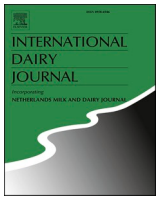
International Dairy Journal 162 (2025) 106162

Contents lists available at ScienceDirect

International Dairy Journal

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



Research paper

Simulated gastrointestinal protein digestion of sheep and goat milk infant formulae

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ARTICLE INFO

Keywords:
In vitro dynamic digestion
Caseins
Whey proteins
Casein phosphopeptides
Bioactive peptides

ABSTRACT

While cow and goat milks are used in manufacturing infant formulae (IFs), sheep milk is not allowed in European Union. To assess its suitability, we studied, by a peptidomics approach, the *in vitro* gastro-intestinal protein digestion of commercial sheep and goat IFs. In both IFs, after 120 min in the stomach, caseins were found massively degraded, while residual whey proteins were detected. In the intestine, β-lactoglobulin, and α-lactalbumin were found resistant to enzymes, particularly in sheep IFs. Compared to goat IF, sheep IF caseins showed a higher degradability, with higher number of released peptides and % protein coverage, particularly for κ- and α_{s1}-casein. On the contrary, whey proteins were found more hydrolysed in goat IF. In the intestine, for both IFs, the peptide profiles resembled those of the stomach, except for α-lactalbumin. Several bioactive peptides were identified, among these casein phosphopeptides. Overall, protein digestion parameters of sheep IF are comparable with goat IF.

1. Introduction

Breast milk is the ideal source of nutrition for neonates. However, when breastfeeding is not possible or insufficient, infant formulae (IFs) act as the main alternative, providing the necessary nutrients for infants' healthy growth and development. IFs are made from a carefully selected mix of ingredients designed to reproduce the composition and nutritional benefits of breast milk. Most of commercial IFs are based on cow milk as main source of proteins, mixed with vegetable oils, and micronutrients. Besides cow milk, IFs produced with milk of small ruminants are available in the market. While goat milk as ingredient for IFs is commonly accepted, the use of sheep milk is permitted in countries such as New Zealand and China (Maryniak, Sancho, Hansen, & Bøgh, 2022) but not yet allowed in the European Union (EU; Commission Directive, 2013/46/EU).

In cow milk IF, the casein:whey proteins ratio (CN:WP~80:20 for ruminant milk) is adjusted by adding WP in proportions to reach the ratio CN:WP~40:60 of human milk (Lai et al., 2023). WP are standard products obtained in whey processing plants and commonly used as food ingredients (Lai et al., 2023; Lucena, Alvarez, Menéndez, Riera, & Alvarez, 2006). Milk proteins are an important source of peptides with biological activity. Bioactive peptides, including those phosphorylated, derived from ruminant milk proteins, have been shown to exhibit various health-promoting functional properties (Bakshi et al., 2023; Santos, Silva, Grácio, Pedroso, & Lima, 2024). During digestion, bioactive peptides can be released in the gastric compartment upon the action of pepsin in a low pH environment, and/or in the intestinal compartment upon the action of trypsin and other enzymes.

The study of infant formula composition for neonatal nutrition is a dynamic field of research, driven by ongoing innovations in formulations and its crucial role in supporting neonatal health and nutrition. In a previous work, the compositional analysis of ovine milk-based ingredients for IF, obtained on a pilot scale, revealed the presence of elements of nutritional interest for the needs of neonates related to the specific features of sheep milk (Lai et al., 2023). However, to better formulate an IF or to approve a new formulation, the study of the digestion mechanisms is of paramount importance. Being difficult to conduct such studies in infants, several *in vitro* alternatives have been proposed and validated (Shani-Levi et al., 2017). The multicompartment *in vitro* dynamic digestion system DIDGI® (INRAE, Rennes, France), was

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https://doi.org/10.1016/j.idairyj.2024.106162
Received 25 September 2024; Received in revised form 6 December 2024; Accepted 7 December 2024
Available online 10 December 2024
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designed to track the food hydrolysis mechanisms in the stomach and the small intestine. Parameters such as transit time, the pH, addition of digestive secretions, and stirring are computer-controlled. The main disadvantage is that absorption of food components in the small intestine is not taken into account (Mackie, Mulet-Cabero, & Torcello-Gómez, 2020). The *in vitro* dynamic DIDGI® system has been also designed simulate infant digestion (Ménard, Picque, & Dupont, 2015).

Peptidomics allows to identify peptides in various biological matrices and to study proteins and protein digestion (Dalabasmaz, de la Torre, Gensberger-Reigl, Pischetsrieder, & Rodríguez-Ortega, 2023; Tagliazucchi, Martini, Shamsia, Helal, & Conte, 2018; Yu, Qi, & Jin, 2019). Using a high-resolution mass spectrometry-based proteomics approach, peptides derived from digested proteins can be detected and assigned to master proteins. This bottom-up shotgun approach relies on analyzing peptides, with well-established methods for their separation, fragmentation, and interpretation.

In our previous work, based on the hypothesis that sheep milk-based IF is as digestible as goat milk-based IF, we compared the hydrolysis profiles of complex lipids during *in vitro* digestion of commercial sheep and goat IFs. The results revealed comparable digestion patterns (Casula et al., 2024). With the same aim, in this study we focused on the protein fraction of IFs. To this goal, we compared, *via* a peptidomic approach, the set of peptides released during dynamic *in vitro* digestion of 0–6 months IFs manufactured using sheep and goat whole milk. By the DIDGI® system, samples were collected during gastric and intestinal digestion, and then analysed by a liquid chromatography high resolution mass spectrometry platform. Quali-quantitative changes of peptides were registered along digestion times and compartments. We mapped the identified peptides on the protein amino acid sequences to reveal proteolysis patterns in both IFs. Finally, we screened for the presence of peptides with potential biological activities. Phosphorylated peptides were also identified and studied.

2. Materials and methods

2.1. Infant formulae

Powdered infant formulae, produced with either sheep or goat milk, were supplied by Blue River Dairy LP (Invercargill, New Zealand). IFs, formulated to meet the nutritional needs of 0–6 month-old infants, were manufactured using whole milk (10–20 %), whey protein concentrates obtained from the same milk species (55–65 %), and vegetable oils (20 %). For both IFs, total proteins were 10–11 %, lipids 25–26 %, and carbohydrates 54–56 %, expressed as % of dry matter. Whey proteins were the 6.7 % of dry matter, with ratio CN/WP of 40/60. Before experiments, IF powders were rehydrated as indicated on the label.

2.2. In vitro dynamic digestion

Gastrointestinal digestion of IFs was simulated by the DIDGI® *in vitro* dynamic system adapted to reproduce the digestive conditions of newborns (Ménard et al., 2014; De Oliveira et al., 2016; Casula et al., 2024). The digestive parameters, such as the gastric acidification curve, and the enzymes flow were controlled by the StoRM® software (INRA, Grignon, FR). Moreover, to simulate the gastric and intestinal emptying, during digestion, the meal content in the digesta samples was under control, resulting in a gradual decrease of the ratio (*v/v*) of meal to secretions with time, therefore a dilution factor value (meal/secretion) was recorded for samples at each time point. The gastric and intestinal digestions lasted 180 min each, and samples were collected at 8 different time points, specifically in the gastric (G) and intestinal (I) compartments at 40, 80, 120, and 180 min. Samples named G0 corresponded to rehydrated IFs, acidified at pH = 3, and diluted in the same conditions as the gastric samples but without enzyme addition. Digestive enzymes and physicochemical parameters used are reported in Supplementary Table S1. Porcine pepsin (P6887; 3090 U mg^{-1}), porcine pancreatin (P7545; 83 U of lipase mg^{-1}), bovine bile extract (B3883; 1 mmol g^{-1}), and the enzyme inhibitors pepstatin A (P5318), pefabloc (76307) and 4-bromophenyl boronic acid (B75956) were obtained from Sigma Aldrich (St. Quentin Fallavier, France). Rabbit gastric extract (RGE), 74 U mg^{-1} of lipase and 754 U mg^{-1} of pepsin were provided by Lipolytech (Marseille, France). Enzyme inhibitors were immediately added to the collected samples before freezing at −20 °C.

