowska et al., 1999).

The stability of BCM7 may depend on the type of enzymes present in cheese. Haileselassie et al. (1999) investigated potentially bioactive peptides including BCM7 in Cheddar cheese extract with the addition of the enzyme, Neutrase isolated from *B. subtilis*. BCM7 was identified but the amount was not reported. However, no BCM7 was detected in samples: (a) prepared from the Cheddar cheese extract with added Neutrase plus a crude extract of *L. casei* isolated from mild Cheddar cheese or (b) the Cheddar cheese extract with added Neutrase plus a peptidase isolated from *Lactococcus lactic* and *Asperillus oryzae*. Haileselassie et al. (1999) suggested that the presence of BCM7 in the cheddar cheese extract with Neutrase was as a result of β-casein hydrolysis by the Neutrase. The absence of BCM7 in the two latter samples may have been as a result of BCM7 hydrolysis by proteases to a level lower than the threshold level of the analysis. Thus, the formation and degradation of BCM7 may involve proteases derived from bacteria used in the starter culture.

The characterization of BCM precursors (i.e. longer peptide chains containing the BCM amino acid sequence) in cheese may support our understanding of the steps involved in BCM formation. In general, proteases digest β-casein into larger peptides including BCM precursors, and subsequently some of precursors are degraded into BCMs by specific peptidases. One BCM precursor found in Crescenza (Smacchi and Gobbetti, 1998) and in Cheddar and Jarlsberg cheese (Stepaniak et al., 1995) after ripening for 8–10 days was β-CN f58-72 (Smacchi and Gobbetti, 1998). This peptide may be further digested into BCM13 (β-CN f60-72) as reported by Jinsmaa and Yoshikawa (1999) and other related BCMs if cheese is aged for a longer period.

As previously described, the formation of BCM7 in cheese depends on β-casein variants present in the milk used for producing the cheese; A1 and B β-casein variants being those most likely to contribute to release of BCM7. Most studies above investigated BCM7 and other related BCMs in commercial cheeses that were not characterized for the β-casein variant in the milk used, except for the study of De Noni and Cattaneo (2010). According to De Noni and Cattaneo (2010), A1, A2, and B variant of β-casein were detected in all cheese samples, so it was unclear which of the variants contributed to the occurrence of BCM7 following fermentation. For elucidating which variant(s) is (are) the source of BCM7 during cheese making, it will be necessary to investigate the formation of BCM7 and other related BCMs in cheese produced from milk of single β-casein variants in future research.

Yoghurt

Yogurt is a popular dairy product usually fermented by two lactic acid bacteria, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (Tamime and Robinson, 1999). Major steps in yoghurt processing include thermal treatment, fermentation, and chilled storage. In addition to inducing changes in pH and viscosity, the yoghurt culture can hydrolyze the milk proteins into peptides with bioactivity. A number of bioactive peptides have been isolated from yoghurt, such as ACE-I peptides (Donkor et al., 2007; Kunda et al., 2012) and antihypertensive peptides (Schieber and Brückner, 2000; Muguerza et al., 2006; Kunda et al., 2012). However, no study has reported on the formation or degradation of BCMs in yoghurt. The lack of reliable information about BCMs in yoghurt may be in part due to difficulty in identification and quantification of the low levels of these peptides within the complex yoghurt matrix (EFSA, 2009).

As reported by Donkor et al (2007), β-casein-derived peptides were isolated from yoghurt inoculated with yoghurt starter culture and probiotic organisms (*L. casei* L26, *L. acidophilus* L10 and *B. lactis* B94). These peptides included different β-CN fragments (β-CN f193-198, f25–29, f69–73 and f84–86) but neither BCMs nor precursors of BCM were identified. Similarly, Schieber and Brückner (2000) identified 30 peptides in yoghurt fermented with *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*, mainly produced from A1 β-casein. These authors did observe two precursors of BCM, β-CN f57–68, and β-CN f57–72, containing histidine at position 67 of the parent

β-casein. This finding suggested that precursors of BCM are more likely to be formed from A1 and B β-casein variant than A2 β-casein (Schieber and Brückner, 2000).

According to Kunda et al. (2012), a large number of peptides were formed from both A1 and A2 β-casein variants during yoghurt manufacture, among which a peptide containing the BCM7 sequence, namely β-CN f58–72 was identified. In addition, some peptides identified in yoghurt were released from only the A2 β-casein variant, namely β-CN f59–68 and β-CN f59–70 and these peptides may be further degraded into BCM13, BCM9, and BCM10, respectively. Furthermore, the authors found β-CN f60–72 was liberated from A1 β-casein variant, indicating that yoghurt starter cultures may hydrolyze A1 β-casein variant into BCMs. Several di-peptides have been also identified in yoghurt, such as β-CN f60–61 and β-CN f62–63. Both di-peptides were released from A1 and A2 β-casein variants. As previously described in Figure 1, amino acids at position 61 and 63 on the chain of parent β-casein are proline residues. Therefore, this finding indicated that yoghurt bacteria-derived enzymes are likely to digest the peptide bonds between proline and other amino acid residues, leading to degradation of BCMs. This degradation of BCMs during yoghurt processing may involve the activity of X-prolyl dipeptidyl aminopeptidase (PepX). PepX is derived from LAB and is specifically hydrolyzes peptide bonds between proline and other residues (Gobbetti, 2002). Similarly, Sabeena Farvin et al. (2010) identified peptide fragments in commercial yogurt within 24 hours of production. Peptides detected included fragments of α_{s1}, α_{s1}, κ-casein, with a majority of fragments from β-casein, from which BCM9 was released from A2 β-casein but BCM7 was not found. The formation of BCMs in cheese processing suggests BCMs may be formed and then degraded during yoghurt processing. Kunda et al. (2012) and Sabeena Farvin et al. (2010) did not isolate and characterize starter cultures present in the yoghurt they investigated, which is a key factor influencing the production and degradation of peptides, including the final BCMs content of the product.

Yogurt inoculated with a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* was isolated and characterized by De Noni and Cattaneo (2010) in a BCM7 study. The yoghurts did not contain BCM7, although A1, A2, and B β-CN variants were identified in yoghurts. The same results were reported by Schieber and Brückner (2000) investigating yoghurt kept 21 days at 4°C. Thus, it has been suggested that the mixture of starter culture *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is not capable of proteolysis of β-casein to form BCMs. This finding was also supported by work on Taleggio cheese using *L. delbrueckii* ssp. *bulgaricus* and *S. thermophiles* as starter culture, that did not contain BCM7 (De Noni and Cattaneo, 2010). However, both the yoghurt and Taleggio cheese used for the analysis of BCM7 were kept for 30 days and 35 days, respectively (De Noni and Cattaneo, 2010). As reported by Atlan et al. (1990) PepX is located in cytoplasm, so these long periods of aging possibly resulted in cell lysis, leading to release of PepX that during storage of yoghurt degraded and BCMs formed. In addition, during fermentation, the proteases associated with the cell wall of the starter culture or contaminating bacteria can hydrolyze caseins into large peptides and possibly even BCM7, which subsequently are transported into the bacteria cell and then hydrolyzed into a large number of smaller peptides, e.g., di and tri-peptides (Nielsen et al., 2009). Consequently, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* used for producing yoghurt may digest β-casein into BCM7 and other related BCMs; however, these may be subsequently degraded into smaller peptides.

The stability of BCMs in yoghurt may be influenced by the symbiotic growth of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. It is well known that the growth of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is strongly related, because products released by each organism stimulate the growth of the other (Tamime and Robinson, 1999). For instance, some peptides and amino acid formed by *L. delbrueckii* ssp. *bulgaricus* stimulate the growth of *S. thermophilus* (Courtin et al., 2002). Therefore, BCMs may be released by *L. delbrueckii* ssp. *bulgaricus* protease activity and subsequently degraded by *S. thermophilus*, and vice versa. Nevertheless, Gobbetti et al. (2000) have demonstrated that only the strain of *L. delbrueckii* ssp. *bulgaricus* SS1, when used for fermentation of yoghurt, produced several fractions originating from β-CN such as β-CN f6–14, β-CN f7–14, β-CN f73–82, β-CN f74–82, and β-CN f75–82 but no BCMs. Similarly, Papadimitriou et al. (2007) identified β-CN f176–180 and β-CN f1–8 in sheep yoghurt inoculated with only *L. delbrueckii* ssp. *bulgaricus* Y 10.13. It is unknown when and if BCMs are released or degraded in yoghurt processing as using starter cultures *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* but this needs further investigation.

IMPACT OF DAIRY PROCESSING ON FORMATION AND DEGRADATION OF BETA-CASOMORPHINS

Effect of Thermal Treatment

Thermal treatment is an important step in dairy processes from a food safety and quality point of view. In addition to killing both spoilage and pathogenic bacteria, thermal treatments result in the modification of the milk protein conformation that beneficially affects the texture and viscosity of fermented dairy products (Krasaekoopt, 2003; Hattem et al., 2011). In yoghurt production, secondary heating in addition to pasteurization is commonly performed at 90–95°C for five minutes (EFSA, 2009) causing denaturation of whey proteins, and promotion of the interaction between caseins and whey proteins; particularly κ-casein and β-lactoglobulin. The formation of this complex influences proteolysis during fermentation (El-Zahar et al., 2003). Furthermore, heat treatments contribute to the breakdown of parent proteins to form lower molecular weight peptides (Gaucheron et al., 1999).

Formation of peptides from caseins is not only by enzymatic (Lotfi, 2004; Meltretter et al., 2008) but also through

fragmentation of the protein backbone during heat treatment of milk (Meltretter et al., 2008). Meltretter et al. (2008) identified five new peptides not present in the raw milk, after it was heated at 120°C for 30 minutes. Unknown peptides were identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) at mass-to-charge (*m/z*) ratios 974.4, 2218.7, 3730.1, 4297.8, and 4436.8 and they were reported to have originated from caseins. None of the *m/z* values matched those of BCMs indicating that no BCMs were formed by simply heating raw milk. During heat treatment of milk at 120°C for 30 minutes, enzymes are inactivated and the pH of the milk is unlikely to change greatly, therefore the formation of peptide by enzyme and acid hydrolysis can be excluded. On the other hand, during thermal treatment of milk the Maillard reaction, which is a reaction between a reducing sugar (lactose) with amino acids, may form radicals that can attack the peptide bonds within the protein to cleave the bonds and yield new peptides (Meltretter et al., 2008).

The formation of new peptides during heat treatment of milk will depend on the thermal strength of peptide bonds of β-casein. Gaucheron et al. (1999) reported the formation of low molecular weight peptides in raw skim milk heated at 120°C for 30 minutes. Ten peptides, not present in raw milk, were identified; two of which were liberated from β-casein, including β-CN f1–14 and β-CN f1–16 but neither BCMs nor precursors of BCMs were found. According to the authors, peptides released after heat treatment are mainly a result of the hydrolysis of peptide bonds involving either aspartic acid (Asp), asparagine (Asn), or glutamic acid (Glu) bound to any other amino acid; bonds known to be more susceptible to hydrolysis during heat treatment (Gaucheron et al., 1999). Gaucher et al. (2008) found Asn containing peptide bonds were also susceptible to thermally induced breakdown. Gaucher et al. (2008) identify several pro-BCMs in UHT milk stored for six months at 20°C, namely; β-CN f54–68, β-CN f54–69, β-CN f55–65, β-CN f55–68, and β-CN f57–68 derived from the action of the enzymes elastase and cathepsins. Peptides, β-CN f54–68, β-CN f55–68, and β-CN f57–68, resulted from hydrolysis of a peptide bond involving Asn. Therefore, it appears that peptide bonds containing Asn are more susceptible to hydrolysis than those not containing this amino acid residue during heat treatment of milk. Schieber and Brückner (2000) also found β-CN f57–68 in yoghurt which may have been a result of milk heat treatment which is a common processing step in yogurt production.

Recently, the formation and stability of BCM7 in heated milk has attracted attention of researchers (De Noni and Cattaneo 2010; Cieślińska et al. 2012). De Noni and Cattaneo (2010) were the first to measure BCM7 content in commercial pasteurized, UHT, and in-bottle heat-treated milk. The authors showed that BCM7 was not detected in these products, even though the products contained A1 and B β-casein variants which contain the BCM7 sequence. Similarly, Cieślińska et al. (2012) quantified the content of BCM7 by ELISA in varieties of milk containing pure A1, A2 and mixture of A1 and A2 β-casein variants after pasteurization (95°C/20 minutes) and sterilization (117°C/5 minutes). BCM7 content tended to decrease after heating of the milk but not due to the breaking down the peptide bonds of BCM7 but because of the reaction between lactose and amino acids residues of BCM7, which reduced the binding affinity of the modified BCM7 to the antibody used in the ELISA assay, leading to underestimated results for BCM7 levels (Cieślińska et al., 2012). Thus, thermal treatment may modify the β-casein and influence formation of BCM7 in further processing of dairy products.

The previous thermal treatment history of the milk plays a crucial role in proteolysis during subsequent fermentation and aging of fermented dairy products (El-Zahar et al., 2004). Hayaloglu et al. (2010) investigated the peptide profile of Malatya cheese made from raw milk and from milk pasteurized at 72°C for 30 seconds. The authors suggested that pasteurization of milk results in a decrease in peptide content during ripening, with considerably higher concentration of peptides being reported in cheese made from raw milk than from pasteurized milk; however, the actual peptides produced were not identified. The release of peptides may be due primarily to proteolysis by nonstarter bacteria-derived protease activity in the raw milk as pasteurization of milk at 72°C for 30 seconds inactivated nonstarter culture bacteria. In contrast, Mendia et al. (2000) reported that the level of proteolysis, mainly of β-casein, was higher in cheese made from pasteurized ewe milk than that made from raw milk.. The action of plasmin which is more active in milk that has been pasteurized than in unheated milk may be the reason for the contrast in results (Mendia et al., 2000; Benfeldt and Sørensen, 2001), although plasmin will be inactivated upon severe heat treatment of milk. Although a number of studies have investigated the effect of thermal treatment of milk on proteolysis (Rynne et al., 2004; Hayaloglu et al., 2010; Hougaard et al., 2010), its effect on modification of protein, leading to formation of BCMs in the final fermented dairy product is still unknown.

Effect of Fermentation Process

Fermentation is an extremely important step in the processing of milk into cheese and yoghurt. In addition to a drop in pH, the fermentation process causes a series of changes in chemical composition of milk resulting from proteolysis, glycolysis, and lipolysis. These reactions are dependent on the type of starter culture, incubation temperature and time, and maturation time.

The choice of starter culture is a crucial factor in determining the attributes of fermented dairy products. During fermentation, different strains of starter culture can release different bioactive peptides from milk protein, including BCMs liberated from β-caseins (Table 3). Some BCMs isolated from cheeses have been described in Table 2; however, these studies did not characterize the starter cultures used for producing

Table 3 BCMs released by different bacteria

β-casomorphins	Starter culture	Origin	References
β-casomorphin 4	A mutant of *Lactobacillus helveticus*	Bovine milk	Matar and Goulet (1996)
β-casomorphin 5	A mixture of *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*[a]	Bovine milk	Sienkiewicz-Szłapka et al. (2009)
β-casomorphin 7	A mixture of *Streptococcus salivarius* ssp. *thermophilus*, *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*[b]	Bovine milk	De Noni and Cattaneo (2010) Sienkiewicz-Szłapka et al. (2009) Jarmolowska et al. (1999)
β-casomorphin 9	A mixture of *Lactococcus lactis* spp. *Lactis* and *Leuconostoc mesenteroides* ssp. *dextranicum*	Bovine milk	Gómez-Ruiz et al. (2004)

[a]Starter culture used for Gouda cheese making (Messens et al., 1999).
[b]Starter culture used for Brie cheese making (Lefier et al., 2000).

the cheeses. As previously described, the use of *L. casei* L26, *L. acidophilus* L10, and *B. lactis* B94, for producing probiotic yoghurt (Donkor et al., 2007) and *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus* for fermentation of traditional yoghurt (Schieber and Brückner, 2000; De Noni and Cattaneo, 2010) does not result in the formation of BCM7 and other related BCMs. According to Atlan et al. (1990) and Meyer and Jordi (1987), a mixture of starter cultures, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* used for traditional yoghurt making contains a high level of PepX that may degrade proline-rich BCMs into a large number of smaller peptides. Meyer and Jordi (1987) reported that the pH optimum of PepX isolated from *S. thermophilus* was in the range of 6.5–8.2 and that this enzyme is inactivated at pH below 5.0. For PepX isolated *L. delbrueckii* ssp. *bulgaricus* optimal conditions were pH 7.0 and 50°C (Atlan et al., 1990). Yoghurt fermentation also involves a drop in pH from 6.8 to 6.3 during first stage of fermentation and if BCM7 is released at this stage, it may be degraded by PepX. Consequently, when BCM7 is formed or degraded in yoghurt inoculated with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* remains unknown.

In addition to type of starter culture, the pH at which the fermentation stops is a key factor affecting proteolysis subsequent during cold storage. In fermented milk manufacture, fermentation commonly ends at pH 3.5–4.6 before subsequent cold storage. According to Nielsen et al. (2009), milk inoculated with *L. lactis* strains and *L. helviticus* strains fermented to pH 4.6 and 4.3 resulted in large peptides being released from β-casein after 7-day cold storage, but no BCMs. The authors also observed no change in peptide profile during storage for milk inoculated with *S. thermophilus* fermented to pH 4.6 and 4.3. This study indicated that protease derived from *S. thermophilus* was not active during cold storage. In another study, Paul and Somkuti (2009) showed that peptides were slightly degraded by *S. thermophilus* and moderately hydrolyzed by *L. delbrueckii* spp. *bulgaricus* at pH 4.5 indicating that any BCMs formed during yogurt fermentation might also be slightly degraded at pH 4.5 during storage. Currently, there is very little published research on the effect of pH, especially a pH of 3.5, on the release or degradation during cold storage of BCMs in yoghurt inoculated with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*.

Effect of Cross Linking

In dairy technology, it is well-known that heat treatment (85–90°C for five minutes) increases the viscosity of yoghurt due to the interaction between κ-casein and whey protein by di-sulphide bonding. Due to the lack of cysteine in β-casein, inter/intra-molecular disulphide bonds formation during heat treatment are not possible for this protein. However, modification of β-casein can be performed by enzymatic covalent cross-linking (Monogioudi et al., 2009) via tyrosine, lysine, and glutamine residues through the addition of tyrosinase and transglutaminase (TGase) (Monogioudi et al., 2009).

TGase affects the texture and proteolysis of yoghurt (Yuksel and Erdem, 2010; Sanli et al 2011). Yuksel and Erdem (2010) reported that peptide content of yoghurt decreases when TGase is added to the milk, but there was no difference in the peptide profile between TGase-treated and untreated milk. Furthermore, peptide content in milk treated with TGase that was still active during fermentation was less than that in milk treated with Tgase that had been inactivated during fermentation. The formation of inter-molecular cross-linking during fermentation of yoghurt is related to the increase in lactic acid that leads to a decrease in stability of casein micelles (Yuksel and Erdem, 2010). As described in Table 1, BCMs contain tyrosine residues that have the potential to form enzyme-catalyzed cross-linking, therefore, treatment of milk with cross-linking enzymes may reduce the production of BCMs from β-casein.

METHODS FOR IDENTIFICATION AND QUANTIFICATION OF BETA-CASOMORPHINS

Milk and dairy products are complex matrices, in which proteins, lactose, and lipids can interfere with separation and detection of target peptides, leading to erroneous quantitative and qualitative results. Reversed-phase high performance

Table 4 Methods for analysis of BCMs

Beta-casomorphines	Matrix	Sample preparation	Analytical technique	Limits of detection	References
BCM7	Cow milk	Cieślińska et al., 2007	ELISA	Not reported	Cieślińska et al. (2012)
BCM5	Cheese	Extraction chloroform/ methanol,	ELISA	11.10^{-3} μg/mL BCM-5	Sienkiewicz-Sz1apka
BCM7		centrifugation, fat removal, and lyophilization and purification on C_{18}T SPE cartridge		13.10^{-3} μg/mL BCM-7	et al. (2009)
BCM7	Cheese	Extraction chloroform/methanol, gel filtration	Ion exchange chromatography	Not reported	Jarmołowska et al., (1999)
BCM3	Cheese	Extraction chloroform/ methanol,	HPLC-UV (220, 254, 280 nm)	2 μg/mL (BCMs)	Muehlenkamp and
BCM4		centrifugation, fat removal, and			Warthesen (1996)
BCM5		lyophilization			
BCM6					
BCM7					
BCM5	Human colostrum, milk	Extraction methanol/chloroform followed by	HPLC-UV (280 nm)	0.8 μg/mL BCM-5	Jarmołowska
BCM7		shaking, centrifugation, lyophilization, and purification on C_{18} SPE cartridge		0.6 μg/mL BCM-7	et al. (2007)
BCM7	Cow milk	Enzymatic digestion Chromatographic	HPLC-UV (280 nm)	Not reported	Jinsmaa and
BCM9		fractionation/purification			Yoshikawa (1999)
BCM13					
BCM21					
BCM4	Fermented milk	Extraction chloroform/ methanol, absorption/ desorption on charcoal/evaporation	HPLC-UV (220 nm) LC-MS MS scan (200–800 m/z)	Not reported	Matar and Goulet (1996)
BCM5	Milk-based infant formulas	milk coagulation, centrifugation, filtration and	HPLC-MS/MS	Not reported	De Noni (2008)
BCM7		lyophilization	MRM transitions (m/z)		
			BCM-5 580.3→408.2; 286.0		
			BCM-7 790.3→381.1; 530.2		
BCM5	Cheese, milk, yoghurt, infant formulas,	Parrot et al. (2003); Donkor et al. (2007)	HPLC-MS/MS	Not reported	De Noni and
BCM7	dried milk		MRM transitions (m/z)		Cattaneo, (2010)
			BCM-5 580.3→408.2; 286.0		
			BCM-7 790.3→381.1; 530.2		
BCM5	Cheese and milk	Extraction, fat removal, purification on	HPLC-QIT-MS	Not reported	Juan-Garcia
BCM7		polymeric SPE cartridge	Q-IT in full scan and MS^n		et al. (2009)
BCM10					
BCM11					
BCM7	Enzyme modified cheese	Centrifuge, methylene chloride and water,	HPLC-API-MS	Not reported	Haileselassie
		concentrated by Speed Vac			et al. (1999)
BCM11	Cheese	Kuchroo and Fox (1982)	HPLC-QIT-MS operated in	Not reported	Rizzello et al. (2005)
			MS scan and MS/MS full scan		
BCM9	Cheese	Homogenization with water following by	HPLC-GPC	Not reported	Saito et al. (2000)
		centrifuge, dialyzation, lyophilization	Fast atom bombardment-MS		
BCM9	Cheese	Extraction with deionized water,	HPLC TOF-MS	Not reported	Toelstede et al. (2008)
BCM10		centrifugation, paper filtration,	LC-MS/MS		
		lyophilization			
BCM9	Yoghurt	Homogenization, remove sugar and lactic acid	LC-MS/MS	Not reported	Sabeena Farvin
		on cation exchanger columns			et al. (2010)
BCM13	Yoghurt	Extraction, trisodium citrate dehydrate and	*micro*LC-TOF-MS	Not reported	Kunda et al. (2012)
		DTT, centrifuge, purification on SPE cartridge			

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liquid chromatography (RP-HPLC) has been used widely in separation of peptides and amino acids. For example, RP-HPLC-UV (Muehlenkamp and Warthesen, 1996) and ion-exchange chromatography (Jarmolowska et al., 1999) have been used for the separation and quantification of BCM7 present in cheese while HPLC-UV has been also used for determining BCM7 content in human milk (Jarmołowska et al., 2007). A limitation of RP-HPLC-UV is that peptides with similar physico-chemical and spectrophotometric absorption properties can co-elute with BCMs, increasing the absorption values (Muehlenkamp and Warthesen, 1996; Sienkiewicz-Szłapka et al., 2009), resulting in an overestimation of the BCM7 content (Cass et al., 2008). In addition, methods employing UV-Vis adsorption may lack the sensitivity required to quantify low levels of BCM7 found in some dairy products. The limit of quantification for BCM7 analyzed by RP-HPLC-UV is approximately 2 μg/mL of cheese extract making accurate quantification often not possible (Muehlenkamp and Warthesen, 1996). Despite these deficiencies, RP-HPLC-UV is a well-established, relatively cheap, and user-friendly analytical technique. These characteristics all contribute to make the technique very popular among others.

Currently, HPLC coupled with mass spectrometry (MS) represents "the-state-of-the-art" method for identification and quantification of peptides in complex matrices such as dairy product extracts. Different mass spectrometry techniques such as tandem mass spectrometry (MS/MS), quadrupole ion-trap mass spectrometry (QIT-MS), and time of flight mass spectrometry (TOF-MS) have been applied successfully to assess BCMs content in dairy products (Table 4). Each technique has its own and unique advantages. The selectivity and sensitivity of tandem mass spectrometry, especially when operated in multiple reaction monitoring (MRM) mode, allows accurate quantification of BCM5 and BCM7 at low levels (low μg/g) in the presence of multiple co-eluting interferences. For example, using LC-MS/MS, Haileselassie et al. (1999) identified several potentially bioactive peptides, including BCM7, from an enzyme-modified Cheddar cheese (EMC) prepared using a neutral protease produced from *B. subtilis*. Using LC-MS, Rizzello et al. (2005) analyzed the water-soluble extracts of an Italian goat cheese. BCM9 was identified in the extract. Formation of BCM7 following 10–60 minutes digestion of milk with pepsin at pH 2.0 and β-casein degradation have also been monitored using LC-MS (Schmelzer et al., 2007). More recently, applications of HPLC-MS/MS for quantifying BCM5 and BCM7 in dairy products (De Noni, 2008; De Noni and Cattaneo, 2010) have been also reported. By using HPLC-MS/MS, De Noni and Cattaneo (2010) quantitatively determined BCM5 and BCM7 in cheese, milk, fermented milk, infant powder, and extracts of these products after in vitro simulated gastro-intestinal digestion. The authors showed that the level of BCM7 that could be detected was as low as 0.01 μg/g.

Quadrupole ion trap mass spectrometry features multiple tandem MS spectra (MS^n), allowing, to some extent, structural elucidation of unknown peptides. In a similar fashion to tandem mass spectrometry, QIT-MS can be also used for quantitative assessment of BCM5 and BCM7 in single reaction monitoring (SRM) mode. For example Juan-Garcia et al. (2009) examined BCMs in cheese and milk using a nanoelectrospray quadrupole ion-trap mass spectrometer providing a fast and easy means of analysis of these compounds in different matrices. The main fragmentation pathways for BCMs and structures for the major fragment ions that were recorded in the MS^n spectra, were also reported.

Time of flight mass spectrometry (TOF-MS), being a high accuracy and high resolving power mass spectrometry technique, is of particular value for confirmation and when unknown compounds need to be unequivocally identified in complex matrices. This is usually achieved by combining elemental formula information (i.e. $C_xH_yO_zN_j\ldots$) from high mass accuracy experiments with structural information from fragmentation experiments. For example, using HPLC TOF-MS, Toelstede et al. (2008) found BCM9 and BCM10 in the water-soluble extract of a matured Gouda cheese. The possibility of interfacing a matrix-assisted laser desorption ion source (MALDI) to a TOF-MS allowed Cass et al. (2008) to analyze opioid-derived exogenous or endogenous peptides in urines of children with autism, and found that peaks previously analyzed by HPLC-UV were erroneously identified as opioid peptides. Hence the need of high resolution for identification and confirmation work. Righetti et al. (1997) confirmed the identity of opioid peptides by N-terminal sequencing followed by MALDI-TOF mass spectrometry.

One alternative method for BCMs determination is ELISA (Sienkiewicz-Szłapka et al., 2009; Cieślińska et al., 2012). Using ELISA, levels of BCM7 and BCM5 in cheese as low as 0.04 and 0.05 μg/g, respectively, could be accurately quantified. However, heat treatment of milk may modify the conformation of BCM7 by interaction between lactose and amino acid residues, leading to reduction in the binding affinity of the modified BCM7 to the antibody and underestimation of the BCM level (Cieślińska et al., 2012). This may be a reason why Cieślińska et al. (2012) observed the decrease of BCM7 content when determined by ELISA during heat treatment of milk.

Thus, HPLC coupled to different ranges of mass spectrometry technologies is a suitable method for quantifying BCMs in complex dairy matrices at low level as well as for confirmation and identification of unknown peptides in the same matrices. The possibility to extend the application of HPLC-MS to determine BCMs content in biological fluids such as urine does exist (Cass et al., 2008) as this technique has been proven rather flexible, accommodating the analysis of a wide range of matrices.

CONCLUSION

BCM is a group of opioid peptides released from β-casein by proteolysis, among which BCM7 is a peptide released from

β-casein variants containing histidine residue at position 67 (β-casein A1 and B variants). Consumption of milk containing variant A1 and B β-casein may increase the risk of human disease such as type 1 diabetes and heart disease but an important precursor to clinical studies is the an understanding of the conditions that cause BCM formation and degradation. BCMs may be formed in all fermented dairy products, cheese, and yoghurt, but may be degraded during processing so as not detectable in the product as consumed. Processing factors that might affect the formation and/or degradation of BCMs include heat treatment, fermentation, ripening, and cold storage. Due to the lack of knowledge on the presence of BCM7 and other related BCMs in fermented milks, especially yoghurt, it is now necessary to further study the effects of combinations of processing factors on the formation and/or degradation of BCM7 and other related BCMs in these popular products. Whether BCMs do form in yoghurt and the amount of BCM forming or degrading at different processing steps needs further investigation and possibly will depend on the heat treatment and fermentation process used, but it remains an intriguing unknown. The application of analytical methods employing tandem mass spectrometry or high resolution mass spectrometry is strongly recommended to confidently identify and accurately quantify BCMs in complex dairy product matrices.

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CHAPTER 3: DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE ACCURATE DETERMINATION AND QUANTIFICATION OF BETA-CASOMORPHIN 5 AND BETA-CASOMORPHIN 7 IN YOGHURT

3.1 Introduction

Yoghurt is a complex food matrix containing proteins, sugars, fatty acids and peptides that can interfere in detection of analytes of interest. In addition, yoghurt may also contain a number of peptides with different sizes and different sequences, but similar physicochemical properties, which could cause overestimation of results for a target peptide due to co-eluting with other peptides when analysed by conventional HPLC coupled with UV-Vis detection (Sienkiewicz-Szłapka et al., 2009). In recent years, a number of studies have reported the presence of ACE-I peptides (Donkor et al., 2007; Kunda et al., 2012), antioxidant peptides (Sabeena Farvin et al., 2010) and antimicrobial peptides (Poyrazoglu Coban et al., 2012) in yoghurt. The majority of these peptides originate from beta-caseins (β-CNs). Furthermore, yoghurt has also been reported to contain the opioid peptides known as BCMs (Jarmolowska, 2012).

