Peptidome comparison following gastrointestinal digesta of bovine versus caprine milk serum

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ABSTRACT

Infant formula is used as a supplement for newborns. Although bovine milk-based infant formulas dominate the market, caprine milk-based infant formula has attracted increasing attention because of its lower allergenicity. This study compared the digestive peptidome of bovine and caprine milk serum proteins by using in vitro infant simulating conditions. The result showed that the degradation pattern of milk proteins was similar, whereas the digestive rates of milk proteins differed between bovine and caprine milks. Several proteins, such as α -lactalbumin (LALBA), β -lactoglobulin (LGB), serum amyloid A protein (SAA1), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), and lactotransferrin (LTF), released more peptides during digestion of caprine milk serum than during digestion of bovine milk serum; however, more peptides derived from α _{S1}-casein (CSN1S1) were found in bovine digesta. In addition, antimicrobial-related peptides were mostly only found in caprine intestinal digesta. The results of this study may be useful in understanding the digestion characteristics of milk serum proteins and providing guidance on the improvement of infant formula.

Key words: bovine milk serum protein, caprine milk serum protein, digestion, peptidome

INTRODUCTION

Milk is the primary source of food for infants, which provides proteins, lipids, and carbohydrates to meet the nutrition and health requirements of infants. Infant formula may be used as a supplement or substitute for a number of reasons, and bovine milk products dominate the infant formula market. Recently, caprine milk-based

infant formula has attracted increasing attention as an alternative because of its high digestibility and lower allergenicity to infants (Clark and Moro García, 2017).

There are some differences in nutrient composition between human, bovine, and caprine milks, especially in the protein fraction. The ratio of caseins to whey in bovine and caprine milks is much higher than that of human milk (Mayer and Fiechter, 2012; Chatterton et al., 2013). Most infant formulas are modified to match the human milk ratio by adding milk serum proteins (de Wit, 1998). Milk serum proteins not only provide essential amino acids (Nilsson et al., 2007), but also have some biological functions, such as immune system stimulation, nutrition system effects (Madureira et al., 2007), and fatty acid transport and synthesis (Lu et al., 2014). However, milk serum protein differs between bovine and caprine milk, which result in differences in the digestibility and biological function between the 2 species. First, the milk serum protein composition of bovine and caprine milk is different. For example, the concentration of the polymeric immunoglobulin receptor isoform 1 is significantly higher in caprine milk than in bovine milk (Hernández-Castellano et al., 2016). Second, the AA sequences of proteins are different in these 2 species. For instance, the AA sequence of bovine β -lactoglobulin (LGB) shows 5.6% nonhomology with caprine LGB based on Uniprot database (<https://www.uniprot.org/>). Moreover, 12 polymorphic variants of LGB have been found in bovine milk to date, whereas only 2 polymorphic variants are found in caprine LGB (Selvaggi et al., 2014). The genetic variability of proteins also leads to differences in AA sequences. Finally, bovine and caprine milk proteins differ in structure. It was found that the binding affinities of caprine α -lactalbumin (LALBA) to monoclonal antibodies were different from bovine LALBA (Kaminogawa et al., 1989), which may indicate that the structure of LALBA differs in these 2 species.

Variations in milk composition may lead to differences in the digestibility of milk proteins between bovine and caprine species. Recently, several studies have reported

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the digestive properties of bovine and caprine milk. One study suggested that bovine and caprine caseins showed similar digestion profiles, but the peptide cleavage sites were different in the 2 species (Hodgkinson et al., 2019). Another study compared digestibility of bovine and caprine milk simulating gastric conditions of infants and young children, and found that caprine caseins were more sensitive to digestion than bovine caseins (Hodgkinson et al., 2018). Bovine milk digesta had a higher dipeptidyl peptidase IV inhibitory activity compared with caprine milk digesta (Tagliazucchi et al., 2018). Infant formula made with caprine milk forms smaller clots of protein and oil droplets compared with that made from bovine milk (Ye et al., 2019). Because these studies used whole milk to simulate in vitro digestion, the digestive properties of milk serum proteins are limited. Peptidomics is a relevant tool for assessing protein digestion; it can be used not only to trace released peptides but also to provide the biological activities of the identified peptides (Dupont, 2017). The objective of this study was to identify the peptides released from bovine and caprine milk serum proteins by simulated infant gastrointestinal digestion and compare the digestive peptidome of milk serum proteins between the 2 species.

MATERIALS AND METHODS

Materials

Fresh Holstein bovine milk was purchased from a local producer (Tianzi Dairy Co. Ltd., Wuxi, Jiangsu, China). Fresh Saanen caprine milk was purchased from a producer (Caiyang Husbandry Co. Ltd., Hangzhou, Zhejiang, China). Porcine pepsin (cat. no. P6887; enzyme activity 3,203 U/mg of protein), and porcine pancreatin (cat. no. P1750; 4×USP) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were of analytical grade.

