



# Heat treatment and homogenization of bovine milk loosened gastric curd structure and increased gastric emptying in growing pigs

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## ABSTRACT

During gastric digestion, bovine milk forms a curd, which consists largely of proteins and lipids. However, it is unknown how processing-induced changes to curd structure affects the gastric emptying of milk proteins and lipids. This study aimed to determine the impact of heat treatment and homogenization on gastric curd formation, and gastric emptying of dry matter (DM), proteins and lipids from bovine milk fed to pigs as a human model. Growing pigs ( $n = 180$ , mean  $\pm$  standard error of the mean (SEM) bodyweight  $22.4 \pm 0.13$  kg) consumed raw, or pasteurized non-homogenized (PNH), or pasteurized homogenized (PH), or ultra-high temperature treated homogenized (UHT) milk diets. A protein-lipid-free lactose (PLFL) solution was also fed as a test diet. At 0, 20, 60, 120, 180 and 300 min postprandially the entire gastrointestinal tract was dissected out. The gastric chyme (curd and liquid) fractions were collected after separation using a mesh screen. The DM, protein, and lipid contents of these fractions were quantified. Confocal, transmission electron microscopy, cryo-scanning electron microscopy and rheological analyses were conducted to determine the micro- and macrostructure of the curd. Overall, both heat treatment and homogenization influenced the *in vivo* gastric curd structure formed of bovine milk, although to different extents. The gastric emptying of DM, proteins, and lipids increased with the extent of processing. Gastric emptying rates of DM and proteins followed the pattern UHT > PH > PNH = raw, while emptying rates of lipid also differed between PNH and raw milk. Curd structure was the main gastric parameter affected in PNH milk.

## 1. Introduction

Milk has been a nutritional staple in the human diet for thousands of years and is consumed worldwide from infancy to adulthood. While milk from caprine and ovine species are popular in some Asian, African and Mediterranean areas, bovine milk is the most widely consumed milk in Western countries (Food and Agriculture Organisation of the United Nations: FAOSTAT Statistical Database, 2022). Bovine milk is an excellent source of nutrition, typically providing around 30 g/L of protein, 35 g/L of fat, calcium, and other minerals (Eigel et al., 1984; Jensen et al., 1991). Before consumption, commercial milk is often processed to improve safety, often through heat treatment (pasteurization and

ultra-high temperature (UHT) treatment) and homogenization. Processing induces various changes to the native milk protein and lipid structures present in milk (Corredig & Dalgleish, 1999; Dalgleish, 1990; Elfagm & Wheelock, 1978; Meisel & Hagemester, 1984; Tunick et al., 2016; Wada & Lönnerdal, 2014; Ye et al., 2016b; Ye, Singh, et al., 2004). For example, during heat treatment, structural changes to the whey proteins result in denaturation and association with caseins (Ye et al., 2016a). Homogenization disrupts the milk fat globule membrane and restructures the milk fat globule, resulting in smaller fat droplets and the adsorption of casein and whey proteins onto the surface of the droplets (Lee & Sherbon, 2002).

During gastric digestion, milk forms a curd, which consists mainly of proteins and lipids (Roy et al., 2021). *In vitro* digestion studies with milk

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### Abbreviations

AOAC	American Association of Chemists
BW	body weight
cryo-SEM	cryo-scanning electron microscopy
DM	dry matter
GIT	gastrointestinal tract
NRC	National Research Council
PH	pasteurized homogenized
PNH	pasteurized non-homogenized
PLFL	protein-lipid-free lactose
SEM	standard error of the mean
TEM	transmission electron microscopy
UHT	ultra-high temperature homogenized
USDA	United States Department of Agriculture

**Table 1**

Processing conditions and source of the milk types and protein-lipid-free lactose solution used in the pig study.

Diet	Processing conditions	Source and diet preparation
Raw	N/A	Local supplier; consumed as sourced
Pasteurized non-homogenized (PNH)	75 °C, 15 s	Commercially available trim milk mixed with commercially available fresh cream <sup>a</sup>
Pasteurized homogenized (PH)	75 °C, 15 s, 160 bar	Commercially available; consumed as sourced
Ultra-high temperature homogenized (UHT)	140 °C, 4 s, 160 bar	Commercially available; consumed as sourced <sup>b</sup>
Protein-lipid-free lactose (PLFL)	12% lactose in distilled water	Commercially available powdered lactose; solution prepared on-site

<sup>a</sup> Identical process was used to pasteurize the skim milk and cream.

<sup>b</sup> A preheating step (95 °C, 90 s) was used to stabilize protein before UHT processing.

**Table 2**

Ingredient and calculated chemical composition of the human-type diet provided to the pigs at lunch and dinner.<sup>a</sup>

Ingredient	g fresh/kg DM	g DM/kg
Milk	617	92
Whole grain bread (diced)	636	540
Peeled hard-boiled egg (minced)	740	186
Raw carrots (minced)	370	36
Canned, chopped fruits	555	108
Sucrose	12	12
Premix of vitamins and minerals <sup>b</sup>	6.2	6.2
Sodium chloride	3.6	3.6
Calcium carbonate	10	10
Dicalcium phosphate	6.2	6.2
<b>Calculated composition</b>		
Protein		171
Calcium		1.5
Phosphorus		4.2
Energy (kcal/kg)		3369

<sup>a</sup> The chemical composition of the ingredients to formulate the diet was obtained from the USDA National Nutrient Database (<https://ndb.nal.usda.gov/>).

<sup>b</sup> Vitamin and mineral premixes were obtained from Vitec Nutrition Ltd (Auckland, New Zealand) and supplied (per kg of diet as-fed): Mn, 45 mg; Zn, 80 mg; Cu, 25 mg; Co, 0.5 mg; Se, 0.3 mg; Fe, 100 mg; I, 1.0 mg; Choline, 100 mg; all-trans retinylacetate, 3.0 mg; cholecalciferol, 0.05 mg;  $\alpha$ -tocopherol, 50 mg; menadione, 2.0 mg; thiamin, 1.0 mg; riboflavin, 3.0 mg; nicotinic acid, 15 mg; pantothenic acid, 20 mg; pyridoxine, 2.0 mg; cyanocobalamin, 0.01 mg; folic acid, 0.5 mg; biotin, 0.1 mg.

have shown that the formation and structure of the gastric curd were influenced by processing, due to changes in native protein and lipid structures as described above (Li et al., 2022; Mulet-Cabero et al., 2019; Ye et al., 2017, 2019). For example, heat-induced aggregation can disrupt gastric curd formation by restricting the ability of proteins to form a network (Ye et al., 2016b, 2017). Such aggregation and subsequent effects on curd formation are greater in UHT processed bovine milk than pasteurized bovine milk (Ye et al., 2019). In addition, the adsorption of caseins and whey proteins onto the surface of homogenized fat droplets allows for incorporation of the fat droplets into the gastric curd matrix (Gallier et al., 2013; Ye et al., 2017). It is unknown if the structural changes in the gastric curd caused by milk processing can influence the gastric emptying rate of dry matter (DM), proteins, and lipids into the small intestine. Such a relationship has been suggested in a recent review by Huppertz and Chia (2021). However, such a relationship still needs to be proven in a valid *in vivo* model.

These observations led to the hypothesis that heat-treated and homogenized bovine milk exhibit faster gastric emptying rates of DM, proteins, and lipids due to forming a softer curd with a more open protein network. This study aimed to quantify the gastric emptying of DM, proteins, and lipids of heat-treated and homogenized bovine milk along with the associated curd formation in a growing pig model of human metabolism. Bovine milk was selected as a milk model as the effects of heat treatment and homogenization on its native protein and lipid structures have been well characterized (Anema & Klostermeyer, 1997; Elfagm & Wheelock, 1978; Guyomarç'h et al., 2003; Lee & Sherbon, 2002; Morr, 1989; Oldfield et al., 1998; Smits & Van Brouwershaven, 1980; Wada & Lønnerdal, 2014; Ye, Anema, & Singh, 2004). The growing pig was selected as a model for humans based on digestive and physiological similarities of the gastrointestinal tract (GIT) between both species (Rowan et al., 1994).

