Food Chemistry 119 (2010) 560–566

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Occurrence of β-casomorphins 5 and 7 in commercial dairy products and in their digests following *in vitro* simulated gastro-intestinal digestion

Ivano De Noni *, Stefano Cattaneo

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, via G. Celoria 2, Milano 20133, Italy

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form 9 June 2009
Accepted 26 June 2009

Keywords:
β-Casomorphins
In vitro gastro-intestinal digestion
Dairy products
HPLC–MS/MS

ABSTRACT

The occurrence of β-casomorphin-5 (BCM5) and β-casomorphin-7 (BCM7) was investigated in commercial dairy products and in their digests, following *in vitro* simulated gastro-intestinal digestion (SGID), by means of HPLC–MS. The analysed dairy products were as follows: 10 cheeses (Gorgonzola, Caprino, Brie, Taleggio, Gouda, Fontina, Cheddar and Grana Padano 10-, 15- or 25-m ripened); 4 samples of drinking milk (unprocessed, pasteurised, UHT and in bottle-sterilised); 2 yoghurts and 4 fermented milks containing probiotics; 7 infant formulas; and 4 dried milk-derivatives (skim milk powder, calcium caseinate and milk protein concentrates). β-Casomorphin-5 was not detected in dairy products, either prior to or after SGID. β-Casomorphin-7 was detected only in cheeses with the exception of Taleggio, Caprino and Grana Padano samples. Peptide amount was in the range 0.01–0.15 mg kg^{-1} the highest level being recovered in Brie sample. Following SGID, BCM7 formed in all dairy samples or increased up to 21.77 mg kg^{-1} in digests of cheeses. The peptide level ranged from 0.29 to 1.23 mg kg^{-1}in fermented milks and from 3.46 to 22.18 mg kg^{-1} in dried milk-derivatives. Digests of commercial infant formulas contained BCM7 at concentrations of 0.04–0.21 mg l^{-1}. For the first time, this work reports quantitative values for BCM5 and BCM7 in a range of dairy products providing evidence that, during processing, only proteolytic systems involved in manufacturing and ripening of cheese can potentially hydrolyse β-CN to BCM7. Nevertheless, formation or further release of BCM7 is mainly promoted by the action of gastrointestinal proteinases during *in vitro* digestion irrespective of the type of dairy product.

1. Introduction

Caseins and whey proteins from bovine milk represent a source of biologically active peptides which may exert "pharmacological" activities. Milk-derived biopeptides are encrypted in an inactive form within the sequences of bovine milk proteins from which they are potentially released during fermentation or *in vivo* or *in vitro* digestion. In recent literature, the bioactivity of milk-derived peptides has been extensively reviewed and several activities (opioid, blood pressure-lowering, antimicrobial and immunomodulatory) have been reported (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006, 2007; Morris & FitzGerald, 2008; Murray & FitzGerald, 2007).

β-Casomorphins (BCMs) are exogenous opioid peptides deriving from proteolysis of β-casein (β-CN) and they were originally isolated from an enzymatic digest of casein (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). Amongst BCMs, β-casomorphin-7 (BCM7) and β-casomorphin-5 (BCM5) represent fragments f60–66 and f60–64 of bovine β-CN, respectively. The release of BCM7 during hydrolysis of bovine β-CN seems to be dependent on the presence of some variants of this protein, such as A^1 and B. These variants have histidine at position 67, whereas proline is present in variant A^2. This amino acidic substitution likely prevents the hydrolysis of the Ile^{66}–Pro^{67} bond during proteolysis thus hindering the release of BCM7 from β-CN A^2 (Hartwig, Teschemacher, Lehmann, Gauly, & Erhardt, 1997; Jinsmaa & Yoshikawa, 1999). Some ecological studies suggested that BCM7 or related peptides may increase the risk for the development of some human diseases such as juvenile diabetes type I, ischaemic hearth disease, autism and schizophrenia (Elliott, Harris, Hill, Bibby, & Wasmuth, 1999; Laugesen & Elliott, 2003a, 2003b; McLachlan, 2001). Recently, the European Food Safety Authority (EFSA) published a review of the available scientific literature that addresses possible health effects of BCMs, in particular BCM7 (EFSA, 2009). EFSA concluded that a cause and effect relationship cannot be established between the dietary intake of BCM7 and non-communicable diseases. The same review recognised that BCM7 can exert biological activities such as regulatory effects on gastro-intestinal motility and on gastric and pancreatic secretions. Despite this, insufficient knowledge is available about the actual levels of BCM5 and BCM7 which can originate from the action of diverse proteolytic systems involved in fermen-

* Corresponding author. Tel.: +39 02 50316680; fax: +39 02 50316672.
E-mail address: ivano.denoni@unimi.it (I. De Noni).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.058

tation or digestion of milk products. This work is aimed at determining the levels of BCM5 and BCM7 in a range of dairy products and in their digests following *in vitro* simulated gastro-intestinal digestion (SGID). For this purpose, HPLC and tandem mass spectrometry (MS/MS) were used for separation and quantification of the two BCMs.

2. Materials and methods

2.1. Standard bovine BCM5 and BCM7

Standard bovine BCM5 (Tyr-Pro-Phe-Pro-Gly) and BCM7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) were purchased from Bachem (Bubendorf, Switzerland).

