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Impact of porcine brush border membrane enzymes on INFOGEST *in vitro* digestion model: A step forward to mimic the small intestinal phase

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ABSTRACT

Brush border membrane (BBM) enzymes greatly affect the bioaccessibility and bioavailability of food nutrients. Despite their physiological importance, a step simulating the final stage of intestinal digestion has not yet been included in the harmonized protocols for *in vitro* digestion, primarily due to the challenges of replicating the dynamics of intestinal degradation. Herein, we propose an advancement toward a more physiologically relevant method, complementing the harmonized static gastric-duodenal digestion INFOGEST model with the missing small intestinal phase. BBM hydrolase activity, incubation time, at pH 7.2 were established to reproduce the small intestinal conditions. Skim milk powder, as a model of protein food, was subjected to the *in vitro* static digestion. Immediately after the duodenal phase, digesta were supplemented with BBM vesicles purified from pig jejunum. To comply with the dynamic nature of intestinal digestion and balance the spontaneous inactivation of hydrolases, BBM supplements were added every two hours throughout 6 h incubation time. Peptide degradation was monitored at each stage of digestion by amino acid analysis, free α-amino group assay, HPLC, LC-MS/MS. Hydrolysis by BBM peptidases led to a significant increase of free amino acids, reflecting the known level of amino acid adsorption (>90 %) in humans after eating milk proteins. LC-MS/MS analysis demonstrated that BBM hydrolases erode progressively the peptides released by gastro-duodenal processing up to stable sequence motifs. The approach described is particularly relevant when the endpoint is identifying the peptide sequences that cannot be further hydrolysed by digestive enzymes or to determine the amino acid bio-accessibility.

1. Introduction

In vitro digestion models are valid methodologies developed to replicate the intricate processes of human digestion. These models are designed to simulate the three primary physiological phases of digestion: oral, gastric, and duodenal. Each phase encompasses specific static and mechanical actions that contribute to the breakdown and absorption of nutrients from food (Hur et al., 2011).

Over the last decades, several *in vitro* digestion models have been devised to closely mimic the human digestion process (Li, Yu, Wu & Chen, 2020). The collaborative efforts within the INFOGEST network have led to an international consensus on conditions to be adopted for *in vitro* digestion (Minekus et al., 2014). This consensus validated a reliable and reproducible framework for reproducing the enzyme activities, digestive juices, and pH values at oral, gastric and duodenal stages of *in vivo* digestion with physiological relevance (Brodkorb et al., 2019). Since its introduction to the scientific community, the static *in vitro* INFOGEST protocol has been widely applied to predict the metabolic fate of food components. This standardized protocol has significantly enhanced our understanding of digestion of nutrients (Zhou, Tan & McClements, 2023) and the interplay between diet and gut microbiota (Le Feunteun et al., 2021; Borewicz & Brück, 2024), and has facilitated the development of food products tailored to specific dietary needs or health conditions (Bavaro et al., 2021; Mackie, Mulet-Cabero &Torcello-Gómez, 2020; Mamone et al., 2022; Menard et al., 2023). Despite its robustness, the static INFOGEST model does not reproduce the dynamics of the digestion process nor the effect of small intestinal brush border membranes (BBM) hydrolases that are crucial for the final stages of nutrient digestion before absorption. Actually, BBM enzymes include oligopeptidases, oligosaccharidases and lipases, which further hydrolyse upstream generated products into simpler compounds, thus greatly affecting the bioaccessibility and bioavailability of food components

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(Donowitz et al., 2007; Hooton, Lentle, Monro, Wickham & Simpson, 2015; Holmes & Lobley, 1989; Mamone & Picariello, 2023; McConnell, Benesh, Mao, Tabb & Tyska, 2011). Notably, small intestinal enterocytes are among the fastest turnover cell lines in our organism and the vesicles shed in the mucosal periapical space contribute to luminal digestion (Hooton et al., 2015). Therefore, neglecting the role of BBM enzymes in *in vitro* digestion studies could lead to an incomplete understanding of nutrient digestion and absorption processes (Mamone & Picariello 2023).

A more relevant approach to simulate the small intestinal digestion in conjunction with the INFOGEST method has been recently proposed by incorporating only the intestinal porcine aminopeptidase N, which is responsible for most of the exopeptidase activity of the small intestinal mucosa and for the release of free amino acids from peptides (Martineau-Côté, Achouri, Pitre, Karboune & L'Hocine, 2023). Implementing this protocol with enzyme mixtures that include glycosidases and lipases in addition to peptidases could further increase the relevance. However, the panel of BBM hydrolases is highly complex, consisting of more than 200 enzymes (Mamone & Picariello, 2023). Reproducing this complexity through a mix of selected and commercially available enzymes is challenging and disadvantageous, whereas BBM vesicles can be purified time- and cost-effectively from porcine intestinal mucosa.

Caco-2 cell model is another valuable tool for assessing nutrient transport and metabolism after the *in vitro* INFOGEST digestion. In particular, differentiated Caco-2 polarized monolayers replicate the intestinal absorption and metabolism because of their ability to mimic processing and translocation of nutrients (Iftikhar, Iftikhar, Zhang, Gong & Wang, 2020) and to express BBM enzymes on the apical side (Howell, Kenny & Turner, 1992). The profile of intestinal hydrolases of differentiated Caco-2 is grossly comparable to that of jejunal enterocytes although the activity of some degrading enzymes from BBM of Caco-2 cells could be low due to their colonic origin (Chantret et al., 1994; Ölander, Wiśniewskii, Matsson, Lundquist & Artursson, 2016). The common approaches to improve biocompatibility of the INFOGEST *in vitro* digestion with the study of downstream degradation and absorption using Caco-2 monolayers have been recently reviewed (Kondrashina et al., 2023).

Efforts to include the intestinal phase with BBM of human or animal origin in static digestion models have been previously carried out. The use of human BBM enzymes in the context of *in vitro* digestion models was successful in identifying the immunodominant gliadin peptides that are involved in the elicitation of celiac disease (Di Stasio et al., 2020a; Mamone et al., 2007; Gianfrani et al., 2015; Hausch, Shan, Santiago, Gray & Khosla, 2002; Shan et al., 2002). Porcine BBM peptidases were proved to hydrolyse casein- and whey proteins-derived peptides rapidly, to generate several smaller peptides and free amino acids (Ozorio et al., 2020; Picariello et al., 2015; Vivanco-Maroto et al., 2022). Similarly, BBM enzymes were employed to assess the behaviour of raw and processed food allergens, demonstrating that the digestion phases might not abolish their allergenicity (Claude et al., 2019; Di Stasio et al., 2017; Di Stasio et al., 2020b; Di Stasio et al., 2020c; Mamone et al., 2015; Nitride et al., 2022). Investigating peptides during *in vitro* digestion with BBM allowed the identification of digestion resistant bioactive sequences that may have significant health benefits (Asledottir et al., 2019; Asledottir et al., 2023; Mamone et al., 2019). Unlike the INFOGEST oral-gastroduodenal digestion which results in an unrealistically low degree of protein hydrolysis, the integration with small intestinal BBM enzymes yields levels of free amino acids that are compatible with those adsorbed *in vivo*, generally exceeding 90 % by weight of the ingested protein fraction (Dupont & Tomè, 2020, Picariello et al., 2015). Additionally, the use of BBM enzymes in the *in vitro* digestion model, produces outcomes that more closely resemble human *in vivo* digestion of oligosaccharides (Hernandez-Hernandez, 2019; Gallego-Lobillo, Ferreira-Lazarte, Hernandez-Hernandez, Villamie, 2020). Despite its physiological significance, which is also underlined in the INFOGEST protocol (Minekus et al., 2014; Brodkorb et al., 2019), a digestion step mimicking the small intestinal digestion is still often omitted, mainly due to the challenge in accurately replicating the dynamic of intestinal degradation. The first drawback is that, unlike other digestive enzymes, BBM hydrolases are not available commercially, impeding the definition of harmonized conditions for the *in vitro* small intestinal digestion. Secondly, there is no consensus on the conditions of use of BBM enzymes, especially regarding incubation times and the enzyme activity-to-substrate ratio. Nevertheless, these limitations are common to other systems devised to simulate the last pre-absorptive or absorptive phase, such as Caco-2 cell monolayers, *ex vivo* intestinal tissues or organoids (Costa, de Carvalho, de Oliveira & Madureira, 2024).