## 2. Methods

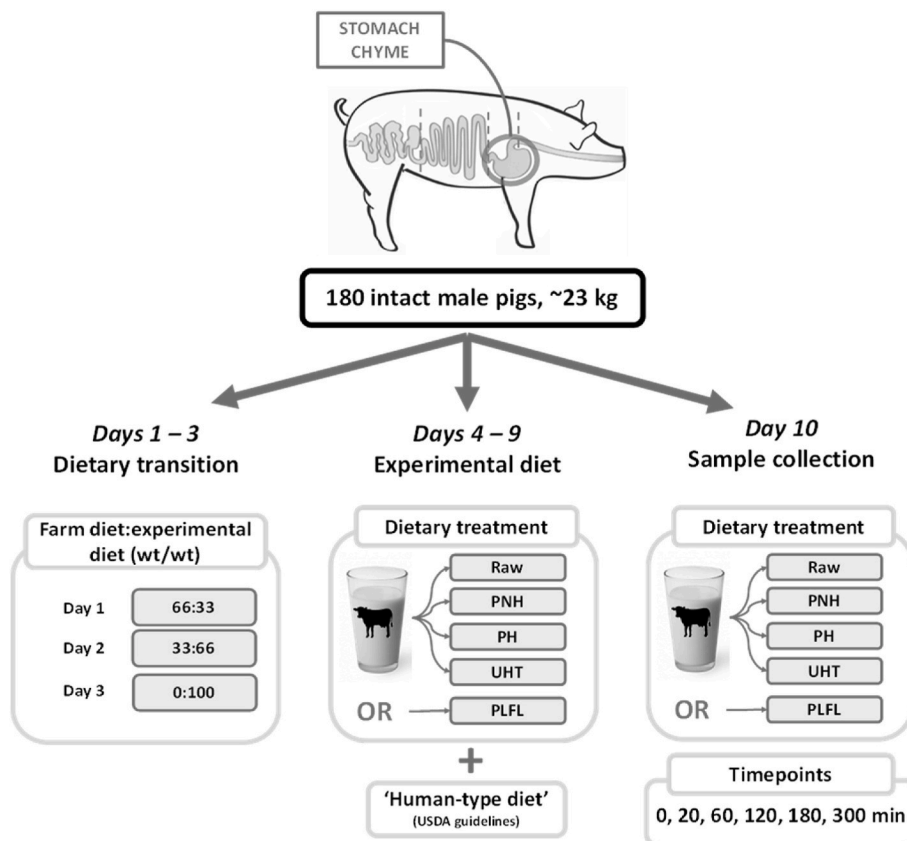
### 2.1. Animals, housing, and dietary treatments

This study was approved by the Massey University Animal Ethics Committee (application no. 19/83). Locally sourced Large White x Landrace entire male pigs (n = 180; 36 pigs per experimental diet; 6 pigs per experimental diet x timepoint combination), bodyweight 22.4 ± 0.13 kg, mean ± standard error of the mean (SEM)) were housed in individual metabolic crates at the Animal Production Unit of Massey University, Palmerston North.

The experimental treatments were raw, pasteurized non-homogenized (PNH), pasteurized homogenized (PH), or ultra-high temperature treated homogenized (UHT) bovine milk. The PH, PNH, and UHT milk types were commercially available processed products (Table 1), while the raw milk was locally sourced (Gorge Fresh Organics, Palmerston North, New Zealand). The raw, PH and UHT milk diets were fed as sourced, whereas PNH was prepared using simple manual agitation to combine pasteurized trim milk with pasteurized cream to the same lipid content as the PH milk. Another treatment included in the study was a protein-lipid-free lactose (PLFL) solution, which consisted of 12% lactose in distilled water. The PLFL treatment was included to allow correction for endogenous gastric luminal materials (Moughan & Rutherford, 2012), and for chyme remaining from the previous meals based on other studies showing some residual meal after an overnight fast (Roy et al., 2022).

### 2.2. Experimental design

A human-type meal (Table 2) was formulated based on the USDA's chemical composition data to meet the NRC nutrient requirements for growing pigs (NRC, 1998). The human-type diet and meal times were also designed to reflect typical Western meal composition and consumption patterns.



**Fig. 1.** Experimental diagram of the pig study. Pigs were gradually transitioned from the farm diet to the experimental diet in one-third increments over three days. The experimental diet was then consumed for seven days. On day 10, pigs consumed their milk diets and were euthanized at various time points. The entire gastric chyme was collected. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized; PLFL, protein lipid free lactose solution.

On arrival, pigs were randomly allocated to each of the five dietary treatments. Over the first three days, the experimental treatments were gradually introduced by replacing the commercial diet provided on the farm (day 1; 33% experimental diet, 66% farm diet; day 2; 66% experimental diet, 33% farm diet; day 3 100% experimental diet) (Fig. 1). On arrival, two meals (12:00 and 16:00 h) were equally fed at a daily DM level of 4% based on individual body weight.

After the dietary transition, the pigs consumed their treatments at 09:00 h (500 mL of raw, PH, PNH, UHT or PLFL) and human-type meals at 12:00 h and 16:00 h for 7 days (Fig. 1; Table 2). The pigs in the PLFL group received UHT milk for the first 3 days after the dietary transition, followed by the PLFL treatment for the final 4 days, as the PLFL diet for 7 days would not provide sufficient nutrition. During the first two days, the meals were kept in the feeder for up to 15 min, before refusals, if any, were collected and weighed. The feeders were then washed and kept empty until the next feeding. From day three onwards, all pigs consumed their meals quickly and within 5–10 min the feeders could be cleaned. This feeding method can be used in pigs as they have the digestive capacity for 'meal-eating' (Miller & Ullrey, 1987; Rowan et al., 1994). Thus, by the sampling day, pigs were able to consume their milk meals in approximately 2 min.

On day 10, pigs were fed lunch and dinner meals from 12:00 and 16:00 h respectively, at staggered times to accommodate the same fasting period at the sampling on day 11 (Fig. 1). On day 11, fasted pigs (16 h) were either euthanized at time 0 min or fed 500 mL of milk type or PLFL solution for breakfast before being euthanized at either 20, 60, 120, 180, or 300 min. Indigestible marker titanium dioxide ( $\text{TiO}_2$ ,  $0.38 \pm 0.3$  g) (Sigma Aldrich, St. Louis, MO, USA) was added to the breakfast meal to estimate meal digestibility. The  $\text{TiO}_2$  results were used for other analyses that are not reported here.

Each pig was anesthetized 15 min before its euthanasia time with a mix of Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Virbac, Hamilton, NZ) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine,

both 100 mg/mL (Phoenix Pharm NZ, Auckland, New Zealand). The final solution contained 50 mg/mL of each drug and was administered at a dose rate of 30–40  $\mu\text{L}$  of the mixed solution/kg BW by intramuscular injection in the neck area. Following sedation, each pig was intravenously administered a second dose of the cocktail (30  $\mu\text{L}$ /kg BW) to induce deep anesthesia. Once anesthetized, the pigs were euthanized by an intracardiac injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300, Provet NZ Pty Ltd, Christchurch, New Zealand).

The abdomen was opened, and the stomach was clamped at the esophageal and pyloric ends prior to being removed. The stomach was carefully removed with minimal movement, washed, dried, and weighed full before being opened by an incision along the lesser curvature. The pH of the gastric contents (chyme) at the proximal and distal regions was recorded, after which the solid (curd) and liquid fractions were separated using a 1 mm sieve. The pH of the liquid fraction was measured, and a photograph of the curd in the sieve was taken. The liquid fraction was weighed. Samples were collected from each fraction for confocal microscopy and from the solid fraction for rheology. Selected curd samples were collected for transmission electron microscopy (TEM) and cryo-scanning electron microscopy (cryo-SEM). The remaining material of each fraction was thoroughly mixed, frozen, freeze-dried, weighed, and ground. Samples were stored at  $-20^\circ\text{C}$  until analysis.

### 2.3. Rheological analysis

Rheological measurements were conducted on milk and gastric curd samples within an hour of obtaining the samples from the pigs, following an existing protocol with minor modifications (Mulet-Cabero et al., 2019). An AR-G2 magnetic bearing rheometer (TA Instruments, Crawley, West Sussex, UK) fitted with a 40 mm diameter parallel steel plate geometry was used. Approximately 5 g of gastric curd was placed in the rheometer geometry, and the samples were pressed to the system's

**Table 3**

Chemical composition of raw and commercially processed bovine milk, and the protein-lipid-free lactose solution fed to the pigs.

