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Suckling Rat Pup Model: Do Caprine Milk Lactoferrin and Immunoglobulins Have Different Digestion and Absorption Properties from That of Human and Bovine Species?

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ABSTRACT: This study aimed to investigate the digestion and absorption properties of caprine milk serum proteins in comparison to human and bovine species by using rat pups to mimic preterm infants. The results indicate that caprine lactoferrin (LTF) had a shorter retention time in the intestine and released a greater number of fragments, resembling human milk LTF more closely. In contrast, caprine immunoglobulins (Igs) were similar to bovine Igs and both exhibited a longer retention time in the intestine. For absorption, caprine Igs could be absorbed intact, which was similar to human and bovine Igs, whereas caprine LTF fragments were found in jejunum but not in plasma of rat pups. This is similar to bovine LTF but differed from human LTF as human LTF could be absorbed intact in plasma of rat pups at 20 min. In addition, the absorption rate of peptides and amino acids from caprine milk serum was similar to that of human milk serum, which was higher than that from bovine milk serum. This study aimed to enhance our understanding of the differences in bioavailability of LTF and Igs derived from caprine, human milk, and bovine milk, thereby offering guidance for selecting protein sources for premature infants.

KEYWORDS: *milk serum protein, lactoferrin, immunoglobulins, in-vivo rat pups, digestion and absorption*

INTRODUCTION

The World Health Organization (WHO) defines preterm birth as a birth occurring before 37 weeks of gestation. This condition represents a significant public health challenge, accounting for an estimated 10% of births globally.[1] Breast milk is recognized as the optimal source of nutrition for premature infants.[2] However, when breastfeeding is not feasible, infant formulas that utilize bovine or caprine milk as a protein source can provide a suitable alternative for delivering essential nutrition to preterm infants.[3] Preterm infants have considerably higher protein requirements than their full-term counterparts,[4] and their gastrointestinal systems are not yet fully developed, resulting in a diminished capacity to digest protein.[5] Additionally, preterm infants may exhibit less developed immune systems[6] and increased intestinal permeability.[7] Caprine milk protein has demonstrated faster gastric emptying,[8] improved digestibility,[9] and reduced allergenicity[10] compared to bovine milk protein. Caprine milk protein is more easily digested and absorbed, and its advantages are evident in several key areas. Consuming caprine milk leads to improved protein efficiency and enhanced food conversion efficiency as well as a more favorable nitrogen balance[11] and increased amino acid absorption[12] compared to bovine milk. Clinical experiments have demonstrated that the fecal microbial sequences, as well as the *Lachnospiraceae* sequences, of infants who are fed caprine milk formula exhibit greater similarity to those of breastfed infants compared to those fed bovine milk formula.[13] Therefore, caprine milk protein has the potential to provide better nutritional and immune protection for preterm infants.

Milk serum proteins, the predominant proteins in human milk, play a crucial role in providing vital nutritional and health benefits to infants.[14−16] Lactoferrin (LTF), a key immune component found in human milk serum, is an iron-binding glycoprotein with diverse biological functions such as enhancing iron absorption, exhibiting antibacterial, antiviral, antioxidant, and anti-inflammatory properties.[17] Another vital immune-related protein present in milk serum is immunoglobulin (Ig), which confers passive immunity to infants through placental transfer and breastfeeding.[15] Milk serum contains various types of Igs, including immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM). Notably, approximately 90% of the Igs present in human milk serum is IgA.[18] In contrast, bovine and caprine milk serum exhibit the highest relative content of IgG.[19,20] Specifically, IgG, the primary Ig in the blood, is associated with long-term immunity and aids in clearing pathogens by activating the complement cascade. Furthermore, research indicates that IgG in breast milk contributes to reducing infections in infants.[21]

Both LTF and Igs in human milk possess a strong resistance to digestion and can maintain their structural integrity within the infant's gastrointestinal tract.[22,23] These proteins can exert their functions within the intestinal tract and can also be

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absorbed into the bloodstream to offer protection. LTF can be transported to target organs via the Intelectin-1 (ITLN1) receptor on intestinal brush border cells.[24] Furthermore, the neonatal Fc receptor (FcRn) present in intestinal epithelial cells facilitates the transintestinal epithelial absorption of IgG.[25] Recent research has highlighted the absorption of maternal milk-derived IgG into the bloodstream of nursing pups through the FcRn receptors in the gut, providing systemic defense against specific pathogens.[26] In our previous research, we employed an in vitro simulated infant digestion model to compare the digestibility of IgG and LTF in human, bovine, and caprine milk, revealing that caprine IgG and LTF exhibited greater resistance to digestion than their bovine counterparts.[27] However, there is limited information regarding the in vivo absorption of caprine LTF and Igs.

Selecting an appropriate animal species as an ideal model for studying immune protein absorption in preterm infants presents significant challenges. Suckling rats are frequently utilized to investigate intestinal characteristics in premature infants.[28] The intestines of premature infants are underdeveloped, resulting in increased intestinal permeability compared to term infants.[29] The rat, a late-maturing species, is also relatively immature at birth and displays a permeable intestine throughout lactation.[30] Notably, both FcRn and ITLN1 receptors are present in the intestines of preterm infants[28,31] and are also expressed in the small intestine of suckling rats. Furthermore, the rat FcRn receptor can bind to human IgG,[32] while the rat ITLN1 receptor has the capacity to bind to human LTF.[33] In summary, suckling rats serve as an excellent model for simulating human preterm infants, particularly for investigating the in vivo absorption of Igs and LTF.

The objective of this study was to compare the digestion and absorption characteristics of caprine, human, and bovine milk serum proteins using rat pups as a model, with a specific focus on two immune-related proteins, LTF and Igs. The digestive properties of the proteins were analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), along with the analysis of amino acids absorbed into the bloodstream. Additionally, Western blot analysis was employed to monitor the hydrolysis of LTF and Igs in various compartments (stomach and upper and lower small intestine), as well as their subsequent absorption into the bloodstream. The findings of this study are expected to provide valuable insights into enhancing protein digestion and absorption in preterm infants.

MATERIALS AND METHODS

Extraction of Milk Serum Protein. After approval from the Medical Ethics Committee of Jiangnan University, mature breast milk was collected from eight healthy mothers in Wuxi City, Jiangsu Province. Fresh Holstein bovine colostrum (second day after calving) was collected from a local dairy in Wenzhou City, Zhejiang Province, while fresh Saanen caprine colostrum (second day after calving) was collected from a local dairy in Hangzhou City, Zhejiang Province.

