Impact of *in vitro* static digestion method on the release of β-casomorphin-7 from bovine milk and cheeses with A1 or A2 β-casein phenotypes

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Abstract

Beta-casomorphin-7 (BCM7) represents the fragment Val^{60}-Ile^{66} of bovine β-casein (β-CN), and there is evidence that it is more easily released during gastrointestinal digestion (GID) of A1 β-CN variant, in comparison to the A2 variant. This study aimed at investigating the effect of type of enzymes and the protease/protein (P/S) ratio on BCM7 release during the intestinal step of *in vitro* static GID of bovine milk and cheeses with A1 or A2 β-CN phenotypes. BCM7 occurred in digests of both A1 and A2 samples, being the release more marked for A1 counterparts. Nonetheless, the BCM7 release depended on both the type of GID enzymes and the-P/S ratio. These findings highlight the importance of GID conditions which may affect the outcomes for possible differences between A1 and A2 milk based on BCM7 release during *in vitro* GID.

Keywords: β-casomorphin-7; A1 and A2 bovine β-casein; milk; cheese; *in vitro* static gastrointestinal digestion

1. Introduction

Beta-casomorphin-7 (BCM7) is the peptide representing the fragment Val^{60}-Ile^{66} of bovine β-casein (β-CN), the most abundant CN fraction in cow's milk. It was firstly identified after enzymatic digestion of CN, and it appeared to display an opioid-like activity in guinea pig ileum (Brantl & Teschemacher, 1979; Brantl, Teschemacher, Henschen, & Lottspeich, 1979). Jinsmaa and Yoshikawa (1999) firstly reported that enzymatic release of BCM7 by gastrointestinal proteases *in vitro* depended on the genetic variant of β-CN. Indeed, this CN presents the largest polymorphism (Farrell et al., 2004), being the A1 and A2 variants the most widespread among dairy herds. The difference between these two variants concerns a single amino acid substitution in a position 67 of the mature protein sequence: proline for β-CN A2 and histidine for β-CN A1. It was found that pepsin and leucine aminopeptidase were responsible for the N-terminus cleavage, while only pancreatic elastase was able to release BCM7 from C-terminus when His was in a position 67 (A1 variant) (Jinsmaa and Yoshikawa, 1999). The hypothesis was that peptide bond was resistant to enzymatic cleavage in A2 instead of A1. Despite further researchers strengthened this theory, the release of BCM7 has been also observed during *in vitro* and *ex vivo* enzymatic hydrolysis of the A2 variant (Asledottir et al., 2017; Asledottir et al., 2018; De Noni, 2008; Ul Haq, Kapila R. & Kapila S., 2015). Nonetheless, the yield of BCM7 was significantly lower than that recorded after the digestion of A1 variant (Asledottir et al., 2017; Asledottir et al., 2018; Cieślińska et al., 2007; Cieślińska et al., 2012; Duarte-Vazquez et al., 2017). Recently, the release of BCM7 was observed during digestion of A2-type milk using an *in vitro* semi-dynamic protocol for GID (Lambers, Broeren, Heck, Bragt, & Huppertz, 2021). These authors demonstrated that slightly different BCM7 amounts were found in digests of A1 and A2 raw milk samples.

Since the studies of Brantl and collaborators (Brantl & Teschemacher, 1979; Brantl et al., 1979), several research groups investigated the biological consequences of the opioid activity of BCM7 with particular interest towards its potential to influence the digestive system (Brooke-Taylor, Dwyer, Woodford, & Kost, 2017; Daniloski et al., 2021a; Daniloski, McCarthy & Vasiljevic, 2021b; Ho, Woodford, Kukuljan, & Pal, 2014; Kay et al., 2021; Küllenberg de Gaudry et al., 2019; Woodford, 2021). In 2009, BCM7 received special attention also by EFSA, which stated that "a cause-effect

relationship between the oral intake of BCM7 or related peptides and aetiology or course of any suggested non-communicable diseases cannot be established" (EFSA, 2009). Based on the literature of the last decade, recent review articles confirmed such conclusions providing only evidence supporting the biological activity of BCM7 at intestinal level *in vivo* (Brooke-Taylor et al., 2017; Küllenberg de Gaudry et al., 2019; Summer et al., 2020).

