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Structural changes in milk from different species during gastric digestion in piglets

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ABSTRACT

This study investigated the structural and physicochemical changes that occur in milk, a naturally designed complex structured emulsion, during gastric digestion using the bottle-fed piglet as an animal model. The gastric digestions of cow, goat, and sheep milk were compared in male piglets euthanized at different postfeeding times to collect the stomach chyme. The cow and noncow milks separated into curd (aggregated caseins) and liquid (mostly soluble whey) phases in the piglet's stomach. For milk from all the species, the curd remained longer in the stomach because of its slow disintegration, whereas the liquid phase emptied readily. The majority of the fat globules were found to be entrapped within the protein network of the curd. The rate of release of fat globules was strongly dependent on the breakdown of the surrounding protein network of the curd. The consistency of the gastric curds changed as digestion progressed, with goat and sheep milk curds having relatively softer curd consistency and less fused protein networks, especially toward the end of digestion. This might have led to the lower protein and fat retention in the goat and sheep milk curds and relatively faster gastric emptying of these nutrients from goat and sheep milk in comparison to cow milk. This in vivo study provided new and enhanced understanding of the mechanisms of the gastric digestion of milk from different species. It may have implications for developing bioinspired structures for the controlled digestion and delivery of nutrients.

Key words: milk, in vivo piglet, protein coagulation, structure, gastric digestion and emptying

INTRODUCTION

Milk undergoes significant changes in its physicochemical and microstructural properties during gastric digestion (Mulet-Cabero et al., 2020a; Roy et al., 2020a; Ye et al., 2020). It has been suggested that such changes have an effect on the gastric emptying rates of different nutrients (such as proteins and fats) (Mulet-Cabero et al., 2020a; Huppertz and Chia, 2021; Ye, 2021), which may influence their subsequent rates of absorption in the small intestine (Montoya et al., 2018). Our previous in vitro studies on understanding the dynamic gastric digestion of the milk from different species (cow, goat, and sheep) have shown that all milks separate into a semi-solid curd (caseins) and a liquid (soluble nutrients such as whey) in a human gastric simulator. The curd formed entraps a significant amount of fat globules, which are gradually released by the breakdown and hydrolysis of the curd structure (protein network) by pepsin and mechanical shearing during gastric digestion, thus influencing the release of proteins and fats (Roy et al., 2021a,b). Some in vivo studies have reported solid curd formation from raw cow or pig milk in piglets (Cranwell et al., 1976; Decuypere et al., 1978; Blakeborough et al., 1986), minipigs (Meisel and Hagemeister, 1984), and rats (Ye et al., 2019). Such structural changes observed during in vitro and in vivo studies are expected to influence the rates of nutrient delivery to the small intestine, and have also led to the widely accepted concept of slow (i.e., caseins) and fast (i.e., whey proteins) emptying milk proteins with relevance to human digestion (Mahe et al., 1996; Boirie et al., 1997; Boutrou et al., 2013).

There is a lack of characterization and understanding of the structural changes that occur in the milk matrix during dynamic gastric digestion in vivo. Only a little information on curd formation in cow milk in vivo is available (Braude et al., 1970; Ye et al., 2019), but there is no in vivo information on curd formation in noncow milks such as goat milk and sheep milk, and its effect on nutrient retention in the stomach. As the

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milk from cow, goat, and sheep vary in physicochemical properties such as casein micelle and fat globule size (Claeys et al., 2014; Roy et al., 2020a), we hypothesize that their curd formation characteristics would also be different, which may influence their stomach emptying rates. Thus, the objective of this study was to investigate the mechanisms of the dynamic gastric digestions of the raw whole milk from 3 different species (cow, goat, and sheep), primarily focusing on their dynamic coagulation behaviors in the stomach. The study also determined the effect of milk coagulation on gastric emptying under physiological conditions using the bottle-fed (suckled) piglet as an animal model (Moughan and Rowan, 1989; Moughan et al., 1992, 1994). Piglets that are exclusively milk-fed using bottles with rubber teats have been previously used to study the digestion of human milk and infant formula (Moughan et al., 1991; Darragh and Moughan, 1998; Rutherfurd et al., 2006a). The piglet was chosen as a model because the physiology and metabolism of its gastrointestinal tract is similar to that of the human infant (Moughan et al., 1992).

MATERIALS AND METHODS

Diets

Spray-dried cow, goat, and sheep whole milk powders were purchased from Davis Food Ingredients, Dairy Goat Co-operative, and Spring Sheep Milk Co., respectively. A vitamin and mineral premix that was formulated for piglets was procured from Nutritech International Ltd. Raw whole milk from cow, goat, and sheep was procured from the Massey University no. 4 dairy farm, Dairy Goat Co-operative and Phoenix Goats, and Neer Enterprises Ltd., respectively.

Animal Study

All procedures involving animals were approved by the Massey University Animal Ethics Committee (MUAEC protocol 18/97). The researchers and technicians involved in the study were aware of all the stages of the experiment starting from group allocation to data analysis. Any data related to the study are available from the researchers involved in this study.

Animals, Housing, and Experimental Set-Up

A pictorial overview of the procedure adopted for the animal trial is given in Figure 1. A total of 66 Large White × (Landrace × Large White) entire male piglets [7–8 d of age; mean BW on arrival 3 kg (range 1.9–4.2 kg)] were obtained from a local commercial farm (Aorere Farms Partnership, Whanganui, New Zealand). The piglets were ear tagged, weighed, and housed individually in purpose-built plastic metabolism crates. The piglets were provided with clean toys that were changed daily. Once a day, the piglets were also put together for 1 h to play with each other under supervision to provide social contact. Technicians and researchers interacted with all the piglets several times daily. The animals were kept in a temperature-controlled room maintained at 28 ± 2°C with a 16 h:8 h light:dark cycle from the day of arrival (d 1) at the Massey University Animal Physiology Unit (Palmerston North, New Zealand). The piglets were allocated at random to the 3 dietary treatments (cow, goat, and sheep milk) on d 1 such that there were 22 piglets per milk type (or milk species), with 2 piglets per treatment considered "spare" in case any did not acclimatize to the experimental conditions. Throughout the animal study, the piglets were monitored for their health, changes in BW, body temperature (~38–40°C), diet intake, meal refusals, as well as for any adverse signs such as dehydration (concentrated urine, skin tenting, and constipation) and scouring. Defecation frequency and fecal consistency was also monitored and recorded daily.

Feeding Frequency

The piglets were weighed on arrival and then every 2 d, and their daily ration was adjusted to ensure an intake of 345 g of reconstituted whole milk powder diet (liquid) per kilogram of BW per day (Darragh and Moughan, 1995; Rutherfurd et al., 2006b). For the first 6 d of the study (acclimatization period), the piglets were trained to drink from a sterilized bottle with a rubber teat and were fed their daily ration across 17 meals at 1-h intervals from 0600 to 2200 h. The milk diet was prewarmed to ~37°C before feeding to the piglets. The aim was to have the piglets consuming >80% of their daily ration (Darragh and Moughan, 1995) before the commencement of the experimental period, which started on d 7. Intensive daily monitoring and interaction with the piglets was undertaken to acclimatize the piglets to human interaction, bottle-feeding, dietary treatments, and daily intake. Any piglet that did not acclimatize by d 6 was withdrawn from the study. From d 6, 20 piglets per milk treatment continued in the study, with the remaining spare piglets rehomed. The use of soft rubber teats ensured that the piglets suckled their milk, thus preserving the suckling reflex. From d 7 onward, the amount of food received by the piglets at each meal was gradually increased by decreasing the frequency of feeding to reach the target meal intake on

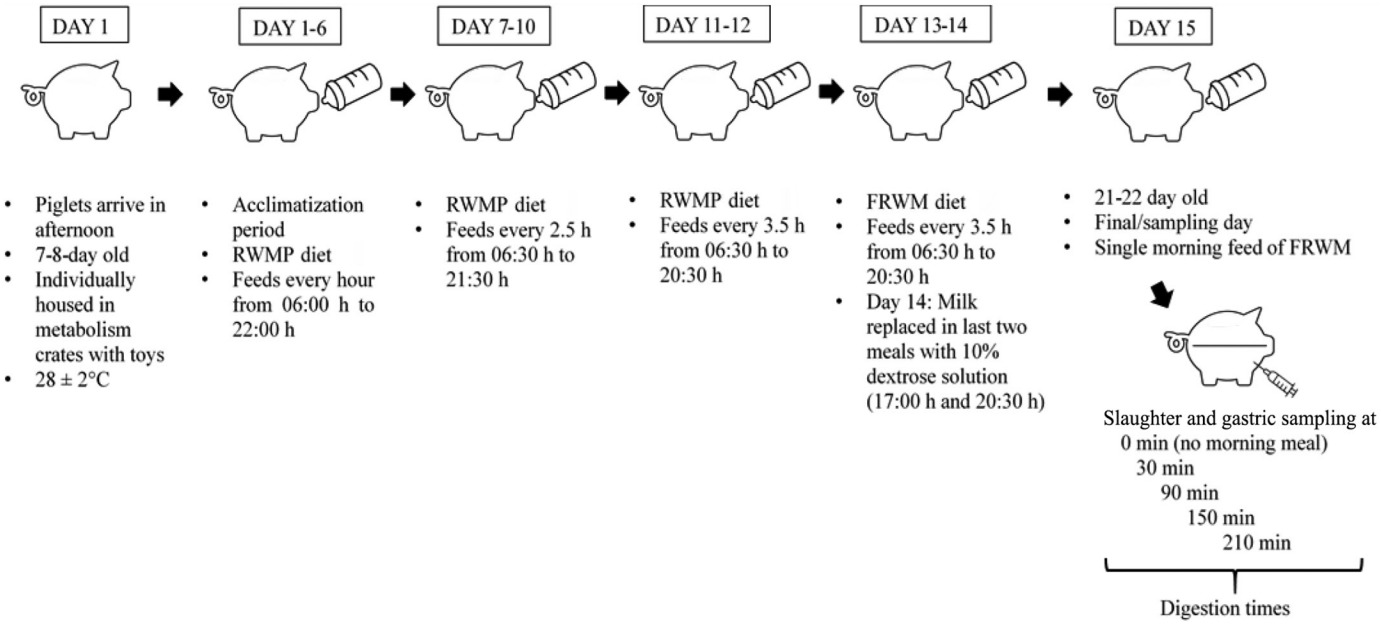

Figure 1. The in vivo piglet study. RWMP = reconstituted whole milk powder diet (cow, goat, and sheep milk-fed groups) with vitamin and mineral premix; FRWM = fresh raw whole milk diet (cow, goat, and sheep milk-fed groups) only.

the last experimental day (d 15). Thus, from d 7 to 10, the daily ration was given as 7 meals at 2.5-h intervals from 0630 to 2130 h, and, from d 11 to 14, the daily ration was given as 5 meals at 3.5-h intervals from 0630 to 2030 h.

Diet Schedule

The piglets were fed the reconstituted whole milk powder diet (including the vitamin and mineral premix supplement) from d 1 to 12. From d 13 onward, the piglets received their respective fresh raw whole milk diet to start adapting them to any changes in taste when shifting from a reconstituted whole milk powder diet to a fresh raw whole milk diet. Fresh raw milk was not fed to the piglets from the start of the study because of the limited supply of fresh raw goat and sheep milk.