2.3. Gel electrophoresis of sheep and goat infant formulae during gastro-intestinal digestion

For all the digesta samples (from G0 to I180) separation of proteins was performed by gel electrophoresis as previously described (Ménard et al., 2018). SDS-PAGE was performed on digesta samples using 4–12% polyacrylamide NuPAGE Novex bis-Tris 15-well precast gels (Invitrogen, Carlsbad, CA, USA). Ten µg of meal proteins were diluted with NuPAGE® LDS buffer and then treated with DL-dithiothreitol and deionized water. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight marker. Gels were fixed in 30 % (*v/v*) ethanol, 10 % (*v/v*) acetic acid and 60 % (*v/v*) deionized water and were rinsed in deionized water before staining with Bio-Safe Coomassie stain (Bio-Rad Laboratories, France). Discoloration of gels was performed with water. Image analyses of gels were carried out using Image scanner III (GE Healthcare Europe GbmH, Velizy-Villacoublay, France). Densitometry on bands was performed by measuring their grey intensity using the software Image Quant TL™ (GE Healthcare Europe183 GbmH, Velizy-Villacoublay, France), allowing a semi-quantitative analysis of proteins (Ménard et al., 2018).

2.4. Quantification and identification of total proteins and peptides during gastro-intestinal digestion

For all collected digesta samples (from G0 to I180, for a total of nine time points), detection and quantitation of total proteins and, as a rule, all substances containing two or more peptidic bonds was performed by bicinchoninic acid (BCA) colorimetric test (Thermo Scientific™ Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific, San Jose, CA, USA) at 562 nm. Calibration was based on bovine serum albumin (BSA) absorbance. Results are reported in Supplementary Fig. S1, together with the dilution factor expressed as the ratio meal/secretion. This latter considers the continuous gastric and intestinal emptying and the gradual addition of enzymes and digestive fluid, as controlled by the StoRM® software (see Supplementary Table S1).

After BCA quantification, fresh aliquots of all digested samples were diluted with formic acid (FA) 0.1 %. One µg of Total Protein Concentration (TPC) for each sample was analysed with LTQ-Orbitrap Elite (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Ultimate 3000 nano-HPLC (Dionex, Sunnyvale, CA, USA). Peptides were capture with a trap column (5 mm × 300 µm) (Thermo Fisher Scientific, San Jose, CA, USA) at 10 µl min^{-1} of 0.1% FA. EASY-spray TM C18 nanoHPLC column (150 mm × 50 µm, particle size of 2 µm) (Thermo Fisher Scientific, San Jose, CA, USA) was used for peptides elution with solvent A, 0.1% FA in water and B, 0.1 % formic acid in acetonitrile-water80/20 (*v/v*). Peptides were separated at flow rate of 300 nl min^{-1} using the following chromatographic gradient: B 4 % 0–3 min; B 4–50 %, 3–70 min; B 50–80 %, 70–90 min; B 90 %, 90–100 min; B 4 % 101–120 min. Full MS studies were conducted in positive ion mode, with mass range of 350–1600 *m/z*, and resolution of 120.000. Source voltage and capillary temperature were set at 1.7 kV and 275 °C, respectively. The three most abundant ions were fragmented using collision-induced dissociation (CID) at 35% normalized energy for 10 ms, 5 *m/z* isolation width, and 0.25 activation q. Spectra collected with Xcalibur software (ThermoFisher Scientific) were analysed using Proteome Discoverer (PD) software (version 2.5, Thermo Fisher Scientific). SEQUEST HT search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA) used the UniProt-KB sheep and goat sequence

database (459 entries, release 2021_01, and 120 entries, 2021_1, respectively). For peptides identification mass tolerance of 10 ppm, and fragment ion mass tolerance of 0.6 Da were used. Only peptides with a minimum length of 6 amino acids and with high confidence were considered. The serine/threonine phosphorylation post-translational modifications were studied. Biological activity of peptides was manually searched in the literature.

3. Results

3.1. Peptide analysis

The total amount of proteins and peptides along all the digestion steps was depicted as column bars together with the dilution factor (Supplementary Fig. S1). This latter is an index of the dilution of the meal in the digesta samples, due to the continuous addition of digestive fluids and emptying. As can be observed in Supplementary Fig. S1, the total amount of proteins and peptides decreased steadily from the first minutes in the gastric compartment, and the trend was more pronounced than the dilution factor, indicating that proteins and polypeptides were promptly hydrolysed.

The peptide profiles of digested sheep and goat IF samples were obtained by UHPLC-Orbitrap-HRMS and assigned to their master proteins. Data from caseins and from the whey proteins β-lactoglobulin (β-lac) and α-lactalbumin (α-lac) were collected and analysed. In sheep IF and goat IF samples, we identified a total of 1777 non-redundant peptides assigned to caseins and to the whey proteins α-lac and β-lac. The 82 % of identified peptides were derived from caseins. The extent of similarities and differences of peptide sequences between sheep and goat IF digested samples were depicted in Venn diagrams (Fig. 1). The Venn diagrams indicate that 549 and 400 peptides are unique for sheep and goat IF, respectively, and that 828 peptides are in common. Sheep IF