To date, negative effects of A1 milk (and derived products) have been not recognized by official health bodies (EFSA, 2009). Nonetheless, the A1/A2 milk hypothesis is still receiving attention as demonstrated by numbers of research articles concerning this topic and commercial strategies of milk companies. Moreover, several methods were recently proposed and compared for accurate assessing of the dairy product β-CN phenotypes (Fuerer et al, 2020; Giglioti et al., 2020; Mayer, Lenz, & Halbauer, 2021). At the same time, static, semi-dynamic and dynamic *in vitro* gastrointestinal digestion (GID) protocols have been developed (Mulet-Cabero et al., 2020; Xavier and Mariutti, 2021) and largely used to unveil the potential release of BCM7 from dairy foods (De Noni and Cattaneo 2010; De Noni, Stuknytė, & Cattaneo, 2015; Duarte- Vázquez et al., 2017; Lambers et al., 2021). The *in vitro* GID methods aim to overcome the ethical, technical and financial issues related to *in vivo* studies with humans and animals. For these reasons, a standardized *in vitro* protocol (INFOGEST 1.0) was developed within the framework of the COST action INFOGEST (Minekus et al., 2014), and it attained an international consensus. Another *in vitro* static method (INFOGEST 2.0) (Brodkorb et al., 2019) introduced some changes to the INFOGEST 1.0.

Even if there is evidence that BCM7 is more easily released during GID of A1-like dairy products, less known is the role played by the *in vitro* static GID method on this phenomenon. Based on the above-mentioned, the aim of this work was to study the influence of type of intestinal enzymes and the protease-to-(sample)protein (P/S) ratio on the BCM7 release during *in vitro* static GID. To this purpose, different GID conditions were used to digest *in vitro* bovine milk and cheeses with A1 or A2 β-CN phenotypes.

2. Materials and methods

2.1. Milk and cheese samples

Milk was collected from two groups (10 herds each) of Holstein-Friesian cows having the genotype β-CN A^1A^1 (A1) or A^2A^2 (A2). To this aim, each cow was genotyped according to β-CN A1 and A2 variants as described by Caroli, Chessa & Erhardt (2009) with the EuroGenomics genotyping beadchip, utilizing the Infinium assay technology (Illumina, San Diego, CA, USA).

Milk collected from single cows of A1 or A2 group was blended to form representative A1 and A2 bulk milk batches. The phenotype of the two batches was confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) as described by Visser, Slangen and Rollema (1991).

The raw A1 and A2 milk batches were used for preparing mozzarella (a fresh pasta filata cheese) and a hard cooked cheese ("grana type", hereafter referred as grana) at a cheese factory according to the procedures reported in Fig. 1. Cheese makings were carried using 250–300 L of milk. All milk and cheese samples were stored at -24 °C until they were subjected to chemical characterization and *in vitro* static GID.

The determination of protein, fat, lactose and dry matter contents of the studied dairy samples was carried out adopting the International standards ISO: 8968-1 (2014), 3433 (2018), 22662 (2007) and 2920 (2004), respectively. The main compositional features of A1 and A2 milk batches and derived cheese samples were reported in Table 1.

The quantity of β-CN in A1 or A2 milk and cheese samples was calculated by considering the chromatographic peak area of β-CN, the protein content of milk and cheeses, and their CN content, which was considered equal to 80% and 95% of total protein content for milk and cheeses, respectively. Due to proteolysis occurring in ripening, RP-HPLC of grana and hence quantitation of β-CN amount was carried out on cheese after brining. Analyses were run in triplicate and mean values were reported.

2.2. In vitro static gastrointestinal digestion (GID)

Raw whole A1 and A2 milk samples, mozzarella and grana cheeses were digested *in vitro* using the three static GID methods INFOGEST 1.0 (Minekus et al., 2014), INFOGEST 2.0 (Brodkorb et al., 2019) (named here and forward NP and HP, respectively) and LP. The latter one differs from

the HP in pancreatin-to-protein ratio as reported in Table 2. Oral phase was the same in all methods and carried out according to the HP method. Salivary, gastric and intestinal simulated fluids (SSF, SGF and SIF, respectively) were prepared according to the HP GID method.