Milk Serum Preparation

The separation of serum protein was based on a previous study with some modifications (Liao et al., 2011). Milk samples were centrifuged at $1,500 \times g$ for 10 min at 10°C (Heraeus Multifuge XIR, ThermoFisher Scientific, San Jose, CA). The pH of the obtained skim bovine milk and caprine milk were adjusted to 4.6 and 4.0, respectively. The samples were kept at 4°C for 1 h. They were centrifuged at $13,000 \times g$ for 30 min twice. Then the transparent liquid was collected and the bicinchoninic acid (BCA) assay kit (BCA protein assay kit, Thermo Scientific Pierce, Rockford, IL) was used to determine the milk serum protein concentration.

SDS-PAGE

We performed SDS-PAGE on the whole milk and milk serum samples. For each sample, 12.5 µg was electrophoresed through 12% polyacrylamide gel. The gels were stained with Coomassie Blue R-250 and destained.

In Vitro Gastrointestinal Digestion

Digestion samples were prepared using a modified procedure (Liu et al., 2016; Su et al., 2017; Brodkorb et al., 2019). Briefly, the protein concentration of milk serum was adjusted to 5 mg/mL. Then, the pH of both milk sera was adjusted to 4.0 and the sera were mixed with simulated gastric fluid (1:1 vol/vol, 22.76 U of pepsin per mg of milk protein, pH 4.0, 0.15 M NaCl). The samples were incubated for 2 h at 37°C. The pH of digesta was increased to 7 to terminate pepsinolysis. Subsequently, an equal volume of simulated intestinal fluid [pancreatin:protein ratio of 1:50 (wt/wt), pH 6.5, 2 mM sodium taurocholate, 2 mM sodium glycodeoxycholate, 50 mM KH_2PO_4] was added to the gastric digesta. Intestinal digestion was performed at 37°C for 2 h. The intestinal digested samples were heated at 100°C for 5 min and then stored at -80°C for further analysis. For each sample, aliquots were taken after 0 and 2 h of gastric digestion and after 2 h of intestinal digestion. All digestive procedures were run in duplicate.

Degree of Hydrolysis

The degree of hydrolysis (DH) was measured using the standardized α -phthalaldehyde (OPA) spectrophotometric assay (Mulet-Cabero et al., 2019). Trichloroacetic acid was added to the samples to a final concentration of 3.12% for insoluble protein precipitation. Then, the samples were centrifuged at $10,000 \times g$ for 30 min. The supernatant was collected and filtered through a nylon membrane with a pore size of 0.22 µm (Xinya Inc., Shanghai, China). The OPA reagent was prepared as following: 3.81 g of sodium tetraborate was added to 80 mL of distilled water. When it was dissolved, 88 mg of dithiothreitol and 0.1 g of SDS were added and mixed. Finally, 80 mg of OPA was dissolved in 3 mL of ethanol and added to the mixture, and the solution was made up to 100 mL with distilled water.

Standard L-leucine solution was used to make a calibration curve. Ten microliters of standard/sample was mixed with 200 µL of OPA reagent. The mixtures were measured at 340 nm using a Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific, Karlsruhe, Germany) after standing for 15 min. The calculation of DH was according to a previous study (Nielsen et al., 2001).

Extraction of Peptides

Before injection, digested samples were precipitated by addition of 200 g/L trichloroacetic acid (1:1 vol: vol; Nielsen et al., 2018). The supernatant from each sample was collected after centrifugation ($4,000 \times g$ for 10 min, 4°C), and dithiothreitol was incorporated with each sample to a final concentration of 10 mM for 30 min at 56°C . Next, iodoacetamide was added at a final concentration of 55 mM and incubated for 45 min at 25°C . After that, the samples were desalted using C18 columns and filtered with a 10-kDa ultra-centrifugal filter. Peptide fractions were obtained after freeze-drying (LaboGene, Lyngby, Denmark).

Determination of Peptides

Peptide analysis was performed on a Q-Exactive HF X (Thermo Scientific, Waltham, MA) equipped with an UltiMate 3000 UHPLC system (Thermo Scientific). Two micrograms of peptides was loaded onto a trap column ($300 \mu\text{m} \times 5 \text{ mm}$, μ -Precolumn, Thermo Scientific) connected to an in-house packed RP C18 analytical column ($75 \mu\text{m} \times 25 \text{ cm}$, $3\text{-}\mu\text{m}$ particle). The liquid chromatography (LC) eluent was initially set to 95% solvent A (2% acetonitrile and 0.1% formic acid) and 5% solvent B (98% acetonitrile and 0.1% formic acid) at 300 nL/min. The linear gradient was designed as follows: 5% solvent B for 5 min, 5 to 25% solvent B for 40 min, 25 to 35% solvent B for 5 min, 35 to 80% solvent

B for 2 min, and held in 80% solvent B for 2 min. The mass scan was 350 to 1,500 m/z with a resolution of 60,000, and the automatic gain control (AGC) target was set to 3×10^6 . The tandem mass spectrometry (MS/MS) scan was 100 m/z with a resolution of 15,000; the AGC target was set to 1×10^5 .

Data Analysis

Raw files were analyzed using MaxQuant (1.5.3.30; <https://maxquant.net/maxquant/>) using 2 databases (*Bos taurus*, *Capra* genus) downloaded from Uniprot (<https://www.uniprot.org/>). The following parameters were used: enzyme = unspecific; minimal peptide length = 7; peptide mass tolerance = 4.5 ppm; fragment mass tolerance = 20 ppm; carbamidomethyl for fixed modification; oxidation of methionine and N-terminal for variable modifications. Maximum false discovery rates (FDR) were set to 1% on peptide spectrum match level. Peptides sharing the same AA sequence but with different modifications were considered different peptides for number counts and intensity.