BCM5 and BCM7 are two important BCMs. Their presence has been extensively studied in milk and fermented milk products due to their potential involvement in human chronic diseases. Many studies have reported the occurrence of BCM7 or BCM5 in different types of cheese (De Noni & Cattaneo, 2010; Norris et al., 2003; Sienkiewicz-Szłapka et al., 2009). However, the presence of these two peptides in yoghurt and milk has been disputed over the years, possibly due to lack of analytical method sensitivity and selectivity. In addition, food processing may alter the BCM content. Recently, ELISA has been applied to quantify BCM7 in commercial yoghurt and other fermented milk products, milk and heated milk. The level of the peptide found was between 29 and 54 ng/mg protein in yoghurt and fermented milk (Jarmolowska, 2012); from 1.1 to 2.1 ng/mL in milk and from 2.9 to 7.4 ng/mL in heated milk (Cieślińska et al., 2012). However, there has been no study reporting analysis of BCM5 in yoghurt by ELISA.

Using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), De Noni and Cattaneo (2010) detected neither BCM5 nor BCM7 in yoghurt and milk. Nevertheless, the latter was found in milk when analysed by

HPLC-quadrupole ion-trap mass spectrometry (HPLC-QIT-MS) (Juan-García et al., 2009) or ultra-performance liquid chromatography (UPLC) coupled with hybrid triple quadrupole linear ion trap mass spectrometry (Thiri et al., 2012).

In LC-MS quantitative analysis, the choice of the calibration technique plays an important role in the accuracy and precision (i.e. trueness) of the analytical results. Serial mixtures of standard solutions for external calibration have previously been used for the simultaneous quantification of BCM5 and BCM7 (De Noni & Cattaneo, 2010; Juan-García et al., 2009). However, this approach could result in ion suppression that ultimately leads to a decrease in the response of target analytes (Jessome & Volmer, 2006) and therefore, resulting in an underestimation of content of the analytes in the sample. When using external standards however, it is necessary for the calibration samples to be identical in composition to test samples to compensate fully for any matrix effects (Jessome & Volmer, 2006). This requirement is a challenging task in the case of analysis of complex matrices such as milk and yoghurt.

Alternatively, using deuterated homologues as internal standards is a technique allowing easy identification and quantification of target analytes in complex matrices. Stable isotope-labelled compounds allow compensation for matrix effects and loss of target analytes during sample preparation, leading to significant reduction of data variability and improvement of accuracy and precision of results (Jessome & Volmer, 2006). To date, only one study has reported the application of stable isotope-labelled BCM as the internal standard for the quantitative determination of BCM7; however this study was performed on plasma, not on milk or milk products (Song, Zaw, Amirkhani, Clarke, & Molloy, 2012). Currently, no study has been reported on the use of deuterated homologues for simultaneous determination of BCM5 and BCM7 in yoghurt or milk.

In this study, a highly sensitive and selective LC-MS/MS method was developed for the simultaneous determination of BCM5 and BCM7 in yoghurt. The successful validated method has then been applied for the identification and quantitation of these two peptides in commercial and laboratory scale manufactured yoghurts (Chapters 4, 5 and 6). This method has also been used for the simultaneous analysis of BCM5 and BCM7 in different types of milk (Chapter 4).

3.2 Materials and methods

3.2.1 Chemicals and materials

UHT milk used for production of yoghurt was supplied by A2 Dairy Products Australia Pty Ltd (Botany, NSW, Australia). Yoghurt culture (YO-MIX^{TM}) is a freeze-dried powder containing two common LAB, *L. delbrueckii* spp. *bulgaricus* and *S. thermophilus* produced by Danisco (France). Standards of BCM5 (purity 97.8%), BCM7 (purity >98.7%) and the deuterated standards [^{2}H10] BCM5 (BCM5-d_{10}) and [^{2}H10] BCM7 (BCM7-d_{10}) - deuterium enrichment > 99%, 2H enrichment at phenylalanine position - were purchased from Auspep Pty. Ltd. (Tullamarine, VIC, Australia). The ultrapure water was purified using an IBIS Technology (Perth, WA, Australia) Ion Exchange System followed by a Elga Purelab Ultra System (Sydney, NSW, Australia). Methanol (ChromAR HPLC grade) was purchased from Mallinckrodt (New Jersey, USA); formic acid (purity 99%) was purchased from Ajax FineChem (Sydney, NSW, Australia). All chemicals and solvents were of HPLC-grade.

3.2.2 Solutions and calibration standards

Stock solutions of BCM5, BCM7 and deuterated standards BCM5-d_{10} and BCM7-d_{10} (1 mg/mL) were prepared by dissolving 5 mg of each compound in a 5 mL of ultrapure water and then dispensed evenly into HPLC vials and stored -20 ^{o}C. Working stock solutions (10 ng/µL and 25 ng/µL) of BCM5, BCM7; and deuterated standards BCM5-d_{10}, BCM7-d_{10} (10 ng/µL) were prepared by diluting stock solutions in ultrapure water. Serial mixed standard solutions of BCM5 and BCM7 in the range 0.01-25 ng/µL, spiked with 1 ng/µL of deuterated standards, were prepared from the working stock solutions. The working solutions and serial mixed standard solutions were prepared freshly before each analysis.

3.2.3 Production of yoghurt

One litre of UHT milk was heated to 43 ^{o}C in an incubator. The warm milk was inoculated with 0.003% (w/v) of YO-MIX^{TM} and mixed thoroughly. The inoculated milk was incubated at 43 ^{o}C until pH reached to the value of 4.6. After fermentation, the yoghurt was cooled down and stored at 4 ^{o}C overnight. This yoghurt was used for the subsequent development and validation of the analytical method.

3.2.4 Extraction of water soluble peptides

The extraction was performed as described in Donkor et al. (2007) with some modifications. Yoghurt (10.0000 ± 0.0001 g) was accurately weighted into a centrifuge tube and then centrifuged at 15,000×*g* at 4 ^{o}C for 30 min (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). The supernatant was decanted into a clean centrifuge tube and then drops of 1M HCl (or 1M NaOH) slowly added to adjust to pH 4.6. The pH adjusted supernatant was allowed to stand for 5 min at room temperature to completely precipitate caseins then centrifuged at 14,000×*g* for 20 min at 4 ^{o}C. The supernatant was collected by decantation and subsequently filtered through a 0.45 µm membrane filter. Evaporation of the filtered extract to a final volume of 1 mL was carried out using a gentle nitrogen flow over the extract surface. The concentrated extract was filtered through a 0.45 µm membrane filter directly into an HPLC vial before injection into the LC system. The extraction was performed in triplicate.

3.2.5 Liquid chromatography-tandem mass spectrometry conditions

Experiments were performed using an 1100 Agilent LC system (Palo Alto, CA, USA) coupled with a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer fitted with an electrospray ionisation source operated in positive mode. Chromatographic separation of the analytes and the deuterated homologues was carried out using a Kinetex C18 LC-MS column (100 mm × 2.1 mm, 2.6 µm, 100 Å, Phenomenex, Sydney, Australia) by injection of 5 µL of exract at a flow rate of 100 µL/min. The column was protected by a KrudKatcher Ultra HPLC in-line filter (0.5 µm depth filter x 0.004 inch ID). The mobile phase consisted of methanol (MeOH) (A) and ultrapure water (B), both containing 0.1% of formic acid. The chromatography runs commenced with 30% (A) for 3 min, followed by a 15 min linear gradient to 100% (A). The mobile phase remained at 100% (A) for 15 min to elute analytes from the column. Afterwards, the initial conditions were re-established within 1 min and the column re-equilibrated for 15 min before injecting the next sample. To minimise potential carryover, the needle of the injector was rinsed for 30 seconds with MeOH before and after each injection.

Optimum MS signals were achieved by tuning the ESI capillary and cone voltages to 3.25 KV and 25 V, respectively. Hexapole1, aperture and hexapole2 were set to 0.0

V, 0.1 V and 0.2 V, respectively. Desolvation temperature and source temperature were set to 325 °C and 135 °C, respectively. Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as desolvation and nebulizer gas; cone gas flow was set to 40 L/h, while the desolvation gas flow was set to 765 L/h. High purity argon (99.997% purity) (BOC Gases, Perth, Australia) was used as collision gas (P = 2.7×10^{-4} kPa). Both quadrupoles (Q1 and Q3) were set at unit mass resolution; ion energy on Q1 and Q3 was set to 1.0, while the multiplier was set at 750 V. Quantification was performed by calculating the ratio of the peak area of the analytes to the peak area of the corresponding deuterated standards. Concentrations were calculated by QuanLynx 4.0 software comparing the peak area ratios from the extracts to peak area ratios from the calibration standards.

3.2.6 Validation of the analytical method

Parameters of the analytical method that need to be validated consisted of instrumental linearity, instrumental detection limits (IDLs) and instrumental quantification limits (IQLs), peak identification criteria (retention time and MRM ratio), accuracy, precision, inter-day reproducibility, method limits of detection (LODs), method limits of quantitation (LOQs) and matrix effects.

3.2.6.1 Liquid chromatography-tandem mass spectrometry performance

Serial standard solutions were repetitively injected into the LC-MS/MS system to assess its performance. The determination of linear range was performed by analysing standard solutions in concentrations from 0.01 to 25 ng/µL (Section 3.2.2). Low concentrations of standard solutions were used for evaluation of IDLs and IQLs. IDLs and IQLs were calculated at signal-to-noise (S/N) ratio of 3 and S/N of 10, respectively. Standard solution at 1 ng/µL was used to determine the variability of the MRM ratio and of the retention time (repeat injection, n=15).

3.2.6.2 Accuracy and precision

Accuracy and precision of the method were assessed by a recovery study of BCM5 and BCM7 spiked in yoghurt. Yoghurt samples (10.000 ± 0.0001 g) were spiked with standard solutions of BCM5 and BCM7 at five different levels of concentration (1, 10, 100, 1000 and 2500 ng/g) in triplicate. In addition, the internal standards at a concentration level of 100 ng/g were also added to the yoghurt samples. Accuracy and precision were expressed as recovery relative to the internal standards (%) and

relative standard deviation (%RSD), respectively. Blank yoghurt samples (10.0000 ± 0.0001 g) in triplicate with the internal standards added at a concentration of 100 ng/g were used for QA/QC purpose. All samples were extracted as described in Section 3.2.4 and injected into the LC-MS/MS system with conditions as described in Section 3.2.5.

3.2.6.3 Inter-day reproducibility

Inter-day reproducibility was determined by repeated analysis (n=3) on two consecutive days of three yoghurt samples spiked with BCM5 and BCM7 at levels of 10, 100 and 1000 ng/g. Inter-day reproducibility was expressed as %RSD.

3.2.6.4 Method limit of detection and limit of quantitation

Yoghurt samples spiked with standard BCM5 and BCM7 at the low levels of 1 ng/g and 10 ng/g were used to estimate LODs and LOQs. LODs and LOQs were calculated from the concentration equivalent to S/N of 3 and S/N of 10, respectively, on smoothed chromatographic traces (Busetti, Linge, Blythe, & Heitz, 2008).

3.2.6.5 Evaluation of matrix effects

The matrix effect was assessed by comparison of response of the analytes in ultra-pure water with that in yoghurt extract at the same concentration. Blank yoghurt extracts and ultra-pure water were added the mixture solution of BCM5 and BCM7 at 1,000 ng/mL and then analysed by LC-MS/MS with above described conditions. The matrix effect was calculated as followed (Eq.1):

$$\mathrm{ME}\ (\%) = \frac{\mathrm{As} - \mathrm{A}m}{\mathrm{As}} \times 100 \quad (1)$$

Where: - ME is the matrix effect (%)

- A*s* is the area of BCM5 or BCM7 added in ultrapure water (standard solution)

- A*m* is the area of BCM5 or BCM7 added in a yoghurt blank extract (matrix).

3.3 Results and discussions

3.3.1 Optimisation of MS/MS conditions

The mixture of standard solution of BCM5 and BCM7 at level of 5 ng/mL in MeOH:H_2O (50:50) containing 0.1% of formic acid was used to optimise: 1)

formation of protonated parent ions minimising secondary adducts in the ion source; 2) fragmentation of selected parent ions (MS/MS) in the collision cell and 3) detection of product ions in multiple reaction monitoring (MRM) mode.

The molecular weight of BCM5, BCM7 and their deuterated standards BCM5-d_{10} and BCM7-d_{10} are 579.65, 789.93, 590 and 800 Da, respectively (Auspep Pty. Ltd. Victoria, Australia). Therefore, initial experiments were performed in MS scan mode (Scan range: 50-1000 *m/z*). It could be observed that most intense precursor ions in the presence of 0.1% of formic acid were the proton adducts $[M+H]^+$. In addition, other precursor ions such as sodium or MeOH adducts usually present in non-acidic/methanolic solutions were substantially weaker. Thus, $[M+H]^+$ precursor ions (Table 5) were selected for fragmentation in product ion scan mode in further MS/MS experiments. The MS/MS spectra of BCM7 have been reported by De Noni (2008). The fragmentation spectra of BCM5 and the main product ions identified in the present study are showed in Fig. 6.

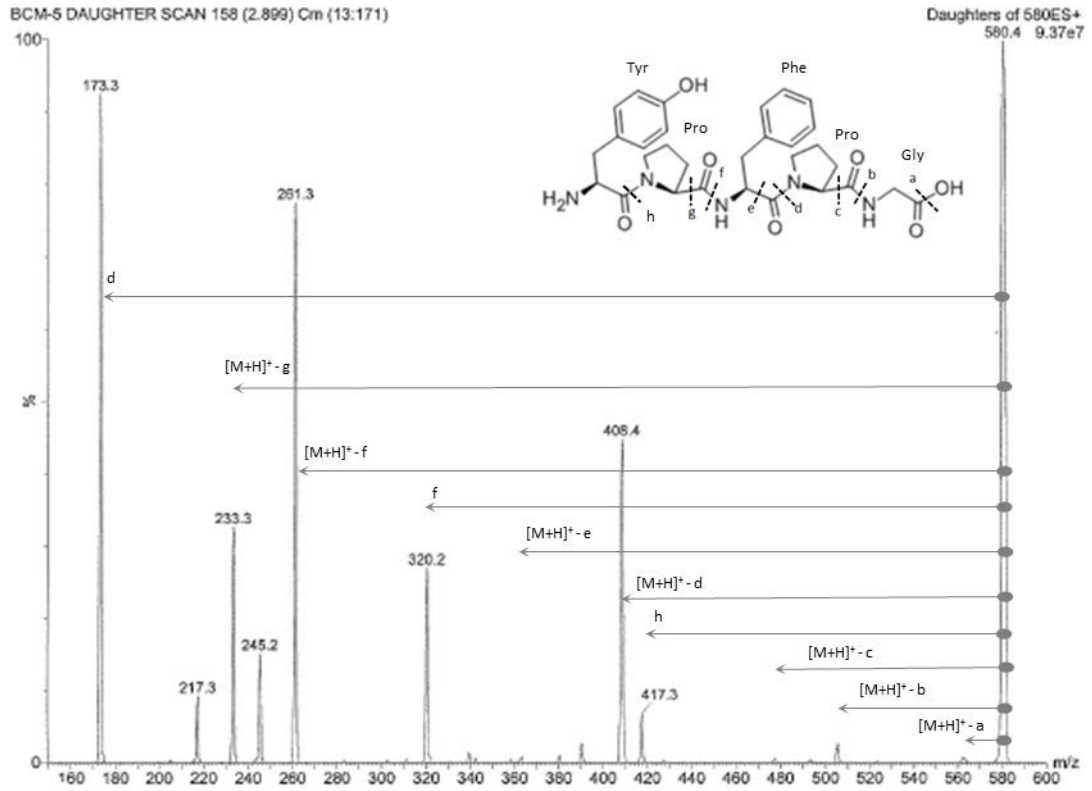

Figure 6. Product ions spectra showing the fragmentation of BCM5 obtained at variable collision energy values. Tyrosine (Tyr); proline (Pro); phenylalanine (Phe) and glycine (Gly) are indicated in the chemical structure of BCM5

After optimisation of the collision energy, which controls the degree of fragmentation of the parent ions in the collision cell (Q2), the most intense characteristic transitions were identified from the product ion spectra (data not shown). Each BCM and its internal standard was identified using two MRM transitions, which means that one parent ion fragmented into two product ions (Table 5). This approach provides four identification points, which are sufficient to fulfil the criteria to identify and confirm the presence of a target analyte in food matrices (EC, 2002). In addition, the presence of the target analytes was also confirmed by monitoring the MRM ratio and retention time (t_R) against standard solutions (Table 6).

Table 5. Precursor and product ions, and collision energy values optimised for the analysis of BCM5 and BCM7 under ESI(+) MS/MS in MRM mode. MRM transitions were grouped in one window. Dwell time was 80 ms for all the transitions monitored.

Compounds	Precursor ions (*m/z*)	Product ions (*m/z*)	Collision energy (arbitrary units)
BCM5	580.4	173.3[a]	30
		408.4	25
BCM5-d_{10}	590.4	173.3[a]	30
		418.4	25
BCM7	790.7	229.3[a]	50
		530.4	35
BCM7-d_{10}	800.7	229.3[a]	50
		540.4	35

[a] The product ions from the transitions used for quantification

3.3.2 Instrumental performance and peaks identification criteria

For assessment of LC-MS/MS instrument performance and identification of peaks of target analytes, standard solutions of BCM5 and BCM7, and their internal standards BCM5-d_{10} and BCM7-d_{10} at level of 1 ng/µL were injected into the LC-MS/MS system. The LC-MS/MS chromatogram is showed in Fig. 7.

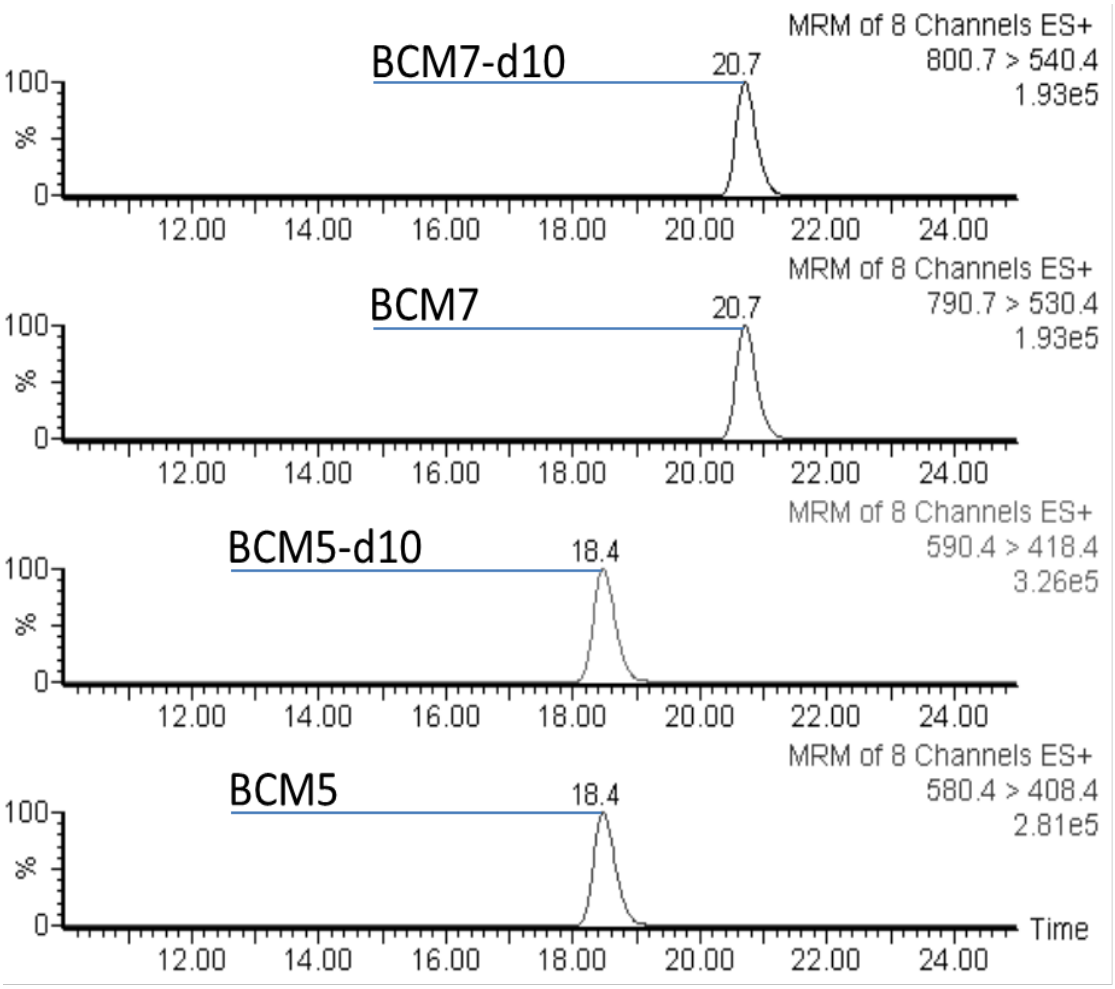

Figure 7. A typical LC-MS/MS chromatogram of BCM5, BCM7 and their deuterated standards (BCM5-d_{10} and BCM7-d_{10})

The linear range was tested using calibration standards in range between 0.01 ng/µL (0.05 ng on column) and 25 ng/µL (125 ng on column). The calibration curve of ratios of peak area of analyte to peak area of deuterated standard against analyte concentrations was constructed. Values of regression coefficient (R^2) for both BCM5 and BCM7 were higher than 0.9980 in the range between 0.05 and 50 ng on column (Table 6), indicating good linearity in the range of concentrations tested. IDLs for BCM7 and BCM5, estimated at S/N of 3, were 0.007 and 0.010 ng on column, respectively. IQLs for BCM7 and BCM5, estimated at S/N of 10, were 0.024 and 0.036 ng on column, respectively. MRM ratio variability (%RSD) and t_R variability (SD) were less than 8.8% and less than 0.2 min (12 s), respectively, indicating reproducible fragmentation of the parent ions in the collision cell and a reproducible chromatographic separation.

Table 6. Instrumental performance parameters obtained by repeat injections of standard solutions into the LC-MS/MS system.

Compounds	Linear range [a]	R^2	IDL[a] S/N = 3	IQL[a] S/N = 10	MRM ratio (±%RSD)	t_R (min±SD)
BCM5	0.05–50	0.9985	0.010	0.036	3.0±7.8	18.2±0.2
BCM7	0.05–50	0.9986	0.007	0.024	2.6±8.8	20.6±0.1

[a] ng on column

IDL: instrumental detection limit; IQL: instrumental quantitation limit; MRM: multiple reaction monitoring; t_R: retention time.

3.3.3 Accuracy and precision

In order to calculate accuracy and precision of the method, yoghurt samples were spiked with the mixture of standard solutions of BCM5 and BCM7 at 5 different levels of concentration levels (Table 7). Accuracy was in the range between 95 and 106% for BCM5 and between 103 and 109% for BCM7 (Table 7). Relative standard deviation ranged between 1-6% for BCM7, and 1-7% for BCM5, with the exception of the value for BCM5 spiked at the concentration of 1 ng/g where relative standard deviation was higher (Table 7).

Table 7. Recovery and precision study for BCM5 and BCM7

Compounds	Spiked levels (ng/g)	Recovery (%, n=3)	Precision (%RSD, n=3)
BCM5	1	106	16
	10	104	7
	100	106	2
	1000	95	2
	2500	97	1
BCM7	1	103	5
	10	109	4
	100	107	6
	1000	106	1
	2500	104	1

3.3.4 Inter-day reproducibility

The inter-day reproducibility of the method was determined by repeated measurements of yoghurt spiked with three different levels of BCM5 and BCM7 on two consecutive days (Table 8). Relative standard deviation ranged between 2.0 and 6.4% for BCM5 and between 3.2 and 6.1% for BCM7 (Table 8), indicating good inter-day reproducibility of the adopted analytical method.

3.3.5 Method limits of detection and method limits of quantitation

Calculation of LODs and LOQs were based on analysis of BCM5 and BCM7 in yoghurt samples spiked with these two peptides at levels of 1 ng/g and 10 ng/g. LODs were 0.5 ng/g and 0.25 ng/g for BCM5 and BCM7, respectively. LOQs for BCM5 and BCM7 were 1.7 ng/g and 0.85 ng/g, respectively. To date, LODs and LOQs for BCM5 and BCM7 in yoghurt have not been previously reported, so it is difficult to compare these results with previously published ones.

3.3.6 Evaluation of matrix effects

In the present study, matrix effects were corrected with the inclusion of deuterated homologues; this approach is by far the most effective method to account for matrix effects as the analytes and the corresponding co-eluting deuterated homologues are subject to almost identical environments. Matrix effects were responsible for causing about 26% and 40% signal suppression for BCM5 and BCM7, respectively. It is possible to reduce signal suppression using additional clean-up of samples (e.g. on-line or off-line SPE). This approach could result in improvement of LODs.

3.4 Conclusions

The results presented in this chapter demonstrate that preparation of samples using high speed centrifugation and 0.45 µm membrane filters followed by analysis by LC-MS/MS is a sensitive, selective and robust analytical protocol that can be applied for the simultaneous determination of BCM5 and BCM7 in a complex matrix, such as yoghurt. The use of isotope labelled homologues as the internal standards in the analytical method produced the highest confidence in the quantitative assessment of BCM5 and BCM7 levels in complex matrices, effectively correcting for 1) analyte loss encountered during sample extraction and 2) matrix effects encountered during the analysis of the samples.

Table 8. Inter-day reproducibility data determined by repeating measurements on two different days of yoghurt samples spiked at three different levels of BCM5 and BCM7

Compounds	spiked level (ng/g)	Analysis (day 1, n=3) (ng/g)	Analysis (day 2,n=3) (ng/g)	Average (ng/g)	Inter-day reproducibility (%RSD)
BCM5	10	10.4	11.2	10.8	6.4
	100	106	106	106	2.0
	1000	950	1000	974	3.6
BCM7	10	10.7	11	10.8	6.1
	100	110	111	110	3.4
	1000	1041	1077	1059	3.2

The method reported in this chapter has been used for the determination of BCM5 and BCM7 in commercial yoghurts as well as yoghurts prepared in our laboratory (see Chapters 4, 5 and 6 for more information). In addition, the present method has been also modified to allow the measurement of BCM5 and BCM7 in milk (see Chapter 4 for more information).

3.5 Papers

A part of content of this chapter has been published in the following journal article: D. D. Nguyen, V. A. Solah, S. K Johnson. J. W. A Charrois and F. Busetti (2014). Isotope dilution liquid chromatography–tandem mass spectrometry for simultaneous identification and quantification of beta-casomorphin 5 and beta-casomorphin 7 in yoghurt. Food Chemistry 146, 345-352.

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Analytical Methods

Isotope dilution liquid chromatography–tandem mass spectrometry for simultaneous identification and quantification of beta-casomorphin 5 and beta-casomorphin 7 in yoghurt

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ABSTRACT

A highly selective and sensitive liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous identification and quantification of beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in yoghurt. The method used deuterium labelled BCM5-d_{10} and BCM7-d_{10} as surrogate standards for confident identification and accurate and quantification of these analytes in yoghurt. Linear responses for BCM5 and BCM7 (R^2 = 0.9985 and 0.9986, respectively) was observed in the range 0.01–10 ng/μL. The method limits of detection (MLDs) in yoghurt extracts were found to be 0.5 and 0.25 ng/g for BCM5 and BCM7, respectively. Analyses of spiked samples were used to provide confirmation of accuracy and precision of the analytical method. Recoveries relative to the surrogate standards of these spikes were in the range of 95–106% for BCM5 and 103–109% for BCM7. Precision from analysis of spiked samples was expressed as relative standard deviation (%RSD) and values were in the range 1–16% for BCM5 and 1–6% for BCM7. Inter-day reproducibility was between 2.0–6.4% for BCM5 and between 3.2–6.1% for BCM7. The validated isotope dilution LC–MS/MS method was used to measure BCM5 and BCM7 in ten commercial and laboratory prepared samples of yoghurt and milk. Neither BCM5 nor BCM7 was detected in commercial yoghurts. However, they were observed in milk and laboratory prepared yoghurts and interestingly their levels decreased during processing. BCM5 decreased from 1.3 ng/g in milk to 1.1 ng/g in yoghurt made from that milk at 0 day storage and <MLQ at 1 and 7 days storage. BCM7 decreased from 1.9 ng/g in milk to <MLQ in yoghurts immediately after processing. These preliminary results indicate that fermentation and storage reduced BCM5 and BCM7 concentration in yoghurt.

1. Introduction

Beta-casomorphins (BCMs) are a group of structurally similar peptides containing a sequence of 4–11 amino acids (Kamiński, Cieślińska, & Kostyra, 2007). The first three amino acids, tyrosine, proline and phenyalanine in the peptide, are conserved (Muehlenkamp & Warthesen, 1996) and arise from enzymatic hydrolysis of beta-caseins (β-CNs) (De Noni & Cattaneo, 2010). These peptides are released from the parent protein by cleavage at position 60 (tyrosine), and a second cleavage of residues at positions 63–70. For example, a peptide with cleavages at position 60 (tyrosine) and position 66 (isoleucine) is beta-casomorphin 7 (Fig. 1), which was first isolated from bovine casein (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). BCMs have morphine-like activity, and therefore are classified as opioid peptides (Kamiński et al., 2007). BCM5 and BCM7 (Fig. 1) have strong opioid activity (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981; Kamiński et al., 2007; Kálmán, Cserháti, Valkó, & Neubert, 1992). Epidemiological evidence suggests that consumption of milk containing A1 beta-casein, which releases BCM7 on hydrolysis, is linked to an increased risk of type-1 diabetes and heart disease (Elliott, Harris, Hill, Bibby, & Wasmuth, 1999; Laugesen & Elliott, 2003; McLachlan, 2001). However, the European Food Safety Authority (EFSA) concluded there were insufficient data to determine a causal relationship between exposure to BCM7 and other related BCMs and non-communicable diseases (EFSA, 2009). Therefore, the reported presence of BCM5 and BCM7 in dairy products needs further research due to their putative link to elevated chronic disease risk.