	Raw <sup>a</sup>	PNH <sup>b</sup>	PH <sup>b</sup>	UHT <sup>b</sup>	PLFL <sup>c</sup>
Protein, g/L	41	36	33	34	0
Fat – total, g/L	44	34	34	33	0
Carbohydrates, g/L	46	46	47	44	117
Sodium, mg/L	481	320	328	319	0
Calcium, mg/L	1340	1203	1149	1154	5.2

PNH, pasteurized non-homogenized milk; PH, pasteurized homogenized milk; UHT, ultra-high temperature treated homogenized milk; PLFL, protein-lipid free lactose solution.

<sup>a</sup> Locally sourced, single farm, bulk milk.

<sup>b</sup> Commercially sourced.

<sup>c</sup> Prepared on-site.

default gap of 2000  $\mu\text{m}$ . After pressing, approximately 2 g of the sample that moved out of the geometry was removed. The analysis was conducted on the remaining 3 g of the sample. Time sweep tests were performed at a frequency of 1 Hz, strain of 0.5, for 20 min at 37 °C.

#### 2.4. Microscopy

Confocal microscopy and TEM analyses were conducted at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, New Zealand). A confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany) was used to determine the microstructures of fresh curd and liquid samples. The fresh samples were immediately stained and imaged. Approximately 200  $\mu\text{g}$  of the sample was transferred into an Eppendorf tube, and 5  $\mu\text{L}$  of 1.0% (w/v) Fast Green and 10  $\mu\text{L}$  of 0.1% (w/v) Nile Red were added to stain protein (He–Ne laser with an excitation at 633 nm) and oil (argon laser with an excitation line of 488 nm) phases, respectively, for at least 10 min. The stained samples were placed on concave confocal microscope slides (Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with coverslips, and observed using magnifications of  $\times 40$  and  $\times 100$  oil immersion lenses.

TEM availability limited the number of samples which could be imaged. Thus, milk and fresh curd samples from pigs that represented a milk type and post-feeding times of 20, 120, and 180 min were randomly selected throughout the six periods of the study. Selected samples were prepared and stored until analysis. However, PH milk and curd samples were accidentally discarded before being analyzed. Sample preparation was carried out as described by Li et al. (2021) and imaged with a Tecnai G2 Spirit BioTWIN (FEI company, Czech Republic) paired with a Veleta TEM camera (Olympus SIS Germany).

Cryo-SEM was conducted at the MacDiarmid Institute for Advanced Materials and Nanotechnology (Victoria University, Wellington, New Zealand). Randomly selected raw, PH, and UHT curd samples at 20 min and 180 min were snap-frozen in nitrogen slush directly after collection. Limited microscope availability did not allow for analysis of all curd samples. Based on other imaging carried out in this study, the PNH curd samples were not imaged using cryo-SEM. The frozen samples were loaded into a cryo unit Gatan Alto 2500 (Gatan Inc., Pleasanton, CA, USA) fitted onto the electron microscope at  $-120$  °C. The fractured surfaces of the curds were sputter-coated with platinum under a current of 10 mA for 240 s. They were then inserted into a JEOL 6500F scanning electron microscope (JEOL Ltd, Tokyo, Japan) for imaging with accelerating voltages of 6 and 8 kV and a probe current of 22 nA. Multiple images were collected for each sample, and a representative image is presented in the results section.

#### 2.5. Chemical compositional analysis

The milk types, curd, and liquid fraction samples were analyzed for DM (AOAC 990.19 (AOAC, 2006)) and protein (nitrogen  $\times 6.38$ ) content

(Koletzko & Shamir, 2006) using the Dumas method (AOAC 968.06 (AOAC, 2006)). The lipid content of a representative sample set (milk, liquid fraction, and curd fraction) was quantified using the Mojonnier method (AOAC 989.05 (AOAC, 2006)) and a modified petroleum ether/diethyl ether extraction. A significant correlation between both methods to determine lipid content was observed ( $R^2 = 0.997$ , Supplementary Fig. S1). Based on this high correlation, the remaining samples (350) were quantified using the modified method.

#### 2.6. Modified petroleum ether/diethyl ether lipid extraction

Freeze-dried and ground curd and liquid fractions were weighed (1 g) in a glass tube before mixing with 5 mL petroleum ether and 5 mL diethyl ether. Solvents were added, vortexed for 30 s, stood for 5 min and centrifuged at 3000 rpm for 7 min at room temperature. The solvent fraction was then discarded after removal using a water aspirator system (approximately 10 torr). The complete procedure was repeated twice to remove residual lipid. The fraction remaining in the tube was left for 16 h at room temperature. The samples were then vortexed to help loosen the remaining material and incubated at 105 °C for 1 h to remove solvent residues. The samples were then cooled for 1 h in a desiccator and weighed to calculate the lipid content.

#### 2.7. Statistical analyses

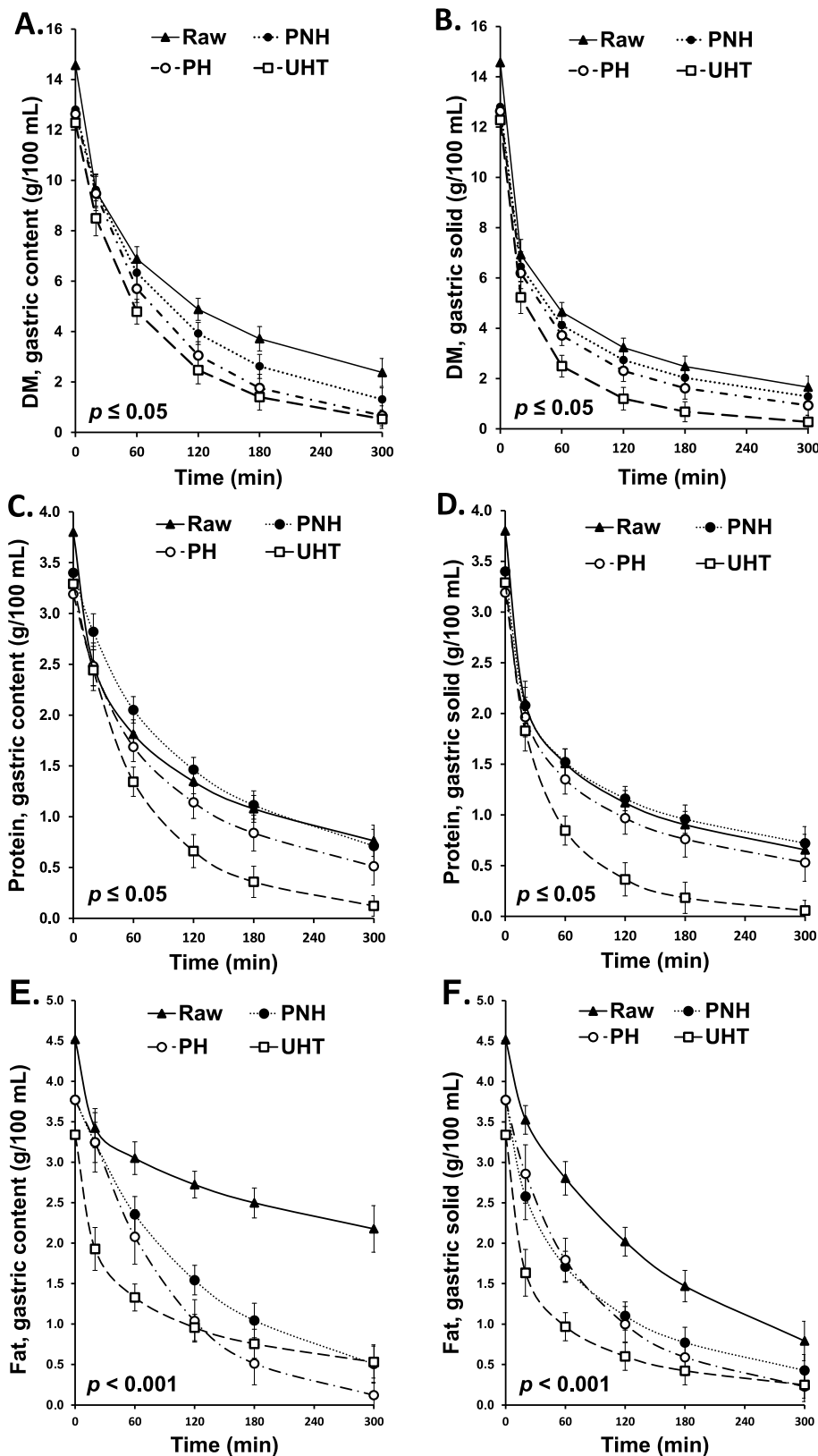
The present animal trial was designed to investigate the gastric digestion of milk, as well as the small intestinal digestibility and the appearance of amino acids in the circulatory system. Thus, it was important to cater to the greatest required number of animals. Based on previous studies with similar outcomes (Butteiger et al., 2013; Chen et al., 1962; Gaudichon et al., 1994; Montoya et al., 2018), six pigs were required to detect a difference in mean values powered over 80% at  $P < 0.05$ .