During the first 7 d of feeding, milk powder was reconstituted to provide iso-caloric and iso-volumetric amounts of each diet to the piglets on a BW basis (345 g of liquid meal per kilogram of BW per day). Thereafter, the reconstituted milk given to the piglets was balanced for protein, as protein is considered to be the main milk component that influences curd formation in the stomach (Roy et al., 2021b). Thus, from d 8 to 12 (reconstituted milk), the piglets received equal amounts of protein (2 g of protein per kilogram of BW in each single meal) and equal volumes of diet (345 g of liquid meal per kilogram of BW per day).

From d 13 to 15 (fresh raw milk), the piglets received their respective milk volumes based on equal amounts of protein per kilogram of BW, as for the previous days. On d 14, the piglets were fed their fresh whole milk for the first 3 meals of the day (i.e., at 0630, 1000, and 1330 h). During the last 2 meals (i.e., at 1700 and 2030 h), the piglets were fed a 10% dextrose solution that was made using cooled boiled water (equivalent energy in relation to the previous 3 meals). This was done to ensure a minimum 18-h gap between the last milk meal on the presampling day (d 14) and the milk meal on the sampling day (d 15). It was expected that this gap would minimize the presence of milk components remaining from the presampling day(s) in the stomachs of the piglets. On d 15, the piglets received one meal of fresh raw milk in the morning (2 g of protein per kilogram of BW, Table 1) before being killed at set times postfeeding. Considering the sampling time required for

Table 1. Amounts (g/kg of BW per piglet) of cow, goat, and sheep raw whole milk diet ingested in the last meal on the sampling day (15) before slaughter

Intake	Cow	Goat	Sheep
Fresh milk	55.3	63.1	31.9
Protein	2.0	2.0	2.0
Fat	2.2	2.0	2.0
Lactose	2.5	2.5	1.3
DM	7.2	7.1	5.6
Gross energy (kcal/kg of BW)	41.9	38.6	34.3

each piglet (~15 min), the piglets received their last meal at 30-min intervals to ensure that they were killed at the assigned postprandial time. For each milk type, 4 piglets did not receive any milk on d 15 (i.e., 0 time) to determine if any food from the meals on the previous day was retained in the stomach.

Euthanasia

The time periods on the sampling day were calculated from the time the piglet finished suckling the milk (the piglets ingested their milk within 2–3 min) until the time of euthanasia as follows: at time 0 (piglets fed no milk meal) and at 30, 90, 150, and 210 min after completion of the fresh raw milk meal [n = 4 per species (or milk type) and time combination]. The piglets were anesthetized with Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Zoetis Inc.) reconstituted with 2.5 mL of ketamine and 2.5 mL xylazine, both 100 mg/mL. The final solution contained 50 mg/mL of each drug. The solution was administered at a dose rate of 0.4 mL of the mixed solution/10 kg of BW by intramuscular injection. Once anesthetized, the piglets were killed by an intracardiac injection of a lethal dose (0.3 mL/kg of BW) of pentobarbitone (Pentobarb 300, Provet NZ Pty Limited).

Sample Collection

Following euthanasia, the abdomen was opened, and the stomach was immediately secured with clamps at the esophagus and the pyloric sphincter. The stomach was removed, any blood was rinsed off with deionized water, and the stomach was dried using absorbent paper towels. The stomach was dissected laterally from the esophageal end to the duodenal end, with a single incision through the middle of the superior face of the stomach, and the gastric contents were removed and collected. The stomach was weighed full and again empty to determine the total amount of contents at each time point (0, 30, 90, 150, and 210 min). This provided data on the wet weight of the total stomach contents at each time point. The curd and the liquid phases of the chyme were then collected separately, and their pH was measured. The liquid phase of the gastric contents was collected after being sieved through a 1-mm sieve and the weight of the liquid part was recorded. A similar method had been used previously by Meisel and Hagemeister (1984) during a study on the effect of different commercial processing of cow milk in minipigs. The weight of the wet curd was determined by deducting the weight of the liquid from the weight of the total stomach contents. Visual images of the curd were taken. The total time between slaughter and completion of the sampling of the stomach contents for each piglet was ~15 min. Samples for immediate analysis (i.e., rheology and microscopy) were stored on ice and analyzed or processed within 2 h of euthanasia. Microscopic analysis of gastric chyme samples from a maximum of 2 piglets for each time point per treatment was conducted due to the limited time available for fresh sample processing. The remaining samples of curd and liquid phases were kept on dry ice during the collection process and then stored at −20°C before being freeze-dried. The freeze-dried samples were weighed, ground, and stored at −20°C.

Analysis

Chemical Analysis of Milk and Stomach Chyme. The chemical composition [DM, fat, protein (total nitrogen × 6.38), and ash] of each milk and gastric chyme sample was determined following AOAC International (2005) protocols. Total DM (or moisture), fat, protein (total nitrogen × 6.38), and ash were analyzed using the air oven-drying method 990.19 (AOAC International, 2005), Mojonnier method 989.05 (AOAC International, 2005), Dumas method 968.06 (AOAC International, 2005), and gravimetric method 945.46 (AOAC International, 2005), respectively. The lactose content of the milk was determined using a spectrophotometric enzymatic kit (catalog no. 10176303035) from R-Biopharm AG. The gross energy content of the milk was measured using a Leco AC500 bomb calorimeter (Leco Corporation). The total calcium and inorganic phosphorus contents of the milk samples were analyzed on an RX Daytona Plus analyzer (Randox Laboratories) using Randox reagents CA 8309 and PH 8328, respectively (Roy et al., 2021a).

Fat Globule Size Distribution. The size of the milk fat globules was measured using a static light scattering technique, on a Malvern MasterSizer 2000 Hydro MU (Malvern Instruments Ltd.) with 2 laser sources, as described by Roy et al. (2021a). The refractive indexes used were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.33 for water. The absorption coefficient at both wavelengths was considered to be 0.0001 (Michalski et al., 2001; Ménard et al., 2010). After initial trial and error, the milk samples were diluted approximately 4 times, with an EDTA-SDS buffer solution containing 2% SDS [≥99.0% (GC), Sigma Aldrich Co. LLC] and 50 m*M* EDTA, pH 6.7, to dissociate the casein micelles and to disrupt any flocs of fat globules before the measurements (Ye et al., 2002). A small amount of sample was added to the measurement cell (with around 800 mL of water) to reach around 10%

obscuration. A general-purpose (spherical) analysis model was used and a pump speed of 2,000 rpm was maintained.

Casein Micelle Size. The mean hydrodynamic diameter (nm; i.e., the Z-average diameter, an intensity-based calculated value) of the casein micelles was measured by a dynamic light scattering technique using a Zetasizer Nano ZS (Malvern Instruments Ltd.), as given in the protocol described by Roy et al. (2021a). The raw skim milk samples (obtained by centrifuging whole milk at 3,500 × *g* for 20 min) were diluted 100 times with a calcium imidazole buffer solution (pH 6.7) containing 20 m*M* imidazole, 5 m*M* $CaCl_2$, and 30 m*M* NaCl (Anema, 1997; Anema and Li, 2003a,b). The diluted milk samples were filtered using a 0.45-μm syringe filter to remove large particles just before analysis. The samples were equilibrated for about 120 s and then measured at 25°C in a particle sizing cell using back scattering technology at a detection angle of 173°. A general purpose (normal resolution) analysis model was used. The calcium imidazole buffer was used as a common diluent for easier comparative analysis of all skim milk samples.

pH. The pH of the milk samples and of the gastric contents (curd and liquid) at each digestion time point were measured using a CyberScan pH 510 pH/mV/°C meter (Eutech Instruments). All the pH values given in this paper correspond to the pH of the liquid phase, unless specified otherwise.

Confocal Laser Scanning Microscopy. High-resolution imaging of the microstructure of the proteins and fat globules in whole milk, curd and liquid samples was performed using a Leica SP5 upright confocal microscope (Leica Lasertechnik GmbH) as per the protocol described by Roy et al. (2021a). A 1.0% (wt/vol) solution of Fast Green (dye content 90%) in water was used to stain protein and a 0.1% (wt/vol) solution of Nile Red in acetone was used to stain fat (He–Ne laser with an excitation line at 633 nm). Curd samples collected at different time points were immediately cut into thin sections using a surgical blade. The thinly sliced curd samples and the liquid samples were then stained with a 1:1 mixture of both dyes for about 10 min. Just before staining, the mixture of dyes was centrifuged at 12,641 × *g* (Microcentrifuge MiniSpin plus, Eppendorf AG) and filtered through a 0.22-μm filter (13 mm, PVDF, Thermo Fisher Scientific) to remove any crystals formed in the dye mixture. The stained samples were then placed on double concave microscope slides (clear glass, ground edges, 26 mm × 76 mm, 1.2–1.3 mm thick; Sail, Sailing Medical-Lab Industries Co. Ltd.), covered with coverslips, and examined with a 63× oil immersion objective (numerical aperture = 1.4). Care was taken to avoid areas near the sides or close to the top of the microscope slides while imaging. Multiple fields were viewed and typical micrographs are presented.

Transmission Electron Microscopy. The liquid samples (5–50 μL) were injected into agarose tubes (5–7 mm in length, prepared from 3% low-temperature-gelling agarose) using an auto-pipette, and the ends were sealed with agarose to form an enclosed capsule, whereas the curd samples were trimmed to the correct size and shape. Both the liquid samples and the curd samples were then processed as described by Gallier et al. (2013). The samples were placed into a 3.0% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.2) for at least 24 h at 4°C and were then washed 3 times for 45 min with the same buffer. The agarose-embedded samples were then fixed in 1.0% osmium tetroxide and placed in 0.1 *M* sodium cacodylate buffer for 1 h at room temperature, overnight at 4°C, and for another hour at room temperature. The samples were then washed in buffer again, as mentioned above. The dehydration process was then carried out through a graded acetone series (25, 50, 75, 95, 100, 100, and 100%; 30 min for each series). The samples were then put into 50:50 resin:acetone and placed on a stirrer overnight at 4°C. This was replaced by fresh 100% resin (Procure 812, ProSciTech) and the samples were stirred for 8 h at 4°C. This step was repeated 4 more times. The samples were then embedded in molds with fresh resin and cured in a 60°C oven for 48 h, after which the block was trimmed down to the selected area and cut using a diamond knife (Diatome Ltd.) at 100 nm. These samples were stretched with chloroform and mounted on grids using a Coat-Quick "G" glue pen (Daido Sangyo Co. Ltd.). The grids were then stained in saturated uranyl acetate in 50% ethanol for 4 min, followed by washing with 50% ethanol and Milli-Q water and then stained in lead citrate for a further 4 min. This was followed by a wash in Milli-Q water. The stained specimens were then examined under a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Company) equipped with an Olympus SIS VELETA camera.

