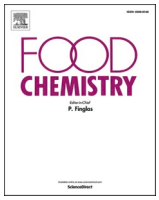
Food Chemistry 506 (2026) 148157

Contents lists available at ScienceDirect

Food Chemistry

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



Release of β-casomorphin-7 in A1/A1 and A2/A2 dairy systems: Insights from simulated semi-dynamic gastrointestinal digestion

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ARTICLE INFO

Keywords:
β-Casomorphin-7
A1/A1 and A2/A2 dairy products
In vitro gastrointestinal digestion
Opioid activity
INFOGEST

ABSTRACT

The study aimed to determine the amount of β-casomorphin-7 released during semi-dynamic *in vitro* gastrointestinal digestion of A1/A1 and A2/A2 milk, yogurt, and Cheddar cheese. Samples were collected at 10 and 120 min during the intestinal phase for each of the four gastric emptying points, following the INFOGEST digestion model. β-Casomorphin-7 was quantified using liquid chromatography coupled to triple quadrupole mass spectrometry. The levels of β-casomorphin-7 were similar in A1/A1 (8.65–11.72 μg/100 g protein) and A2/A2 (9.64–15.29 μg/100 g protein) milk digests, higher in A1/A1 yogurt, while A1/A1 cheese digest exhibited the highest levels among all samples (36.19–62.73 μg/100 g protein), nearly ten times higher than A2/A2 cheese digest. After *in vitro* gastrointestinal digestion of a serving size of the studied dairy products the released amounts may not align with levels required for *in vivo* opioid activity. However, further human clinical trials are warranted.

1. Introduction

Encoded within the β-casein polypeptide chain in a latent state, numerous peptides can be released through enzymatic hydrolysis (Brantl et al., 1981). The liberated peptides can influence the functionality of milk and dairy products and possibly affect human health in both, advantageous and disadvantageous manners (de Vasconcelos et al., 2023). One group of peptides that can be released is known as β-casomorphins, identified as active binders to μ-receptors in the digestive, nervous, and immune systems. These peptides share a common initial N-terminal sequence, consisting of the following three amino acids: tyrosine, proline and phenylalanine (Hautefeuille et al., 1986).

Within this family, β-casomorphin-7, first identified by Henschen et al. (1979), has been the most studied and discussed peptide, particularly in bovine dairy products (Nguyen et al., 2015). β-Casomorphin-7, a fragment of β-casein (fragment [f]: tyrosine^{60} isoleucine^{66}), consists of seven amino acids (tyrosine-proline-phenylalanine-proline-glycine-proline-isoleucine) and has a molecular weight of 790 Da (Markoska et al., 2021). It potentially exerts morphine-like activity, with an estimated opioid activity of 55.00 μmol/L in guinea pig ileum, though its specific activity is approximately 250 times lower than normorphine (Brantl et al., 1979; Henschen et al., 1979). Its physiological potential for various health conditions, including digestive discomfort, diabetes, cardiovascular diseases, neurological disorders, and pulmonary inflammation, has been widely speculated (Summer et al., 2020). However, both the European and New Zealand Food Safety Authorities (De Noni et al., 2009; Swinburn, 2004) have concluded that there is insufficient evidence to definitively relate β-casomorphin-7 to these health outcomes. More recent systematic reviews have reached similar findings, indicating that while no conclusive associations were established, the possibility of such an association cannot be entirely ruled out (Brooke-Taylor et al., 2017; Daniloski, Cunha, et al., 2021; Küllenberg

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https://doi.org/10.1016/j.foodchem.2026.148157
Received 21 May 2025; Received in revised form 12 December 2025; Accepted 22 January 2026
Available online 23 January 2026

de Gaudry et al., 2019). This ambiguity arises because β-casomorphin-7 is identical regardless of which genetic variant of its precursor, β-casein, it is derived from. Although many β-casein variants exist, the most commonly secreted in bovine milk are β-caseins A1 and A2 (Daniloski, McCarthy, & Vasiljevic, 2021). The only distinction between these two proteins arises from the point mutation in the sixth chromosome of the *CSN2* gene, resulting in the replacement of proline in β-casein A2 with histidine in β-casein A1 at position 67 of the polypeptide chain (Aschaffenburg, 1963).

Nearly five decades ago, a study conducted by Brantl et al. (1979) regarding the resistance of proline-rich peptides to enzymatic cleavage, laid a foundational understanding of β-casein digestion and the release of β-casomorphin-7. Subsequent studies by Jinsmaa and Yoshikawa (1999), and most recently by Markoska (2023), have highlighted that the additional proline in β-casein A2 tightly packs the molecule, shielding the isoleucine^{66} - proline^{67} bond and potentially reducing protease access, which hinders the release of β-casomorphin-7. Despite this, β-casomorphin-7 was released during gastrointestinal digestion of milk (Asledottir et al., 2018; Lambers et al., 2021; Nguyen et al., 2021; Nguyen Busetti et al., 2015), yogurt (Nguyen et al., 2014; Nguyen et al., 2018a, 2018b), and cheese (Cattaneo et al., 2023; De Noni et al., 2015; Muehlenkamp & Warthesen, 1996) with both β-casein variants, albeit, more readily liberated from dairy products with β-casein A1.

The exact conditions, including temperature, pH, enzyme activity or β-casein variant under which β-casomorphin-7 is released remain unclear. However, recent research suggests that the structural conformation of this peptide (Markoska et al., 2021), along with the gastric digestion of dairy products formulated from milk samples that shared the same genetic variants for all major whey proteins and the caseins, except for β-casein, may shed light on the variations in gastrointestinal digestion of products containing β-casein A1 *versus* A2 (Daniloski, Hailu, et al., 2024; Daniloski, Page, et al., 2024; Daniloski, Vasiljevic, et al., 2024). In addition to β-casein sequence and variant, the gastrointestinal fate of β-casomorphin-7 might be influenced by the complexity of dairy food matrices. Factors such as casein micelle packing, colloidal calcium phosphate content, milk-fat-globule-membrane integrity, and processing-induced microstructures (*e.g.*, heat treatment, homogenization, and gelation) can modulate protease accessibility and digestion kinetics, thereby affecting the extent of β-casomorphin-7 release and its potential bioavailability (Barbé et al., 2013; Everett, 2025; Le Feunteun et al., 2014; Mulet-Cabero et al., 2024). Thus, this study aimed to elucidate the extent to which the genetic variant of β-casein and the dairy matrix influence the release of β-casomorphin-7 during the gastrointestinal digestion of A1/A1 and A2/A2 skim milk, yogurts, and Cheddar cheeses processed at conditions adapting industrially relevant processing procedures at pilot scale.

2. Materials and methods

2.1. Skim milk powder, yogurt, and Cheddar cheese samples

Milk used to produce the skim milk powder, yogurt and cheese was collected from individual Irish Holstein cows from Moorepark Dairy Farm at the Teagasc Animal and Grassland Research and Innovation Centre (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland). The cows were homozygous for the β-casein A1/A1 and A2/A2, as determined by an in-house developed DNA-based (allele-specific primer) protocol (Fitzgerald et al., 1999) and further confirmed by the Irish Cattle Breeding Federation database (www.icbf.com). To minimize the bias, apart from having either β-casein A1/A1 or A2/A2, all cows selected further contained the same α-lactalbumin, β-lactoglobulin, α_s- and κ-casein variants.