Statistical Analysis and Functional Analysis

Peptides detected in 2 replicates can be considered identified, and the peptides not detected in both duplicates were deleted from the data. Log₂ scale transformation was performed in Perseus (<https://maxquant.net/perseus/>), which was also used to carry out statistical analysis, including multiple ANOVA and 2-samples *t*-test. The biological functions of identified peptides were analyzed according to Milk Bioactive Peptide Database (<http://mbpdb.nws>; Nielsen et al., 2017). Peptides in the samples with 100% homology to database peptides were reported. For DH, statistical analysis was performed using the independent-samples *t*-test in SPSS 24.0 (IBM Corp., Armonk, NY). Between different groups, $P < 0.05$ was considered a significant difference.

RESULTS

Extraction of Milk Serum Proteins

The milk serum proteins of bovine and caprine milks were isolated by removing the fat layer and precipitating the caseins. The abundance of casein bands in milk serum decreased significantly after treatment, suggesting successful removal of caseins (Figure 1).

Hydrolysis of Proteins

Figure 2 shows the DH of milk serum samples before and after digestion. The DH increased in bovine and

Figure 1. Sodium dodecyl sulfate-PAGE of whole milk proteins and milk serum proteins. M = molecular weight standard, lane 1 = bovine whole-milk proteins, lane 2 = bovine milk serum proteins, lane 3 = caprine whole-milk proteins, and lane 4 = caprine milk serum proteins. LGB = β -lactoglobulin; LALBA = α -lactalbumin.

caprine milk serum samples during digestion. Significant statistical differences ($P < 0.01$) were observed in milk serum between the 2 species at the beginning and after 2 h of gastric digestion. A significant increase ($P < 0.01$) in DH was observed in gastric digesta compared with the raw milk serum in both bovine and caprine species. In addition, DH continued to increase significantly ($P < 0.01$) from gastric digesta to intestinal digesta in both milk species.

Identified Peptides and Proteins

Figure 3 shows the Venn diagram of peptides identified in the 6 samples (peptides detected in 2 replicates were considered identified). In total, 5,676 unique peptides deriving from bovine and caprine milk serum proteins were identified (Figure 3A). Of the total number, 2,402 and 3,698 originated from bovine and caprine milk serum protein, respectively (Figure 3B, 3C). For both species, the number of identified peptides was much higher in gastric digesta than in intestinal digesta. Overall, 39 and 101 overlapping peptides were identified in fresh milk serum and gastric and intestinal digesta of bovine and caprine milks, respectively. Most of these peptides originated from α ₁-casein (CSN1S1), β -casein (CSN2), and LGB in bovine milk serum samples (Supplemental Table S1; <https://doi.org/10.3168/jds.2020-18471>), whereas they were from CSN1S1, α ₂-casein (CSN1S2), CSN2, BLG, and serum amyloid A protein (SAA1) in caprine milk serum samples (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18471>). These peptides were thought to be survivors of milk serum peptides after gastrointestinal digestion.

The protein origin of identified peptides is given in Figure 4. Proteins were divided into the following cat-

Figure 2. Degree of hydrolysis of bovine and caprine milk serum before and after gastrointestinal digestion. *Results at different digestion times are significantly different ($P < 0.01$). Different letters (a, b) indicate a significant difference ($P < 0.01$) between species after their respective digestion period. Error bars represent SD.

egories: major milk serum proteins (LALBA, LGB), caseins [CSN1S1, CSN1S2, CSN2, and κ -casein (CSN3)], minor proteins [SAA1, lactotransferrin (LTF), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), lactoperoxidase (LPO), polymeric immunoglobulin receptor (PIGR), osteopontin (SPP1), butyrophilin subfamily 1 member A1 (BTN1A1)], and other proteins. The number of identified peptides from caseins and major milk serum proteins made up a large part of the total identified peptides. In addition, CSN2 and LGB were the dominant proteins in

Figure 3. Venn diagram showing the unique peptides identified in bovine and caprine milk serum before and after a 2-h gastrointestinal digestion.

these 2 species, respectively. In contrast, the number of endogenous peptides derived from SAA1 was much higher in caprine than in bovine milk serum. The number of peptides derived from LPO and BTN1A1 was higher in gastric digesta of caprine milk than of bovine milk, and the number of peptides derived from SAA1, GLYCAM1, and LTF was higher in both gastric and intestinal digesta of caprine milk than in those of bovine milk.

Quantitative Level of Identified Peptides

The exclusively identified peptides (i.e., only identified in one species, caprine or bovine) in gastric and intestinal digesta in bovine and caprine milk serum are shown in Figure 5. In general, the total number of exclusive peptides in caprine milk serum digesta was

much higher than that in bovine milk serum digesta. However, the proteins of origin of exclusive peptides varied between bovine and caprine milk serum. For instance, the number of exclusive peptides derived from LALBA, CSN2, SAA1, GLYCAM1, LPO, PIGR, LTF, and BTN1A1 was greater in caprine than in bovine milk serum, whereas exclusive peptides from CSN1S1, CSN1S2, CSN3, and LGB were relatively more numerous in bovine gastric digesta than in caprine gastric digesta. With respect to the number of exclusive peptides in intestinal digesta, we found only a few peptides in bovine intestinal digesta (16) and many more (181) derived from proteins including LGB, SAA1, and LTF in caprine intestinal digesta (Figure 5).