Rheological Analysis. An AR-G2 magnetic bearing rheometer (TA Instruments) fitted with a 40-mm diameter parallel steel plate geometry was used; a procedure similar to that reported by Mulet-Cabero et al. (2019) was followed. Gastric curd (~5 g) was placed in the rheometer geometry and the sample was pressed to the system's default gap of 2,000 μm. After pressing, ~2 g of the sample had moved out of the geometry; it was removed and the analysis was then conducted on the remaining ~3 g of sample. Time sweep tests were

performed on the gastric curds at a frequency of 1 Hz and a strain of 0.5 at 37°C. The complex modulus (G*, Pa = oscillation stress/strain) values obtained after 15 min of measurement are reported.

Protein Hydrolysis Profile by Tricine-SDS-PAGE. Reducing tricine-SDS-PAGE gels were prepared for freeze-dried milk samples and for freeze-dried gastric curd and liquid samples from each piglet. Finely ground freeze-dried powder (15 mg) was mixed with 1 mL of tricine sample buffer [0.2 *M* Tris-HCl buffer, pH 6.8; 40% glycerol, 2% SDS, 0.04% Coomassie brilliant blue G-250, β-mercaptoethanol (0.05%, vol:vol)], heated in a boiling water bath for 5 min, cooled to room temperature, and then centrifuged at 6,418 × *g* for 3 min. A small volume of this mixture was immediately loaded on to a 16.5% Criterion Tris-tricine gel (Ref 3450064, Bio-Rad) to achieve a protein concentration of 20 μg in each well. The gels were run at a constant voltage of 150 mV for 85 min using a Criterion cell (Bio-Rad Laboratories Pty Ltd.), after which they were removed, stained, destained, and scanned as described by Ye et al. (2016a).

Statistical Analysis. Statistical analyses were in general performed using the MIXED Model procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc.).

No previous in vivo study in piglets have been reported to quantify the gastric digestion characteristics of different milks. Thus, reported information on infant formula fed to piglets was used to calculate the sample size. Based on the nitrogen retained [e.g., 79.0, 39.2, and 10.1% at 30, 90, and 210 min postfeeding, respectively, with a pooled standard deviation of 13.5% (Bouzerzour et al., 2012)] and the viscosity of the clots formed [e.g., 0.16 and 0.08 Pa·s at 60 and 120 min, respectively, with a pooled standard deviation of 0.032 (Tari et al., 2018)] over time in the stomach of piglets fed either a cow milk or whey protein–based formula, a power higher than 0.836 is reached with 3 to 4 animals. Thus, based on the power analysis, 4 replicates (i.e., piglets) at each time point per milk diet were used.

The chemical composition (DM, CP, crude fat, ash, lactose, calcium, phosphorus, protein-to-fat ratio, and gross energy) of the 3 batches of each milk type from different species used during the piglet study were compared using a one-way ANOVA.

Gastric emptying rates were calculated after making a correction for the amounts of DM and chemical components present in the stomach from previous meals (time 0); animals with excess amounts of diet in the stomach (outliers) were removed before conducting any statistical analysis. The relative retentions (DM, CP, and crude fat) postfeeding were determined according to a power exponential model:

$$\text{Relative retention or amount retained}_{Time} = \alpha_0 \exp - (\kappa \times \text{time})^{\beta},$$

where α_0 is the proportion remaining at time 0 (100% for relative retention). The parameters κ (slope of the curve), β (index for the shape of the curve; results not shown as they did not differ across the milk types), and $T_{1/2}$ (half gastric emptying time) were estimated using the Proc NLIN procedure of SAS. The parameters κ and β were used to determine $T_{1/2}$ $\{T_{1/2}\ (\text{min}) = (1/\kappa) \times [\log(1/0.5)]^{(1/\beta)}\}$. For all analyzed response variables, fitted curves of the reduced (i.e., no difference between species) or full nonlinear model were compared using the *F*-test. For all variables, the full nonlinear model better described the responses, unless otherwise specified.

For the pH and the rheological properties, models containing species, time (30–210 min as either a categorical or a numerical variable), and species-by-time interaction as fixed effects were used. When the interaction effect was not statistically significant, it was removed from the model.

The model diagnostics for each response variable were tested after combining the PROC UNIVARIATE and the ODS GRAPHICS procedures of SAS before comparing the means. When a response variable did not fulfill the model assumptions of normality and homoscedasticity, a transformation of the raw data was conducted. When the *F*-value of the model was significant ($P < 0.05$), the means were compared using the adjusted Tukey's test. The results are reported as mean ± standard error of the mean.

RESULTS AND DISCUSSION

Physicochemical Characteristics (Chemical Composition, Fat Globule Diameter, and Casein Micelle Diameter) of the Different Milks

The comparative chemical compositions (per 100 g) of the fresh cow, goat, and sheep whole milk are reported in Table 2. Sheep milk contained significantly higher ($P < 0.05$) levels of TS (DM), protein, fat, and minerals (calcium and phosphorus) than both goat milk and cow milk. Goat milk had significantly lower levels ($P < 0.05$) of TS, protein, fat, and calcium than cow milk. The phosphorus content of goat milk was similar to that of cow milk. There were no statistically significant differences in the ash content and the protein-to-fat ratio of the milk across species. The lactose content of cow milk was the highest followed by sheep milk and then goat milk. The volume-weighted ($\boldsymbol{d_{43}}$) and surface-weighted ($\boldsymbol{d_{32}}$) mean diameters of the fat globules in the

Table 2. Chemical compositions of cow, goat, and sheep raw whole milk (g/100 g of milk)[1]

Component	Cow	Goat	Sheep
DM	13.1 ± 0.13[b]	11.23 ± 0.13[c]	17.60 ± 0.13[a]
Protein	3.62 ± 0.05[b]	3.17 ± 0.05[c]	6.27 ± 0.05[a]
Fat	4.06 ± 0.11[b]	3.23 ± 0.11[c]	6.31 ± 0.11[a]
Lactose	4.56 ± 0.04[a]	3.92 ± 0.04[c]	4.17 ± 0.04[b]
Ash	0.77 ± 0.02	0.80 ± 0.01	0.86 ± 0.08
Protein-to-fat ratio	0.89 ± 0.02	0.98 ± 0.02	0.99 ± 0.02
Calcium[2]	125.68 ± 3.4[b]	112.40 ± 3.4[c]	188.16 ± 3.4[a]
Inorganic phosphorus[2]	88.13 ± 0.39[b]	80.84 ± 3.56[b]	139.21 ± 6.39[a]
Gross energy (kcal/100 g of milk)	75.81 ± 0.74[b]	61.11 ± 0.74[c]	107.50 ± 0.74[a]

[a–c]Values within a row with different superscripts are significantly different ($P < 0.05$).
[1]Values are reported as mean ± SEM, n = 3.
[2]Milligrams per 100 mL of milk.

different whole milk samples are reported in Table 3. The d_{43} of the fat globules in goat milk (~3.6 μm) was smaller than those in cow milk (~4.4 μm) and sheep milk (~4.3 μm). Similarly, the d_{32} of the fat globules in goat milk (~2.9 μm) was smaller than those in cow milk (~3.8 μm) and sheep milk (~3.5 μm). The Z-average diameter (nm) of the casein micelles (Table 3) in cow milk was significantly ($P < 0.05$) lower (~158 nm) than those in sheep milk (~180 nm) and goat milk (~190 nm). The natural physicochemical compositions of the cow, goat, and sheep whole milk differed considerably from each other, and the trends observed in this study were similar to those reported by others (Barlowska et al., 2011; Claeys et al., 2014; Roy et al., 2020b).

Coagulation of Milk in the Piglet Stomach

Representative photographs of the piglet stomach and the gastric contents (chyme) collected are shown in Figure 2. The gastric chyme consisted of a curd and a liquid phase; the liquid was removed by sieving the gastric chyme through a 1-mm sieve, as shown in Figure 2A. The quantities of liquid and curd differed at different digestion times. The stomach appeared to be full at 30 min and to be nearly empty at 210 min (Figure 2B). The milk from all species formed a curd within 30 min of feeding, at which time the pH was ~5.9 (for both the separated gastric curd and the liquid, Figure 2B). The curds formed at 30 min appeared to be fragile and soft. With an increase in the digestion time, accompanied by a decrease in the gastric pH (to ~3.0 at 210 min of digestion), the curds became more compact, tighter, and smaller in size. This was a similar trend for the curds from the milk of all species when observed visually. The curds remaining in the piglet's stomach during the later stages of digestion appeared to be slimy and yellowish in color, which could have been due to coating of the curd by gastric mucus and bile reflux, respectively. Some previous studies have also reported the presence of bile pigments in the stomach contents because of reflux of the intestinal contents during milk digestion in pigs or piglets (Braude et al., 1970; Cranwell, 1985).

The pH of the gastric contents decreased during digestion (Figure 2B); however, the pH of the curd remained relatively higher than that of the liquid phase, especially after 30 min of digestion. The higher pH of the curd could have been due to the hindered diffusion of gastric acid into the curd (Roy et al., 2021a,b) and to a relatively high concentration of phosphate or hydrogen phosphate ions in the casein-rich curd fraction (Salaün et al., 2005). The gradual decrease in the gastric pH of the milk-fed piglets (as observed in our study) was similar to that reported by Moughan et al. (1991), who also noted a gradual decrease in the gastric pH of bottle-fed piglets (gastric-cannulated) of similar age to a pH of ~2.5 after 240 min of ingestion

Table 3. Average diameters of fat globules and casein micelles in cow, goat, and sheep milk[1]

Property	Cow	Goat	Sheep
Fat globule size			
d_{43} (μm)	4.38 ± 0.06[a]	3.61 ± 0.02[b]	4.29 ± 0.03[a]
d_{32} (μm)	3.77 ± 0.06[a]	2.86 ± 0.02[c]	3.49 ± 0.02[b]
Casein micelle size			
Z-average diameter (nm)	158.41 ± 1.68[c]	189.98 ± 3.5[a]	179.55 ± 0.73[b]

[a–c]Values within a row with different superscripts are significantly different ($P < 0.05$).
[1]Values are reported as mean ± SEM, n = 3. d_{43} and d_{32} = volume-weighted and surface-weighted mean diameter, respectively.