Milk from various species mentioned above was centrifuged at 1500 rpm for 10 min at 4 °C to separate the skim milk from the upper milk fat layer. The pH of the skim milk was adjusted to 6.5, and chymosin was added at a concentration of 0.01% (w/w). Following a 45 min incubation at 35 °C, the mixture was centrifuged at 13,000 rpm for 30 min to collect the supernatant. The supernatant was then placed in a 7000 Da dialysis bag, with deionized water being replaced every 4 h. Finally, the solution was freeze-dried to obtain the milk serum protein powder.

In Vivo Oral Absorption Studies. The in vivo digestion of milk serum proteins was investigated using suckling rat pups. Animal experiments were conducted with the approval of the Jiangnan University Institutional Animal Ethics Committee (JN.No20220430S0090707[142]). Sprague−Dawley dams with litters of 7 day-old pups were procured commercially and housed in an SPF-level barrier facility with consistent temperature (21−25 °C) and humidity (55% ∼ 65%). When the pups reached 14 days of age, they were separated from their dams for 6 h. On day 14, pups of comparable body weight were selected and randomly assigned to 18 cages, with 6 pups per cage. Each type of milk serum protein treatment was replicated across 6 cages. Previous studies indicate that the protein concentration in rat milk ranges from 70 to 130 mg/mL.[34−36] A rat milk substitute containing approximately 90 mg/mL of milk protein has been shown to adequately meet the nutritional needs of suckling rats.[36] Consequently, the milk serum protein powder was dissolved in sterile distilled water to achieve a protein concentration of 90 mg/mL. The pups were orally administered with one of three types of milk serum protein at a 1:36 v/w ratio (milk in mL to body weight in g).[37] Pups were anesthetized with isoflurane at 0, 20, 60, 120, 180, or 240 min after intubation.

Sample Collection. Blood was collected via heart puncture and centrifuged at 4 °C at 4000 rpm for 15 min, and the plasma was then extracted and stored at −80 °C. Stomach contents were collected, and the small intestine was evenly divided into upper and lower halves. Each section was flushed with 1 mL of prechilled saline. Subsequently, the rinsed jejunal tissue was snap-frozen in liquid nitrogen.

SDS-PAGE Analysis of Gastrointestinal Digesta. After the gastrointestinal contents of young rats were freeze-dried, a certain volume of deionized water containing protease inhibitors was added to reconstitute them. The solution was mixed with sample buffer (containing 5% β-mercaptoethanol) in equal volumes, boiled for 3 min, cooled, and loaded. A 5% stack and 12% separating gel were prepared with a starting voltage of 80 V and an adjustment voltage of 120 V when the sample enters the separator. The gel was stained with Coomassie Brilliant Blue (R-250) and photographed using a fully automated chemiluminescence system after destaining.

Identification of Igs or LTF in Gastrointestinal Digesta, Jejunal Tissue, and Plasma. Western blot analysis was conducted to detect LTF and Igs in digesta, jejunal tissue, and plasma.[27] 20 mg of jejunal tissue, 250 μL of lysate, 5 μL of protease inhibitor, and 5 μL of phosphatase inhibitor were homogenized in a grinder, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The resulting supernatant was then assessed for protein concentration using a BCA kit. The gastrointestinal digesta, jejunal tissue, and plasma were separated by SDS-PAGE. Following SDS-PAGE, the proteins were transferred onto a 0.45 μm nitrocellulose membrane at 100 V for 90 min. Subsequently, the membrane was incubated in blocking solution for 15 min at room temperature. Primary antibodies specific to human, bovine, and caprine IgG and LTF were then added and allowed to incubate overnight at 4 °C. After washing the membrane with TBST, a secondary antibody was applied and incubated for 2 h at room temperature. The membrane was washed again with TBST before a mixture of liquid A and liquid B from the ECL reagent was applied to the NC membrane. Finally, the membrane was inserted into an automatic chemiluminescence system for visualization.

In-Gel Digestion. Plasma from control rat pups was analyzed by using SDS-PAGE, and the striped gel was cut into pieces. After decolorization and reductive alkylation, the gel block underwent treatment with 50 mM NH_4HCO_3. Subsequently, the block was incubated in a 20 ng/uL trypsin solution at 37 °C for 16 h to allow digestion. The resulting peptide extract was then freeze-dried. Prior to analysis, 12 μL of a 0.1% formic acid solution was added to the peptide sample. The redissolved peptide was separated using a Thermo high-performance liquid chromatography (HPLC) system EASY-nLC 1200. Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B was a mixture of 0.1% formic acid and 80% acetonitrile. Peptide samples were separated on a column (C18, 15 cm long, 150 μm inner diameter, 1.9 μm resin, Dr. Maisch GmbH) and then subjected to analysis using a Q Exactive HF-X mass