Five mL of milk (169–176 mg total protein), or 5.00 g of grinded mozzarella (1055–1115 mg total protein) or grana (1755–1900 mg total protein) cheeses were supplemented with 5 mL SSF. Cheese samples and SSF were mixed in a mincer at pH 7.0 for 2 min to reproduce the salivary phase of digestion. The gastric phase was performed by adding 10 mL SGF, porcine pepsin (2000 U/mL digest) and, in the case of HP and LP methods, rabbit gastric lipase (60 U/mL) along with 1M HCl to reach pH 3.0. The gastric phase was simulated at 37 °C for 2 h in slight stirring. Subsequently, 20 mM bile salts dissolved in 20 mL of SIF were added to each digest. Upon completion of the gastric phase, the following intestinal enzymes were used: NP, porcine trypsin (100 U/mL digest), bovine chymotrypsin (25 U/mL digest), pancreatic lipase (2000 U/mL digest) and colipase (1:1 molar ratio colipase:pancreatic lipase); HP, porcine pancreatin (8 x USP, the same trypsin activity as the NP ~~protocol~~ method); LP, porcine pancreatin (8 x USP) at different P/S ratio as reported in Table 2.

The intestinal phase was performed at 37 °C for 2 h at pH 7.0 (by adding 1 M NaOH) in slight stirring. The samples were immediately frozen at the end of the GID. The activities of the enzymes were determined according to Brodkorb et al. (2019). The enzymes were from Merck (Darmstadt, Germany). Each digestion was carried out in triplicate.

The effect of the P/S ratio on the BCM7 release was evaluated by digesting 5.00 g or 0.76 g of mozzarella applying the NP method and analysing the related digests taken at 0.5 h time interval during the intestinal phase (2 h).

2.3. Determination of protein breakdown

To assess the extent of protein breakdown during GID of mozzarella, the amount of the soluble nitrogenous fraction was determined by analysing the permeate deriving from the 3 kDa ultrafiltration of the samples before and the end of the GID according to the NP method. The ultrafiltration permeates were obtained using a stirred ultrafiltration cell equipped with a regenerated

cellulose membrane (Amicon, Merck, Darmstadt, Germany). Undigested milk and digests were directly ultrafiltered, whereas undigested cheeses were suspended in water prior to ultrafiltration.

The protein breakdown (as % of total nitrogen, N_T) was calculated by Eq. (1):

$$(N_{GID} - N_b) - N_{SB}/N_T \times 100 \quad (1)$$

where:

N_{GID}, N (nitrogen) content of the UF (3 kDa) permeate of the samples after GID;

N_b, N content of the UF (3 kDa) permeate of the blank sample (enzymes and simulated digestive fluids) after GID;

N_{SB}, N content of UF (3 kDa) permeate of the samples before GID;

N_T, total N content of the samples.

The N content of each fraction was determined by Kjeldahl method according to the International standard ISO 8968-2014.

2.4. UPLC/HR-MS analyses

UPLC/HR-MS analysis was conducted to identify and quantify the presence of BCM7. Before UPLC/HR-MS analysis, digests were ultrafiltered using an Omega polyethersulfone UF membrane (cut-off 3 kDa) in a Nanosep Advance device (Pall, Port Washington, NY, USA). An Acquity UPLC module (Waters, Milford, MA, USA) with Aeris PEPTIDE XB-C18 column (150×2.1 mm, 1.7 µm) (Phenomenex, Torrance, CA, USA) was coupled to a Q Exactive instrument (Thermo Fisher Scientific, San Jose, CA, USA) interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The column was kept at 40 °C. The eluents were 0.1% (v/v) formic acid (FA) in MilliQ-treated water (solvent A) and 0.1% (v/v) FA in acetonitrile (solvent B). A linear elution gradient was applied (14% to 28% of solvent B in 14 min) at a flow rate of 0.3 mL min^{-1}. Mass spectrometer parameters were set as previously described (Cattaneo et al., 2020). Targeted selected ion monitoring (t-SIM) and data dependent tandem MS analysis ($ddMS^2$) method was applied. Identification and quantification of BCM7 was conducted using the Xcalibur software (v3.0,