We analyzed the overlapping peptides between groups using the 2-samples *t*-test (Figure 6). The peptide intensity was used to determine the statistical significance of

Figure 4. Distribution of protein origin for the peptides detected before and after gastrointestinal digestion in bovine milk serum (A), in bovine gastric digesta (B), in bovine intestinal digesta (C), in caprine milk serum (D), in caprine gastric digesta (E), and in caprine intestinal digesta (F). LALBA = α -lactalbumin, LGB = β -lactoglobulin, CSN1S1 = α _{S1}-casein, CSN1S2 = α _{S2}-casein, CSN2 = β -casein, CSN3 = κ -casein, SAA1 = serum amyloid A protein, GLYCAM1 = glycosylation-dependent cell adhesion molecule 1, LPO = lactoperoxidase, PIGR = polymeric immunoglobulin receptor, SPP1 = osteopontin, BTN1A1 = butyrophilin subfamily 1 member A1, LTF = lactotransferrin.

Figure 5. Exclusively identified peptides in gastric and intestinal digesta between bovine and caprine milk serum in bovine gastric digesta (A), in bovine intestinal digesta (B), in caprine gastric digesta (C), and in caprine intestinal digesta (D). LALBA = α -lactalbumin, LGB = β -lactoglobulin, CSN1S1 = α _{S1}-casein, CSN1S2 = α _{S2}-casein, CSN2 = β -casein, CSN3 = κ -casein, SAA1 = serum amyloid A protein, GLYCAM1 = glycosylation-dependent cell adhesion molecule 1, LPO = lactoperoxidase, PIGR = polymeric immunoglobulin receptor, SPP1 = osteopontin, BTN1A1 = butyrophilin subfamily 1 member A1, LTF = lactotransferrin.

peptides, and significantly different peptides are shown as red (upregulated) and green dots (downregulated) based on the criteria of a fold change >1.5 or <0.67 and $P < 0.05$. A total of 147 peptides were downregulated and 32 were upregulated in the bovine gastric group compared with the bovine milk serum group; 26 peptides were downregulated and 8 upregulated in the bovine intestinal group compared with the bovine milk gastric group. For caprine milk serum, 91 peptides were downregulated and 41 upregulated in the gastric group compared with the milk serum group (Table 1). A total of 81 peptides were downregulated and 52 were upregu-

lated in the caprine intestinal group compared with the caprine gastric group. Of the upregulated peptides in gastric digesta compared with milk serum, the number of peptides derived from CSN1S2, CSN2, and SAA1 was much higher in caprine than in bovine milk serum; in contrast, the number of peptides corresponding to LTF in milk serum was much higher in bovine than in caprine milk serum (Table 1). With respect to the significantly different peptides in intestinal and gastric groups, the number differed between the species, including peptides derived from CSN1S1, LALBA, CSN2, GLYCAM1, and SAA1 (Table 1).

Figure 6. Volcano plots of differential peptides in bovine gastric group versus bovine milk serum group (A), bovine intestinal group versus bovine gastric group (B), caprine gastric group versus caprine milk serum group (C), and caprine intestinal group versus caprine gastric group (D). Red and green dots represent upregulated and downregulated peptides, respectively. S = milk serum sample, G = gastric sample, I = intestinal sample; 2h represents 2-h digestion.

The profile of peptide release over time across the 2 species is presented in Figure 7. The peptides originating from LALBA in bovine and caprine serum before digestion were different (Figure 7A). The peptides primarily originated from 2 sections of the caprine LALBA sequence, at AA positions f72–79 and f112–122. The endogenous peptides in bovine LALBA were primarily from 2 large segments (f20–78, f105–137). Although the peptide sequences in bovine and caprine LALBA after gastric digestion covered almost the entire protein, the abundance of peptides was higher in caprine than in bovine gastric digesta, particularly for sequences f20–49 and f108–136. The endogenous peptides of bovine LGB primarily originated from 4 sections, at AA f19–37, f47–72, f92–100, and f140–175 (Figure 7B). The sequences of the endogenous peptides of LGB were different in caprine milk serum than in bovine milk serum, which were dominated by f20–38, f51–60, and f76–122. The overlap in peptides found in intestinal digesta and

entire protein was greater in caprine than in bovine samples.