Statistical analyses were conducted using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). First, a two-factor linear model including milk, time (as either a categorical or a numerical variable), and their interaction as fixed effects, was used to assess curd strength. For pH of the proximal and distal chyme, a linear mixed model including the fixed factors described above, and the pig (as repeated measures in space) included as a random effect, was used. Next, the log-likelihood ratio test was used to select the best polynomial model, and then compare models with time as numerical (selected polynomial) and categorical variables. The best model had time as a numerical variable for all response parameters.

In some pigs, part of the meal at 16:00 h (human-type diet) remained in the stomach despite the 16 h fasting period. Where the gastric content did show more than 10% of the human-type diet remained, these samples were excluded from DM and protein analyses of the chyme, as the fibrous remnant material could influence the curd (e.g., formation, structure, and degradation) and subsequent gastric emptying (Bornhorst et al., 2014). Gastric contents with less than 10% of the human-type diet were corrected using average DM and protein contents found in the PLFL group for each post-feeding time. Based on this, 20 pigs were removed from the analyses of DM and protein stomach emptying rates. The remaining parameters (rheometry, confocal microscopy, TEM, cryo-SEM) were quantified in the samples from these pigs using portions of curd samples free from the remnants of the 16:00 h meal. A correction for lipids was omitted, as the lipid content of the remaining chyme for the pigs fed the PLFL solution was negligible. Excluded pigs were five for raw, one for PNH, six for PH, and eight for UHT.

The power exponential model was used to analyze the retention of DM, proteins, and lipids (on a 100 mL milk basis) in the stomach as detailed by Montoya et al. (2014) (protein as an example):

$$\text{Remaining}_{\text{Protein}} (\text{g}) = \alpha_0 \exp - (\kappa \times \text{time})^\beta$$



**Fig. 2.** Gastric emptying of total gastric contents and gastric curd (solid) collected at different post-feeding times of growing pigs fed different processed bovine milk types. **A and B:** total and curd dry matter (DM); **C and D:** total and curd protein; **E and F:** total and curd lipid. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. Values are means  $\pm$  SEM,  $n = 4-6$ . The starting values for the curd were assumed to be the content of the milk consumed. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

where the parameter  $\alpha_0$  is the amount of DM (g), protein, or lipid in the milk before being consumed,  $\kappa$  is the logarithmic slope of the curve (g/min),  $\beta$  is a dimensionless index for the shape of the curve, and time is in minutes. The parameters  $\kappa$  and  $\beta$  were then used to determine the half gastric emptying time ( $T_{1/2}$ ):

$$\text{Gastric } T_{1/2 \text{ Protein}} \text{ (min)} = (1/\kappa) \times (\log[1/0.5])^{(1/\beta)}$$

For analysis of the gastric emptying of the curd, it was assumed that the initial values (i.e.,  $\alpha_0$ ) were the same as the milk consumed.

**Table 4**

Gastric emptying parameters of the power exponential model ( $\kappa$ ,  $\beta$ ) and half-time ( $T_{1/2}$ ) of gastric emptying of total and gastric curd (solid) dry matter, protein, and lipid collected at different post-feeding times of growing pigs fed different processed bovine milk types.

	Total gastric content			Curd		
	$\kappa \times 10^{-3}$ (g/min)	$\beta$	$T_{1/2}$ (min)	$\kappa \times 10^{-3}$ (g/min)	$\beta$	$T_{1/2}$ (min)
<b>DM</b>						
Raw	9.1 ± 1.4 <sup>c</sup>	0.57 ± 0.10 <sup>d</sup>	57 ± 15.7 <sup>ab</sup>	21 ± 4.2 <sup>c</sup>	0.41 ± 0.075 <sup>c</sup>	19 ± 8.0 <sup>a</sup>
PNH	12 ± 1.4 <sup>c</sup>	0.68 ± 0.10 <sup>c</sup>	49 ± 4.6 <sup>a</sup>	27 ± 5.6 <sup>c</sup>	0.42 ± 0.078 <sup>c</sup>	15 ± 4.1 <sup>a</sup>
PH	15 ± 2.0 <sup>b</sup>	0.76 ± 0.12 <sup>a</sup>	42 ± 5.4 <sup>ab</sup>	32 ± 6.9 <sup>b</sup>	0.44 ± 0.084 <sup>b</sup>	14 ± 3.6 <sup>a</sup>
UHT	18 ± 2.6 <sup>a</sup>	0.70 ± 0.12 <sup>b</sup>	32 ± 4.9 <sup>b</sup>	49 ± 12 <sup>a</sup>	0.51 ± 0.12 <sup>a</sup>	10 ± 3.5 <sup>a</sup>
<b>Protein</b>						
Raw	10 ± 1.8 <sup>c</sup>	0.46 ± 0.09 <sup>c</sup>	45 ± 14 <sup>ab</sup>	16 ± 3.8 <sup>b</sup>	0.38 ± 0.09 <sup>b</sup>	25 ± 11 <sup>a</sup>
PNH	8.4 ± 1.2 <sup>c</sup>	0.59 ± 0.10 <sup>b</sup>	64 ± 9.6 <sup>a</sup>	15 ± 3.5 <sup>b</sup>	0.36 ± 0.09 <sup>b</sup>	24 ± 10 <sup>a</sup>
PH	13 ± 2.3 <sup>b</sup>	0.54 ± 0.10 <sup>b</sup>	40 ± 6.2 <sup>b</sup>	21 ± 5.4 <sup>b</sup>	0.39 ± 0.10 <sup>b</sup>	19 ± 5.5 <sup>a</sup>
UHT	19 ± 2.6 <sup>a</sup>	0.72 ± 0.13 <sup>a</sup>	32 ± 5.3 <sup>b</sup>	34 ± 6.5 <sup>a</sup>	0.62 ± 0.14 <sup>a</sup>	16 ± 4.6 <sup>a</sup>
<b>Lipid</b>						
Raw	1.2 ± 0.1 <sup>d</sup>	0.50 ± 0.21 <sup>b</sup>	228 ± 79 <sup>a</sup>	6.6 ± 0.1 <sup>c</sup>	0.94 ± 0.19 <sup>a</sup>	95 ± 17 <sup>a</sup>
PNH	7.9 ± 0.1 <sup>c</sup>	0.85 ± 0.19 <sup>b</sup>	93 ± 11.04 <sup>a</sup>	13 ± 0.3 <sup>b</sup>	0.60 ± 0.15 <sup>ac</sup>	43 ± 12 <sup>b</sup>
PH	11 ± 0.2 <sup>b</sup>	1.04 ± 0.31 <sup>a</sup>	51 ± 9.69 <sup>b</sup>	13 ± 0.3 <sup>b</sup>	0.79 ± 0.21 <sup>b</sup>	50 ± 12 <sup>b</sup>
UHT	21 ± 0.7 <sup>a</sup>	0.38 ± 0.11 <sup>b</sup>	11 ± 5.1 <sup>c</sup>	38 ± 1.6 <sup>a</sup>	0.42 ± 0.13 <sup>abc</sup>	7.5 ± 4.0 <sup>c</sup>

DM, dry matter; PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. Values are means ± SEM, n = 4–6. Means with different superscript letters within a column for DM, protein, or lipid differ ( $P \leq 0.05$ ). Values are reported on a g/100 mL milk basis.  $\kappa$ , the slope of the curve, and  $\beta$ , the index for the shape of the curve, were used to determine the half gastric emptying time ( $T_{1/2}$ ). The parameter alpha values ( $\alpha_0$ ), the amount of DM, protein, or lipid in the milk before being consumed, used were: DM, 13.0 g/100 mL; protein, 3.5 g/100 mL; lipid, 4.0 g/100 mL.