The production of skim milk, yogurt, and cheese followed distinct methods, with skim milk and yogurt made from reconstituted skim milk powders (Daniloski, Hailu, et al., 2024; Daniloski, Vasiljevic, et al., 2024), while cheese was made from raw milk followed by standardization and pasteurization (Daniloski, Page, et al., 2024). Skim milk powders were rehydrated in Milli-Q water, stirred for 1 h, and left to hydrate overnight at 4 °C. Skim milk and yogurt were produced using rehydrated milk which was mixed from eight A1/A1 cows and ten A2/A2 cows, respectively, with three technical replicates for each. Yogurt production involved heating the rehydrated skim milk to 85 °C for 10 min, cooling it to 43 °C, and inoculating it with a starter culture (2% *w/w*) of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (YC-380, Chr. Hansen, Hørsholm, Denmark). The fermentation process continued at 43 °C until a pH of 4.60 was achieved. For Cheddar cheese production, raw milk was mixed from ten A1/A1 cows and ten A2/A2 cows, respectively, and standardized to a protein-to-fat ratio of 0.95, pasteurized at 72 °C for 15 s (MicroThermics®, Raleigh, NC, USA), and inoculated with starter cultures containing *Lactococcus lactis sub. Lactis and Lactococcus lactis sub. Cremoris (R-604, Chr. Hansen, Hørsholm, Denmark), and Lactobacillus helveticus (LH-B02, Chr. Hansen, Hørsholm, Denmark)*. Chymosin (CHY-MAX Plus, Chr. Hansen, Hørsholm, Denmark) was added for curd formation, followed by cooking at 38 °C, cheddaring, salting, and pressing. The cheese was ripened for 180 days, with three biological replicates conducted for each β-casein phenotype (A1/A1 and A2/A2).

Determination of protein and overall composition of the milk, yogurt and cheese samples have earlier been comprehensively explained in studies from the same research group (Daniloski, Hailu, et al., 2024; Daniloski, Page, et al., 2024; Daniloski, Vasiljevic, et al., 2024). All chemicals used in this study were purchased from Sigma (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) unless otherwise indicated.

2.2. Identification of β-casein variants by reversed phase high performance liquid chromatography

Milk protein variants in the samples were verified using Reversed Phase High Performance Liquid Chromatography (RP-HPLC) with the RP-HPLC data presented previously (Daniloski, Hailu, et al., 2024; Daniloski, Page, et al., 2024; Daniloski, Vasiljevic, et al., 2024). An Agilent 1200 RP-HPLC system featuring a quaternary pump, heated column compartment, temperature-controlled autosampler, and a multiwavelength detector was employed, with data processed using ChemStation software (Agilent Technologies, Ballytrasna, Co. Cork, Ireland). Separation and identification of individual milk proteins were performed on a Poroshell 300SB-C_{18} column (2.1 mm diameter, 75 mm length, 5 μm) paired with a Zorbax Poroshell guard column (1.0 mm diameter, 17 mm length, 5 μm), both from Agilent Technologies, Ireland. The elution process used two mobile phases: a mixture of acetonitrile, milli-Q water, and TFA (10:89.9:0.1, *v*/v/v) for phase A, and a second mixture (90:9.9:0.1, v/v/v) for phase B (Bobe et al., 1998).

2.3. In vitro semi-dynamic gastrointestinal digestion

The milk, yogurt, and cheese samples were subjected to a dynamic phase with samples removed at four different gastric emptying points (GE 1–4). The GE samples were collected from the digests at 5.30 (GE 1), 10.80 (GE 2), 16.00 (GE 3) and 21.76 min (GE 4) for milk and yogurt and at 15.79 min (GE 1), 31.58 min (GE 2), 47.28 min (GE 3) and 63.16 min (GE 4) for Cheddar cheese samples. The gastric digestion time for each sample was calculated based on its composition, considering caloric values (protein, lipid, and carbohydrate content), as well as portion size. Since the samples included skimmed milk, yogurt, and full-fat Cheddar cheese, the digestion times varied accordingly. The total gastric digestion time for each sample was determined using the calculations provided by the INFOGEST protocol (Mulet-Cabero et al., 2020). Subsequently, each gastric digest aliquot went through intestinal digestion, as illustrated in Fig. 1. The intestinal digestion followed the standardized semi-dynamic INFOGEST protocol (Mulet-Cabero et al., 2020), which included the preparation of simulated intestinal fluid (SIF) and the appropriate proportions of the other intestinal phase components (Brodkorb et al., 2019). The general process involved mixing 5 mL

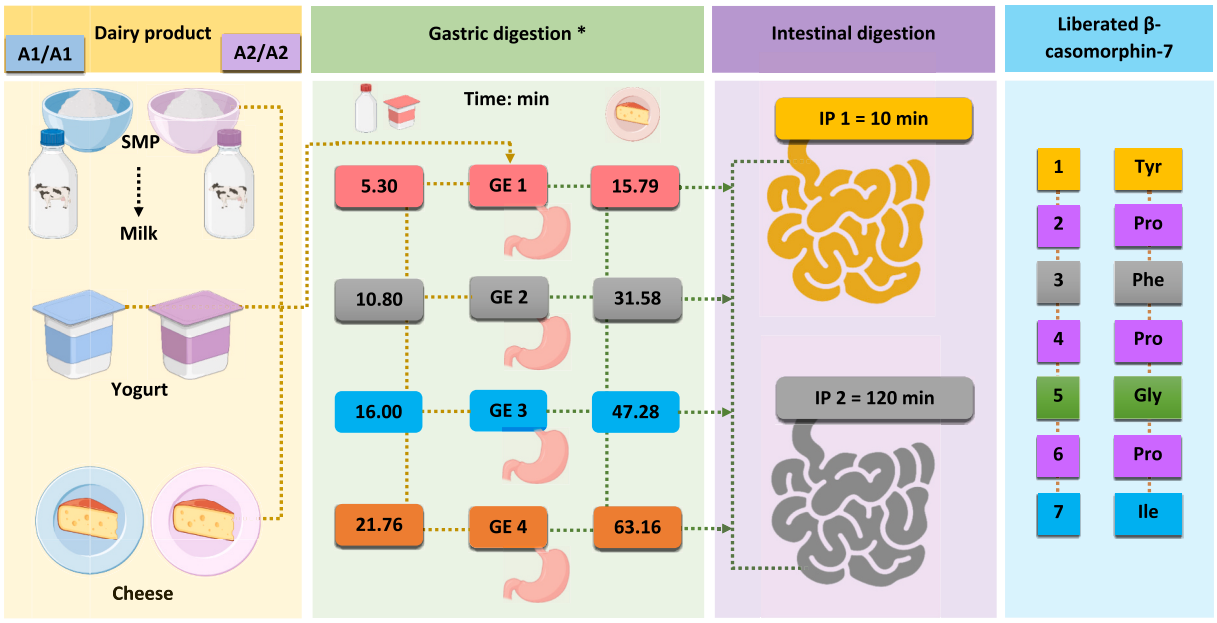

Fig. 1. Overview and flow diagram of the INFOGEST simulated semi-dynamic *in vitro* gastrointestinal digestion method used in the study. *Skim milk powder* **(SMP)**, *gastric emptying point* **(GE),** and *intestinal phase* **(IP)**. * For the gastric digestion emptying points please refer to the previously published data (Daniloski, Hailu, et al., 2024; Daniloski, Page, et al., 2024; Daniloski, Vasiljevic, et al., 2024).