Bioactivity of Peptides After Simulated Gastrointestinal Digestion

Tables 2 and 3 display bioactive peptides of bovine and caprine milk serum after simulated gastrointestinal digestion. Of all the identified peptides, 41 and 30 peptides of bovine and caprine milk serum were found to have bioactivities, including angiotensin-converting enzyme (**ACE**) inhibition and antimicrobial functionalities. The 10 peptides common to these 2 species are noted in Tables 2 and 3. Of these common proteins, 5 were derived from CSN2 (ACE-inhibitory, antimicrobial, and immunomodulatory activities), 2 from LALBA (dipeptidyl peptidase IV inhibitory activity), 2 from LGB (ACE-inhibitory activity), 1 from CSN1S2 (antimicrobial activity). Antimicrobial and

Table 1. The number of differential peptides (down- or upregulated) between bovine and caprine milk serum in fresh milk serum, gastric digesta, and intestinal digesta

Protein	Bovine gastric group vs. bovine milk serum group		Bovine intestinal group vs. bovine gastric group		Caprine gastric group vs. caprine milk serum group		Caprine intestinal group vs. caprine gastric group	
	Down	Up	Down	Up	Down	Up	Down	Up
LALBA	2	1	0	0	0	0	9	2
LGB	2	0	5	0	0	0	6	17
CSN1S1	22	1	3	0	11	1	1	0
CSN1S2	11	1	1	1	16	5	2	1
CSN2	21	4	2	3	26	14	26	12
CSN3	2	1	1	0	1	2	3	0
SAA1	1	0	1	0	32	15	10	10
GLYCAM1	8	3	1	0	0	3	8	5
LPO	2	0	0	0	1	0	0	0
PIGR	6	2	2	1	0	0	1	0
SPP1	2	0	0	0	2	0	1	0
BTN1A1	5	1	0	0	2	1	0	0
LTF	2	4	2	1	0	0	5	0
Other	61	14	8	2	0	0	9	5

¹LALBA = α -lactalbumin; LGB = β -lactoglobulin; CSN1S1 = α_{S1} -casein; CSN1S2 = α_{S2} -casein; CSN2 = β -casein; CSN3 = κ -casein; SAA1 = serum amyloid A protein; GLYCAM1 = glycosylation-dependent cell adhesion molecule 1; LPO = lactoperoxidase; PIGR = polymeric immunoglobulin receptor; SPP1 = osteopontin; BTN1A1 = butyrophilin subfamily 1 member A1; LTF = lactotransferrin.

ACE-inhibitory peptides were found in bovine caseins; however, no bioactive peptides were found in caprine CSN1S1 milk serum or gastrointestinal digesta samples. Eleven bioactive peptides were found in caprine intestinal digesta samples, whereas only 3 were identified in bovine intestinal digesta.

DISCUSSION

Although the protein digestibility of whole milk between species has been reported previously, this is the first study to investigate the peptidome between bovine and caprine species based on milk serum samples in an in vitro infant digestion system. In total, we identified 5,676 peptides in bovine (2,402) and caprine (3,698) samples, which is much higher than the number of peptides (over 350) previously identified in bovine and caprine milk from simulated infant gastric digestion (Hodgkinson et al., 2019), and much higher than the number of peptides (384) identified in bovine milk after in vitro standardized gastro-pancreatic digestion (Tagliazucchi et al., 2016). The higher number of peptides identified can likely be attributed to sample preparation. In this study, we used the milk serum protein of 2 species to mimic infant digestion, which removed the influence of relatively high abundant milk proteins (caseins). Therefore, the number of digested peptides was relatively higher compared with digesta from whole milk. On the other hand, tracing the dynamics of digestion from fresh milk serum to intestinal digestion also probably explained the higher number of identified pep-

tides in this study. For instance, a previous study only detected peptides in gastric samples (Hodgkinson et al., 2019), whereas our study used milk serum samples, gastric samples, and intestinal samples to comprehensively demonstrate the endogenous peptides and peptides released from gastrointestinal tracts. Another reason why we identified more peptides could be attributed to the LC-MS/MS analysis. In this study, we used Q Exactive HF MS/MS for analysis, whereas the cited articles mentioned above used maxis impact HD Q-TOF mass spectrometer. In addition, we used *Bos taurus* and *Capra* genus databases when comparing different species. The database used for matching may explain the differences in the numbers of identified peptides between species. At the beginning of digestion, more peptides were identified in caprine than in bovine milk serum samples (Figure 3), which may explain why high DH values were obtained in raw caprine milk serum samples (Figure 2). The number of peptides increased from milk serum samples to gastric samples in both species, which agrees with the findings of a previous study on gastric digestion in term infants (Dallas et al., 2014). The DH results from milk serum samples to gastric samples show the similar trend in both species (Figure 2). In addition, both the number of identified peptides (Figure 3) and the DH values (Figure 2) were higher in caprine than in bovine gastric digesta, which indicates that caprine milk serum proteins have a higher digestibility during gastric digestion. A decrease in peptide counts from gastric samples to intestinal samples was observed in both species. This was probably related

Figure 7. Schematic presentation of peptides formed before and after gastrointestinal digestion for (A) α -lactalbumin (LALBA) and (B) β -lactoglobulin (LGB). Green indicates low relative abundance and red indicates high relative abundance of detected AA found in peptides found in digesta samples. S = milk serum sample, G = gastric sample, I = intestinal sample; 2h represents 2-h digestion.

to a limitation of the method because our approach could only identify peptides longer than 7 AA; short peptides released after intestinal digestion were not detected in this study. Therefore, an increase in DH was observed from gastric samples to intestinal samples in both species (Figure 2), which is consistent with results of a previous study (Tagliazucchi et al., 2018).