BCM7 is found in bovine milk (Cieślińska, Kaminski, Kostyyra, & Sienkiewicz-Szłapka, 2007; Cieślińska et al., 2012), in human milk (Jarmołowska et al., 2007), in cheeses (De Noni & Cattaneo, 2010; Jarmolowska, Kostyra, Krawczuk, & Kostyra, 1999; Norris, Coker, Boland, & Hill, 2003; Sienkiewicz-Szłapka et al., 2009) and in

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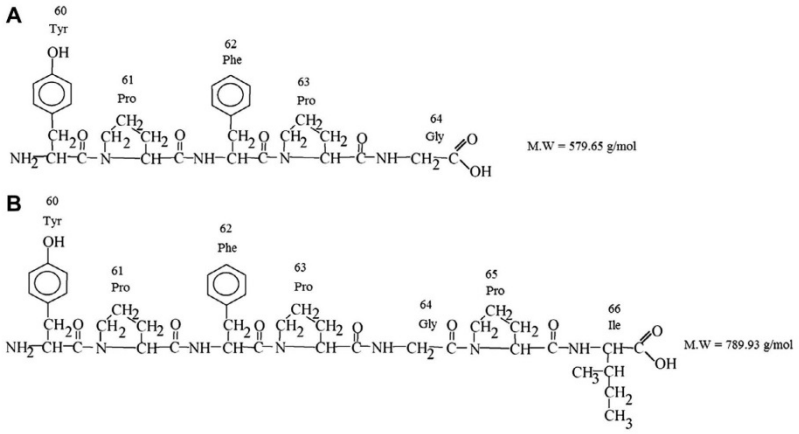

Fig. 1. Structure of BCM5 (A) and BCM7 (B). (Adapted from Juan-García, Font, Juan, & Picó, 2009.)

commercial yoghurt (Jarmolowska, 2012). In contrast, De Noni and Cattaneo (2010) showed that BCM7 was not present in yoghurt purchased from a market. Therefore, the presence of BCM7 in yoghurt may be affected by processing steps in yoghurt manufacturing or levels may be below the limit of detection of previous analytical methods used.

Yoghurt is a popular dairy product usually fermented by two lactic acid bacteria (LAB), *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Tamime & Robinson, 1999, chap. 6). In addition to containing angiotensin-converting enzyme inhibitory (ACE-I) peptides (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007; Kunda et al., 2012), antihypertensive peptides (Kunda et al., 2012; Muguerza et al., 2006; Schieber & Brückner, 2000), and antioxidant peptides (Sabeena Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010), yoghurt may also contain BCM peptides (Jarmolowska, 2012). However, yoghurt is a complex food matrix (EFSA, 2009), in which protein, sugar or lactic acid can interfere with the identification and quantification of target analytes such as peptides.

Reverse-phase high performance liquid chromatography (RP-HPLC) coupled to an ultraviolet–visible (UV–Vis) detector is used widely for separation and quantification of BCM5 and BCM7 in milk and dairy products (Jarmołowska et al., 2007; Muehlenkamp & Warthesen, 1996). A limitation of RP-HPLC-UV for detection and quantification of BCMs is that peptides with similar physicochemical and spectrophotometric absorption properties can co-elute with BCM5 and BCM7, increasing their apparent absorption values (Muehlenkamp & Warthesen, 1996; Sienkiewicz-Szłapka et al., 2009) resulting in an overestimation of BCM7 (Cass et al., 2008; Sienkiewicz-Szłapka et al., 2009) and BCM5 concentration (Sienkiewicz-Szłapka et al., 2009). Additionally, analytical methods employing UV–Vis detector may lack the sensitivity required to quantify the low levels (e.g. 2 μg/ml of cheese extract) of BCM7 and BCM5 found in dairy products (Muehlenkamp & Warthesen, 1996).

More recently, enzyme–linked immune sorbent assays (ELISA) have been applied to detect and quantify BCM5 and BCM7 in bovine milk and dairy products (Cieślińska et al., 2012; Jarmolowska, 2012; Sienkiewicz-Szłapka et al., 2009). However, during milk processing, heat treatment of milk may modify the conformation of BCM7 by interaction between lactose and amino acid residues, leading to a reduction in the binding affinity of the modified BCM7 to the ELISA antibody, resulting in an underestimation of the BCM7 concentration (Cieślińska et al., 2012). RP-HPLC coupled with mass spectrometry (MS) represents "the-state-of-the-art" method for identification and quantification of peptides in complex matrices. Cass et al. (2008) applied matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) to analyse opioid-derived exogenous or endogenous peptides in urine, and found that peaks previously analysed by RP-HPLC-UV were erroneously identified as BCM7. Alternatively, tandem mass spectrometry (MS/MS) operating in multiple reaction monitoring (MRM) mode allows accurate quantification of BCM7 in plasma at low levels (ng/ml) (Song, Zaw, Amirkhani, Clarke, & Molloy, 2012). Currently, there is no report in the literature on LC–MS/MS applications for the simultaneous quantification of BCM5 and BCM7 in different processing stages of yoghurt manufacture.

The choice of calibration method plays an important role in LC–MS quantitative analysis. External calibration using standards can be used. However, this approach could result in ion suppression, leading to a decrease in the response of target analytes (Jessome & Volmer, 2006). In addition, using external standards requires that calibration samples are identical in composition to test samples, to compensate fully for matrix effects (Jessome & Volmer, 2006). To date, the use of UHT milk extract for dissolving synthesised BCM5 and BCM7 as calibration samples has been reported by De Noni and Cattaneo (2010) who used LC–MS/MS coupled to electrospray ionisation (ESI) for quantifying BCM5 and BCM7 in yoghurt. The extracts of UHT milk, however, are not identical to those of yoghurt because after fermentation, many compounds in milk are degraded into different ones in yoghurt, for instance, lactose is degraded into lactic acid. Therefore, the difference in matrix may affect ion suppression, leading to variable results.

Inclusion of deuterated homologues in LC–MS/MS quantitative analysis is an alternative technique that allows easy identification and quantification of target analytes in complex matrices. Stable isotope-labelled compounds allow compensation for matrix effects and loss of target analytes during sample preparation, so their use can significantly reduce data variability and improve accuracy and precision of the analytical determination (Jessome & Volmer, 2006). Recently, Song et al. (2012) used LC–MS/MS and applied stable isotope-labelled BCM7 as the surrogate standard for quantification of BCM7 in plasma at low concentrations (ng/ml).

To date, however, there are no reported studies using stable isotope-labelled BCM7 and BCM5 as the surrogate standards for simultaneous determination of these peptides in yoghurt.

The aim of this study was to develop a sensitive and selective method for the accurate determination of BCM5 and BCM7 in yoghurt. For separation and detection, the method employed LC coupled to MS/MS operated in MRM mode. Stable isotope-labelled BCM5 and BCM7 were used as surrogate standards for confident identification and accurate and quantification purposes. The LC–MS/MS method was also tested for the identification and quantification of target BCMs in commercial and laboratory prepared yoghurts.

2. Materials and methods

2.1. Chemicals and materials

Yoghurt culture (YO-MIX™) containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* was purchased from Danisco Australia Pty Ltd. (Botany, NSW, Australia). UHT milk and A1 skim milk powder were supplied by A2 Dairy Products Australia Pty Ltd. (Botany, NSW, Australia). BCM5 (purity 97.8%), BCM7 (purity >98.7%) and the deuterated standards [^{2}H10] BCM5 (BCM5-d_{10}) and [^{2}H10] BCM7 (BCM7-d_{10})-deuterium enrichment >99%, ^{2}H enrichment at phenylalanine position-were obtained from Auspep Pty (Tullamarine, Victoria, Australia). Ultrapure water used for laboratory purposes as well as LC mobile phase was purified using an IBIS Technology (Perth, Australia) Ion Exchange System followed by Elga Purelab Ultra System (Sydney, Australia). Methanol (ChromAR HPLC grade) was purchased from Mallinckrodt (New Jersey, USA); formic acid (purity 99%) was purchased from Ajax FineChem (Sydney, Australia).

2.2. Solutions and calibration standards

Stock solutions of analytes of BCM5, BCM7 and deuterated standards BCM5-d_{10}, BCM7-d_{10} were prepared by dissolving 5 mg of each compound in a 5 ml flask of ultrapure water (nominal concentration of 1 mg/ml). Working stock solutions (10 and 25 ng/μL) were prepared by diluting stock solutions in ultrapure water. Serial mixed standard solutions of BCM5 and BCM7 ranging from 0.01 to 25 ng/μL (nominal concentrations 0.01, 0.025, 0.05, 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, 10, 12.5 and 25 ng/μL) were prepared from the working stock solutions. A working solution (nominal concentration of 10 ng/μL) of deuterated standards was also prepared by dilution of BCM5-d_{10} and BCM7-d_{10} stock solutions. All the solutions, as well as analytical and surrogate standards were stored at −20 °C to minimise degradation.

2.3. Laboratory-prepared yoghurts

Yoghurt was prepared by inoculating YO-MIX™ into the UHT milk preheated at 43 °C in an incubator. The inoculated milk was then incubated at 43 °C until pH 4.6 was reached. After fermentation, yoghurt was cooled down and stored at 4 °C. This product was used for validation of analytical method and is referred to as "laboratory prepared yoghurt". In order to prepare yoghurt for the application of analytical method, 65 g of A1 skim milk powder was dissolved in 0.5 litre of deionised water that was pre-boiled for 10 min and then cooled down to 55 °C. After thorough mixing, 100 ml of reconstituted milk was placed into two tightly capped centrifuge tubes and stored at −20 °C prior to analysis. The rest of the reconstituted milk was cooled down to 43 °C and inoculated with 0.003% (w/v) of YO-MIX™. The inoculated milk was placed into seven centrifuge tubes, which were then capped, and fermented at 43 °C until the pH reached the value of 4.6. One tube was used to measure pH during the course of fermentation. When pH reached the required value, two tubes were stored −20 °C for further analysis. Two tubes were stored for 1 day and two others were stored for 7 days at 4 °C. All these tubes were stored −20 °C until extraction of water soluble peptides.

2.4. Extraction of water soluble peptides

The preparation of yoghurt extracts was carried out as described in Donkor et al. (2007) with some minor modifications. Ten grams of yoghurt were weighted (±0.1 g) into a centrifuge tube, then centrifuged at 15,000×g at 4 °C for 30 min. The supernatant was decanted into a clean centrifuge tube and the extract was adjusted to pH 4.6 with 1 M HCl (or 1 M NaOH). After re-centrifugation at 14,000×g for 20 min at 4 °C, the supernatant was collected by decantation and filtered through a 0.45 μm membrane filter. The filtered extract was evaporated to a final volume of 1 mL using a gentle nitrogen flow over the extract surface. The concentrated extract was filtered through a 0.45 μm membrane filter into a vial.

2.5. Liquid chromatography–tandem mass spectrometry conditions

The LC–MS/MS system used in this study consisted of an 1100 Agilent (Palo Alto, CA, USA) LC system and a Micromass (Manchester, UK) Quattro Ultima Triple Quadrupole Mass spectrometer fitted with an electrospray ion source (ESI) operated in positive ionisation mode. Chromatographic separation was achieved using a Kinetex C18 LC–MS column (100 mm × 2.1 mm, 2.6 μm, 100 Å) from Phenomenex (Sydney, Australia) at a flow rate of 100 μL/min. A KrudKatcher Ultra HPLC in-line filter (0.5 μm depth filter × 0.004 inch ID) was used to protect the analytical column. The mobile phase was methanol (MeOH) (A) and ultrapure water (B) both containing 0.1% of formic acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 15 min linear gradient to 100% (A). The mobile phase remained at 100% (A) for 15 min to elute analytes from the column. Afterwards, the initial conditions were re-established within 1 min and the column re-equilibrated for 15 min before injecting the next sample. To minimise potential carryover, before and after each injection, the needle of the injector was rinsed for 30 s in the injection port with MeOH. The injected volume was 5 μL.

Optimum MS signals were achieved by tuning the ESI capillary and cone voltages to 3.25 kV and 25 V, respectively. Hexapole1, aperture and hexapole2 were set to 0.0, 0.1 and 0.2 V, respectively. Desolvation temperature and source temperature were 325 and 135 °C, respectively. Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as desolvation and nebulizer gas; cone gas flow was set to 40 L/h, while the desolvation gas flow was set to 765 L/h. High purity Argon (99.997% purity) (BOC Gases, Perth, Australia) was used as collision gas ($P = 2.7 \times 10^{-4}$ kPa). Both quadrupoles (Q1 and Q3) were set at unit mass resolution; ion energy on Q1 and Q3 was set to 1.0, while the multiplier was set at 750 V. Surrogate standards, BCM5-d_{10} and BCM7-d_{10} were included in the analytical method to correct results for any losses encountered during sample extraction and to correct for matrix effects (Jessome & Volmer, 2006). Quantification was performed by rationing the peak area of the analytes to the peak area of the corresponding deuterated standards. Concentrations were calculated by QuanLynx 4.0 software comparing the peak area ratios from the extracts to peak area ratios from the calibration standards.

2.6. Validation of the analytical method

Evaluation of instrumental linearity, instrumental detection limits (IDLs) instrumental quantification limits (IQLs), peak

identification criteria (retention time and MRM ratio), accuracy, precision, inter-day reproducibility, method limits of detection (MLDs), method limits of quantification (MLQs), and matrix effects were undertaken to validate the analytical procedure.

2.6.1. LC–MS/MS performance

Repetitive injections of standard solutions were used to assess LC–MS/MS instrument performance. The linear range was tested using calibration standards spanning from 0.01 ng/μL up to 25 ng/μL. Injections of low concentrations standard solutions were used to assess IDLs and IQLs. IDLs were estimated at signal-to-noise (S/N) ratio equal to 3 while IQLs were estimated at S/N = 10. Repeat injections ($n = 15$) of a solution at 1 ng/μL were used to determine the variability of the MRM ratio and of the retention time.

2.6.2. Accuracy and precision

Accuracy and precision of the analytical method were assessed by recovery experiments of BCMs in yoghurt. Yoghurt samples (10 g) were spiked with standard solutions of BCM5 and BCM7 at five different levels of concentration (1, 10, 100, 1000 and 2500 ng/g) in triplicate. Surrogate standards were also spiked at a concentration level of 100 ng/g. Accuracy was expressed as recovery relative to the surrogate standards, while precision was expressed as relative standard deviation (%RSD). Blank samples (10 g of yoghurt spiked with 100 ng/g of deuterated standards) were also processed in triplicate along with each batch of samples for QA/QC purposes.

2.6.3. Inter-day reproducibility

Repeated measurements on two different days of three yoghurt samples spiked with 10, 100 and 1000 ng/g of BCM5 and BCM7 were conducted. The %RSD of these measurements was used to assess the inter-day reproducibility of the analytical method.

2.6.4. Method limit of detection and method limit of quantification

Yogurt samples spiked with low levels of BCM5 and BCM7 (1 ng/g and 10 ng/g) were extracted and analysed; the results were used to estimate MLDs and MLQs. MLDs were calculated from the concentration equivalent to S/N of three, while MLQs were calculated from the concentration equivalent to S/N of ten (Busetti, Linge, Blythe, & Heitz, 2008; Foley & Dorsey, 1984) on smoothed chromatographic traces.

2.6.5. Evaluation of matrix effects

In order to study the degree of signal suppression caused by the presence of the matrix, blank yoghurt extracts were spiked at 1000 ng/mL with BCM5 and BCM7 and analysed by LC–MS/MS. Results were compared to injections of analytical standards at the same concentration levels. Matrix effects were calculated as followed (Eq. (1)):

$$\text{Matrix effects}(\%) = (A_s - A_m)/A_s \times 100 \quad (1)$$

where A_s is the area of the analyte spiked in ultrapure water (standard solution) and A_m is the area of the analyte spiked in a yoghurt blank extract (matrix).

2.7. Analysis of commercial and laboratory prepared samples of yoghurt and reconstituted milk

Ten commercial yoghurts were purchased from a local supermarket located in Perth, Western Australia. Three yoghurts contained *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, other yoghurts contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and Bifidobacterium or contain *L. acidophilus*, Bifidobacterium and *L. casei*. The expiry dates of all yoghurts ranged from 13 and 29 days. The samples were extracted immediately after purchasing.

Commercial and laboratory prepared yoghurts and reconstituted milk samples were prepared as described in Section 2.4. The laboratory prepared yoghurts and reconstituted milk were thawed at room temperature and thoroughly mixed before sampling. Ten grams of reconstituted milk was gradually acidified with 1 M HCl to pH 4.6 and allowed to rest for 10 min. Before extraction, yoghurt and reconstituted milk samples were spiked with surrogate standards BCM5-d_{10} and BCM7-d_{10} at a concentration level of 100 ng/g. In addition, 10 g of yoghurt and 10 g of reconstituted milk spiked with both 1000 ng of BCM5-d_{10} and 1000 ng of BCM7-d_{10} as well as 100 ng of BCM5 and 100 ng of BCM7 were used as quality control samples (QCs). The QCs were extracted as described in Section 2.4. BCM5 and BCM7 were analysed using the validated LC–MS/MS method as described in Section 2.5. All samples were extracted and analysed in duplicate.

3. Results and discussions

3.1. Optimisation of MS/MS conditions

Solutions consisting of BCM5 and BCM7 at 5 ng/μL in MeOH:H_2O (50:50) with 0.1% of formic acid were infused into the mass spectrometer to optimise: (1) formation of protonated parent ions minimising secondary adducts; (2) fragmentation of selected parent ions (MS/MS) and (3) detection of product ions in MRM mode.

Initial experiments were conducted in MS scan mode (50–1000 *m/z*). In the presence of 0.1% of formic acid, the most intense precursor ion observed for BCM5 and BCM7 was the proton adduct $[M+H]^+$; other characteristic ESI precursor ions such as the sodium or MeOH adducts usually present in non-acidic/methanolic solutions were substantially weaker. The $[M+H]^+$ precursor ions were therefore selected for further MS/MS experiments. Fragmentation experiments were conducted in product ion scan mode. While the MS/MS spectra of BCM7 have been reported previously (De Noni, 2008), the fragmentation spectrum of BCM5 is reported in Fig. 2 along with assignment of the main product ions observed.

After optimisation of the collision energy, which controls the degree of fragmentation of the parent compound in the collision cell (Q2), the most intense characteristic transitions were identified from the product ion spectra (data not shown). Two MRM transitions (one parent ion fragmenting into two product ions) were used for the identification of each compound and surrogate standard (Table 1). This approach provides four identification points, which are sufficient to fulfil the criteria for identifying and confirming the presence of a target analyte in food matrices (EC, 2002). In addition, the MRM ratio and retention time (t_R) were also monitored against standard solutions for additional confidence.

3.2. Instrumental performance and peaks identification criteria

Injections of standard solutions were used to assess LC–MS/MS instrument performance. An example of a LC–MS/MS chromatogram of a 1 ng/μL standard solution of BCM5, BCM5-d_{10}, BCM7 and BCM7-d_{10} is showed in Fig. 3.

The linear range was tested using calibration standards spanning from 0.01 ng/μL (0.05 ng on-column) to 25 ng/μL (125 ng on-column). For both BCM5 and BCM7 tested, calibration curves showed good linearity in the range 0.05–50 ng on-column, with R^2 values typically higher than 0.9985 (Table 2). Injections of low concentrations standard solutions were used to assess IDLs and IQLs. IDLs for BCM7 and BCM5, estimated at S/N ratio equal to 3, ranged from 0.007 to 0.010 ng on-column, respectively. For BCM7

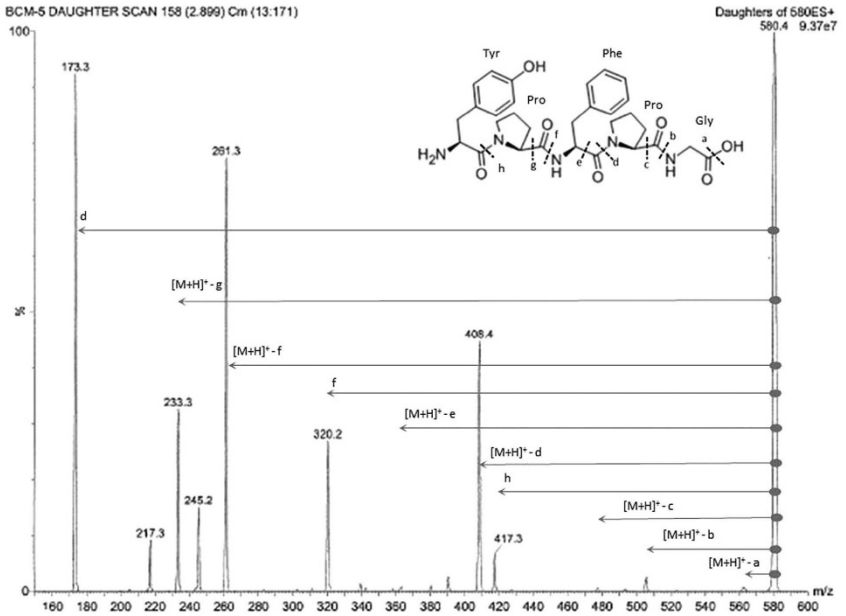

Fig. 2. Product ions spectrum showing the fragmentation of BCM5 obtained at variable collision energy values. The amino acids tyrosine (Tyr), proline (Pro), phenylalanine (Phe), and glycine (Gly) are also indicated in the chemical structure of BCM5.

Table 1
Mass spectrometry: precursor ions, product ions and collision energy values optimised for the analysis of BCM5 and BCM7 under ESI(+) MS/MS in multiple reaction monitoring (MRM) mode. MRM transitions were grouped in one window. Dwell time was 80 ms for all the transitions monitored.

Compounds	Precursor ion (*m/z*)	Product ions (*m/z*)	Collision energy (arbitrary units)
BCM5	580.4	173.3[a]	30
		408.4	25
BCM5-d_{10}	590.4	173.3[a]	30
		418.4	25
BCM7	790.7	229.3[a]	50
		530.4	35
BCM7-d_{10}	800.7	229.3[a]	50
		540.4	35

[a] The productions from the MRM transitions used for quantification.

and BCM5, IQLs were estimated at S/N = 10, and ranged from 0.024 to 0.036 ng on-column, respectively. Repeat injections (n = 15) of a solution at 1 ng/μL were used to determine the variability of the MRM ratio and of the retention time. MRM ratio variability, measured as %RSD, was less than 8.8% indicating reproducible fragmentation of the parent ions in the collision cell; t_R variability, measured as standard deviation (SD) was less than 12 s, indicating a reproducible chromatographic separation.

3.3. Accuracy and precision

Accuracy and precision of the analytical method were determined by experiments on yoghurt samples spiked with increasing concentration of BCM5 and BCM7. Accuracy was expressed as recovery percentage relative to the surrogate standards while precision was expressed as %RSD. Accuracy was in the range 95–106% for BCM5 and 103–109% for BCM7 (Table 3). Relative standard deviation of these recoveries was between 1–6% for BCM7, and 1–7% for BCM5 exception for the value at spiked concentration of 1 ng/g (%RSD = 16) (Table 3). No correction to recovery was necessary since blank samples were found not to contain BCM5 and BCM7 in detectable amounts.

3.4. Inter-day reproducibility

The inter-day reproducibility of the LC–MS/MS analytical method was determined by repeated measurements on two different days of three yoghurt samples spiked with different amount of BCM5 and BCM7 (Table 4). Relative standard deviation values were 6.4% and 6.1% for BCM5 and BCM7 respectively; indicating good reproducibility of the method (Table 4).

3.5. Method limits of detection (MLDs) and method limits of quantification (MLQs)

Average sample based MLDs and MLQs in yoghurt extracts were calculated from analysis of samples spiked with 1 ng/g and 10 ng/g of BCM5 and BCM7. MLDs were found to be 0.5 ng/g for BCM5 and 0.25 ng/g for BCM7. Meanwhile, MLQs for BCM5 and BCM7 were found to be 1.7 ng/g and 0.85 ng/g, respectively. These results are difficult to compare with published methods because, to the best of our knowledge, MLDs and MLQs for BCM5 and BCM7 in yoghurt have not been reported previously (Nguyen, Johnson, Busetti, & Solah, 2013).

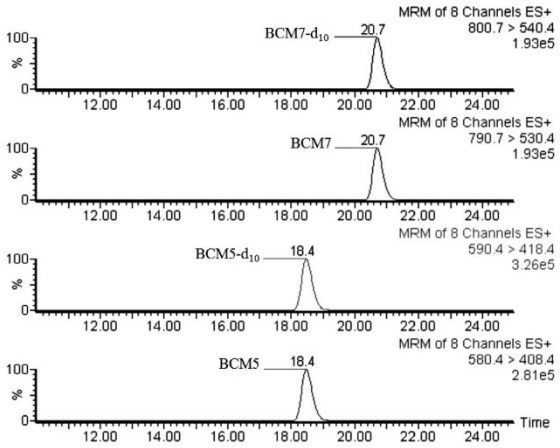

Fig. 3. Typical LC–MS/MS chromatograms of BCM5 and BCM7 and their corresponding deuterated homologues BCM5-d_{10} and BCM7-d_{10}. The ion source was an ESI operated in positive ion mode, while the mass spectrometer was a triple quadrupole operated in MRM mode.

Table 2
Instrument performance parameters obtained by repeat injections of standard solutions into the LC–MS/MS system.

Compounds	Linear range[a]	R^2	IDL[a,b] S/N = 3	IQL[a,c] S/N = 10	MRM ratio (± %RSD)	t_R (min ± SD)
BCM-5	0.05–50	0.9985	0.010	0.036	3.0 ± 7.8	18.2 ± 0.2
BCM-7	0.05–50	0.9986	0.007	0.024	2.6 ± 8.8	20.6 ± 0.1

[a] ng on-column.
[b] IDL: instrumental detection limit.
[c] IQL: instrumental quantification limit; MRM: multiple reaction monitoring; t_R: retention time.

Table 3
Recovery and precision study for BCM5 and BCM7.

Compounds	Spiked concentration (ng/g)	Recovery (%) $n = 3$	Precision (%RSD) $n = 3$
BCM5	1	106	16
	10	104	7
	100	106	2
	1000	95	2
	2500	97	1
BCM7	1	103	5
	10	109	4
	100	107	6
	1000	106	1
	2500	104	1

3.6. Evaluation of matrix effects affecting detection of BCM5 and BCM7 in yoghurt

The efficiency of ESI sources to ionise polarisable analytes in real samples, is often affected by the matrix, which can be responsible for suppressing the absolute response of analytes when analysed in LC–MS/MS. If no correction is made, matrix effects often lead to an underestimation in the concentration of the analytes of interest in real samples. In order to address matrix effects, different approaches have been proposed (Jessome & Volmer, 2006). For example, De Noni and Cattaneo (2010) reported the use of matrix matched calibration standards to take into account the presence of the matrix when quantifying BCM7 in UHT milk. This is a valid and effective method to account for matrix effects especially when deuterated standards are unavailable or prohibitively expensive.

In the present work, matrix effects were corrected for with the inclusion of deuterated homologues. This approach is by far the most effective method to account for matrix effects as the analytes and the corresponding co-eluting deuterated homologues are theoretically subject to identical matrix effects. In order to study the degree of signal suppression caused by the presence of the matrix, blank yoghurt extracts were spiked at 1000 ng/mL with BCM5 and BCM7 and analysed by LC–MS/MS. Results were compared to injected analytical standards at the same concentration levels. Matrix effects were responsible for causing about 26% signal suppression for BCM5 and about 40% signal suppression for BCM7. It is possible that additional clean up (e.g. on-line or off-line solid-phase extraction) for sample matrix would decrease observed signal suppression, which could improve detection limits.

3.7. Application of the validated method

The validated method was applied to the identification and quantification of BCM5 and BCM7 in ten commercial yoghurts, laboratory prepared yoghurt and reconstituted milk. Neither BCM5 nor BCM7 was detected in commercial products, however both peptides were identified and quantified in reconstituted milk and laboratory prepared yoghurts. The content of BCM5 decreased

Table 4
Inter-day reproducibility data determined by repeat measurements on two different days of yoghurt samples ($n = 3$) spiked at three different concentrations of BCM5 and BCM7.

Compounds	Nominal spiked concentration (ng/g)	Analysis day 1 (ng/g) $n = 3$	Analysis day 2 (ng/g) $n = 3$	Average (ng/g)	Inter-day reproducibility (%RSD)
BCM5	10	10.4	11.2	10.8	6.4
	100	106	106	106	2.0
	1000	950	1000	974	3.6
BCM7	10	10.7	11	10.8	6.1
	100	110	111	110	3.4
	1000	1040	1080	1060	3.2

Table 5
BCM5 and BCM7 concentrations in reconstituted milk and laboratory prepared yoghurts. Results for quality control (QC) samples are also reported.

Samples	BCM5 (ng/g)	BCM7 (ng/g)
Reconstituted milk	1.3[a]	1.9[a]
Yogurt, 0 day storage	1.1[a]	<MLQ
Yoghurt, 1 day storage	<MLQ	<MLQ
Yoghurt, 7 day storage	<MLQ	<MLQ
QC (reconstituted milk)	94 ± 3[b]	93 ± 1[b]
QC (yoghurt produced from reconstituted milk)	95 ± 1[c]	98 ± 1[c]

[a] Mean values, $n = 2$.
[b] Recoveries (%) are the average ± %RSD of $n = 2$ milk samples spiked at 100 ng/mL.
[c] Recoveries (%) are the average ± %RSD of $n = 2$ yoghurt samples made from reconstituted milk spiked at 100 ng/g.

from 1.3 ng/g in milk to 1.1 ng/g in yoghurt at 0 day storage and to less than the MLQ at 1 and 7 day storage. BCM7 content decreased from 1.9 ng/g in milk to less than the MLQ in yoghurts at all storage days (Table 5). It appears, BCM5 and BCM7 present in the milk have degraded into small peptides during fermentation and storage of yoghurt.

Recently, there have been a few studies measuring BCM5 and BCM7 in commercial yoghurts. De Noni and Cattaneo (2010) attempted to measure both peptides in these products, but neither BCM5 nor BCM7 was found. Nevertheless, BCM7 has been quantified between 0.86–2.45 μg/g in natural and probiotic yoghurt, levels decrease considerably during storage (Jarmolowska, 2012).

Kunda et al. (2012) have identified several di-peptides such as β-CN fragment 60–61 and β-CN fragment 62–63 in commercial yoghurt. As can been seen in Fig. 1, amino acids at position 61 and 63 on the chain of parent β-CNs are proline residues. This finding shows that yoghurt bacterial enzymes are likely to digest the peptide bonds between proline and other amino acid residues, leading to degradation of BCM5 and BCM7 during storage. The degradation of these peptides during yoghurt processing may involve the activity of X-prolyl dipeptidyl aminopeptidase (PepX), which is an enzyme derived from LAB that specifically hydrolyses peptide bonds between proline and other residues (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002). Furthermore, PepX activity in dairy products fermented with LAB has demonstrated to increase during storage (Otte, Lenhard, Flambard, & Sørensen, 2011).

4. Conclusions

A highly selective and sensitive analytical method employing LC–MS/MS was successfully developed and validated for the simultaneous determination of BCM5 and BCM7 in a complex matrix, yoghurt. For the highest confidence in the identification and quantification of BCM5 and BCM7, two isotopically labelled homologues were included in the method as surrogate standards. The method showed very low MLQs, (1.7 ng/g for BCM5 and 0.85 ng/g for BCM7) making it suitable for the analysis of the low level of BCMs in yoghurt .