of digest (collected during gastric digestion) with SIF, bile, calcium dichloride dihydrate ($CaCl_2H_4O_2$) solution, 2 M sodium hydroxide (NaOH, to adjust the pH to 7.0), milli-Q water, and pancreatin solution. The quantities of pancreatin, bile salts, and $CaCl_2H_4O_2$ were adjusted based on the volume of the gastric aliquots to achieve final concentrations of 100 U/mL (trypsin activity), 10 mmol/L, and 0.6 mmol/L, respectively. Milli-Q water was added to reach a total volume of 10 mL, maintaining a 1:1 ratio between chyme and intestinal fluids. The samples were placed in a rotor (SB3 Model, Stuart, Bibby Scientific, UK) and stirred at 15 rpm inside an incubator (BF56, Binder GmbH, Germany) at 37 °C for up to 120 min. For each gastric emptying point, two intestinal digestion phases were collected at 10 min (intestinal phase 1 = IP 1) and 120 min (intestinal phase 2 = IP 2), resulting in a total of 8 intestinal emptying points for each biological replicate. It is worth noting here that IP 1 from GE 1 represents an early digestive process, *i.e.*, poorly digested gastric chyme enters the small intestine (shortly after the duodenum, into the jejunum), whereas IP 2 marks the end of the intestinal phase for that particular GE, resembling the ileum (Mulet-Cabero et al., 2020). Aliquots of 5 mL from each intestinal phase were mixed with Pefabloc® (5 mM) to inhibit enzyme activity, snap-frozen in liquid nitrogen, and immediately stored at −80 °C for subsequent analysis (Fig. 1). This step was performed at the end of the intestinal digestion phase (after 120 min) to preserve the digested peptides, including potential β-casomorphin-7, without further enzymatic breakdown. Enzyme controls, such as digestion in the absence of enzymes or with inactivated enzymes, were not included prior to digestion. While we cannot completely exclude the possibility of pre-hydrolysis of proteins in the products before digestion, any such effect would likely vary between products due to differences in composition and processing. The primary focus of this study was on the dynamics of β-casomorphin-7 release during gastrointestinal digestion. To simplify the presentation of results and differentiate the samples, each sample was denoted as: a genetic variant of β-casein (A1/A1 or A2/A2), followed by the gastric emptying point (GE) and corresponding intestinal phase (IP), and finally the dairy product (*e.g.*, A1/A1 GE 1 IP 1: Milk).

2.4. Identification and quantification of β-casomorphin-7

To quantify β-casomorphin-7 across all samples, multiple reaction monitoring (MRM) mass spectrometry was utilized. Protein digests were analyzed on an Agilent 1260 Infinity LC system coupled with a 6460 Triple Quad (QQQ) mass spectrometer (Agilent Technologies, Waldbronn, Germany). Peptide separation was performed using a Zorbax C18 column (2.1 mm × 50 mm, 1.8 μm, Agilent Technologies) at 45 °C, following the parameters outlined by Asledottir et al. (2017) and Le et al. (2020). Q1/Q3 transition (*m/z* values) for β-casomorphin-7 (790.2/383.1 and 790.2/530.0) were used for data acquisition. Quantification was conducted using a calibration curve, where the x-axis represented standard concentrations, and the y-axis reflected the ratio of analyte peak area to the internal standard, processed *via* MassHunter Quantitative Analysis software (Agilent Technologies). Each sample was measured in duplicate, with an optimal collision energy of 30 eV. Serial concentrations of the standard ranged from 25 to 250 fmol/μL. Isotope-labeled internal standards (C^{13} and N^{15} at Phenylalanine), at a concentration of 50 fmol/μL, were spiked into both standard solutions and digested samples. Both the standard and isotope-labeled β-casomorphin-7 peptides, with over 97% purity, were obtained from ThermoFisher Scientific (Biopolymers, Ulm, Germany) as AQUA Ultimate peptides. The MRM method underwent validation for linearity, repeatability, limit of detection (LOD), and limit of quantification (LOQ). Duplicate measurements were taken for digested milk, yogurt, and cheese samples, each with distinct β-casein phenotypes.

2.5. Data processing

The statistical analysis was performed using Minitab statistical software (Version 20; Minitab, Pennsylvania, USA) with a one-way analysis of variance (1-way ANOVA) to evaluate the effect of phenotype (A1/A1 or A2/A2 genetic variant) on the gastrointestinal digests for each dairy product. Post-hoc pairwise comparisons were conducted using Tukey's test to identify significant differences between the groups. For each sample, the mean concentration and standard deviation of β-casomorphin-7 in the reaction mixture were calculated from three independent runs and expressed as micrograms (μg) per 100 mg (mg) of

the starting protein in milk, yogurt, and cheese. The figures and statistical annotations were generated using OriginPro 2023 (v95E; OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Estimated β-casomorphin-7 contents were calculated for each sample to align with recommended dairy intakes outlined in food-based dietary guidelines (Comerford et al., 2021). In addition, the amount of bioaccessible β-casomorphin-7 in the jejunal effluents after 120 min of intestinal digestion was determined for all four gastric emptying time points to assess its potential physiological activity.

3. Results and discussion

3.1. Composition of milk, yogurt, and Cheddar cheese

As confirmed by the genotyping assay of the cows (www.icbf.com), RP-HPLC analysis of the protein haplotype verified that the bulk milk batches, regardless of the dairy product, exclusively contained either β-casein A1/A1 or A2/A2 phenotypes, but also κ-casein A/A, αs_2-casein A/A, αs_1-casein B/B and β-lactoglobulin A/B (Fig. 2). The compositions (total protein, calcium, phosphorus, and fat content) of reconstituted skim milks, yogurts, and Cheddar cheeses are also presented in Fig. 2. No differences were observed in the protein content between A1/A1 and A2/A2 samples in milk and yogurt, respectively and both products had similar protein levels. This similarity is expected since all samples were rehydrated to achieve comparable protein levels. However, the relative κ-casein levels were marginally higher in A1/A1 milk and yogurt compared to A2/A2 samples (Daniloski, Hailu, et al., 2024; Daniloski, Page, et al., 2024). Regarding the chemical composition of the Cheddar cheese samples produced from A1/A1 and A2/A2 cheese milk, no significant differences were observed after 180 days of ripening, likely due to the standardization of the protein-to-fat ratio during cheese milk preparation (Fig. 2).

