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Digestion of micellar casein in duodenum cannulated pigs. Correlation between *in vitro* simulated gastric digestion and *in vivo* data

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ABSTRACT

Correlation and validation of the results of simulated gastrointestinal digestion of food compounds towards *in vivo* data is essential. The objective of this work was to monitor the digestion of milk micellar casein in the porcine upper intestinal tract and to match the outcome with the gastric *in vitro* digestion following the Infogest harmonized protocol. In pig duodenum, small amounts of intact caseins were present in all samples, while caseins were observed up to 60 min of gastric *in vitro* digestion. The peptide profile generated after *in vitro* and *in vivo* digestion showed clear similarities with specific overrepresented regions rich in proline and other hydrophobic residues. The statistical comparison of the *in vivo* and *in vitro* peptidome resulted in satisfactory correlation coefficients, up to 0.8. Therefore, the *in vitro* protocol used was a robust and simple model that provides a similar peptide profile than that found in porcine duodenum.

1. Introduction

Food, after being ingested, suffers a series of complex physical and chemical modifications during digestion that will originate the release and absorption of nutrients. Food proteins are hydrolyzed by gastric and pancreatic enzymes, and epithelial brush border membrane peptidases into a complex mixture of peptides and free amino acids that transit and are progressively absorbed along the small intestine (Goodman, 2010). In fact, several released peptides during the digestion of proteins could entail physiological implications, acting as signals in regulatory functions of the organism (Rutherfurd-Markwick, 2012; Santos-Hernández, Miralles, Amigo, & Recio, 2018) or pose a toxicological risk through the formation of epitopes capable of inducing allergic reactions (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015).

In this context, it is critical to know the changes that food proteins undergo in the stomach in relation to degradation and the identification of resistant protein regions during the digestive process. Gastric acid denatures and unfolds food proteins to make them more accessible to the action of pepsin. This endopeptidase has a higher specificity for hydrolyzing peptide bonds C-terminal to aromatic amino acids and leucine, and degrades 10 to 15% of the common dietary proteins into oligopeptides (Antonov, Rumsh, & Tikhodeeva, 1974; Luo, Boom, & Janssen, 2015). However, the behaviour of food proteins and susceptibility to the action of pepsin varies with the protein source depending mainly on amino acid sequence, tertiary structure and post-translational modifications (Wang, et al., 2017). While globular and compact proteins like β-lactoglobulin are resistant to the action of this gastric enzyme, other proteins with a looser structure like milk caseins undergo disaggregation of micellar structure with submicelle formation and clotting, and are hydrolysed by the action of pepsin into smaller polypeptides (Dallas et al., 2017). Due to clotting in the stomach, transit rate to the intestinal tract of milk casein is delayed with regard to other food proteins. This clotting/aggregation behavior of caseins is also influenced by the manufacture conditions of the product including heat treatment, drying or gelation. In a previous study with mini-pigs cannulated at duodenum and jejunum, it was found that the structure of dairy products (stirred or not stirred gels and rennet or acid gels) strongly affected gastric kinetics of protein digestion (Barbé, Le Feunteun, et al., 2014). It was proposed that milk rennet gels had longer residence time at the stomach due to the formation of a coagulum with higher stiffness than acid gels (Barbé, Ménard, et al., 2014). The coagulation behavior and kinetics of protein hydrolysis of different milk protein ingredients has been evaluated by using an *in vitro* dynamic model. The formation of different structured curds under gastric conditions conditioned the course of protein digestion with skim milk

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powder exhibiting slower hydrolysis rate than sodium caseinate (Wang, Ye, Lin, Han, & Singh, 2018). Similarly, it has been reported that the consistency of the casein coagulum from UHT milk was softer than that formed from pasteurized milk and this affects protein hydrolysis at the end of the gastric digestion (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019). Casein obtained by isoelectric precipitation or by enzymatic hydrolysis with chymosin has long been used as a popular food ingredient in many applications. A more recently adopted technology consists in milk microfiltration and diafiltration to obtain products with high protein content where 85–95% corresponds to casein. This micellar casein is successfully applied in cheese making and in high-protein nutritional and sport beverages, among others. Although the technological properties of micellar casein powders and concentrates have been evaluated (Crowley et al., 2018), the kinetics of protein hydrolysis in the stomach has not been attempted.

Pig is an animal model well suited to predict protein digestibility in humans (Deglaire, Bos, Tomé, & Moughan, 2009). This model has been previously employed for studying the resistance of selected milk protein fragments in the neonate (Bouzerzour et al., 2012) or the kinetics of protein digestion with regard to the heating or gelation process (Barbé et al., 2013). Although methods evaluating *in vivo* digestion, both in humans or animals, contribute the most to get relevant physiological results, in order to give a general prediction of any nutrient digestibility, it is necessary to develop *in vitro* models of gastrointestinal simulation (Bohn et al., 2018). *In vitro* methods employ less manpower and are expected to reduce the variation associated with inter-individual variability. The development of the harmonized model of digestion in the Infogest network based on physiologically relevant conditions provides a consensus set of conditions that has already proved to produce reproducible results in inter-laboratory trials (Egger et al., 2016). This *in vitro* digestion protocol provides also comparable results with skim milk powder digestion in pigs (Egger et al., 2017) and with casein and whey protein digests obtained in human jejunum (Sanchón et al., 2018). The suitability of the protocol to mimic the behaviour of micellar casein in the upper intestinal is hypothesized.