This validated method was applied to analyse BCMs in commercial and laboratory prepared yoghurts and in milk. The absence of BCM5 and BCM7 in commercial yoghurts tested may have been due to degradation of these peptides during processing or storage. In the laboratory prepared yoghurt and milk tested, fermentation and storage lowered the content of BCM5 and BCM7.

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CHAPTER 4 IDENTIFICATION AND QUANTIFICATION OF BETA-CASOMORPHINS IN COMMERCIAL MILK PRODUCTS

4.1 Introduction

Bovine milk has been found to contain β-CN with 13 variants (Kamiński et al., 2007) that differ at various amino acid positions (Fig. 3), among which, β-CN A1 and β-CN A2 variants are commonly found in bovine milk. The ratio of β-CN A1 and β-CN A2 variants in milk depends on the cow breed from which the milk was obtained. Holstein-Friesian cows produce milk containing higher levels of β-CN A1 than β-CN A2 variant (Tailford et al., 2003), while Guernsey and Jersey breeds predominantly produce β-CN A2 variant (Bell et al., 2006; Merriman, 2009). Milk that contains both variants in equal amount is known as "normal" milk, while milk containing β-CN A2 variant only is known as A2 milk (Woodford, 2007).

In Australia, the most common dairy cow breed is Friesian Holstein, accounting for 65-70% of dairy cows. Other breeds include the Jersey, the Holstein/Jersey cross, Brown Swiss and Ayrshire. In addition, local breeds such as the Australian Red and the Illawarra are also important in dairy industry in Australia (Dairy Australia, 2013). In recent years, consumers and the media in Australia have been interested dairy products containing the majority of β-CN A2 variant, which was reported to not release BCM7 (De Noni, 2008; Haq, Kapila, & Kapila, 2015) or if formed, it will be at a very low rate (Korhonen & Marnila, 2013). Therefore, this milk should either not contain this peptide or contain it at very low level. Furthermore, intake of this milk should not release BCM7 during digestion and thus A2 milk is reported by manufacturers to possibly assist in digestion well-being. However, there has been no previous study of Australian milk on the concentration BCM5 and BCM7 in β-CN A_2 variant-rich milk or other normal milk.

Currently, identification of BCM5 and BCM7 in milk, yoghurt and milk powder has also been equivocal. Several studies have reported BCM7 in milk, yoghurt and milk powder (Cieślińska et al., 2012; Jarmolowska, 2012; Juan-García et al., 2009; Thiri et al., 2012) while BCM5 has been reported in infant formula that contains modified skimmed cow milk (Jarmołowska et al., 2007) and high protein supplement containing milk protein (Wocior, 2008). However, neither BCM5 nor BCM7 was detectable in infant formulas containing skim milk (De Noni & Cattaneo, 2010).

The aim of study in this chapter was to investigate the levels of BCM5 and BCM7 in commercial yoghurt, pasteurised milk and skim milk powder using the highly sensitive method of LC-MS/MS; the development of which was reported in Chapter 3. Furthermore, confirmation of the presence of BCM5 and BCM7 in some milk samples was performed by LC-HRMS.

4.2 Materials and methods

4.2.1 Materials and chemicals

Ten samples of commercial yoghurt, in December 2012, and 14 samples of pasteurised milk, from May to July 2014, produced from Australian milk by different manufacturers, were purchased from supermarkets around Australia. A skim milk powder supplied by A2 Dairy products Australia Pty Ltd was reported to be pasteurised before thermal evaporation and then spray drying. All products were randomly coded before sampling (Table 9). Other materials and chemicals used in this investigation have been previously described in Section 3.2.1.

4.2.2 Methods

4.2.2.1 Preparation of yoghurt extracts

The extraction of water soluble peptides from commercial yoghurt was carried out as described in Section 3.2.4.

4.2.2.2 Preparation of milk and skim milk powder extracts

The extraction of water soluble peptides from pasteurised milk was performed as follows: 25.0000 g (±0.0001 g) of pasteurised milk and 25.0000 g (±0.0001 g) of reconstituted skim milk (1.3/10: skim milk powder/water–w/v) were spiked with 500 ng of a mixture of BCM5-d_{10} and BCM7-d_{10}. In addition, a separate 25.0000 g (±0.0001 g) sample of pasteurised or reconstituted milk were each spiked with 100 ng of a mixture of BCM5 and BCM7 plus 500 ng of the mixture of BCM5-d_{10} and BCM7-d_{10}; these samples were used as quality control samples (QCs). All samples were gradually acidified with 1 M HCl to pH 4.6 and then left at room temperature for 10 min for precipitation of caseins, followed by centrifugation at 15,000 × g for 30 min at 4 °C. The supernatants were decanted into tubes and then the tubes were re-centrifuged at 14,000 ×g for 20 min at 4 °C.

Table 9. Specification of milks and yoghurts and milk powder used for investigation of determination of BCM5 and BCM7 contents

Products	Location of process	Type of milk	Expiration time (day) [a]	Protein content [b]			Strains of bacteria [c]
				Total	β-CN A2	B-CN A1	
Yoghurt							
Yg 1	WA, Australia	Cow milk	30	46	n.a	n.a	*S. thermophilus* and *L. bulgricus*
Yg 2	WA, Australia	Cow milk	13	46	n.a	n.a	*S. thermophilus* and *L. bulgricus*
Yg 3	WA, Australia	Cow milk	18	58	n.a	n.a	*S. thermophilus, L. acidophilus* and *Bifidobacterium*
Yg 4	WA, Australia	Cow milk	16	51	n.a	n.a	*S. thermophilus, L. bulgricus, L. acidophilus* and *Bifidobacterium*
Yg 5	WA, Australia	Cow milk	19	102	n.a	n.a	*S. thermophilus, L. bulgricus, L.acidophilus, bifidus* and *L. casei*
Yg 6	WA, Australia	Cow milk	30	47	n.a	n.a	*S. thermophilus* and *L. bulgricus*
Yg 7	WA, Australia	Cow milk	22	50	n.a	n.a	*S. thermophilus, B. lactis* and *L. acidophilus*
Yg 8	WA, Australia	Cow milk	22	46	n.a	n.a	*L. acidophilus, bifidus* and *L. casei*
Yg 9	WA, Australia	Cow milk	16	36	n.a	n.a	*S. thermophilus, L. acidophilus* and *Bifidobacterium*
Yg 10	WA, Australia	Cow milk	14	50	n.a	n.a	*L. acidophilus, Bifidobacterium* and *Lactobacillus GG*
Milk							
PM 1	South Brisbane, QLD	Cow milk	5	36	n.a	n.a	-
PM 2	South Brisbane, QLD	Cow milk	5	32	n.a	n.a	-
PM 3	Wagga Wagga, NSW	Cow milk	4	34	n.a	n.a	-
PM 4	Harvey, WA	Cow milk	12	33	n.a	n.a	-
PM 5	Margaret River, WA	Cow milk	9	33	n.a	n.a	-
PM 6	Northcliffe, WA	Cow milk	8	32	n.a	n.a	-
PM 7	Perth, WA	Cow milk	13	31	10	n.a	-
PM 8	Perth, WA	Cow milk	2	33	n.a	n.a	-

PM 9	South Brisbane, QLD	Cow milk	3	36	n.a	n.a	-
PM 10	Manning Valley, NSW	Cow milk	4	31	n.a	n.a	-
PM 11	Penrith, NSW	Cow milk	4	34	7	4	-
PM 12	Docklands, Vic	Cow milk	4	34	n.a	n.a	-
PM 13	Riverina, NSW	Cow milk	7	31	10	n.a	-
PM 14	Perth, WA	Cow milk	12	31	10	n.a	-
Skim milk powder							
SMP	Keysborough, VIC	Cow milk	30	n.a	n.a	n.a	-

QLD: Queensland; NSW: New South Wale; Vic: Victoria; WA: Western Australia

Yg: Yoghurt; PM: Pasteurised milk; SMP: Skim milk powder

n.a.: not available

[a] Days to expiration on the day of extraction

[b] Protein content reported by manufacturers: Yoghurt (mg/g), milk (mg/mL)

[c] Name of strains of bacteria showed on the labels of the products

The supernatants were then filtered through Whatman filter paper n° 541 before clean-up by SPE. The SPE cartridges (Oasis HLB, 500 mg/6mL, Waters Tokyo, Japan) were conditioned with 9.5 mL of MeOH followed by 9.5 mL of ultrapure water. The filtrates were diluted with 150 mL of ultrapure water, mixed thoroughly and then loaded onto the conditioned SPE cartridges. The loaded SPE cartridges were washed with 3.5 mL of 10% MeOH in ultrapure water (v/v) to elute interferences, followed by drying under vacuum of 20 mmHg for 10 min at room temperature to remove excess moisture. Retained peptides were then eluted using 3×4 mL of MeOH. A delay of 1 min between each aliquot of solvent was used to ensure that the stationary phase was efficiently soaked with the eluting solvent. The peptide-containing extracts were evaporated at 30 °C under a gentle stream of nitrogen using a dry block heater with nitrogen blowdown to a final volume of 0.5 mL. Extracts were then transferred into brown glass vials and analysed by LC-MS/MS and LC-HRMS. All samples and QCs were extracted and analysed in duplicate.

4.2.2.3 Identification and quantification of BCM5 and BCM7 by liquid chromatography-tandem mass spectrometry

a. Calibration standards

Mixed standard solutions of BCM5 and BCM7 ranging from 0.1 to 1000 ng/mL (0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 ng/mL) also containing deuterated standards BCM5-d_{10} and BCM7-d_{10} at 1000 ng/mL were serially diluted with ultra-pure water from the stock solutions prepared as previously reported in Section 3.2.2 . Calibration standards were prepared on the day of use.

b. Liquid chromatography-tandem mass spectrometry conditions

Chromatographic separation of BCM5 and BCM7 was carried out as previously described in Section 3.2.5 with some minor modifications. BCM5 and BCM7 were separated using a Kinetex C18 column (Phenomenex, USA, 150 mm × 2.1 mm, 100Å, 2.6 µm particle diameter) with a guard column (2.1 mm x 10 mm) of the same stationary phase. The mobile phase consisted of MeOH (A) and ultrapure water (B) both containing 0.1% formic acid at a flow rate of 100 µL/min. Chromatographic runs began with 30% (A) for 3 min, followed by a 15 min linear gradient to 95% (A). The mobile phase remained at 95% (A) for 10 min to elute analytes from the column.

The initial conditions were re-established within 1 min and the column re-equilibrated for 10 min before injecting the next sample. The injection volume was 25 µL. The tuning of the electrospray ion source and the mass spectrometry conditions for detection of BCM5 and BCM7 were as previously described in Section 3.2.5.

4.2.2.4 Identification and quantification of BCM5 and BCM7 by liquid chromatography-high resolution mass spectrometry

a. Calibration standards

Serial concentrations of calibration standards were prepared as described in Section 4.2.2.3a

b. Liquid chromatography-high resolution mass spectrometry conditions

Separation and identification of BCM5 and BCM7 in milk extracts by LC-HRMS were carried out using an Accela 600 LC system coupled to a high resolution mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific Corporation, Waltham, USA) fitted with an electrospray ion source operated in positive ionisation mode (+eV). BCM5 and BCM7 were chromatographically separated as described in Section 4.2.2.3b. To minimise potential carryover, the needle of the injector was rinsed thoroughly within the injection port with MeOH before and after each injection. ESI and $HRMS^n$ settings are reported in Table 10.

Full calibration of the LTQ Orbitrap XL in the 150-2000 *m/z* range was conducted weekly with the positive ion calibration solution provided by Thermo Scientific. Optical lenses were optimised with a standard solution of BCM5 and BCM7 prior each measurement. For increased mass accuracy on the LTQ Orbitrap XL, a plasticiser interfering peak present in the LC mobile phase at *m/z* 214.0887 (n-butyl benzenesulfonamide, $C_6H_5SO_2NH(CH_2)_3CH_3$, $[M+H]^+$=214.0896 *m/z*), was used for the lock mass function. For screening and quantification, two different MS acquisition modes were combined in the same analytical run. The screening analysis was conducted operating the LTQ Orbitrap XL mass spectrometer in full-scan mode from 70-1000 *m/z* with a mass resolution of 60.000 (@ 400 *m/z*). In addition, all of the target analytes and deuterated standards were specifically analysed for quantitation in single reaction monitoring (SRM) mode using the LTQ ion trap mass

spectrometer. The following SRM transitions and collision induced dissociation (CID) energies were used: BCM5 (580.3→408.3 *m/z*; CID=25); BCM5-d_{10} (590.3→418.3 *m/z*; CID=25); BCM7 (790.4→383.2 *m/z*; CID=23); BCM7-d_{10} (800.4→383.2 *m/z*; CID=23). The presence of the detected peptides was confirmed in the sample extracts by operating the LTQ Orbitrap XL in $HRMS^2$ mode, where the mass spectrometer is forced to isolate the parent compound, fragment it in the LTQ ion trap and then scan for the product ions in the Orbitrap mass analyser. A mass resolution of 30000 (@ 400 *m/z*) was used for the fragmentation experiments. Along with the chromatographic retention time, for BCM5 and BCM7 identification the deviation of the measured mass (i.e. parent compound and fragments) was compared against the theoretical mass (< 5ppm, relative error) and, fragmentation pattern of BCM5 and BCM7 in the samples was compared with that obtained from analysis of standard solutions. Data was processed using Xcalibur QualBrowser 2.0.7 SP1, TraceFinder 3.1 and MassFrontier 7.0.

Table 10. Summary of ESI and HRMSn parameters used for target analysis of BCM5 and BCM7 in milk extracts

Parameter	Setting
Spray voltage (kV)	4.0
Capillary voltage (V)	-35
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	-110
Scan range (*m/z*)	70-1000
Ion trap full MS automatic gain control target	3E4
Ion trap MS^n automatic gain control target	1E4
Fourier transform full MS automatic gain control target	2E5
Fourier transform MS^n automatic gain control target	1E5
Ion trap and fourier transform micro scans	3
Ion trap full MS Max ion time (ms)	10
Fourier transform MS^n Max ion time (ms)	200
MS^2 isolation window (*m/z*)	1

Arb: arbitrary units; ms: milliseconds

4.3 Results

4.3.1 Content of beta-casomorphin 5 and beta-casomorphin 7 in pasteurised milk and skim milk powder

4.3.1.1 Analysis of sample extracts by liquid chromatography-tandem mass spectrometry

SPE is a useful technique used widely in quantitative analysis. Preparation of samples by SPE followed by LC-MS/MS analysis can reduce interferences causing ion suppression overall improving LODs. However, added analysis time and costs are associated with SPE use. Thus, before a real quantification of the peptides in various pasteurised milk and skim milk powder samples by both LC-MS/MS and LC-HRMS, a preliminary work was conducted. In this regard, a sample of commercial UHT milk and reconstituted milk were prepared as described in Section 3.2.4 and then analysis by LC-MS/MS. In the preliminary samples of commercial UHT and reconstituted milk, LC-MS/MS chromatograms detected BCM7 but not BCM5 (Figure not shown), however chromatogram peaks of BCM7 were relatively poor. Therefore, in order to improve LODs, SPE was used for extraction and purification of peptides instead of 0.45 µm membrane filters before injection into the LC-MS/MS and LC-HRMS systems.

Figure 8 shows that peaks of BCM7 and BCM7-d_{10} are unequivocally detected in seven samples of pasteurised milk (PM 1, PM 2, PM 3, PM 4, PM 5, PM 6 and PM 7) and the reconstituted milk. The levels of BCM7 were between 0.25 ng/g and 2.16 ng/g, whereas BCM5 was below LOD in all samples of pasteurised milk (Table 11). This result for BCM5 agrees with those of De Noni and Cattaneo (2010) and Juan-García et al. (2009).

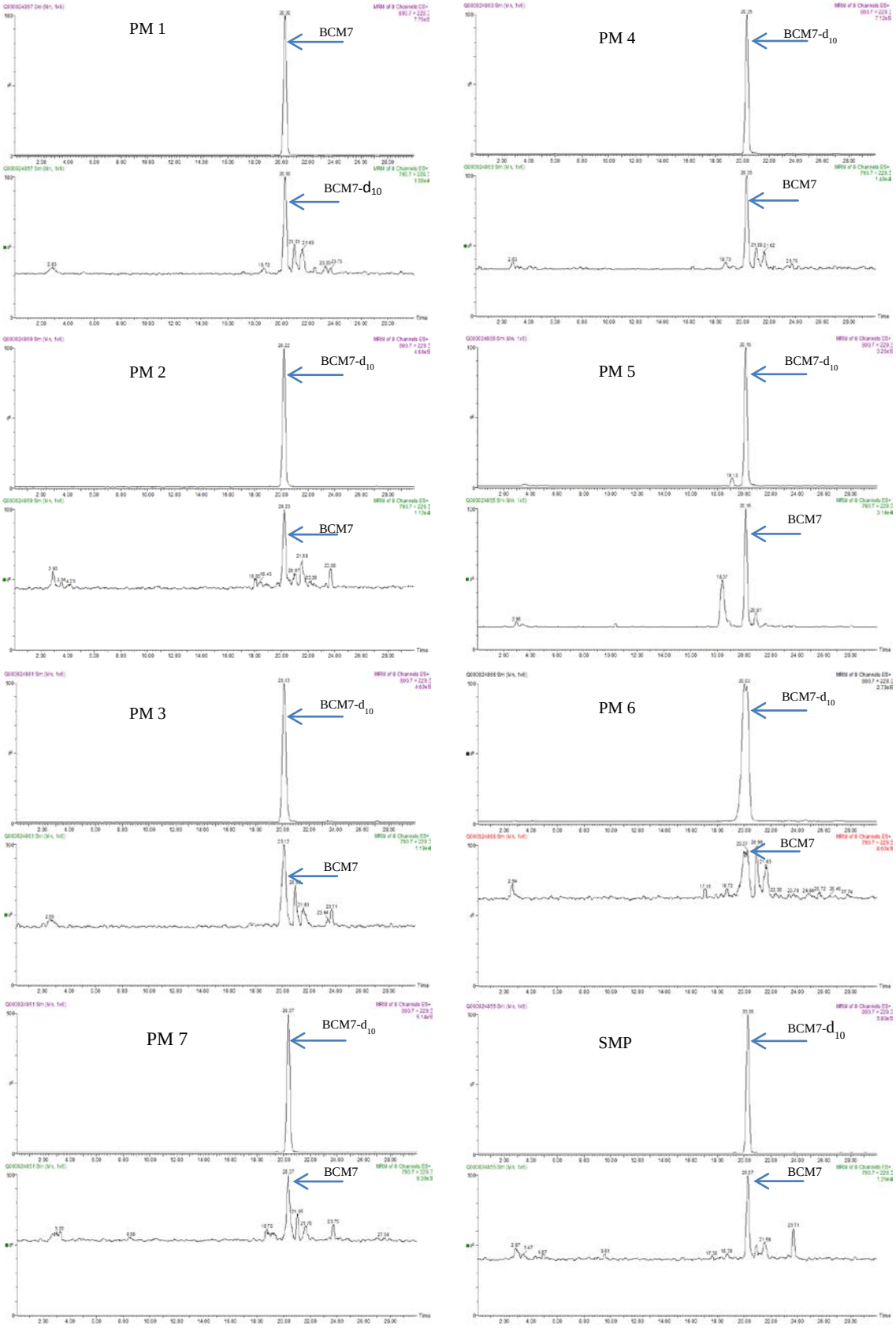

Figure 8. LC-MS/MS chromatograms of BCM7 and its deuterated standard (BCM7-d_{10}) in different milk extracts

Table 11. BCM5 and BCM7 levels in dairy products analysed by LC-MS/MS

Milk products	BCM7 level (ng/g)[a]	BCM5 level (ng/g)
Milk		
PM 1	0.44±0.01	< LOD
PM 2	0.40±0.01	< LOD
PM 3	0.53±0.01	< LOD
PM 4	0.40±0.00	< LOD
PM 5	2.16±0.05	< LOD
PM 6	0.32±0.01	< LOD
PM 7	0.25±0.01	< LOD
Skim milk powder		
SMP	0.34±0.00[b]	< LOD
QCs	108.6±1.7[c]	97.4±0.4[c]

[a] average levels (n=2) ± standard deviation

[b] BCM7 level in reconstituted milk from skim milk powder

[c] average recovery (n=2) ± standard deviation

PM: Pasteurised milk

SMP: Skim milk powder

LOD: Limit of detection

QCs: Quality control samples

4.3.1.2 Analysis of sample extracts by liquid chromatography-high resolution mass spectrometry

In this experiment, three pasteurised milks (PM 3, PM 5 and PM 6) previously analysed by LC-MS/MS were selected based on their characteristic high, medium and low content of BCM7 (Table 11) and re-analysed by LC-HRMS. In addition, seven further samples of pasteurised milk (PM 8, PM9, PM10, PM11, PM12, PM13 and PM 14) purchased from various supermarkets in Australia (Table 9) were analysed by LC-HRMS.

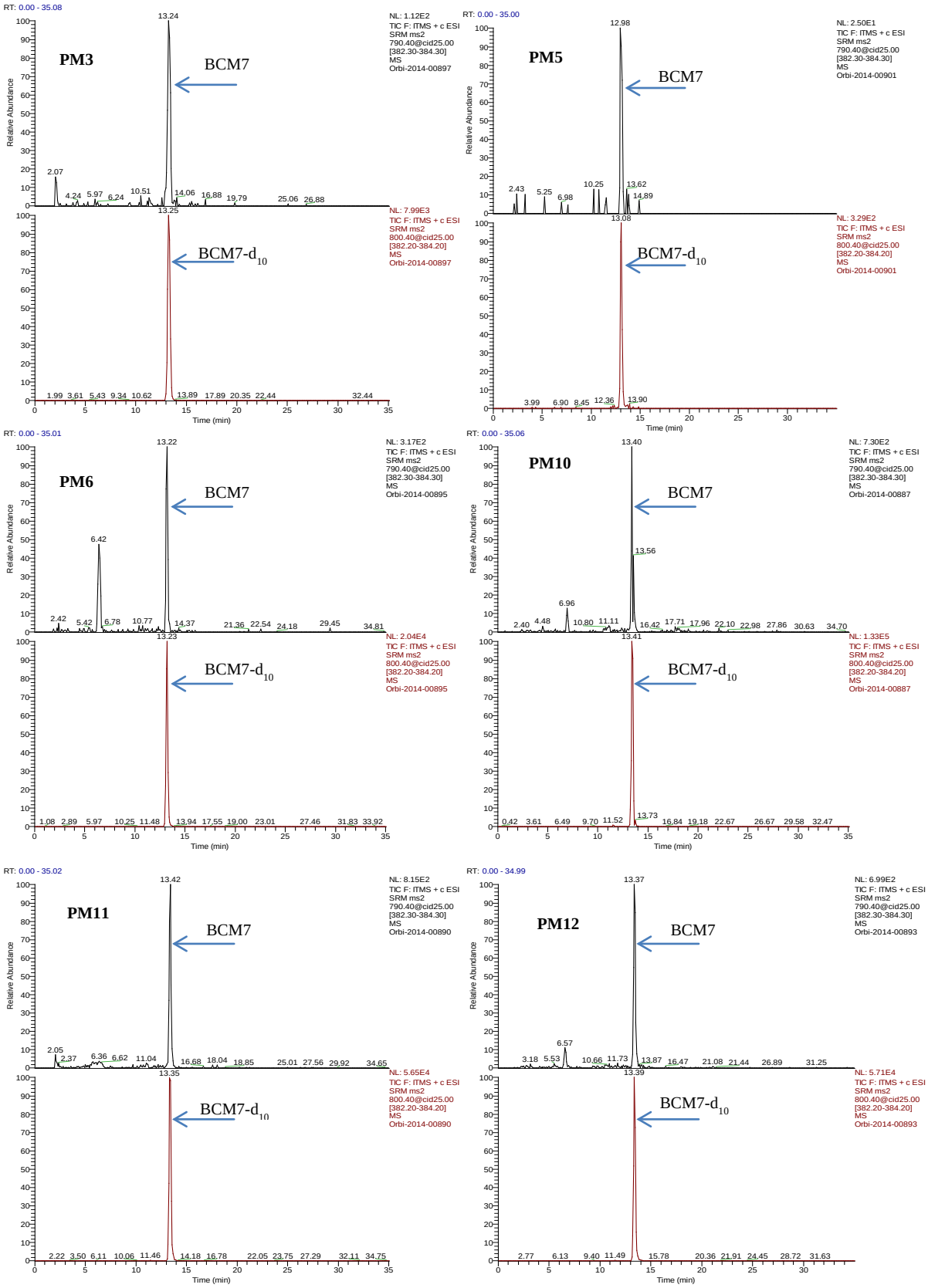

Figure 9. LS-HRMS chromatograms of BCM7 and its deuterated standard (BCM7-d_{10}) in different milk extracts

Figure 9 shows that the LC-HRMS chromatographic peaks of BCM7 and its deuterated standard are detected in six samples of pasteurised milk including three products previously analysed by LC-MS/MS, namely PM 3, PM 5, PM 6, PM 10, PM 11 and PM 12. Furthermore, the high resolution mass spectrometry data with a mass accuracy relative error below 5 ppm unequivocally confirmed the presence of this peptide in these six samples of milk (Table 12). The content of BCM7 in PM 3, PM 5 and PM 6 showed in Table 12 also compares well to the levels reported by analysis using LC-MS/MS (Table 11). The content of BCM7 in PM 8, PM 9, PM 13 and PM 14 (Figure not shown) was below LOD (Table 12). BCM5 was below LOD in all samples of pasteurised milk.

Table 12. Mass and level of BCM7 identified in different samples of pasteurised milk by LC-HRMS.

Products	$[M+H]^+_{teo}$ (Da)	$[M+H]^+_{exp}$ (Da)	Relative error (ppm)	BCM7 level[a] (ng/g)
PM 3	790.4134	790.4123	1.4	0.50±0.04
PM 5	790.4134	790.4122	1.4	2.38±0.09
PM 6	790.4134	790.4116	2.3	0.41±0.01
PM 8	790.4134	n.d	n.a	<LOD
PM 9	790.4134	n.d	n.a	<LOD
PM 10	790.4134	790.4111	2.9	0.13±0.01
PM 11	790.4134	790.4103	3.8	0.39±0.01
PM 12	790.4134	790.4122	1.4	0.38±0.01
PM 13	790.4134	n.d	n.a	<LOD
PM 14	790.4134	n.d	n.a	<LOD
QCs	790.4134	790.4120	1.7	102.4±0.1[b]

[a] Average values of two analysis (n=2) ± standard deviation

[b] Average recoveries (n=2) ± standard deviation

$[M+H]^+_{exp}$: Experimental molecular ion mass; $[M+H]^+_{teo}$: Theoretical molecular ion mass

PM: Pasteurised milk

n.d: Not detected

n.a: Not available

QCs: Quality control samples

4.3.2 Content of beta-casomorphin 5 and beta-casomorphin 7 in commercial yoghurts

LC-MS/MS chromatograms showed that BCM5 and BCM7 were not detected peaks in the commercial yoghurts (Figure not shown). This result agrees with the findings of De Noni and Cattaneo (2010). As discussed in Chapter 3, using SPE for cleaning-up of yoghurt supernatants before analysis by LC-MS/MS can improve LODs. However, LC-MS/MS analysis showed that all samples of yoghurt seemed not to contain BCM5 and BCM7 (<LOD) in this investigation. Thus, none of the yoghurt samples in the present study were subjected to clean-up by SPE before analysis by LC-MS/MS. The absence of BCM5 and BCM7 in the commercial yoghurts may have been due to degradation of these peptides, which possibly were originally present in the milk, during yoghurt manufacture and storage. Investigation of this potential phenomenon will be reported in Chapter 5 and 6.

4.4 Discussion

4.4.1 Occurrence of beta-casomorphin 5 and beta-casomorphin 7 in pasteurised milk and reconstituted skim milk powder

Milk extracts of UHT milk and reconstituted milk were prepared by using 0.45 µm membranes for cleaning-up supernatant and then analysed by LC-MS/MS. LC-MS/MS chromatograms showed that BCM7 was detected in these two samples of milk; however, the S/N of the peaks corresponding to BCM7 were generally low resulting in “noise” chromatographic traces. Milk is a complex food matrix in which proteins, fat, lactose or pre-existing peptides can cause interference during determination of BCM5 and BCM7 by chromatographic and mass spectrometric methods. In order to improve the S/N of the peaks and corresponding LODs of BCMs (Chapter 3), SPE was used for extraction and cleaned-up of supernatants before injecting into the LC-MS/MS system. After SPE extraction and clean-up, LODs resulted 0.4 ng/g and 0.2 ng/g for BCM5 and BCM7, respectively which were lower than those achieved by direct injection of supernatant (Chapter 3). The LC-MS/MS chromatograms show that peaks of BCM7 and its deuterated homologue (BCM7-d_{10}) were found in all samples of pasteurised milk and reconstituted milk (Fig. 8). The levels of BCM7 are given in Table 11.

Although low resolution LC-MS/MS is a highly sensitive and selective analytical technique currently used worldwide for the quantitative assessment of polar/apolar organic compounds, it lacks the capability of providing information regarding the accurate mass of parent compounds and fragments. The results obtained as using LC-HRMS show that LODs were 0.25 ng/g and 0.1 ng/g for BCM5 and BCM7, respectively. LC-HRMS data showed that BCM7 presents in a range of various pasteurised milks and skim milk powder at different levels (Table 12).

Enzyme and acid action are key factors in the hydrolysis of β-CNs to peptides in milk and dairy products (Meltretter et al., 2008). In addition, heat treatment also plays an important role in the breakdown of proteins to peptides (Meltretter et al., 2008). Heat treatment of milk is commonly carried out at either 72 – 75 °C for 15 s (high temperature-short time; HTST) or at 125 – 138 °C for 2 s (ultra-pasteurisation) in production of pasteurised milk and at extra low heat (<70 °C) or low heat (70 °C for 15 s) before the evaporation and spay drying processes used in skim milk powder manufacture (Gosta, 1995). Under these heat treatment regimes, the degradation of β-CNs to BCM7 by acid-induced hydrolysis is very unlikely since little change in pH of the milk (6.5-6.7) occurs. Meltretter et al. (2008) used MALDI-TOF-MS to identify five new peptides, not present in raw milk, after heating at 72, 85 and 120 °C for 10, 20 and 30 min respectively; however none of these peptides was reported as BCM7. Furthermore, these authors did not report any *m/z* matching with that of BCM7 in commercially pasteurised, ultra-pasteurised or UHT milk. Similarly, using LC-MS/MS, Gaucher et al. (2008) did identify pro-BCMs such as β-CNf(54-68), β-CNf(54-69), β-CNf(55-66), β-CNf(55-68) and β-CNf(57-68) but no BCM7 in UHT milk. De Noni and Cattaneo (2010) did not detect BCM5 and BCM7 in unprocessed milk, commercial pasteurised, sterilised UHT milk and skim milk powder used LC-MS/MS. Therefore, BCM7 found in some samples of pasteurised milk in this research may be due to its pre-existence in raw milk, possibly released by endogenous or exogenous enzyme action, rather than to formation during the pasteurisation process.