3.2. Presence of β-casomorphin-7 in gastrointestinal digested reconstituted skim milk

The current study found β-casomorphin-7 present in all milk gastrointestinal digests despite the β-casein phenotype (Figs. 3A and B). The intestinal phase one of digestion (upon 10 min) revealed a clear difference in the release of β-casomorphin-7 in both milk types. In A1/A1 milk digests, the β-casomorphin-7 concentrations ranged between 3.21 and 3.68 μg/100 mg protein. However, A2/A2 milk digests exhibited significantly lower β-casomorphin-7 levels, ranging from 1.15 to 1.53 μg/100 mg protein ($p < 0.05$, Fig. 3A). In the first intestinal phase, Tukey's *post hoc* test showed that A1/A1 GE 1 and GE 3 were similar, with GE 4 exhibiting somewhat lower value and GE 2 lying between the other A1/A1 digests. All A2/A2 GE samples (GE 1 - GE 4) showed consistently lower values than the A1/A1 digests ($p < 0.05$). By the second intestinal phase (120 min), the release of β-casomorphin-7 increased for both milk variants. Interestingly, while the A2/A2 digests showed an overall increase, they surpassed the A1/A1 digests with a slightly greater, though non-significant, amount of β-casomorphin-7 per 100 mg protein. As a result, no pairwise differences were detected in the second intestinal phase ($p > 0.05$; Fig. 3B). While Asledottir et al. (2018) found that after 120 min of intestinal digestion the highest β-casomorphin-7 concentration was seen in digested A1/A1 milk (4.0 mg/g β-casein) followed by A2/A2 milk (1.4 mg/g β-casein), in the current study, the levels of β-casomorphin-7 based on the β-casein amount in both milk types were much lower. Namely, at the end of the gastrointestinal digestion, A1/A1 digest had β-casomorphin-7 levels ranging between 8.65 and 11.72 μg/100 mg protein (0.27–0.37 mg/g β-casein), while A2/A2 digest had its levels between 9.64 and 15.29 μg/100 mg protein (0.29–0.46 mg/g β-casein). These findings are notably higher than the recently reported concentration of 0.005 mg/g β-casein in A2/A2 skim milk (Smolenski et al., 2025). Hence, the amounts of β-casomorphin-7 detected in the final intestinal digests contrast with earlier findings from the gastrointestinal digestion of A2/A2 milk, which reported higher or lower levels of this peptide, potentially due to variations in the digestion models employed across studies as well as

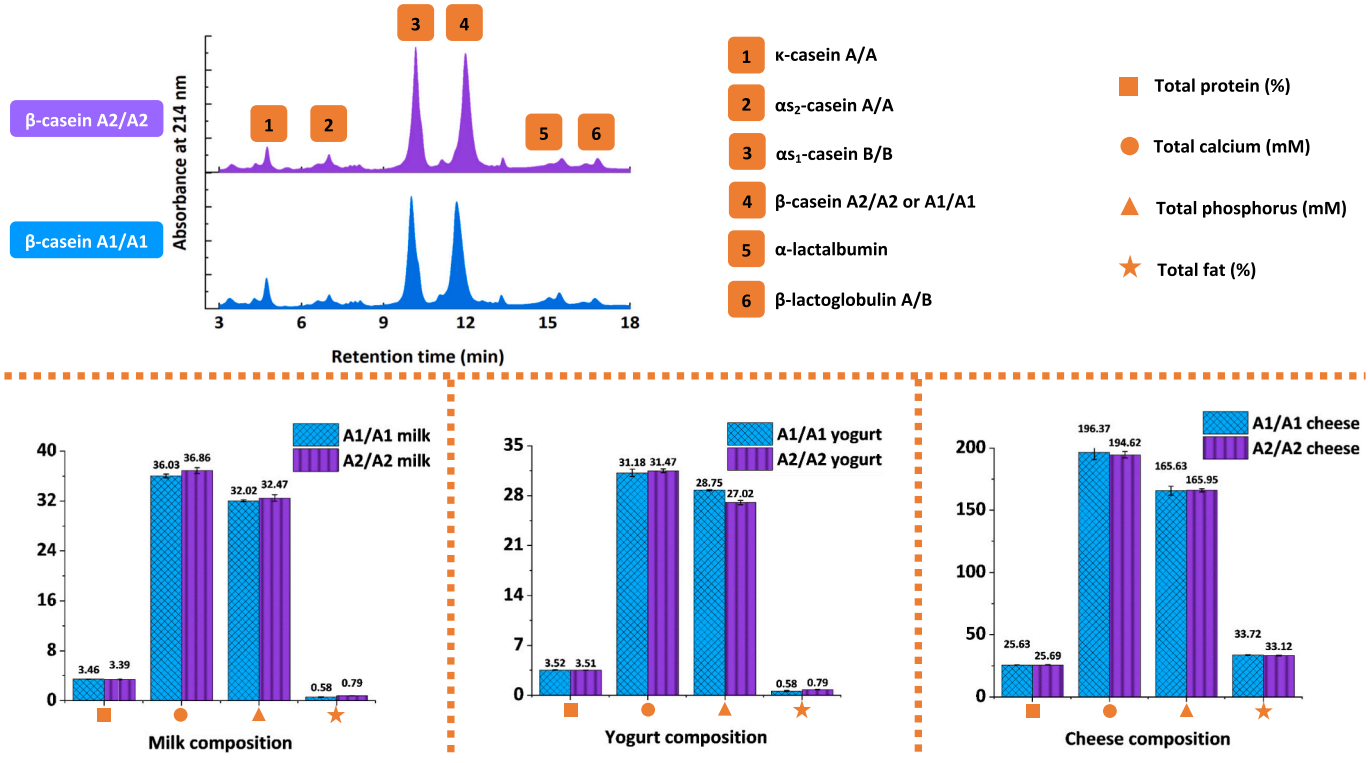

Fig. 2. Composition and RP-HPLC chromatographic profiles used for identification of the A1/A1 and A2/A2 milk, yogurt, and Cheddar cheese.

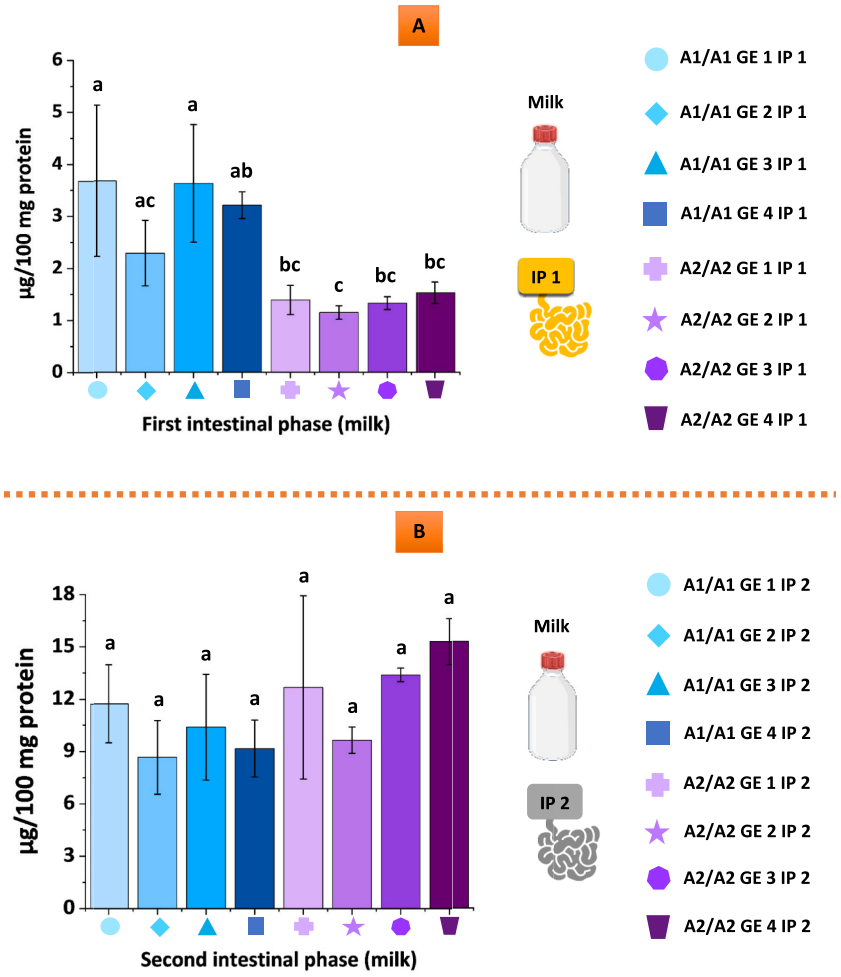

Fig. 3. Release of β-casomorphin-7 from A1/A1 and A2/A2 reconstituted skim milks subjected to *in vitro* semi-dynamic gastrointestinal digestion (gastric emptying point - GE and intestinal phase - IP). A) Intestinal phase 1 and B) Intestinal phase 2. Different letters represent digests that significantly differ according to Tukey's *post hoc* test ($p < 0.05$).

differences in the type of skim milk used, being natural or reconstituted (Asledottir et al., 2018; Cattaneo et al., 2023; Cieślińska et al., 2007; Lambers et al., 2021).