Recently, we proposed an optimized method to purify BBM vesicles from specimens of pig jejunum (Mamone & Picariello, 2023). Proteomic analysis demonstrated that BBM vesicles are primarily enriched in hydrolases, including peptidases and glycosidases, with lipases to a lesser extent. Porcine BBM enzymes share significant structural and functional similarities with human BBM enzymes, making them a practical and relevant substitute for use in *in vitro* digestion models (Mamone & Picariello, 2023). Therefore, using porcine BBM may bridge the gap left by the unavailability of human BBM enzymes.

The study aimed to propose an advancement towards a more physiologically relevant method by complementing the harmonized static INFOGEST gastric-duodenal digestion model with the missing phase of BBM digestion. Skim milk Powder (SMP) was chosen as a model matrix for *in vitro* digestion. To better mimic the dynamic nature of intestinal digestion and account for the spontaneous inactivation of purified hydrolases, the digesting SMP sample was supplemented with porcine BBM enzymes during the small intestinal phase (Hooton, 2018). Following digestion, proteins and peptides were characterized by mass spectrometry, while free amino acid release was monitored by HPLC-fluorescence detection. This approach could significantly enhance our understanding of how food proteins are digested and made available for human intestinal absorption.

2. Materials and methods

2.1. Chemicals and reagents

SMP, provided in the context of the INFOGEST network was composed of 42.3 % protein, 0.89 % fat, and 49.8 % (w/w) lactose (Egger et al., 2016). Porcine digestive enzymes (pepsin, trypsin, chymotrypsin, pancreatic α-amylase, pancreatic lipase), chemicals used for the preparation of digestion buffers (potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate and calcium chloride), Dowex 50WX8 cation exchange resin (100–200 mesh), HPLC-grade solvents and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St Louis, MO, USA). The AccQ-Fluor (AQC) Reagent Kit and sodium borate buffer were provided by Waters Co. Ltd. (Milford, MA, USA). A standard solution containing 17 protein amino acids and 4 additional non-protein amino acids was purchased from Waters Co. Ltd. (Milford, MA, USA). Reagent for 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay and calf intestinal alkaline phosphatase enzyme were provided by Thermo Scientific (San Jose, CA, USA).

2.2. BBM purification

BBM vesicles were purified from porcine jejunum according to a previously optimized protocol (Mamone & Picariello, 2023). After purification, BBM vesicles were aliquoted and stored at −80 °C. Soon before use, BBM vesicles were thawed on ice cold bath and peptidase activity was checked as detailed below (paragraph 2.3).

2.3. In vitro digestion of SMP

SMP was digested with the model-2 of the INFOGEST protocol making use of individual enzyme in the duodenal phase (Brodkorp et al., 2019). The composition of digestive juices, origin of enzymes and their activities were the same described in the harmonized protocol (Minekus et al., 2014). The SMP (1 g) was reconstituted in 10 mL of deionized H_2O. The oral phase was skipped due to the very short residence times of liquid foods in the oral cavity and the absence of starchy matrices in the SMP (Minekus et al., 2014; Egger et al., 2016).

A 2 mL of reconstituted SMP was mixed with 2 mL of simulated gastric juice containing pepsin (2000 U/mL) and incubated for 2 h at 37 °C. Subsequently, the pH was raised to 7.0 with 1 M NaOH and 4 mL of intestinal juice, containing trypsin (100 U/mL), chymotrypsin (25 U/mL), lipase (2000 U/mL), and α-amylase (200 U/mL) were added and incubated for 2 h at 37 °C. Immediately after duodenal digestion, the chyle was cooled on an ice-cold bath, ten-fold diluted with PBS (pH 7.4) and the pH was adjusted to 7.2 with 1 M HCl. To simulate the digestion with BBM enzymes, 300 μL aliquots of the diluted chyle were separately incubated at 37 °C for 1 h and 2 h, with 3.9 μL of BBM vesicles with aminopeptidase N activity of 1300 μU/μL. Separate aliquots were further supplemented with an additional 3.9 μL of BBM extracts after 2 h and 4 h of incubation, for a total of 4 h and 6 h incubation times, respectively. The experimental workflow is outlined in Fig. 1. Details about the calculation are provided in Supplementary Information Table S1.

The activity of aminopeptidase N in BBM was determined using L-leucine *p*-nitroanilide as the substrate (3.0 mM concentration). A final concentration of 13 μU/μL of BBM completely degraded 100 μM angiotensin I, selected as a model susceptible peptide, within 4–5 h, as verified by HPLC monitoring (Picariello et al., 2015). After the intestinal phase, the reaction was stopped by immersion in boiling water for 5 min and stored at –20 °C until further analysis. A parallel, control gastro-duodenal digestion was carried out exactly in the same condition above but omitting the SMP. All digestive phases were performed under constant gentle mixing.

2.4. HPLC analysis of peptide fraction

Digested SMP was analysed by RP-HPLC before and after BBM phase, using an HPLC 1260 Infinity II Prime LC System modular system (Agilent, Palo Alto, CA, USA). Digests (50 μg) were diluted with 0.1 % TFA and separated by C18 column (Aeris PEPTIDE, 3.6 μm, 250 × 2.10 mm i.d., Phenomenex, Torrance, CA, USA). Eluent A was 0.1 % TFA (v/v) in Milli-Q water; eluent B was 0.1 % TFA (v/v) in acetonitrile. The column was equilibrated at 5 % B. Peptides were separated by applying a linear 5–70 % gradient of B over 90 min at a 0.2 mL/min flow rate. Chromatographic separation was performed at 40 °C, using a thermostatic column holder. The column effluent was monitored at 214 and 280 nm using a multi-wavelength UV–Vis detector.

2.5. Determination of free amino group (TNBS assay)

The quantitative measurement of free amino group was performed by the TNBS assay (Adler-Nissen, 1979). Briefly, 5 μg of digesta sample was incubated in 0.1 M sodium carbonate solution pH 8.5 containing 0.01 % (w/v) of TNBS solution (Thermo Scientific, USA) at 37 °C for 2 h. Afterwards, 1 N HCl and 10 % SDS were added to stop the reaction. The absorbance at 335 nm of the resulting solutions was measured and converted into the concentration of free amines by comparison against the leucine standard curve. The standard was assayed under reaction conditions identical to those utilized for the samples. Experiments were performed in triplicate.

2.6. Free amino acid (FAA) analysis

FAA were analyzed by HPLC-fluorescence detection after pre-column derivatization. The lyophilized sample was dissolved in 90 μL of 0.1 M HCl. A volume of 10 μL of α-aminobutyric (1 mM) was added as internal standard. FAA were extracted using a column packed with Dowex 50 W-X8 according to Cukier et al., (2018) and then lyophilized. For FAA derivatization, the AQC powder was dissolved in acetonitrile at a final 3 mg/mL concentration. Then, 10 μL of 0.1 M HCl, 70 μL of 0.2 M borate buffer (pH 8.8) and 20 μL of the AQC solution were added to the FAA sample and incubated for 10 min at 55 °C.

FAA were quantified with the same HPLC system above, equipped with a fluorescence detector 1260-VLD (excitation at 250 nm and emission at 395 nm). Separation was carried out using the reverse phase column AccQ-Tag (Waters Co. Ltd., Milford, MA, USA) 3.9 × 150 mm, 4 μm particle diameter. Eluents were: A, 20 mM sodium acetate, 4 mM

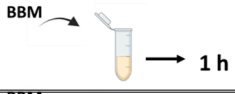
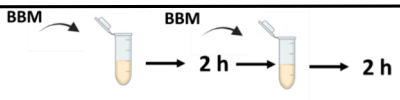
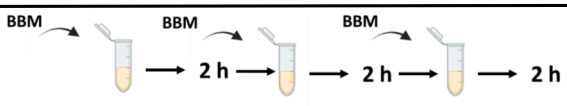
	SAMPLE NAME	INTESTINAL PHASE (BBM ADDITION	DIGESTION TIME (INTESTINAL PHASE)	SCHEMATIC RAPRESENTATION OF BBM ADDITION DURING INTESTINAL PHASE
1	GD	None	-	
2	GD-BBM 1h	13 μU/100 μg	1 h	
3	GD-BBM 2h	13 μU/100 μg	2h	
4	GD-BBM 4h	Two supplements (13 μU/100 μg) each 2h	4h	
5	GD-BBM 6h	Three supplements (13 μU/100 μg) each 2h	6h	

Fig. 1. This figure illustrates the periodic addition of BBM every 2 h up to a total of 6 h, to closely mirror the physiological conditions of the intestinal tract. This approach is critical for understanding the interactions and efficiencies of digestive processes under conditions that closely resemble those in humans. Details are provided in Supplementary Information Table S1.

triethylamine pH 5.89; B, acetonitrile. The gradient eluting condition was: 0–0.5 min, 0–1 % B; 0.5–22 min, 1–5 % B; 22–28 min, 5–9 % B; 28–28.1 min, 9–12 % B; 28.1–35 min, 12–15 % B; 35–45 min, 15–25 % B; 45–45.1 min, 25–85 % B; 45.1–56 min, 85 % B; 56.1–58 min, 0 % B, at a flow rate of 1 mL/min.