The normal distribution and the homogeneity of variance were evaluated for each statistical analysis, and the difference was declared significant if  $P \leq 0.05$ . Apart from the curd strength data ( $G^*$ ), which was natural log-transformed, all other data was normally distributed and showed homogeneity of variance. For the factorial models, means were compared using an adjusted Tukey test, while for the non-linear model parameters, means were compared using a  $t$ -test.

### 3. Results

The pigs adapted well to the experimental diets and after the third day, they consumed all meals offered. One pig was excluded during the study due to coprophagia. Throughout the study, the remaining pigs were healthy.

The chemical composition of the milk treatments is shown in Table 3. The protein content of the milk samples ranged from 33 to 41 g/L, and the lipid content ranged from 33 to 44 g/L. The carbohydrate (average 46 g/L), sodium, and calcium contents were similar among milk treatments.

The gastric digestion of all milk types resulted in the formation of separable solid (curd) and liquid fractions, but the consistency and structure of the curd visually varied between treatments (Supplementary Fig. S2). After 300 min of digestion, there was insufficient gastric content in five of the six pigs fed the UHT milk to conduct any analyses.

#### 3.1. Gastric emptying of processed bovine milk

The total gastric DM (Fig. 2a), protein (Fig. 2c), and lipid (Fig. 2e) emptying rates differed ( $P \leq 0.05$ ) across milk types, but in general, they followed similar patterns. For instance, the pigs fed the UHT milk had faster ( $P \leq 0.05$ ) gastric emptying rates of DM, protein, and lipid, compared to the other milk types. Pigs that received the raw and PNH milk had a slower ( $P \leq 0.05$ ) gastric emptying of DM, protein, and lipid, compared to the UHT and PH milk types. Similar patterns were observed when only the curd was considered (Fig. 2b, d, and f). In addition, the gastric emptying of DM, protein and lipid in the liquid fraction was similar ( $P > 0.05$ ) between the pigs fed the different milk types (data not shown).

The differences in gastric emptying of the total (or curd) DM, protein, and lipid of the processed bovine milk types led to different kinetic parameters ( $\kappa$  and  $\beta$ ) and  $T_{1/2}$  and followed the pattern UHT > PH > PNH = raw (Table 4). The  $\kappa$  of DM, protein, and lipid in both the total content and curd of UHT milk was at least twice that ( $P \leq 0.05$ ) of the raw milk. The differences in  $\kappa$  and  $\beta$  for total gastric content and curd were reflected in the  $T_{1/2}$ . For instance, the pigs fed the raw milk needed 228 min to empty half of the lipid consumed, while the pigs fed the UHT milk needed 11 min. In addition, high variability was observed in the amount of total gastric lipid of the raw milk, which can be explained by large amounts of lipid in the liquid fraction. (Supplementary Figs. S5c–d).

#### 3.2. Structural changes in gastric contents of processed bovine milk

After 20 min of gastric digestion, a protein network was formed for all milk types with differences in density, structure, and lipid distribution as measured using confocal microscopy (Fig. 3) or TEM (Fig. 4). In the liquid fraction, the average size and amount of lipid droplets appeared visually lower in the PH and UHT milk types, compared to the raw and PNH milk types (Fig. 5).

After 20 min, the raw and PNH curds formed a tight, more continuous protein network with larger, less distributed pores, whereas the PH and UHT curds were looser and crumblier, with a less dense network (Fig. 3). For the raw, PNH and UHT curds, TEM in Fig. 4 supported this observation. Although the PNH curd was looser than the raw curd, after 120 min, larger empty areas were observed within the network formed in the raw curd (Fig. 4). At 300 min of digestion, the raw curd continued to be tight, whereas the protein network of the PNH curd began loosening (Fig. 3). Throughout digestion, the UHT curd network remained fragmented and open (Figs. 3 and 4).

In contrast to pigs fed PH and UHT milk, separated fat globules appeared to be suspended around, rather than inside of, the protein network in the curd of pigs fed raw and PNH milk (Fig. 3). As a result, the lipid in the raw and PNH curds formed comparatively large conglomerations, while the lipid in the PH and UHT curds showed minimal and smaller lipid conglomerations (Fig. 3). This pattern continued up to 120 min of digestion. After 180 min of digestion, the lipid conglomerations in the curd of pigs fed the raw milk remained large and intact, but the lipid conglomerations of the curd of the pigs fed PNH were reduced in size and amount (Fig. 3).

The cryo-SEM images further confirmed differences between the curd protein network formed by the digestion of raw, PH and UHT milk (Supplementary Figs. S3 and S4). For instance, at 20 and 180 min, the raw curds had fat globules embedded within pockets in the protein network, whereas the PH and UHT curds had smaller fat droplets bound to the protein network (Supplementary Fig. S4). At 180 min of digestion, the protein network around the fat globules in the raw milk curd was tighter compared to 20 min, with less space and smaller cavities visible (Supplementary Fig. S3). Over the digestion, the thickness of the protein network formed by the PH and UHT milk curds appeared to increase. However, the apparent density of the network appeared to increase for the PH curd and decrease for UHT milk curds (Supplementary Fig. S3).