Therefore, the specific objectives of this work were: (a) to study the behaviour of milk micellar casein along the digestion process in the upper intestinal tract through the use a porcine model cannulated in duodenum, an experimental design that permits to collect early duodenal effluents in the course of the stomach emptying and (b) to compare the casein degradation products upon the application of the Infogest harmonized digestion protocol to those delivered *in vivo* in order to evaluate the digestion model.

2. Materials and methods

2.1. Samples and reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Commercial micellar casein was purchased from Ingredia S.A, Arras, France (Prodiet 85B, batch no. 141179). Its protein content was 77.84% measured by Kjeldahl.

2.2. Animal experiments

All procedures were in accordance with the European Community guidelines for the use of laboratory animals (L358-86/609/EEC). The study was approved by the local committee for ethics in animal experimentation. The animals were 10 pigs Large White × Landrace × Piétrain of about 40 kg live weight. Two weeks before starting the trials, they were surgically fitted with a T-shaped cannula in the duodenum (10 cm downstream from the pylorus). Following the operation, the animals were housed in individual slatted pens within a ventilated room with controlled temperature (21 °C). During convalescence following the surgical procedure and also between the sampling days, pigs were fed with 800 g/d of a pig feed concentrate (Cooperl Arc Atlantique®, Pelstan, France) containing 16% proteins, 1% fat, 4% cellulose and 5% mineral matter. Animals had free access to water.

Within the experimental period (two weeks), the days of sampling were separated by at least 2 days. Test meal (casein powder reconstituted in water 5% (w/v) in sufficient quantity to cover the protein daily requirements of 50 g/kg) was offered to the pigs for 10 min. Chromium-EDTA (110.8 ppm, w/w) was added to the meal as marker of the liquid phase, as previously described (Barbé et al., 2013). The animals had no access to water from 1 h before to 7 h after the meal delivery. Duodenum effluents were collected in plastic bottles 15 min before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after ingestion of test meal. The sampling was stopped when 40 mL were collected or after a maximum of 3 min of sampling time. A protease inhibitor (Pefabloc®, Sigma-Aldrich, St. Louis, MO, USA at 2.5 mM final concentration) was added to the collecting bottles. The effluents were weighed and freeze-dried.

2.3. In vitro simulated digestion

Casein was digested following the oral and gastric phase of the *in vitro* harmonized protocol (Brodkorb et al., 2019). The protein concentration in the simulated oral fluid was 6% (w/v) and human amylase was not used in this step due to the absence of starch in the substrate. Gastric phase was initiated by the addition of pepsin from porcine gastric mucosa (EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA) mixed in simulated gastric fluid (2000 U/mL of digesta), at a ratio of 50:50 (v/v). Samples were collected at different times of digestion (10, 20, 30, 60, 90 and 120 min) and the reaction was stopped by adjusting the pH to 7 with NaOH 1 M. Digests were snap freezed in liquid nitrogen, freeze-dried and kept at −20 °C until analysis.

2.4. Analysis of digests

2.4.1. Chromium and nitrogen analysis in duodenal effluents

The chromium content was determined using an inductively coupled plasma mass spectrometry (ICP-MS) instrument NexION 300XX (Perkin-Elmer, Waltham, MA, USA). Nitrogen content was determined by elemental analysis in a LECO CHNS-932 analyzer. The analyses were performed at the Mass spectrometry and Elemental analysis units from the Interdepartmental Investigation Service from the Autonomous University of Madrid.

2.4.2. Protein characterization by SDS-PAGE, in-gel digestion and identification.

Protein load of duodenal effluents and *in vitro* gastrointestinal digests was adjusted to 1 mg of protein/mL based on the nitrogen content of the samples determined by elemental analysis. Freeze-dried digests were treated as previously reported (Cruz-Huerta, García-Nebot, Miralles, Recio, & Amigo, 2015). A molecular weight marker (Precision Plus Protein™ Unstained standard, Bio-Rad Laboratories, Hercules, CA, USA) and undigested casein (0.8 mg of protein/mL) were included on each gel. Thereafter, the gels were stained with Coomasie Blue (Instant blue, Expedeon, Swavesey, UK) and images were taken with a Molecular Imager®VersaDoc™ MP 5000 system (Bio-Rad Laboratories, Hercules, CA, USA) and processed with Quantity One®1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Coomassie Blue-stained bands were manually excised and decolored with 25 mM ammonium bicarbonate and acetonitrile (1:1; v:v). Then, the bands were reduced with 10 mM 1,4-dithiothreitol (DTT, Sigma-Aldrich) in 25 mM ammonium bicarbonate for 1 h at 56 °C prior to 30 min incubation with 55 mM iodoacetamide at room temperature. Bands were washed (×2) with 25 mM ammonium bicarbonate: acetonitrile (1:1; v:v) and then dehydrated in 100% acetonitrile. The in-gel digestion was carried out at 37 °C, overnight, with trypsin (V5117, Promega Biotech Ibérica S. L., Spain) at a final concentration of