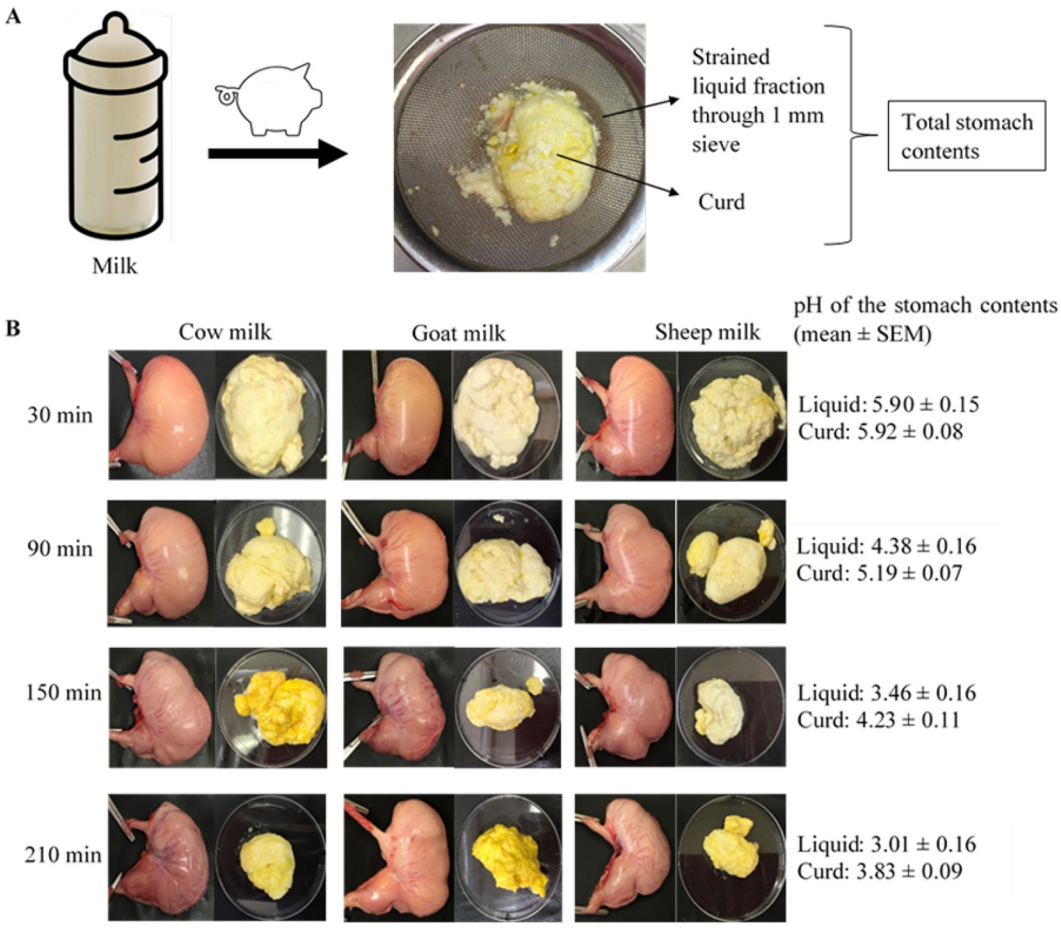

Figure 2. Photographs of the gastric chyme collected from the piglet's stomach: (A) an example of the stomach contents; (B) piglet's stomach (before dissection) and the curds obtained at different digestion time points along with the pH of the stomach contents. Only a representative photograph from one piglet at each time point is shown. For changes in the pH of the stomach contents (curd and liquid phases), n = 2–4: the species × time interaction was not significant ($P > 0.05$); the effect of species was not significant ($P > 0.05$); the effect of time was significant ($P < 0.05$).

of a cow-milk-based formula. The trends in the gastric pH observed in our in vivo study were similar to those reported by Mason (1962) in human infants fed breast milk. Mason (1962) reported a mean pH of 6.4 at 30 min postfeeding, which gradually decreased to 3.2 at 210 min in infants who were feeding on mother's breast milk. The extent of the pH decrease was probably dependent on the composition and quantity of the diet and the frequency of feeding as well as the acid secretion capacity of the stomach. It has been reported that, along with hydrochloric acid, the presence of lactic acid (because of the gastric fermentation of lactose) may also contribute to the acidity of the stomach contents of piglets (Cranwell et al., 1976).

The formation of a curd during the gastric digestions of the raw whole milk from the different species (Figure 2) is due to aggregation of the casein micelles (Ye et al., 2016a, 2017, 2019; Roy et al., 2021a); the whey proteins remain soluble in the liquid part of the gastric contents (Roy et al., 2021a,b). The 3-wk-old suckling piglet is considered to have significant chymosin activity (along with some pepsin activity; Moughan et al., 1992). It has been reported that during the first few hours after birth, some human infants may have a chymosin-like enzyme (which is not pepsin) along with pepsin (Henschel et al., 1987), but this is considered to disappear from the gastric fluid within 10 d of birth and is not found in adult gastric fluid (Dallas et al., 2012). As the mechanisms of action of chymosin and pepsin are similar in relation to milk clotting (Guinee and Wilkinson, 1992; Moschopoulou, 2011; Leite Júnior et al., 2015), the formation of the casein curd at around pH 6.0 (as observed in this study) is due to the specific cleavage of the Phe105–Met106 peptide bond of κ-casein by both chymosin and pepsin, resulting in destabilization and aggregation of the casein micelles (Jollès, 1966; Tam

and Whitaker, 1972). In addition, both chymosin and pepsin have some activity in the pH range 5.5 to 6.3 (Piper and Fenton, 1965; Crabbe, 2004), which indicates that, although complete phase separation of the milk was observed in the stomach of the piglets at 30 min (pH ~5.9), the process of milk coagulation might have been initiated much earlier.

The gastric coagulation of casein has also been previously reported in casein-dominant infant formula during digestion in piglets (Tari et al., 2018). Braude et al. (1970) observed curd formation in the stomach contents of 28-d-old pigs after 15 min of feeding homogenized cow milk, and complete separation of the curds and the soluble or whey fraction at 30 min postfeeding. Cranwell et al. (1976), while studying the gastric secretions and the fermentation of pig milk in 1- to 33-d-old piglets, reported no physical changes to the pig milk when the stomach contents were collected at 2 to 3 min postsuckling. However, soft and gelatinous curds were observed at 5 min postsuckling. Cranwell et al. (1976) also reported that the curds became granular in appearance, although still remaining soft during the subsequent 30 min of digestion. They further reported that, during the next 30 min (i.e., ~60 min in total), the pig milk curds became drier and crumbled. Some similar changes were observed in the present study for the cow, goat, and sheep whole milk curds.

The dynamic changes in the milk from the 3 species in the stomach of the piglets (in terms of curd formation, compaction and disintegration, Figure 2) were due to a combination of factors, such as gastric shearing (contraction) action, proteolytic enzyme (chymosin and pepsin) activity, and the acidic environment of the stomach (continuous secretion of hydrochloric acid into the stomach, reducing the pH of the chyme).

Characteristics of Stomach Chyme (Curd and Liquid Phases)

Confocal Laser Scanning Microscopy. The microstructures of the gastric curds and the liquid phase obtained from the milk of the 3 species at 30, 90, 150, and 210 min, as observed using confocal laser scanning microscopy, are depicted in Figures 3 and 4. All milk samples (cow, goat, and sheep) showed a compact curd protein matrix (in green), with a significant proportion of the fat globules (in red) entrapped (or embedded) within the curd protein matrix. Spherical, coalesced, and nonspherical fat globules (larger in size than those present in the fresh milks) were observed in the curds at all digestion time points (Figure 3). But the degree of fat globule coalescence in the curd appeared to be higher at longer digestion times (i.e., at 210 min compared with 30 min). The changes in the protein network of the curd with digestion time were not obvious possibly because the curd protein network was dense and cohesive. However, these differences were more obvious at an ultrastructural scale using transmission electron microscopy (**TEM**; explained in the next section).

Compared with the fat globules in the gastric curds, the fat globules in the liquid phase appeared to maintain their spherical shape and showed a low degree of coalescence (Figure 4). In addition, some small casein aggregates were also observed in the liquid phase obtained from all species. The extensive coalescence of the fat globules and the nonglobular forms of fat observed within the curds were not present in the liquid phase, although there was an increase in the proportion of larger fat globules in the liquid phase of the chyme with increasing digestion time (Figure 4).

The observed coalescence of the fat globules may potentially have been due to the proteolytic and lipolytic actions of pepsin (and chymosin) and gastric lipase in the piglet stomach (pH 3–6), respectively. Pepsin can hydrolyze some of the milk fat globule membrane (**MFGM**) proteins to peptides, which, being surface active, remain at the interface, leading to modification of the interfacial protein composition and making the interfacial structure weaker (Ye et al., 2011; Gallier et al., 2012). Because of this alteration to the structure of the MFGM, gastric lipase can access the triglyceride core of the milk fat globules and the products (such as diglycerides and free fatty acids) generated from partial lipolysis of the triglycerides can accumulate at the interface or even replace some of the interfacial phospholipids (Gallier et al., 2013). These changes in the milk fat globule structure that are induced by interfacial protein hydrolysis and gastric lipolysis decrease the interfacial stability and thus reduce the repulsive forces between the fat globules, making them more susceptible to coalescence under the shearing action of the stomach (Singh and Gallier, 2017; Gallier and Singh, 2020). The presence of a dense protein matrix around the fat globules and the high entrapped fat phase volume concentration in the gastric curd may have increased the frequency of contacts between the fat globule surfaces, leading to breakdown of the native or altered (stabilized by peptides, diglycerides, or phospholipids) membrane and thus enhancing coalescence (Roy et al., 2021a). The liquid fraction is readily emptied from the stomach as well as diluted with gastric secretions, rendering it less prone to the shearing action of the stomach and the action of the gastric enzymes (compared with the fat globules entrapped within the curd). Gallier et al. (2013) also reported an increase in size of the fat globules in raw (nonhomogenized) cream