Amino acids were identified based on the retention time of the corresponding standards. For each identified FAA, the peak area was normalized to the peak area of the internal standard. The quantification of individual amino acids was based on the response of the fluorescence signal of the corresponding standard, converted into units of concentration through normalized area obtained for each compound, using the internal standard.

2.7. Mass spectrometry analysis and database search

Prior to mass spectrometry analysis, an aliquot of the digests, corresponding to approximately 20 μg of SMP proteins, was dephosphorylated by adding calf intestine alkaline phosphatase (1 μU enzyme:μg protein) and incubated at 37 °C for 8 h. Peptides were purified using a C18 spin column (Thermo Scientific, San Jose, CA, USA), according to the manufacturer's instructions, eluting with 70 % acetonitrile (v/v).

Mass spectrometry analysis of peptides in digests was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), coupled on-line with an Ultimate 3000 ultra-high-performance liquid chromatography instrument (Thermo Scientific, San Jose, CA, USA). Purified peptides were diluted in 100 μL of 0.1 % (v/v) formic acid solution, loaded through a 5 mm long, 300 μm internal diameter pre-column (LC Packings, San Jose, CA, USA) and separated by an EASY-Spray- PepMap™ 100 C18 column (2 μm, 15 cm–75 μm; 3 μm particles; 100 Å pore size (Thermo Scientific, San Jose, CA, USA). Eluent A was 0.1 % formic acid (v/v) in Milli-Q water and eluent B was 0.1 % formic acid (v/v) in acetonitrile. The column was equilibrated with 5 % eluent B. Peptides were separated by a 4–50 % eluent B gradient over 50 min (300 nL/min). The mass spectrometer operated in data-dependent mode, and all MS1 spectra were acquired in the positive ionization mode by scanning the 200–1600 m/z range. A maximum of 10 of the most intense MS1 ions were fragmented in MS/MS mode at a resolving power of 17,500 full width at half maximum (FWHM), applying a 10 s dynamic exclusion. The resolving power was set at 70,000 FWHM, using automatic gain control (AGC) target of 1 x 10^6 ions and 100 ms as a maximum ion injection time (IT) to generate precursor spectra. The fragmentation of mono-charged ions was allowed, while selection of background ions was impeded with an exclusion list, which was prepared based on the peak list obtained from a blank LC-MS/MS analysis. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific) (De Cicco et al., 2019).

LC-MS/MS runs resulting from triplicate analyses of digests produced in triplicate (nine runs for time point) were used for peptide identification using the Andromeda search engine of the MaxQuant bioinformatic suite (version 2.2.0.). Explorative searches were taxonomically restricted to Bos taurus in the UniProtKB database (downloaded in December 2023). Subsequently, to limit the computational demand for the identification of nonspecifically cleaved peptides, the searches were refined using a manually-constructed protein database containing the 30 most abundant cow-milk gene products inferred from proteomic-based investigations (Picariello, et al., 2019). For all the searches, the following parameters were used: mass tolerance value of 10 ppm for the precursor and 0.02 for the fragment ions; no proteolytic cleavage specificity; Met oxidation, pyroglutamic acid formation at N-terminal Gln. Peptide spectrum matches (PSMs) were filtered using the target decoy database approach with an e-value of 0.01 peptide-level false discovery rate (FDR), corresponding to a 99 % confidence score.

2.8. Peptide visualization

Peptide maps were visualized using the Peptigram web application (http://bioware.ucd.ie/peptigram/) (Manguy et al., 2017). Relative peptide abundances were obtained from the MS signal ion counts. In the resulting peptigrams, sequence entries and their corresponding abundances, represented with different shades of green, were aligned to the parent protein sequence.

2.9. Statistical analysis

Analyses of FAA and TNBS were carried out in triplicate using the software GraphPad Prism version 9.0 (GRAPH PAD software Inc, California, USA). Values reported are means and standard deviations (SD). The least significant difference (LSD) test at a 95 % confidence level ($p < 0.05$) was performed to identify differences among samples. Significant differences were determined by analysis of one-way ANOVA, followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Setting parameters of the BBM intestinal digestion

The effectiveness of incorporating the intestinal phase by BBM enzymes in the static *in vitro* model was evaluated using SMP because it is a well-characterized protein food matrix which has been extensively exploited to develop and harmonize the INFOGEST protocol (Brodkorb et al., 2019; Egger et al., 2016; Egger et al., 2017; Egger et al., 2018; Egger et al., 2019). It is also worth noting that the duodenal phase was performed according to INFOGEST model-2 (Brodkorb et al., 2019), which involves the use of selected pancreatic enzymes (i.e., trypsin, chymotrypsin, amylase, and lipase) instead of pancreatin to prevent the inclusion of high amounts of proteins and FAA that may interfere with the analytical evaluation of the ability of BBM to degrade SMP peptides.

The determination of physiologically relevant parameters for the incorporation of porcine BBM enzymes raised the main challenge of this study. Key parameters needed to be established, including the amount of BBM to be added, the incubation time, and the pH. After the 2-hour gastric phase and immediately at the end of the 2-hour duodenal phase, the digest (chyle) was supplemented with porcine BBM hydrolases. Importantly, the addition of BBM enzymes was not preceded by inactivation of the duodenal enzymes. This approach aimed to more closely mimic physiological conditions, as the activity of digestive enzymes along the small intestine continues uninterrupted. The peptide substrate to BBM enzymes ratio was established according to Shan et al. (2002) and Haush et al., (2002), who confirmed the physiological relevance of the conditions and the use of human intestinal biopsies. These authors demonstrated that human BBM enzymes with an aminopeptidase N activity of 13 μU/μL hydrolysed control peptides that are susceptible to degradation at a concentration of 100 μM within 1–5 h. Thus, in preliminary experiments, we verified that the same aminopeptidase N activity of porcine BBM (13 μU/μL) hydrolysed completely a 100 μM solution of angiotensin I, used as a control peptide, within 4–5 h, which is a realistic small intestinal transit timeframe (Picariello et al., 2015; Claude et al., 2019). In line with Shan et al., (2002), the activity of BBM was calculated based on the reaction volume.

To comply with the dynamic nature of intestinal digestion and to balance the spontaneous inactivation of hydrolases (Hooton, 2018), digesting batches were supplemented with the same concentration of BBM in the course of the experiments every two hours, up to 6 h incubation time, as depicted in Fig. 1. This approach aims to consider the dynamic nature of the intestinal system, especially from the duodenal to the ileum regions. Indeed, in humans the advancement of chyle from the small intestine to the large intestine occurs within 3–10 h. This movement is characterized by a progressive decrease in the concentration of digested nutrients as they are absorbed, along with an increasing concentration and activity of BBM enzymes from the proximal to the distal small intestine, which are crucial for maximizing the efficiency of nutrient digestion and absorption (Picariello, Ferranti, Addeo, 2016). It

has to be underlined that in terms of BBM peptidase activity-to-protein substrate, Shan et al., (2002) used a significantly higher ratio than our conditions. Even in the aliquots incubated with three sequential refreshes of BBM enzymes, our BBM peptidase activity-to-protein substrate is much lower than Shan et al., 2002. The pH was statically adjusted to 7.2, as a weighted average value considering the dynamic fluctuation of pH in the small intestinal tract, which could vary between 6 in the duodenal to 7.4 in the ileum (Fallingborg. 1999). Clearly, in real systems, the pH can vary depending on many factors, among which is the buffering capacity of the food.

Hydrolysis of SMP was monitored during the gastric and duodenal phases, and at different time points during the BBM phase. These digest samples were then analyzed using LC-MS/MS-based peptidomics.