The content of BCM7 varies in samples of pasteurised milk (Tables 11 and 12). As indicated in Table 9, samples of pasteurised milk were produced by different dairy companies around Australia, as a result raw milk was very likely to have been supplied from different dairy herds. Therefore, the differences in the concentrations

of BCM7 observed are probably related to differences in the genetic make-up of each herd (e.g. different breeds). The present study showed that three samples of pasteurised milk, namely PM 7, PM 13 and PM 14, did not contain BCM7 or only contained it at a low concentration (Tables 11 and 12). According to manufacturers, these three samples of pasteurised milk contained β-CN A2 variant at 10 mg/mL (Table 9). Fox and McSweeney (1998) indicated that there is about 9.8 mg/mL of β-CN in bovine milk. As a result, the samples PM 7, PM 13 and PM 14 in the present work may only contain the β-CN A2 variant. This finding indicates that BCM7 may not be generated from β-CN A2 variant or only formed at a very low concentration. These results agree with that reported by Korhonen and Marnila (2013). The results also showed that two other samples of pasteurised milk (PM 8 and PM 9) were not detected BCM7 (Table 12), suggesting that these samples of milk probably contain only the β-CN A2 variant. Therefore, the raw milk supplied for production of PM 8, PM 9, PM 13 and PM 14 may have been mainly collected from herds of A2/A2 cows.

Seven of the milk samples (PM 1, PM 2, PM 3, PM 4, PM 6, PM 11 and PM 12) containing BCM7 (Tables 11 and 12) were not claimed by their manufacturers to contain specific β-CN variants with the exception of PM 11 that was claimed to contain both β-CN A2 and β-CN A1 variant (Table 9). Consequently, the raw milk used for production of this milk is likely to have been collected from herds of A1/A2 cows. In the present work, one sample, PM 5 had BCM7 at the highest concentration (Table 12) and thus, may have been collected from herds with a greater predominance of A1/A1 cows.

There is currently a DNA test for identification of A2/A2 cows available in Australia and New Zealand, but it is not used widely for dairy farmers or industry. Several milk manufacturers in Australia have applied this test to segregate individual cows into A2/A2, A1/A2 and A1/A1 herds. Therefore, milk collected from these segregated herds could contain different ratios of β-CN A2 to β-CN A1 variant, leading to the difference in content of BCM7 in different samples of pasteurised milk.

4.4.2 .Occurrence of beta-casomorphin 5 and beta-casomorphin 7 in commercial yoghurts.

The release of BCM7 during *in vitro* digestion of the β-CN A1 variant (De Noni, 2008; Haq et al., 2015; Jinsmaa & Yoshikawa, 1999) and during *in vivo* digestion of milk protein (Boutrou et al., 2013) has been reported. In addition, a number of studies have reported the finding of BCM7 and other related BCMs in some varieties of cheese (De Noni & Cattaneo, 2010; Jarmolowska et al., 1999; Norris et al., 2003). However, the occurrence of these peptides in yoghurt is still equivocal. This is probably due to the lack of sensitivity of analytical methods previously used for analysis of BCMs, and the effects of the source of raw milk, different strains of yoghurt culture, and days of storage on the formation and degradation of these peptides. The present experiment focused on identification and quantification of BCM5 and BCM7 in various yoghurts produced by different dairy manufacturers from Australian milk (Table 9) by LC-MS/MS.

As presented in Chapter 3, LC-MS/MS using BCM5-d_{10} and BCM7-d_{10} as internal standards was successfully developed for identification and quantification of BCM5 and BCM7 at very low levels (sub-ng/g) in yoghurt. This method was applied to ten commercial cow milk yoghurts, fermented with varieties strains of culture at various days of storage (Table 9). Both BCM5 and BCM7 were lower than LOD (see Chapter 3) in all yoghurts, indicating that either these peptides were (a) never formed or present in yoghurts or in the milk used for their manufacture or (b) were formed or present at some stage but were degraded during yoghurt fermentation and storage. Investigations on these hypotheses will be reported in Chapters 5 and 6.

4.5 Conclusions

Application of LC-MS/MS and LC-HRMS using BCM5-d_{10} and BCM7-d_{10} as internal standards successfully simultaneously identified and quantified BCM5 and BCM7 in commercial milk products: yoghurt, pasteurised milk and skim milk powder. Furthermore, purification of extracts using SPE before injecting to LC-MS/MS and LC-HRMS system is a useful technique for improvement of LOD. This is the first study on investigation of BCM5 and BCM7 in a large number of various milk products made from Australian milk. The concentration of BCM7 at different levels in a range of pasteurised milk and skim milk powder indicates that the raw milk may be collected from herds varying in their composition of A2/A2, A1/A1 or

A1/A2 genotypes. The presence of BCM7 in pasteurised milk and skim milk powder may provide evidence that BCM7 may be degraded by bacteria derived enzymes during yoghurt process and thus, the hydrolysis of BCM7 during fermentation and storage of yoghurt will be studied further in Chapter 5. This finding provides new knowledge for further studies concerning A2/A2, A1/A1 or A1/A2 genotypes and BCM7; and the potential bioactivity of BCM7 as intake of milk. In addition, the impact of heat treatments to these milk containing pure β-CN variants on their concentrations of BCM7 should be further investigated.

4.6 Papers

Part of this study has been published in the following journal article: D.D. Nguyen, F. Busetti, S.K. Johnson and V.A. Solah (2015). Identification and quantification of native beta-casomorphins in Australian milk by LC-MS/MS and LCHRMS. *Journal of Food Composition and Analysis, 44*, 102-110.

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Article Type: Original Research Article

Keywords: beta-casomorphin 5, beta-casomorphin 7, pasteurised, milk, LC-MS/MS, Orbitrap

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Abstract: Beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in 14 commercial pasteurised milks around Australia were investigated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-high resolution (Orbitrap) mass spectrometry (LC-HRMS). Collision induced dissociation HRMS was used to confirm the presence of BCM7 in milk extracts. The accurate mass-to-charge ratio (m/z) and relative abundance of BCM7 parent ion and fragments in milk extracts matched with that obtained from the analysis of a BCM7 standard solution. The deviation against the theoretical values of the measured m/z of BCM7 (parent ion and fragments) all resulted below 5 ppm (relative error). BCM5 was below limit of detection (LOD) for all milks, while BCM7 was between 0.13 and 2.38 ng/g in ten milks and below LOD in the others. The concentrations of BCM7 may depend on ratio of beta-casein A1 to beta-casein A2 variants in the commercial milk which varies according to the herd compositions.

Identification and quantification of beta-casomorphins in Australian milk by LC-MS/MS and LC-HRMS.

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Abstract

Beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in 14 commercial pasteurised milks around Australia were investigated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-high resolution (Orbitrap) mass spectrometry (LC-HRMS). Collision induced dissociation HRMS was used to confirm the presence of BCM7 in milk extracts. The accurate mass-to-charge ratio (*m/z*) and relative abundance of BCM7 parent ion and fragments in milk extracts matched with that obtained from the analysis of a BCM7 standard solution. The deviation against the theoretical values of the measured *m/z* of BCM7 (parent ion and fragments) all resulted below 5 ppm (relative error). BCM5 was below limit of detection (LOD) for all milks, while BCM7 was between 0.13 and 2.38 ng/g in ten milks and below LOD in the others. The concentrations of BCM7 may depend on ratio of beta-casein A1 to beta-casein A2 variants in the commercial milk which varies according to the herd compositions.

Keywords: beta-casomorphin 5, beta-casomorphin 7, pasteurised, milk, LC-MS/MS, Orbitrap

Chemical compounds studied in this article: Beta-casomorphin 5 (PubChem CID:122318); beta-casomorphin 7 (PubChem CID: 121946)

1. **Introduction**

Casein is one of the major milk proteins, consisting of α_{s1}, α_{s2}, β and κ-casein and is a source of bioactive peptides that can be released by endogenous enzyme activity in milk, by digestive enzymes in the gastrointestinal tract or during food processing (Sienkiewicz-Szłapka et al., 2009). Several bioactive peptides with opioid properties, designated as beta casomorphins (BCMs), originate from β-casein (β-CN).

Beta casein of bovine milk has 209 amino acids with 13 variants, A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and G (Kamiński et al., 2007). Among the variants, β-CN A1 and β-CN A2 variant are frequently present in bovine milk. Both variants differ only at amino acid position 67, where the histidine residue on β-CN A1 variant is replaced by a proline residue on β-CN A2 variant. Normal milk is usually found to contain both β-CN A1 and β-CN A2 variants and so is designated as A1/A2 milk (Nguyen et al., in press). However, milk containing only the β-CN A1 or only the β-CN A2 variant is known as A1 milk or A2 milk (Woodford, 2007).

During the past decade, a potential relationship between consumption of milk containing β-CN A1 variant and the increased risk of human disease has been reported and then disputed. A number of studies have reported that BCM5 and BCM7 (Fig.1), which are released from β-CN A1 variant, are risk factors for the development of type 1 diabetes, autism in children, sudden infant death and ischemic heart diseases (Birgisdottir et al., 2006; Elliott et al., 1999; McLachlan, 2001; Wasilewska et al., 2011). However, other reports have concluded that there is no strong evidence for any cause-effect relationship (EFSA, 2009; Swinburn, 2004; Truswell, 2005). Claeys et al (2013) suggested casein peptide fragments may have a positive role such as antimicrobial and antiviral properties.

49 *In vitro* studies have demonstrated that BCM7 is released from β-CN A1 variant by digestion
50 with gastrointestinal enzymes (De Noni, 2008; Haq et al., 2015; Jinsmaa & Yoshikawa,
51 1999). However, BCM5 is not formed from either β-CN A1 or β-CN A2 variant by digestion
52 with these enzymes (De Noni, 2008; Haq et al., 2015). In a human study, BCM7 was
53 identified in jejunal effluent after intake of bovine milk protein; however the β-CN variant
54 status of the milks was unknown (Boutrou et al., 2013).
55 Recently, investigations related to the presence of BCM5 and BCM7 in both milk and
56 processed milk products such as yoghurt and milk powder have been a topic of great interest.
57 According to De Noni & Cattaneo (2010), neither BCM5 nor BCM7 were identified in milk,
58 yoghurt, milk powder, milk protein concentrate and infant formulas. In contrast, other studies
59 have identified BCM7 in milk, yoghurt and milk powder (Cieślińska et al., 2012;
60 Jarmolowska, 2012; Juan-García et al., 2009; Thiri et al., 2012) and BCM5 has been reported
61 in infant formulas and high-protein concentrate (Jarmołowska et al., 2007; Wocior, 2008).
62 However, none of these studies has reported an accurate molecular mass of the peptide
63 BCM5 and BCM5, and confirmed their structure through high resolution fragmentation
64 experiments to provide robust evidence of their identification.
65 In Australia consumer and media interest in A2 milk, a product rich in β-CN A2 variant, and
66 other milk products containing natural A2 protein has increased in recent times. However,
67 there has been no previous study in Australia reporting the concentration of BCM5 and
68 BCM7 in these pasteurised milks as well as normal milks.

69 The aim of the present study was to use a highly sensitive isotope dilution LC-MS/MS for
70 simultaneous quantification of BCM5 and BCM7 in commercial pasteurized milk. On the
71 other hand, this study also used LC-HRMS for identification and quantification of these
72 casomorphins in some milk samples. LC-HRMS was used for confirmation of the presence of

BCM7 and BCM5 by high mass resolution and high mass accuracy fragmentation experiments.

2. Materials and methods

2.1 Materials and chemicals

Fourteen pasteurised milks were purchased from supermarkets around Australia between May and July 2014. The products were randomly coded PM 1 to PM 14 (Table 1) and extracted immediately or stored at -80 °C until extraction. BCM5 (purity 97.8%), BCM7 (purity >98.7%) and their deuterated standards [^{2}H10] BCM5 (BCM5-d_{10}) and [^{2}H10] BCM7 (BCM7-d_{10}) (deuterium enrichment > 99%, 2H enrichment at phenylalanine position) were purchased from Auspep Pty (Tullamarine, Australia). Ultrapure water was purified using a IBIS Technology (Perth, Australia) Ion Exchange System followed by the Elga Purelab Ultra System (Sydney, Australia). Methanol (MeOH) was purchased from Mallinckrodt (New Jersey, USA); formic acid (purity 99%) was purchased from Ajax FineChem (Sydney, Australia). All chemicals were of HPLC grade.

2.2 Preparation of milk extracts

Twenty five grams (±0.0001 g) of each sample were spiked with 500 ng of mixture of BCM5-d_{10} and BCM7-d_{10}. Samples were gradually acidified with 1N HCl to pH 4.6, left at room temperature for 10 min for complete precipitation of caseins and then centrifuged at 15,000×*g* at 4 ºC for 30 min. The supernatants were filtered through a Whatman filter paper N° 541 before extraction and clean-up by solid-phase extraction (SPE). The SPE cartridges (Oasis HLB, 500 mg/6 mL from Waters, Tokyo, Japan) were conditioned with 9.5 mL of MeOH, followed by 9.5 mL of ultrapure water. The supernatants were diluted to 150 mL with ultrapure water, mixed thoroughly and then loaded onto the SPE cartridges. The loaded SPE cartridges were then washed with 4.5 mL of 10% MeOH in ultrapure water (v/v) to elute interfering compounds. The washed cartridges were then dried under vacuum (-20 mmHg)

98 for 10 min to remove excess moisture. Retained peptides were then eluted from the cartridges
99 using 3×3.5 mL of MeOH. A delay of 1 min between each aliquot of MeOH was used to
100 ensure that the cartridge stationary phase was fully soaked with the eluting solvent. The
101 peptide-containing eluates were combined and then evaporated at 30 °C to final volume of
102 0.5 mL, under a gentle stream of nitrogen using a dry block heater with nitrogen blowdown.
103 These concentrated extracts were then transferred into brown glass vials for analysis. Quality
104 control samples (QCs, n=2) consisted of 25 g (±0.0001 g) of a selected pasteurized milk,
105 spiked with 100 ng of mixture of BCM5 and BCM7, and 500 ng of deuterated standards.
106 Instrumental and laboratory contaminations were monitored by analysis of mobile phase
107 blanks, injector blanks and procedural blanks (i.e. 150 mL of ultrapure water spiked with
108 deuterated standards and processed through SPE. All samples including pasteurised milk
109 extracts, QCs as well as procedural blanks were processed in duplicate.

110 2.3 Analysis of BCM5 and BCM7

111 *2.3.1 Calibration standards*

112 Stock solutions (1 mg/mL) of BCM5, BCM7 and their deuterated standards (BCM5-d_{10} and
113 BCM7-d_{10}) were prepared by dissolving 5 mg of each compound in ultrapure water to a
114 volume of 5 mL in a volumetric flask. Stock solutions were stored at -80 °C until use. Mixed
115 standard solutions of BCM5 and BCM7 ranging from 0.1 to 1000 ng/mL of each (i.e. 0.1,
116 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 ng/mL) also containing
117 BCM5-d_{10} and BCM7-d_{10} at 1000 ng/mL, were freshly prepared before use in ultrapure water
118 by serial dilution of the stock solutions.

119 *2.3.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions*

120 The LC-MS/MS conditions adopted for the identification and quantification of BCM5 and
121 BCM7 were as previously described in Nguyen et al. (2014), with some minor modifications.
122 In brief, chromatographic separation of BCM5 and BCM7 was achieved using a Kinetex C18

123 column (Phenomenex, USA, 150 mm × 2.1 mm, 100Å, 2.6 µm particle diameter) using a
124 guard column (2.1 mm x 10 mm) of the same stationary phase. The mobile phase constituted
125 of MeOH (A) and ultrapure water (B) both containing 0.1% formic acid at a flow rate of 100
126 µL/min. Chromatographic runs began with 30% (A) for 3 min, followed by a 15 min linear
127 gradient to 95% (A). The mobile phase remained at 95% (A) for 10 min to elute analytes
128 from the column. The initial conditions were re-established within 1 min and the column re-
129 equilibrated for 10 min before injecting the next sample. The injection volume was 25
130 µL. The tuning of the electrospray ion source as well as the mass spectrometry conditions
131 adopted for detection of BCMs remained the same as previously reported by Nguyen et al.
132 (2014).
133 *2.3.3 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) conditions*
134 An Accela 600 LC system coupled to a high resolution mass spectrometer (LTQ Orbitrap
135 XL, Thermo Fisher Scientific Corporation, Waltham, USA) fitted with an electrospray ion
136 source (ESI) operated in positive ionization mode (+eV) was used for LC-HRMS analysis.
137 For the reverse phase chromatographic separation, an aliquot of extract (25 µL), was injected
138 onto a Kinetex C18 column (Phenomenex USA, 100 mm × 2.1 mm, 100Å, 2.6 µm particle
139 diameter) using a guard column (2.1 mm x 10 mm) of the same stationary phase. The mobile
140 phase consisted of MeOH (A) and ultrapure water (B), both containing (0.1% V/V) of formic
141 acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 15 min linear
142 gradient to 95% (A). The mobile phase remained at 95% (A) for 5 min to elute analytes and
143 interferences from the column. The initial conditions were re-established within 1 min and
144 the column re-equilibrated for 10 min before injection of the next sample. To minimise
145 potential carryover, the needle of the injector was rinsed thoroughly within the injection port
146 with MeOH before and after each injection. ESI and $HRMS^n$ settings are reported in Table
147 S1, with further details available in the supporting information.

148 Full calibration of the LTQ Orbitrap XL in the 150-2000 *m/z* range was conducted weekly
149 with the positive ion calibration solution provided by Thermo Scientific. Optical lenses were
150 optimised with a standard solution of BCM5 and BCM7 prior each measurement. For
151 increased mass accuracy on the LTQ Orbitrap XL, a plasticizer interfering peak present in LC
152 mobile phase at *m/z* 214.0887 (n-butyl benzenesulfonamide, $C_6H_5SO_2NH(CH_2)_3CH_3$,
153 $[M+H]^+$=214.0896 *m/z*), was used for the lock mass function. For screening and
154 quantification, two different MS acquisition modes were combined in the same analytical run.
155 The screening analysis was conducted operating the LTQ Orbitrap XL mass spectrometer in
156 full-scan mode from 70-1000 *m/z* with a mass resolution of 60.000 (@ 400 *m/z*). In addition,
157 all of the target analytes and deuterated standards were specifically analysed for quantitation
158 in single reaction monitoring (SRM) mode using the LTQ ion trap mass spectrometer. The
159 following SRM transitions and collision induced dissociation (CID) energies were used:
160 BCM5 (580.3→408.3 *m/z*; CID=25); BCM5-d_{10} (590.3→418.3 *m/z*; CID=25); BCM7
161 (790.4→383.2 *m/z*; CID=23); BCM7-d_{10} (800.4→383.2 *m/z*; CID=23). The presence of the
162 detected peptides was confirmed in the sample extracts operating the LTQ Orbitrap XL in
163 $HRMS^2$ mode, where the mass spectrometer is forced to isolate the parent compound,
164 fragment it in the LTQ ion trap and then scan for the product ions in the Orbitrap mass
165 analyser. A mass resolution of 30000 (@ 400 *m/z*) was used for the fragmentation
166 experiments. Along with the chromatographic retention time, for substance identification the
167 deviation of the measured mass (i.e. parent compound and fragments) was compared against
168 the theoretical mass (< 5ppm, relative error) and, the measured isotope pattern (i.e.
169 fragmentation pattern) of BCMs in the samples was compared with that obtained from
170 analysis of standard solutions. Data was processed using Xcalibur QualBrowser 2.0.7 SP1,
171 TraceFinder 3.1 and MassFrontier 7.0.

172 **3 Results**

173 3.1 Concentration of BCM5 and BCM7 in milk extracts determined by LC-MS/MS analysis
174 Previously, isotope dilution LC-MS/MS has been successfully applied to simultaneously
175 identify and quantify BCM5 and BCM7 in reconstituted milk and yoghurt extracts (Nguyen
176 et al., 2014). In the present study, various pasteurised milks collected from supermarkets in
177 around Australia were extracted, cleaned-up by SPE and analysed by LC-MS/MS operated in
178 multiple reaction monitoring (MRM) mode.
179 The LC-MS/MS chromatograms show that BCM7 is present in all of the pasteurized milks
180 analysed (Fig. 2), whereas BCM5 was below limit of detection (LOD). The concentrations of
181 BCM7 in the pasteurised milk extracts ranged from 0.25 to 2.16 ng/g (Table 2). The samples,
182 containing the lowest (PM 7) and highest (PM 5) concentrations, were both produced in
183 Western Australia.
184 3.2 Concentration of BCM5 and BCM7 in milk extracts by LC-HRMS analysis
185 LC-HRMS was used for identification and quantification of BCMs in PM 3, PM 5 and PM 6,
186 which were previously analysed by LC-MS/MS and selected based on their high, medium
187 and low content of BCM7 (Table 2), and in PM 8, PM 9, PM 10, PM 11, PM 12, PM 13 and
188 PM 14.
189 The LC-HRMS chromatograms (Fig. 3) show that BCM7 was found in some of the milk
190 extracts, whereas BCM5 was below LOD in all of the samples analysed. The concentrations
191 of BCM7 in PM 3, PM 5 and PM 6 determined by LC-HRMS ranged from 0.41 to 2.38 ng/g
192 (Table 2), and resulted very similar values (within experimental error limits) to those
193 previously determined by LC-MS/MS (Table 2). BCM7 was also detected in three other milk
194 samples, PM 10, PM 11 and PM 12. However, concentration of this peptide was below LOD
195 in PM 8, PM 9, PM 13 and PM 14 milk extracts (Table 2).
196 **4. Discussion**

197 4.1 Analytical performance and identification criteria adopted for the analysis of BCMs in
198 milk extracts
199 For the identification and quantitation of BCM5 and BCM7 in the milk extracts using the LC-
200 MS/MS method (Micromasss Ultima Triple Quadrupole system), a MRM approach was used,
201 the mass spectrometer was set to select the parent ion, fragment it in the collision cell, and
202 select two daughters for detection. The most sensitive daughter ion is then used for
203 quantification while the secondary daughter ion is used for confirmation purposes.
204 According to the identification performance criteria defined by the European Community
205 (2002) for quantitative mass spectrometric detection, a minimum of three identification points
206 are required. Using LC–MS/MS to monitor one parent ion and two daughter ions 'earns'
207 1+(1.5×2)=4 identification points and therefore fulfils these criteria. In addition, the MRM
208 ratio of the parent to daughter transitions was monitored. The standard deviations of MRM
209 ratio, as defined by European Community (2002) on the performance of analytical methods,
210 were also well within permitted tolerances (data not shown).
211 For the identification and quantitation of BCM5 and BCM7 in the milk extracts using the LC-
212 HRMS method (LTQ Orbitrap XL system), two different MS acquisition modes were
213 combined in the same analytical run. Given that the LTQ Orbitrap XL system consists of both
214 a low resolution ion trap and a high resolution Orbitrap mass spectrometer, these two mass
215 spectrometers can be used simultaneously for parallel detection of ions and fragments of
216 interest. For the ion trap, a single reaction monitoring approach was used where the ion trap
217 mass spectrometer is set to select the parent ion, trap it and fragment it, and trap and select
218 one daughter for detection. In addition, a high resolution MS^2 full scan spectra was acquired
219 in the Orbitrap. This gives important information such as high accuracy *m/z* values as well as
220 elemental composition of the molecular ions and fragments analysed. Using LC–MS^2 (Ion
221 Trap, 1 parent and 1 daughter) in combination to LC-$HRMS^2$ (Orbitrap, 1 parent 2 daughters)

9

222 'earns' 1+1.5+2+(2×2)=8.5 identification points and therefore fulfils the European
223 Commission (2002) for quantitative mass spectrometric detection criteria.
224 The results of the identification points "earned" by the two mass spectrometry techniques
225 adopted are summarised in Table S2 available as supporting information.
226 Quantification of BCM5 and BCM7 in the milk extracts was achieved using commercial
227 available deuterated standards of the peptides, [^{2}H10] BCM5 and [^{2}H10] BCM7 with 2H
228 enrichment at phenylalanine position. The use of deuterated standards gives the highest
229 possible confidence of the "trueness" (i.e. accuracy and precision) of the analytical results.
230 Deuterated homologues are very effective to 1) account for recovery losses occurring during
231 sample preparation (i.e. extraction and clean-up by SPE) and 2) correct for matrix effects
232 causing analytes signal suppression/enhancement occurring during the ionisation stage (i.e. in
233 the electrospray ionisation source). For example, the QCs analysed by LC-MS/MS showed
234 relative recoveries of 97.4±04% and 108.6±1.7% for BCM5 and BCM7, respectively. The
235 QCs analysed by LC-HRMS showed relative recoveries of 92.9±0.4% and 102.7±0.4% for
236 BCM5 and BCM7, respectively. Deuterated standards also are useful to correct for analyte
237 retention time shifts caused by matrix effect occurring during the LC separation stage. In
238 general, for both LC-MS methods, retention time shifts were minimal and in the order of tens
239 of seconds (e.g. see retention times in Table 3).
240 In a previous study, LC-MS/MS using stable isotope-labelled BCMs (BCM5-d_{10} and BCM7-
241 d_{10}) as surrogate standards was successfully applied to identify and quantify BCM5 and
242 BCM7 at very low concentrations (sub-ng/g) in reconstituted milk extracts (Nguyen et al.,
243 2014). The method employed direct injection into the LC-MS/MS system of samples extracts
244 which were previously cleaned-up by 0.45 μm filter membranes and dried down to a final
245 volume of 1 mL. Milk is a complex food matrix in which proteins, fat, lactose or pre-existing
246 peptides can interfere with analysis of target peptides such as BCM5 and BCM7. Therefore,

247 in the present study, in order to improve detection limits, the milk extracts were cleaned-up
248 by SPE prior to analysis by LC-MS/MS and LC-HRMS. This improved the limit of detection
249 (LOD) by about 20% for both BCM5 and BCM7 on the triple quadrupole system (i.e. LOD
250 0.4 ng/g for BCM5; LOD 0.2 ng/g for BCM7) while LOD measured by LC-HRMS was in
251 the order of 0.25 ng/g for BCM5 and 0.1 ng/g for BCM7.

252 4.2 Occurrence of BCM5 and BCM7 in milk

253 Beta-casomorphins are opioid peptides originally isolated from the enzymatic digest of
254 bovine casein (Brantl et al., 1979). Among them, BCM5 and BCM7 are two peptides with the
255 highest opioid properties (Brantl et al., 1981; Kálmán et al., 1992) and their ingestion have
256 been linked with the increased risk of non-communicable diseases in human (Birgisdottir et
257 al., 2006; Elliott et al., 1999; McLachlan, 2001; Wasilewska et al., 2011). In human studies,
258 Kost et al. (2009) identified BCM7 in the blood of infants fed formula containing cow milk
259 and it was claimed that the higher concentrations of bovine BCM7 in the blood of the infants
260 correlated with delays in psychomotor development.

261 The generation of BCM7 during *in vitro* digestion of β-CN A1 variant (De Noni, 2008; Haq et
262 al., 2015; Jinsmaa & Yoshikawa, 1999) and *in vivo* human digestion of milk protein (Boutrou
263 et al., 2013), has been confirmed by experimental evidence. Furthermore, the occurrence of
264 BCM7 and other related BCMs in cheese has also been reported in a number of studies (De
265 Noni & Cattaneo, 2010; Jarmolowska et al., 1999; Norris et al., 2003). However, the
266 occurrence of these peptides in pasteurized milk, yoghurt or milk powder is still equivocal.
267 Juan-García et al. (2009) used liquid chromatography (LC) coupled to a quadrupole ion-trap
268 mass spectrometry (QIT-MS) to detect BCM7 at 1.4 ng/mL in commercial milk. Using a
269 similar analytical approach, Thiri et al. (2012) quantified BCM7 at 0.2 ng/mL to 0.5 ng/mL in
270 ultra-high temperature (UHT) milk. However, De Noni & Cattaneo (2010) developed a
271 method using LC-MS/MS for quantification of BCM7 in milk and dairy products and using
272 this analytical approach did not detect BCM7 in similar milk products.