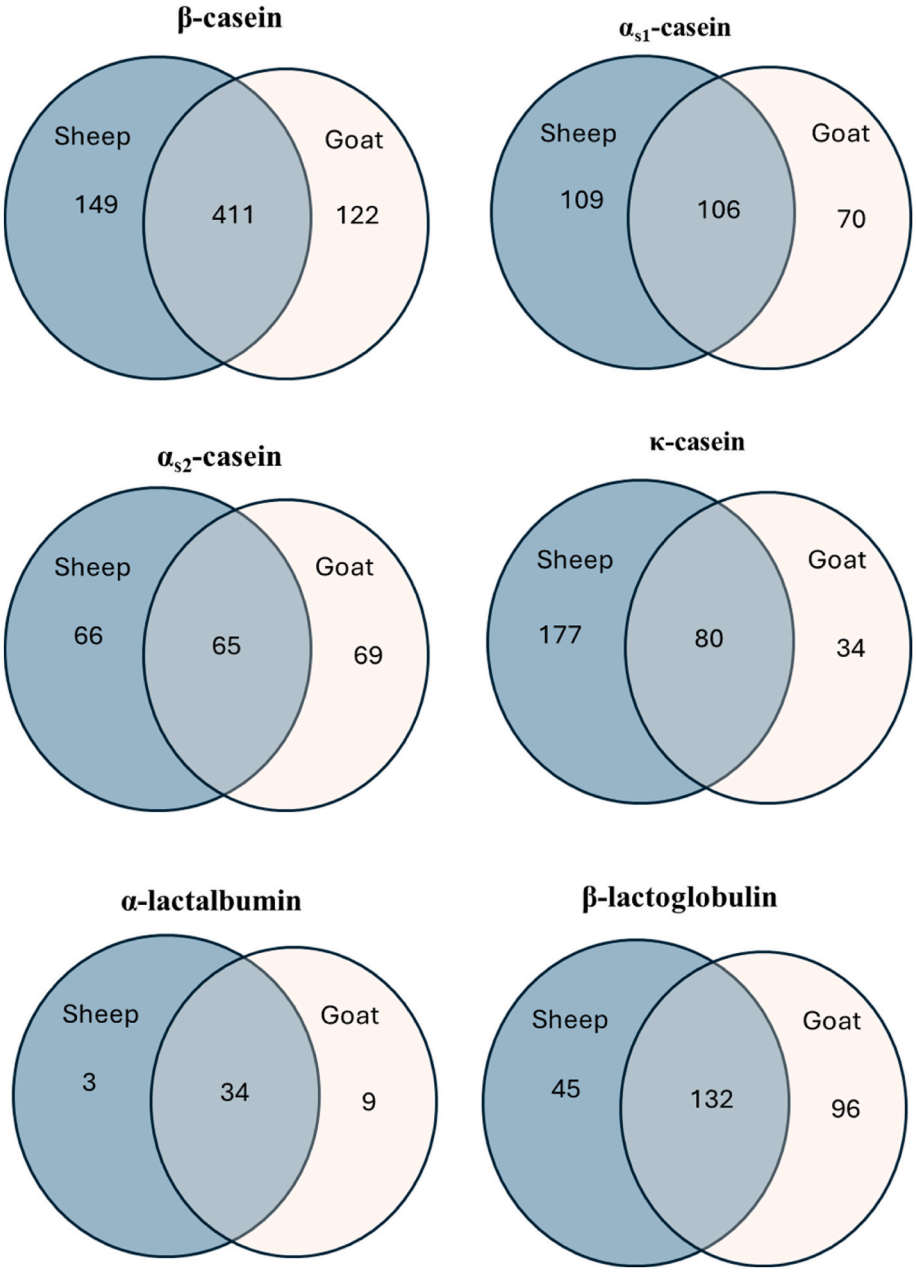

Fig. 1. Venn diagrams depicting the number of unique and common non redundant peptides in sheep IF and goat IF proteins along the whole *in vitro* digestion process.

showed a higher number of peptides from α_{s1}-casein and κ-casein, while goat IF was higher in peptides released by whey proteins. The numbers of peptides in samples collected along the gastro-intestinal digestion are reported as column plots for sheep and goat IF (Fig. 2). For all caseins and β-lac the number of peptides is greater in the gastric compartment, with a peak at G180 (180 min in the gastric compartment from beginning of digestion). On the contrary, for α-lac more peptides were released during intestinal digestion.

3.2. Protein coverage

The percent coverage of main proteins of sheep and goat IF was calculated. Coverage refers to the proportion of the protein covered by the detected peptides and indicates how much of the protein sequence has been hydrolysed during digestion. Sequence alignments of sheep and goat proteins, highlighting similarities and differences in their amino acid sequences are reported in Supplementary Fig. S2, together with the protein percentage identity matrices (Supplementary Table S2). Percent coverage of proteins was reported in Table 1 for the gastric and intestinal compartments and as heat maps depicting the identified peptides in the protein sequences (Figs. 3 and 4). In general, digestive enzymes differently act on specific cleavage sites of protein sequence, and the involved regions release of a larger number of peptides. Accordingly, the identified peptides covered specific regions of the protein amino acid sequences, with a similar pattern for sheep and goat IF. Regarding casein digestibility, β-casein sequence was the most hydrolysed with more than 90% of coverage in the gastric compartment

Table 1
Peptide percent coverage of proteins for sheep and goat IF samples. Mean values over 5 time points for the gastric compartment (G0, 40, 80, 120, 180 min) and 4 time points for the intestinal compartment (I40, 80, 120, 180 min).

	Sheep IF		Goat IF	
	Compartment			
Protein	Gastric	Intestinal	Gastric	Intestinal
β-casein	92	78	92	82
α_{s1}-casein	76	44	61	46
α_{s2}-casein	63	31	64	36
κ-casein	67	51	60	49
α-lactalbumin	26	29	24	30
β-lactoglobulin	53	59	62	64

(Table 1 and Fig. 3). Sheep and goat IF showed a similar pattern for β-casein (amino acid sequence similarity of β-casein in goat and in sheep milk is 99.52%, Supplementary Table S2), where the cleavage sites most frequently contained K, L, and Q amino acids. For α_{s1}-casein similar cleavage sites were observed for sheep and goat IF, with a higher coverage for sheep particularly in the gastric compartment, starting from G0. In the gastric compartment, α_{s2}-casein of sheep IF exhibited main cleavage sites between 20 and 40 positions of the amino acid sequence, while in goat IF more peptides were found at the C-terminal (from 200 on). κ-casein showed a different cleavage pattern in sheep and goat IF. In sheep IF the peptide pattern indicates the presence of the two

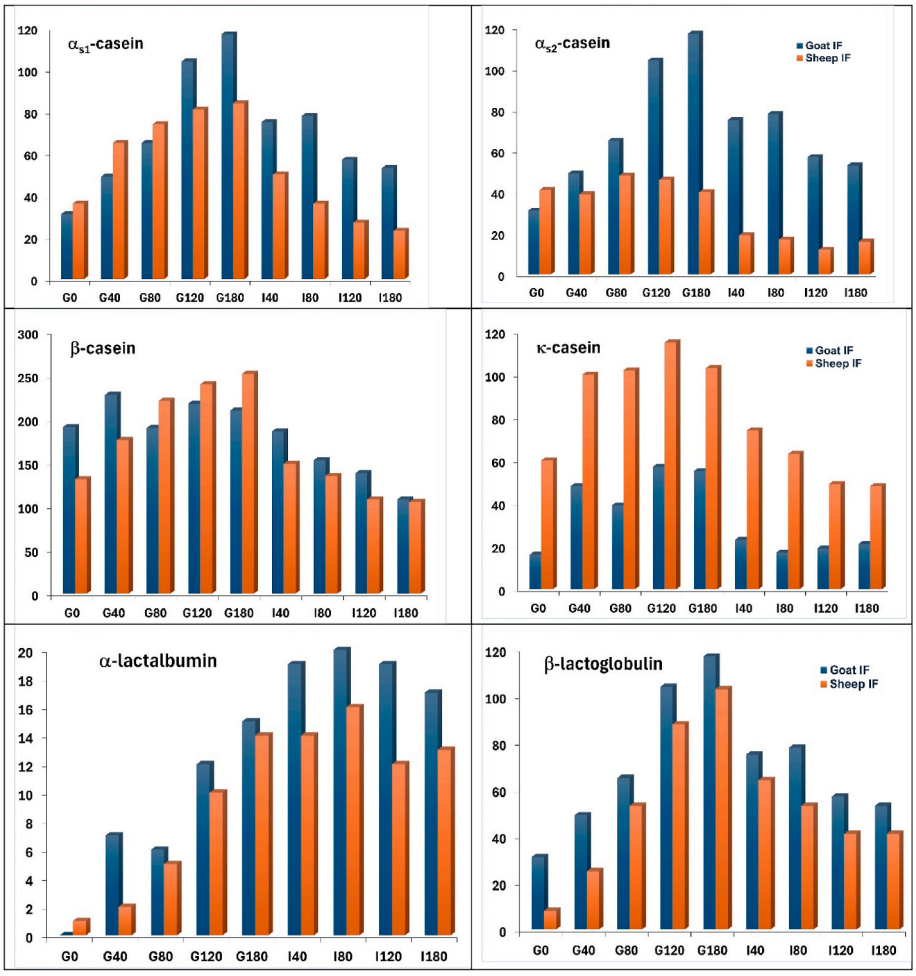

Fig. 2. Number of non redundant peptides (**y-axis**) of proteins in sheep IF and goat IF at the different time points during digestion in the gastric (G0, G40, G80, G120, G180) and the intestinal (I40, I80, I120, I180) compartments (**x-axis**).