Thermo Fisher Scientific) and the synthetic peptide as an external standard (5 points calibration curve). Peak areas were calculated from extracted t-SIM chromatograms of BCM7 with 3 ppm mass tolerance. Results were expressed as means ± standard deviations. Beta-casomorphin-7 was quantified in 3 kDa-ultrafiltered digests of three digest replicates and each acquired by UPLC/HR-MS in triplicate runs.

2.5. Statistical analyses

Significance of the results was analyzed by one-way analysis of variance (ANOVA) followed by a post hoc *t* test. ANOVA was performed with Daniel's XL Toolbox add-inn for Excel, version 6.60, by Daniel Kraus, Würzburg, Germany (available at: http://xltoolbox.sourceforge.net/). P-value < 0.05 indicated statistical significance. Three independent experiments were performed, and all results were expressed as the mean ± standard deviation in this study.

3. Results and discussion

3.1. Determination of BCM7 release from A1 and A2 milk and derived cheeses

In the present work, we evaluated the release of BCM7 from raw bovine milk with A1 or A2 β-CN phenotypes and derived cheeses (mozzarella and grana) after *in vitro* static GID. The A1 and A2 batches of bulk milk intended for mozzarella or grana cheese production presented a similar gross composition (Table 1). The lower fat content of the milk batches used to produce grana cheese was due to the natural creaming step adopted prior to cheese making (Fig. 1). As expected from the genotyping assay of cows, the RP-HPLC analyses of β-CN phenotype confirmed the two bulk milk batches to contain exclusively A1 or A2 β-CN variant. Based on the same RP-HPLC approach, β-CN represented about 38% of the total CN, as percentages of total chromatographic peak areas of CN fractions of the two types of milk (data not shown; chromatograms are provided only for the referee).

In mozzarella cheese, proteolysis is ~~almost~~ limited to splitting of caseinomacropeptide from κ-CN due to rennet action, and only a minor part of κ-CN is lost in whey during milk coagulation (Walstra, Wouters, & Geurts, 2005). As a matter of fact, the proportion of β-CN on total casein in

mozzarella was only slightly higher (about 41%) than in milk. Due to proteolysis occurring in ripening, the evaluation of the phenotype and amount of β-CN was assessed on grana only after brining. The latter almost overlapped that revealed for mozzarella samples. Overall, the quantity of β-CN in milk and cheeses (calculated as described in the Materials and methods) was in the ranges 1.02–1.07, 8.61–8.69, and 13.67–14.41 g/100 g in milk, mozzarella, and grana samples, respectively (Table 1).

Most of the available *in vitro* GID methods are based on static conditions (Xavier and Mariutti, 2021). They generally use fixed pH values for gastric and duodenal step of GID, whereas they can present differences in other GID parameters. To better resemble *in vivo* pH and kinetic conditions, semi-dynamic protocols have been developed, including that developed within the COST Action INFOGEST in 2020 (Mulet-Cabero et al., 2020) based on the physiological conditions previously published in the standardized static protocols INFOGEST 1.0 and 2.0 (Minekus et al., 2014; Brodkorb et al., 2019). As previously mentioned, applications of the semi-dynamic INFOGEST protocol regarded also BCM7 release from bovine raw A1 and A2 milk samples (Lambers et al., 2021). Nonetheless, even the semi-dynamic methods show limitations related to some variations like the type of equipment used for simulating digestion and gastrointestinal motility. As a matter of fact, despite of the method, even small difference in *in vitro* GID parameters could affect outcomes regarding occurrence of molecules with nutritional, physiological, or pathological interest.