Semi-dynamic gastrointestinal models, such as the one used in the present study, more closely mimic physiological conditions by gradually acidifying the gastric phase, modulating enzyme secretion over time, and dynamically adjusting gastric emptying (Mulet-Cabero et al., 2020). These features influence casein micelle destabilization and protease accessibility (Mulet-Cabero et al., 2024), producing different temporal patterns of β-casein hydrolysis and altering access to the region surrounding the variant-defining position 67, from which β-casomorphin-7 is released. In contrast, static models impose fixed pH and constant enzyme concentrations, which can either accelerate or suppress specific cleavage events and often lead to higher early peptide release (Xavier & Mariutti, 2021).

Furthermore, the structural and physicochemical differences between pasteurized natural and reconstituted skim milk can influence digestion behavior (Jiang ZhuMao et al., 2018; Le Feunteun et al., 2014). While both types of milk are thermally processed, the skim milk powder used to make the reconstituted skim milk undergoes an additional dehydration-heat cycle during spray drying, which promotes whey protein denaturation and association with casein micelles, often resulting in larger and more turbid micelles (Guan et al., 2012; Kelly & Fox, 2016; Martin et al., 2007). In pasteurized natural milk, caseins coagulate in the stomach, with whey proteins remaining soluble and sequentially delivered into the intestine (Fitzpatrick et al., 2024; Le Feunteun et al., 2014; Mulet-Cabero et al., 2019). By contrast, in reconstituted milk, a significant fraction of whey proteins form heat-induced aggregates that are insoluble at low pH, disrupting native micelle structure and colloidal calcium phosphate integrity (Kelly & Fox, 2016). These processing-induced alterations partly disrupt native micelle architecture and colloidal calcium phosphate integrity (Martin et al., 2007; Martin et al., 2008), generating a matrix that might behave differently during gastric coagulation (Daniloski, Hailu, et al., 2024; Mulet-Cabero et al., 2019; Ye et al., 2016; Ye et al., 2019) and subsequent β-casein proteolysis (Nguyen et al., 2021). *In vitro* digestion studies show that while the overall protein digestibility of reconstituted milk is often comparable to natural pasteurized milk, reconstituted systems typically exhibit reduced bioactive protein content and altered peptide diversity due to processing-induced denaturation (Li et al., 2022; Li et al., 2024; van Lieshout et al., 2020). These matrix-dependent effects must therefore be considered when interpreting differences in β-casomorphin-7 release across different studies.

3.3. *Presence of β-casomorphin-7 in gastrointestinal digested yogurt*

In contrast to the milk samples, A1/A1 yogurt digests from either intestinal phase one or two showed significantly higher levels of β-casomorphin-7 compared to their A2/A2 counterparts ($p < 0.05$, Fig. 4A and B). More specifically, in the first intestinal phase, the A1/A1 yogurt digests released between 2.16 and 5.64 µg of β-casomorphin-7 per 100 mg of protein, with A1/A1 GE 1 IP 1 being the most distinct. In

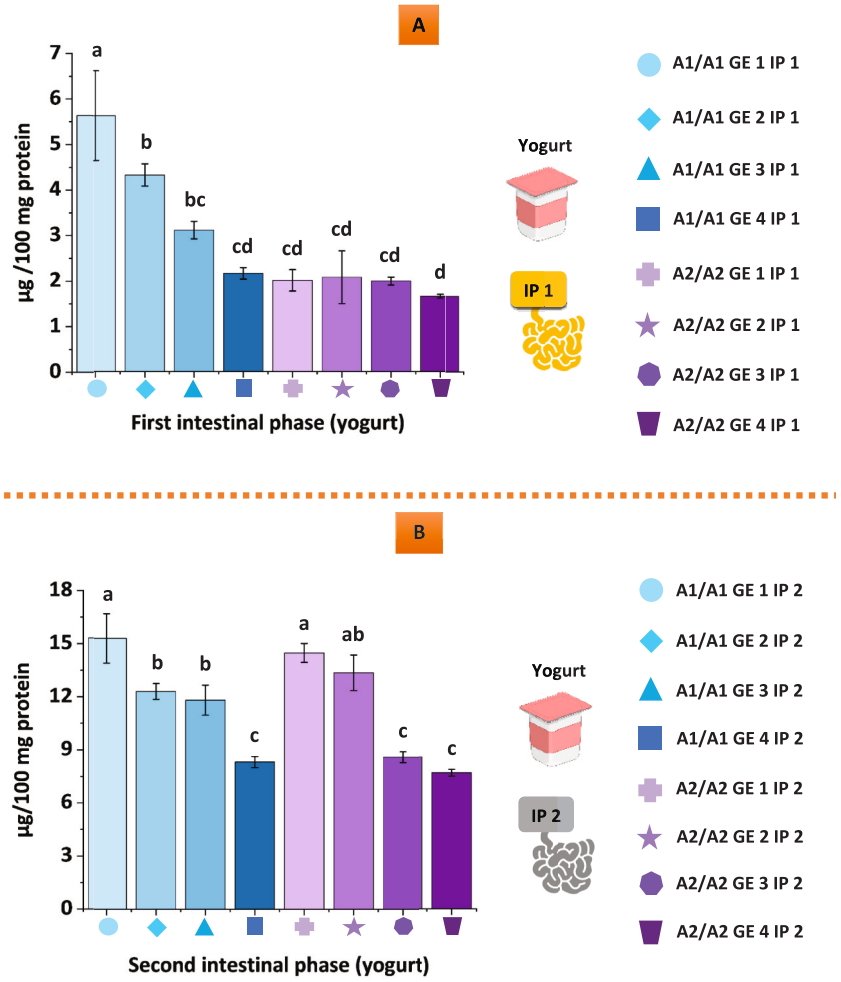

Fig. 4. Release of β-casomorphin-7 from A1/A1 and A2/A2 yogurts submitted to *in vitro* semi-dynamic gastrointestinal digestion (gastric emptying point - GE and intestinal phase - IP). A) Intestinal phase 1 and B) Intestinal phase 2. Different letters represent digests that significantly differ according to Tukey's *post hoc* test ($p < 0.05$).

contrast, no significant differences were observed among the A2/A2 digests across the GE points ($p > 0.05$),where β-casomorphin-7 levels were lower compared to the A1/A1 digests ($p < 0.05$), ranging from 1.66 to 2.08 µg/100 mg protein (Fig. 4A). This trend persisted in the second intestinal phase, where β-casomorphin-7 in A1/A1 yogurt digests was almost 6% higher than in A2/A2 digests ($p < 0.05$, Fig. 4B). Nguyen et al. (2014) and De Noni and Cattaneo (2010) found little to no presence of β-casomorphin-7 in undigested yogurts.