2.2. Samples

The dairy products considered in the present work were as follows: 10 cheese samples (Gorgonzola, Caprino, Brie, Gouda, Taleggio, Fontina, Cheddar, and Grana Padano 7-, 15- or 25-m old); 4 samples of drinking milk (unprocessed, pasteurised, UHT and in bottle-sterilised); 2 yoghurts and 4 fermented milks containing probiotics; one sample of skim milk powder and one sample of calcium caseinate; 2 milk protein concentrates; and 7 infant formulas (4 powder and 3 liquid products) suitable for the normal full-term newborn infants during the first six months of life. Labels of powder infant formulas did not report quantitative information about protein ingredients All samples were collected at the Italian market in September–December 2008 and they represented industrial products. The main characteristics of cheeses, fermented milks and infant formulas are reported in Table 1.

2.3. Capillary zone electrophoresis (CZE) of dairy samples

CZE was used to ascertain the presence of the most common variants of β-CN, namely A^1, B and A^2, in commercial dairy samples. For this purpose, the method reported in De Noni (2008) was used.

2.4. Preparation of water-soluble peptides extracts

Pre-formed BCMs were searched in the water-soluble peptides extracts (WSEs) from undigested dairy products. WSEs were obtained according to the procedures described below. Each sample was subjected to three replicate extractions which were further submitted to HPLC–MS/MS analysis.

Cheese. WSE was prepared according to Parrot, Degraeve, Curia, and Martial-Gros (2003) with modifications. Twenty grams cheese were homogenised in 40 ml of water using an Ultraturrax apparatus. The mixture was kept at 40 °C for 1 h under gentle stirring and then centrifuged at 10,000×*g* for 30 min at 10 °C. The WSE was acidified to pH 4.60 with HCl 2 M and centrifuged at 5000×*g* for 20 min at 4 °C. *Milk*. Fifty millilitre milk was acidified to pH 4.60 and the WSE recovered after centrifugation at 5000×*g* for 20 min at 4 °C. *Fermented milk*. The procedure described by Donkor, Henriksson, Singh, Vasiljevic, and Shah (2007) was adopted with modifications. Fifty millilitre fermented milk was centrifuged at 10,000×*g* for 20 min at 4 °C. The recovered WSE was adjusted to pH 4.60 and then centrifuged at 5000×*g* for 20 min at 4 °C. Milk powder, sodium caseinate and milk protein concentrates. Ten grams powder was reconstituted to 100 ml water, adjusted to pH 4.60 and then centrifuged at 5000×*g* for 20 min at 4 °C. *Infant formulas*. Solutions of powder products were prepared by dissolving the powder according to label's recommendations and using distilled water. One hundred millilitre liquid or reconstituted formulas were centrifuged at 10,000×*g* for 20 min at 4 °C. The recovered WSE was adjusted to pH 4.60 and then centrifuged at 5000×*g* for 20 min at 4 °C.

Prior to HPLC–MS/MS analysis, all WSEs were submitted to ultrafiltration (cut-off 3 kDa, Millipore, Billerica, MA) in order to remove high molecular mass peptides. For this purpose, 30 ml WSE were ultrafiltered and 25 ml permeate were collected and immediately analysed or stored at −40 °C until HPLC–MS/MS.

Table 1
Main ingredients and protein content (% w/w) of commercial samples of cheeses, fermented milks and infant formulas submitted to determination of β-casomorphin-5 and β-casomorphin-7.

	Ingredients	Protein content
Cheeses		
Brie	Whole pasteurised milk	16.3
Caprino	Whole pasteurised milk	13.5
Cheddar	Whole pasteurised milk	17.1
Fontina	Whole unprocessed milk	24.3
Gorgonzola	Whole pasteurised milk	18.9
Gouda	Whole pasteurised milk	23.4
Grana Padano (10-m old)	Partially skimmed unprocessed milk	30.8
Grana Padano (17-m old)		34.6
Grana Padano (25-m old)		36.0
Taleggio	Whole pasteurised milk	21.8
Fermented milks		
1. Yoghurt	Skimmed milk, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*	4.4
2. Yoghurt	Skimmed milk, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*	4.0
3. Probiotic	Skimmed milk, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *Bifidobacterium*	4.9
4. Probiotic	Skimmed milk, sucrose, *L. delbrueckii* ssp. *Bulgaricus*, *S. thermophilus, L. casei*	2.8
5. Probiotic	Skimmed milk, *L.casei*	1.4
6. Probiotic	Skimmed milk, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus, Bifidobacterium*, *L. casei, L. acidophilus*	2.8
Infant formulas		
1. Powder	Milk whey and skim milk	11.3
2. Powder	Skim milk and milk whey	12.5
3. Powder	Skim milk and milk whey	9.5
4. Powder	Skim milk and milk whey	11.0
5. Liquid	Skim milk and milk whey	1.2
6. Liquid	Skim milk and milk whey	1.3
7. Liquid	Skim milk and milk whey	1.3

2.5. Determination of protein content and assessment of proteolysis on cheeses

Protein content of dairy products was determined by the Kjeldahl method (IDF-International Dairy Federation, 1993).

Proteolysis was assessed on cheeses by the determination of soluble nitrogen after treatment with trichloroacetic acid (TCA; 120 g l^{-1}). Grated cheese (4 g) was suspended in 100 ml deionised water at 40 °C. The mixture was homogenised with an Ultraturrax apparatus, stirred for 1 h at 40 °C. After addition of TCA (12% w/v final concentration), the suspension was centrifuged (10,000*g* for 30 min at 5 °C) and the supernatant filtered through a Schleicher and Schuell 595 folded filter. Nitrogen soluble in 12% TCA of the filtrate was determined by the Kjeldahl method (IDF, 1993).