In the present study, we subjected bovine raw milk and cheeses with A1 or A2 β-CN phenotypes to static *in vitro* GID according to HP method (Brodkorb et al., 2019). The use of pancreatin instead of single intestinal enzymes (trypsin and chymotrypsin) and the use of rabbit gastric lipase in the gastric phase differentiated the HP and LP methods from NP (Minekus et al., 2014). As reported in Materials and methods and in Table 2, the LP procedure differs from the HP in pancreatin-to-protein ratio. The different P/S ratios (Table 2) during GID resulted from the different protein content of the sample (Table 1). The use of a lower pancreatin activity (measured as trypsin activity) justifies the lowest P/S ratio in the LP method.

We revealed the BCM7 in all milk digests, despite of the β-CN phenotype and the applied GID method (Fig. 2A). Considering the amount of β-CN in milk, the quantity of this peptide was expressed as mg BCM7/g β-CN. As found in our previous studies (De Noni, 2008; De Noni &

223 Cattaneo, 2010), the content of BCM7 was only a negligible part of the total theoretical quantity
224 releasable from complete digestion of β-CN. Nonetheless, the amounts of BCM7 differed according
225 to the applied GID in BCM7 formation between A1 and A2 samples appeared to be somewhat
226 smaller than those described by Asledottir et al. (2018). These last authors reported the release of
227 BCM7 from bovine milk containing the variants A1, A2, F or I of β-CN during *ex vivo* gastrointestinal
228 digestion. BCM7 released from all variants, although the highest amounts of BCM7 were found in
229 the digested A1 milk sample. Four mg BCM7/g β-CN were detected in milk containing variant A1
230 after 120 min duodenal digestion, compared to about 1.4 mg/g β-CN from milk with variant A2.
231 Comparable findings arose from the study of Duarte-Vázquez et al. (2017), who found approximately
232 3-fold more BCM7 in cow's milk with A1/A2 variants (2.11±0.19 mg/100 mL) than in A2 milk
233 (0.74±0.008 mg/100 mL) after *in vitro* simulated GID.
234 Interestingly, the BCM7 amounts in HP and LP digests were lower than those recorded in
235 the NP counterparts, especially for the A2 milk samples (Fig. 2A). In detail, the BCM7 amounts found
236 in A1 milk samples were about 1.3- and 2.6-fold lower for HP and LP digests, respectively, in
237 comparison to their NP-digested counterparts. In A2 samples, the quantity of BCM7 decreased by a
238 factor of 5.2 and 7.3, respectively. It can be hypothesized that the low amount of BCM7 resulted from
239 degradation of BCM7 likely occurring when pancreatin is used, due to the presence of proteases
240 other than trypsin and chymotrypsin (e.g., elastase) potentially capable of degrading BCM7
241 (Asledottir et al., 2019). Asledottir et al. (2019) reported BCM7 to be partly digested by
242 gastrointestinal human enzymes, as several fragments were detected after digestion of synthetic
243 BCM7 using human gastrointestinal juices. Apart from breakdown, the low levels of BCM7 recorded
244 when the LP method was applied could also be explained by the scarce release of this peptide due
245 to the low intestinal protease activity.
246 In the present work, we also applied the three GID methods to mozzarella and grana cheeses
247 (Fig. 2B–D). Different amounts (0.43±0.01, 0.34±0.02 mg and 0.14±0.001 mg BCM7/g β-CN) of
248 BCM7 were released in the NP, HP and LP digests of A1-type mozzarella (Fig. 2B). Very small levels
249 of the peptide characterized the A2 digests (0.08±0.03 mg BCM7/g β-CN in NP, and 0.02±0.001 mg
250 BCM7/g β-CN in both LP and HP) (Fig. 2B). As shown in Fig. 2C, quite similar amounts of BCM7

were found in the digests of A1 grana cheese ripened for 3 months in comparison to mozzarella considering the same digestion method (0.40±0.003, 0.42±0.004 and 0.11±0.002 mg BCM7/g β-CN in NP, HP and LP, respectively). In the A2 counterparts, the BCM7 amount was in the range 0.02–0.04 mg BCM7/g β-CN. The BCM7 values observed in the 3-month A2 grana cheese also characterized the digests of the A2 sample ripened for 6 months (0.01–0.04 mg BCM7/g β-CN) (Fig. 2D). Contrarily, we revealed higher amounts of the peptide in the digest of 6-month ripened A1 grana cheese (0.48±0.004, 0.56±0.001 and 0.35±0.004 mg BCM7/g β-CN upon NP, HP and LP digestions, respectively).