3.2. Release of FAA from digested SMP

Analysis of FAA sampled at various time points during the intestinal phase provided insights into the kinetics of peptide degradation (Fig. 2). To verify that FAA were released from SMP during digestion, a control sample (blank) containing only gastro-duodenal enzymes without SMP was analyzed in parallel. The analysis confirmed that FAA introduced with the digestive enzymes contribute negligibly to the overall FAA amount (< 1 %) (Supplementary Fig. S1). In contrast, the use of pancreatin in the duodenal phase might introduce high amounts of FAA in the reaction batch interfering with the determination of FAA released from the digesting substrate. The concentrations of most individual amino acids varied significantly throughout the BBM digestion time course compared to the gastro-duodenal sample. However, some exceptions were observed for arginine, leucine, and phenylalanine, which showed a progressive, though not statistically significant, release during BBM digestion.

At early incubation time with BBM enzymes, no significant differences were observed for FAA compared to the gastro-duodenal sample, with the exception of alanine, which exhibited a significant release after 1 h of BBM incubation. After 4 h of digestion, with two refreshing supplements of BBM enzymes added every 2 h, a significant increase in the concentration of serine, aspartic acid, histidine, lysine, and glycine was observed. After 6 h of digestion, that is three BBM enzyme supplements every 2 h, a notable release of valine, phenylalanine, isoleucine, tyrosine, and glutamic acid was evident.

An interesting trend was observed for proline as it showed a delayed release during BBM digestion, probably because it requires the dual sequential enzymatic action of dipeptidyl peptidase IV followed by an *exo*-peptidase. This finding is consistent with the known resistance of proline to enzymatic hydrolysis (Hernández-Ledesma, del Mar Contreras & Recio, 2011), which prevents the degradation of many proline-rich peptides.

Overall, these results highlight the significant contribution of aminopeptidases and carboxypeptidases from BBM enzymes in releasing amino acids from a complex mixture of peptides as digestion progresses.

3.3. HPLC profiles of digested SMP

The RP-HPLC-UV monitoring of the digestion steps highlighted the relevant impact that BBM hydrolases had on gastro-duodenal SMP digests (Fig. 3). As expected, after simulated gastric and gastro-duodenal digestion of SMP, the HPLC chromatogram exhibited complex patterns of small- and medium-sized peptides. After 1 h of BBM digestion, the intensity of peaks decreased, while after 2 h of BBM only a few peaks were still detectable. During the extensive time course of BBM digestion with 2 and 3 supplements of BBM at 4 h and 6 h, respectively, SMP peptides were intensely degraded. The intestinal phase including BBM endo- and exopeptidases resulted in the hydrolysis of SMP proteins more extensively compared to the duodenal and gastric phases. The addition

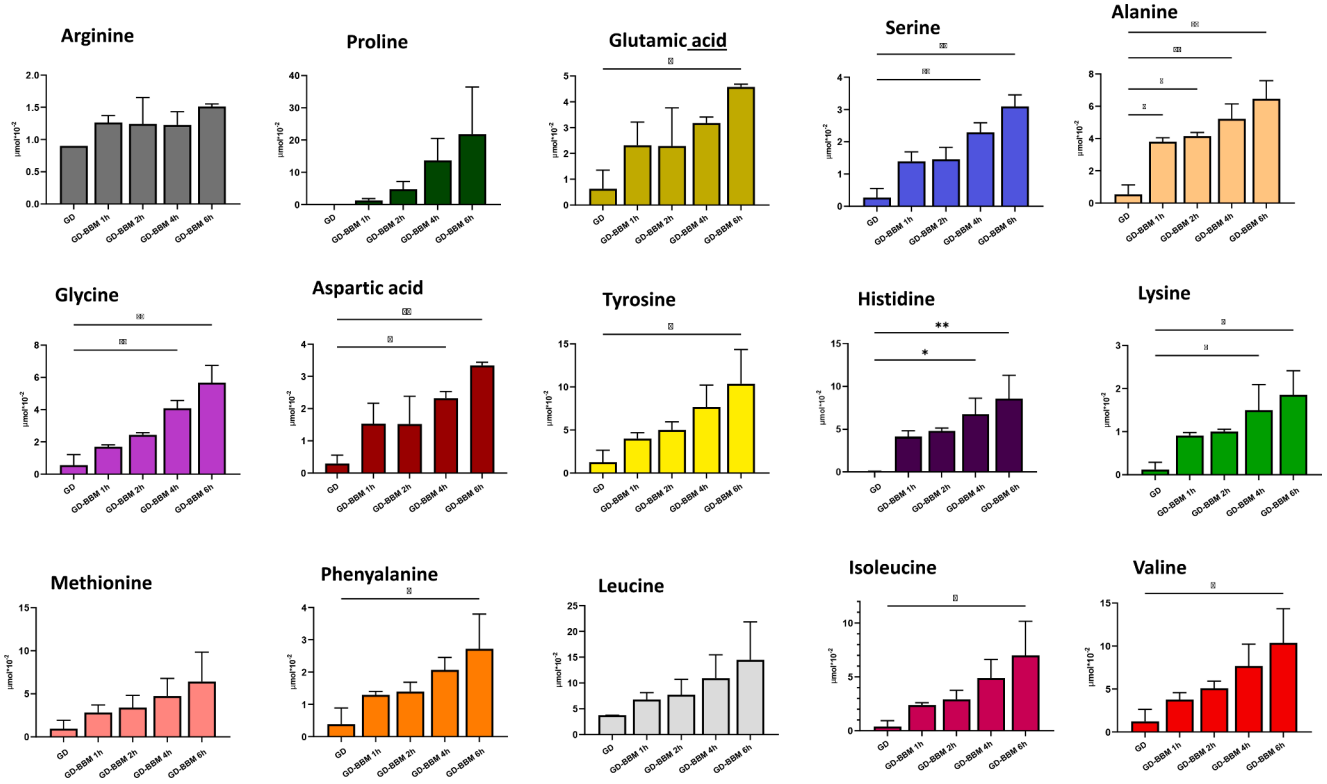


Fig. 2. Release of free amino acids (FAAs) over time during *in vitro* BBM hydrolase digestion, with error bars representing variation within 2 standard deviations. Asterisks (*) denote significant differences ($p \leq 0.05$) observed between BBM and gastro-duodenal (GD) digestion time points. FAA were analysed after GD digestion, at 1 h and 2 h of incubation with BBM, at 4 h and at 6 h with two or three supplements of BBM added every 2 h, respectively. Different superscripts *, ** are significantly different at $p < 0.05$, and $p < 0,01$ respectively by Dunnett's multiple comparisons test.

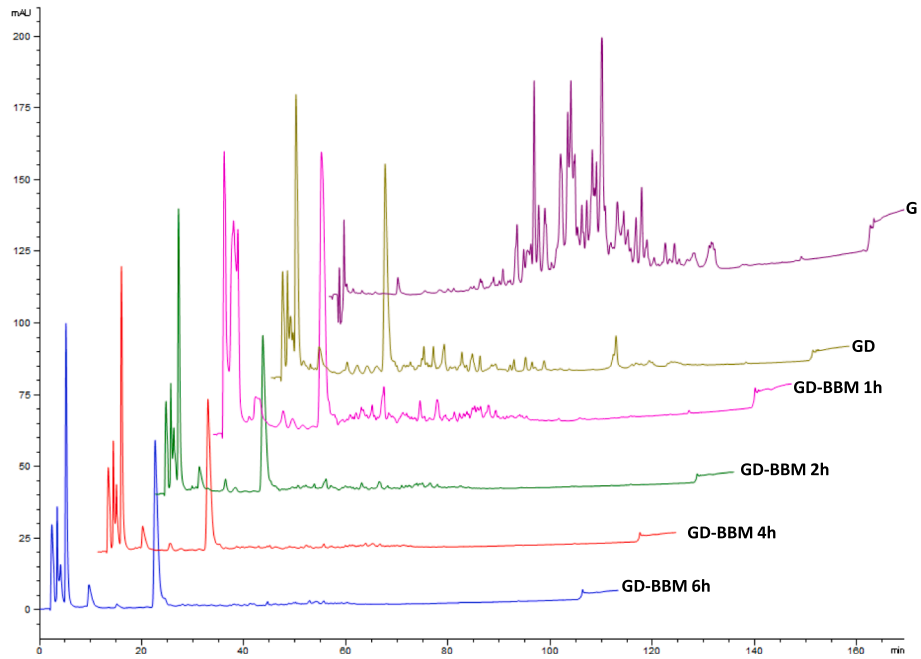


Fig. 3. RP-HPLC-UV (214 nm) comparison of peptides arising from the *in vitro* digestion of SMP proteins. Digests were analysed after gastric (G) and gastro-duodenal (GD) digestion, at 1 h and 2 h of incubation with BBM, at 4 h and 6 h with two or three supplements of BBM added every 2 h, respectively.

of further aliquots of BBM enzymes enhanced the hydrolysis process, as small-sized peptides were no longer detectable in the HPLC-UV chromatograms. These results were consistent with the increased release of FAA over the time course of digestion, confirming the action of BBM peptidases on SMP small sized peptides. Notably, the intense peak of free tryptophan (HPLC peak at 24 min, confirmed by DAD and with the parallel analysis of the authentic standard) dominated all the chromatographic profiles, confirming that the level of free tryptophan can be exploited as an indicative parameter of the hydrolysis degree of food proteins (Picariello et al., 2015).