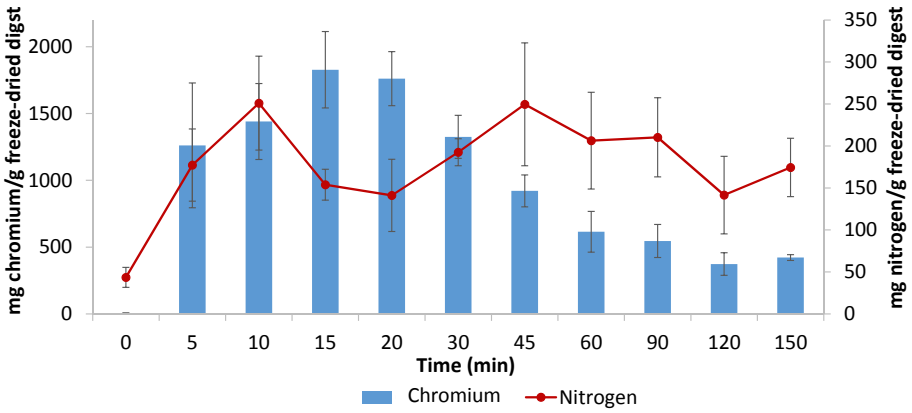

Fig. 1. Chromium-EDTA concentration (blue bars) and nitrogen content (red trace) of pig duodenal effluents from 5 to 150 min of digestion after oral administration of caseins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

12.5 ng/μL in 25 mM ammonium bicarbonate. Soluble peptides were recovered by centrifugation at 7000 × *g* for 1 min and bands were washed twice with acetonitrile: 10% formic acid (1:1; v:v) and centrifuged; the supernatants were merged and dried using a Speed Vac prior to reconstitution in acetonitrile 33% containing trifluoroacetic acid 0.1%. An Autoflex SpeedTM (Bruker Daltonics, Bremen, Germany) was used. Samples were spotted into a MALDI target plate with α-cyano-4-hidroxycinnamic acid matrix. Ions were detected in positive reflection mode and were collected from the sum of 100–600 lasers shots. Protein calibration Standard I, (Bruker Daltonics) was employed for external calibration of spectra. Monoisotopic peaks generated using FlexAnalysis software were fragmented using the lift method for MS/MS analysis. For peptide identification the MASCOT v2.4 software (Matrix Science) Server 2.1 and Biotools version 2.1 (Bruker Daltonics) were used.

2.4.3. Peptidomic analysis

Freeze-dried digests were reconstituted in solvent A (water:formic acid, 100:0.1, v/v) and centrifuged at 13000 × *g*, 10 min. 50 μL of supernatant was injected for each sample. Additionally, samples were submitted to a selective precipitation by adding calcium chloride (1% w/v) and ethanol (50% v/v) to prepare an enriched phosphopeptide fraction (Sánchez-Rivera et al., 2014).

HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranea Sea C_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain). HPLC system was connected to an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with an electrospray ionization source. A linear gradient from 0 to 45% of solvent B (acetonitrile:formic acid, 100:0.1, v/v) in 120 min was performed for peptide elution and the flow was set at 0.2 mL/min. Spectra were recorded over the mass/charge (*m/z*) range 100–1200 and the target mass was set at 900 *m/z*.

Data processing was done by using Data Analysis (version 4.0, Bruker Daltonics GmbH, Bremen, Germany). Peptide identifications were obtained using the MASCOT v2.4 protein search engine (Matrix Science) against a homemade database of major genetics variants of bovine caseins. A homemade database of porcine digestive enzymes was also used (Tables S1 and S2 in Supplementary material, respectively). No enzyme was selected. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. No post-translational modifications were considered. The matched MS/MS spectra were interpreted by using Biotools version 3.2 (Bruker Daltonics). The relative abundance of peptides was that provided by the software output. A manual revision was done for each identification peptide spectrum, regardless of its *P* value. The analysis of enzymatic cleavages was performed in the peptide lists by EnzymePredictor tool (Vijayakumar et al., 2012).

2.4.4. Analysis of free amino acids

Protein from freeze-dried digests was precipitated with 4-sulphosalicylic acid (12.5 mg/mg of protein) by allowing to stand 1 h on ice. Centrifugation for 15 min at 15000 × *g*, 4 °C, was followed by filtration of the supernatant by 0.45 μm membranes and adjusting to pH 2.2 with 0.3 M NaOH. Amino acid analysis was performed on a Biochrom 30 amino acid analyser (Biochrom Ltd, Cambridge, UK). Results were expressed as mmol of amino acid per kg of digest. Under our analysis conditions, tryptophan, glutamine and asparagine are not determined.

2.5. Statistical analysis

Spearman correlation matrices were built on the basis of frequency of appearance of amino acids belonging to peptides, ranging from 1 to 30. Statistica software (StatSoft Incl., Tulsa, OK, USA) was used for the calculations.