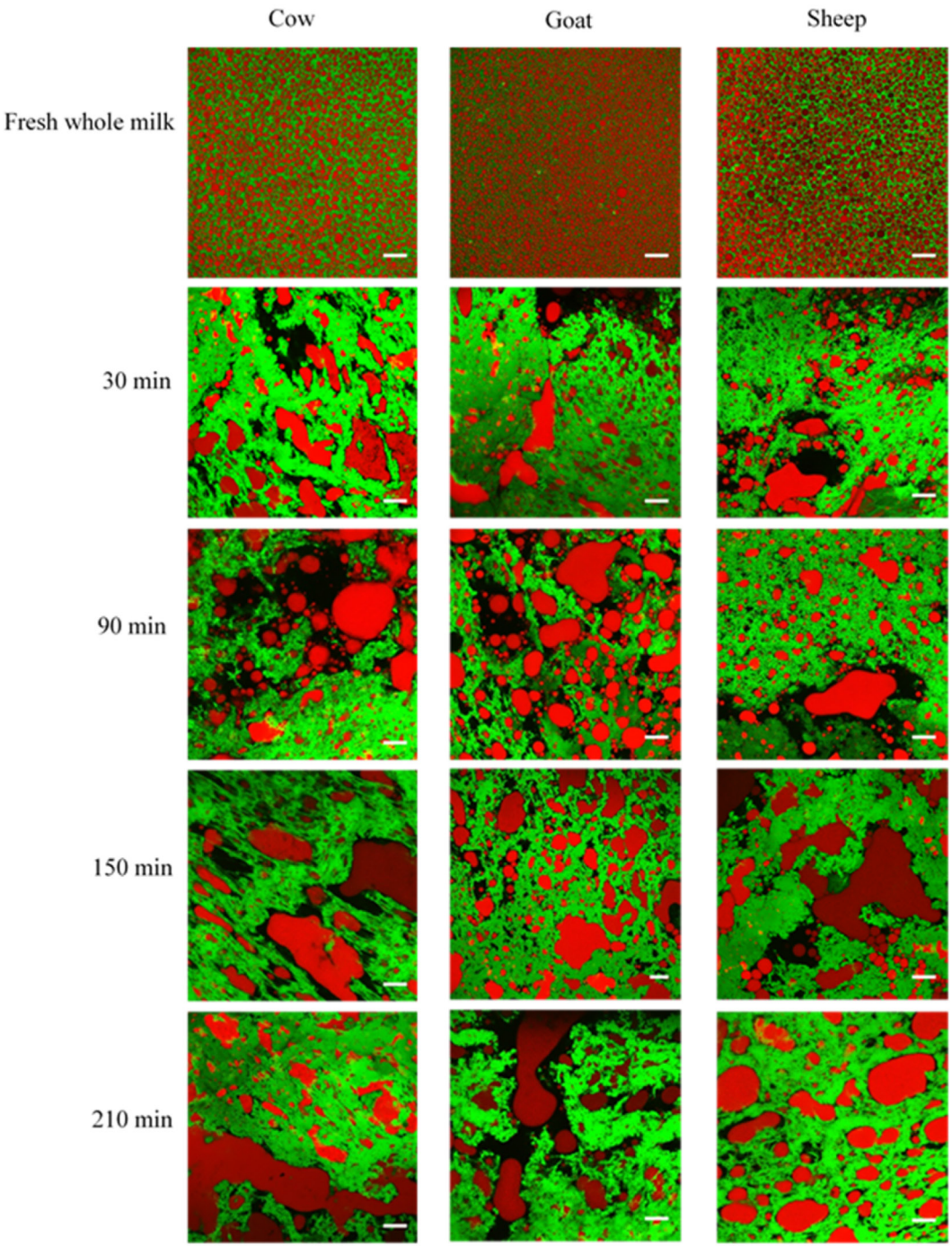

Figure 3. Confocal micrographs of the cow, goat, and sheep whole milk curds remaining in the piglet's stomach at different digestion times. Green = protein matrix; red = fat globules. Scale bars represent 25 μm. Only one representative micrograph from one piglet at each time point is shown.

during digestion in the stomach of adult rats. There are no direct reports on the microstructural changes in the proteins and the fat for raw cow, goat, and sheep milk during gastric digestion in vivo.

TEM. The states of the protein network and the fat globules within the gastric curds, as observed using TEM, are shown in Figure 5. The light electron-dense structures in the micrographs represent fat and the dark electron-dense areas represent protein. In fresh milk, the proteins and the fat globules were uniformly dispersed. At 30 min of digestion, the casein micelles had aggregated to form a loose network, with fat glob-

ules entrapped within this network. As the digestion progressed, with the decrease in the stomach pH (as discussed above), the casein aggregates appeared to fuse together and become denser. In addition, the milk fat globules appeared to be disrupted, with a high degree of coalescence. The fat globules located inside the protein matrix were not stained properly; this may have been due to the partial hydrolysis by gastric lipase of

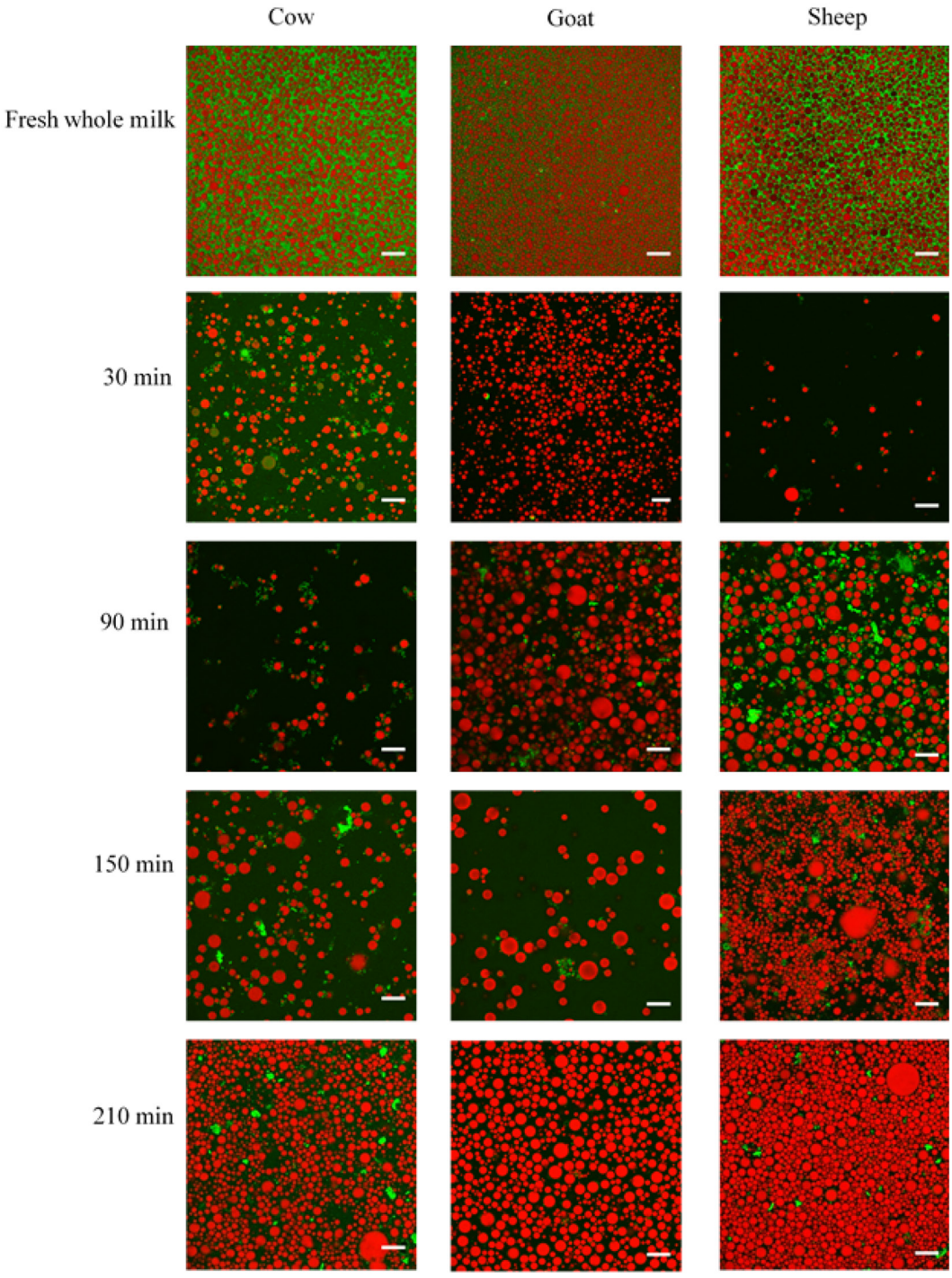

Figure 4. Confocal micrographs of the gastric liquid from the cow, goat, and sheep whole milk remaining in the piglet's stomach at different digestion times. Scale bars represent 25 μm. Only one representative micrograph from one piglet at each time point is shown.

triglycerides inside the fat globules. These observations were similar for the milk curds from all species. However, there were differences in the appearance of the protein network during digestion among the 3 milks. The extents of fusion and compaction of the protein network with increasing digestion time were less obvious in the goat and sheep milk curds than in the cow milk curds. Some remnants of MFGM material (void

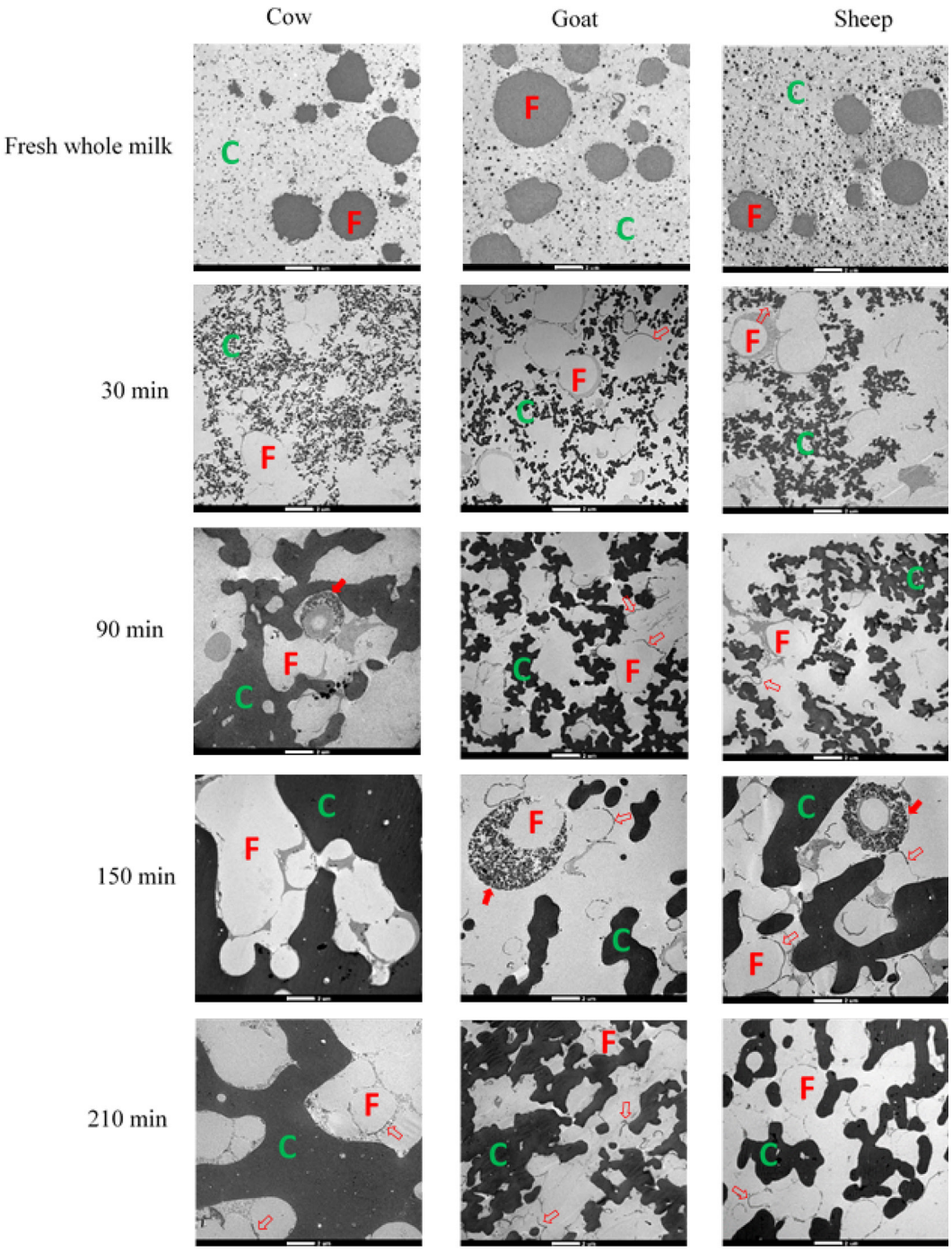

Figure 5. Transmission electron micrographs of the gastric curds from the cow, goat, and sheep whole milk remaining in the piglet's stomach at different digestion times. Scale bars represent 2 μm. C represents casein; F represents fat; void arrows point toward broken milk fat globule membrane (or fragments); filled arrows point toward the accumulation of hydrolytic products. Only one representative micrograph from one piglet at each time point is shown.