2.6. In-vitro simulated gastro-intestinal digestion (SGID)

Digestions of different dairy products were carried out using pepsin and Corolase PP™ according to the protocol reported in De Noni (2008). Each commercial dairy sample was submitted to three replicate digestions on the same day. The mean amount of released BCM5 and BCM7 determined by means of LC–MS/MS was considered.

2.7. HPLC–MS/MS analysis

Separation and identification of BCM5 and BCM7 in WSEs of undigested dairy products and in their digests from *in vitro* SGID were performed by means of HPLC–MS/MS as reported by De Noni (2008). Calculation of BCM5 and BCM7 amounts was carried out by summing up the intensities of y_3 and b_3 ions or y_4 and y_5 ions for BCM5 and BCM7, respectively. WSEs from either Grana Padano cheese (17-m old) or UHT milk were spiked with synthetic BCM5 or BCM7 and used as an external standard for quantification of BCM5 and BCM7.

3. Results and discussion

3.1. Occurrence of BCM5 and BCM7 in commercial dairy products

Formation and fate of BCM5 and BCM7 have been mainly investigated adopting enzymatic hydrolysis of bovine β-CN using digestive proteinases and peptidases (Jinsmaa & Yoshikawa, 1999; Macaud, Zhao, Ricart, & Piot, 1999). On the contrary, scant information concerns the actual levels of BCM5 and BCM7 in undigested milk products or in their digests following enzymatic treatment or SGID (Jarmolowska, Kostyra, Krawczuk, & Kostyra, 1999; Muehlenkamp & Warthesen, 1996; Jarmolowska et al., 2007). Recently, an HPLC–MS/MS method has been developed for quantifying BCM5 and BCM7 in infant formulas (De Noni, 2008). In the present work, the same analytical approach was applied to different commercial dairy products, including cheese, fermented milk, drinking milk, infant formulas and dried derivatives of milk. The potential effect of such dairy matrices on the ionisation and fragmentation efficiencies of BCM5 and BCM7 during LC–MS/MS analysis was investigated by analysing WSEs from either UHT milk or Grana Padano cheese (17-m old) spiked with the same amounts of synthetic BCM5 or BCM7. The results from LC–MS/MS analyses demonstrated that the same fragmentation pattern and ionisation efficiencies were obtained for both spiked WSEs. Accordingly, the sum of the intensities of y_3 and b_3 ions of BCM5 and y_4 and y_5 ions of BCM7 were used for the quantification of the two peptides in commercial dairy products.

As BCM7 seems to be released only from A^1 and B variants of β-CN (Hartwig et al., 1997; Jinsmaa & Yoshikawa, 1999), the commercial dairy products considered in the present work were initially assessed for the presence of these variants by means of CZE. In addition to the A^2 variant, all products contained A^1, B variants of β-CN (not shown) and, for this reason, they might potentially release BCM7 following fermentation and/or digestion. According to peak areas of CZE patterns, B and A^1 variants accounted for 2–5% and 50–60% of β-CN in infant formulas and in fermented or drinking milks. The similar proportion of these variants would not affect the actual levels of BCM7 in the final products. It was not possible to calculate the ratio amongst variants in cheese samples since some protein breakdown occurred in ripening. Levels of BCM7 in these samples could be potentially related to the proportion of different β-CN variants present in the milk taken for cheesemaking.

In the present work, neither BCM5 nor BCM7 were detected in unprocessed milk. In literature, only the study of Cieslinska, Kaminski, Kostyra, and Sienkiewicz-Szlapka (2007) reports the presence of BCM7 in unprocessed bovine milk. This milk sample was not characterised for bacterial and somatic cell counts. Indeed, the release of BCMs or their precursors in unprocessed milk may be influenced by the proteolysis associated with the microbial and somatic cells activities. Nevertheless, different works did not report the release of BCM5 or BCM7 in mastitic milk samples (Napoli, Aiello, Di Donna, Prendushi, & Sindona, 2007; Wedholm et al., 2008) or during *in vitro* digestion of β-CN with cathepsin B (Considine, Healy, Kelly, & McSweeney, 2004). In the present work, BCM5 and BCM7 were not seen in pasteurised, UHT and in-bottle sterilised samples as well. By means of LC–MS/MS, Gaucher, Mollé, Gagnaire, and Gaucheron (2008) identified pro-BCMs but not BCM5 or BCM7 in UHT milk stored at 20 °C for 6 months. Pro-BCMs (f54–68, f54–69, f55–65, f55–68, f57–68) derived from the action of cathepsins and elastase on β-CN.

Four fermented milks containing probiotics and two yoghurts were analysed 30-d after manufacturing. Bacterial strains present in these samples are listed in Table 1. Yoghurt contains a mixed culture of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* with the former being more proteolytic. A recent study demonstrated that 21 strains of *L. delbruekii* ssp. *bulgaricus* isolated from fermented milks were able to degrade *in vitro* 80–90% of β-casein after 72–96 h incubation in a sodium caseinate solution (Tzvetkova et al., 2007). Despite this, the two yoghurts considered in the present work did not contain BCM5 or BCM7. The same findings were reported by Kahala, Pahkala, and Pihlanto (1993) for two Finnish yoghurts and by Schieber and Brückner (2000) for yoghurt made from heated milk and stored at 4 °C for 3 weeks. Schieber and Brückner (2000) observed an intensive proteolysis of caseins whilst only pro-BCMs sequences (f57–68 and f57–72) were recognised by means of HPLC–MS and peptide sequencing. According to Donkor et al. (2007), proteolysis in fermented milk that contain both yoghurt culture and probiotics (*L. acidophilus*, *L. casei* and *Bifidobacterium lactis*) is significantly higher as compared to the milk fermented with the yoghurt culture only. In the present work, BCM5 or BCM7 were not found in fermented milks containing both probiotic strains and yoghurt culture or *L. casei* only (Table 1). Also Kahala et al. (1993) did not find BCMs in two fermented milks containing *L. acidophilus* and *Lactobacillus GG* or a mixed mesophilic culture, including *L. lactis* ssp. *cremoris*.