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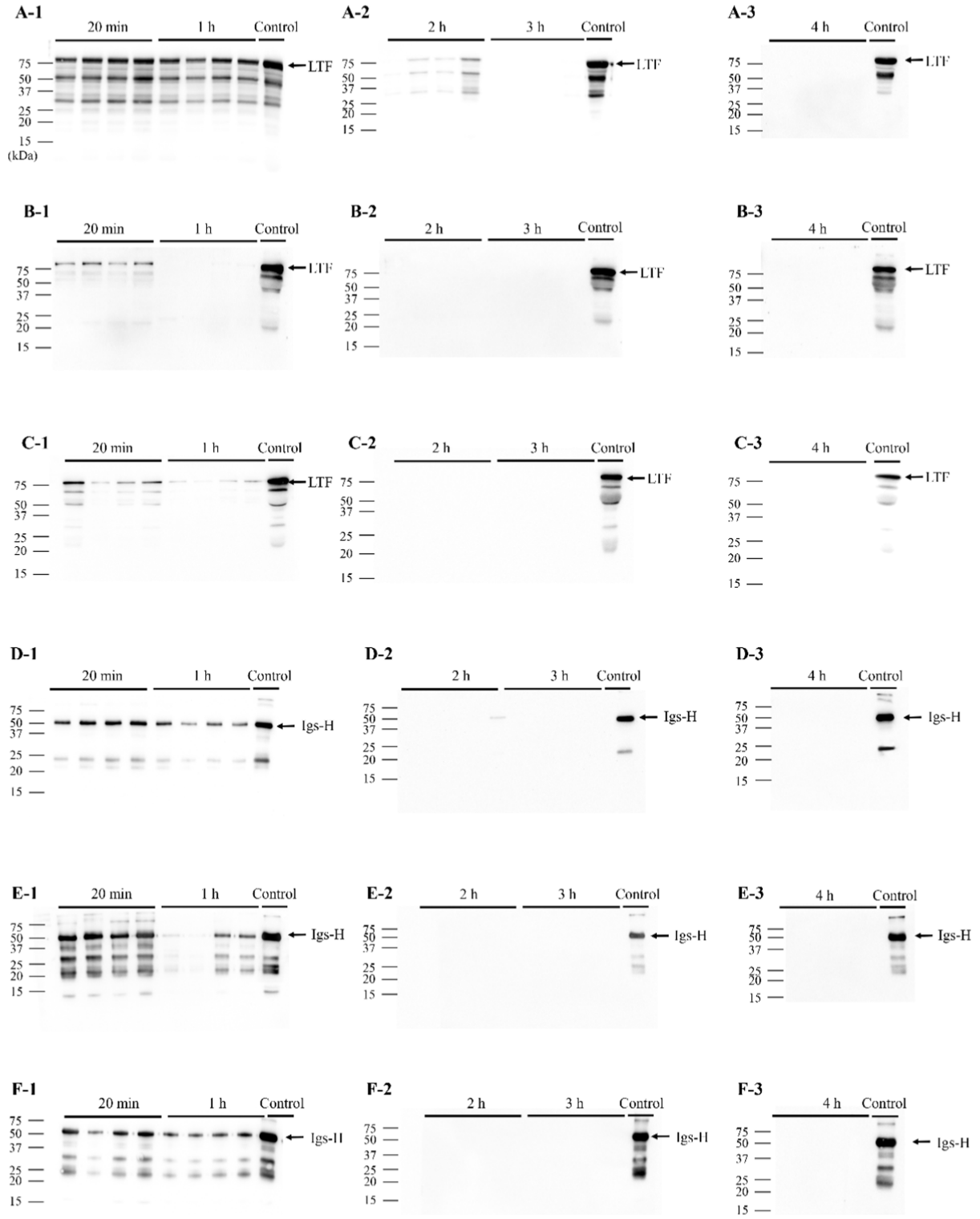

Figure 1. Western blot analysis of LTF and Igs-H in gastric digesta of rat pups. (A,D) Human milk serum group. (B,E) Bovine milk serum group. (C,F) Caprine milk serum group. LTF: lactoferrin, Igs-H: immunoglobulins heavy chain. Control: undigested control sample. Four biological replicates were collected for each sample.

spectrometer (Thermo Fisher). The mass spectrometry was conducted with a precursor ion scanning range of 300−1400 m/z. The first-order mass spectrometry resolution was set at 120,000 at 200 m/z with an automatic gain control (AGC) target of 3×10^6. HCD fragmentation mode was employed with a collision energy of 27%, and the secondary mass spectrometry resolution was 7500 at 200 m/z. An IProteome one-stop data analysis cloud platform was utilized for identification and quantitative analysis using the RAW files of mass spectrometry. The database (*Rattus norvegicus*) from NCBI (https://www.ncbi.nlm.nih.gov/) was downloaded for the search.

RT-PCR Analysis of FcRn, ITLN1, ZO-1, and Occludin in Jejunal Tissue. Total RNA was extracted from the jejunum using the Fast Pure Cell/Tissue Total RNA Isolation Kit V2, followed by assessment of purity and concentration with an ultramicrovolume spectrophotometer. Subsequently, cDNA was synthesized following the protocol for HiScript III RT SuperMix for qPCR. The ChamQ

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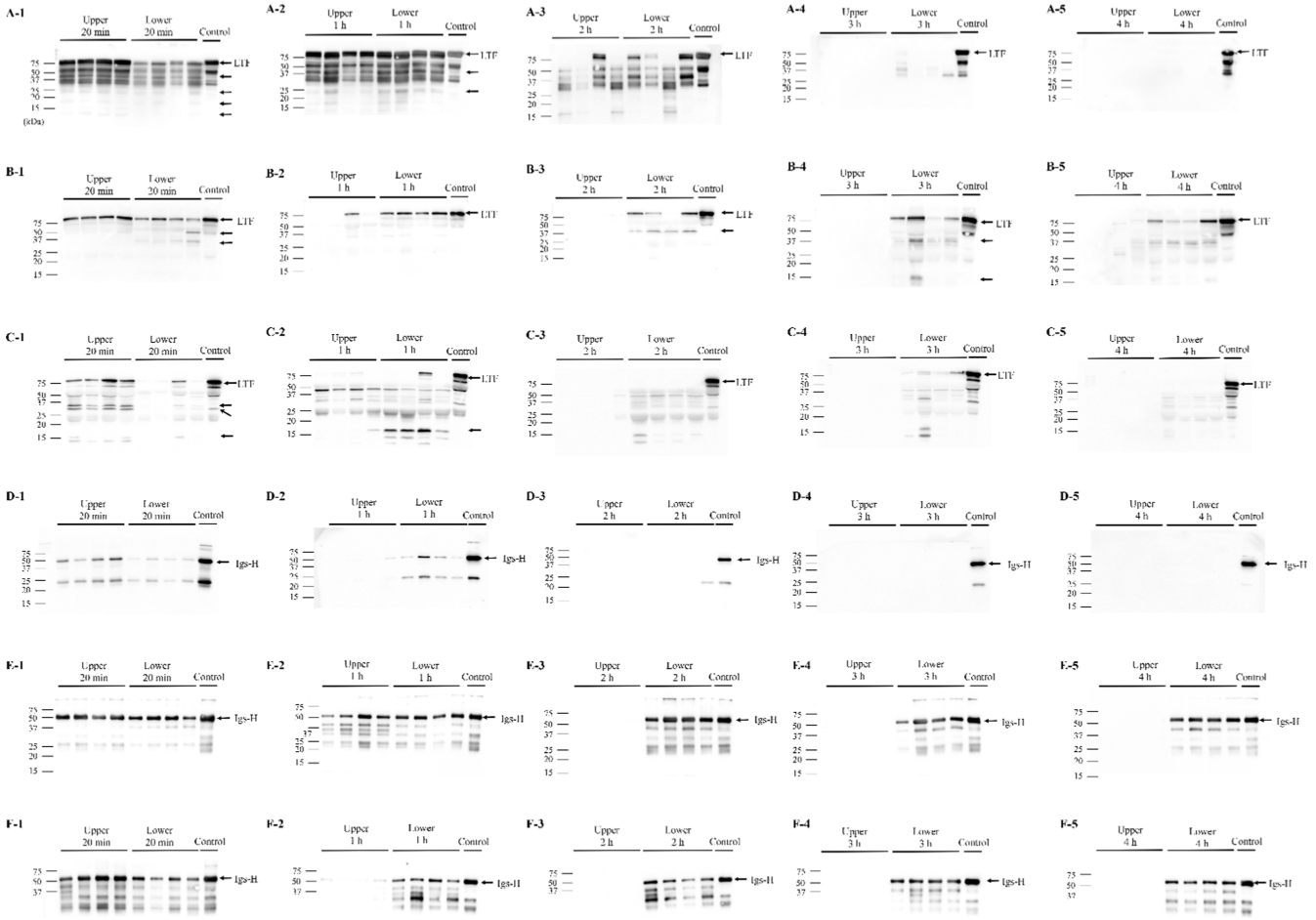