3.4. *Presence of β-casomorphin-7 in gastrointestinal digested Cheddar cheese*

During both intestinal phases, A1/A1 Cheddar cheese exhibited the highest β-casomorphin-7 release among all tested dairy products (Figs. 3–5). In the first intestinal phase, A1/A1 cheese liberated β-casomorphin-7 levels approximately 10-fold higher than those observed in A2/A2 cheese (Fig. 5A). Tukey's *post hoc* test revealed no significant differences in the first intestinal phase among GE 1 - GE 3 within the A1/A1 genotype, whereas GE 4 showed higher values than the other A1/A1 time points ($p < 0.05$). In the same intestinal phase, the A2/A2 digests (GE 1 - GE 4) possessed notably lower values compared with all A1/A1 digests ($p < 0.05$). During the second intestinal phase, while the liberation of β-casomorphin-7 from A1/A1 cheeses peaked, ranging from 36.19 to 62.73 µg/100 mg protein, the A2/A2 cheese showed substantially lower β-casomorphin-7 release during this phase, with values between 3.63 and 7.63 µg/100 mg protein (Fig. 5B). *Post hoc* comparisons showed that there were no significant differences among the A1/A1 GE 1 - GE 4 digests in the second intestinal phase. However, within the same phase, A2/A2 GE 1 - GE 4 digests had significantly lower values and were statistically distinct from all A1/A1 digests ($p < 0.05$).

Currently, only a few studies have investigated and specified the formation of β-casomorphin-7 upon the gastrointestinal digestion of Cheddar cheese (De Noni et al., 2015; De Noni & Cattaneo, 2010; Haileselassie et al., 1999). De Noni and Cattaneo (2010) measured β-casomorphin-7 levels in cheese subjected to *in vitro* digestion using Corolase PP™ as the intestinal enzyme. The study reported, that the β-casomorphin-7 content in the cheese (aged between 7 and 25 months) after gastrointestinal digestion was 15.22 mg/kg cheese (De Noni & Cattaneo, 2010). This was similar to the current results for β-casomorphin-7 levels in A2/A2 gastrointestinal digest, which ranged between 3.63 and 7.63 µg/100 mg protein (9.30–14.2 mg/kg cheese). In a subsequent study by De Noni et al. (2015), 4-month-old Cheddar cheese after static *in vitro* gastrointestinal digestion yielded 1.26 mg/kg cheese of β-casomorphin-7, in almost 10-times lower amounts as compared to the results obtained in the current study. Also, Haileselassie et al. (1999), by using Neutrase protease, identified β-casomorphin-7 in Cheddar cheese, yet in a concentration below the detection threshold. Nonetheless, in all three studies, the β-casein phenotype of the cheeses was unknown. The gastrointestinal digestion of Cheddar cheese used in the above studies contrasts with the semi-dynamic method used in the present study. The present digestion method used resembles more closely the human

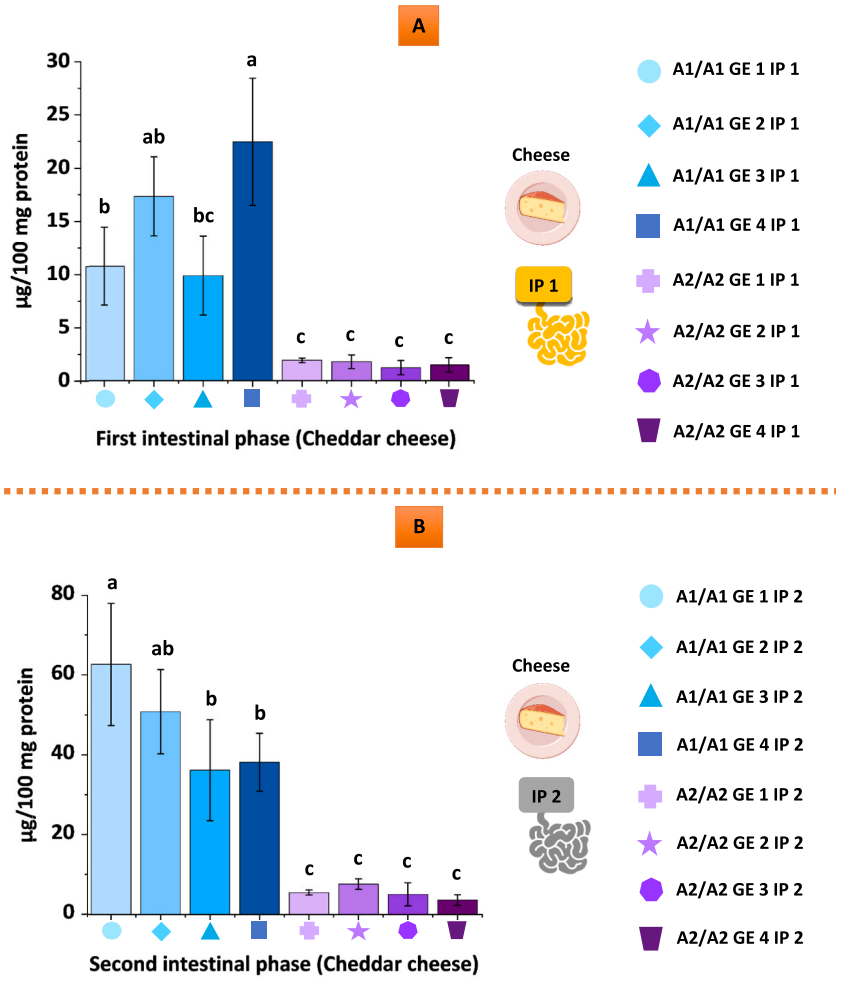

Fig. 5. Release of β-casomorphin-7 from A1/A1 and A2/A2 Cheddar cheeses submitted to *in vitro* semi-dynamic gastrointestinal digestion (gastric emptying point - GE and intestinal phase - IP). A) Intestinal phase 1 and B) Intestinal phase 2. Different letters represent digests that significantly differ according to Tukey's *post hoc* test ($p < 0.05$).

gastric phase as it involves the gradual addition of gastric fluids and enzymes, as well as the gradual emptying of the gastric chyme (Miralles et al., 2021). Hence, using the semi-dynamic gastrointestinal digestion model gives a possibility to study dynamic and kinetic processes during the gastrointestinal transit (Mulet-Cabero et al., 2020).

4. β-Casomorphin release: possible mechanism, digestive insights, and health significance

Several notable differences were observed in this study when comparing the liberated amounts of β-casomorphin-7 from the gastrointestinal digests of A1/A1 and A2/A2 dairy products. While A1/A1 and A2/A2 reconstituted skim milk yielded similar levels of β-casomorphin-7 after 120 min of intestinal digestion (second intestinal phase, $p > 0.05$, Fig. 3), both fermented A2/A2 dairy products (yogurt and Cheddar cheese) liberated fewer amounts of this heptapeptide after the final intestinal digestion compared to A1/A1 dairy products ($p < 0.05$, Figs. 4 and 5). This is an important finding for dairy manufacturers and may be related to the fermentation process and the dairy matrix, which lead to the generation of significantly higher levels of β-casomorphin-7 in cheese and yogurt compared to its amount in milk (Figs. 3–5). The starter cultures used in yogurt and cheese processing play a principal role in lowering the pH and breaking down milk proteins into shorter peptides or even releasing free amino acids by exopeptidases throughout fermentation, storage, and cheese maturation (Fox et al., 1998). Even though *Lactobacillus delbrueckii* subsp. *bulgaricus* is weakly proteolytic, *Streptococcus thermophilus* has an appreciable proteolytic activity (Elfahri et al., 2014). Additionally, they have a cell envelope-associated proteinase, such as X-prolyl dipeptidyl aminopeptidase (PepX), that has an affinity towards hydrolyzing proteins and peptides containing proline residues (Muehlenkamp & Warthesen, 1996; Sousa et al., 2001).