273 Although quadrupole ion traps and triple quadrupole mass spectrometers are regarded
274 worldwide as highly sensitive and selective analytical techniques, they lack peak resolving
275 power as well as the capability of providing information regarding the accurate mass of
276 parent compounds and fragments.
277 The present study focused on the identification and quantification of BCM5 and BCM7 in
278 various commercial pasteurised milks manufactured in Australia (Table 1). Figure 2 and 3
279 show the LC-MS chromatographic peaks of BCM7 and its deuterated standard detected in
280 pasteurised milk extracts. The high resolution mass spectrometry data (Table 3), in particular
281 the mass accuracy values obtained on the parent ion and fragments, unequivocally confirm
282 the presence of this peptide in the milks extracts analysed (i.e. PM 3, PM 5, PM 6, PM 10,
283 PM 11 and PM 12), with a mass accuracy relative error below 5 ppm. The fragmentation
284 pattern of BCM7 is also consistent to that previously reported by De Noni (2008) using an
285 ion trap mass spectrometer. For BCM5, the LC-MS/MS as well as LC-HRMS
286 chromatograms shown no detection of this peptide in any milk products analysed (Figure not
287 shown). The absence of BCM5 in milk extracts results consistent with previous studies (De
288 Noni & Cattaneo, 2010; Juan-García et al., 2009).
289 In principle, BCM7 in pasteurised milk can be released from β-CNs at the residues 60-66 by
290 either enzymatic or acid-induced hydrolysis, or heat treatment. Conventionally,
291 pasteurisation is commonly carried out at either 72 – 75 °C for 15 s, known as high
292 temperature-short time (HTST), or 125 – 138 °C for 2 s, designated as ultra-pasteurisation
293 (Gosta, 1995). Under these pasteurisation regimes, the hydrolysis of β-CNs to BCM7 by
294 acid-induced hydrolysis can be excluded since little change in pH of the milk occurs. Several
295 studies have demonstrated that heat treatment seems very unlikely to fragment β-CNs to
296 BCM7. Using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
297 (MALDI-TOF-MS), Meltretter et al. (2008) demonstrated that five new peptides, which were

298 not present in raw milk, were identified in milk after heating at 72, 85 and 120 ºC for 10, 20
299 and 30 min respectively, but none of these peptides was identified as BCM7. Furthermore,
300 these authors did not report any *m/z* matching with that of BCM7 in commercially
301 pasteurised, ultra-pasteurised and UHT milk. Meltretter et al. (2008) also did not identified
302 BCM7 in extended shelf life (ESL) milk stored at 4 °C for 3 weeks and in UHT milk stored
303 room temperature for 3 months. Similarly, using LC-MS/MS, Gaucher et al. (2008) did not
304 identify BCM7 in UHT milk stored at 20 °C for 6 months. Recently, De Noni &
305 Cattaneo(2010) were also unable to detect BCM7 in commercially pasteurised, sterilised and
306 UHT milk. Therefore, the presence of BCM7 in some of the pasteurised milks in the present
307 study is likely due to its pre-existence in the raw milk, possibly formed by endogenous or
308 exogenous enzyme action, rather than to formation during the pasteurisation process.
309 However, to date, enzymes that contribute to the formation of BCM7 have been unknown.
310 As indicated in Table 2, the concentration of BCM7 varies in between the milk samples.
311 Since the samples were produced by different dairy companies around Australia, the raw
312 milks were highly likely to have been supplied from different dairy herds. Therefore, the
313 differences in the concentrations of BCM7 observed are possibly related to differences in the
314 genetic make-up of each herd (e.g. different breeds).
315 It has previously been demonstrated that BCM7 is not released from β-CN A2 variant by the
316 action of enzymes (De Noni, 2008; Haq et al., 2015; Jinsmaa & Yoshikawa, 1999) or if
317 released, it is only formed at a very low concentration (Korhonen & Marnila, 2013).
318 Interestingly, the present study shows that three samples, namely PM 7, PM 13 and PM 14,
319 reported by the manufacturer to contain β-CN A2 variant at 10 mg/ml (Table 1), do not
320 contain BCM7 or only contain it at a low concentration (Table 2). The data of Fox &
321 McSweeney (1998) showed that bovine milk contains approximately 9.8 mg/ml of β-CN.
322 Thus, PM 7, PM 13 and PM 14 in the present work probably contain only contain β-CN A2

323 variant. Two samples, PM 8 and PM 9, which do not contain BCM7 (Table 2), also may
324 contain only β-CN A2 variant. Therefore, these results indicate that PM 7, PM 8, PM 9, PM
325 13 and PM 14 may have been mainly collected from herds of A2/A2 cows.
326 Table 2 show that the samples, namely PM 1, PM 2, PM 3, PM 4, PM 6, PM 11 or PM 12
327 contain BCM7 at medium concentrations. These samples were not reported by manufacturers
328 to contain β-CN A2 variant and β-CN A1 variant, exception with PM 11 reported a ratio of β-
329 CN A2 variant to β-CN A1 variant of 7/4 (Table 1). Consequently, these milks are likely to
330 be from herds of A1/A2 cows. PM 5 found BCM7 at highest concentration (Table 2) may
331 have been collected from herds of more A1/A1 cows than A1/A2 or A2/A2 cows.
332 Australian dairy cow herds are mainly Friesian Holstein accounting for 65-70% of dairy
333 cows. Other breeds include the Jersey, the Holstein/Jersey cross, Brown Swiss, Ayrshire and
334 local breeds, the Australian Red and the Illawarra (Dairy Australia, 2013). Friesian Holstein
335 typically carries the A1 and A2 allele in equal amounts but individual herds may carry the
336 allele at levels that are quite different to the average for their breed (Woodford, 2007a). Milk
337 originating from European breeds such as Holstein-Friesian mainly contains β-CN A1 variant
338 (Tailford et al., 2003), while Guernsey and Jersey breeds commonly produce a high
339 concentration of β-CN A2 variant (Bell et al., 2006; Merriman, 2009). Currently, there is the
340 patented test for identification of A2/A2 cows available in Australia (Woodford, 2007b) but
341 this is not freely available for dairy farmers or industry. Some milk manufacturers in
342 Australia have applied this technique to segregate individual cow into A2/A2, A1/A2 and
343 A1/A1 cow herds. Therefore, milk collected from segregated herds can contain different ratio
344 of β-CN A2 to β-CN A1 variant, which is a factor contributing on the difference in
345 concentrations of BCM7 in different milk.
346 5. **Conclusions**

This study has demonstrated that both LC-MS/MS and LC-HRMS methods using stable isotope-labelled BCMs successfully identified and quantified BCM5 and BCM7 in pasteurised milk. The preparation of extracts using SPE before analysis by LC-MS/MS and LC-HRMS is a useful approach for improvement of detection limits. The present work is the first study to investigate BCMs in various numbers of pasteurised milk in Australia. The results have confirmed the presence of BCM7 in the 0.13 - 2.38 ng/g range in some pasteurised milk samples, while BCM5 was below limit of detection in all of the samples. The concentration of BCM7 may be affected by the ratio of β-CN A1 to β-CN A2 variant in the milks. The different concentrations of BCM7 in milk from various processing plants indicates that raw milk may be collected from herds varying in their composition of A2/A2, A1/A1 or A1/A2 cows. In order to elucidate the impact of β-CN variant on BCM7, it will be necessary to investigate the concentrations of BCM7 in fresh milk containing pure β-CN A1 or pure β-CN A2 variants. In addition, the impact of heat treatments to these milks containing pure β-CN variants on their concentrations of BCM7 should be further investigated.

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472

Table(s)1

Table 1. Milk samples used for analysis of beta-casomorphin 5 and beta-casomorphin 7

Sample Code	Processing Location	Specification	Protein content			Days to expiration[b] (days)
			Total[a] (mg/mL)	β-casein A_2 variant[a] (mg/mL)	β-casein A_1 variant[a] (mg/mL)	
PM 1	South Brisbane, QLD	Full cream milk	36	n.a	n.a	5
PM 2	South Brisbane, QLD	Full cream milk	32	n.a	n.a	5
PM 3	Wagga Wagga, NSW	Full cream milk	34	n.a	n.a	4
PM 4	Harvey, WA	Full cream milk	33	n.a	n.a	12
PM 5	Margaret River, WA	Full cream milk	33	n.a	n.a	9
PM 6	Northcliffe, WA	Full cream milk	32	n.a	n.a	8
PM 7	Perth, WA	Reduced fat milk	31	10	n.a	13
PM 8	Perth, WA	Reduced fat milk	33	n.a	n.a	2
PM 9	South Brisbane, QLD	Full cream milk	36	n.a	n.a	3
PM 10	Manning Valley, NSW	Full cream milk	31	n.a	n.a	4
PM 11	Penrith, NSW	Full cream milk	34	7	4	4
PM 12	Docklands, Vic	Full cream milk	34	n.a	n.a	4
PM 13	Riverina, NSW	Reduced fat milk	31	10	n.a	7
PM 14	Perth, WA	Reduced fat milk	31	10	n.a	12

QLD: Queensland; NSW: New South Wales; Vic: Victoria; WA: Western Australia. PM: Pasteurised milk. n.a.: not available. [a]Protein content calculated from data on the product label [b]Days to expiration on extraction

Table 2. Beta-casomorphin 5 and beta-casomorphin 7 concentrations in milk extracts as analysed by LC-MS/MS and LC-HRMS.

Sample	BCM5 [a] (ng/g)	BCM7 [a] (ng/g)
LC-MS/MS		
PM 1	<LOD	0.44
PM 2	<LOD	0.40
PM 3	<LOD	0.53
PM 4	<LOD	0.40
PM 5	<LOD	2.16
PM 6	<LOD	0.32
PM 7	<LOD	0.25
QCs	97.4±0.4[b]	108.6[b]
LC-HRMS		
PM3	<LOD	0.50
PM5	<LOD	2.38
PM6	<LOD	0.41
PM8	<LOD	<LOD
PM9	<LOD	<LOD
PM10	<LOD	0.13
PM11	<LOD	0.39
PM12	<LOD	0.38
PM13	<LOD	<LOD
PM14	<LOD	<LOD
QCs	92.9±0.4[b]	102.4[b]

[a] Mean (n=2)

[b] Mean (%) recoveries (n=2) PM: Pasteurised milk

QCs: Quality control samples

LOD: Limit of detection

Table 3. Mass to charge ratios (*m/z*), retention time and elemental formula observed from analysis in $HRMS^2$ scan mode of a standard solution of beta-casomorphin 7 (1 ng/µL) and of milk extracts.

Sample type	RT (min)	Specie	Elemental formula	Theoretical value (*m/z*)	Experimental value (*m/z*)	Relative error (ppm)
Standard solution	13.54	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4112	-2.8
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4009	-2.5
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.296	-2.5
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.2282	-1.9
PM 3	13.45	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4118	-2.0
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.3996	-4.2
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.2958	-2.9
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.228	-2.3
PM 5	13.28	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4121	-1.6
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4005	-3.5
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.296	-2.5
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.2281	-1.9
PM 6	13.47	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4119	-1.9
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4009	-2.5
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.2957	-3.0

		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	283.2279	-2.5
PM 10	13.40	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4121	-1.6
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4008	-2.6
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.2957	-3.0
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.2281	-2.0
PM 11	13.37	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4110	-3.0
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4014	-1.8
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.2958	-2.8
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.2281	-2.2
PM 12	13.45	$[M+H]^+$	$C_{41}H_{56}O_9N_7$	790.4134	790.4108	-3.3
		Frag#1	$C_{41}H_{54}O_8N_7$	772.4028	772.4015	-1.7
		Frag#2	$C_{27}H_{40}O_6N_5$	530.2973	530.2958	-2.9
		Frag#3	$C_{18}H_{31}O_5N_4$	383.2289	383.228	-2.3

Frag#1, 2, 3: Fragment 1, 2, 3; RT: chromatographic retention time; *m/z*: mass-to-charge ratios; ppm: parts per million (relative error).

Table(s)2

Table S1. Summary of ESI and HRMS^n parameters used for target analysis of beta-casomorphin 5 and beta-casomorphin 7 in milk extracts.

Parameter	Setting
Spray voltage (kV)	4.0
Capillary voltage (V)	-35
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	-110
Scan range (*m/z*)	70-1000
IT full MS AGC target	3E4
IT MS^n AGC target	1E4
FT full MS AGC target	2E5
FT MS^n AGC target	1E5
Ion trap and FT micro scans	3
IT full MS Max ion time (ms)	10
FT MS^n Max ion time (ms)	200
MS^2 isolation window (*m/z*)	1

Arb: arbitrary units; ms: milliseconds; IT: Ion trap; FT Fourier Transform (Orbitrap mass spectrometer); AGC: Automatic Gain Control.

Table S2: Identification points earned by the mass spectrometry techniques adopted

MS technique	Identification points earned per ion	Triple quadrupole MS	Ion Trap MS	Orbitrap MS
LR-MS^n parent ion	1.0	1.0	1.0	-
LR-MS^n daughter ion	1.5	1.5 × 2	1.5 × 1	-
HR-MS^n parent ion	2.0	-	-	2.0 × 1
HR-MS^n daughter ion	2.0	-	-	2.0 × 2
Total	-	4	2.5	6

LR: low resolution; HR: high resolution; MS^n: multiple stage fragmentation

Figure(s)

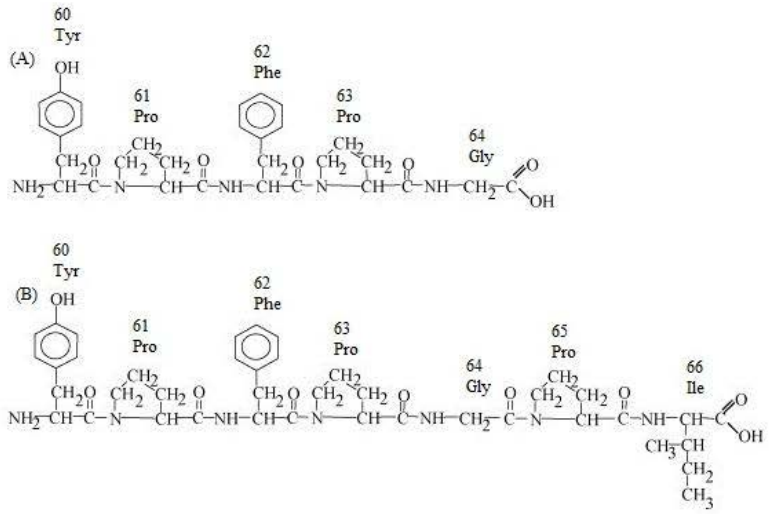

Figure 1. Structure of beta-casomorphin 5 and beta-casomorphin 7 (Nguyen et al., 2014)

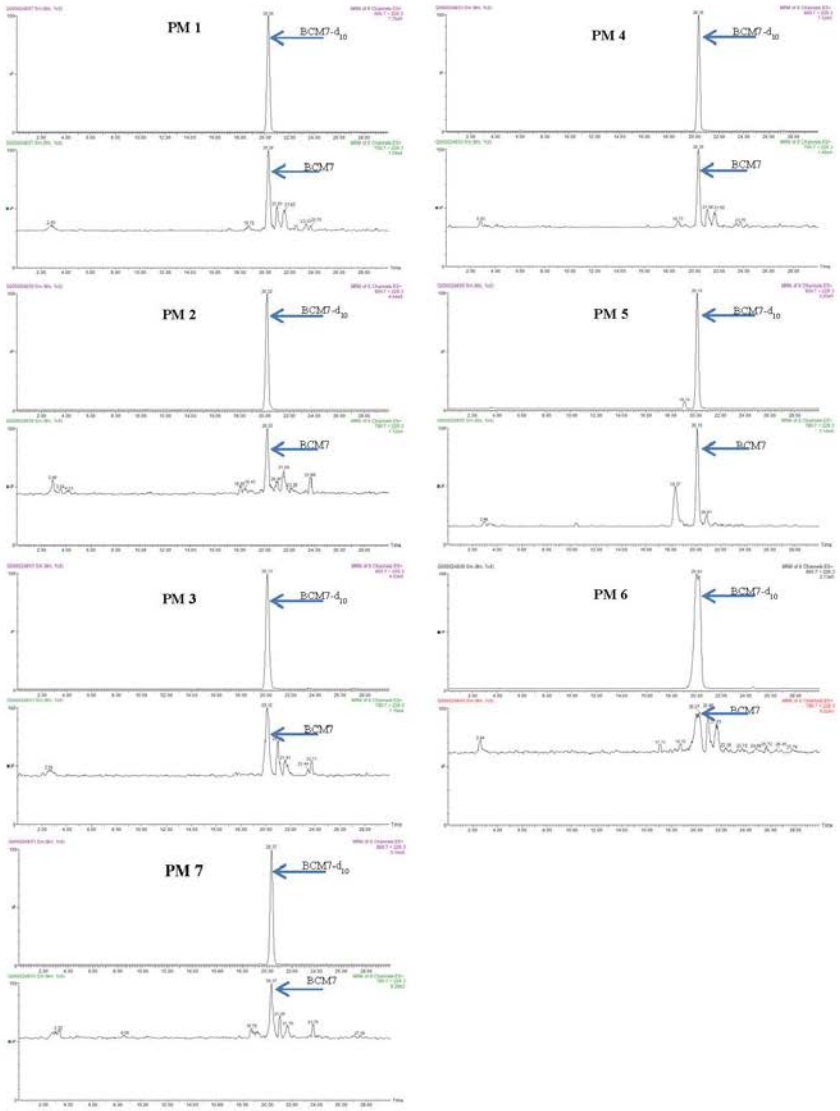

Figure 2. Liquid chromatography-tandem mass spectrometry chromatograms showing the detection of beta-casomorphin 7 and its deuterated standard in the milk extracts

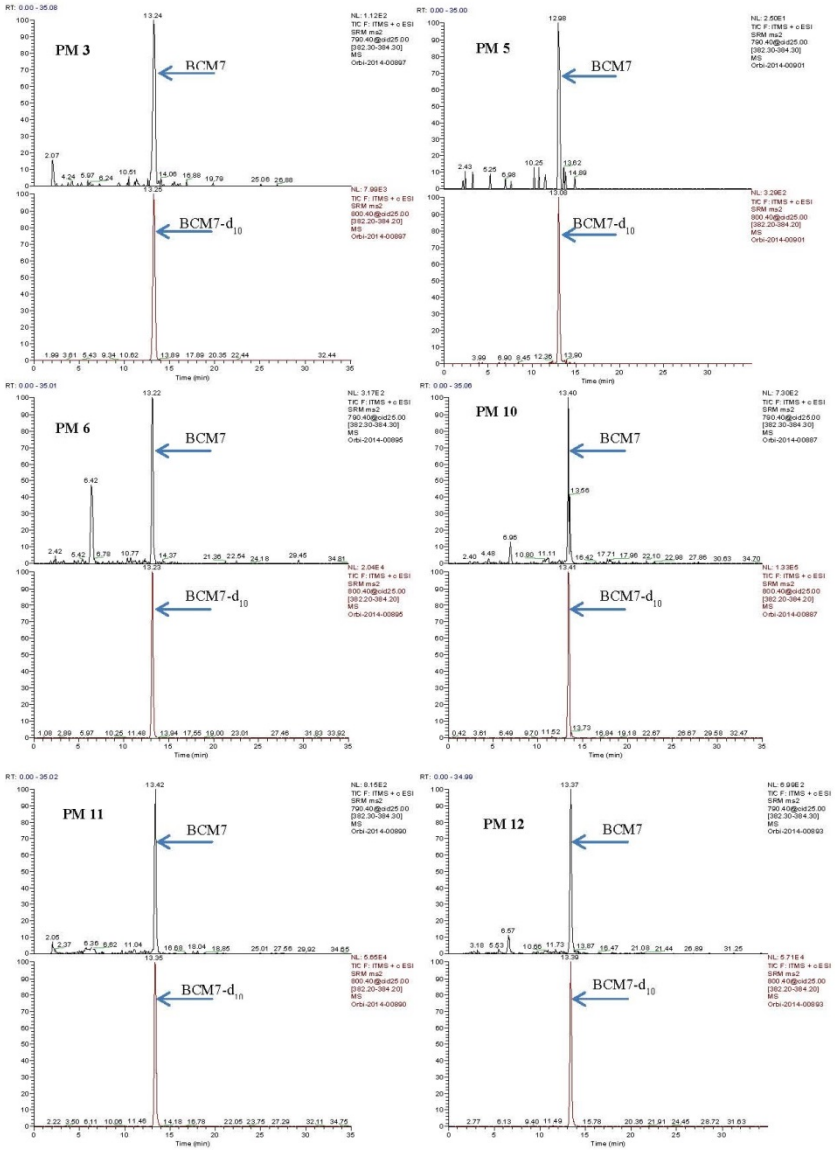

Figure 3. Liquid chromatography-high resolution mass spectrometry chromatograms showing the detection of beta-casomorphin 7 and its deuterated standard in milk extracts

CHAPTER 5 EFFECT OF FERMENTATION AND STORAGE DURING YOGHURT PROCESSING ON DEGRADATION OF BETA-CASOMORPHINS

5.1 Introduction

In recent years, fermented milk and yoghurt products containing peptides with biological activity such as ACE-I and antioxidant action have been widely studied (Donkor et al., 2007; Otte et al., 2011; Sabeena Farvin et al., 2010). These peptides are mainly released from milk proteins by bacteria during fermentation and storage processes (Nielsen et al., 2009).

Yoghurt is commonly fermented with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* at 42-45 °C until the pH drops to 4.3-4.5 (Paul & Somkuti, 2009). During fermentation of milk, caseins are hydrolysed into large peptides by the cell wall-associated proteinases of bacteria. Subsequently, these large peptides are transported into the bacterial cells and hydrolysed into a large number of shorter peptides, including peptides with opioid activity (Sabeena Farvin et al., 2010), by intracellular peptidases (Nielsen et al., 2009). During cold storage, peptides with bioactive property are also produced (Donkor et al., 2007; Papadimitriou et al., 2007), but some other peptides are hydrolysed (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000). BCMs are proline-rich peptides (Table 1). Proline-containing peptide bonds are known to be resistant to hydrolysis by digestive enzymes, but are unstable with PepX activity produced from most dairy cultures (Donkor et al., 2007). Interesting data is becoming available on transport of peptide(s) across gut wall. Osborne et al. (2014) showed that BCM-7 was hydrolyzed by cell surface peptidases of caco-2 cells. However, the degradation of BCM5 and BCM7 during fermentation of milk and subsequent storage of yoghurt has received little attention in the scientific literature.

In the present project, the measurement of BCM5 and BCM7 in commercial yoghurts fermented a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, and other LAB were carried out as detailed in Chapter 4 and none of the investigated yoghurts contained BCM5 and BCM7. Similarly, De Noni and Cattaneo (2010) did not detect BCM5 and BCM7 in commercial yoghurts that had been stored for 30 days before analysis using LC-MS/MS. In contrast, BCM7 has been reported to present in commercial yoghurts and its level was observed to decrease during storage using

ELISA (Jarmolowska, 2012). However, from these studies, it remains unclear if BCM7 is released during fermentation, and if, in turn, it is degraded during storage.

The aim of study described in this chapter was to (a) investigate the degradation of BCM5 and BCM7 analysed by LC-MS/MS in yoghurt during fermentation of reconstituted milk with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and (b) determine the effect of storage of the yoghurt fermented to different pH values on the level of BCM5 and BCM7.

5.2 Materials and methods

5.2.1 Materials and chemicals

Skim milk powder, produced in Rakaia, New Zealand, was supplied by A2 Dairy Products Australia Pty Ltd (Botany, NSW, Australia). Other materials and chemicals used for the determination of BCM5 and BCM7 in this study have been previously described in Section 3.2.1. Gly-pro-pNA triflouroacetate salt was purchased from Auspep Pty. Ltd. (Tullamarine, VIC, Australia). 4-nitroaniline (pNA) was a product of Sigma-Aldrich (China).

5.2.2 Methods

5.2.2.1 Production of yoghurt

Yoghurt made from the skim milk powder was prepared by the processes illustrated in Fig. 10. Reconstituted milk was firstly prepared by dissolving the skim milk powder in deionised water (1.3/10; w/v) and thoroughly mixed by a stirrer. The water had been boiled for 10 min and then cooled to 55 °C before use. The reconstituted milk was inoculated with 0.003% (w/v) of YO-MIX^{TM} and thoroughly mixed by stirring with a clean spoon. The inoculated milk was divided into 13 tubes, sealed and incubated at 43 °C until the pH dropped to the required values of pH 4.8 and 4.5. One tube was used for measurement of pH during fermentation. When the pH reached each of the two required values, six tubes were removed from the incubator. Two tubes were then extracted immediately (or stored at -20 °C prior to extraction if immediate extraction was not possible) and the four others were stored for one day (2 tubes) and seven days (2 tubes) at 4 °C and then stored at -20 °C until extraction.

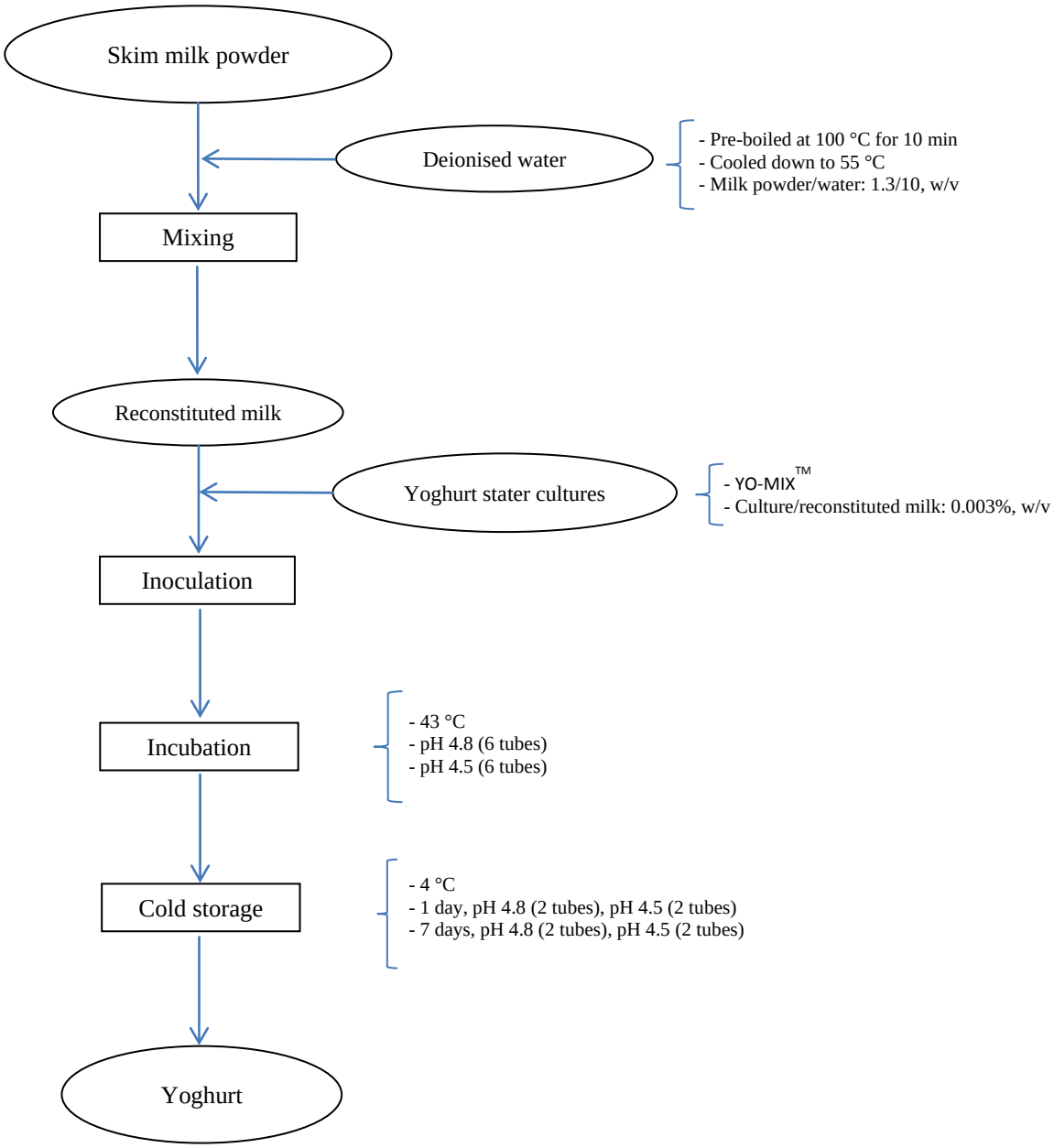

Figure 10. Production of yoghurt from skim milk powder

5.2.2.2 Extraction of water soluble peptides

Extraction of water soluble peptides from the yoghurt was carried out as follows**:** the yoghurt previously stored at -20 °C was thawed at room temperature for approximately 30 min and thoroughly mixed by inversion of tubes. Twenty five grams (±0.0001 g) of yoghurt was spiked with 500 ng of BCM5-d_{10} and 500 ng of BCM7-d_{10}. In addition, a separate 25.0000 g (±0.0001 g) sample of the yoghurt was spiked with 500 ng of mixture of deuterated standards and 100 ng of mixture of BCM5 and BCM7 standard and used for QCs. The extraction was performed as described in Section 3.2.4. The supernatants were then filtered through Whatman

filter paper n° 541 instead of 0.45µm membrane filters. The filtrates were then purified using SPE cartridges (Strata-X, 500 mg/6 mL, Phenomenex, NSW, Australia). The SPE clean-up was carried out as described in Section 4.2.2.2. All samples and QCs were extracted in duplicate.

5.2.2.3 Identification and quantification of beta-casomorphin 5 and beta-casomorphin 7 by liquid chromatography-tandem mass spectrometry

a. Calibration standards

Preparation of standard solutions was carried out as described in 4.2.2.3a

b. Liquid chromatography-tandem mass spectrometry conditions

BCM5 and BCM7 in the yoghurt extracts were determined using the LC-MS/MS method described in Chapter 3 with some minor modifications of LC conditions as follows: Chromatographic separation ofBCM5 and BCM7 was achieved using a Waters XBridge C18 column (50 mm x 2.1 mm i.d., 3.5 µm particle size, 135A pore diameter) (Waters Tokyo, Japan) at a flow rate of 250 µL/min. An XBridge BEH Sentry guard cartridge (Waters) was also used to protect the analytical column. The mobile phase was MeOH (A) and ultrapure water (B) both containing 0.1% formic acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 10 min linear gradient to 95% (A). The mobile phase remained at 95% (A) for 10 min to elute analytes from the column. The initial conditions were re-established within 1 min and the column re-equilibrated for 9 min before injecting the next sample. The injection volume was 25 µL. The same mass spectrometry conditions were used as described in Chapter 3.

5.2.2.4 Measurement of PepX activity

a. Preparation of extract

The extraction was performed as described by Otte et al. (2011) with some minor modifications. Twenty grams (±0.0001 g) of the yoghurt samples were placed in a centrifuge tube and then thoroughly mixed by inversion with 1 mL of 1 M sodium citrate. The tube was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was decanted into another centrifuge tube and stored at 20 °C until analysis. Before

measuring PepX activity, the frozen supernatant was thawed at room temperature and centrifuged at 15,000 × g for 10 min at 4 °C.

b. Measurement of PepX activity

The PepX activity was carried out by a method adapted from Miyakawa et al. (1991). Gly-Pro-pNA was used as a substrate and dissolved in 0.1M NaH_2PO_4-NaOH pH 6.5 at 1 mM. The substrate solution and the supernatant were separately pre-incubated at 37 °C for 5 min. The reaction mixture consisting of 0.5 mL of Gly-Pro-pNA and 0.5 mL of the supernatant was mixed and then incubated at 37 °C for 5 min. The reaction was stopped by addition of 1 mL of 30% acetic acid. The absorbance was measured at 410 nm using UV-1800 spectrophotometer (Shimadzu, Canby, OR, USA). Serial standard solutions of pNA in the range 2-200 µM was used for the calibration curve. The PepX activity was defined as nmole of pNA released per min per mL of yoghurt.

5.3 Results

5.3.1 Degradation of beta-casomorphin 5 and beta-casomorphin 7 during fermentation

In the present study, the identification and quantification of BCM5 and BCM7 in the unfermented reconstituted milk used for manufacture of the yoghurt was performed in order to assist in the understanding of the release/degradation of these peptides during yoghurt fermentation and storage. The extraction of water soluble peptides was carried out as previously described in Section 4.2.2.2 and analysed by LC-MS/MS as described in Section 5.2.2.3. Figure 11 shows chromatograms of BCM7 and its deuterated standard BCM7-d_{10} in the unfermented reconstituted milk extract, suggesting that BCM7 presents itself in the reconstituted milk made from the skim milk powder. The content of BCM7 is shown in Table 13. In contrast, BCM5 was undetectable in the milk extract (Figure not shown).