Figure 2. Western blot analysis of LTF and Igs-H in the small intestinal digesta of rat pups. (A,D) Human milk serum group. (B,E) Bovine milk serum group. (C,F) Caprine milk serum group. LTF: lactoferrin, Igs-H: immunoglobulins heavy chain. Control: undigested control sample. Four biological replicates were collected for each sample.

Universal SYBR qPCR master mix, along with primers and cDNA, were combined and amplified in a real-time PCR instrument with the following parameters: predenaturation at 95 °C for 30 s, cycling reactions at 95 °C for 10 s and 60 °C for 10 s for 40 cycles, and a cooling step at 37 °C for 30 s. GAPDH served as the reference gene, and the relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for both the reference gene and the target gene can be found in Table S1.

Determination of Amino Acids in Plasma. The procedure for determining free amino acids involved thawing frozen plasma on ice, treating it with trichloroacetic acid solution, allowing it to stand for 1 h, centrifuging the mixture to remove precipitated proteins, and filtering the resulting supernatant through a 0.22 μm membrane.

For the determination of free amino acids in the hydrolyzed samples, 200 μL of the supernatant obtained above was placed in a hydrolysis tube with 2 mL of 6 M HCl, hydrolyzed under nitrogen at 120 °C for 22 h, evaporating the nitrogen posthydrolysis, reconstituting the sample with 0.1 M HCl, and filtering it through a 0.22 μm membrane. The analysis of both free and hydrolyzed amino acids was performed using an Agilent 1100 HPLC system.

Determination of LTF Content in Serum Using ELISA. The levels of LTF in the serum of rat pups were determined by using ELISA kits specific for human LTF (Abcam, UK), bovine LTF (Bethyl Laboratories, USA), and caprine LTF (MyBioSource, CA).

Statistical Analysis. The experimental results were expressed as the mean ± standard deviation. All statistical analyses were conducted using SPSS version 25.0. Statistical significance was assessed using independent-sample t tests, one-way analysis of variance (ANOVA), or two-way ANOVA, as appropriate. The data profiling among species during digestion were analyzed for significance at $P < 0.05$ using two-way ANOVA, with digestion time and species as the factors.

RESULTS

Isolation of Milk Serum Protein. Bovine and caprine colostra are rich in IgG and thus were selected for this study. The composition of whole milk protein and isolated milk serum protein is illustrated in Figure S1. Milk serum protein was semiquantified as a percentage and calculated based on the intensity of the gel. The proportions of serum proteins in human, bovine, and caprine milk are approximately 65%, 45%, and 53%, respectively. In human milk serum, the bands corresponding to lactoferrin (LTF) and α-lactalbumin (α-LA) exhibited the greatest intensity, while the bands associated with immunoglobulin heavy chain (Ig-H) and β-lactoglobulin (β-LG) were most intense in both bovine and caprine milk serum. Albumin (ALB) was detected in all of the samples. Weaker bands of casein were observed in human milk serum, whereas a darker protein band around 25 kDa was present in both bovine and caprine milk serum.

Digestion of LTF and Igs in the Stomach of Pups. Although primary antibodies against human, bovine, and caprine IgG were employed to monitor changes in IgG during digestion, the heavy chains of various immunoglobulin types exhibit significant similarities that may lead to cross-reactivity. Therefore, we use "Igs" instead of "IgG" when presenting the

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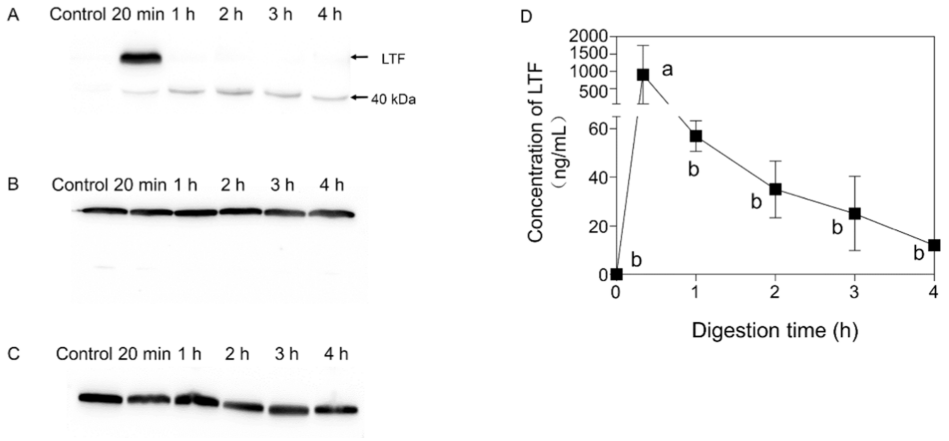

Figure 3. LTF detection in plasma of rat pups by using Western blot and ELISA analysis. (A) Western blot of human LTF. (B) Western blot of bovine LTF. (C) Western blot of caprine LTF. (D) ELISA analysis of human LTF, different lowercase letters indicated significant differences ($P < 0.05$). LTF: lactoferrin.