Compared to all other caseins, β-casein is found to carry the highest amount of proline residues (17%) which are evenly distributed along its polypeptide chain (34 and 35 proline residues are found in β-casein A1 and A2, respectively) (Huppertz et al., 2018). The additional proline residue in β-casein A2 affects its structure and may reduce the protein's susceptibility to breakdown during digestion compared to β-casein A1. This effect is likely because proline being an imino acid inhibits the protein hydrolysis at the N-terminus by bacterial aminopeptidases (De Noni et al., 2015; Nguyen et al., 2015). The reduced susceptibility can also be attributed to the high rigidity imposed by the proline cyclic structure, that prevents rotations around the N-Cα bond leading to lower structural flexibility of the protein (Daniloski, McCarthy, Huppertz, & Vasiljevic, 2022). Thus, the peptide bond in β-casein A2 between isoleucine[66] and proline[67] is suggested to be more resistant to enzymatic cleavage, than the corresponding bond in β-casein A1 between isoleucine[66] and histidine[67] (De Noni et al., 2015; Muehlenkamp & Warthesen, 1996; Nguyen et al., 2015). To confirm these statements, Markoska (2023) used molecular modeling to evaluate the tertiary structure of two peptides, each containing 11 amino acids, derived from β-casein A1

($f_{60–70}$ with f_{67} = histidine) and β-casein A2 ($f_{60–70}$ with f_{67} = proline). The analysis revealed that the β-casein A1 undecapeptide (11 amino acid residues) adopted a more open conformation, whereas the β-casein A2 undecapeptide exhibited a closely packed tertiary structure. Specifically, in β-casein A2, the internalized isoleucine^{66} - proline^{67} bond may be less accessible to proteases (Markoska, 2023). The restricted access likely reduces the extent of cleavage in β-casein A2, leading to a lower release of β-casomorphin-7 (Nguyen et al., 2018b). This mechanistic difference directly reflects the observed data, where A2/A2 yogurt and cheese consistently showed lower β-casomorphin-7 release than their A1/A1 counterparts throughout the intestinal phase (Figs. 4 and 5). However, given the complexity of milk, yogurt, and cheese matrices, extrapolating these findings to contexts beyond these dairy systems may yield results that are less applicable or relevant.

Recent research revealed that subtle differences in the dairy protein matrix may significantly impact both gastric and intestinal digestion properties of the products (Everett, 2025; Huppertz et al., 2024; Lamothe et al., 2017; Le Feunteun et al., 2014; Mulet-Cabero et al., 2024). While no significant differences were observed between the milks alone, the yogurt and, particularly, the cheese matrices appeared to influence the release of β-casomorphin-7 in A1/A1 and A2/A2 gastrointestinal digests. The firmer texture of A1/A1 yogurt compared to A2/A2 yogurt was accompanied by a higher degree of heat-induced β-lactoglobulin aggregation (Daniloski, Page, et al., 2024). Heat processing typically causes partial unfolding of whey proteins, exposing buried cleavage sites and making them more susceptible to digestive enzymes (Barbé et al., 2013). This increased enzymatic accessibility possibly accelerated gastric protein breakdown in A1/A1 yogurt (Daniloski, Vasiljevic, et al., 2024), resulting in a faster and potentially greater release of peptides, including β-casomorphin-7, during gastrointestinal digestion, as observed in the current study (Fig. 4). Specifically, Fig. 4 shows that A1/A1 yogurt released higher amounts of β-casomorphin-7 across the intestinal timepoints, especially during the IP 1 than A2/A2 yogurt, directly supporting the proposed mechanism of enhanced whey-protein aggregation-driven digestibility. At elevated temperatures, such as those involved in yogurt processing, β-casein A2 may function as a more potent molecular chaperone than β-casein A1 (Raynes et al., 2015). By interacting with partially unfolded whey proteins *via* hydrophobic domains, β-casein A2 can inhibit their thiol-disulfide interchange with other whey proteins and with κ-casein, thereby reducing the heat induced protein aggregate formation (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022). This interaction, in turn, could lower gastrointestinal digestibility of samples with β-casein A2.

Beyond high-temperature treatments, β-caseins with higher proline content, such as β-casein A2, may contribute to stabilizing the inherently unstable monomers of native κ-casein by binding to and shielding their hydrophobic surfaces (Thorn et al., 2005). If this mechanism applies to the A2/A2 cheese examined in this study, it could have contributed to reduced κ-casein interactions with digestive enzymes, leading to its increased presence in the gastric digest (Daniloski, Vasiljevic, et al., 2024) and ultimately altering the accessibility of intestinal proteases. It is well established in the literature, that undigested food proteins can impede the accessibility of intestinal proteases to cleavage sites (Picariello et al., 2023). This can reduce the efficiency of enzymatic hydrolysis in the intestine, limiting peptide release. However, the study of Sheng et al. (2021) reported that neither post-translational modifications nor the presence, quantity, or genetic variants of κ-casein significantly impacted the intestinal digestion of AA, AB, and BB milks. While κ-casein may not have influenced cheese matrix disintegration during gastrointestinal digestion, Lamothe et al. (2017) found that harder cheeses, such as the A2/A2 Cheddar cheese in this study (Daniloski, Vasiljevic, et al., 2024), exhibited greater resistance to protein and lipid hydrolysis during *in vitro* gastrointestinal digestion. Specifically, in solid gastric cheese matrices, proteolysis during the intestinal phase gradually increased to an average of 89% at 120 min, whereas in softer gastric cheese matrices, it reached approximately 98% over the same period (Lamothe et al., 2017). These findings align with the current study, where the firmer A2/A2 cheese gastric digest (Daniloski, Vasiljevic, et al., 2024) released less β-casomorphin-7 than its A1/A1 counterpart during gastrointestinal digestion (Fig. 5). In Fig. 5, the A1/A1 cheese consistently shows higher β-casomorphin-7 release at both intestinal phases compared with A2/A2 cheese, mirroring the expected behavior of a softer, more digestible matrix and reinforcing the proposed matrix-driven protease-accessibility mechanism.

The amount of β-casomorphin-7 released during gastrointestinal digestion varies depending on the type of dairy product and its typical serving size. In this study, milk (45 mL), yogurt (45 mL), and Cheddar cheese (20 g) were digested using a semi-dynamic *in vitro* INFOGEST model (Mulet-Cabero et al., 2020). These portion sizes were selected to reflect standard daily consumption levels in humans (Comerford et al., 2021) (Table 1). A standard serving of milk (250 mL) contained approximately 2.80 g of β-casein, while yogurt (200 mL) had around 2.15 g of the same protein. Following gastrointestinal digestion, milk and yogurt released approximately 1 mg and 0.40 mg β-casomorphin-7 per serving, respectively, regardless of the β-casein variant (Table 1). On the other hand, the gastrointestinal digestion of A1/A1 Cheddar cheese corresponded to 6.43 mg of β-casomorphin-7 released per 40 g (daily recommended intake) A1/A1 Cheddar cheese, which was about eight times more than the amount released by the same serving of A2/A2 cheese (Table 1). These amounts could be used to determine the levels of intact β-casomorphin-7 after exposure to digestive peptidases.