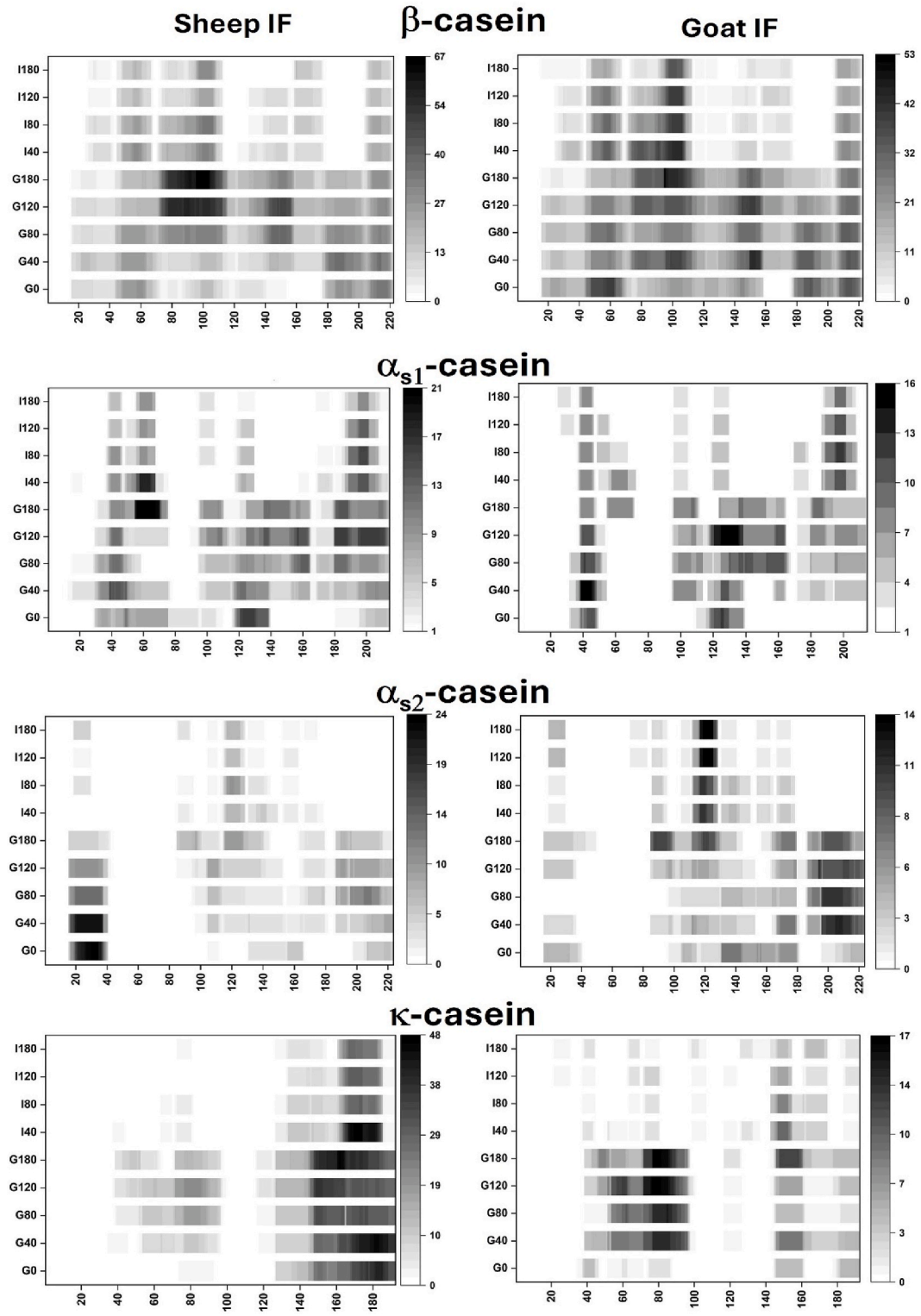

Fig. 3. Heat maps depicting the identified peptides in the casein amino acid sequences (***x*-axis**) for the gastric (G0, G40, G80, G120, G180) and the intestinal (I40, I80, I120, I180) compartments (***y*-axis**) in sheep IF and goat IF. Intensity of grey colour represents frequency of involved amino acid sequences (maximum intensity corresponds to normalized maximum frequency). For details of the amino acid sequences see Supplementary Fig. S2A. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

distinct domains, *i.e.*, the C-terminal, called caseinomacropeptide (CMP), and the N-terminal known as para-κ-casein, separated by the very specific cleavage at Phe-Met (126–127). In sheep IF, during digestion, the C-terminal fraction generated much more peptides than the N-terminal counterpart (90-40 amino acid positions), particularly in the gastric phase. In goat IF, the opposite is true since the N-terminal generated more peptides than its counterpart. Additionally, the number of peptides released by κ-casein in goat IF was lower than in sheep IF (Fig. 2). Compared to the main milk proteins, κ-casein scores the lowest % of similarity in sheep and goat milk (95.83 of similarity,

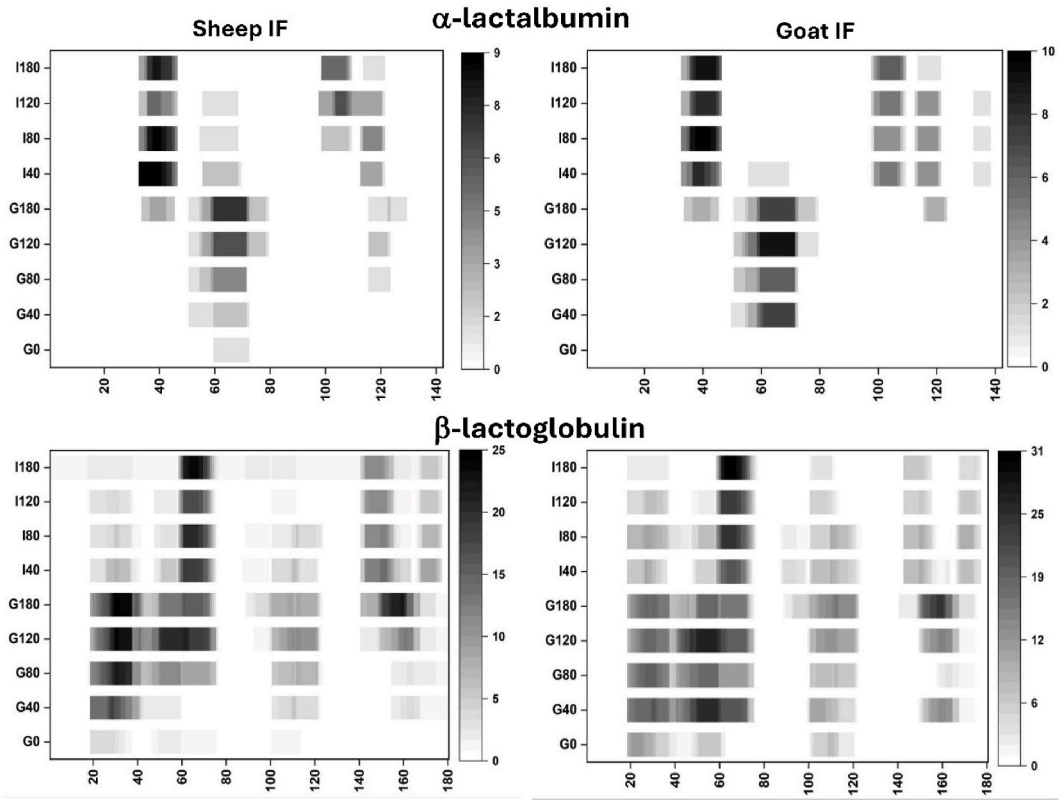

Fig. 4. Heat maps depicting the identified peptides in the whey protein amino acid sequences (***x*-axis**) for the gastric (G0, G40, G80, G120, G180) and the intestinal (I40, I80, I120, I180) compartments (***y*-axis**) in sheep IF and goat IF. Intensity of grey colour represents frequency of involved amino acid sequences (maximum intensity corresponds to normalized maximum frequency). For details of the amino acid sequences see Supplementary Fig. S2B.