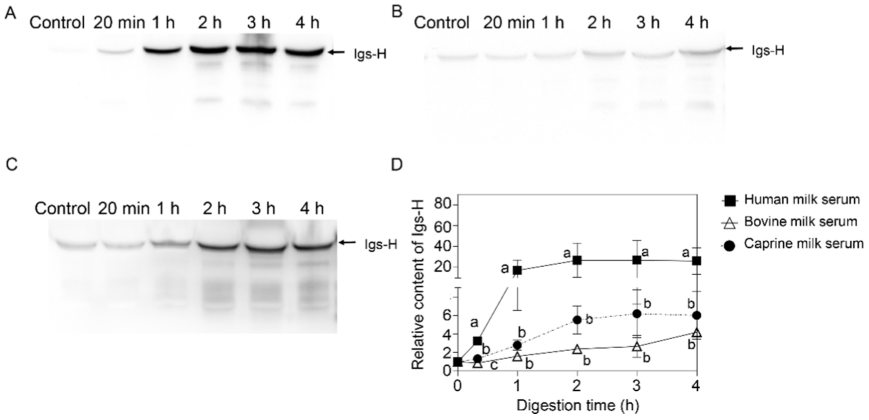

Figure 4. Western blot analysis of Igs-H in plasma of rat pups. (A) Human milk serum group. (B) Bovine milk serum group. (C) Caprine milk serum group. (D) Relative quantification via Western blot. Igs-H: immunoglobulins heavy chain. Different lowercase letters indicated significant differences at each time point ($P < 0.05$). Two-way ANOVA showed that the main effects were all significant ($P < 0.001$ for time; $P < 0.001$ for species; $P < 0.001$ for factor interaction). Control: rat pups without any treatment.

results. The digestion of LTF and Igs in the stomach of pups is illustrated in Figure 1. Multiple bands were observed in the undigested milk serum lanes, potentially indicating nonspecific binding or endogenous peptide fragments of LTF and Igs in the milk serum. Notably, LTF and Igs were kept intact during gastric digestion (Figure 1). After 1 h, the intensities of LTF and Igs bands in the caprine and human milk serum were darker than those in the bovine milk serum group. Only a small amount of human LTF was detected in the stomach of pups after 2 h but all disappeared after 3 h, suggesting that both caprine and bovine LTF were emptied from the stomach after 2 h whereas it took 3 h for human LTF.

Digestion of LTF and Igs in the Small Intestine of Rat Pups. The digestion of LTF in the small intestine of rat pups is illustrated in Figure 2A−C. At 20 min, intact LTF bands were observed in caprine, human, and bovine groups, while bands of LTF fragments were much more in caprine and human than in the bovine group. At 1 h, intact caprine LTF was barely detectable in the small intestine, but strong LTF bands (15−50 kDa) in its partially fragmented forms were observed in caprine and human milk serum. At 4 h, intact caprine and human LTF or their fragment bands were almost undetectable, whereas bovine LTF and its fragments were found in the lower half of the small intestine.

The intensity of intact LTF bands throughout the entire small intestine of the pups was analyzed, and the results are presented in Figure S2A. The relative content of caprine and bovine LTF gradually decreased, while for human LTF, it initially increased and then decreased. At 20 min, the relative content of caprine and bovine LTF was significantly lower compared to human LTF. By 3 and 4 h, the relative content of caprine and human LTF was significantly higher than that of bovine LTF.

The degradation of three sources of Igs in the small intestine of rat pups is illustrated in Figure 2D−F. Throughout the digestion process, the bands of Igs fragments appeared light. At 1 h, caprine and human Igs were concentrated in the lower half of the small intestine, while bovine Igs were distributed throughout the small intestine. At 2 h, substantial amounts of caprine and bovine Igs were found in the lower half of the small intestine, whereas human Igs were nearly undetectable. The results presented in Figure S2B indicate that the relative content of Igs in the small intestine of the three groups gradually decreased over digestion. At various time points during digestion, the relative content of caprine and bovine Igs in the small intestine was notably higher compared to that of human Igs.

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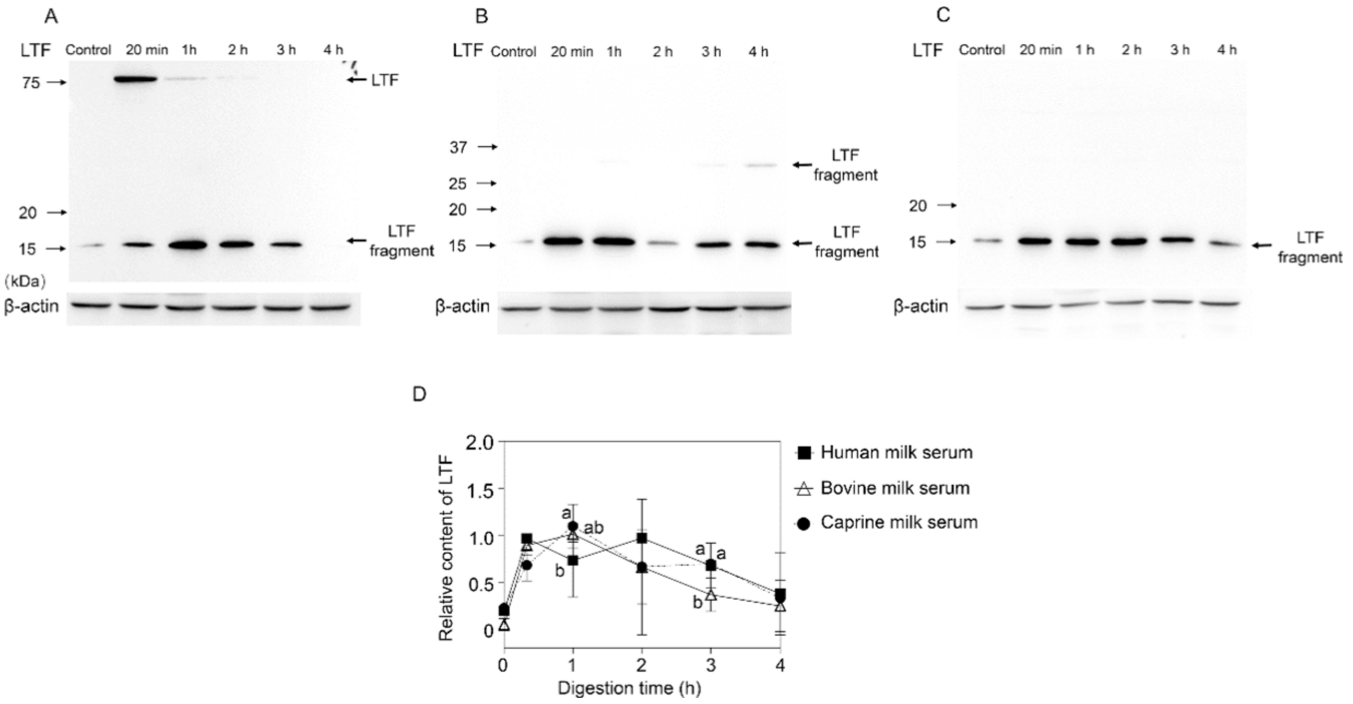