To date, data concerning the levels of BCM7 and BCM5 in cheeses refer to only a few studies (Jarmolowska et al., 1999; Muehlenkamp & Warthesen, 1996). As found in the present work, these studies did not report the presence of BCM5 in cheese. On the contrary, Jarmolowska et al. (1999) reported BCM7 as being present in extracts from Brie cheese samples using HPLC–UV detection. This peptide was present at amounts ranging from 5 to 15 mg kg^{-1} cheese. As shown in Table 2, the sample of Brie considered here presented a notably lower (0.15 mg kg^{-1}) BCM7 content. Neverthe-

Table 2
β-Casomorphin-7 (BCM7) level in commercial cheese samples (mean of three replicate digestions ± standard error).

	BCM7 (mg kg^{-1})
Grana Padano (10-m old)	0.00
Grana Padano (17-m old)	0.00
Grana Padano (25-m old)	0.00
Brie	0.15 ± 0.02
Caprino	0.00
Fontina	0.04 ± 0.01
Gouda	0.10 ± 0.02
Gorgonzola	0.01
Taleggio	0.00
Cheddar	0.11 ± 0.02

less, it was the highest amount recovered in the studied cheese samples. This finding could be somehow related to proteolysis occurring upon the action of enzymes produced by fungi present as felt on the cheese surface. Muehlenkamp and Warthesen (1996) did not detect BCM7 in Brie cheese using HPLC coupled to UV detection. The same author did not find BCM7 in other types of cheese, including Swiss type, Blue, Limburger and Cheddar. In the present work, BCM7 was present at 0.11 mg kg^{-1} level in the Cheddar sample. This cheese is manufactured using a starter culture consisting in a mixture of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* (Robinson, 1995). According to Muehlenkamp and Warthesen (1996), the proteolytic system of *L. lactis* ssp. *cremoris* provokes a 50% reduction of BCM7 level during *in vitro* test carried out in a 1.5% NaCl medium at pH 5.0, after 6–15 weeks. Taking the pH value (5.0–5.1) and salt concentration (1.8–2.0%) of Cheddar cheese into consideration, BCM7 would expected to be degraded to some extent by strains of subspecies of *L. lactis* present in the starter culture of Cheddar cheese. On the contrary, according to Singh, Fox, and Healy (1995, 1997), the persistence in Cheddar cheese of several peptides including the BCM7 sequence, such as BCM9 (β-casein 60–68), suggests that peptidases from LAB have no or little activity in this cheese. Taleggio, a PDO Italian smear-coated cheese, did not contain BCM7 (Table 2). This cheese is manufactured from pasteurised milk and using *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* as starter culture which undergoes cell lysis after 35-d maturation. Since BCM7 was not found in Taleggio, the proteinases/peptidases activity of this LAB does not seem capable to release BCMs during ripening. As fungal growth in curd produces intense proteolysis, strong breakdown of proteins and peptides has to be expected in Gorgonzola cheese, an Italian PDO blue-cheese. This could explain why only a very small level (0.01 mg kg^{-1}) of BCM7 was found in the cheese (Table 2). Caprino is a fresh cheese made from pasteurised milk employing mesophilic starter cultures, including subspecies of *L. lactis.* As reported in Table 2, Caprino did not contain BCM7. This result could be supported by findings of Mierau, Kunji, Venema, and Kok (1997) and Juillard et al. (1995). According to these authors, proteinases from L. *lactis* ssp. *cremoris* can extensively hydrolyse β-CN resulting in more than 100 oligopeptides other than BCM7.

No other data concerning the actual amounts of BCM7 in cheeses can be attained from the literature so far whilst Gouda, Fontina and Grana Padano cheese samples were also analysed in the present work (Table 2). Gouda cheese presented a BCM7 level (0.10 mg kg^{-1}) similar to that recovered in Cheddar cheese. This finding is not surprising as a similar starter LAB and maturation period can characterise the two cheeses. As reported by Singh et al. (1995, 1997) for Cheddar cheese, BCM9 was also found in Gouda (8-m aged) by Saito, Nakamura, Kitazawa, Kawai, and Itoh (2000). Similarly, Toelstede and Hofmann (2008) revealed BCM9 and BCM10 in the WSE of a matured Gouda cheese. BCM7 was not detected in the three samples of Grana Padano cheese 10-, 17- or 25-m aged (Table 2). In manufacturing of this cheese, and in Parmigiano Reggiano as well, a natural starter mainly consisting of *L. helveticus* is added to unprocessed milk prior to renneting. Peptidase and proteinase systems of this starter lead to strong casein breakdown during ripening and, after 10-m maturation, about 20% of protein nitrogen in Parmigiano Reggiano and Grana Padano cheeses is represented by free amino acids (Resmini, Pellegrino, Pazzaglia, & Hogenboom, 1985). Nevertheless, according to Addeo et al. (1992) long peptides (*e.g.* 20–21 residues) containing the BCM7 sequence are present in matured (up to 15 months) Parmigiano Reggiano cheese. The presence of such peptides demonstrates the resistance of this β-CN region to degradation by proteinase and peptidase systems of LAB starter involved in the maturation of these two hard cheeses. Small BCM7 amounts (0.04 mg kg^{-1}) has been recovered in Fontina (Table 2). This Italian PDO cheese is made from unprocessed milk and maturation (>70-d) relies on the activity of a natural flora of mesophilic and thermophilic bacteria originally present in milk. This activity leads to scant proteolysis and usually only 1–2% of protein nitrogen is represented by free amino acids (Pellegrino, Hogenboom, Pazzaglia, & Todesco, 1995).