Supplementary Table S2). Results suggest that, as opposite of goat IF, CPM was present in sheep IF since the beginning of digestion, thus more easily releasing peptides. In general, for all caseins, the patterns of peptides found in the intestinal compartment were similar to that of the stomach.

For what concerns whey proteins, the peptide profiles were similar for sheep and goat IF (Fig. 4). As also reported in Table 1, percent coverage of β-lactoglobulin was much higher than in α-lactalbumin, and in both compartments goat IF showed a higher coverage than sheep IF. In the gastric environment, the most frequent cleavage sites of α-lac were from 50 to 72 amino acid position involving mostly F_{50}, F_{72}, and L_{71}, and between 115 and 130 amino acid position, with L_{115} the most prone to hydrolysis. In the intestine α-lac showed new areas of coverage, and lysine was the mostly involved (K_{32}, K_{35}, K_{98}, K_{112}, and K_{113}). While in α-lactalbumin specific amino acids were sites of cleavages, on the contrary, β-lac showed different amino acids involved in cleavages, the most frequents were A_{18} and I_{19}, V_{59}, Y_{60}, V_{61}, G_{70}, N_{71}, and L_{72}.

The beginning of digestion was set at G0 (sample before enzymes addition). Considering that IF were in liquid form, the oral phase was skipped, as indicated by the international consensus (Minekus et al., 2014). At G0, peptides attributed to milk proteins were detected, suggesting that hydrolysis of the master proteins already occurred, even before gastric enzymes addition. Coverage % and number of non-redundant peptides for all examined proteins at G0 are reported in Table 2 β-casein showed the greatest number of peptides (191 and 131, for goat and sheep IF, respectively) with the highest coverage (86%). α-lac scored the lowest coverage (0 and 9, for goat and sheep IF, respectively) and only one peptide was found in sheep IF. α_{s1}-casein showed a higher hydrolysis susceptibility in sheep IF than in goat IF (97.21% of similarity, data UniProt-KB). κ-casein showed a similar coverage in the two IFs, but with a higher number of peptides in sheep IF.

Table 2
Peptide percent coverage of proteins and number of non-redundant peptides for sheep IF and goat IF samples collected at the beginning of digestion (G0).

Protein	Sheep IF		Goat IF	
	Coverage %	# Peptides	Coverage %	# Peptides
β-casein	86	131	86	191
α_{s1}-casein	64	36	28	24
α_{s2}-casein	50	41	62	31
κ-casein	45	60	41	16
α-lactalbumin	9	1	0	0
β-lactoglobulin	34	8	42	31

3.3. Semiquantitative analysis of protein content

SDS-PAGE for gastric and intestinal phases are shown in Supplementary Figs. S3A and B, and results of the quantitative analysis of the protein contents during the gastrointestinal digestion steps are shown as column plots in Fig. 5. In the gastric compartment, the protein hydrolysis degree for the two IFs was overall similar. At 80 min of gastric digestion (G80), casein content appeared to be drastically reduced to the 90 % (<10 % remaining), and at the end to the gastric phase (G180) they were barely detectable (Fig. 5). At G180, the residual content of β-lac was ~10 % for sheep and goat IF, while 4 % and 3 % were detected for α-lac. During intestinal digestion, hydrolysis of the residual proteins after the gastric steps takes place. For sheep IF, after 40 min in the intestine the amount of α-lac dropped to the 50% and then remained stable until the end of digestion, while after 40 min in the intestine in goat IF α-lac showed a smaller amount (25%) than sheep IF and this value dropped until a final value of 8 % (Fig. 5). At I40, β-lac content was evaluated as 73% and 30%, for sheep and goat IF, respectively and

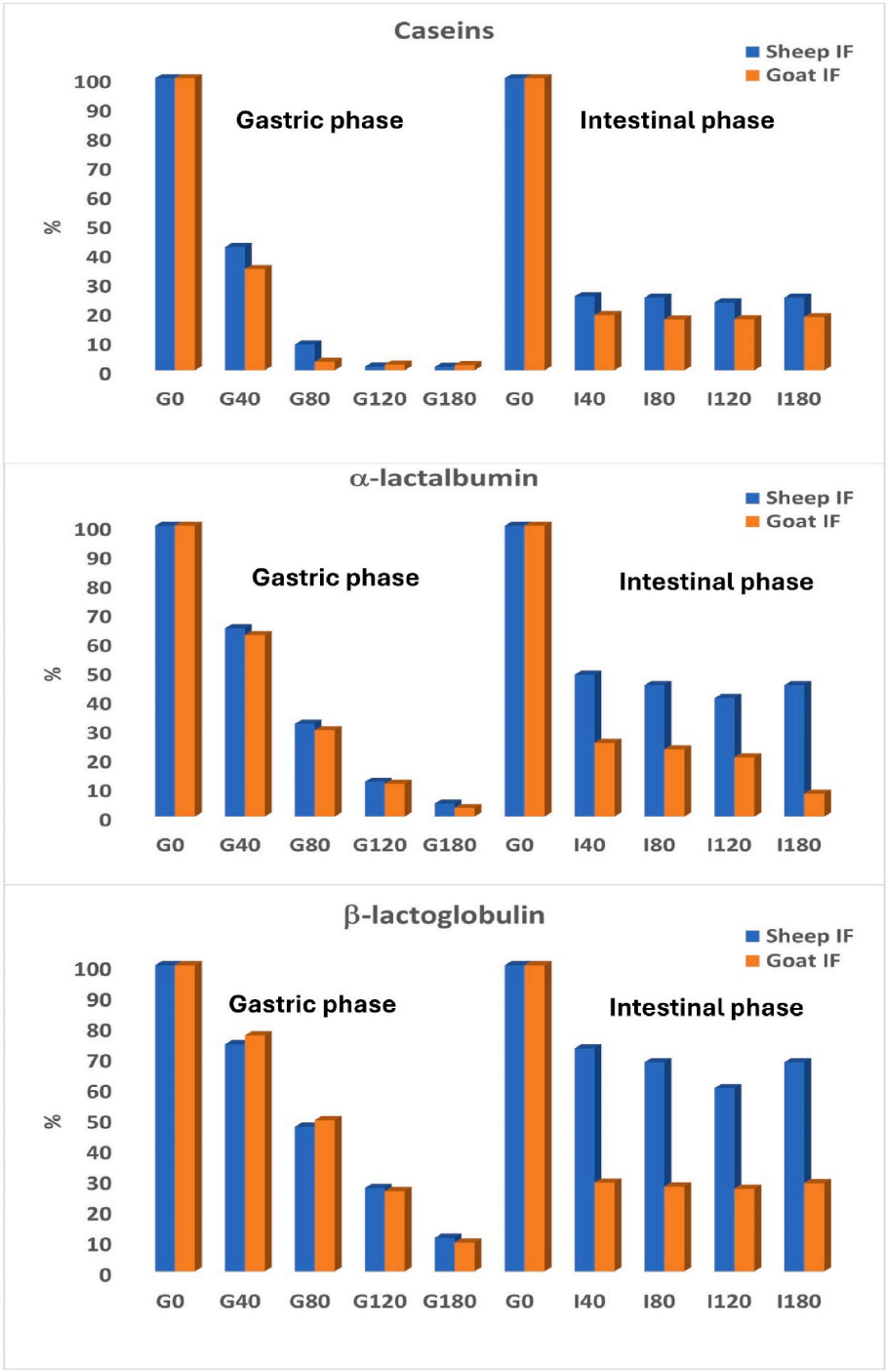

Fig. 5. SDS-PAGE protein profile during digestion of sheep IF and goat IF in the gastric (G40, G80, G120, G180) and the intestinal (I40, I80, I120, I180) compartments. Calculated percent of the residual intact proteins for caseins, α-lactalbumin, and β-lactoglobulin, with respect to G0. Data were obtained from densitometric analysis of the SDS PAGE gels.

remain stable until the end of intestinal digestion (Fig. 5).