Peptides Derived from Major Milk Proteins

Although most of the caseins in the milk were removed, a large number of peptides derived from caseins were identified in all samples. This could be due to endogenous casein peptides and free caseins (caseins not in the form of micelles) present in milk serum (Fig-

ure 1). Another reason for the high number of peptides from caseins in gastric digesta is likely the different digestibility between caseins and milk serum proteins. Their flexible and open structure makes free caseins more sensitive to proteolysis (Dupont et al., 2010), and the rapid degradation of caseins has been reported in a previous study (Picariello et al., 2010). The different amount of caseins in milk serum of these 2 species may be due to the distinct casein micelle structure. The casein micelles in these 2 species have different average sizes, protein compositions, and levels of hydration (Ingham et al., 2018). Peptides from CSN1S1 and CSN2 were present in high proportions in bovine milk serum, whereas peptides from CSN1S2 and CSN2 had high abundance in caprine milk serum, which agrees with

a previous study (Tamime et al., 2011). Of the unique peptides of bovine gastric digesta, the number of peptides derived from CSN1S1 and CSN1S2 were higher in bovine than in caprine. In contrast, the number of unique peptides derived from bovine CSN2 was lower than that from caprine CSN2. A previous study verified that sequence coverage for bovine caseins (CSN1S1, CSN2S2, CSN2) after gastric digestion was higher than that for caprine caseins (Hodgkinson et al., 2019). These results may indicate that the extent of gastric digestion of CSN1S1 and CSN1S2 is higher in bovine than in caprine milk. On the other hand, more peptides originating from CSN2 were observed to increase or decrease significantly in the caprine intestinal group compared with the caprine gastric group, suggesting that caprine

Table 2. Bioactivity of peptides before and after gastrointestinal digestion in bovine milk serum

Protein ¹	Query peptide	Serum	Gastric	Intestinal	Function ²
LALBA	IVQNNDSTEYGLF ³		✓		DPP-IV inhibitory
	LCSEKLDQ	✓	✓	✓	DPP-IV inhibitory
	LKGYGGVSLPEW	✓			ACE inhibitory
	TKCEVFRE		✓		DPP-IV inhibitory
LGB	WLAHKALCSEKLDQ ³	✓			DPP-IV inhibitory
	DAQSAPLR		✓		Antimicrobial
	DAQSAPLRVY ³		✓		ACE inhibitory
	LDAQSAPLR ³		✓		ACE inhibitory
CSN1S1	LIVTQTMK		✓		Cytotoxic
	LSFNPTQ		✓		ACE inhibitory
	TPEVDDEALEK		✓		Antimicrobial, DPP-IV inhibitory
	FFVAPFPEVFGK	✓	✓		ACE inhibitory
CSN1S2	IKHQGLPQEV		✓		Antimicrobial
	LEQLRLKKY	✓			Antimicrobial
	LRLKKYKVPQL		✓		Antimicrobial
	SDIPNPIGSENSEK		✓		Antimicrobial
CSN2	VLNENLLR		✓		Antimicrobial
	IVLNPWDQVK ³		✓		Antimicrobial
	NMAINPSKENLCSTFCK	✓	✓		ACE inhibitory
	TKVIPYVRYL		✓		Antimicrobial
CSN3	EPVLGPVRGPF ³	✓	✓		ACE inhibitory
	KVLPVPQK		✓		Antioxidant
	KYPVEPFTESQSLTL		✓	✓	ACE inhibitory
	LNVPGEIVE	✓			ACE inhibitory
CSN3	LVYFPFGPIPNLSPQ	✓	✓		ACE inhibitory
	LYQEPVLGPVRGPFPIIV		✓		Immunomodulatory
	MPFPKYPVEP		✓		ACE inhibitory
	NIPPLTQTPV	✓			ACE inhibitory
CSN3	PVVVPPFLQPE ³		✓		Antimicrobial
	QEPVLGPVRGPFPIIV		✓		ACE inhibitory
	RELEELNVPGEIVESLSSEESITR		✓		Immunomodulatory, caseinophosphopeptide
	TEDELQDKIHPF ³	✓			Antimicrobial
CSN3	VENLHLPLPL		✓	✓	ACE inhibitory
	VLPVPQKAVPYPQR		✓		Antimicrobial
	VPYPQRDMPIQAF		✓		Antimicrobial
	VYFPFGPIP		✓		Prolyl endopeptidase inhibitory, PEP inhibitory
CSN3	YFPFGPIP		✓		ACE inhibitory, DPP-IV inhibitory
	YQEPVLGPVR ³		✓		ACE inhibitory, immunomodulatory
	YQEPVLGPVRGPFPI ³		✓		Antimicrobial
	MAIPPCKNQDKTEIPTINT		✓		Antimicrobial
CSN3	YYQQKPVA		✓		Antimicrobial

¹LALBA = α -lactalbumin; LGB = β -lactoglobulin; CSN1S1 = α _{S1}-casein; CSN1S2 = α _{S2}-casein; CSN2 = β -casein; CSN3 = κ -casein.

²DPP-IV = dipeptidyl peptidase IV; ACE = angiotensin-converting enzyme; PEP = prolyl oligopeptidase.

³The 10 peptides common to both bovine and caprine samples.