In order to assess the potential relationship between degree of proteolysis and BCM7 occurrence, cheeses were assessed for levels of 12% TCA soluble nitrogen (TSN). This nitrogen fraction generally includes small peptides (less than 2–20 residues) the shortest of them (<7 residues) are soluble irrespective of parameters, such as hydrophobicity, that could affect peptide solubility (Yvon, Chabanet, & Pélissier, 1989). As shown in Fig. 1a, the level of TSN and hence the extent of proteolysis varied a lot amongst cheeses and it was not related to BCM7 amounts (expressed as mg kg^{-1} protein). Indeed, the highest peptide level characterised the Brie cheese sample which presented the lowest level of TSN. The contrary applied for samples of Grana Padano that did not contain BCM7 although they showed high TSN levels. Taking these results into account, it can be supposed that the release and fate of BCMs in cheese might vary as a function of specific proteolytic phenomena occurring during cheesemaking and ripening. In this regard, additional investigations are needed to ascertain whether the specificity of proteinases and peptidases action of starter and non starter bacteria could determine the occurrence and the level of BCM7 in the final cheese.

To our knowledge no literature reports on BCM5 and BCM7 levels in powdered milk-derivatives. Samples of skim milk powder, calcium caseinate and milk protein concentrates did not contain BCM5 or BCM7. The same applied for the seven infant formulas analysed in the present work.

3.2. Release of BCM5 and BCM7 following in vitro SGID of dairy products

In the present work, pepsin and Corolase PP™ were used for SGID at the end of which none of the analysed dairy samples released BCM5. On the contrary, dairy products which did not contain this peptide prior to SGID released BCM7 following digestion. In particular, this level increased to 1.16 mg kg^{-1} in unprocessed milk. A recent study of Cieslinska et al. (2007) reported the release of BCM7 from unprocessed milk samples after 24 h digestion with pepsin. According to these authors, the recovered amount in an homozygote A^2 milk was four times lower than that released from a homozygote A^1 milk. β-Casomorphin-7 level was in the range 0.60–0.96 mg kg^{-1} in pasteurised, UHT and in-bottle sterilised milks. To our knowledge, only De Noni (2008) has considered the effect of milk processing conditions on the release of BCM7 during subsequent SGID. In this study, industrial indirect-UHT treatments did not modify the extent of the release of BCM7 and, during SGID, comparable peptide amounts formed