3.4. Bioactive peptides

Several peptides with a plethora of biological activities are released from milk proteins, particularly caseins (Santos et al., 2024). During gastrointestinal digestion, biological active peptides may be released from proteins or from inactive or less active peptide precursors. However, active peptides may be also degraded by pepsin and pancreatic enzymes losing their biological activity. Comparing data with literature reports, we identified 37 bioactive peptides that derived from cleavage of caseins, results are reported in Table 3. No bioactive peptides were found for whey proteins, however, in both IFs, we found α-lac peptides with the opioid-like sequences YGLF (69–72 amino acid sequence) terminal motif (Pihlanto-Leppälä, 2000). Analogously, in β-lac peptides, the terminal motives YLLF (120–123) and HIRL (164–167), reported to have opioid-like effects (Pihlanto-Leppälä, 2000), were detected.

Table 3
Casein peptides with biological activity.

Peptide (sequence, [start-end])	Lenght[a]	Master Protein	Sheep IF	Goat IF	Activity	Ref
ENLLRF [33–38]	6	α_{s1}-casein	Y[b]	Y	ACE-inhibitory	Quiros et al. (2007)
RYLGYL [105–110]	6	α_{s1}-casein	N	Y	Opiod	Silva and Malcata (2005)
YIPIQY [46–51]	6	κ-casein	Y	Y	ACE-inhibitory, Antioxidative	Gómez-Ruiz, Ramos, & Recio (2007)
DKIHPF [62–67]	6	β-casein	Y	Y	ACE-inhibitory, Protein transport inhibitor	Quiros et al. (2007)
YPVEPF [129–134]	6	β-casein	Y	Y	ACE-inhibitory, Antioxidant, Opiod	Cicchi et al. (2023); Qian, Zheng, Zhao, & Zhao (2022)
PYVRYL [218–223]	6	α_{s2}-casein	Y	Y	Antimicrobial ACE-inhibitory, Antioxidative,	López-Expósito, Quirós, Amigo, and Recio (2007)
LKKISQ [180–186]	6	α_{s2}-casein	Y	N	ACE-inhibitory	López-Expósito et al. (2007)
NENLLRF [32–38]	7	α_{s1}-casein	Y	Y	ACE-inhibitory and anticoagulant activities	Tu et al. (2021)
VAPFPEV [40–46]	7	α_{s1}-casein	Y	Y	ACE-inhibitory	Kopf-Bolanz et al. (2014)
YTDAPSF [188–194]	7	α_{s1}-casein	Y	N	ACE-inhibitory	Tagliazucchi, Shamsia, Helal, and Conte (2017)
SDIPNPI [195–201]	7	α_{s1}-casein	Y	Y	ACE-inhibitory, Antioxidant, Opiod	Quian et al., 2022; Cicchi et al. (2023)
IPIQYVL [47–53]	7	κ-casein	Y	Y	Antioxidant	Farvin et al. (2010)
FPPQSVL [172–178]	7	β-casein	Y	Y	ACE-inhibitory	Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio (2005)
LHLPLPL [148–154]	7	β-casein	Y	Y	ACE-inhibitory	Quiros et al. (2007)
YQKFPQY [105–111]	7	α_{s2}-casein	Y	Y	ACE-inhibitory, Antioxidant	Miguel, Contreras, Recio, & Aleixandre (2009); Silva, Pihlanto, & Malcata (2006)
DAYPSGAW [172–179]	8	α_{s1}-casein	Y	Y	ACE-inhibitory,	Kopf-Bolanz et al. (2014)
VAPFPEVF [95–109]	8	α_{s1}-casein	Y	Y	ACE-inhibitory	Kopf-Bolanz et al. (2014)
VLNENLLR [30–37]	8	α_{s1}-casein	Y	N	Antimicrobial	Hayes, Ross, Fitzgerald, Hill, and Stanton (2006)
RDMPIQAF [196–203]	8	β-casein	Y	Y	ACE-inhibitory	Yamamoto, Akino, and Takano (1994)
VVAPFPEVF [39–47]	9	α_{s1}-casein	Y	Y	ACE-inhibitory	Dalabasmaz et al. (2023)
TAQVTSTEV [184–192]	9	κ-casein	Y	Y	ACE-inhibitory	Dalabasmaz et al. (2023)
PVVVPPFLQ [96–104]	9	β-casein	Y	Y	ACE-inhibitory, Antioxidant, Opiod, and anticoagulant activities	Quian et al., 2022; Cicchi et al. (2023); Tu et al. (2021)
EVLNENLLRF [29–38]	10	α_{s1}-casein	Y	N	ACE-inhibitory	Pihlanto (2013)
LGPVRGPFPI [211–220]	10	β-casein	N	Y	ACE-inhibitory	Robert, Razaname, Mutter, and Juillerat (2004)
TGPIPNSLPQ [78–87]	10	β-casein	Y	Y	ACE-inhibitory	Quiros et al. (2007)
VLGPVRGPFP [210–219]	10	β-casein	N	Y	ACE-inhibitory, Neuropeptide	Quiros et al. (2007); Miguel, Recio, Ramos, Delgado, & Aleixandre (2006)
YQKFPQYLQY [105–114]	10	α_{s2}-casein	Y	Y	ACE-inhibitory	Xue et al. (2018)
ARHPHPHLSF [117–126]	11	κ-casein	Y	Y	Antioxidant, ACE-inhibitory	Dalabasmaz et al. (2023)
EPVLGPVRGPFP [208–219]	12	β-casein	Y	Y	ACE-inhibitory, Neuropeptide	Hayes et al. (2007)
GVPKVKETMVPK [109–120]	12	β-casein	Y	Y	ACE-inhibitory	Quiros et al. (2007)
PVLGPVRGPFPI [209–220]	12	β-casein	Y	Y	ACE-inhibitory, Antioxidant, Opiod	Quian et al., 2022; Cicchi et al. (2023)
YQEPVLGPVRGPF [206–218]	13	β-casein	Y	Y	ACE-inhibitory	Robert et al. (2004)
HKEMPFPKYPVEPF [121–134]	14	β-casein	Y	N	ACE-inhibitory	Robert et al. (2004)
TQTPVVVPPFLQPE [93–106]	14	β-casein	Y	Y	Antioxidant	Farvin et al. (2010)
YQEPVLGPVRGPFPI [206–220]	15	β-casein	Y	Y	Antimicrobial	Birkemo, O'Sullivan, Ross, and Hill (2009)
FQSEEQQQTEDELQDK [48–63]	16	β-casein	Y	Y	Antithrombotic	Tu et al. (2017)

[a] Number of amino acids.
[b] Peptide found (Y) or not found (N) in sheep IF and goat IF.