Table 3. Bioactivity of peptides before and after gastrointestinal digestion in caprine milk serum

Protein	Query peptide	Serum	Gastric	Intestinal	Function ²
LALBA	DKVGINY		✓	✓	ACE inhibitory
	DKVGINYW		✓		ACE inhibitory
	ILDKVGINY			✓	DPP-IV inhibitory
	IVQNNSTSEYGLF ³		✓		DPP-IV inhibitory
	LAHKALCSEKL		✓		DPP-IV inhibitory
	WLAHKALCSEKLDQ ³		✓	✓	DPP-IV inhibitory
LGB	DAQSAPLRVY ³	✓		✓	ACE inhibitory
	GLDIQKVAGT	✓		✓	Antimicrobial
	IIAEKTKIPAVF	✓	✓	✓	Antimicrobial
	IQKVAGTW		✓	✓	DPP-IV inhibitory, ACE inhibitory
	LDAQSAPLR ³		✓		ACE inhibitory
	VEELKPTPEGNLE		✓		Antimicrobial
CSN1S2	ALNEINQFYQK	✓			ACE inhibitory
	IVLNPWDQVK ³	✓			Antimicrobial
CSN2	EPVLGPVRGPF ³	✓			ACE inhibitory
	GVPKVKETMVPK	✓	✓		ACE inhibitory
	HKEMPFPK	✓	✓	✓	Antimicrobial
	KETMVPK	✓			Antimicrobial
	LVYPFTGPIPN	✓	✓		ACE inhibitory
	PFTGPIPNSLPQ		✓		Antimicrobial
	PVVVPPFLQPE ³		✓		Antimicrobial
	REQEELNV	✓			Antimicrobial
	TEDELQDKIHPF ³	✓		✓	Antimicrobial
	VLGPVRGPF	✓	✓		ACE inhibitory
CSN3	YQEPVLGPVR ³	✓	✓		ACE inhibitory, immunomodulatory
	YQEPVLGPVRGPFPI ³	✓	✓	✓	Antimicrobial
	INNQFLPPY		✓	✓	DPP-IV inhibitory
	IVNTVDNPEASSE		✓		Antimicrobial
	MAIPPKKDQDKTEVPAINT		✓		Antimicrobial
	PTVHSTPTTE		✓		Antimicrobial

¹LALBA = α -lactalbumin; LGB = β -lactoglobulin; CSN1S1 = α _{S1}-casein; CSN1S2 = α _{S2}-casein; CSN2 = β -casein; CSN3 = κ -casein.

²DPP-IV = dipeptidyl peptidase IV; ACE = angiotensin-converting enzyme.

³The 10 peptides common to both bovine and caprine samples.

CSN2 is further digested in the small intestine. Identifying a bioactive peptide in the gastrointestinal tract is not enough to demonstrate its biological effect. Some peptides need to pass through the intestinal epithelium to exert their activity. However, peptides might be hydrolyzed by endogenous peptidases (Dupont, 2017). It is meaningful to focus on peptides that are active in the gut with no need to be absorbed. In addition, CSN2 is the most important precursor of peptides, including some functional peptides. In caprine intestinal samples, 3 peptides originating from CSN2 have antimicrobial activity. The presence of these antimicrobial peptides in the gut may be important to prevent gastrointestinal infections in infants (Dallas et al., 2013).

In addition to caseins, a large number of peptides derived from LALBA and LGB were also identified. More digested peptides from caprine LALBA and LGB were found during gastrointestinal digestion. Caprine LGB is digested faster than bovine LGB during in vitro digestion (Almaas et al., 2006), which was consistent with our study. Despite the number of identified peptides, the relative abundance of peptides from LALBA and LGB was higher in caprine than in bovine milk

serum digesta (Figure 7), indicating higher digestibility of LALBA and LGB in caprine milk than in bovine milk. It is worth noting that the high-abundance peptides (GLDIQKVAGT and IIAEKTIPAVF) found in caprine LGB have been reported to have antimicrobial activity. Synthetic peptides of these 2 sequences inhibit *Escherichia coli* K12 (Almaas et al., 2011). These 2 peptides may also play an important role in preventing gastrointestinal infections.

The digestibility of LALBA and LGB showed a similar trend in bovine and caprine milk, although the digestibility rate differed between the two proteins, probably because of differences in protein structure between species. When comparing dimers in bovine and caprine LGB, the root mean square deviation value was different because of the distinct orientations between the 2 monomers in these 2 species (Crowther et al., 2014). It has been shown that LALBA in bovine and caprine milk have different binding capacities to monoclonal antibodies (Kaminogawa et al., 1989). The LALBA sequences in the 2 species are highly homogeneous, and differences in the binding capacity of LALBA are related to the difference in conformation. Therefore, the

digestibility differences in LALBA between bovine and caprine milk serum is likely related to the differences in their conformation.