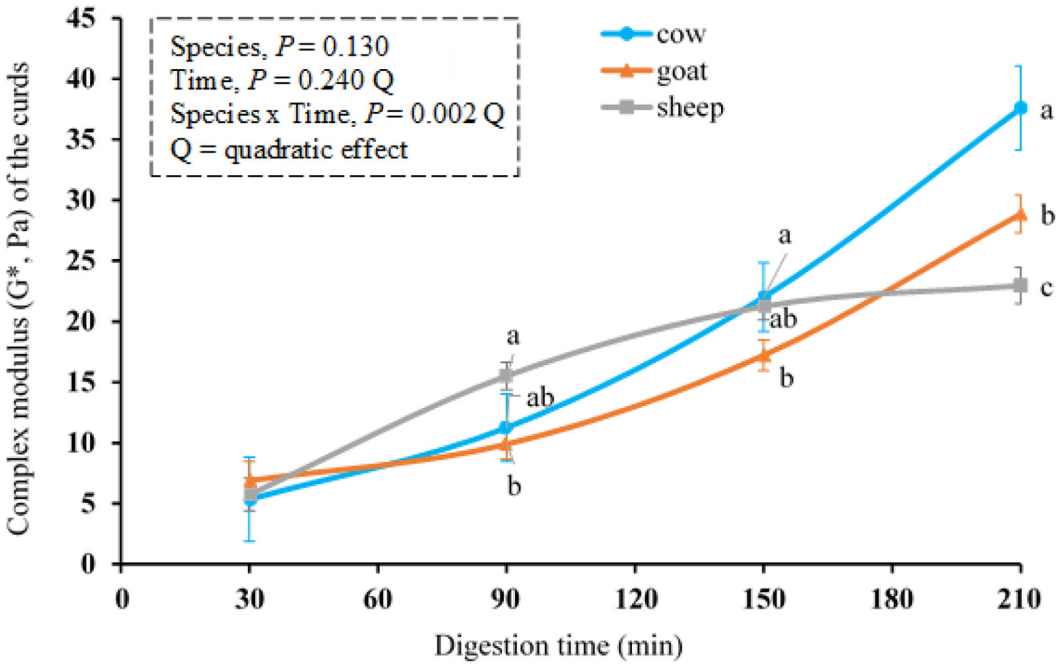

Figure 6. Changes in the complex modulus (G*, Pa) of the remaining gastric curd in the piglet's stomach at different digestion times. Values are reported as mean ± SEM, n = 3 to 4 piglets per species and time combination; outliers removed. Different letters (a–c) above the mean values represent significant differences among the curds at a given digestion time point ($P < 0.05$); the statistical significance of differences for a particular milk sample across different digestion times is not shown. For y-axis data, square root transformation of the raw data was required to fulfill the model assumptions.

arrows) were also apparent, particularly in the goat and sheep milk curds.

The TEM confirmed the confocal laser scanning microscopy observations on protein aggregation, fat globule entrapment, and fat globule coalescence. These ultrastructural changes in the state of the proteins and the fat globules in the gastric curds, as observed by TEM, were similar to those observed by Berendsen and Blanchette-Mackie (1979) and Berendsen (1982) in 12- to 24-h-old suckling rats (rat milk). These studies also found fat globules surrounded by an interconnected casein network, broken MFGM, and a homogeneous compact mass of fused caseins.

Fragments of MFGM, milk fat globules, and some protein aggregates were observed in the liquid fraction of the gastric chyme of milk from all species (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.19130216.v1). This indicated that, although most of the fat was entrapped within the curds, some of the detached or broken MFGM fragments were released into the liquid part of the gastric contents. It is expected that the MFGM fragments observed in the liquid phase would rapidly transit the gastric phase into the small intestine; this may be an important factor in delivering some of the key bioactive nutrients associated with the MFGM to the body.

Rheological Changes in the Gastric Curds

The complex modulus (G*) values of the gastric curds of the milk from the 3 species remaining in the stomach at the different digestion times were determined using small deformation rheology (Figure 6). G* is a measure of the resistance to deformation of a viscoelastic sample (in this case, the curd formed in the stomach). A lower G* value indicates a softer consistency of the curd and vice versa. For G*, there was a statistically significant ($P < 0.01$) interaction between species and time, meaning that, overall, G* differed over time but that the effect of time was different between the species. The G* values of the curds from the milk of the different species were similar at 30 min of digestion; as the digestion progressed, the G* values of the curds increased but at different rates for the different species. In general, the average G* values of the curds from goat milk and sheep milk remained lower than that from cow milk after 150 min of digestion. It appeared that the G* value of the sheep milk curds reached a plateau at around 150 min of digestion, whereas the G* values of the cow and goat milk curds increased further beyond 150 min of digestion (Figure 6).

The rheological changes observed in the gastric curds of the milk from the different species during diges-

tion (Figure 6) complemented the visual observations (Figure 2) and the TEM observations (Figure 5). The increase in the G* values of the milk curds over time was due to the compaction of the curd as digestion progressed (Figures 2 and 5). This occurred because of the stomach contraction forces and the gradual decrease in pH to below the isoelectric point of the caseins (pH 4.6) in the stomach during the 210 min of gastric digestion (progressive solubilization of colloidal calcium phosphate as well as loss of the entrapped liquid from the curds). There are no other direct quantitative in vivo reports for the dynamic changes in the consistency of milk curds in the stomach.

The differences observed in the consistency of the curds of the milk from the different species in this study may be explained by the changes in the protein network of the curd observed using TEM. The less fused protein networks of the goat and sheep milk curds may be a reason for their lower G* values (i.e., lower curd consistency) and the relatively soft curds at 210 min compared with the cow milk curds. These lower levels of curd consistency in the goat and sheep milk may be due to their lower amounts of α_{S1}-casein (Bell and Vlahopouloua, 1995) along with their larger casein micelle size compared with cow milk; however, this needs to be further investigated in detail.

It should be pointed out that small amount of curd from the presampling day diet was present along with the fresh milk curd from the sampling day in some of the piglet's stomach; the curds from the presampling day diet were very hard and thus were distinguishable from the fresh milk curds. Only the fresh milk curd sections were carefully sampled for rheological analysis. Some of the curds from the piglets killed at 150 min were also hard and could not be pressed to achieve the required gap for rheological analysis under the parallel plate geometry of rheometer, and thus, these piglets were removed as outliers during statistical data analysis.

Changes in Protein Profiles

The protein hydrolysis profiles of the gastric curds and the liquid phases at different digestion times, determined using tricine-SDS-PAGE under reducing conditions, are shown in Figures 7 and 8. It was evident that higher proportions of caseins than of whey proteins were present at all time points in all curd samples (Figures 7A–7C). The band intensities of the caseins appeared to be similar at all digestion times, and a para-κ-casein band was observed at all digestion time points. The para-κ-casein band is considered to be derived from the hydrolysis of κ-casein to para-κ-casein by milk-clotting enzymes (Ye et al., 2016b, 2019). Thus, in the case of the piglets, it was expected that both chymosin and pepsin would have been involved in the formation of para-κ-casein. Faint β-LG and α-LA bands were also observed in all curds. This could have been due to the liquid phase entrapped in the curds, which was expelled slowly during digestion, leading to a decrease in the intensities of the β-LG and α-LA bands as the digestion progressed. Also, traces of inherent milk high molecular weight proteins (such as lactoferrin, serum albumin, and immunoglobulins) were found in the curds at initial digestion times; they seem to disappear with further digestion.

Compared with the aggregated caseins of the curd samples (Figure 7), the liquid phase samples showed intense β-LG and α-LA bands (Figures 8A–8C). Small amounts of intact caseins were also found in the liquid phases at all digestion times. Other whey proteins (such as lactoferrin, serum albumin, and immunoglobulins) were also present, with intense bands in the 30-min samples, but these proteins almost disappeared after 210 min of digestion. The disappearance of the proteins from the liquid phase is expected to be mainly due to gradual hydrolysis, as the SDS-PAGE for all digestion time points was performed on equal protein concentrations; the continuous gastric emptying may have also played a role. Some peptides were also seen in the liquid phases, especially from 90 min onward, and these may have been generated from the slow hydrolysis of the caseins during gastric digestion along with hydrolysis of the intact whey proteins. Although the changes in the protein profiles of the curds and the liquid phases were observed to be similar for all milks in this study, there may have been differences in the extent of protein hydrolysis and in the number and type of peptides generated from the different milks; this needs to be investigated further.

No in vivo studies on the protein hydrolysis profile of goat and sheep milk during gastric digestion have been reported, but a few in vivo studies have reported the hydrolysis profile of cow milk proteins (Bouzerzour et al., 2012; Tari et al., 2018; Ye et al., 2019). Tari et al. (2018) also observed extensive hydrolysis of the caseins (including appearance of para-κ-casein) in the clotted gastric contents of piglets fed pasteurized cow-milk-protein-based infant formula (containing different ratios of caseins to whey proteins). Tari et al. (2018) also reported that β-LG and α-LA remained fairly undigested in the soluble fraction (liquid phase) collected until 120 min after feeding the piglets. Bouzerzour et al. (2012) observed similar gradual hydrolysis of native β-LG and α-LA in the stomach of piglets over a period of 210 min of gastric digestion after feeding an infant