3.5. Phosphorylated peptides

Phosphorylation is the most common post-translational modification of proteins. Caseins have different sites of phosphorylation mainly on serine (S) and threonine (T). Peptides with phosphorylated motif, called casein phosphopeptides (CPPs), are released upon proteolysis of caseins and have been reported to exhibit different bioactivities (Yu et al., 2019). Numbers of non-redundant CPPs found in sheep and goat IF along the whole digestion process are reported in Supplementary Table S3. Bioactive peptides with the three adjacent phosphorylated residues -SerP-SerP-SerP-Glu-Glu (SSSEE) show multifunctional bioactivities (Miquel et al., 2005; Yu et al., 2019). This phosphorylated motif was found in β-casein of sheep and goat IF, where 111 and 119 phosphorylated peptides were identified, respectively. Sites of phosphorylation, for both IFs, were S_{32}, S_{33}, S_{34}, S_{37}, (motif in peptide SSSEES at position 32–37), S_{50} (motif SEE 50–52), and T_{27}. For α_{s1}-casein, 24 non-redundant CPPs for goat IF (phosphorylations at S_{56}, S_{61}, S_{63}, S_{130}, S_{137}, and T_{64}) and 58 for sheep IF (phosphorylations at S_{27}, S_{90}, S_{56}, S_{61}, S_{63}, S_{130}, S_{137}, and T_{64}) were found, containing between 1 and 3 phosphorylated not adjacent residues *per* peptide. No phosphorylated peptides were found holding the sequence SSSSSEE at position 79–85. For α_{s2}-casein, 27 and 52 CPPs were found for goat and sheep IF, respectively. For goat IF, between 3 and 1 phosphorylations at S_{23}, S_{24}, S_{25}, S_{145}, S_{147}, S_{151}, S_{159} were found *per* peptide, sites of phosphorylation in sheep IF were: S_{24}, S_{25}, S_{32}, S_{145}, S_{147}, S_{159}, and T_{160}. The motif SSSEES, with 3 adjacent phosphorylations at position 23–25 was found in goat α_{s2}-casein but not in sheep IF, lacking this latter the third phosphorylation at S_{23}. Release of CPPs for κ-casein showed a very different pattern between sheep and goat IF. A total of 21 non redundant κ-casein CPPs were found for goat IF mainly with 1 phosphoryl group *per* peptide at S_{148}, S_{165}, S_{172}, S_{180}, S_{189}, and T_{190}, While for sheep κ-casein, 118 CPPs were found (S_{148}, S_{156}, S_{172}, S_{175}, S_{178}, S_{189}, and T_{190}) bearing

between 3 and 1 phosphoryl groups, CPPs were mostly released from the C-terminal CMP of κ-casein. CPPs from caseins bearing 1 to 3 phosphoryl groups *per* peptide were found in the intestinal tract for both IF. No phosphorylated peptides were found for α-lac and β-lac.

4. Discussion

In infant formulae, milk and milk whey protein concentrate are the primary source of proteins. In ruminant milk, main proteins are caseins and albumins. Literature reports indicate that goat milk and sheep milk have, on average, a protein content of 3.7 and 5.5 g 100 g^{-1} (2.4 and 4.7 g 100 g^{-1} of casein), respectively, with sheep milk exhibiting a higher content of total proteins compared to goat milk (Balthazar et al., 2017). Tagliazucchi et al. (2018) measuring the amount of released free amino groups, studied and compared *in vitro* gastrointestinal digestibility of camel, cow, goat, and sheep skimmed milk. Results showed that proteins of goat milk were degraded faster than those of the other milks. However, authors argued that, during digestion, the ratio enzyme-to-substrate may have affected the hydrolysis of milk proteins, especially for sheep milk having the highest protein content (Tagliazucchi et al., 2018). These results cannot be compared with ours, since in the studied IFs, we had similar total protein content for sheep and goat IF, with a controlled WP/CN of 60/40.

Pepsin and trypsin are the main proteolytic enzymes used in our protocol. Pepsin was used in stomach compartment, accompanied by a low pH. Trypsin was activated in the intestine, in alkaline environment. Pepsin hydrolyses peptide bonds between hydrophobic amino acids, such as leucine (L), and phenylalanine (F), whereas trypsin preferred cleavage sites are at the C-terminal side of arginine (R) and lysine (K) residues (The UniProt Consortium, 2021). It has been reported that, for low-heat-treated products, caseins, exhibiting a looser and flexible structure, are more susceptible to the action of pepsin than whey proteins; during gastric digestion they form a solid coagulum, which causes a delay in gastric emptying toward the small intestine. On the contrary, native β-lactoglobulin and α-lactalbumin, which have a compact and globular structure, remain soluble in the stomach and enter into the small intestine more rapidly than casein (Ménard et al., 2018). By an *in vitro* model of infant formula digestion, after 60 min of gastric digestion only the 10 % of caseins were found in the stomach (Ménard et al., 2018). Consistently, at the end of the gastric digestion phase (G180) we found almost no traces of caseins, while whey proteins were found less degraded. Moreover, as reported in Table 2, all caseins showed a higher number of peptides and a greater coverage in the gastric compartment, compared to the intestinal compartment, confirming higher susceptibility to pepsin and low pH. However, it must be also considered that if significant hydrolysis already occurred in the gastric compartment, subsequent hydrolysis in the intestinal compartment may likely resulted in the formation of free amino acids and peptides shorter than the detection limit of the method used in this study (threshold was set at 6 residues)

In particular, we found that β-casein, the most abundant casein in ruminant milk (61.6 and 54.8 % of caseins, for sheep and goat milk respectively, (Balthazar et al., 2017), with 99.52 % of similarity (Supplementary Table S2), was the casein fraction most prone to hydrolysis, showing high peptide coverage, in both gastric and intestinal compartment (up to 93% in the gastric compartment), with a comparable rate of released peptides for sheep and goat IF digested samples. α_{s1}-casein and α_{s2}-casein showed higher sequence coverage in the gastric compartment, with a higher number of peptides for α_{s1}-casein in sheep IF. Importantly, human milk shows low amount of α_{s1}-casein, while does not contain α_{s2}-casein, lacking the gene that encodes this protein (Meng, Uniacke-Lowe, Ryan, & Kelly, 2021). α_{s1}-casein of cow milk has been indicated as a major allergen in children (Ruiter et al., 2007). Sheep and goat milk contain α_{s1}-casein (6.7 and 5.6 % of total casein, respectively) and α_{s2}-casein (22.8 and 19.2%, for sheep and goat milk respectively (Balthazar et al., 2017), with a similarity of protein sequence of 97.20 % and 98.21 %, for α_{s1}-casein and α_{s2}-casein, respectively. *In silico* studies (Masoodi & Shafi, 2010) revealed also considerable identity in chemical properties between goat and sheep α_{s1}-casein and α_{s2}-casein and a considerable difference with cow proteins, indicating that sheep milk may be a convenient alternative for children allergic to cow milk.

It has been reported that sheep milk contains a lower percentage of κ-casein than goat milk (8.9 and 20.4 % of total casein, for sheep and goat milk, respectively) (Balthazar et al., 2017), and that similarity of κ-casein is 95.32. In sheep IF, peptide coverage of the κ-casein sequence showed the presence of the two distinct domains: the N-terminal para-κ-casein and the C-terminal CMP. Several health beneficial effects of CMP have been reported (Sharma, Rajput, & Mann, 2013). Moreover, we found that, along all the digestion steps since the beginning of digestion, the number of released κ-casein peptides was higher in sheep IF compared to goat IF. These findings are in accordance with Yang et al. (2024) that reported a higher hydrolysis of κ-casein by pepsin in sheep milk than in goat milk. In particular, a higher number of phosphorylated peptides, all originating from CMP, were detected in sheep IF than in goat IF.