Figure 5. Western blot analysis of lactoferrin in jejunum tissue of rat pups. (A) Human milk serum group. (B) Bovine milk serum group. (C) Caprine milk serum group. (D) Relative quantification via Western blot. LTF: lactoferrin. Different lowercase letters indicated significant differences at each time point ($P < 0.05$). Two-way ANOVA showed that the main effect of time was significant ($P < 0.001$), while the main effect of time was not significant ($P > 0.05$), and there was no significant interaction between the two main effects ($P > 0.05$).

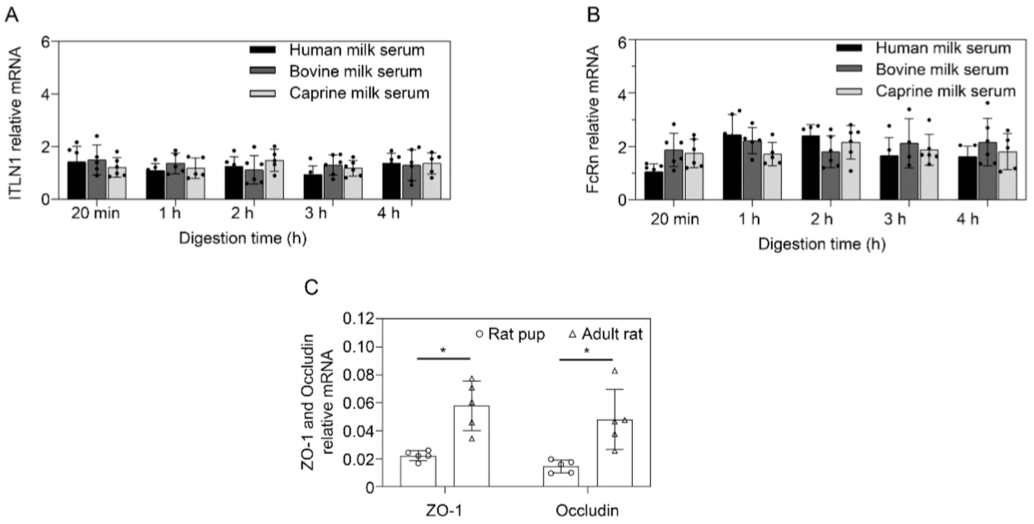

Figure 6. MRNA levels by RT-PCR. (A) ITLN1 mRNA level in the jejunum tissue of rat pups. (B) FcRn mRNA level in the jejunum tissue of rat pups. (C) ZO-1 and Occludin1 in the jejunum tissue of rat pup and adult rat. *Indicates that there is a significant difference in mRNA expression between young and adult mice ($P < 0.05$).

Absorption of LTF and Igs into the Blood of Young Rats. Caprine or bovine LTF was not detected in the plasma. Human LTF was found to be absorbed to the plasma of rat pups in intact form after administration for 20 min but was undetectable after 1 h (Figure 3A). Degradation fragments of human LTF (approximately 40 kDa) was observed in plasma, with bands becoming darker and then lighter during digestion. The protein in rat pups' plasma nonspecifically binding with the antibody (Figure 3B,C) was identified as rat serum transferrin through in-gel digestion combined with MALDI-TOF. ELISA was then used to confirm absorption of the three types of LTF by rat pups, with results depicted in Figure 3D. The concentration of human LTF absorbed by pups initially increased, peaked after 20 min of digestion, and then declined.

The absorption of human, bovine, and caprine Igs in rat pups was examined using Western blot (Figure 4A–C). The band intensity of Igs in plasma increased over time in all three species. The caprine and bovine Igs bands reached their highest intensity after 2 h, while human Igs band was the highest after 1 h. The changes of Igs band intensity of three species were further calculated, as shown in Figure 4D. At each time point, the relative content of caprine Igs in plasma was significantly lower than that of human Igs ($P < 0.05$) but slightly higher than that of bovine Igs ($P > 0.05$).

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Uptake of LTF by the Jejunum of Young Rats. Figure 5A−C illustrates the relative abundance of human, bovine, and caprine LTF absorbed in the jejunal tissue of rat pups, respectively. A substantial amount of LTF degradation fragments (approximately 15 kDa) were detected in the jejunal tissues from all three species. Furthermore, only intact human LTF protein was identified in the jejunal tissue after 20 min. As the time increased, the relative content of LTF and its fragments initially increased and then declined, as depicted in Figure 5D. The relative content of caprine LTF in the jejunum after 1 h was significantly higher than that of human LTF ($P < 0.05$) and was comparable to that of bovine LTF. Furthermore, the relative contents of caprine LTF and human LTF were similar after 3 h, and both were significantly higher than that of bovine LTF ($P < 0.05$).

Expression of Intestinal Receptors and Barrier Functions Related Genes. There was no significant difference in the relative expression levels of the ITLN1 receptor and FcRn receptor in jejunal tissue of the various groups of rat pups (Figure 6A,B). However, the expression of tight junction proteins (ZO-1 and Occludin) in the intestinal tract of 14 day-old rats was notably lower compared with that of adult rats (Figure 6C).

SDS-PAGE Analysis of Stomach and Small Intestinal Digesta. In addition to LTF and Igs, the digestibility of other milk serum proteins at various time points (20 min, 1, 2, 3, and 4 h) were also analyzed through SDS-PAGE (Figure S3). Over the course of digestion, the intensity of protein bands declined gradually in all three species and almost disappeared at 3 h. After 20 min and 1 h of gastric digestion, both caprine and bovine β-lactoglobulin (β-LG) remained intact, while almost no intact caprine α-lactalbumin (α-LA) was detected; however, intact bovine α-LA was observed in the milk serum protein digesta. In contrast, human α-LA was found to be intact at 20 min, 1 h, and 2 h of gastric digestion.