3.4. Evaluation of the hydrolysis degree through TNBS assay

The degree of protein hydrolysis during *in vitro* digestion was determined by measuring the free amino groups released utilizing the TNBS assay with leucine as the standard for comparison (Adler-Nissen 1979). As shown in Fig. 4, the free α-amino groups released during BBM digestion significantly increased over the digestion time course. However, after 1 h and 2 h of BBM digestion, the amount of free α-amino groups did not show a significant difference compared to the gastro-duodenal SMP digests. The lag in the progress of hydrolysis can be attributed to the slow activity of BBM enzymes on larger peptide fragments, which limits the release of FAA at the early stages of digestion. However, after 4 h and 6 h of BBM digestion (with supplements of BBM enzymes added every 2 h), the concentration of FAA became significantly higher ($p < 0.05$) than that in the gastro-duodenal samples, as a consequence of combined endo- and *exo*-peptidase activity.

3.5. Analysis of digested SMP by LC-MS/MS

The LC-MS/MS analysis of gastro-duodenal digests, both pre- and post-BBM incubation, revealed insights into the peptide degradation during the intestinal phase. The detailed list of peptide sequences arranged by SMP protein is reported in Supplementary Table S2. The "peptigram analyses" from raw LC-MS/MS data illustrated the distribution of peptides identified at various digestion stages for each protein, with the intensity of the green shade corresponding to the ion count for individual peptides, as described by Manguy et al. (2017) (Fig. 5).

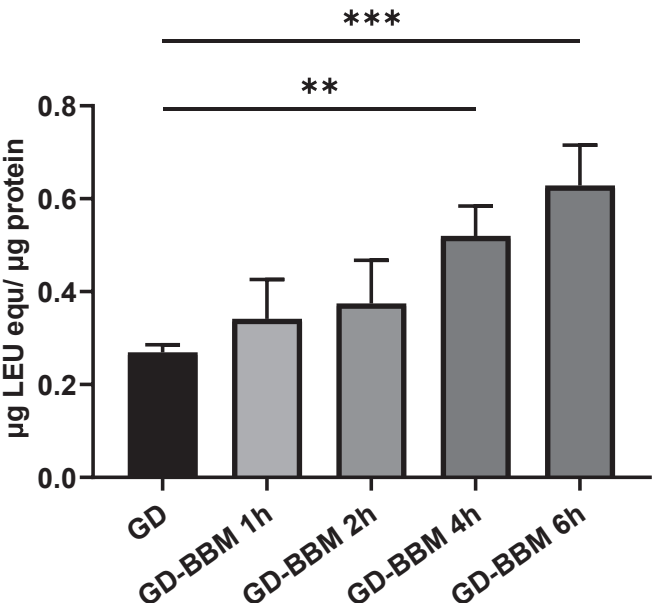


Fig. 4. Degree of hydrolysis expressed as µg leucine equivalent/µg SMP proteins, determined during gastro-duodenal (GD) and BBM hydrolases digestion. Data are mean values and standard deviations from the three analytical replicates. The degree of hydrolysis was measured after GD digestion, at 1 h and 2 h of incubation with BBM, at 4 h and at 6 h with two or three supplements of BBM added every 2 h, respectively. Different superscripts **, *** are significantly different at $p < 0,01$ and $p < 0.005$ respectively by Dunnett's multiple comparisons test.

Compared to the gastric-duodenal digest, BBM hydrolysis resulted in peptide ladders consisting of sequences progressively shortened at the N- and C-termini, highlighting the dominant activity of *exo*-peptidases. Only a few intrinsically stable peptide regions of β-casein, αs1-casein, and αs2-casein survived the complete proteolytic breakdown by BBM

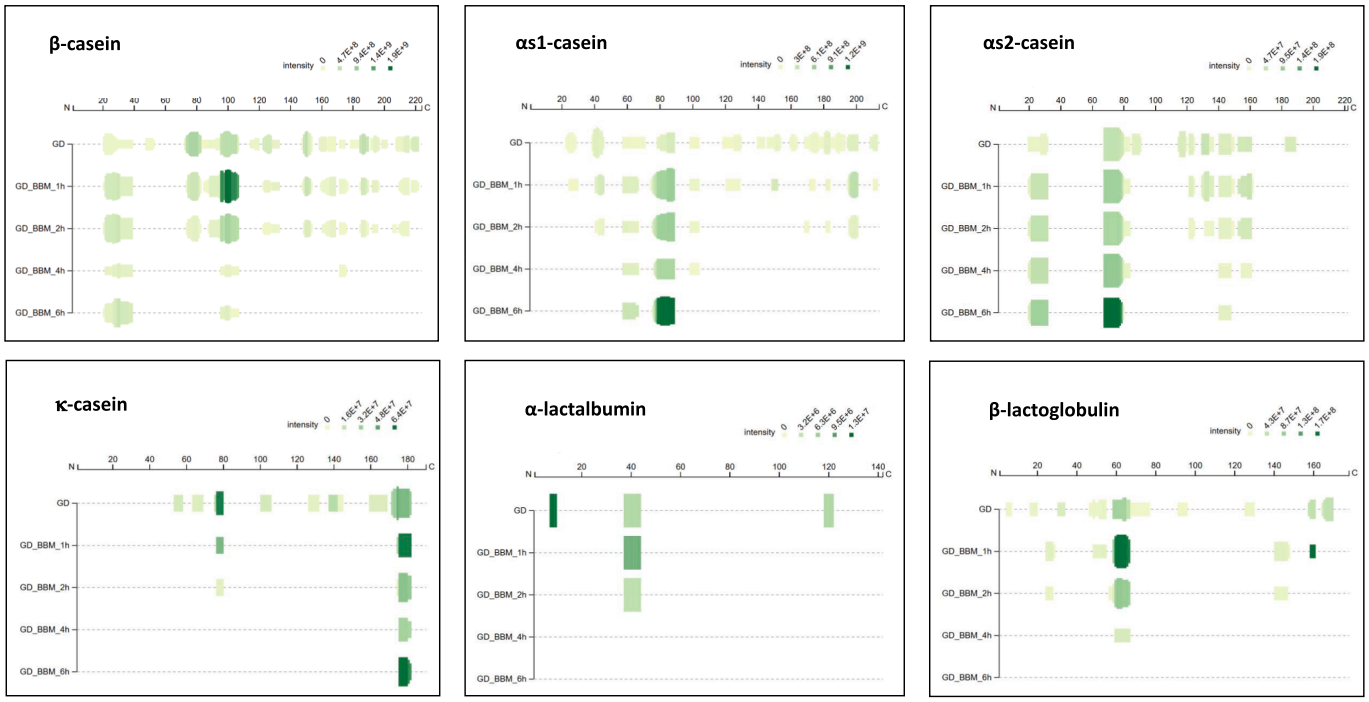


Fig. 5. Peptide profiles of representative precursor proteins including β.Casein, αs1-casein, αs2-casein, κ-casein, α-lactalbumin and β-lactoglobulin identified in gastro-duodenal (GD) digests and after 1 h, 2 h, 4 h and 6 h incubation with BBM. Sample at 4 h and 6 h resulted from supplementation with two and three doses of BBM every 2 h respectively. The intensity of the green colour is proportional to the MS peptide ion count and reflect the sum of the peptide intensities overlapping the relevant position. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

enzymes. A common feature among these proteins was that most of the resistant peptides were located in regions containing multi-phosphorylated clusters, which showed high resistance to digestion due to their acidic properties (Picariello et al., 2010). The increasing intensity of peptides belonging to the multi-phosphorylated casein regions observed during hydrolysis with BBM is likely a consequence of improved detectability due to the action of BBM alkaline phosphatase.