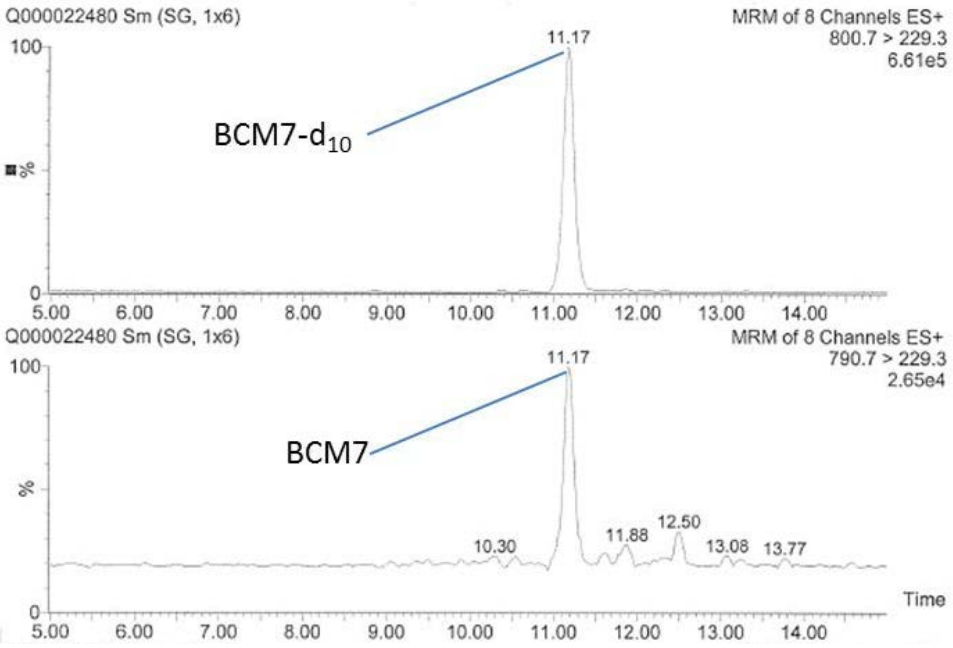

Figure 11. LC-MS/MS chromatograms of BCM7 and its deuterated standard (BCM7-d_{10}) in the reconstituted milk

Figure 12 shows the LC-MS/MS chromatogram of BCM7 in the yoghurt fermented to pH 4.8. BCM7 content was found to decrease during fermentation from pH 6.5 to 4.8 (Table 13). After a more prolonged fermentation for about 1 hour to pH 4.5, BCM7 was below LOD (Figure not shown). BCM5 was also below LOD in yoghurt fermented to any pH values (4.5 and 4.8) (Figure not shown).

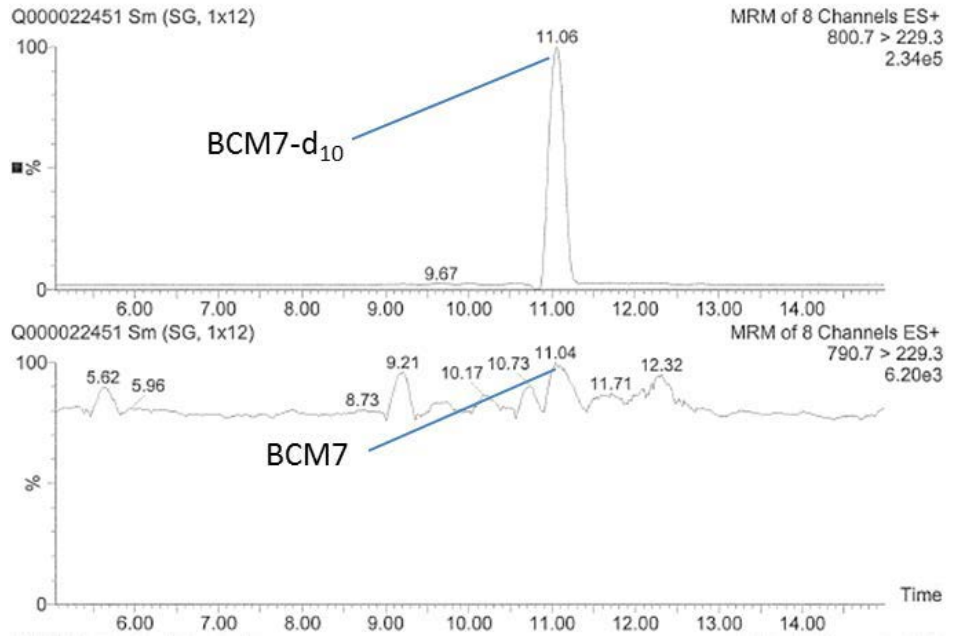

Figure 12. LC-MS/MS chromatograms of BCM7 and its deuterated standard (BCM7-d_{10}) in yogurt made from the reconstituted milk (pH=4.8, 0 day of storage)

5.3.2 Degradation of beta-casomorphin 5 and beta-casomorphin 7 during storage

Variation in the level of BCM5 and BCM7 during storage was carried out by determining their level in the yoghurt at pH 4.8 and 4.5 after one and seven days of storage. No detectable level of BCM5 was found in the yoghurt at any day of storage (Figure not shown). At end fermentation pH 4.8, BCM7 presented in yoghurt stored for one day (Fig. 13), but was not detected when yoghurt was stored for seven days. Table 13 shows that BCM7 content decreased after one day of cold storage. At the end of fermentation at pH 4.5, BCM7 was below LOD in yoghurt after one and seven days of storage (Figure not shown).

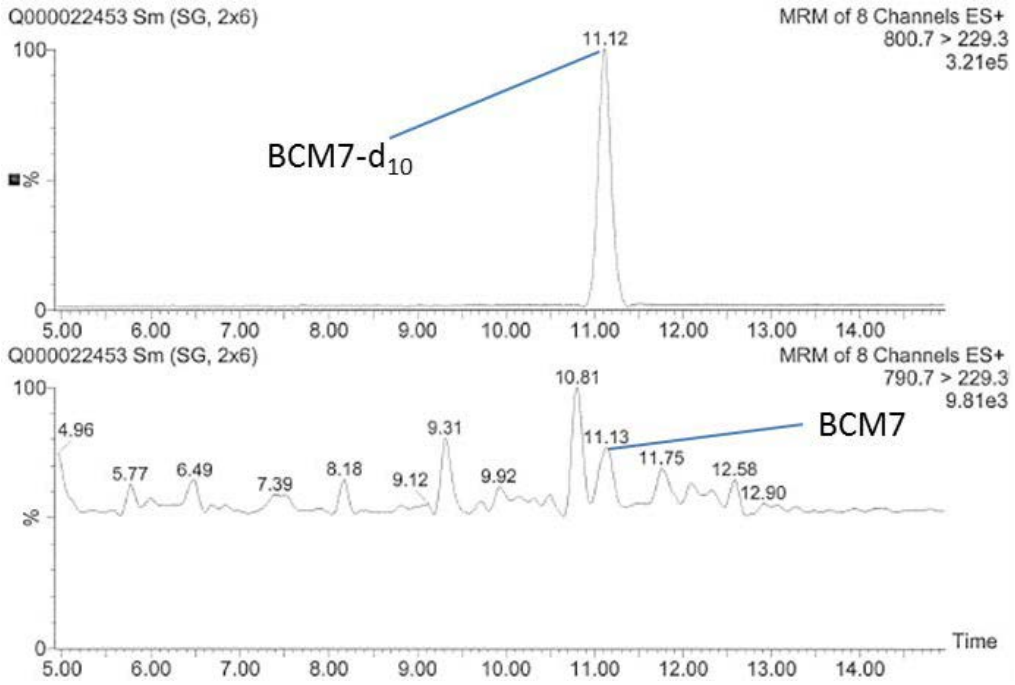

Figure 13. LC-MS/MS chromatograms of BCM7 and its deuterated standard (BCM7-d_{10}) in yogurt made from the reconstituted milk (pH=4.8, one day of storage)

5.3.3 PepX activity

The PepX activity of the milk fermented to pH 4.8 and 4.5 and in yoghurt stored for various periods is showed in Figure 14. There was an increase in the PepX activity during fermentation, from 6.14 to 8.28 nmole pNA.min^{-1}.mL^{-1}as fermentation pH dropped from 4.8 to 4.5. For the yoghurt samples at pH 4.8, the PepX activity increased to 7.54 nmole pNA.min^{-1}.mL^{-1} in the sample stored for one day. However,

after seven days of storage the PepX activity increased very slightly to 7.62 nmole pNA.min^{-1}.mL^{-1}.

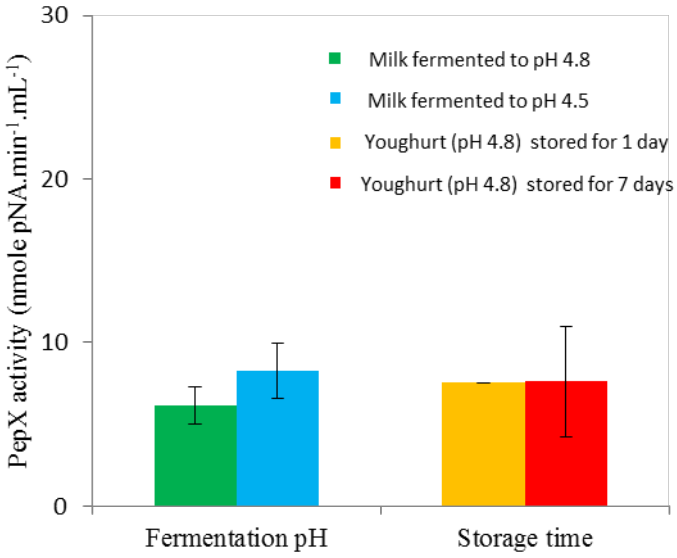

Figure 14. PepX activity (nmole pNA.min-1.mL-1) in milk fermented to pH 4.8 and 4.5 and in yoghurt stored for one and seven days

5.4 Discussion

Fermentation of milk in yoghurt processing causes the drop in pH to target values, normally 4.3-4.5, leading to changes in viscosity and texture of yoghurt, resulting in the desired final product characteristics (Beal, 1999). During fermentation, bacteria derived proteases hydrolyse caseins into large peptide fragments, which in turn are degraded by peptidases into shorter peptides (Nielsen et al., 2009). Most peptides that contain proline residue are hydrolysed by bacteria derived PepX activity (Donkor, Henriksson, Vasiljevic, & Shah, 2007). BCM7 is a 3 proline-containing peptide (Fig. 1). The proline in second position helps BCM7 stablise with aminopeptidase activity produced from bacteria, however it is known to be hydrolysed by PepX (Hafeez et al., 2013). The present results show that BCM7 content decreases during fermentation and completely hydrolysed as it fermented to pH 4.5 (Table 13). The decrease in BCM7 content correlates to the increase in the PepX activity during fermentation (Fig. 14), suggesting that the degradation of BCM7 is likely to be a result of PepX activity produced from yoghurt cultures.

Most dairy cultures used for fermentation of milk are LAB possessing PepX activity (Donkor et al., 2007). *Lactococcus lactis* spp. *cremoris* produces PepX activity associating to cell wall of bacterium and this enzyme has been reported to efficiently degrade BCM7 (Kiefer-Partsch, Bockelmann, Geis, & Teuber, 1989). Similarly, Matar and Goulet (1996) have demonstrated that a strain of *L. helveticus* completely hydrolysed BCM7, whereas a mutant strain of *L. helveticus* that does not possess PepX only partly hydrolysed BCM7 to BCM4. Interestingly, *L. delbrueckii* ssp.*bulgaricus* and *S. thermophilus* has also been reported to be capable of producing extracellular PepX (Hafeez et al., 2013; Meyer & Jordi, 1987).

In yoghurt processing, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* rapidly symbiotically grew during fermentation. It has been documented that lysis is minimal and cell numbers increase during fermentation (Beal, 1999; Donkor et al., 2007). In the present work, the fermented milk samples were centrifuged at 15,000 × *g* for 20 min, leading to the absence of bacteria cells in the extracts. Therefore, PepX activity measured in the extract at fermentation pH 4.8 and 4.5 is extracellular enzyme. The increase in PepX activity during fermentation could be due to more bacteria cells that could release more extracellular PepX.

S. thermophilus is an important bacterium in first stage of fermentation of milk into yoghurt. In addition to the production of pyruvic acid, formic acid, folic acid, fatty acids and CO_2 for the growth of *L. delbrueckii* ssp. *bulgaricus* (Settachaimongkon et al., 2014), *S. thermophilus* strains also produce peptides with ACE-I property during fermentation (Miclo et al., 2011). However, Hafeez et al. (2013) have reported that strains of *S. thermophilus* completely degraded BCM7 after a 4-6 hour incubation. These periods of incubation are similar to those in the present study; 4.5 and 5.5 hours for fermentation to pH 4.8 and 4.5, respectively (Table 13). However, the *in vitro* medium in the study of Hafeez et al. (2013) is different from that in yoghurt. In contrast to *S. thermophilus*, the degradation of BCM7 by *L. delbrueckii* ssp. *bulgaricus* in an *in vitro* study has not been reported. In yoghurt processing, the role of each bacterium in degradation of BCM7 has received very little attention in scientific literature. Therefore, studies on the capacity of each bacterium for hydrolysis of BCM5 and BCM7 during yoghurt processing will be described in Chapter 6.

Table 13. BCM5 and BCM7 content of reconstitute milk and yoghurt fermented to different pHs

Samples	Fermentation time (h)	BCM7 ng/g	BCM5 ng/g
Reconstituted milk pH 6.5	0	1.4[a] ± 0.00	<LOD
Reconstituted milk fermented to pH 4.8	4.5	0.29[a] ± 0.01	<LOD
Reconstituted milk fermented to pH 4.5	5.5	<LOD	<LOD
QCs (reconstituted milk samples)		102[b] ± 1	103[b] ± 1

a Mean values, ng/g (n = 2)

[b] Recovery (%) is the average ±%RSD of reconstituted milk samples (n=2) spiked with 100 ng/g of BCM5 or BCM7 .

QCs: Quality control sample

LOD: Limit of detection

The formation/degradation of peptides in yoghurt depends on the period of storage. Some peptides may be degraded and others are formed during prolonged storage (Donkor et al., 2007). The present study shows that cold storage has influence on the stability of BCM7. This peptide was degraded in yoghurt samples at pH 4.8 stored for one day and completely hydrolysed after seven days of storage (Table 14). The results also show that PepX activity increases after one day of storage (Fig. 14), suggesting that PepX activity is likely a factor causing decrease in BCM7 content during storage. The increase in PepX activity in yoghurt samples at pH 4.8 may be due to growth of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* during cold storage (Donkor et al., 2007).

The present work also shows that neither BCM5 nor BCM7 was found in yoghurt at pH 4.5 stored for any storage period. This finding agrees with De Noni and Cattaneo (2010) who measured BCM5 and BCM7 in commercial yoghurts. Similarly, Schieber and Brückner (2000) have identified some pro-BCMs in yoghurt fermented with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and stored for 21 days, but neither BCM5 nor BCM7 was identified. However, Jarmolowska (2012) found BCM7 in commercial yoghurt and observed that the content of this peptide decreased during storage.

As can be seen in Fig. 1, BCM7 is a parent peptide of BCM5, so it can be a hypothesised that BCM7 is hydrolysed into BCM5 by peptidases during fermentation and storage of yoghurt. However, Hafeez et al. (2013) clearly show the release of X-Pro dipeptides from N-terminal side to BCM7 and not the C-terminal side. Moreover, chromatograms from the present study do not show any peak of BCM5 (Figure not shown), indicating that this peptide was not released from hydrolysis of BCM7. Two di-peptides described as β-CNf60-61 and β-CNf62-63 have previously been identified in commercial yoghurts (Kunda et al., 2012). Furthermore, Hafeez et al. (2013) have reported that BCM7 is cleaved at peptide bonds Pro^{61}-Phe^{62} and Pro^{63}-Gly^{64} (see Fig. 1) to form β-CNf60-61, β-CNf62-63, β-CNf64-66 and β-CNf62-66 when BCM7 was incubated with strains of *S. thermophilus* for 22 hours at 42 °C. However, BCM5 is found in some cheese produced by fermentation of milk with other LAB (Sienkiewicz-Szłapka et al., 2009). This indicates that the formation of BCM5 may depend on strain of bacteria used in dairy processing (Jarmolowska, 2012).

Table 14. BCM5 and BCM7 content in yoghurt made from reconstituted milk after different storage days

Cold storage (day)	BCM7 (ng/g)		BCM5 (ng/g)	
	pH 4.8	pH 4.5	pH 4.8	pH 4.5
1	0.17[a] ± 0.01	<LOD	<LOD	<LOD
7	<LOD	<LOD	<LOD	<LOD
QCs (yoghurt samples)	104[b] ± 1.0		104[b] ± 2.0	

[a] Mean values, ng/g (n = 2)

[b] Recovery (%) is the average ±%RSD of yoghurt samples (n=2) spiked with 100 ng/g of BCM5 or BCM7 .

QCs: Quality control sample

LOD: Limit of detection

5.5 Conclusions

This study demonstrated that fermentation and storage in yoghurt processing have a strong influence on the degradation of BCM7. The content of BCM7 decreases during fermentation and completely hydrolysed as fermentation of milk to pH 4.5.

BCM7 in yoghurt produced by fermentation of milk to pH 4.8 decreased during cold storage and completely degraded after seven days of storage. The results also show that yoghurt produced by fermentation of milk to pH 4.5, which is a characteristic of final yoghurt, does not contain BCM7, with regard to various periods of storage. The degradation of BCM7 is likely a result of bacteria derived PepX. The content of BCM5 and BCM7 found in milk were zero and very low, respectively, leading to a hard determination of the remaining of their content as fermentation of milk to pH 4.5. Addition of standard BCM5 and BCM7 to milk for investigation of their remaining as milk fermented to pH 4.5 and during cold storage will be reported in Chapter 6.

5.6 Papers

The results of this study were presented at the 2nd International Dairy Federal Symposium on Microstructure of Dairy Products and 5th International Dairy Federal Symposium on Science and Technology of Fermented Milk on 3-7 March 2014 in Melbourne, Australia (Appendix 1).

CHAPTER 6 DEGRADATION OF BETA CASOMORPHINS BY *L. delbruekii* spp. *bulgaricus* and *S. thermophilus* DURING YOGHURT MANUFACTURE

6.1 Introduction

L. delbruekii spp. *bulgaricus* and *S. thermophilus* are two LAB widely used as a starter culture for co-fermentation of milk in yoghurt manufacture (Tamime & Robinson, 1999). *L. delbruekii* spp. *bulgaricus* produces peptides and essential amino acids that stimulate the growth of *S. thermophilus* (Courtin et al., 2002; Shihata & Shah, 2000). In turn, *S. thermophilus* produces pyruvic acid, formic acid, folic acid, fatty acids and CO_2 for *L. delbruekii* spp. *bulgaricus* (Settachaimongkon et al., 2014). Therefore, co-cultivation results in the rapid growth of these bacteria in the milk and hence, rapid acidification (Courtin et al., 2002). Co-fermentation of milk by these bacteria during manufacture of yoghurt also produces various bioactive peptides such as ACE-I peptides (Donkor et al., 2007; Kunda et al., 2012; Papadimitriou et al., 2007), antioxidant peptides (Sabeena Farvin et al., 2010), antimicrobial peptides (Poyrazoglu Coban et al., 2012) and opioid peptides (Jarmolowska, 2012).

In addition to *L. delbruekii* spp. *bulgaricus* and *S. thermophilus*, yoghurt can also be fermented with other LAB such as *L. acidophilus, L. casei, Bifidobacterium* or *L. paracasei* spp. *paracasei* (Donkor et al., 2007; Papadimitriou et al., 2007; Shihata & Shah, 2002). The previous study in this research project (Chapter 4) showed that none of the commercial yoghurts fermented with various cultures contained BCM5 or BCM7. In chapter 5 of this thesis it was reported that BCM7 was degraded during fermentation of milk from pH 6.5 to pH 4.8 and then to 4.5 using a mixture of *L. delbruekii* spp. *bulgaricus* and *S. thermophilus.* In addition, this peptide is also completely degraded when the pH 4.8 yoghurt was stored for seven days (Chapter 5). This degradation may be the reason why BCM5 and BCM7 was not present in the commercial yoghurts analysed in chapter 4. Similarly, Jarmolowska (2012) observed that there is a decrease in content of BCM7 in commercial yoghurt containing *L. delbruekii* spp. *bulgaricus* and *S. thermophilus* during storage, but BCM7 was still found in yoghurt stored for 18 days. However, the role of individual bacterial strain in the degradation of BCM7 in yoghurt has received little attention in the scientific literature.

L. delbruekii spp. *bulgaricus* and *S. thermophilus* can be used individually for fermentation of milk. Both bacteria have been demonstrated to be capable of hydrolysing antimicrobial and antihypertensive peptides to varying extents (Paul & Somkuti, 2009; Somkuti & Paul, 2010). Interestingly, *S. thermophilus* possess PepX that hydrolyses BCM7 to smaller peptides in an *in vitro* study (Hafeez et al., 2013). There is little research reporting on the degradation of BCM7 by *S. thermophilus* or *L. delbruekii* spp. *bulgaricus* during yoghurt manufacture at pH 4.5. Also, research on the degradation of BCM5 by these bacteria has not been reported.

The aim of this study was to investigate the influence of *S. thermophilus* and *L. delbruekii* spp. *bulgarius* individually on the degradation of BCM5 and BCM7 when milk fermentation to pH 4.5, which is characteristic of final yoghurt, and during its cold storage. To conduct this study, standards of BCM5 and BCM7 were spiked into milk and their concentration measured during yoghurt production.

6.2 Materials and Methods

6.2.1 Materials and chemicals

UHT milk was purchased in November 2014 from a supermarket in Perth, Western Australia. Yoghurt cultures (LB 340 LYO and CHOOZIT^{TM} TA 52 LYO, Danisco, France) were obtained as freeze-dried powders. LB 340 LYO contains *L. delbrueckii* spp. *bulgraricus* and CHOOZIT^{TM} TA 52 LYO contains *S. thermophilus*. Other materials and chemicals used in this study have been previously described in Section 3.2.1.

6.2.2 Methods

6.2.2.1 Production of yoghurt

The yoghurt manufacturing process is illustrated in Fig. 15. UHT milk was warmed in an incubator until it reached 43 °C. The warmed milk was added the mixture of standard BCM5 and BCM7 at 40 ng/g (1,000 ng/25 g of yoghurt) and separate batches of milk were inoculated with either 0.06% (w/w) of *S. thermophilus*, 0.06% (w/w) of *L. delbrueckii* spp. *bulgraricus* or 0.06% (w/w) of mixture of *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* (50/50, w/w). The inoculated milk was divided into seven sterilised tubes, sealed and fermented at 43 °C until the pH dropped to 4.5. One tube was used for measurement of pH during fermentation. When the pH reached the required value, two tubes were extracted immediately (or stored at -20 °C

to preserve the sample until extraction) and two tubes each were stored for one and seven days at 4 °C and then preserved at -20 °C until extraction. In addition, six tubes of UHT milk spiked the mixture of standard BCM5 and BCM7 at 40 ng/g were acidified with 1 M HCl until pH 4.5 and stored for 0, 1 and 7 days as described for the fermented samples. The acidified milk was used for investigation of effect of pH on the stability of BCMs independent of any effect due to the bacterial fermentation.

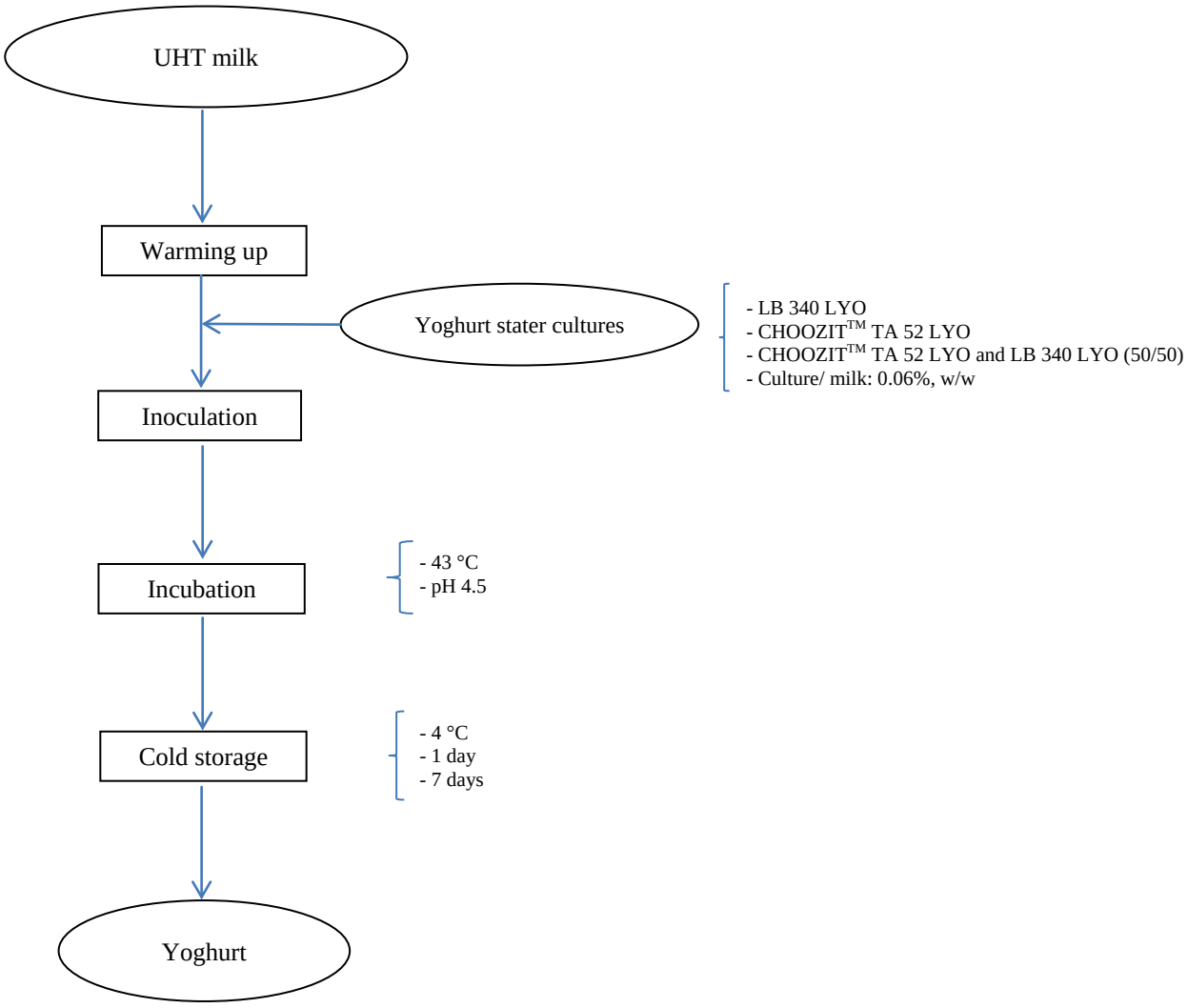

Figure 15. Production of yoghurt from the UHT milk spiked with standard BCM5 and BCM7

6.2.2.2 Extract of water soluble peptides

The extraction was performed in duplicate (n=2) as described in Section 5.2.2.2.

6.2.2.3 Determination of beta-casomorphin 5 and beta-casomorphin 7 by liquid chromatography-high resolution mass spectrometry

Identification and quantification of BCM5 and BCM7 by LC-HRMS were conducted as described in Section 4.2.2.4.

6.3 Results

6.3.1 Degradation of beta-casomorphin 5 by L. delbrueckii spp. bulgraricus and S. thermophilus

In order to investigate the stability of BCM5 in the presence of individual bacterial strains used in yoghurt processing, BCM5 was spiked into the milk and then the milk fermented to pH 4.5 with either *S. thermophilus* or *L. delbrueckii* spp. *bulgraricus or a* mixture of the two and subsequently stored for 0, 1 and 7 days at 4 °C. In comparison, the integrity of BCM5 due to pH alone was investigated by acidification of the BCM5 spiked milk with 1M HCl to pH 4.5 prior to the storage protocol.

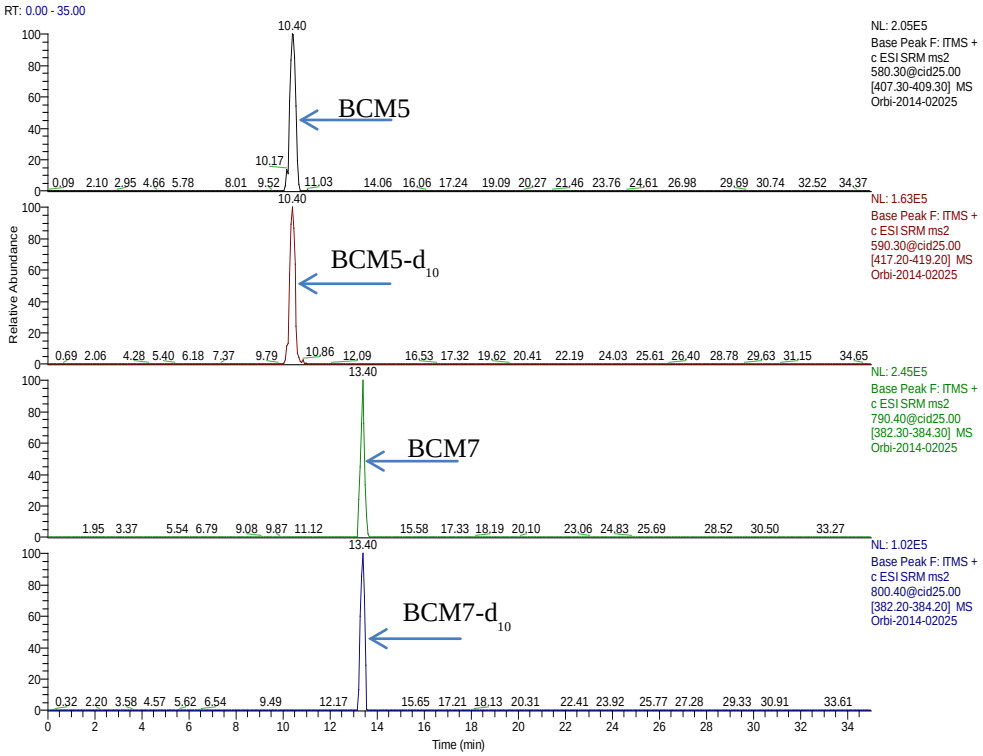

Figure 16. LC-HRMS chromatogram of BCM5 and BCM7 in UHT milk spiked with BCM5 and BCM7 standard and their deuterated standards (BCM5-d_{10} and BCM7-d_{10}).

LC-HRMS chromatogram shows that BCM5 was below LOD in UHT milk (Figure not shown). However, BCM5 was clearly identified in the milk to which had been spiked with a standard of BCM5 (Fig. 16). Immediately after fermentation to pH 4.5, BCM5 was below LOD in spiked milk samples incubated with only *S. thermophilus*, only *L. delbrueckii* spp. *bulgraricus* or the mixture of two these bacteria (Table 15). Similarly, BCM5 was below LOD in yoghurt stored for one and seven days (Table 15). However, LC-HRMS chromatogram shows that BCM5 was detected in milk spiked with a standard of BCM5 when milk was acidified with 1M HCl (Figs. 17, 18 and 19). Calculation of BCM5 concentration indicated that the portion of BCM5 was lost upon acidification of milk to pH to 4.5 and in stored acidified milk (Table 15).