To the best of our knowledge, data concerning the release of BCM7 during *in vitro* GID of mozzarella are not reported in literature to date. Concerning "grana type" cheeses, investigations refer only to our previous studies. De Noni and Cattaneo (2010) reported BCM7 levels in different cheeses digested *in vitro* according to a method using pepsin and Corolase PP™ as gastric and intestinal enzymes, respectively. This method differs from the static methods here adopted in relation to diverse intestinal enzymes and parameters used for GID, digestion fluids, pH and time. Upon this GID, the amount of BCM7 in Grana Padano digests (10–25 month ripened) was 8.79–12.55 mg/kg. This would account for about 0.07–0.10 mg BCM7/g β-CN. De Noni, Stuknytė and Cattaneo (2015) also studied the occurrence of BCM7 in Grana Padano cheese (11-months-old) after *in vitro* GID using the static protocol NP (Minekus et al., 2014). The amount of BCM7 found at the end of intestinal phase was 0.12 mg/kg (i.e., lower than 0.01 mg BCM7/g β-CN). Nonetheless, in both these studies the β-CN phenotype of the cheeses was not assessed.

3.2. Effect of the protease-to-(sample)protein (P/S) ratio on the release of BCM7 during GID

According to the methods adopted in the present work, the same amount (5 g or 5 mL) of cheese or milk was submitted to GID, despite the sample protein content and hence a different P/S ratio during digestion (Table 2). As a matter of fact, the more consistent release of BCM7 deriving from milk digestion in comparison to that arising from cheese digestion (Fig. 2 A–D) could be explained by the adoption of a high P/S ratio during the intestinal phase.

To verify the potential effect of P/S ratio on the release of BCM7, 0.76 g or 5.00 g of mozzarella cheese were also digested with the NP method. Mozzarella (differently from grana) was chosen as its casein matrix is not proteolyzed. Indeed, the curd acidification was attained by adding citric acid to milk, and the cheese was not ripened. The lowest amount (0.76 g) of mozzarella corresponded to a total protein amount (about 170 mg) overlapping that characterizing 5 mL of milk. The NP method was adopted because the intestinal phase is carried out using single enzymes the activities of which have been measured. In the HP method the amount of pancreatin to be used is based only on the determination of trypsin activity, but not on that of the other proteolytic intestinal enzymes. We took the related digests at 0.5 h time intervals during the intestinal phase and quantified their BCM7 content. When the lowest amount of mozzarella was digested, the intestinal P/S ratio was the same as occurred during milk GID. In the presence of both high (Fig. 3A) and low (Fig. 3B) amount of (protein) substrate the release of BCM7 increased during intestinal digestion and reached its maximum at the end (2 h) of intestinal phase for both A1 and A2 samples. The BCM7 content in digests from cheese with the A1 β-CN phenotype was about 7-fold higher than in A2 mozzarella in the presence of high amount of sample. When digesting 0.76 g of A1 or A2 mozzarella cheeses, the rate of BCM7 release was found to be similar between A1 and A2 samples (Fig. 3B). At the end of intestinal digestion, the peptide amount was quite the same in the two digests (0.39±0.01 and 0.41±0.01 mg BCM7/g β-CN, respectively) (Fig. 3B). Interestingly, the peptide amount in the A2 cheese digest increased about seven times when 0.76 g, instead of 5.00 g, of the cheese were digested. On the contrary, we revealed the same final BCM7 level in A1 digests despite of the cheese amount submitted to GID. These findings show that the release of BCM7 during GID of A2 mozzarella was particularly affected by the initial sample amount (and hence P/S ratio).