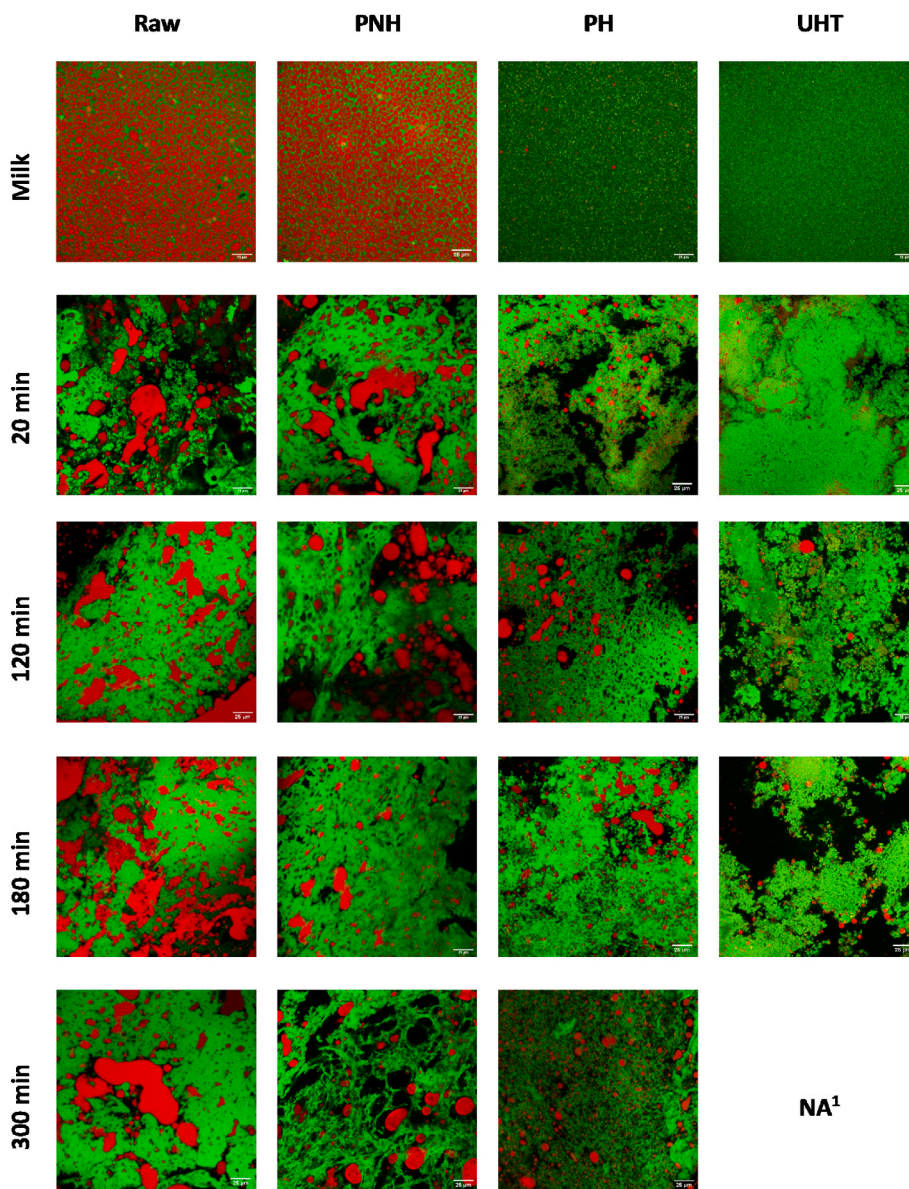


Fig. 3. Representative confocal scanning microscopy images of milk and gastric curd (solid) collected at different post-feeding times of growing pigs fed different processed bovine milk types. Protein appears green, while lipid appears red. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. <sup>1</sup>NA, no available curd sample remained at 300 min. Scale bar is 25  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Up to 120 min of digestion, the liquid fraction of the raw and PNH milk chyme contained a high amount of lipid and larger lipid conglomerations, which decreased thereafter, especially for PNH (Fig. 5). In contrast, the liquid fraction of the PH and UHT chyme showed small lipid conglomerations widely distributed throughout the sample which remained over time (Fig. 5).

### 3.3. Changes to curd strength over time

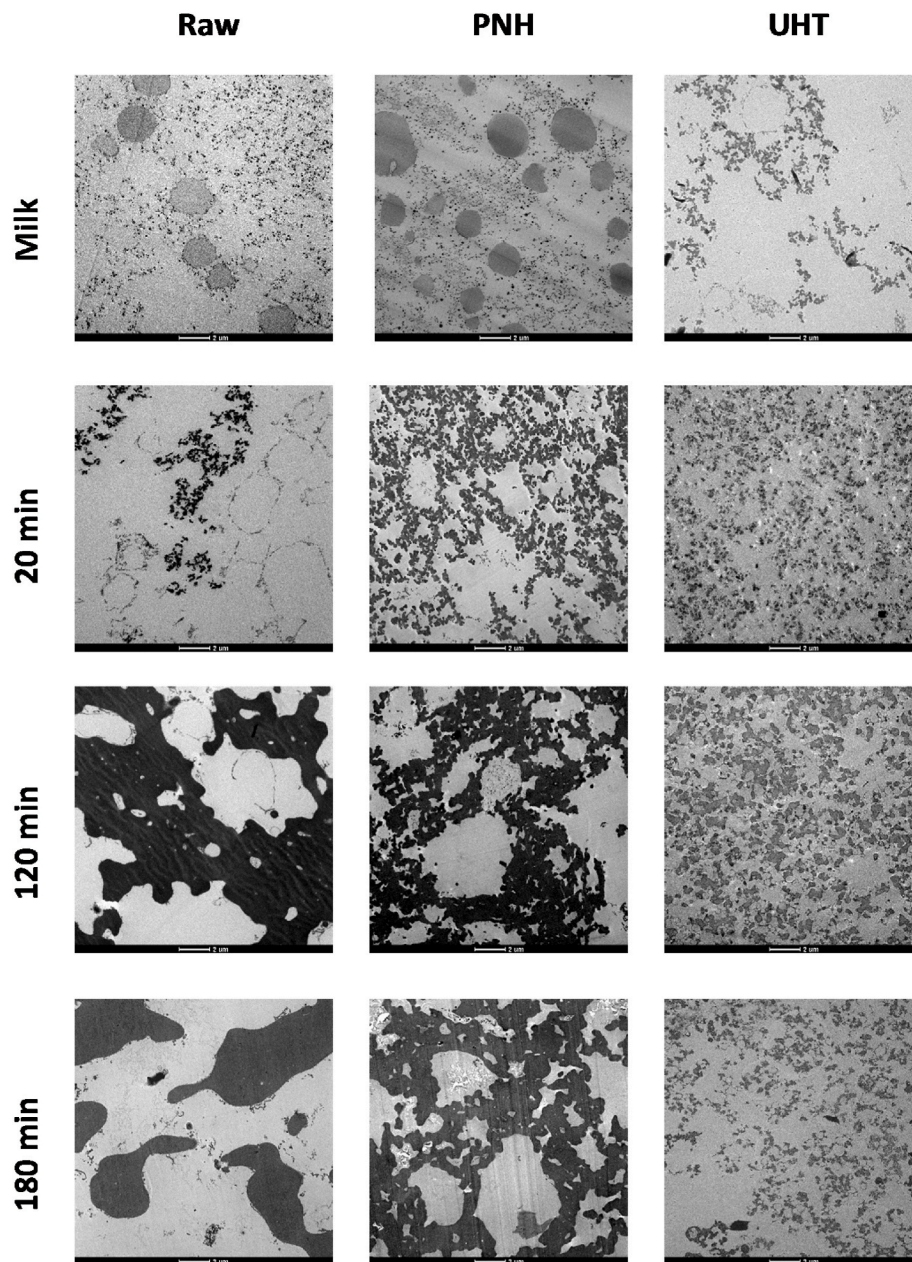
The  $G^*$  value (oscillation stress (Pa) divided by strain) was applied to indicate the strength of the curd formed during gastric digestion. During the first 120 min of digestion, the strength increased similarly between milk types (2.1–3.0 Pa/h for UHT and PNH, respectively) (Fig. 6). However, after 120 min of digestion, homogenization (PH vs PNH) and heating (raw vs PH or UHT vs PNH) led to less firm curd ( $P \leq 0.05$ ) (Fig. 6). As a result, the curd strength for the pigs fed UHT milk decreased from 4.2 Pa to 2.7 Pa after 120 min post-feeding ( $P < 0.05$ ), whereas it remained constant for the pigs fed the other milk types (Fig. 6).

### 3.4. Gastric pH during digestion

The gastric digestion of all milk types resulted in a similar sharp increase of pH of proximal and distal chyme, and of the liquid fraction (Fig. 7a, b, and c) during the first 20 min. Significant changes in chyme pH were also observed across milk types after 20 min and 120 min post-feeding ( $P \leq 0.05$ ). After 120 min until 300 min, the pH of the proximal and distal stomach chyme and liquid fraction of the pigs fed the raw milk decreased by 0.8, 1.2 and 1.1 units respectively ( $P \leq 0.05$ ), while it plateaued ( $P > 0.05$ ) for the pigs fed the PNH, PH and UHT milk types.

## 4. Discussion

This is the first *in vivo* study using the pig as an adult human model to report how heat processing and/or homogenization of whole bovine milk affects the gastric emptying rates of DM, protein, and lipid, and how these results are linked to compositional and structural changes of the solid (curd) and liquid fraction of the gastric chyme. The results support the stated hypothesis that heat-treated and homogenized bovine milk exhibit faster gastric emptying rates of DM, proteins, and lipids due to forming a softer curd with a more open protein network.



**Fig. 4.** Transmission electron microscopy images of milk and gastric curd (solid) collected at different post-feeding times of growing pigs fed different processed bovine milk types. Protein appears in the dark area, while lipid appears in the light grey area. PNH, pasteurized non-homogenized; UHT, ultra-high temperature treated homogenized.

It is important to highlight that the PNH, PH, and UHT milk originated from a different source to the raw milk, which could have affected the comparison to the raw milk, as a result of differences in protein, lipid and mineral composition. For example, differences in the amount of  $\kappa$ -casein present may impact pepsin-induced casein micelle aggregation during curd formation (Horne, 2020). In addition, 11 treatment x time combinations had a smaller sample size ( $n = 4$  or  $5$  vs  $6$ ) for some analyses due to remnant portions of the human-type evening meal received on day 10 in some animals. However, these limitations have been controlled where possible. For example, all processed milk used in this study was bulk milk from herds with similar compositions to minimize compositional variation. Some animals were given a PLFL meal to be able to consider remnants of the last human-type meal. Thus, the limitations were expected to have had minimal effects on the findings.