3. Results and discussion

3.1. Digestion kinetics and protein degradation in vivo

Chromium was added to the casein meal as marker of the liquid phase. Its concentration in the digests underwent an increase up to 20 min and decreased to a steady level from this time (Fig. 1). A return to the basal values could not be observed in the analysed time interval. This is consistent with previous reported data in pig after consumption of a normal diet, where the liquid marker remained in the stomach 300 min post-ingestion with a half time of 48.8 min (Chiang, Croom, Chuang, Chiou, & Yu, 2008). The increased concentration in chromium-EDTA between 5 and 30 min corresponded to the rapid emptying of soluble compounds. However, the protein content of the effluents reached a maximum at shorter time points (around 10 min), decreased up to 20 min, and a second maximum was reached at 45 min. This bimodal curve for the protein content of the effluents is compatible with the gastric emptying of soluble whey proteins present in small amounts in the casein product at short time points and the draining of casein degradation products at longer times.

The protein in the duodenal effluents was characterized by SDS-PAGE. Fig. 2A shows a representative electrophoretic profile of successive duodenal samples taken from a single animal. The oral administered micellar casein also contained β-lactoglobulin (18 kDa), α-lactalbumin (14 kDa) and several bands corresponding to casein fragments with MWs between 10 and 15 kDa, as identified by in-gel digestion and MALDI TOF/TOF analysis. Table S3 in Supplementary data summarizes the identified proteins and protein fragments. Most intense

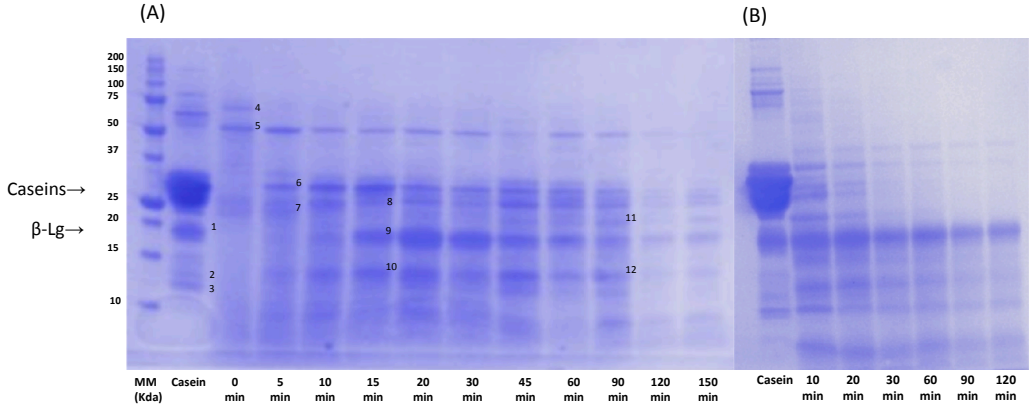

Fig. 2. SDS-PAGE profile of (A) *in vivo* and (B) *in vitro* casein digests. Each lane in A corresponds to an effluent sampling time. Each lane in B corresponds to a gastric digestion time. MM, molecular weight marker. Identified bands:1: β-lactoglobulin, 2: β-casein fragment, 3: β-casein fragment, 4: Porcine serum albumin, 5: Porcine pancreatic α-amylase, 6: α_{S1}-casein + trypsin, 7: α_{S1}-casein fragment + chymotrypsin, 8: κ-casein, 9: β-lactoglobulin, 10: κ-casein + α_{S1}-casein fragment, 11: α_{S1}-casein fragment, 12: κ-casein fragment.

electrophoretic bands observed in the effluents taken 15 min before oral feeding (lane 0), corresponded to porcine serum albumin and pancreatic α-amylase (Fig. 2A). Low amounts of non-degraded caseins could be identified in the 15 to 120 min range, with time points 45, 60 and 90 min showing particular abundance of caseins. Small traces of undigested caseins were also found in duodenum of cannulated pigs at short times after the oral administration of milk by Barbé, Le Feunteun et al. (2014). A study in pigs did not detect intact casein in duodenum after administration of reconstituted skim milk powder, although in that case the total duodenum content was analysed instead duodenal effluents (Egger et al., 2017). In addition to the different sampling method, cannula *vs* intestinal contents, the nature of the product could influence clotting and solubility of the sample. A fraction of the colloidal calcium phosphate may be dissolved during the manufacturing process of the micellar casein used in our study resulting in looser and smaller micellar structure (Wang et al., 2018). In addition, clotted casein is exposed to the action of gastric pepsin and empties slowly from the stomach in the form of degraded products, as reflected in the protein content of the effluents (Fig. 1) and in the bands between 10 and 20 kDa identified as casein fragments SDS-PAGE by in-gel digestion and MALDI-TOF/TOF analysis (Supplementary data).

Moreover, all digests contained bands with MW < 10 kDa corresponding to small size peptides which were identified by HPLC-MS/MS (ion trap, see Section 3.2). The electrophoretic band corresponding to β-lactoglobulin was also present at short time points (up to 30 min) being emptied with soluble phase and showing a similar behaviour than chromium. This indicates that the small amount of β-lactoglobulin present in the casein substrate resists the first digestion stage in the stomach, as it was found in the early duodenal effluents.