Furthermore, SDS-PAGE was utilized to analyze the protein composition of small intestinal digesta throughout the digestion process, as depicted in Figure S4. Initially, larger quantities of protein were observed in the upper segment of the small intestine compared to the lower segment across all species. Subsequently, the intensity of the protein bands was greater in the lower segment than in the upper segment. After 20 min, the β-LG bands observed in the lower part of the small intestine for both bovine and caprine milk were weaker than those in the upper part. However, after 1 h, this difference in β-LG levels between the upper and lower parts of the small intestine diminished. During digestion for both 20 min and 1 h, the α-LA bands in the milk serum protein across the three species exhibited changes similar to those observed in the β-LG bands.

Plasma Amino Acid Patterns. The concentration of free amino acids in the plasma of pups initially rose and then declined following the ingestion of milk serum protein (Figure S5A). At 2 h, the concentration of free amino acids in plasma reached its peak across all three groups. After 20 min, 1 h, and 2 h of digestion, the levels of free amino acids in the plasma of pups fed with human milk serum were significantly higher compared to those fed with bovine and caprine milk serum ($P < 0.05$). The concentration of hydrolyzed amino acids in the plasma of pups in the caprine and human milk serum groups initially increased and then decreased during digestion, while it steadily increased in the bovine milk serum group; see Figure S5B. The levels of hydrolyzed amino acids in the plasma of pups fed with caprine and human milk serum were significantly higher than those fed with bovine milk serum ($P < 0.05$). The concentration of peptides absorbed into the blood increased gradually across all three groups over digestion. At 1, 2, and 3 h, the concentration of peptides in the plasma of the caprine milk serum group exceeded that of the human and bovine milk serum groups ($P > 0.05$, Figure S5C).

DISCUSSION

Suckling rat pups were used as a model of a human infant to investigate the digestion and absorption properties of serum proteins in caprine, human, and bovine milk, focusing on two immunological proteins, LTF and Igs. Our previous in vitro studies showed that caprine IgG was more resistant to digestive enzymes than bovine IgG.[27]

The Western blot analysis indicates a degree of survival of LTF from three species during gastric digestion, followed by nearly complete degradation after intestinal digestion (Figures 1A−C and 2A−C). Some caprine and human LTF may remain intact during small intestinal digestion, consistent with the earlier report.[27] However, most previous studies have indicated that intact bovine LTF is completely degraded following gastrointestinal digestion.[38] In this study, intact bovine lactoferrin was detected in the lower part of the small intestine after 4 h, which contrasts with the findings of the in vitro study. One potential reason for this discrepancy may be the variation in digestive enzymes between this study and the previous investigation, which may include differences in the species' origin and enzyme activity. Another possible explanation is that continuous emptying occurs during in vivo digestion, whereas under in vitro conditions, all digestion products remain stationary in the beaker. Consequently, more intact bovine LTF was detected in vivo than in static in vitro conditions. We observed more degradation fragments of LTF in caprine and human milk serum compared to bovine LTF (Figure 2A−C), and peptide bands of human LTF between 15 and 50 kDa during the intestinal phase were also noted in a previous study.[39] Overall, caprine LTF exhibits a retention time and degradation pattern in the gastrointestinal tract that is more similar to that of human LTF than bovine LTF.

The current results indicate that in comparison to LTF, the three species of Igs exhibit greater resistance to intestinal digestion. This is evidenced by the observation that during the small intestinal digestion stage, almost no degradation fragments of the three species of Igs are detected (Figure 2D−F). IgA is the most abundant Ig in human milk serum, comprising 90% of the total Ig content.[18] IgG can provide immune protection throughout the body. Preterm infants receive lower levels of specific IgG compared to term infants, which increases their risk of pathogen infection during the first two months of postnatal life.[40] The relative concentrations of Igs in caprine and bovine milk were higher than those found in human milk (Figure S1), and intact caprine and bovine Igs were retained in the small intestine for a longer duration than human Igs (Figure 2D−F). This observation contrasts with the previous report[27] and may be attributed to variations in the digestion model employed. Additionally, it is important to consider the absorption rates of different species of Igs into the bloodstream. Consequently, further experiments are necessary to validate these findings.

Rat pups possess the ability to absorb LTF and Igs from the intestinal lumen into the bloodstream through two mechanisms: transport receptors and intestinal leakage. Specifically,

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ITLN1 receptors and FcRn receptors located in the small intestine facilitate the transport of LTF and IgG into the bloodstream, respectively. It is essential to consider both the expression levels and the receptor binding capabilities of these transport receptors. Notably, the relative expression of ITLN1 and FcRn receptors in small intestinal tissue did not exhibit significant changes shortly after ingestion (Figure 6A,B), suggesting that the mRNA expression levels of these protein transporter receptors do not account for the observed differences in LTF and Igs absorption levels. Furthermore, beyond the expression of protein transport receptors, their binding affinities warrant consideration. Human and rat FcRn receptors demonstrate structural similarities; specifically, human FcRn receptors can bind to human, rabbit, and guinea pig IgG, but not to bovine or sheep IgG,[41] which may elucidate why both caprine and bovine IgG are absorbed less efficiently than human IgG. There is a paucity of literature examining the binding affinity of caprine LTF to the rat ITLN1 receptor. Current studies indicate that both human LTF and bovine LTF can specifically bind to rat brush border membrane vesicles.[33] However, neither the intact form nor fragments of caprine or bovine LTF were detected in the blood of rat pups, whereas human LTF was identified. Consequently, the differential ability of LTF from various species to bind to the ITLN1 receptor in rat pups does not account for the observed differences in the uptake. Overall, due to the expression and affinity of the FcRn and ITLN1 receptors, the suckling rat serves as an excellent model for simulating the in vivo absorption of IgG and LTF in human preterm infants.