#### 4.1. Impact of heat treatment and homogenization on gastric emptying

Differences in gastric emptying rates of DM were apparent across milk types. Both mild (pasteurization) and strong (UHT) heat treatments combined with homogenization (raw vs PH, raw vs UHT) increased the emptying rate of total DM content and curd DM content, whereas mild heat treatment alone did not (raw vs PNH). The curd DM content represented 70% of the total gastric DM content for all treatments and post-feeding times. Similar patterns in the gastric emptying of total and curd DM content and total gastric DM content were found in piglets receiving either raw bovine, ovine, or caprine milk (Roy et al., 2022). These findings agree with an observational study with one mini-pig, where similar gastric emptying patterns of DM from raw, pasteurized, and UHT bovine milk were reported (Meisel & Hagemeyer, 1984). However, they did not report gastric curd emptying.

The curd is comprised mainly of casein (Pfeil, 1984) and lipid (Ye

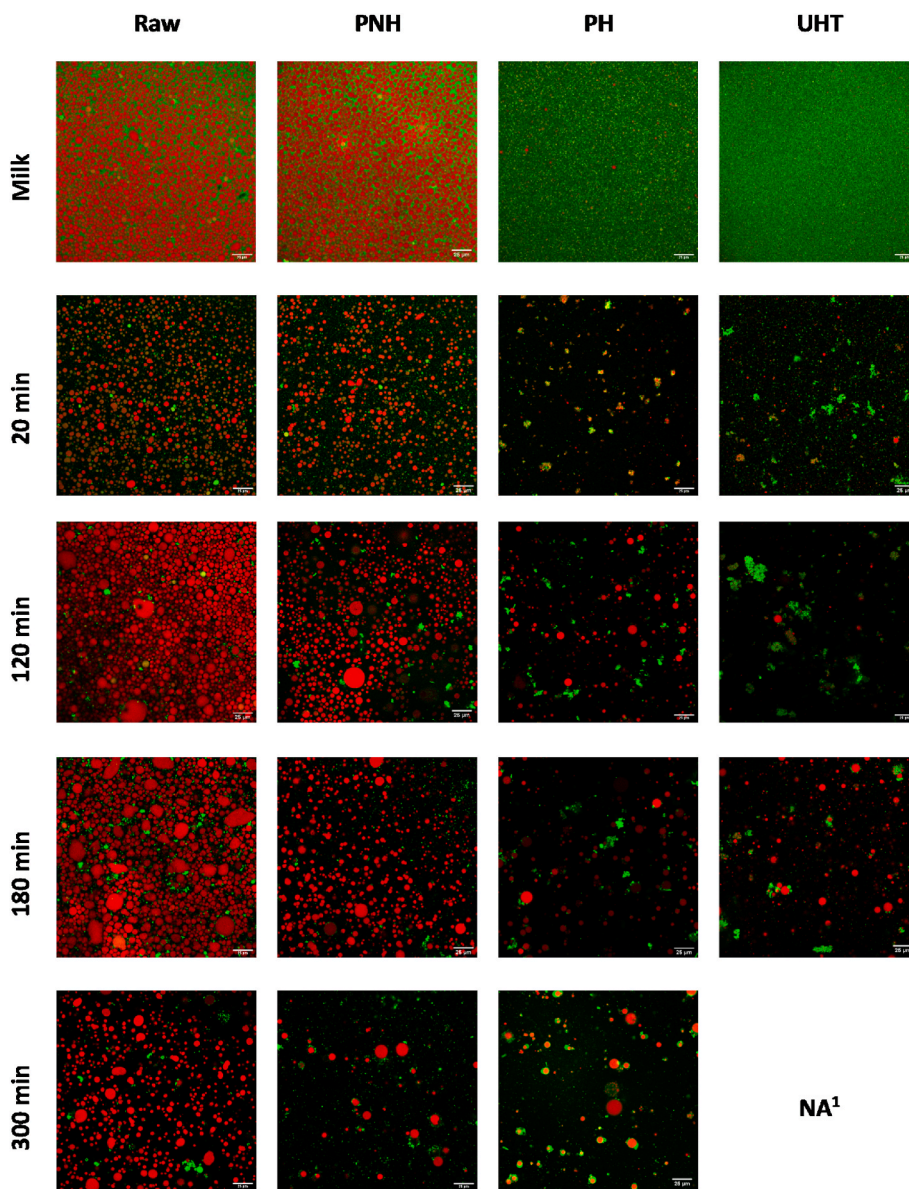


Fig. 5. Representative confocal scanning microscopy images of milk and gastric liquid fraction collected at different post-feeding times of growing pigs fed different processed bovine milk types. Protein appears green, while lipid appears red. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. <sup>1</sup>NA, no available curd sample remained at 300 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2016a), as observed here and reported elsewhere. Thus, the gastric emptying of protein and lipid in the curd can be explained by total and curd DM contents. When compared to raw milk, the gastric emptying rate of protein ( $\kappa_{\text{protein}}$ ) in the total gastric content was affected by both mild and strong heat treatment with homogenization (PH and UHT). However, in the curd, only UHT increased  $\kappa_{\text{protein}}$ . These findings imply that the combination of heat treatment and homogenization increased the total gastric protein emptying, while only strong heat treatment influenced gastric curd emptying.

Similar patterns were observed for  $\kappa_{\text{lipid}}$ , suggesting that the combination of heat treatment and homogenization impacted total gastric  $\kappa_{\text{lipid}}$ , whereas the heat treatment alone affected curd  $\kappa_{\text{lipid}}$ . For example, the  $\kappa_{\text{lipid}}$  of UHT was at least two-fold that of PNH and PH in both the total gastric content and the curd, but differences between  $\kappa_{\text{lipid}}$  for PH and PNH were only significant for total gastric content.

The faster gastric emptying of protein and lipid into the small intestine due to processing might result in faster absorption of milk nutrients. It has been shown that the amount of digested protein (nitrogen) entering the small intestine modulates the rate of absorption in the first half of the small intestine (Montoya et al., 2018). In addition, studies have shown a faster appearance of amino acids in peripheral blood for

healthy human adults who consumed UHT milk compared to pasteurized milk (Lacroix et al., 2008), which could have implications for protein synthesis, insulin regulation and satiety (Dangin et al., 2001; Hall et al., 2003; Tang et al., 2009).

#### 4.2. Impact of processing on curd structure and link to gastric emptying

The differences observed in gastric emptying rates of DM, protein, and lipid could be ascribed to changes in the macro- and micro-structure of the formed curds. The formation of curd from all milk types was mainly induced by the pepsin hydrolysis of  $\kappa$ -casein, which led to the coagulation of casein micelles at around pH 6 after 20 min of gastric digestion. With the further pH decrease to pH 2.5–4 at 300 min of gastric digestion, the structure of the clot became denser, likely due to gastric acid diffusing into the clot and the impact of gastric motility on the curd. In addition, as pH decreases, pepsin activity increases which could have continued altering the structure of curd. These changes in the curd structure during gastric digestion were dependent on the processing condition of milk as the initial structure of the curd was different between the differently processed milk types.