3.2. Peptide and amino acid composition in duodenal effluents

Peptide sequencing was conducted by HPLC-MS/MS by matching with the main casein genetic variants. All identified peptide sequences in the effluents collected at different time points for each individual are detailed in Miralles et al., Data in brief, co-submitted with this manuscript. On the whole, the 652 distinct casein-derived peptides identified over digestion time are listed in the Supplementary Excel file. Their distribution for β-, α_{s1}-, α_{s2}-, and κ-casein was 32, 29, 18 and 20%, respectively. Peptide size ranged between 5 and 34 amino acid residues and peptides masses between 520 and 3502 Da. The identified peptides were mapped and aligned on their parent protein sequences with the Peptigram tool (Manguy et al., 2017). Phosphorylated regions are not included in this mapping since they were analysed separately in an enriched fraction, given their lower ionization capacity. Fig. 3A and 3B show the β- and α_{s1}-casein derived peptides found in the duodenal digests over the sampling time. Peptides from α_{s2}- and κ-casein are shown in Fig. 4. Each vertical bar corresponds to an amino acid, the peptide abundance is represented by the height of the bars, while the intensity of the green colour is proportional to the intensities of overlapping peptides. The protein coverage ranged from 76 to 94% in the case of β-casein, and from 70 to 91% in the case of α_{s1}-casein. For all proteins, a rather similar pattern of peptides with regard to time could be observed, which suggests certain homogeneity of the outflowed material from the stomach. In fact, peptides showed a similar size at the different collected time points. Around 70% of peptides comprised < 10 amino acids, which reflects the susceptibility of caseins to pepsin. For β- and α_{s1}-casein, an important number of peptides with high intensity were found at the C-terminal region of the protein, which may correlate with peptide abundance and/or the ionization capacity of the peptides belonging to these domains. In addition, peptides from regions 24–40 and 53–64 from α_{s1}-casein were abundant in duodenal effluents at all-time points. Lower protein coverage was observed for α_{s2}- and κ-casein, ranged 51–78% and 60–83%, respectively. Those regions with a higher number of overlapping peptides are between residues 89 and 124 from α_{s2}-casein, residues 31 to 89 from κ-casein, and the C-terminal region of κ-casein. The lower coverage observed, especially in the *N*-terminal part of these two proteins can be due to the absence of charged residues in this part of both proteins chains, as compared with α_{s1}- and β-casein.

The analysis of phosphorylated peptides was performed separately in an enriched Ca/ethanol fraction prepared from the duodenal effluents. Several phosphorylated fragments from α_{s1}- and α_{s2}-, β-, and κ-casein could be identified (Fig. 5). In the case of β-casein, the region covered by most phosphopeptides corresponded to ^{30}IEKFQSpEEQQQ^{40}, comprising phosphorylated serine 35. In α_{s1}-casein, the determined sequences covered the three regions where phosphorylations occur, including the phosphorylation sites 46, 48, 64, 66–68, 75, and 115. Other phosphorylated fragments were identified from α_{s2}- and κ-casein. There are only scarce reports on the appearance of phosphorylated peptides in digests from *in vivo* assays. Nevertheless, fragments ^{7}NVPGEIVESpLSpSpSpE^{20} and ^{7}NVPGEIVESpLSpSp^{18} from β-casein had been previously found in human duodenum after milk ingestion after 20 and 40 min, respectively (Chabance et al., 1998). Various sequences comprised in these regions have also been identified in human jejunum effluents (Sanchón et al., 2018) or have proven to be bioavailable after consumption of cheese (Caira et al., 2016). These peptides are expected to form soluble complexes with different minerals such as calcium, zinc, and iron with a positive role in mineral absorption (Cross et al., 2007). Although the role of casein phosphopeptides needs to be elucidated, the present study showed the release of, at least, 70 phosphorylated fragments from casein at early times of intestinal digestion in pig after casein intake.

Most peptides found in cannulated duodenum were compatible with the action of pepsin with a high number of cleavages explained by the action of this enzyme, as calculated by using the EnzymePredictor tool (results not shown). Some of the identified sequences had been previously reported as biologically active peptides (Table S4 in