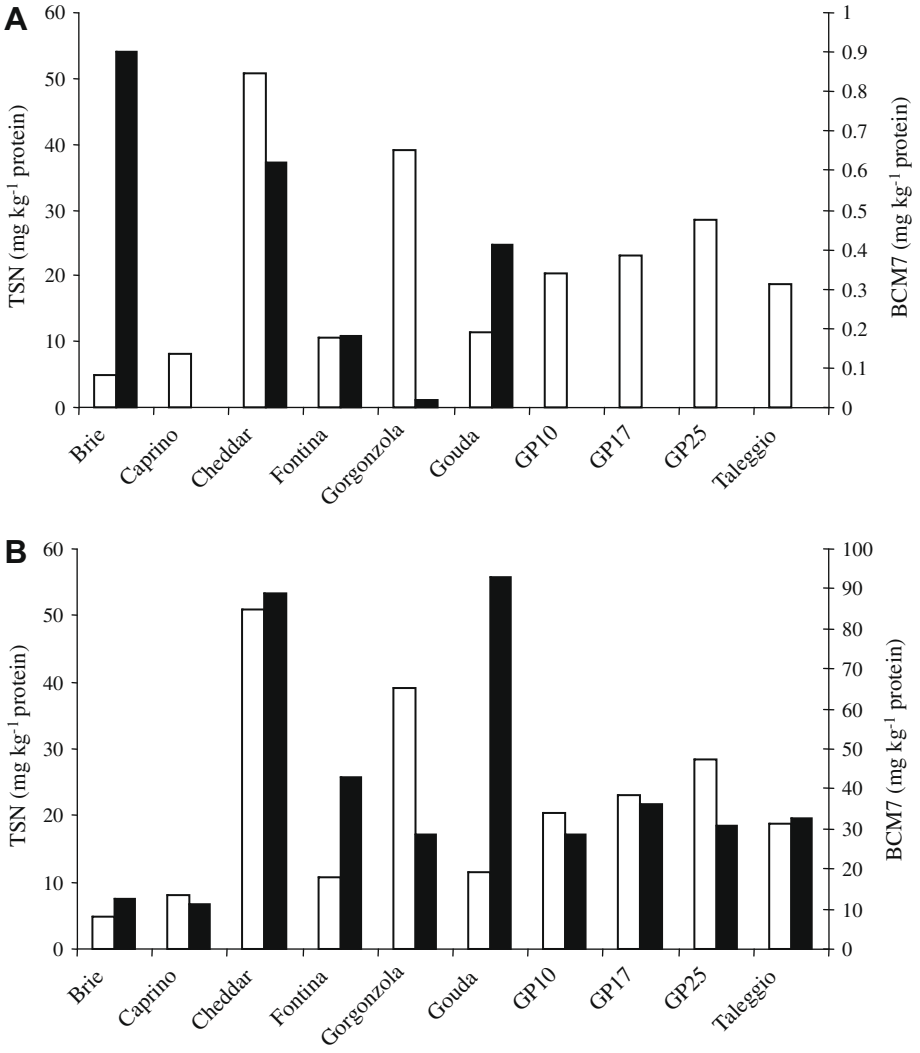

Fig. 1. Level of 12% TCA soluble nitrogen (TSN, empty bars) in undigested cheese samples and corresponding β-casomorphin-7 amount (BCM7, full bars) (A) prior to or (B) after *in vitro* simulated gastro-intestinal digestion (mean value of three replicates). (GP: Grana Padano, 10-, 17- or 25-m old).

from both unprocessed and heat-treated samples. Results obtained in the present work for pasteurised, UHT and in bottle-sterilised milks seem to enforce this finding. BCM7 levels (0.65 and 0.98 mg kg^{-1}) of yoghurt samples were similar to those revealed in either unprocessed or heat-treated milk samples. It can be deduced that neither intense heat treatments (90–95 °C for 5–20 min) usually applied in yoghurt manufacturing (Tamime & Robinson, 1999) modifies the susceptibility of β-CN to proteolysis and hence the release of BCM7. The fermented milks containing probiotics presented BCM7 levels in the range 0.29–1.23 mg kg^{-1}. Use of probiotic strains in fermented milk production may modify the peptide pattern in the final product and the potential release of BCMs during SGID could be affected as well. Nevertheless, a UHT milk fermented by the probiotic *Lactobacillus* GG strain and subsequently digested by pepsin and trypsin was not found to contain BCM7 whilst only peptide sequences such as BCM11 and BCM4 were revealed (Rokka, Syväoja, Tuominen, & Korhonen, 1997).

β-Casomorphin-5 was not present whereas BCM7 formed or increased in cheese samples following *in vitro* digestion (Table 3). As reported above, ripening itself did not release BCM7 in Grana Padano cheeses whilst peptide amount was in the range 8.79–12.55 mg kg^{-1} after SGID (Table 3). The highest BCM7 levels were found in digests of Cheddar and Gouda cheeses, namely 15.22 mg kg^{-1} and 21.77 mg kg^{-1}. Stepaniak, Fox, Sorhaug, and Grabska (1995) isolated the β-CN (58–72) peptide from the WSE of Cheddar cheese. As already mentioned, BCM9, BCM10 and several peptides including the BCM7 sequence were found in Cheddar and Gouda cheeses (Singh et al., 1995, 1997; Saito et al., 2000; Toelstede & Hofmann, 2008). Presence of these short peptides is likely prone to the action of gastrointestinal enzymes and may support the substantial release of BCM7 during SGID of Cheddar and Gouda cheeses. Undigested Brie cheese presented the highest peptide amount and *in vitro* digestion caused a further increase of BCM7 amount, namely from 0.15 mg kg^{-1} to 2.07 mg kg^{-1}. A greater increase (5–10 mg kg^{-1}) of BCM7 level was observed in digests of Fontina and Gorgonzola cheeses. Formation of BCM7 up to 7.15 mg kg^{-1} was found in Taleggio whilst the lowest (1.51 mg kg^{-1}) peptide amount characterised the digest of Caprino cheese. As a matter of fact, the final level of BCM7 in cheeses was mainly affected by proteolysis during SGID as only negligible (<0.15 mg kg^{-1}) amounts of peptide released during maturation of the analysed cheese samples. In this regard, the degree of proteolysis of cheese (evaluated as TSN) can not be clearly regarded as a promoting or hindering factor for BCM7 release during SGID (Fig. 1b).

Table 3
β-Casomorphin-7 (BCM7) level in commercial dairy products and in their digests from *in vitro* simulated gastro-intestinal digestion (mean of three replicate digestions ± standard error).

	BCM7 (mg kg^{-1}, * mg l^{-1})
Drinking milks	
Unprocessed milk	*1.16 ± 0.04
Whole pasteurised milk	*0.64 ± 0.03
Whole UHT milk	*0.96 ± 0.02
Skimmed in-bottle-sterilized milk	*0.60 ± 0.02
Fermented milks	
1. Yoghurt	0.65 ± 0.02
2. Yoghurt	0.98 ± 0.03
3. Probiotic	1.23 ± 0.04
4. Probiotic	0.62 ± 0.03
5. Probiotic	0.29 ± 0.01
6. Probiotic	0.72 ± 0.01
Powdered milk-derivatives	
Skimmed milk powder	3.46 ± 0.03
Sodium caseinate	17.68 ± 0.12
Milk protein concentrate 1	16.99 ± 0.10
Milk protein concentrate 2	22.18 ± 0.10
Cheeses	
Brie	2.07 ± 0.09
Caprino	1.51 ± 0.09
Cheddar	15.22 ± 0.13
Fontina	10.43 ± 0.12
Gorgonzola	5.41 ± 0.08
Gouda	21.77 ± 0.12
Grana Padano (10-m old)	8.79 ± 0.04
Grana Padano (17-m old)	12.55 ± 0.09
Grana Padano (25-m old)	11.06 ± 0.08
Taleggio	7.15 ± 0.07
Infant formulas	
1. Powder	*0.04 ± 0.01
2. Powder	*0.21 ± 0.03
3. Powder	*0.06 ± 0.01
4. Powder	*0.12 ± 0.02
5. Liquid	*0.18 ± 0.03
6. Liquid	*0.13 ± 0.02
7. Liquid	*0.12 ± 0.02

* Refers to results expressed as mg l^{-1} in order to distinguish them from those expressed as mg kg^{-1}. The BCM7 contents of powdered infant formulas are expressed as mg l^{-1} of reconstituted (with water) powder.