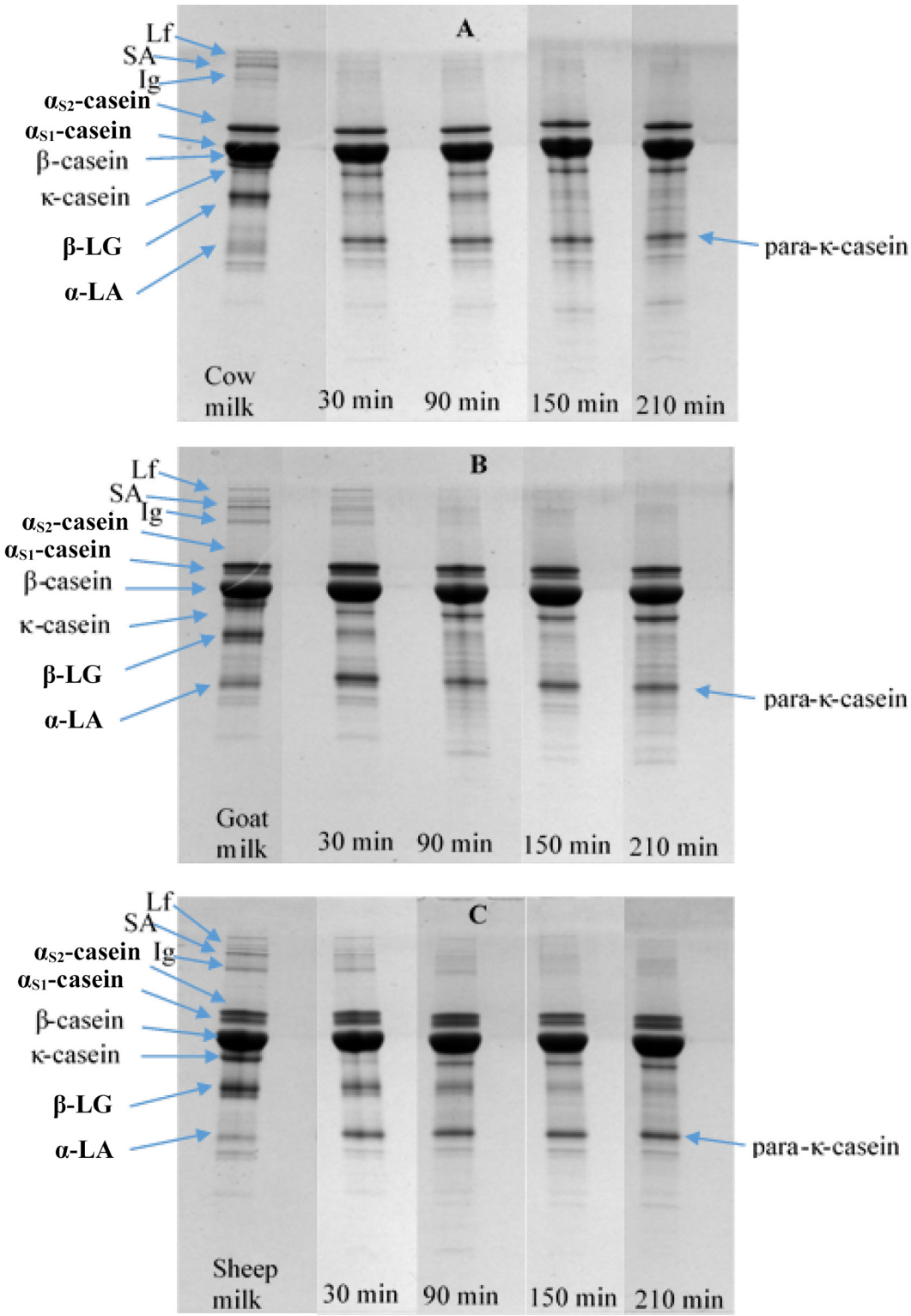

Figure 7. Sodium dodecyl sulfate-PAGE patterns of the freeze-dried milk and curds (20 μg of protein in each lane) obtained during the gastric digestion of whole milk from the piglet's stomach at different digestion times (30, 90, 150, and 210 min). (A) Cow whole milk; (B) goat whole milk; (C) sheep whole milk. SA = serum albumin; Lf = lactoferrin. Only one representative SDS-PAGE gel from one piglet at each time point is shown.

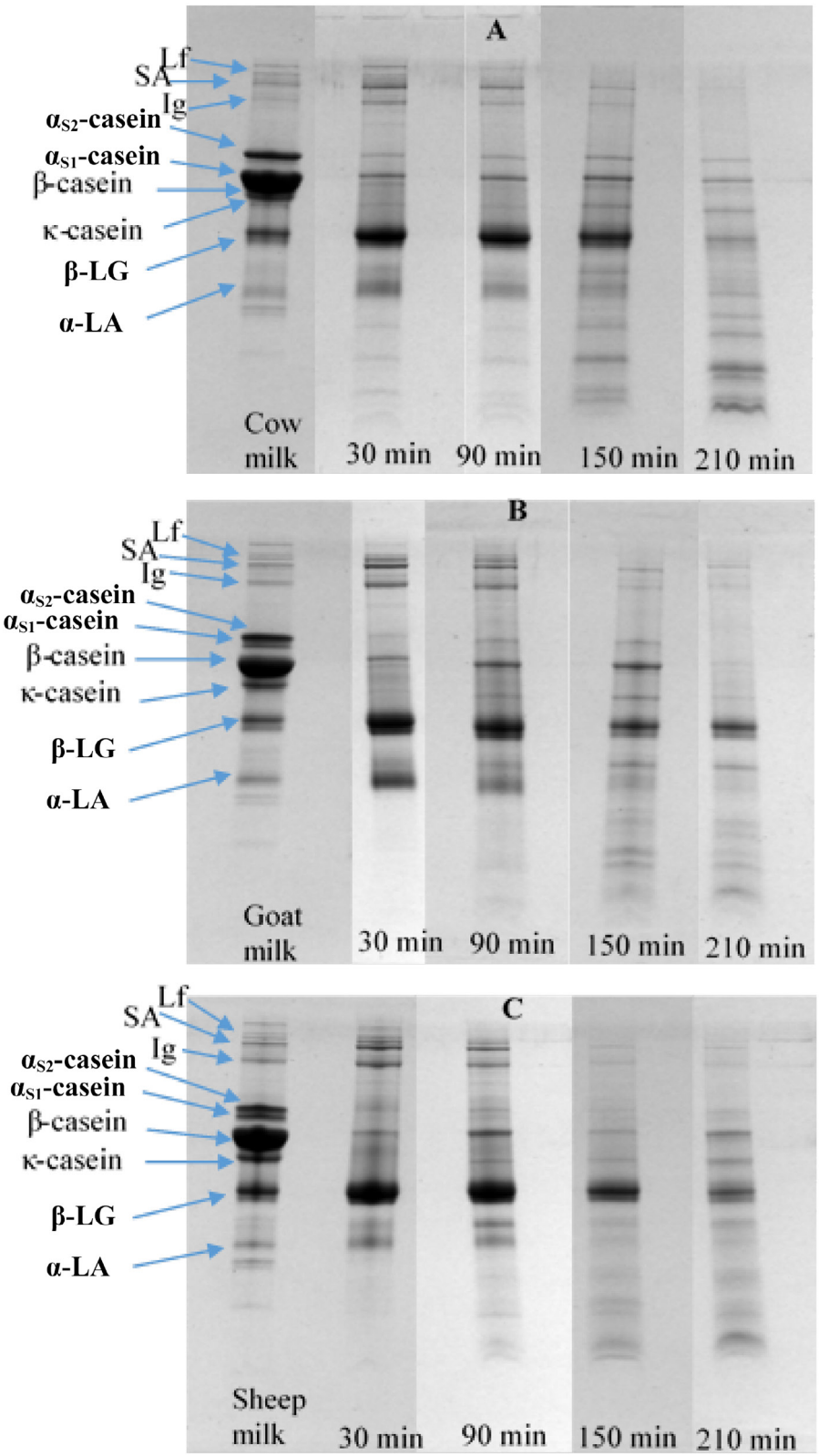

Figure 8. Sodium dodecyl sulfate-PAGE patterns of the freeze-dried milk and liquid fraction (20 μg of protein in each lane) obtained during the gastric digestion of whole milk from the piglet's stomach at different digestion times (30, 90, 150, and 210 min). (A) Cow whole milk; (B) goat whole milk; (C) sheep whole milk. SA = serum albumin; Lf = lactoferrin. Only one representative SDS-PAGE gel from one piglet at each time point is shown.

formula containing a 40:60 ratio of caseins to whey proteins (obtained from the soluble phase of microfiltered and ultrafiltered cow milk). Bouzerzour et al. (2012) also observed rapid hydrolysis of the caseins compared with the whey proteins in the infant formula during gastric digestion. The hydrolysis of caseins observed in these studies (Bouzerzour et al., 2012; Tari et al., 2018) may have been due to the heat treatment applied during the manufacture of infant formula (Wang et al., 2018; Ye et al., 2019; Roy et al., 2021a) as well as the significantly lower protein-to-fat ratio of infant formula (Mulet-Cabero et al., 2020b). These factors would lead to the formation of soft or fragile curds, which could be easily hydrolyzed by proteolytic enzymes and readily emptied from the stomach. The changes in the protein profile of the raw cow milk curd (caseins) observed in this study were similar to those reported by Ye et al. (2019) in adult rats; however, there may be differences in the extent (or degree) of protein hydrolysis in different animal models because of differences in enzyme concentrations (activity), acid secretion capacities (or pH profile), stomach emptying rates, and shear forces of the stomach.

Gastric Emptying

Relative Retention of Milk Proteins and Fats in the Stomach. The relative retention curves (gastric emptying) for protein (Figure 9A) and fat (Figure 9B) of the milk from the different species decreased as the digestion time progressed. However, the relative gastric retention of proteins and fats from both goat milk and sheep milk was lower ($P < 0.01$) than that from cow milk. Accordingly, the $T_{1/2}$ (time required to empty half the total nutrient amounts) followed the order: sheep milk < goat milk < cow milk (Figure 9).

As the total gastric content was composed of the curd and liquid phases, the relative nutrient retentions in these 2 fractions were also calculated (Supplemental Figures S2 and S3, https://doi.org/10.6084/m9.figshare.19130216.v1); this indicated that the major fraction of the nutrients (proteins and fats) in the gastric contents was associated with the curd. The lower protein and fat contents of the liquid phase were expected, because of the formation of the casein curd, which physically entrapped the majority of the fat globules, leading to the higher protein and fat contents of the curd. Based

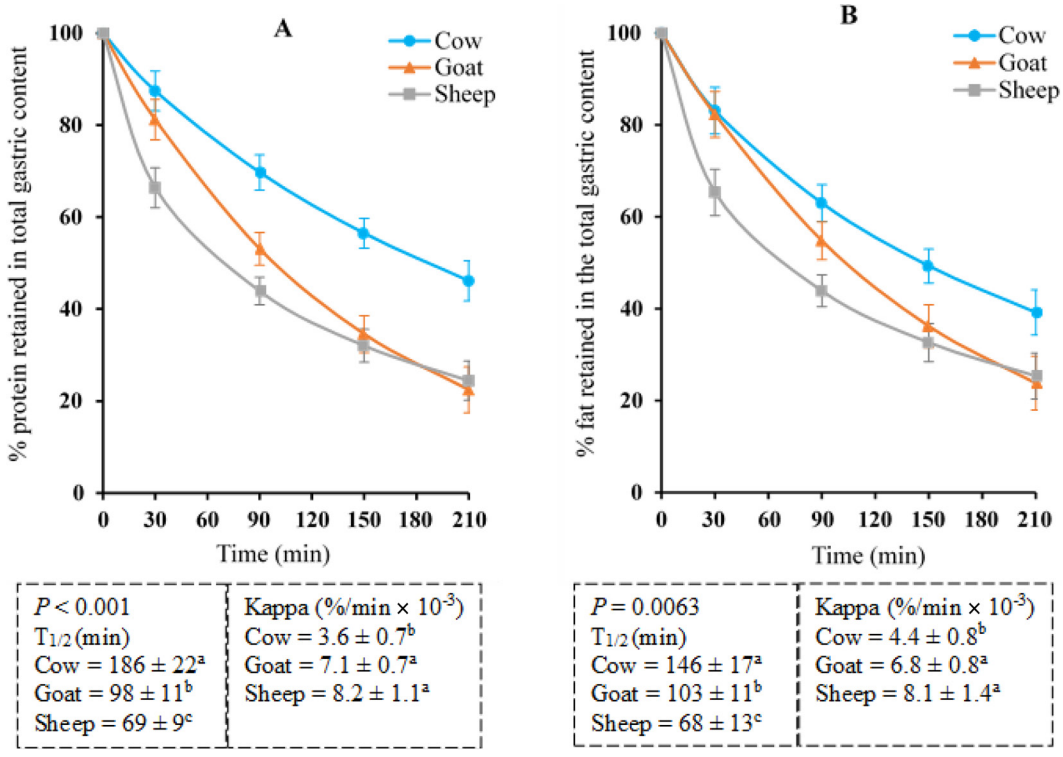

Figure 9. Changes in the (A) protein and (B) fat retained in the total gastric contents during the digestion of different milks in the piglet's stomach. Values are reported as the mean ± SEM, n = 2 to 4 piglets per species and time combination (outliers removed). Kappa is slope of the curve (unit is %/min × 10^{-3}), and $T_{1/2}$ (unit is min) is half gastric emptying time. Mean values with different superscript letters (a–c) differ ($P < 0.05$).