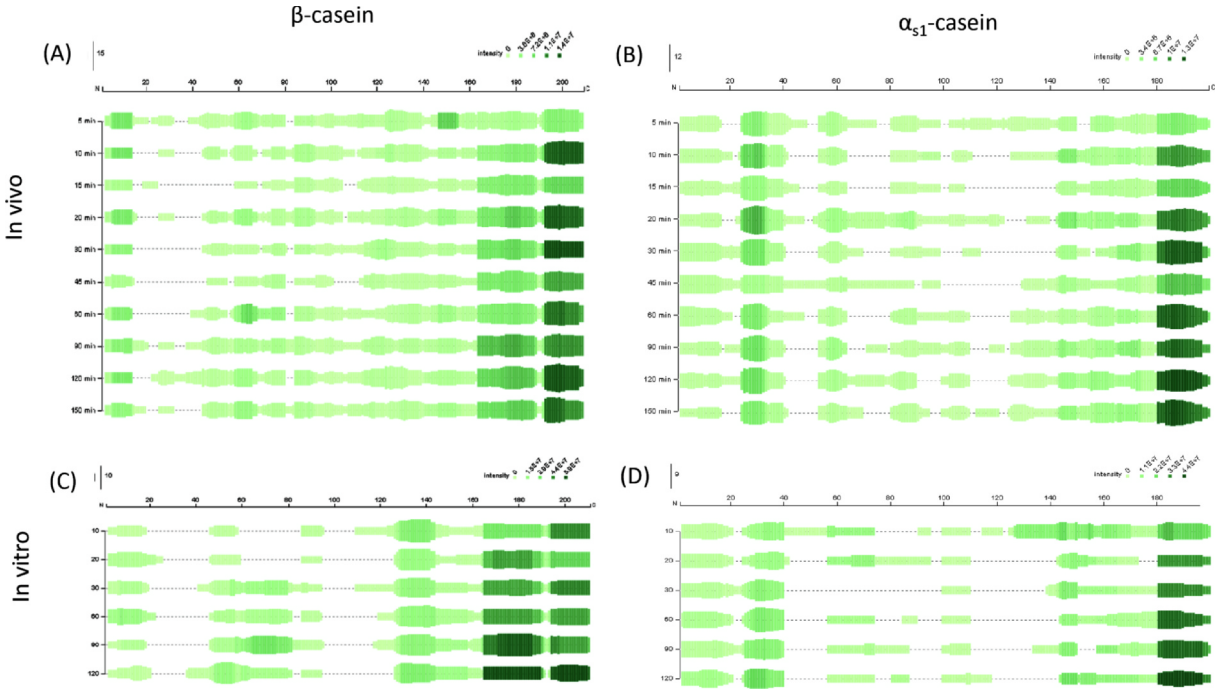

Fig. 3. Peptides derived from β- and α_{s1}-casein in the duodenal (*in vivo*, A, B) and *in vitro* (C, D) digests over sampling time (5 to 150 min) identified by HPLC-MS/MS. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence. Peptide overlapping is represented by the height of the bars while the intensity of the green colour is proportional to the sum of intensities of overlapping peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Supplementary Material). For instance, various peptides found in the digests at all times had been previously described as opioid peptides, such as fragments β-casein ^{114}YPVEPF^{119} (Jinsmaa & Yoshikawa, 1999), α_{s1}-casein ^{144}YFYPEL^{149} (Fernández-Tomé et al 2016), and κ-casein ^{33}SRYPSY^{38} (Yoshikawa et al., 1994). Thirteen sequences reported as antibacterial or antimicrobial were identified in nearly all samples, while antioxidant, antihypertensive or immunomodulatory peptides were observed at certain collection times. Other activities reported for the identified peptides include ACE, DPP-IV and Prolyl-endopeptidase inhibition, increased mucin genes expression, bradykinin potentiation and antithrombin. Some of these peptides might exert their activity by their interaction at the intestinal level (i.e.: opioid, antioxidant), while others are expected to be absorbed to yield their known biological effect (i.e.: antihypertensive, immunomodulatory).

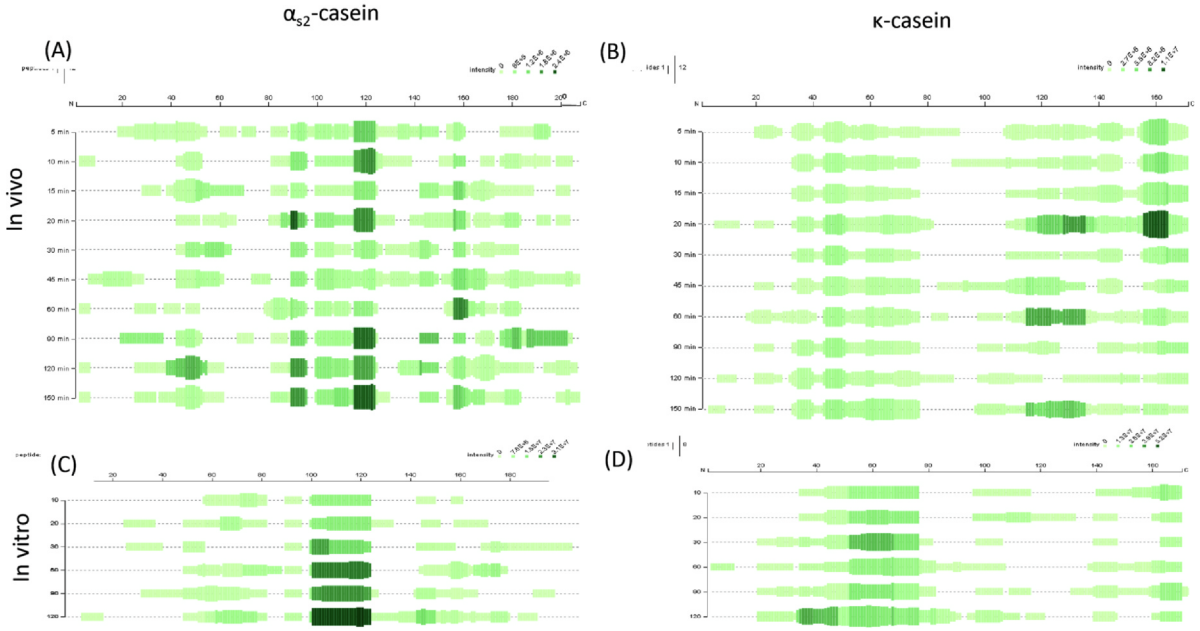

Fig. 4. Peptides derived from α_{s1}- and κ-casein in the duodenal (*in vivo*, A, B) and *in vitro* (C, D) digests over sampling time (5 to 150 min) identified by HPLC-MS/MS. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence. Peptide overlapping is represented by the height of the bars while the intensity of the green colour is proportional to the sum of intensities of overlapping peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Caseinophosphopeptides from β-, α-, and κ-caseins identified in the pig duodenum digests. X, denotes a phosphorylated serine residue.