Thus, to enhance the detectability of phosphorylated casein peptides, after digestion the peptides were subjected to complete dephosphorylation with calf alkaline phosphatase. The enzymatic removal of phosphate groups allowed for a comprehensive characterization of proteolytic-resistant regions in SMP proteins.

Albumin, lactotranferrin, and lactoperoxidase were highly susceptible to BBM hydrolysis (Supplementary Fig. S2). Peptides derived from κ-casein, α-lactalbumin, and β-lactoglobulin survived in low amounts (Fig. 5). After 6 h of digestion, only one peptide from the C-terminal region of κ-casein was identified, likely because the double proline residues confer it resistance to digestion. The TPEVDDE peptide of β-lactoglobulin was remarkably stable against BBM enzyme hydrolysis, as previously documented (Picariello et al., 2013). However, this peptide was completely hydrolyzed at longer incubation times, following supplementation of BBM peptidases. Although the MS acquisition method allowed to include the MS/MS fragmentation of short sequences, dipeptides and most of tripeptides escaped the detection, as their comprehensive characterization would require a dedicate approach. Similarly, possible large fragments or disulphide crosslinked peptides remained undetected (De Cicco et al., 2019).

4. Discussion

The results of this study reinforce the critical role of BBM in the intestinal phase for developing a robust and relevant *in vitro* digestion model. The feasibility of obtaining the entire profile of BBM hydrolases from porcine jejunum, as previously proposed by us (Mamone & Picariello, 2023), enables the definition of standardized digestion conditions, such as enzyme quantity, digestion duration, and pH, thereby increasing the physiological relevance of *in vitro* digestion methods. Porcine BBM vesicles serve as a practical alternative to human BBM hydrolases, because these latter cannot be available for routine use in digestion experiments. Moreover, the use porcine BBM hydrolases aligns with the INFOGEST framework, which uses commercially available proteases of porcine origin (Brokorb et al., 2014, Minekus et al., 2019). Given the similarities between pig and human digestion, including omnivorous diet and monogastric anatomy and physiology, porcine BBM enzymes ensure relevance and applicability for functional studies of human digestion (Mach et al., 2014; Miller & Ullrey, 1987).

Complementing the static INFOGEST *in vitro* digestion method with an intestinal phase with porcine BBM vesicles could be an optimal time-/cost-effective compromise between practicality and relevance offering an informative system for studying the pre-adsorption metabolism of nutrient *in vitro*. The current results were focused on the metabolic fate of food proteins, demonstrating that subjecting the gastro-duodenal digests of SMP to prolonged digestion with refreshing supplementation of BBM vesicles, the intestinal peptidases effectively break down upstream generated peptides into smaller peptides and FAA. Increased levels of FAA compared to the gastro-duodenal sample indicate the progress of protein hydrolysis. After reiterating supplementation of BBM enzymes and overall 6 h incubation, the release of FAA approaches the estimated levels of FAA adsorbed in humans following administration of milk proteins (>90 %), supporting the physiological correspondence of the described model (Dupont & Tomè, 2020). Probably more than for the other digestive compartments, the physiological dynamic of small intestinal digestion is hardly mimicked due to the progressive absorption and subtraction of nutrients, the progressive dilution of residual nutrients, the flux of compounds from the lumen toward the mucosa (and reversed sense) in turn affected by the mucus layer thickness, and the longitudinally varying activity of mucosal hydrolases. The approach of extended digestion time and higher enzyme concentration during BBM digestion appears highly relevant for identifying peptide sequences particularly stable to digestion that cannot be further hydrolyzed by

digestive enzymes and for determining the bioaccessibility of FAA. The method improves the physiological correspondence of the *in vitro* digestion model since it maintains the enzymatic activity relatively stable throughout the experiment compensating for the quick inactivation of purified BBM peptidases (Hooton et al., 2018).

The proposed strategy to reproduce the intestinal phase focuses on the ability of BBM peptidases to hydrolyze oligopeptides. In this regard, assessing the impact of porcine BBM hydrolyses on the metabolism of oligosaccharide and lipid nutrients would be of great interest. BBM hydrolases include oligosaccharidases (among the most abundant hydrolases of the BBM, together with peptidases) and, to a lesser extent, lipases (Mamone & Picariello, 2023). Previous studies demonstrated that pancreatic amylases used to reproduce the intestinal phase, significantly influence the digestibility of polysaccharides of food digested with INFOGEST *in vitro* method (Hernandez-Hernandez, 2019). While the literature may not explicitly report combinations of INFOGEST protocols with intestinal lipase, this enzyme is known to play a crucial role in the digestion of lipids before absorption in the small intestine tract (Nilsson & Duan, 2006). Exploiting the combined action of porcine BBM oligosaccharides and lipases in conjunction with peptidases could lead to improved models of intestinal digestion of real food systems and better insights into the metabolic processes involved.

An additional important consideration is that BBM vesicles contain not only the expected hydrolases but also a complex array of protease inhibitors against pancreatic serine proteases. These inhibitors could modulate the activity of pancreatic enzymes during intestinal digestion, adding another layer of complexity to accurately simulating the digestive process (Mamone & Picariello, 2023).

An accurate validation and comparative process of the model with the *in vivo* digestion is not straightforward. However, the very low abundance and identity of peptides that survive *in vitro* digestion after incubation with BBM are substantially consistent with the patterns of milk-derived peptides found in the blood of human volunteers after ingestion of milk, apart from interindividual variability (Caira et al., 2022). Furthermore, the extensive release of FAA when BBM enzymes are incorporated in the model mirrors the elevate plasma influx of FAA observed *in vivo*, after the intake of highly digestible proteins such as milk.

In conclusion, the proposed integration of the *in vitro* INFOGEST digestion with a small intestinal phase simulated with porcine BBM hydrolases could be a valuable tool for enabling a primary evaluation of the digestibility and quality of novel protein sources and processed food products, prior to the *in vivo* assessment. By incorporating these BBM enzymes, future models can provide more accurate and comprehensive insights into nutrient digestion and absorption, ultimately enhancing our understanding of dietary impacts on human health. This methodological improvement could significantly advance research in food science and nutrition, leading to better dietary recommendations and novel food product development.

CRediT authorship contribution statement

Luigia Di Stasio: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Salvatore De Caro:** Methodology, Data curation, Conceptualization. **Serena Marulo:** Methodology, Data curation, Conceptualization. **Pasquale Ferranti:** Data curation, Conceptualization. **Gianluca Picariello:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Gianfranco Mamone:** Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.115300.

Data availability

Data will be made available on request.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food chemistry, 27*(6), 1256–1262. https://doi.org/10.1021/jf60226a042

Asledottir, T., Picariello, G., Mamone, G., Ferranti, P., Røseth, A., Devold, T. G., & Vegarud, G. E. (2019). Degradation of β-casomorphin-7 through *in vitro* gastrointestinal and jejunal brush border membrane digestion. *Journal of Dairy Science, 102*, 8622–8629. https://doi.org/10.3168/jds.2019-16771

Asledottir, T., Vegarud, G. E., Picariello, G., Mamone, G., Lea, T. L., Røseth, A., Ferranti, P., & Gulbrandsen Devold, T. (2023). Bioactive peptides identified in pea and faba bean after *in vitro* digestion with human gastrointestinal enzymes. *Journal of Functional Foods, 102*, Article 105445. https://doi.org/10.1016/j.jff.2023.105445

Bavaro, S. L., Mamone, G., Picariello, G., Callanan, M. J., Chen, Y., Brodkorb, A., & Giblin, L. (2021). Thermal or membrane processing for Infant Milk Formula: Effects on protein digestion and integrity of the intestinal barrier. *Food Chemistry, 347*, Article 129019. https://doi.org/10.1016/j.foodchem.2021.129019

Borewicz, K., & Brück, W. M. (2024). Supplemented infant formula and human breast milk show similar patterns in modulating infant microbiota composition and function *in vitro*. *International Journal of Molecular Sciences, 25*(3), 1806. https://doi.org/10.3390/ijms25031806