Peptides Derived from Minor Milk Proteins

In addition to the major proteins, numerous peptides originating from minor proteins were identified. Most peptides were derived from SAA1, GLYCAM1, LPO, PIGR, SPP1, BTN1A1, and LTF in both bovine and caprine milk serum. These proteins were resistant to gastrointestinal digestion, as indicated by the number of digested peptides compared with that of caseins. Digestibility rate is usually related to the protein AA sequence, protein structure, and posttranslational modification of the protein. Protein sequences with a high proline content are resistant to proteolysis, mainly because of the reduction in protein chain flexibility (Tagliazucchi et al., 2016). Gluten protein has lower digestibility than milk protein, in part because of its high proline content (Joye, 2019). It is generally believed that protein structure also affects its digestibility. For example, LGB is a dimeric globular protein. This stable conformation gives LGB high resistance to proteolysis. In contrast to LGB, casein molecules are more sensitive to proteolysis due to their flexible structure. The difference in the structure of these 2 proteins leads to their distinct digestibilities. Thermal treatment has a positive effect on the susceptibility of LGB to digestion (Reddy et al., 1988) because susceptible peptide bonds are exposed because of the heat-induced disruption to the structure. Proteins that undergo posttranslational modifications are more resistant to gastrointestinal digestion. The digestibility of milk protein concentrate increased with an increase in dephosphorylation (Liu et al., 2016). A high level of glycosylation in bovine LGB results in limited proteolysis compared with native bovine LGB (Corzo-Martínez et al., 2010).

Serum amyloid A is an acute phase protein in small ruminants (González et al., 2008); it protects newborns by preventing bacteria development (McDonald et al., 2001). The AA sequence of bovine SAA1 shows 94.6% homology with caprine SAA1. The high count of peptides derived from SAA1 in caprine sample was consistent with previous study (Hodgkinson et al., 2019). Caprine serum has a greater abundance of SAA1 compared with bovine (Lu et al., 2018), which may explain why larger number of peptides derived from SAA1 were identified in caprine samples.

Glycosylation-dependent cell adhesion molecule 1 is a secreted molecule detected in blood and milk. More peptides derived from GLYCAM1 were identified in both bovine gastric and intestinal samples than in caprine. The AA sequence of bovine GLYCAM1 shows 89.5%

homology with caprine GLYCAM1. Bovine GLYCAM1 contains 5 partial phosphorylation sites and 3 glycosylation sites, whereas caprine GLYCAM1 contains 2 partial phosphorylation sites and 2 glycosylation sites (Girardet et al., 1995; Lister et al., 1998). The lower content of phosphorylation and glycosylation of caprine GLYCAM1 may explain why it was more sensitive to proteolysis. The differences in the digestibility of GLYCAM1 between bovine and caprine samples is probably related to the differences in AA sequences and post-translational modifications.

Lactoperoxidase has antibacterial effect, which could protect newborns from infections (Goldman and Smith, 1973). The AA sequence of bovine LPO shows 96.5% homology with caprine LPO. It is a single polypeptide chain with several glycosylation sites (Sievers, 1981): 5 and 4 in bovine LPO and caprine LPO, respectively (B S et al., 2018). Because of the high homology between the 2 species, higher glycosylation of bovine LPO may explain why fewer peptides from LPO were identified in bovine gastric digesta.

Lactotransferrin is involved in several biological events, including transportation of iron ions, bacteriostatic effects, and constitution of the innate defense system. The AA sequence of bovine LTF shows 92.2% homology with caprine LTF. Bovine and caprine LTF shared about 90% of their 3-dimensional structure (Sreedhara et al., 2010). Lactotransferrin is also a glycoprotein. Although bovine and caprine LTF have the same number of potential glycosylation sites (Baker and Baker, 2009), some differences do exist in LTF glycosylation pattern between these 2 species. For instance, caprine LTF contains fewer sialylated *N*-glycans (Le Parc et al., 2014). A previous study showed that glycans bound to LTF affect its digestibility (van Berkel et al., 1996). In addition, LTF has a strong iron-binding capacity and the combination of iron and LTF reduces its sensitivity to digestion (Troost et al., 2001). A previous study reported that the iron concentration differed in bovine and caprine milks (Bilandžić et al., 2015), and differences in bovine and caprine LTF structure may lead to different iron-binding capacities. All of these factors may cause different iron saturations of LTF between the 2 species, which affect the digestibility of LTF in bovine and caprine milk. In general, the AA sequence, 3-dimensional structure, glycosylation pattern, and iron strength may result in differences in LTF digestions between the 2 species.

Butyrophilin subfamily 1 member A1, a member of the immunoglobulin superfamily, is related to lipid synthesis and secretion. Bovine and caprine BTN1A1 share a high homology (97.0%) of AA sequence. Both bovine and caprine BTN1A1 are known to be *N*-glycosylated. However, the different band in SDS-PAGE for bovine

and caprine BTN1A1 after periodic acid-Schiff staining or immunoblotting indicates differences in carbohydrate content (Cebo et al., 2010). The different carbohydrate contents may explain why different number of peptides from BTN1A1 were detected in bovine and caprine gastric digesta.

In summary, our results suggest that the digestive profiles of proteins in bovine and caprine serum differ. The difference in the digestive profile of milk serum proteins may be caused by different (1) databases used for searching, (2) protein sequences, (3) posttranslational modification, such as glycosylation and phosphorylation, and (4) physiochemical properties of milk serum, including pH and ion strength. The difference in the digestibility of milk serum proteins between bovine and caprine species can be used to better understand the differences between these 2 species. These results may increase our understanding of the differences in digestibility of goat milk-based infant formula fortified with either goat milk serum or bovine milk serum.

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