Irrespective of this aspect, the rate of appearance of peptides raises the question on the role of the concentration of selected protein fragments with biological activity during digestion. A continuous flow might permit to reach a peak concentration able to overcome the limit threshold and trigger a physiological effect.

In order to portray the global composition of the material coming out from the stomach the profile of free amino acids was also studied over time (Fig. 6). Since this determination was conducted after normalization of the digests by nitrogen content, the average concentration trend is representative of the proportion of amino acids and small peptides in the total protein fraction in the digest. Time point zero in the graph corresponds to free amino acids determined in the effluent taken 15 min prior casein feeding. The global concentration in the samples taken after casein supplementation ranged between 50 and 140 mmol/kg and represented from 0.3 to 1% of the total nitrogen content; a small proportion if compared with the reported amount at the end of the small intestine, 30% (Goodman, 2010). Results in the same order of magnitude have been previously shown for milk proteins in pig stomach and duodenum (Egger et al., 2017). Regarding the amino acid distribution, the duodenal content before casein ingestion showed a marked abundance of glycine followed by alanine and leucine. In contrast, after casein intake, the pattern changed to high occurrence of phenylalanine followed by leucine, lysine, and tyrosine, with average relative shares of 30, 13, 12, and 10%, respectively (Fig. 6). The rest of amino acids were present at much lower amounts while proline concentration was below 0.2 mmol/kg in the duodenal effluents after casein ingestion. In the analysis of the amino acid composition of the identified peptides, it was observed that proline was the most abundant amino acid (Miralles et al., Data in brief). This suggests that this amino acid remains preferentially in the form of peptides. Only minor changes in the amino acid abundance distribution could be detected over time, which is consistent with the mentioned homogeneous composition of the material outflowed from the stomach.

3.3. *In vitro simulated digestion. Comparison with in vivo data*

The *in vitro* oral and gastric digestion of the same substrate was performed according to the standardized Infogest protocol (Brodkorb et al., 2019). *In vitro* gastric digestion of the micellar casein product was tracked by stopping the reaction at 10, 20, 30, 60, 90 and 120 min. Considering that the duodenal effluents are representative of the gastric contents, these were compared with the end point of the *in vitro* gastric

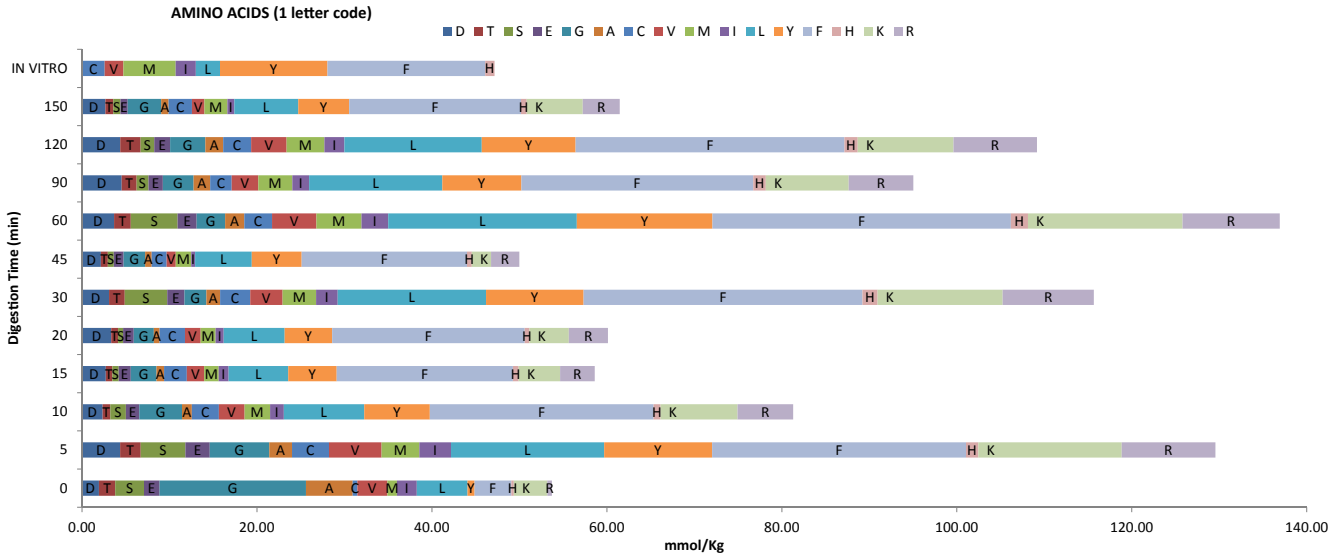

Fig. 6. Distribution of free amino acids in the duodenal (5–150 min) and *in vitro* digests of caseins at 120 min. Tryptophan, glutamine and asparagine were not determined. * Proline showed a value under 0.2 mmol/kg and is not represented.