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., & Recio, I. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Caira, S., Pinto, G., Picariello, G., Vitaglione, P., De Pascale, S., Scaloni, A., & Addeo, F. (2022). *In vivo* absorptomics: Identification of bovine milk-derived peptides in human plasma after milk intake. *Food Chemistry, 385*, Article 132663. https://doi.org/10.1016/j.foodchem.2022.132663

Chantret, I., Rodolosse, A., Barbat, A., Dussaulx, E., Brot-Laroche, E., Zweibaum, A., & Rousset, M. (1994). Differential expression of sucrase-isomaltase clones isolated from early and late passages of the cell line Caco-2: Evidence for glucose-dependent negative regulation. *Journal of Cell Science, 107*(1), 213–225. https://doi.org/10.1242/jcs.107.1.213

Claude, M., Lupi, R., Picariello, G., Drouet, M., Larré, C., Denery-Papini, S., & Brossard, C. (2019). Digestion differently affects the ability of native and thermally aggregated ovalbumin to trigger basophil activation. *Food Research International, 118*, 108–114. https://doi.org/10.1016/j.foodres.2017.11.040

Costa, C. M., de Carvalho, N. M., de Oliveira, D. L., & Madureira, A. R. (2024). A critical review on *in vitro* and *ex vivo* models of the intestinal epithelium of humans and monogastric animals. *Gastrointestinal Disorder, 6*, 337–358. https://doi.org/10.3390/gidisord601002

Cukier, C., Lea, P. J., Cañas, R., Marmagne, A., Limami, A. M., & Hirel, B. (2018). Labeling maize (Zea mays L.) leaves with 15NH4+ and monitoring nitrogen incorporation into amino acids by GC/MS analysis. *Current Protocols in Plant Biology, 3*(3), e20073. doi: 10.1002/cppb.20073.

De Cicco, M., Mamone, G., Di Stasio, L., Ferranti, P., Addeo, F., & Picariello, G. (2019). Hidden “digestome”: Current analytical approaches provide incomplete peptide inventories of food digests. *Journal of Agricultural and Food Chemistry., 67*(27), 7775–7782. https://doi.org/10.1021/acs.jafc.9b02342

Di Stasio, L., Picariello, G., Mongiello, M., Nocerino, R., Berni Canani, R., Bavaro, S., Monaci, L., Ferranti, P., & Mamone, G. (2017). Peanut digestome: Identification of digestion resistant IgE binding peptides. *Food and Chemical Toxicology, 107*, 88–98. https://doi.org/10.1016/j.fct.2017.06.029

Di Stasio, L., Picascia, S., Auricchio, R., Vitale, S., Gazza, L., Picariello, G., Gianfrani, C., & Mamone, G. (2020a). Comparative analysis of *in vitro* digestibility and immunogenicity of gliadin proteins from durum and einkorn wheat. *Frontiers in Nutrition, 7*, 56. https://doi.org/10.3389/fnut.2020.00056

Di Stasio, L., Tranquet, O., Picariello, G., Ferranti, P., Morisset, M., Denery-Papini, S., & Mamone, G. (2020b). Comparative analysis of eliciting capacity of raw and roasted peanuts: The role of gastrointestinal digestion. *Food Research International, 127*, Article 108758. https://doi.org/10.1016/j.foodres.2019.108758

Di Stasio, L., d'Acierno, A., Picariello, G., Ferranti, P., Nitride, C., & Mamone, G. (2020c). *In vitro* gastroduodenal and jejunal brush border membrane digestion of raw and roasted tree nuts. *Food Research International, 136*, Article 109597. https://doi.org/10.1016/j.foodres.2020.109597

Donowitz, M., Singh, S., Salahuddin, F. F., Hogema, B. M., Chen, Y., Gucek, M., Cole, R. N., Ham, A., Zachos, N. C., Kovbasnjuk, O., Lapierre, L. A., Broere, N., Goldenring, J., deJonge, H., & Li, X. (2007). Proteome of murine jejunal brush border membrane vesicles. *Journal of Proteome Research, 6*(10), 4068–4079. https://doi.org/10.1021/pr0701761

Dupont, D., & Tome, D. (2020). *Chapter 20 - Milk proteins: Digestion and absorption in the gastrointestinal tract. Milk proteins* (third ed., pp. 701–714). San Diego: Academic Press.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., & Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International, 88*, 217–225. https://doi.org/10.1016/j.foodres.2015.12.006

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., Dürr, D., Stoll, P., Vergères, G., & Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to *in vivo* pig digestion. *Food Research International, 102*, 567–574. https://doi.org/10.1016/j.foodres.2017.09.047

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., Dürr, D., Stoll, P., Vergères, G., & Portmann, R. (2018). Mass spectrometry data of *in vitro* and *in vivo* pig digestion of skim milk powder. *Data in Brief, 16*(21), 911–917. https://doi.org/10.1016/j.dib.2018.09.089

Egger, L., Ménard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., Vergères, G., Dupont, D., & Portmann, R. (2019). Digestion of milk proteins: Comparing static and dynamic *in vitro* digestion systems with *in vivo* data. *Food Research International, 118*, 32–39. https://doi.org/10.1016/j.foodres.2017.12.049

Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. *Danish Medical Bulletin, 46*(3), 183–196.

Gallego-Lobillo, P., Ferreira-Lazarte, A., Hernandez-Hernandez, O., & Villamiel, M. (2020). Kinetic study on the digestibility of lactose and lactulose using small intestinal glycosidases. *Food Chemistry, 2020*(316), Article 126326. https://doi.org/10.1016/j.foodchem.2020.126326

Gianfrani, C., Camarca, A., Mazzarella, G., Di Stasio, L., Giardullo, N., Ferranti, P., Picariello, G., Rotondi Aufiero, V., Picascia, S., Troncone, R., Pogna, N., Auricchio, S., & Mamone, G. (2015). Extensive *in vitro* gastrointestinal digestion markedly reduces the immune-toxicity of Triticum monococcum wheat: Implication for celiac disease. *Molecular Nutrition & Food Research, 59*(9), 1844–1854. https://doi.org/10.1002/mnfr.201500126

Hausch, F., Shan, L., Santiago, N. A., Gray, G. M., & Khosla, C. (2002). Intestinal digestive resistance of immunodominant gliadin peptides. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 283*(4), G996–G1003. https://doi.org/10.1152/ajpgi.00136.2002

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*(1), 23–35. https://doi.org/10.1016/j.cis.2010.11.001

Hernandez-Hernandez, O. (2019). *In vitro* gastrointestinal models for prebiotic carbohydrates: A Critical Review. *Current Pharmaceutical Design, 25*(32), 3478–3483. https://doi.org/10.2174/1381612825666191011094724

Holmes, R., & Lobley, R. W. (1989). Intestinal brush border revisited. *Gut, 30*(12), 1667. https://doi.org/10.1136/gut.30.12.1667

Hooton, D., Lentle, R., Monro, J., Wickham, M., & Simpson, R. (2015). The secretion and action of brush border enzymes in the mammalian small intestine. *Reviews of Physiology, Biochemistry and Pharmacology, 59*, 118. https://doi.org/10.1007/112_2015_24

Hooton, D.F. (2018). Brush border digestion: development of a physiologically relevant in vitro model: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiology at Massey University, Manawatū, New Zealand (Doctoral dissertation, Massey University).