Intestinal leakage is another factor that warrants consideration. Moreover, due to the incomplete development of the small intestine in young rats, there was a lower relative content of tight junction proteins (Figure 6C), potentially leading to the leakage of macromolecular proteins from the intestinal lumen into the bloodstream. In a previous animal experiment comparing the absorption of human LTF in young mice and adult mice, the results indicated that the uptake of LTF by young mice was primarily due to the leakage of their intestines.[42] The permeability of the small intestine is regulated by cell junctions between adjacent epithelial cells and the apical tight junctions, whereby the intestinal transfer of macromolecules such as Igs and LTF across the intestinal epithelium occurs. However, both the apical and basolateral membranes of intestinal epithelial cells contain a significant number of endogenous proteases. For proteins in the intestinal lumen to be absorbed into the bloodstream in either a complete or partially degraded form, they must possess antidigestive enzyme activity to withstand degradation by these endogenous enzymes. Human LTF and its enzymatically degraded peptide fragments can both bind to the ITLN1 receptor, facilitating absorption into the bloodstream of rat pups. Degradation fragments of LTF (approximately 15 kDa) were identified in the jejunal tissues of rat pups from all three milk types (Figure 5A−C), with minimal detection in the gastrointestinal digesta (Figure 2A−C). This suggests that LTF from different milks can be absorbed and broken down by the jejunum of the rat pups. Only intact human LTF was found in the jejunal tissue, potentially because human LTF is more resistant to enzymatic breakdown compared to bovine and caprine LTF.[27] Although intact forms of Igs from the three species have been detected in the small intestine (Figure 5), the relative amount of caprine Igs absorbed into the bloodstream is lower than that of human Igs, yet higher than that of bovine Igs (Figure 4D), and this may be due to the fact that caprine milk Igs are less resistant to digestion than human milk Igs and stronger than bovine milk Igs. Therefore, we propose that the difference in Igs absorption between bovine and caprine milk is attributable to their varying resistance to digestion.

After LTF and Igs are absorbed into the blood, they remain stable for different periods of time. LTF can bind to hepatocytes and undergo degradation in lysosomes, leading to rapid metabolism in the blood,[33] which could explain why intact human LTF was detected in the blood at 20 min and then disappeared (Figure 3A). FcRn binds to IgG, increasing its stability and protecting it from lysosomal degradation.[43] Unlike LTF, the amount of Igs absorbed into the bloodstream did not decrease, likely due to IgG's longer half-life of approximately 10−22 days across different species.[44] IgG is critically important for premature infants due to its agglutination mechanism against pathogens. However, premature infants do not receive this immune protection to the same extent and are born with an IgG deficiency compared to full-term infants.[45] Consequently, it is essential to supplement premature infants with additional IgG. In these infants, the incomplete development of intestinal barrier function allows macromolecular proteins to enter the bloodstream from the intestinal lumen through tight junctions.[46] More caprine Igs were absorbed into the blood of rat pups compared with bovine Igs (Figure 4), which may mean that caprine Igs may provide more protection for premature infants.

In addition, we evaluated the absorptive properties of milk serum proteins from three species of rat pups. The amino acid profile in the blood after a meal can reflect the digestion and absorption of protein.[47] Blood concentrations of free amino acids initially increased and then decreased during digestion (Figure S5A), aligning with previous research.[48] Hydrolyzed amino acid levels were notably elevated for human and caprine milk serum after 1, 2, and 3 h (Figure S5B), suggesting a faster absorption rate of proteins in human and caprine milk serum into peptides and free amino acids compared to bovine milk serum proteins. Particularly, after 20 min, 1 h, and 2 h of digestion, the concentration of free amino acids in the blood of the human milk serum group surpassed that of the bovine and caprine milk serum groups (Figure S5A), indicating a more efficient absorption of human milk serum proteins by rat pups in a free amino acid form. Premature infants encounter several nutritional challenges due to their insufficient development time. Specifically, their protein requirements are higher than those of full-term infants to support rapid growth and development.[4] However, premature infants possess lower levels of gastric acid, reduced gastric enzyme activity, prolonged gastric emptying times, and impaired protein digestion capabilities.[49] Compared to bovine milk serum protein, the consumption of rapidly digested caprine milk serum protein results in a more rapid increase in postprandial circulating amino acids and peptides. Consequently, caprine milk serum protein may provide more accessible nutrition for premature infants, facilitating better digestion and absorption of nutrients.

For the first time, we used an in vivo digestion and absorption model using 14 day-old rat pups to compare the digestibility and absorbability of serum proteins derived from human, bovine, and caprine milk. However, there are some limitations of our study that warrant consideration. Our conclusions are solely based on serum proteins from bovine

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and caprine colostrum, necessitating further experimental validation of the digestibility and absorbability of serum proteins from bovine and caprine mature milk. Additionally, Western blot relies on specific antibodies to identify target proteins or protein fragments. If the target fragment in the digestion product is small or if the epitope is compromised, the antibody may not effectively recognize it.

In conclusion, an in vivo digestion model was developed using rat pups to compare the digestion and absorption characteristics of serum proteins in human, bovine, and caprine milk. The overall rate of absorption of free amino acids and peptides was found to be faster in human and caprine milk serum, with free amino acids being the main form of absorption for human milk serum protein and peptides being the primary form of absorption for caprine milk serum protein. More degradation fragments from human and caprine LTF were observed in the small intestinal digesta. Intact human LTF was detected in the blood of rat pups, and Igs from human, bovine, and caprine milk were also found to be absorbed into the blood. The relative amount of human Igs absorbed in young rats was significantly higher than those of bovine and caprine Igs, while caprine Igs had a slightly higher absorption rate than bovine Igs. The enhanced antidigestive properties of caprine Igs compared with bovine Igs were identified as the primary reason for the higher absorption rate of caprine Igs in rat pups.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c10539.

Primer sequence, SDS-PAGE of protein in human, bovine, and caprine milk, relative quantification of LTF and Igs-H in small intestinal digesta, SDS-PAGE of proteins in the gastric digesta of rat pups, SDS-PAGE of proteins in the small intestinal digesta of rat pups, and concentration of amino acids in plasma of rat pups (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

LTF. lactoferrin; Ig. immunoglobulin; IgG. immunoglobulin G; ITLN1. intelectin-1; FcRn. neonatal Fc receptor; SDS-PAGE. sodium dodecyl sulfate-polyacrylamide gel electrophoresis; α-LA. alpha-lactalbumin; β-LG. beta-lactoglobulin; Ig-H. immunoglobulin heavy chain; ALB. albumin

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