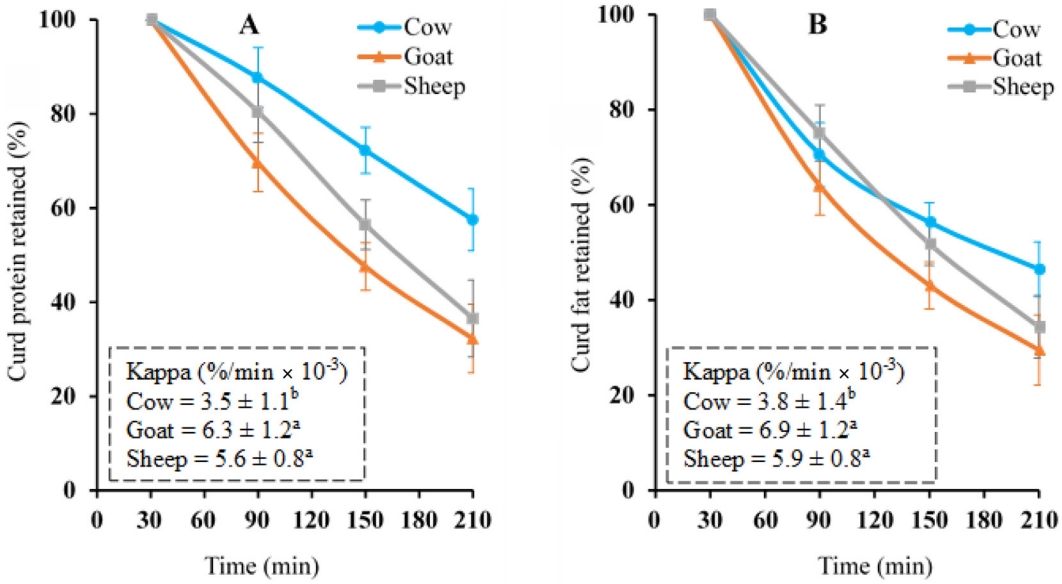

Figure 10. Relative retention of (A) protein and (B) fat in the curds formed from the different milks during digestion in the piglet stomach. Values are reported as mean ± SEM (considering the relative retention of nutrients in the curd at 30 min as 100%; i.e., 30 min is considered to be time 0 for the analysis of curd nutrient retention), n = 2 to 4 piglets per species and time combination (outliers removed). Kappa is slope of the curve (unit is %/min × 10^{-3}). Mean values with different superscript letters (a,b) differ ($P < 0.05$).

on the SDS-PAGE profiles, the emptied liquid fraction from the stomach was mainly whey proteins and was expected to have other water-soluble components such as lactose. With gradual hydrolysis or disintegration of the curd, the proteins and fats would be released into the liquid phase. It should be noted that, because of the continuous gastric emptying of the liquid phase, it was not possible to relate the changes occurring in the protein and fat composition of the curd to the changes in the nutrient content of the liquid phase.

As the curd formed, it retained most of the proteins and lipids at the beginning of digestion (i.e., at 30 min, which was our first sampling time point). The protein and fat retention in the curd during digestion was also analyzed, considering the 30-min digestion time as a starting time point for the curd formed (Figure 10). The results revealed that the protein and fat retained in the goat and sheep milk curds was lower than that retained in the cow milk curd and thus the rates of retention (i.e., κ values) for both protein and fat in their curds followed the order: cow > goat = sheep (Figures 10A and 10B). The relatively open microstructure and softer consistency of the goat and sheep milk curds observed in this study may have been a reason for the relatively lower retention rates of the goat and sheep milk proteins in the stomach. Differences in nutrient retention between species were also observed when the data were analyzed considering the actual curd nutrient retention at 30 min (Supplemental Figure S4, https://doi.org/10.6084/m9.figshare.19130216.v1). Dalziel et al. (2020) used high-resolution X-ray imaging to monitor the movement of metallic beads combined with the solid contents of reconstituted spray-dried whole cow and goat milk powder diets (reconstituted to equal protein contents) in the gastrointestinal tract of 10-wk-old rats. They observed comparatively faster gastric emptying of the metallic beads in rats fed goat milk than in those fed cow milk. In addition, they suggested that the easier expulsion of the beads from the stomach in the presence of goat milk compared with cow milk could have been due to the different physicochemical behaviors of goat and cow milk curds.

It should be noted that as the quantity of fresh milk that the piglets were fed from the 3 milk types was based on the piglets receiving an equal amount of protein per kilogram of BW in this study, the piglets received different volumes and calories per milk type (Table 1). This may have affected the stomach emptying rates. Further studies based on standardized (and isocaloric) milk intakes are necessary to investigate if the differences observed in gastric emptying of different milks under the conditions of the present study were affected by milk volume (and calories).

Relationship Between Protein and Fat Lost from the Curds. As the majority of the fat globules (~60–79%, depending on the species) were entrapped within the protein curd in the stomach, and because there was a gradual decrease in both the protein content and the fat content of the curds (Supplemental Figures S2 and S3, https://doi.org/10.6084/m9.figshare

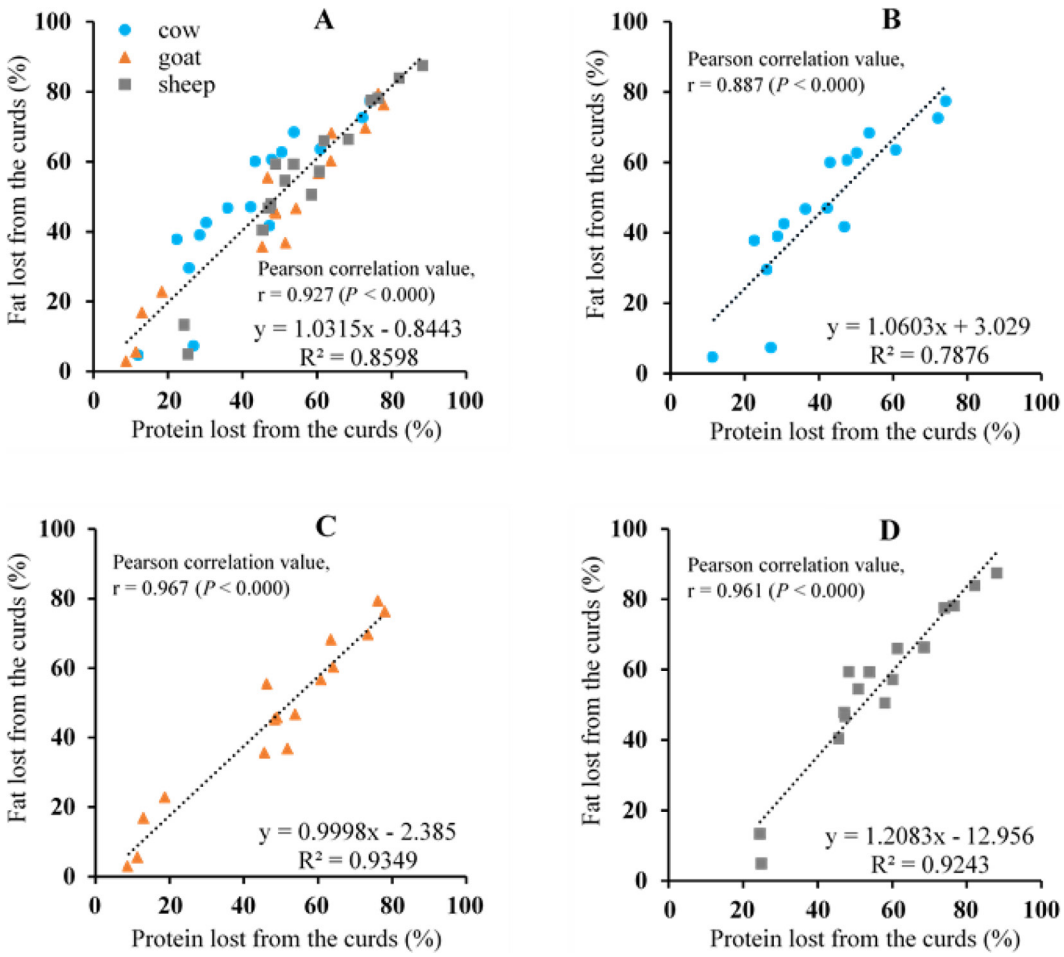

Figure 11. Relationships between the amounts of protein and fat lost from the curds (given as %) during gastric digestion from 30 to 210 min. (A) Whole milk curds from all the different species (n = 60); (B) cow whole milk curds (n = 20); (C) goat whole milk curds (n = 20); (D) sheep whole milk curds (n = 20). The Pearson correlation coefficient value (r), the regression line, and the linear regression equation are depicted. Data points represent the results obtained from each piglet.

.19130216.v1), it was hypothesized that the release of fat from the curd may be dependent on its surrounding matrix. To confirm this, the amounts of protein and fat lost from the curd in the stomach of each piglet were plotted (Figure 11). The results obtained showed a linear dependence between the protein and fat lost from the curds when a linear regression line was fitted for data from all species [i.e., R^2, Pearson correlation (r), and the slopes were found to be ~0.86, ~0.93, and ~1.0 respectively; regardless of the species; Figure 11A]. When data from all species were plotted separately, R^2 was between ~0.79 and 0.93, r was in the range from ~0.89 to 0.97, and the slope was from ~1.0 to 1.2 (depending on the milk species; Figures 11B, 11C, and 11D). This indicated a strong positive linear relationship between the 2 variables, suggesting that the release of fat globules from the coagulated curd was directly related to the loss of proteins during gastric digestion (irrespective of the species). Thus, these in vivo results emphasize that the changes in the curd structure (i.e., compaction, disintegration, and hydrolysis) during gastric digestion were a key factor responsible for the release of fat globules from the curd into the liquid phase.

CONCLUSIONS

This study enhanced our previous understanding and provided new insights into the physicochemical and structural changes that occur during the gastric digestion of milk, one of nature's complex structured foods. The overall mechanisms of the gastric digestions of raw cow, goat, and sheep whole milk were found to be similar. However, compared with cow milk, goat, and sheep milk had relatively high gastric emptying rates for protein and fat under the conditions of this study. This

could be attributed to the softer consistency of goat and sheep milk curds due to the inherent differences in their protein composition and casein micelle sizes. This emphasized that the structural and physical changes occurring in the curd fraction of the gastric contents are a critical factor influencing the delivery of protein and fat to the small intestine. The key mechanistic knowledge generated in this study about the gastric coagulation of different mammalian milks can be applied to design nature-inspired food structures for the controlled digestion and delivery of nutrients. Further studies to understand the effect of different commercial processing conditions on the digestion and absorption of milk from different species are required.

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