Whey proteins form the second group of milk proteins, in ruminant milk primarily consisting in β-lactoglobulin and α-lactalbumin. In IFs, these proteins are supplied as WP concentrate. In an *in vitro* model of infant formula digestion, by SDS-PAGE it was observed that β-lactoglobulin and α-lactalbumin resisted to gastric digestion for 60 min, with a residual level of 82 % and 87 %, respectively (Ménard et al., 2018). In this regard, we found that in our samples, at 180 min of gastric digestion also whey protein content decreased up to the 80–90 % of the initial values. Regarding intestinal proteolysis, previous literature on *in vitro* digestion of milk (De Oliveira et al., 2016) and of infant formula (Ménard et al., 2018) reports that no intact proteins were detected for both the infant and adult models at the end of the intestinal digestion. In our study, although the levels of whey protein in the last stages of gastric digestion were very low, we found that β-lac and α-lac were found resistant to the enzymatic intestinal system, particularly for sheep IF. The different susceptibility of sheep and goat whey proteins to the porcine pancreatin used in this protocol can be due to specie-specific differences between enzymes and substrate, moreover, in these IFs whole milk was used, then also the different lipid profiles (https://doi.org/10.1016/j.foodchem.2024.140850) of sheep and goat milk may have had un impact on protein digestibility. Compared to caseins, β-lac and α-lac showed an overall lower peptide coverage. β-lac in our samples follows the same trend of caseins, with more unique peptides released in the gastric compartment. α-lac released peptides were found more numerous in the intestinal compartment compared to the gastric. Cleavage sites of α-lac suggest a specific action of pepsin (amino acids F and L) and trypsin (sites K), on the contrary β-lac showed less specific cleavage sites. β-lactoglobulin is one of the main components of commercial WP ingredients for infant formulae and represents 60–70 % of total whey proteins in goat and sheep milk (Khan et al., 2019). β-lactoglobulin is not present in human milk (Lucena et al., 2006) and in highly sensitive infants this protein can trigger allergic reactions. Taking into account the low allergenicity of goat milk (Park, 1994), it is worth to consider that sheep β-lactoglobulin has 98.33 % similarity with goat, and similarity with cow is 95% for both sheep and goat β-lactoglobulin (Supplementary Table 2S). Furthermore, in one of the most important allergenic epitopes, the f125–135 β-lac sequence, the Asp^{130} of β-lactoglobulin cow milk is substituted both in goat (Lys^{130}) and sheep (Asn^{130}) milk, lowering in both milk types their allergenic potential (Picariello et al., 2010).

At the beginning of the gastro-intestinal simulated digestion, before enzymes addition (samples G0), we found a number of peptides from caseins, particularly for *β*-casein, indicating that denaturation and hydrolysis of proteins took already place, due to manufacturing treatments or by the action of plasmin and cathepsin D present in milk. Plasmin is the main proteinase of milk (Kelly & Mcsweeney, 2003). The plasmin system has been reported to have a relatively high heat-stability that

may result in a residual activity even after UHT processes (Ismail & Nielsen, 2010). β-casein is the preferred target of plasmin, followed by α_{s2}-casein (Chove, Grandison, & Lewis, 2011). Moreover, we observed that in sheep IF, analysing coverage of κ-casein sequence, the presence of CMP was clearly detectable in sample (G0). The presence of CMP in sheep G0 may be due to the use of WP concentrate obtained from cheese manufacturing or a different cleavage susceptibility between sheep and goat κ-casein.

However, we should consider that in infant formulae the chemical-physical properties of proteins can be subjected to drastic changes due to the processing conditions during manufacturing. Manufacturing of milk powder involves several thermal treatment steps, such as spray drying, heating, and evaporation, that can induce aggregation or denaturation of thermolabile proteins (Bakshi et al., 2023). It was found that the native proteins contained in whey protein isolate remained soluble in the stomach and passed to the intestine, indicating that they were not hydrolysed by pepsin during gastric digestion (Wang, Ye, Lin, Han, & Singh, 2018). In contrast, heated WPs were rapidly hydrolysed by pepsin and aggregated in the stomach (Wang et al., 2018). In agreement, it has been reported that heat treatments on β-lactoglobulin solution increase its digestibility, making it more accessible to pepsin and facilitating the action of trypsin (Deng, Govers, Tomassen, Hettinga, & Wichers, 2020). In this context, in sample G0 we found peptides from β-lac (8 and 34 peptides for sheep IF and goat IF, respectively) indicating that hydrolysis of WP took already place to some extent and massively continued in the gastric compartment. On the contrary, at G0 no unique peptides from α-lactalbumin were found, suggesting a higher stability to manufacturing processes.

During gastrointestinal digestion and/or during food processing, hydrolysis of milk proteins yields amino acid sequences of different length. Some protein fragments, known as bioactive peptides, are endorsed with different biological activities, such as antimicrobial, antihypertensive, antioxidant, immunomodulatory, opioid, and mineral-binding (Quian et al., 2022). From the hydrolysis of β-casein we found the peptide YPVEPF a sleep-enhancing peptide (Quian et al., 2022). SDIPNPI from αs1-casein, and PVVVPPFLQ, PVLGPVRGPFPI and YPVEPF from β-casein, were found to have ACE inhibitor, opioid, and antioxidant activities (Cicchi et al., 2023). κ-casein releases casoxins from para-κ-casein, casoxins are food opioid antagonists (exorphins) (Ul Haq, 2020). In our sheep and goat IF samples we found the bioactive YIPIQYVL fragment (Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010) of casoxin C (YIPIQYVLSR) (Ul Haq, 2020). From CMP we found the sequence MAIPPKKDQD of casoplatelin (Park & Nam, 2015).

A total of 339 and 191 casein phosphopeptides, *i.e.*, peptides with phosphorylated motif derived from post translation modifications of the parent proteins, were detected in sheep IF and goat IF respectively, almost all of them showed phosphorylation at the serine amino acid. Remarkably, for κ-casein we found 118 CPPs for sheep IF *vs.* 21 for goat IF, all originating from the CMP fraction, this probably due by the higher susceptibility to hydrolysis of sheep IF κ-casein, where peptides from CMP were detected since the beginning of digestion. CPPs have been described as having health multifunctional bioactivities, such as anti-oxidant properties and mineral carriers enhancing the bioavailability of calcium, iron, zinc and magnesium (Miquel et al., 2005; Yu et al., 2019). The polar acidic SSSEE phosphorylated domain acts as binding site for minerals such as calcium, iron, and zinc and plays an important role in mineral bioavailability (Miquel et al., 2005).

5. Conclusions

In conclusion, caseins in sheep IF showed better digestibility compared to goat IF, with a higher number of peptides and higher sequence coverage, along all the digestion steps. On the contrary whey proteins in goat IF were found more hydrolysed than in sheep IF. Sheep IF showed a higher number of beneficial casein phosphopeptides. These results indicate that simulated digestion of sheep milk IF is comparable to goat IF, thus endorsing the worldwide use also of sheep milk in manufacturing infant formulae.

CRediT authorship contribution statement

Paola Scano: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Mattia Casula:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Olivia Ménard:** Writing – original draft, Methodology, Investigation, Conceptualization. **Didier Dupont:** Writing – original draft, Methodology, Investigation, Conceptualization. **Cristina Manis:** Writing – original draft, Methodology. **Simone Serrao:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Barbara Manconi:** Writing – original draft, Methodology. **Pierluigi Caboni:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) in order to improve language, syntax, and vocabulary. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Funding

This work was partly supported by the POR FESR Sardegna 2014–2020 Asse I – Azione 1.2.2 Programma di ricerca e sviluppo, Area di specializzazione Agroindustria- “Business Intelligent”.

Declaration of competing interest

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

Acknowledgments

We acknowledge the CeSAR (Centro Servizi d’Ateneo *per* la Ricerca, of the University of Cagliari, Italy) for the LTQ-Orbitrap experiments, performed with a LTQ-Orbitrap Elite (Thermo Fisher Scientific, San Jose, CA, USA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2024.106162.

Data availability

Data will be made available on request.

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