digestion of the same substrate. The electrophoretic bands corresponding to caseins could be clearly detected up to 20 min of the *in vitro* gastric digestion (Fig. 2B). After that time, although faint bands of caseins were still distinguishable at 30 and 60 min, a profile dominated by bands of whey proteins and small molecular weight compounds, together with digestive enzymes was found. The *in vitro* digestion electrophoretic pattern reflects the rapid degradation of caseins and the resistance of β-lactoglobulin to the action of pepsin. The absence of intact casein and the presence of the β-lactoglobulin band in the *in vitro* gastric electrophoretic pattern fits the duodenal effluents outflowed during the first 30 min (Fig. 2A).

The *in vitro* gastric digests were analysed by HPLC-MS/MS under the same conditions that the duodenal contents and the set of identified protein fragments were mapped to their parent proteins with the Peptigram tool over sampling time. As observed in the *in vivo* effluents, the more abundant and intense peptides belonged to the N- and, especially, the C-terminal domains of β- and α_{s1}-casein. In the *in vitro* digests, peptides from regions 41–59 and 122–144 from β-casein and 20–40 from α_{s1}-casein were especially abundant which matched with the peptides found in duodenal contents (Fig. 3 C,D). In the case of α_{s2}-casein, the region 99–124 was remarkably intense while in κ-casein the 52–76 and the C-terminal regions comprised a higher number of peptides, in good correspondence with the *in vivo* pattern, despite a lower number of distinct identified sequences (285) (Fig. 4).

The frequency of appearance of each amino acid as part of β-casein and α_{s1}-casein-derived peptides were used to contrast *in vitro* with *in vivo* results. The comparison of different time points of digestion provided correlation coefficients between 0.66 and 0.79 at the final point of gastric digestion, while the correlation coefficient average between animals ranged from 0.51 to 0.75. The application of this statistical analysis for the comparison of peptidomic results was previously used to evaluate an interlaboratory trial with the Infogest digestion protocol (Egger et al., 2016) or to compare peptides found in human jejunum with those released by this *in vitro* digestion protocol (Sanchón et al., 2018). As expected, the amino acid composition of the *in vivo* and *in vitro* identified peptides was also similar (Miralles et al., Data in brief, submitted). The most abundant amino acid in peptides identified both *in vivo* and *in vitro* is proline reaching a 20%, followed by valine, leucine, glutamic acid, and glutamine, which ranged from 11 to 6%.

The distribution of free amino acids at 120 min of *in vitro* digestion was compared with those determined in the *in vivo* digests (Fig. 6). The total concentration of free amino acids in the *in vitro* gastric digests reached on average 50 mmol/kg, an amount at the lower limit of values observed in the duodenum after casein consumption. This can be attributed to the absence of endogenous amino acids that are present in the *in vivo* samples or to the lack of enzymatic activities giving rise to single amino acids. With regard to amino acid composition, the most abundant free amino acids, phenylalanine and tyrosine, matched with the *in vivo* effluents, whereas lysine, leucine, and arginine were found at lower concentration in the *in vitro* digests. It has been reported that arginine, lysine, tyrosine and phenylalanine are the most readily liberated free amino acids, while cysteine and proline were the least digested in four protein sources comprising casein, cod protein, soy and gluten. The different occurrence of these amino acids had been observed after the sequential hydrolysis with pepsin and pancreatin (Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005). In the present study the pattern is displayed from the early duodenal content in pigs and largely matches with the application of the simulated gastric phase.

4. Conclusion

The analysis of the duodenum effluents shows the behavior of caseins in the stomach, with a nearly emptying of low protein-liquid phase which corresponds to the initial clotting of caseins. With regard to peptide release, many resistant sequences that include physiologically active fragments have been identified and monitored during gastric emptying. On the other hand, the determined free amino acids in the digests ranged from 60 to 140 mmol/kg and showed a distinctive pattern with regard to the basal one, although the fraction of free amino acid represented < 1% of the total nitrogen content. From the point of view of protein degradation, it is only possible to establish a correspondence between the end point of the gastric phase and the *in vivo* profile at certain points. In this case, kinetic studies where emptying of the stomach is considered, should better mimic the evolution of the protein content. In view of the similarities, this *in vitro* gastric protocol could be accepted to simulate protein degradation at duodenal level.

CRediT authorship contribution statement

B. Miralles: Investigation, Formal analysis, Writing - original draft. **J. Sanchón:** Investigation, Formal analysis. **L. Sánchez-Rivera:** Investigation, Formal analysis. **D. Martínez-Maqueda:** Investigation, Formal analysis. **Y. Le Gouar:** Investigation, Formal analysis. **D. Dupont:** Conceptualization, Supervision. **L. Amigo:** Writing - original

draft. **I. Recio:** Conceptualization, Supervision.

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.foodchem.2020.128424.

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