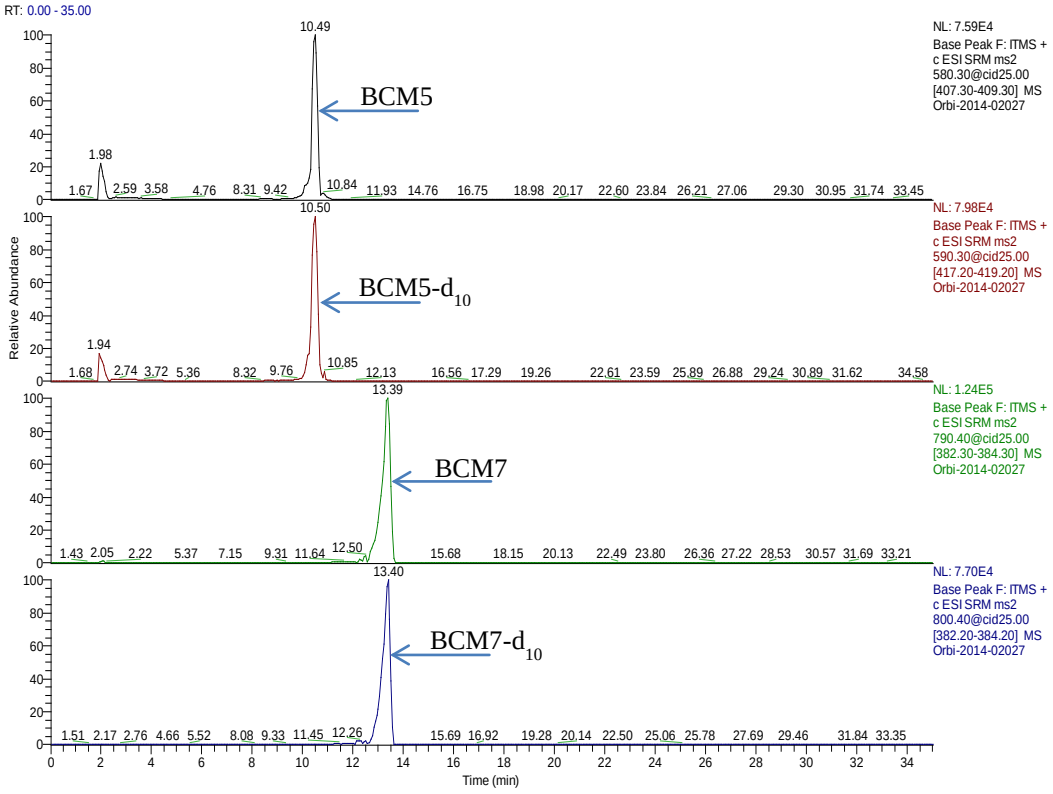

Figure 17. LC-HRMS chromatogram of BCM5 and BCM7 in UHT milk spiked BCM5 and BCM7 standard and their deuterated standards (BCM5-d10 and BCM7-d10) acidified to pH 4.5, zero days of storage

6.3.2 *Degradation of beta-casomorphin 7 by L. delbrueckii* spp. *bulgraricus and S. thermophilus*

It has been previously reported in this thesis (Chapter 5) that BCM7 was found in reconstituted milk at a low level (1.4 ng/g) and its level was partially reduced when milk was fermented to pH 4.8 and completely disappeared during fermentation to pH

4.5. Because the concentration of BCM7 in the milk was low (see Chapter 4 & 5), in the present study, BCM7 was added to UHT milk to study the ability of various bacteria to reduce the levels of this peptide during yoghurt processing.

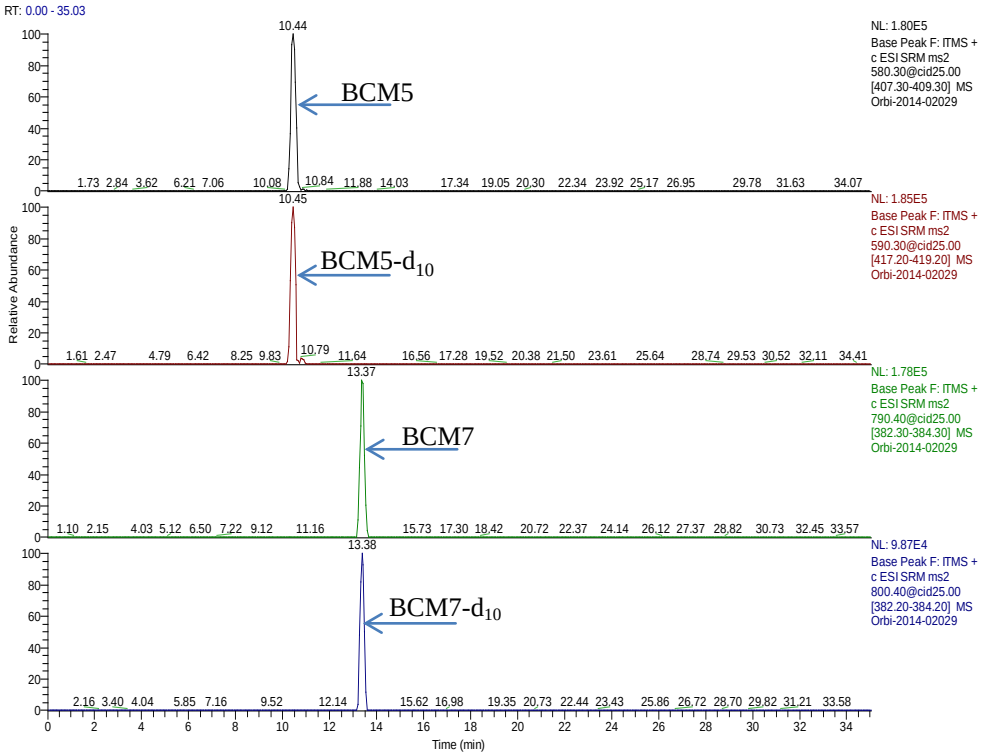

Figure 18. LC-HRMS chromatogram of BCM5 and BCM7 in UHT milk spiked BCM5 and BCM7 standard and their deuterated standards (BCM5-d10 and BCM7-d10) acidified to pH 4.5, 1 day of storage

LC-HRMS analysis shows that BCM7 was below LOD in the UHT milk (Figure not shown). BCM7 found in UHT milk spiked with standard BCM7 (Fig. 16) before yoghurt manufacture was considered as remaining 100 percent. UHT milk spiked with BCM7 fermented with either *S. thermophilus* or *L. delbrueckii* spp. *bulgraricus* or the mixture of these bacteria indicated that BCM7 was completely degraded when fermentation pH reached to 4.5 (Table 15). This peptide was below LOD in these yoghurts stored at 4 °C for one and seven days (Figure not shown). However, there was only a very slight change in the concentration of BCM7 when milk was acidified with 1 M HCl to pH 4.5 and in acidified milk was stored (Table 15). This finding suggests that BCM7 is stable at acidic pH, but may be susceptible to hydrolysis in the presence of the bacteria.

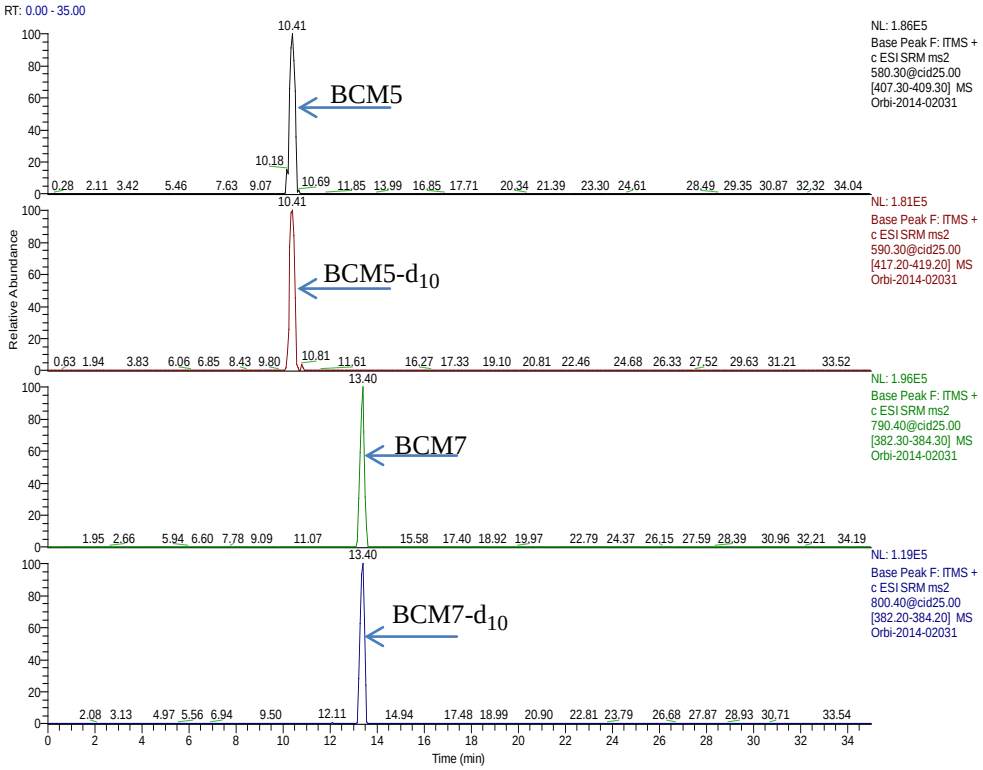

Figure 19. LC-HRMS chromatogram of BCM5 and BCM7 in UHT milk spiked BCM5 and BCM7 standard and their deuterated standards (BCM5-d10 and BCM7-d10) acidified to pH 4.5, seven days of storage

Table 15. Degradation of BCM5 and BCM7 by *S. thermophilus* and *L. delbrueckii* spp. *bulgraricus* during fermentation and storage of yoghurt

Bacteria	End pH	Fermentation time (hour)	Storage (day)	The loss of BCM5 (%)	The loss of BCM7 (%)
No bacteria	pH 4.5 (HCl acidification)	-	0	28.5	0
			1	23	0
			7	25	2.1
S. thermophilus	4.5	9.75	0	100	100
			1	100	100
			7	100	100
L. delbrueckii spp. *bulgraricus*	4.5	10.75	0	100	100
			1	100	100
			7	100	100
S. thermophilus + *L. delbrueckii spp. bulgraricus*	4.5	6.5	0	100	100
			1	100	100
			7	100	100

6.4 Discussion

Fermentation of milk with *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* not only reduces the pH of milk to the required value for yoghurt, but also leads to changes in the peptide components in the final products. Both bacteria used individually or in combination for fermentation, can produce lactic acid to lower pH to 4.3-4.5 that is a characteristic of the final yoghurt (Paul & Somkuti, 2009). *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* can also release proteases/peptidases, which can hydrolyse proteins/peptides to various extents (Paul & Somkuti, 2009).

Previous studies revealed that concentration of BCM5 in milk is lower than LOD and that of BCM7 is from 0.13 and 2.38 ng/g (see Chapters 4 and 5), these initial low levels make it difficult to investigate the even lower levels remaining at the final pH of fermentation (pH 4.5) and in the final yoghurt (yoghurt stored at 4-6 °C for at least one day). In the present study, the UHT milk spiked with standards of BCM5 and BCM7 and then fermented with mixture of *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* revealed that BCM5 and BCM7 were undetectable when pH of milk dropped to 4.5. This result is the same as that reported in Chapter 5. The same findings were observed in the milk inoculated with only *S. thermophilus* or only *L. delbrueckii* spp. *bulgraricus* and then fermented to pH 4.5 (Table 15). These results indicate that the reduction in levels of BCM5 and BCM7 by *S. thermophilus* is similar to that by *L. delbrueckii* spp. *bulgraricus*.

Change in the levels of BCMs strongly depends on enzymes released from *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus.* It has been well documented that PepX is released from LAB and hydrolyses proline-containing peptide bonds (Gobbetti et al., 2002). *In vitro* studies show that *S. thermophilus* produces intracellular and extracellular PepX (Hafeez et al., 2013; Meyer & Jordi, 1987). *L. delbrueckii* spp. *bulgraricus* also has been reported to produce intracellular and extracellular PepX (Atlan et al., 1990; Miyakawa et al., 1991). Previous work in this research project has revealed that PepX released from mixture of *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* can degrade BCM7 during fermentation of milk.

BCM5 and BCM7 are two proline-rich peptides that have a similar structure (Fig. 1). Hafeez et al. (2013) demonstrated that BCM7 is hydrolysed to di-, tri- and tetra-peptides by PepX activity. Furthermore, Hafeez et al. (2013) also revealed that

proline residue in second position of BCM7 sequence prevented the hydrolysis of this peptide by the activity of another aminopetidase. As a result, BCM5 is more likely a substrate for PepX activity, rather than aminopetidase activity. In the present study, incubation with *S. thermophilus* alone or *L. delbrueckii* spp. *bulgraricus* alone gave complete degradation of BCM5 with fermentation to pH 4.5. These findings suggest the degradation of BCM5 is likely to be a result of PepX activity.

In order to investigate the effect of pH alone without the presence of bacterial culture on the levels of BCM5 and BCM7, the milk spiked with BCMs was gradually acidified with 1 M HCl to pH 4.5. LC-HRMS data showed that BCM7 is more stable than BCM5 to this acidification (Table 15). A previously reported *in vitro* study revealed that PepX is stable at pH 4.5 (Miyakawa et al., 1991). Furthermore, as reported in this thesis (Chapter 5), PepX activity was also found in yoghurt at pH 4.5. These findings suggest that the degradation of BCM7 during fermentation is due to PepX activity, while the degradation of BCM5 may involve both PepX activity and effect of low pH independent of bacterial activity.

6.5 Conclusion

This study demonstrated that *L. delbrueckii* spp. *bulgraricus* and *S. thermophilus* used for production of yoghurt are capable of reducing the level of BCM5 and BCM7 during fermentation of milk to produce yoghurt. These peptides are completely degraded by both bacteria when milk fermented to pH 4.5. However, the degradation of BCM7 appears due to the activity of the bacteria, while that of BCM5 appears due to both bacterial activity and an independent effect of acidification. The results also show that BCM5 and BCM7 are not detectable in the yoghurt made from milk fermented with *S. thermophilus, or L. delbrueckii* spp. *bulgraricus,* or a mixture of these two bacteria.

CHAPTER 7 QUANTIFICATION OF BETA-CASOMORPHIN 7 CONTENT IN YOGHURT USING SANDWICH ELISA

7.1 Introduction

In recent years, ELISA has been used for quantification of BCM5 and BCM7 in dairy products (milk, cheese and yoghurt) and urine in several studies (Cieślińska et al., 2012; Jarmolowska, 2012; Sienkiewicz-Szłapka et al., 2009; Sokolov et al., 2014). ELISA is a simple to perform analytical technique capable of assaying a large number of samples simultaneously in a short time. However, the effectiveness of this method depends on the quality of the antibodies used against the target antigen such as BCM7 (Lutter et al., 2011). Furthermore, in yoghurt making, heat treatment of milk may modify the conformation of BCM7, leading to reduction in the binding affinity of the modified BCM7 to the ELISA antibodies. This may lead to underestimation of the BCM7 level in most commercial milks as they are usually treated at high temperature (Cieślińska et al., 2012).

Yoghurt is a complex food matrix containing numerous proteins and peptides that can interfere with the detection of target analytes such as BCM7 (EFSA, 2009; Kunda et al., 2012). Several commercial ELISA kits are available for quantification of BCM7 in plasma, serum, cell culture fluid or other biological fluids, but not for quantification of this peptide in milk and yoghurt. Currently, there is no reported study using commercial ELISA kits for the analysis of BCM7 in milk or yoghurt.

In previous chapters, the successful development and application of LC-MS/MS and LC-HRMS using stable isotope-labelled BCM7 as an internal standard for identification and quantification of BCM7 in yoghurt and milk have been reported. These methods can accurately identify and quantify this BCM at very low levels (sub-ng/g). Several studies have also applied LC-MS/MS methods for quantification of BCM7 in yoghurt and milk (De Noni, 2008; De Noni & Cattaneo, 2010; Juan-García et al., 2009; Thiri et al., 2012).

In the present study, a commercial sandwich ELISA kit was used for determination of BCM7 content in yoghurt to investigate the variation in BCM7 levels during fermentation of milk and in yoghurt after various periods of storage. LC-MS/MS was used as the “gold standard” method to compare and validate ELISA results. This

study will evaluate the applicability of the commercial sandwich ELISA kit for quantification of BCM7 in milk and yoghurt as an alternative method.

7.2 Materials and Methods

7.2.1 Materials and chemicals

The chemicals used have been previously described in Section 3.2.1. The BCM7 sandwich ELISA kit was purchased from Novateinbio (Cambridge, MA, USA).

7.2.2 Preparation of yoghurt

Yoghurt was prepared as described in Section 5.2.2.

7.2.3 ELISA analysis

7.2.3.1 Preparation of extracts

Frozen reconstituted milk and yoghurt samples were thawed at room temperature for approximately 30 min and then thoroughly mixed by vortex. Ten grams (±0.0001) of reconstituted milk were gradually acidified with 1 M HCl to pH 4.6 and then allowed to stand for 10 min at room temperature. Ten grams of yoghurt sample and acidified reconstituted milk were centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was separated by decantation into glass tubes. The pH of the yoghurt supernatant was gradually adjusted to pH 4.6 with 1 M HCl (or 1 M NaOH) and then the sample was allowed to stand at room temperature for 5 min. All extracts were re-centrifuged at 14,000 × *g* for 20 min at 4 ^{o}C followed by filtration through a 0.45 µm membrane filter. The extraction of each sample was performed in duplicate (n=2).

7.2.3.2 Determination of beta-casomorphin 7 content

BCM7 was quantified using the sandwich ELISA kit according to the manufacturer's instructions. A brief description of the procedure is as follows: 50µL of BCM7 standard solutions (0, 3.12, 6.25, 12.5, 50 and 100 pg/mL) and sample extract was placed into each well of a 96 well microplate in duplicate. After adding 100 µL of conjugate to each well, and mixing thoroughly by gentle swirling of the microplate, the microplate was covered and incubated at 37 °C for one hour. The incubation mixture was then removed from the well by aspiration. The wells were washed five times with diluted wash solution. After washing, the plate was inverted onto absorbent paper and left until dry. Fifty microliters of chromogen solution A and 50 µL of chromogen solution B were added into each well and then the covered plate

was incubated at 37 °C for 15 min. The colour reaction was stopped by adding 50 µL of stop solution to each well. Optical density was measured at 450 nm using a microplate reader (BioTek Instruments, Winooski, USA). The concentration of BCM7 defined as ng/g of yoghurt in the samples was determined by comparing the absorbance of the samples with that of the standard curve.

7.2.4 Liquid chromatography-tandem mass spectrometry analysis

7.2.4.1 Purification of extracts

Water soluble peptides from the milk and yoghurt were extracted and cleaned-up by SPE as described in Section 5.2.2.2.

7.2.4.2 Determination of beta-casomorphin 7 content

BCM7 content in the extracts of reconstituted milk and yoghurt was determined using LC-MS/MS as previously described in Section 5.2.2.3.

7.3 Results

7.3.1 Levels of beta-casomorphin 7 in reconstituted milk during fermentation

The level of BCM7 was quantified in unfermented reconstituted milk by both ELISA and LC-MS/MS representing the raw material for yoghurt production. As presented in Chapter 5, LC-MS/MS quantified BCM7 in reconstituted milk before fermentation and after fermentation to pH 4.8 (Figs. 11 and 12). As indicated in Table 16, the level of BCM7 as determined by LC-MS/MS decreased by approximately 80% when the milk was fermented to pH 4.8 and further decreased to <LOD after a more prolonged incubation to pH 4.5. The level of BCM7 in the milk and the yoghurt fermented to pH 4.8 and 4.5 are given in Table 16. Its' level in the milk as determined by ELISA was over two orders of magnitude lower than that obtained using LC-MS/MS. In contrast with the results from LC-MS/MS, BCM7 was observed to increase during fermentation (Table 16).

Table 16. BCM7 levels of milk and yoghurt fermented to different pHs as determined by ELISA and LC-MS/MS

Samples	Levels of BCM7 found by the two methods (ng/g [a])	
	LC-MS/MS [b]	ELISA
Reconstituted milk (pH 6.5)	1.4 ± 0.00	3.37×10^{-3} ± 0.0004
Reconstituted milk fermented to pH 4.8	0.29 ± 0.01	3.52×10^{-3} ± 0.003
Reconstituted milk fermented to pH 4.5	<LOD	12.22×10^{-3} ± 0.0009

[a] Mean values ± standard deviation (SD), n = 2.

[b] Data previously reported in Chapter 5

7.3.2 Levels of beta-casomorphin 7 in yoghurt during cold storage

The levels of BCM7 as analysed by ELISA showed an increase in level in yoghurt at pH 4.8 during cold storage (Table 17). However, its' level decreased in yoghurt at pH 4.5 after seven days of storage. As previously reported in Chapter 5, LC-MS/MS analysis showed that BCM7 was detected in yoghurt at pH 4.8 after one day of storage (Fig. 13), but its level was <LOD after seven days of storage (Table 17). For yoghurt at pH 4.5, the level of BCM7 was <LOD after storage for both one and seven days.

Table 17. BCM7 levels in yoghurt at different storage days as determined by ELISA and LC-MS/MS

Final fermentation pH	Storage time (days)	Levels of BCM7 (ng/g [c])	
		LC-MS/MS [d]	ELISA
4.8	1	0.17 ± 0.01	3.99×10^{-3} ± 0.0002
	7	<LOD	12.78×10^{-3}± 0.0003
4.5	1	<LOD	12.19×10^{-3} ± 0.0009
	7	<LOD	2.19×10^{-3} ± 0.00003

[c] Mean values ± standard deviation (SD), n = 2

[d] Data previously reported Chapter 5

7.4 Discussion

LC-MS/MS used in this project was used as the "gold standard" method for identification and quantification of BCM7 in milk and yoghurt. On the basis of the results in Chapter 5, in the present study, milk or yoghurt containing BCM7 were considered as positive samples. In contrast, milk or yoghurt that did not contain BCM7 were known as negative samples. Sandwich ELISA data shows that BCM7 is not only present in all positive samples, but also in all negative samples. However, BCM7 levels in positive samples analysed by ELISA are much lower than that analysed by LC-MS/MS (Tables 16 and 17).

In the case of the reconstituted milk, casein, whey protein and peptides that are present in the milk can remain in the extract after filtering through the 0.45 µm membrane used prior to sample analysis. Therefore, these compounds may interfere with BCM7 binding to ELISA antibodies. Furthermore, BCM10, which is released by cathepsin B activity, may be found in milk (Considine et al., 2004). Currently, the content of BCM10 in milk has not been reported. However, its' structure is known and is similar to that of BCM7 (see Table 1). In present the study, the ELISA assay used is the sandwich ELISA, which requires BCM7 to contain at least two sites to bind antibodies, one of which binds to the capture antibody and another site that binds to the detection antibody. It can be seen that BCM10 also contained the same potential antibody sites as BCM7 and may have affinity with the antibodies to BCM7. Therefore, the result obtained may include the content of BCM10 in addition to that of BCM7.

ELISA data in this study showed that there is an increase in BCM7 content during fermentation and storage, with exception of yoghurt sample at pH 4.5 stored for seven days (Tables 16 and 19), whereas content of this peptide decreased during these processes using LC-MS/MS (see Chapter 5) and LC-HRMS (see Chapter 6). According to the instructions of the manufacturer, the sandwich ELISA test has good sensitivity, but the present work shows that the selectivity may be problematic owing to potential formation and degradation of peptides, which are structurally closely related to BCM7, during fermentation and storage of yoghurt. Interestingly, several peptides known as pro-BCMs have been reported in yoghurt such as β-CNf57-68, β-CNf57-72, β-CNf58-72, β-CNf59-68, β-CNf59-70, β-CNf59-72, β-CNf60-72 (BCM13) and β-CNf60-68 (BCM9) (Kunda et al., 2012; Plaisancié et al., 2013;

Sabeena Farvin et al., 2010; Schieber & Brückner, 2000). It can be assumed that these peptides contain the same antibody binding sites as those of BCM7. Therefore, an increase in BCM7 content measured during fermentation and storage in the present work may be due to formation of pro-BCMs rather than BCM7.

The formation/degradation of peptides in yoghurt depends on time of storage. Some peptides may be hydrolysed and new ones may be produced during prolonged storage (Gobbetti et al., 2000). BCM7 as measured by LC-MS/MS is completely hydrolysed in yoghurt produced by fermentation of milk to pH 4.5 and stored for seven days (see Chapters 5 and 6). This may indicate that the decrease in BCM7 measured by ELISA in this yoghurt sample is due to degradation of pro-BCMs rather than BCM7 during storage from one to seven days (Table 17).

7.5 Conclusion

Application of commercial sandwich ELISA allows simple and rapid quantification of a target analyte in a large number of samples, however, this test is very unlikely to be used for quantification of BCM7 in a complex matrix such as yoghurt and milk because of its selectivity. More research is needed to determine what precautions are needed to enable ELISA analysis of complex matrices. It would be recommended that a validation process be undertaken using spiking of samples.

In contrast, LC-MS/MS has not only high sensitivity and selectivity for detection and quantification of BCM7, but also can provide simultaneous detection and quantification of other BCMs in milk and yoghurt. With the use of stable isotope-labelled internal standards, LC-MS/MS can be confidently used for detection of BCM7 and other BCMs in milk and yoghurt as well as other dairy products such as cheese, infant formulas and high milk protein powder.

CHAPTER 8 OVERALL CONCLUSIONS AND FUTURE RESEARCH

8.1 Overall conclusions

LC-MS/MS and LC-HRMS are sensitive, selective and robust analytical techniques that can be applied for the simultaneous identification and quantification of BCM5 and BCM7 in milk and yoghurt. Furthermore, using isotope labelled homologues as internal standards in these analytical techniques produced the highest confidence in the determination of BCM5 and BCM7 concentrations. In addition, preparation of samples before LC-MS/MS and LC-HRMS analysis carried out by centrifuging at high speed (14,000-15,000 × g) and then cleaning-up supernatants by SPE instead of filtering supernatants through 0.45 µm membranes can improve LOD methods.

Commercial sandwich ELISA may be a sensitive test used for rapid quantification of BCM7 in a large number of samples of serum, plasma or cell culture fluid. However, this method is very unlikely to be used for quantitative determination of BCM7 in complex food matrix such as yoghurt and milk.

The difference in BCM7 content in various products of Australian pasteurised milk or reconstituted milk seems to not depend on heat treatment of milk. However, it may be affected by β-CN A1 and β-CN A2 variant concentrations in milk collected from cow herds varying in their composition of A2/A2, A1/A1 or A1/A2 genotypes.

Yoghurt processes strongly influence the stability of BCM5 and BCM7. Fermentation of milk with yoghurt cultures, a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus* alone or *S. thermophilus* alone, causes the degradation of BCM5 and BCM7 during incubation at 43 °C and complete hydrolysis of these BCMs when milk is fermented until pH 4.5. BCMs in yoghurt produced by fermentation of milk with yoghurt cultures to pH 4.8 decreases during cold storage and completely degraded after seven days of storage. Yoghurt produced by fermentation of milk to pH 4.5, which is a characteristic of final yoghurt, does not contain BCM5 and BCM7 with regardless various periods of storage.

The degradation of BCM5 and BCM7 in yoghurt processing is likely a result of PepX activity, which is produced by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. However, the degradation of BCM7 is owing to presence of yoghurt cultures, while the stability of BCM5 is affected by yoghurt cultures and pH.

8.2 Future research

In this thesis, the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been reported to provide an extremely sensitive, selective and robust analytical method that was utilised for simultaneous identification and quantification of BCM5 and BCM7 in milk and yoghurt. In addition, the use of isotope labelled homologues of the BCMs as internal standards within these analytical techniques improved accuracy and precision of the determination of BCM5 and BCM7. Therefore, in future work it is proposed that LC-MS/MS and LC-HRMS method using isotope labelled standards will be used for the identification and quantification of BCM5, BCM7 and other related BCMs during simulated (*in vitro*) human gastrointestinal digestion and absorption of caseins and their peptides fragments.

Epidemiological studies showed that the intake of cow milk containing β-CN A1 variant from which BCM7 may be generated, has been linked to type 1 diabetes and ischemic heart disease. Therefore, it has been indicated that BCM7 may be a risk factor for development of these human diseases. Results of this thesis showed that BCM7 was found in various Australian milks. However, a major challenge is the lack of knowledge of (1) degradation and/or formation of BCM7 and other related BCMs in the human gastrointestinal tract as intake of cow milk already containing BCM7 and intake of cow milk containing pure beta casein A1 and A2 variant and (2) the bioavailability of intact BCM7 peptides and other related BCMs across the intestinal wall. Therefore, it is proposed that future research should use an *in vitro* gastrointestinal model to investigate the degradation and formation of BCM7 and other related BCMs after each simulated gastrointestinal stage of enzymatic digestion (stomach, duodenum and jejunum). The potential bioavailability of the intact BCM7 and other related BCM peptides through intestinal absorption will then be assessed using the in vitro Caco-2 cell monolayer model. Throughout this proposed study, the formation of BCM7 and other related BCM peptides will be identified and quantified by the LC-MS/MS and LC-HRMS methods developed in this thesis.

Structural difference between β-CN A1 variant and β-CN A2 variant results in difference in milk functionality between milk homozygous for β-CN A1 variant and β-CN A2 variant. Therefore, it is proposed that future work should investigate effect of milk containing homozygous β-CN A1 variant and β-CN A2 variant on physical

properties of yoghurt. In addition, it should use an in vitro gastrointestinal model to investigate the degradation of β-CN variants and formation of BCM7 and other related BCMs after digestion of these yoghurts with simulated gastrointestinal enzymes.

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APPENDICES

Appendix 1: The 2nd International Dairy Federal Symposium on Microstructure of Dairy Products and 5th International Dairy Federal Symposium on Science and Technology of Fermented Milk on 3-7 March 2014 in Melbourne, Australia

Effect of fermentation and storage on β-casomorphin 7 and β-casomorphin 5 in yoghurt

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The study was to investigate the effect of fermentation and storage on β-casomorphin 7 (BCM7) and β-casomorphin 5 (BCM5) in yoghurts made of skim milk powder and fresh milks. This study was also to investigate these peptides in different sources of milk. BCM7 and BCM5 were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The extracts were purified by 0.45 µm membrane filter (MF) and solid phase extraction (SPE) before injected to LC-MS/MS system. Neither BCM7 nor BCM5 was detected in fresh milks as well as in yoghurt made of fresh milks at all different values of fermentation pH and at all storage days. However, both peptides were quantified in A1 skim milk powder and yoghurt. Their contents decreased during fermentation and storage of yoghurt. As a result, fermentation and storage degrade BCM7 and BCM5, and do not form any β-casomorphin (BCM) in manufacture of yoghurt.