For β-casomorphin-7 to potentially induce any effect in internal organs, absorption in the gut and transport through blood is necessary (Kost et al., 2009). Based on the physiologically active concentration, which is the most important parameter in order to evaluate the β-casomorphin-7 activity (Boutrou et al., 2013), the current study estimated that the β-casomorphin-7 concentrations in the jejunal effluents of milk, yogurt and cheese digests after 120 min of intestinal digestion, ranged from 0.39 to 6.83 μM. These levels can be considered as the amount of bioaccessible β-casomorphin-7 for binding to opioid receptors in the gut or available for transport across the epithelium and transfer into blood circulation (Asledottir et al., 2019). Boutrou et al. (2013), in a single-blind, two-arm parallel trial involving 16 humans, found that consuming 30 g of lyophilized micellar casein as a dietary supplement led to the release of β-casomorphin-7 in the jejunal effluent. The concentration was estimated at 17 μM in an average effluent volume of 304.0 ± 12.6 mL, sufficient for *in vivo* opioid activity, given its half-maximal inhibitory concentration (IC_{50}) of 7–100 μM as measured by the inhibition of electrically evoked contractions of longitudinal muscle of guinea-pig ileum and mouse vas deferens (Yoshikawa et al., 1994).

Table 1
Estimated averaged β-casomorphin-7 levels after the second intestinal phase (120 min) in milk, yogurt, and Cheddar cheese based on adult daily recommended intake*.

Sample	Serving size (mL or g) *	Protein content (mg)	β-casomorphin-7 presence (mg)
A1/A1 milk	250 mL	8648	0.75–1.01
A2/A2 milk	250 mL	8480	0.82–1.30
A1/A1 yogurt	200 mL	7040	0.21–0.39
A2/A2 yogurt	200 mL	7000	0.19–0.36
A1/A1 cheese	40 g	10,252	3.71–6.43
A2/A2 cheese	40 g	10,276	0.37–0.78

* Daily recommended intake levels for milk and dairy products were obtained from authoritative dietary guidelines, including Australia (Dairy Australia), Europe (EFSA), and the United States (Dietary Guidelines for Americans, 2020–2025). Additionally, global recommendations are summarized in the study by Comerford et al. (2021).

Hence, the concentrations of β-casomorphin-7 found in this study were just below previously reported thresholds, suggesting that standard servings of these dairy products are unlikely to produce physiologically significant effects.

Very recently, Gard et al. (2024) proposed that achieving high concentrations of β-casomorphin-7 in the blood might be unlikely. However, they explained that even with a 100% absorption rate through the brush border membrane, consuming approximately 1 L of A1/A1 milk would result in blood concentrations of β-casomorphin-7 up to 13 μM (Gard et al., 2024). Caira et al. (2024) monitored the levels of β-casomorphin-7 in the blood of eight healthy volunteers at various time points within 24 h after they consumed 250 mL of UHT bovine milk with both β-casein variants. While the study did not reveal any presence of β-casomorphin-7 in the human blood, after *ex vivo* incubation of synthetic β-casomorphin-7 with human blood, its presence was evident with a very short half-life (30–35 min) in circulation (Caira et al., 2024). A follow-up study by the same group demonstrated that β-casomorphin-7 was highly susceptible to degradation by human plasma peptidases under physiological conditions (De Pascale et al., 2024). The peptide's *ex vivo* half-life in blood was estimated to be 35–40 min, comparable to other food-derived peptides with rapid elimination or peptide-based drugs without chemical stabilization (De Pascale et al., 2024; Foltz et al., 2010). The authors in both studies suggested that β-casomorphin-7 may lack physiological significance, particularly in healthy individuals.

Although the semi-dynamic adult INFOGEST method employed in this study adhered to well-established *in vitro* digestion protocol from previously published research (Brodkorb et al., 2019; Mulet-Cabero et al., 2020), the findings should be interpreted with caution. Extrapolating gastrointestinal *in vitro* protein digestion results to human protein digestion is inherently challenging due to the complexities involved in replicating physiological conditions (Santos-Sánchez et al., 2024). Simulating human digestion accurately involves addressing several technical issues, including protein and peptide solubility, as well as the exact enzymatic and pH environments encountered in different regions of the human digestive tract (Brodkorb et al., 2019; Egger et al., 2019; Miralles et al., 2021).

5. Conclusion

The present study demonstrated that varying quantities of β-casomorphin-7 were released during *in vitro* semi-dynamic gastrointestinal digestion of milk, yogurt, and Cheddar cheese containing β-casein A1/A1 and A2/A2 variants. β-Casomorphin-7 was consistently released from all milk and yogurt samples regardless of the β-casein variant. However, significantly higher quantities were observed in A1/A1 Cheddar cheese after the final intestinal phase. This study underscores the complexities surrounding the potential systemic effects of β-casomorphin-7 in humans. The estimated levels of β-casomorphin-7 released after *in vitro* semi-dynamic gastrointestinal digestion for all three dairy products, based on the daily serving sizes, seems not to be adequate to exert a potential physiological activity. Together, these findings highlight the need for further *in vivo* research to better understand the physiological relevance of β-casomorphin-7 from dairy consumption. This is particularly important when considering the potential health implications associated with its release, which requires robust scientific validation.

Author contribution

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Davor Daniloski**, **Neha Sharma and Conor J. Fitzpatrick** prepared the methodology, formal analysis and investigation. **Lotte B. Larsen**, **Thao T. Le, and Lotte J. Knudsen** were part of the formal analysis and investigation, reviewed and edited the manuscript. **Nina A. Poulsen**, **Todor Vasiljevic**, **Noel A. McCarthy and André Brodkorb** supervised the study, gave critical feedback and analysis, reviewed and edited the manuscript and secured funding. All authors have contributed to the manuscript and reviewed the final version.

CRediT authorship contribution statement

Davor Daniloski: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Noel A. McCarthy:** Writing – review & editing, Supervision, Funding acquisition. **Todor Vasiljevic:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition. **Neha Sharma:** Writing – review & editing, Formal analysis, Data curation. **Conor J. Fitzpatrick:** Writing – review & editing, Formal analysis, Data curation. **André Brodkorb:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Thao T. Le:** Writing – review & editing, Validation, Formal analysis. **Lotte J. Knudsen:** Validation, Formal analysis. **Lotte B. Larsen:** Writing – review & editing, Visualization, Validation, Formal analysis. **Nina A. Poulsen:** Writing – review & editing, Validation, Supervision, Funding acquisition.

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.

Acknowledgments

Davor Daniloski was supported by both the VU International Postgraduate Research Scholarship (VUIPRS) and Teagasc - the Agriculture and Food Development Authority (Walsh Scholarship Programme, Ireland) that was registered with Victoria University (Funding Agency ID: 2019039). The authors acknowledge the assistance of the team at the **Moorepark Dairy Farm** in Teagasc Animal and Grassland Research and Innovation Centre in collecting milk samples. Some of the figures are created with BioRender.com.

Data availability

No data was used for the research described in the article.

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