After *in vitro* digestion, BCM7 occurred at 3.46 mg kg^{-1} in milk powder. The peptide amounts released from milk protein concentrates (16.99 and 22.18 mg kg^{-1}) and sodium caseinate (17.68 mg kg^{-1}) were similar despite the higher casein content of the latter (Table 3).

The seven commercial infant formulas did not contain BCM5 after *in vitro* digestion whereas BCM7 formed in digests of all samples at concentrations corresponding to 0.04–0.21 mg l^{-1} (Table 3). The lowest BCM7 amount formed in the powder infant formula which presented whey as the main protein ingredient (Table 1). The label of this powder infant formula did not report quantitative information about protein ingredients. Recently, Hernandez-Ledesma, Amigo, Ramos, and Recio (2004) reported BCM7 as present (amount not given) in the UF permeate of a reconstituted infant formula submitted to a SGID protocol similar to that adopted here.

4. Conclusion

For the first time, this work reports quantitative values for BCM5 and BCM7 in a range of dairy products providing evidence that, during processing, only proteolytic systems involved in manufacturing and ripening of cheese can potentially hydrolyse β-CN to BCM7. Nevertheless, formation or further release of BCM7 is mainly promoted by the action of gastrointestinal proteinases during *in vitro* digestion irrespective of the type of dairy product. In this regard, differences in the ratio of the different β-CN variants in the milk taken for manufacture could affect the BCM7 level in the final products after SGID. Overall, these findings provide new basic knowledge for further studies concerning the potential bioactivity of BCMs.

References

Addeo, F., Chianese, L., Salzano, A., Sacchi, R., Cappuccio, U., Ferranti, P., & Malorni, A. (1992). Characterization of the 12% trichloroacetic acid-insoluble oligopeptides of Parmigiano-Reggiano cheese. *Journal of Dairy Research, 59*, 401–411.

Brantl, V., Teschemacher, H., Henschen, A., & Lottspeich, F. (1979). Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. *Hoppe-Seyler's Z Physiological Chemistry, 360*, 1211–1216.

Cieslinska, A., Kaminski, S., Kostyra, E., & Sienkiewicz-Szlapka, E. (2007). Beta-casomorphin 7 in raw and hydrolyzed milk derived from cows of alternative beta-casein genotypes. *Milchwissenschaft, 62*, 125–127.

Considine, T., Healy, Á., Kelly, A. L., & McSweeney, P. L. H. (2004). Hydrolysis of bovine caseins by cathepsin B, a cysteine proteinase indigenous to milk. *International Dairy Journal, 14*, 117–124.

De Noni, I. (2008). Release of beta-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine beta-casein variants and milk-based infant formulas. *Food Chemistry, 110*, 897–903.

Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., & Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal, 17*, 1321–1331.

EFSA, European Food Safety Authority (2009). Review of the potential health impact of β-casomorphins and related peptides. Scientific Report n. 231, 1–107.

Elliott, R. B., Harris, D. P., Hill, J. P., Bibby, N. J., & Wasmuth, H. E. (1999). Type I (insulin-dependent) diabetes mellitus and cow milk: Casein variant consumption. *Diabetologia, 42*, 292–296.

Gaucher, I., Mollé, D., Gagnaire, V., & Gaucheron, F. (2008). Effects of storage temperature on physico-chemical characteristics of semi-skimmed UHT milk. *Food Hydrocolloids, 22*, 130–143.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology, 18*, 163–169.

Hartwig, A., Teschemacher, H., Lehmann, W., Gauly, M., & Erhardt, G. (1997). Influence of genetic polymorphisms in bovine milk on the occurrence of bioactive peptides. Seminar on milk protein polymorphism, IDF Special Issue no. 9702. *International Dairy Federation, Brussels*, 459–460.

Hernandez-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas. *International Dairy Journal, 14*, 889–898.

IDF-International Dairy Federation (1993). Milk. Determination of nitrogen content. Standard 20B.

Jarmolowska, B., Kostyra, E., Krawczuk, S., & Kostyra, H. (1999). β -casomorphin-7 isolated from Brie cheese. *Journal of the Science of Food and Agriculture, 79*, 1788–1792.

Jarmolowska, B., Szlapka-Sienkiewicz, E., Kostyra, E., Kostyra, H., Mierzejewska, D., & Marcinkiewicz-Darmochwal, K. (2007). Opioid activity of Humana formula for newborns. *Journal of the Science of Food and Agriculture, 87*, 2247–2250.

Jinsmaa, Y., & Yoshikawa, M. (1999). Enzymatic release of neocasomorphin and β-casomorphin from bovine β-casein. *Peptides, 20*, 957–962.

Juillard, V., Laan, H., Kunji, E. R., Jeronimus-Stratingh, C. M., Bruins, A. P., & Konings, W. N. (1995). The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes beta-casein into more than one hundred different oligopeptides. *Journal of Bacteriology, 177*, 3472–3478.