The curd of pigs fed raw, PNH, and UHT milk types formed different

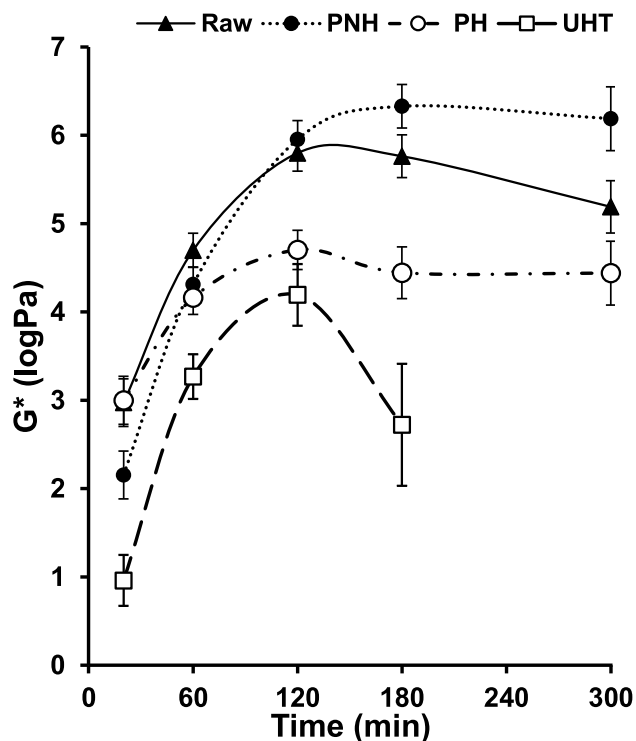


Fig. 6. Strength ( $G^*$ ) of curds collected at different post-feeding times of growing pigs fed different processed bovine milk types. Values are means  $\pm$  standard errors,  $n = 5-6$ . PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. No available UHT curd sample remained at 300 min.

protein networks. When compared to raw milk, heat treatment alone (PNH) led to a more fractionated protein network, and the addition of homogenization (PH) followed by high-intensity heat treatment (UHT) led to progressively more brittle protein networks. Similar results have been shown in an *in vitro* dynamic model, and in rats fed pasteurized and UHT milk and euthanized at various timepoints (Ye et al., 2019).

The differences in protein networks were likely partially due to changes in the initial structure of casein micelles, the bonding of whey proteins with the caseins due to heat treatment and the enzymatic hydrolysis of the resulting protein structures during gastric digestion (Corredig & Dalgleish, 1999; Dalgleish, 1990; Ye et al., 2016b). Other studies also show that heat treatment denatures whey proteins and leads to the formation of  $\kappa$  casein-whey protein aggregates, both free and

associated with the surface of the casein micelles (Anema & Klostermeyer, 1997; Anema & Li, 2003). These aggregates reduce the ability of casein micelles to associate with one another, through steric hindering which in turn results in the formation of a softer, more porous curd (Ye et al., 2016a, 2016b, 2019).

Mild and strong heat treatments combined with homogenization (PH and UHT) led to the formation of a soft curd. However, the extent of heat treatment had a stronger influence on curd disintegration: UHT curd weakened rapidly after 120 min, PH curd did not. Visually, the protein network of the PH curd began disintegrating after 120 min, although the curd strength was maintained. There were no differences between the raw and PNH curd strength during the formation of the curd (up to 120 min) or  $\kappa_{\text{protein}}$ . However, at 120 min, when the strength of the curds was the highest, it was correlated to  $\kappa_{\text{protein}}$ , suggesting that it is one of the factors influencing  $\kappa_{\text{protein}}$ .

The faster curd disintegration and gastric emptying rate of protein could be explained by the weaker, more open curd structure resulting from the protein denaturation during heat treatment. Based on other studies, the more open structure could allow a greater surface area for enzymatic activity (Van Hooydonk, 1987) and a greater ingress of gastric fluid containing pepsin and hydrochloric acid (Kalantzi et al., 2006) into the curd. In another study, pigs fed raw, PH, and UHT bovine milk (one pig per diet and timepoint) had slower hydrolysis of casein in a stronger curd, resulting in slower gastric emptying of casein, compared to a weaker curd (Pfeil, 1984).

The pigs fed the PH or UHT milk treatment had smaller fat globules as homogenization disrupted the native fat globules forming small fat droplets (Robson & Dalgleish, 1984), resulting in a looser curd structure, and faster total gastric  $\kappa_{\text{lipid}}$ , compared to the raw and PNH milk treatments. The cryo-SEM images of the gastric curd showed fat globules suspended amongst networks formed by the protein matrix in the raw milk curd. For the homogenized milk (PH and UHT) treatments, the small fat droplets were incorporated into the protein network, which occurred during curd formation via casein micelle aggregation by adsorption onto and around the fat droplets (Ye et al., 2017). The wide distribution of small fat droplets incorporated throughout the PH and UHT curd protein networks may have influenced curd integrity and strength by restricting the linking of the casein matrix (Gallier et al., 2012), as shown in curd used for cheese production (Kelly et al., 2008) and under gastric conditions (Wang et al., 2018). The smaller surface area of the homogenized fat droplet may have also impacted curd disintegration and subsequent emptying by indirectly increasing proteolysis, as shown in an *in vitro* comparison of non-homogenized and homogenized infant formula (Bourlieu et al., 2015).

The curd from the pigs fed the unhomogenized (raw and PNH) milk treatments had greater gastric lipid coalescence due to tightening of the casein matrix (Gallier et al., 2013). In addition, the coalesced lipids of these milk types were entrapped within the rigid and intact protein

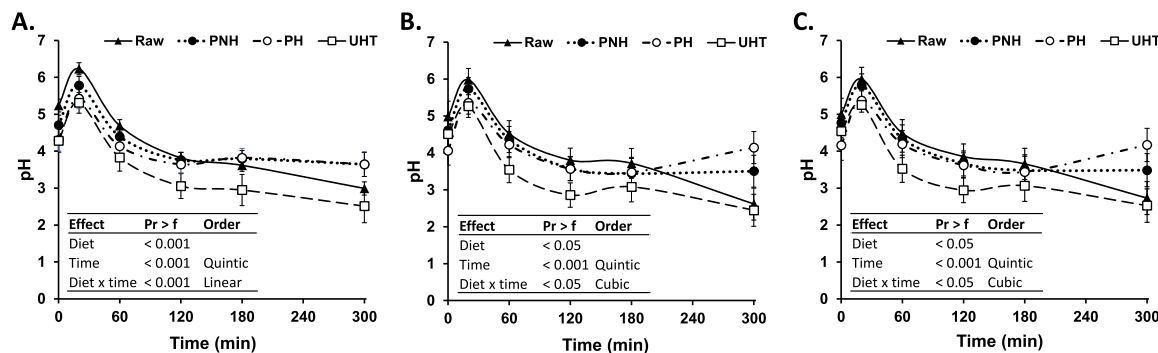


Fig. 7. Changes in gastric pH values observed at different post-feeding times of growing pigs fed different processed bovine milk types. Values are means  $\pm$  standard errors,  $n = 5-6$ . A, proximal chyme; B, distal chyme; C, liquid fraction. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized.

matrix of the curd, resulting in a higher concentration of lipid remaining in the curd and a subsequent smaller  $\kappa_{\text{lipid}}$  when compared to UHT. The  $t_{1/2 \text{ lipid}}$  of raw curd was two-fold larger than that of PNH curd, indicating that pasteurization increases the release of lipid into the small intestine.

## 5. Conclusions

This study demonstrated the impacts of commercial processing treatments of bovine milk on the gastric emptying of DM, protein, and lipid and curd formation in a pig model of adult human metabolism. The UHT milk treatment had the greatest impact on gastric emptying of DM and protein: it was faster with UHT treatment followed by PH and PNH treatments compared to raw milk. In addition, the gastric emptying of lipid was also faster in the pigs fed the PNH treatment than the raw milk treatment.

The differences in gastric emptying of DM, protein and lipid between processing treatments were ascribed to differences in the structure of the curds formed and their disintegration. In particular, heat treatments resulted in a looser protein network, and homogenization led to the incorporation of restructured lipid droplets into the curd matrix.

This study provided a new understanding of the gastric emptying of processed bovine milk DM, protein, and lipid. Whether these effects can improve nutrient absorption in the small intestine and subsequent release into the systemic circulation for metabolism remains to be confirmed.

## Author statement

Natalie Ahlborn: methodology, investigation, formal analysis, writing – original draft, visualization, project administration; Carlos Montoya: conceptualization, methodology, investigation, formal analysis, writing – review and editing, project administration; Suzanne Hodgkinson: investigation, writing – review and editing; Anant Dave: resources (cryo-SEM); Aiqian Ye: validation (microstructure), writing – review and editing; Linda Samuelsson: writing – review and editing; Nicole Roy: funding acquisition, conceptualization, writing – review and editing; Warren McNabb: funding acquisition, resources, conceptualization, writing – review and editing. All authors have read and approved the final manuscript.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2022.108380>.

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