Howell, S., Kenny, A. J., & Turner, A. J. (1992). A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. *Biochemical Journal, 284*, 595–601. https://doi.org/10.1042/bj2840595

Hur, S., Lim, B., Decker, E., & Mcclements, D. (2011). *In vitro* human digestion models for food applications. *Food Chemistry, 125*, 1–12. https://doi.org/10.1016/j.foodchem.2010.08.036

Iftikhar, M., Iftikhar, A., Zhang, H., Gong, L., & Wang, J. (2020). Transport, metabolism and remedial potential of functional food extracts (FFEs) in Caco-2 cells monolayer: A review. *Food Research International, 136*, Article 109240. https://doi.org/10.1016/j.foodres.2020.109240

Kondrashina, A., Arranz, E., Cilla, A., Faria, M. A., Santos-Hernández, M., Miralles, B., Hashemi, N., Rasmussen, M. K., Young, J. F., Barberá, R., Mamone, G., Tomás-Cobos, L., Bastiaan-Net, S., Corredig, M., & Giblin, L. (2023). Coupling in vitro food digestion with *in vitro* epithelial absorption; recommendations for biocompatibility. *Critical Reviews in Food Science and Nutrition, 1–19*. https://doi.org/10.1080/10408398.2023.2214628

Le Feunteun, S., Al-Razaz, A., Dekker, M., George, E., Laroche, B., & van Aken, G. (2021). Physiologically based modeling of food digestion and intestinal microbiota: Sstate of the art and future challenges. An INFOGEST Review. *Annual Review of Food Science and Technology, 12*, 149–167. https://doi.org/10.1146/annurev-food-070620-124140

Li, C., Yu, W., Wu, P., & Chen, X. D. (2020). Current *in vitro* digestion systems for understanding food digestion in human upper gastrointestinal tract. *Trends in Food Science & Technology, 96*, 114–126. https://doi.org/10.1016/j.tifs.2019.12.015

Mach, N., Berri, M., Esquerré, D., Chevaleyre, C., Lemonnier, G., Billon, Y., & Estellé, J. (2014). Extensive expression differences along porcine small intestine evidenced by transcriptome sequencing. *PLoS One1, 9*(2), e88515.

Mackie, A., Mulet-Cabero, A.-I., & Torcello-Gómez, A. (2020). Simulating human digestion: Developing our knowledge to create healthier and more sustainable foods. *Food & Function, 11*(11), 9397–9431. https://doi.org/10.1039/D0FO01981J

Mamone, G., Ferranti, P., Rossi, M., Roepstorff, P., Fierro, O., Malorni, A., & Addeo, F. (2007). Identification of a peptide from alpha-gliadin resistant to digestive enzymes: implications for celiac disease. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 855*(2), 236–241. doi: 10.1016/j.jchromb.2007.05.009.

Mamone, G., Nitride, C., Picariello, G., Addeo, F., Ferranti, P., & Mackie, A. (2015). Tracking the fate of pasta (T. Durum semolina) immunogenic proteins by *in vitro* simulated digestion. *Journal of Agricultural and Food Chemistry, 63*(10), 2660–2667. https://doi.org/10.1021/jf505461x

Mamone, G., Picariello, G., Ramondo, A., Nicolai, M. A., & Ferranti, P. (2019). Production, digestibility and allergenicity of hemp (Cannabis sativa L.) protein isolates. *Food Research International, 115*, 562–571. https://doi.org/10.1016/j.foodres.2018.09.017

Mamone, G., Comelli, M. C., Vitale, S., Di Stasio, L., Kessler, K., Mottola, I., Siano, F., Cavaletti, L., & Gianfrani, C. (2022). E40 glutenase detoxification capabilities of residual gluten immunogenic peptides in in vitro gastrointestinal digesta of food matrices made of soft and durum wheat. *Frontiers in Nutrition, 9*, Article 974771. https://doi.org/10.3389/fnut.2022.974771

Mamone, G., & Picariello, G. (2023). Optimized extraction and large-scale proteomics of pig jejunum brush border membranes for use in *in vitro* digestion models. *Food Research International, 164*, Article 112326. https://doi.org/10.1016/j.foodres.2022.112326

Manguy, J., Jehl, P., Dillon, E. T., Davey, N. E., Shields, D. C., & Holton, T. A. (2017). Peptigram: A web-based application for peptidomics data visualization. *Journal of Proteome Research, 16*, 712–719. https://doi.org/10.1021/acs.jproteome.6b00751

Martineau-Côté, D., Achouri, A., Pitre, M., Karboune, S., & L'Hocine, L. (2024). Improved *in vitro* gastrointestinal digestion protocol mimicking brush border digestion for the determination of the Digestible Indispensable Amino Acid Score (DIAAS) of different food matrices. *Food Research International, 178*, Article 113932. https://doi.org/10.1016/j.foodres.2024.113932

McConnell, R. E., Benesh, A. E., Mao, S., Tabb, D. L., & Tyska, M. J. (2011). Proteomic analysis of the enterocyte brush border. *American Journal of Physiology-Gastrointestinal and Liver. Physiology, 300*(5), G914–G926. https://doi.org/10.1152/ajpgi.00005.2011

Menard, O., Lesmes, U., Shani-Levi, C. S., Araiza Calahorra, A., Lavoisier, A., Morzel, M., Rieder, A., Feron, G., Nebbia, S., Mashiah, L., Andres, A., Bornhorst, G., Carrière, F., Egger, L., Gwala, S., Heredia, A., Kirkhus, B., Macierzanka, A., Portman, R., Recio, I., & Dupont, D. (2023). Static *in vitro* digestion model adapted to the general older adult population: An INFOGEST international consensus. *Food & Function, 14*(10), 4569–4582. https://doi.org/10.1039/d3fo00535f

Miller, E. R., & Ullrey, D. E. (1987). The pig as a model for human nutrition. *Annual Review of Nutrition, 7*, 361–382. https://doi.org/10.1146/annurev.nu.07.070187.002045

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T. O. R. S. T. E. N., Bourlieu, C., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food–an international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Nilsson, A., & Duan, R. D. (2006). Absorption and lipoprotein transport of sphingomyelin. *Journal of Lipid Research, 47*(1), 154–171. https://doi.org/10.1194/jlr.M500357-JLR200

Nitride, C., Vegarud, G. E., Comi, I., Devold, T. G., Røseth, A., Marti, A., Iametti, S., Mamone, G., Picariello, G., Alfieri, F., Adalgisa Nicolai, M., Mills, C., & Ferranti, P. (2022). Effect of sprouting on the proteome of chickpea flour and on its digestibility by *ex vivo* gastro-duodenal digestion complemented with jejunal brush border membrane enzymes. *Food Research International, 154*, Article 111012. https://doi.org/10.1016/j.foodres.2022.111012

Ölander, M., Wiśniewskii, J. R., Matsson, P., Lundquist, P., & Artursson, P. (2016). The proteome of filter-grown Caco-2 cells with a focus on proteins involved in drug disposition. *Journal of Pharmaceutical Sciences, 105*(2), 817–827. https://doi.org/10.1016/j.xphs.2015.10.030

Ozorio, L., Mellinger-Silva, C., Cabral, L., Jardin, J., Boudry, G., & Dupont, D. (2020). The influence of peptidases in intestinal brush border membranes on theabsorption of oligopeptides from whey protein hydrolysate: An ex vivo study using an ussing Chamber. *Foods, 9*(10), 1415. https://doi.org/10.3390/foods9101415

Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Moniva, S., & Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *Journal of Chromatography B, 878*(3–4), 295–308. https://doi.org/10.1016/j.jchromb.2009.11.033

Picariello, G., Iacomino, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., Di Luccia, A., & Addeo, F. (2013). Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins. *Food Chemistry., 139*(1–4), 203–212. https://doi.org/10.1016/j.foodchem.2013.01.063

Picariello, G., Miralles, B., Mamone, G., Sánchez-Rivera, L., Recio, I., Addeo, F., & Ferranti, P. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition & Food Research, 59*(5), 948–956. https://doi.org/10.1002/mnfr.201400856

Picariello, G., Ferranti, P., & Addeo, F. (2016). Use of brush border membrane vesicles to simulate the human intestinal digestion. *Food Research International, 88*, 327–335. https://doi.org/10.1016/j.foodres.2015.11.002

Picariello, G., De Cicco, M., Nocerino, R., Paparo, L., Mamone, G., Addeo, F., & Berni Canani, R. (2019). Excretion of dietary cow's milk derived peptides into breast milk. *Frontiers in Nutrition, 6*, 25. https://doi.org/10.3389/fnut.2019.00025

Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., Sollid, L. M., & Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. *Science, 297*(5590), 2275–2279. https://doi.org/10.1126/science.1074129

Vivanco-Maroto, S. M., Santos-Hernández, M., Sanchón, J., Picariello, G., Recio, I., & Miralles, B. (2022). *In vitro* digestion of milk proteins including intestinal brush border membrane peptidases. Transepithelial transport of resistant casein domains. *Food Research International, 157*, Article 111238. https://doi.org/10.1016/j.foodres.2022.111238

Zhou, H., Tan, Y., & McClements, D. J. (2023). Applications of the INFOGEST *in vitro* digestion model to foods: A review. *Annual Review of Food Science and Technology, 14*, 135–156. https://doi.org/10.1146/annurev-food-060721-012235