To further ascertain the role played by the initial sample (protein) amount on the general proteolysis, we determined the soluble N fraction (as % of N_T) in the above-considered A1 and A2 mozzarella digests at the end of GID applying the NP method (Fig. 3). The β-CN phenotype did not affect the overall protein breakdown, whereas the degree of protein breakdown strongly depended on the adopted P/S ratio: higher P/S ratio translated into a more consistent protein breakdown. Butré, Sforza, Gruppen and Wierenga (2014) demonstrated that an increase in substrate concentration

306 resulted in a decrease in protein breakdown rate during hydrolysis of WPI with a *Bacillus*
307 *licheniformis* protease. The negative effect of high substrate concentration on protein hydrolysis has
308 been demonstrated in other studies carried out by keeping constant the concentration of (different)
309 enzymes and varying the substrate concentration, as revealed for rapeseed protein isolates and
310 micellar casein by Chabanon, Chevalot, Framboisier, Chenu and Marc (2007) and Camacho et al.
311 (1993), respectively.

312 Interestingly, as above mentioned, when a low P/S ratio was adopted (Fig. 3A), the A1 and
313 the A2 mozzarella cheese digestion yielded a markedly different amount of BCM7, although in the
314 presence of the same protein breakdown. These findings could be justified by the fact that the
315 release of BCM7 relies on the cutting action of specific enzymes rather than on the overall degree
316 of protein hydrolysis, similarly to what demonstrated by Spellman, O'Cuinn and FitzGerald (2005)
317 for certain bitter peptides released during enzymatic hydrolysis of whey proteins at different initial
318 total solids' levels.

319

320 **4. Conclusions**

321 This research study confirms the formation of BCM7 during the *in vitro* static GID of both A1
322 and A2 milk and cheese samples. The peptide release was more marked for A1 samples, despite of
323 the adopted GID method. Nonetheless, the amount of the released BCM7, as well as the related
324 differences among A1 and A2 samples, depended on both the quantity of digested protein and the
325 used GID method. These findings raise the need for adopting the same *in vitro* GID conditions to
326 achieve comparable results, especially when the release of bioactive peptides deserves to support
327 certain health benefits for the consumer. From this point of view, based on our findings, the
328 perspective of possible health-related differences between A1 and A2 milk based on the occurrence
329 of BCM7 during *in vitro* GID seems weakened. Differently, it somehow keeps when cheeses are
330 digested *in vitro*. Nonetheless, other digestive parameters (e.g. possible degradation of BMC7 in gut
331 epithelium by brush border enzymes) should be studied to support these findings, and *in vivo* studies
332 are necessary to definitively validate the (different) occurrence of BCM7 revealed in intestinal digests
333 during static *in vitro* GID of A1 and A2 milk and cheese.

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CRediT authorship contribution statement

Stefano Cattaneo: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Ivano De Noni:** Conceptualization, Funding acquisition, Methodology, Data curation, Supervision, Writing - original draft, Writing - review & editing. **Fabio Masotti, Milda Stuknytė:** Investigation, Formal analysis, Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions

Fig. 1. Flow-chart of mozzarella and grana cheese makings.

Fig. 2. Release of β-casomorphin-7 (BCM7) from (A) milk, (B) mozzarella cheese and grana cheese ripened (C) 3 or (D) 6 months presenting the A1 (gray bars) or A2 (white bars) β-casein phenotypes and submitted to *in vitro* static GID applying three different methods (NP, HP and LP). Upper case letters indicate differences ($P < 0.05$) of mg BCM7/g β-CN ratio among three GID methods; lower case letters indicate differences ($P < 0.05$) of mg BCM7/g β-CN ratio between A1 and A2 phenotypes applying the same GID method.

Fig. 3. Release of β-casomorphin-7 (BCM7) during *in vitro* static intestinal digestion of different amounts (A: 5.0 g, B: 0.76 g) of mozzarella cheese obtained from milk presenting the A1 (solid line) or A2 (dotted line) β-casein phenotypes, adopting the NP method. The values close to the symbols indicate the protein breakdown (as % of soluble nitrogen (<3kDa) on total nitrogen) at the end (2 h) of the GID.