Kahala, M., Pahkala, E., & Pihlanto, A. (1993). Peptides in fermented Finnish milk products. *Agricultural and Food Science in Finland, 2*, 379–386.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945–960.

Korhonen, H., & Pihlanto, A. (2007). *Bioactive peptides from food proteins. Handbook of food products manufacturing: Health meat, milk, poultry, seafood, and vegetables*. John Wiley & Sons. pp. 5–38.

Laugesen, M., & Elliott, R. (2003a). Ischaemic heart disease, Type 1 diabetes, and cow milk A^1 β-casein. *Journal of the New Zealand Medical Association, 116*, 1168.

Laugesen, M., & Elliott, R. (2003b). The influence of consumption of A^1 β-casein on heart disease and Type 1 diabetes – the authors reply. *Journal of the New Zealand Medical Association, 116*, 1170.

Macaud, C., Zhao, Q., Ricart, G., & Piot, J. M. (1999). Rapid new casomorphin-like peptide from a peptic casein hydrolysate second order derivative spectroscopy during HPLC. *Chromatography and Related Technologies, 22*, 401–418.

McLachlan, C. N. S. (2001). β-casein A^1, ischaemic heart disease mortality, and other illnesses. *Medical Hypotheses, 56*, 262–272.

Mierau, I., Kunji, E. R. S., Venema, G., & Kok, J. (1997). Casein and peptide degradation in lactic acid bacteria. *Biotechnology and Genetic Engineering Reviews, 14*, 279–301.

Morris, P.E., & FitzGerald, R.J. (2008). Whey proteins and peptides in human health. In: Onwulata, C. (Ed.), Whey Processing, Functionality and Health Benefits (pp. 287–345). UK: Woodhead Publishing.

Muehlenkamp, M. R., & Warthesen, J. J. (1996). Beta-casomorphins: Analysis in cheese and susceptibility to proteolytic enzymes from *Lactococcus lactis* ssp. *cremoris*. *Journal of Dairy Science, 79*, 20–26.

Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design, 13*, 773–791.

Napoli, A., Aiello, D., Di Donna, L., Prendushi, H., & Sindona, G. (2007). Exploitation of endogenous protease activity in raw mastitic milk by MALDI-TOF/TOF. *Analytical Chemistry, 79*, 5941–5948.

Parrot, S., Degraeve, P., Curia, C., & Martial-Gros, A. (2003). In vitro study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. Nahrung/Food, 47, 87–94.

Pellegrino, L., Hogenboom, J. A., Pazzaglia, C., & Todesco, R. (1995). La caratterizzazione analitica del formaggio Fontina sulla base della sua composizione in amminoacidi liberi. *Il Latte, 10*, 1086–1093.

Resmini, P., Pellegrino, L., Pazzaglia, C., & Hogenboom, J. A. (1985). Free amino acids for the quality control of Parmigiano-Reggiano cheese and particularly for the grated product. *Scienza e Tecnica Lattiero-Casearia, 36*, 557–592.

Robinson, R.K. (1995). A colour guide to cheese and fermented milks (pp. 49–51). London, UK: Chapman & Hall.

Rokka, T., Syväoja, E. L., Tuominen, J., & Korhonen, H. (1997). Release of bioactive peptides by enzymatic proteolysis of *Lactobacillus* GG fermented UHT-milk. *Milchwissenschaft, 52*, 675–678.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *Journal of Dairy Science, 83*, 1434–1440.

Schieber, A., & Brückner, H. (2000). Characterization of oligo-and polypeptides isolated from yoghurt. *European Food Research and Technology, 210*, 310–313.

Singh, T. K., Fox, P. F., & Healy, A. (1995). Water-soluble peptides in Cheddar cheese: Isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. *Journal of Dairy Research, 62*, 629–640.

Singh, T. K., Fox, P. F., & Healy, Á. (1997). Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *Journal of Dairy Research, 64*, 433–443.

Stepaniak, L., Fox, P. F., Sorhaug, T., & Grabska, J. (1995). Effect of peptides from the sequence 58-72 of β-casein on the activity of endopeptidase, aminopeptidase, and X-prolyl-dipeptidyl aminopeptidase from *Lactococcus lactis* ssp. *lactis* MG1363. *Journal of Agricultural and Food Chemistry, 43*, 849–853.

Tamime, A. Y., & Robinson, R. K. (1999). *Yoghurt: Science and Technology*. Boca Raton, USA: CRC Press.

Toelstede, S., & Hofmann, T. (2008). Sensomics mapping and identification of the key bitter metabolites in Gouda cheese. *Journal of Agricultural and Food Chemistry, 56*, 2795–2804.

Tzvetkova, I., Dalgalarrondo, M., Danova, S., Iliev, I., Ivanova, I., Chobert, J. M., & Haertle, T. (2007). Hydrolysis of major dairy proteins by lactic acid bacteria from Bulgarian yogurts. *Journal of Food Biochemistry, 31*, 680–702.

Wedholm, A., Moller, H. S., Lindmark-Mansson, H., Rasmussen, M. D., Andren, A., & Larsen, L. B. (2008). Identification of peptides in milk as a result of proteolysis at different levels of somatic cell counts using LC MALDI MS/MS detection. *Journal of Dairy Research, 75*, 76–83.

Yvon, M., Chabanet, C., & Pélissier, J. P. (1989). Solubility of peptides in trichloroacetic acid (TCA) solutions. Hypothesis on the precipitation mechanism. *International Journal of Peptide and Protein Research, 34*, 166–176.