



Comparative lipidomics analysis of in vitro lipid digestion of sheep milk: Influence of homogenization and heat treatment

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ABSTRACT

This study investigated the changes in sheep milk lipids during in vitro gastrointestinal digestion in response to heat treatment (75°C/15 s and 95°C/5 min) and homogenization (200/50 bar) using lipidomics. Homogenized and pasteurized sheep milk had higher levels of polar lipids in gastric digesta emptied at 20 min than raw sheep milk. Intense heat treatment of homogenized sheep milk resulted in a reduced level of polar lipids compared with homogenized–pasteurized sheep milk. The release rate of free fatty acids during small intestinal digestion for gastric digesta emptied at 20 min followed the order: raw \leq pasteurized < homogenized–pasteurized \leq homogenized–heated sheep milk; the rate for gastric digesta emptied at 180 min showed a reverse order. No differences in the lipolysis degree were observed among differently processed sheep milks. These results indicated that processing treatments affect the lipid composition of digesta and the lipolysis rate but not the lipolysis degree during small intestinal digestion.

Key words: fat globules, lipidome, lipid composition, fatty acids, lipolysis

INTRODUCTION

The lipolysis of milk fat is an interfacial process that is dependent on the milk fat globule (MFG) surface composition, the MFG size, and the adsorption of lipolytic enzymes to the surface. The surface of native MFG is composed of MFG membrane (MFGM), which is a trilayer of phospholipids naturally encapsulating MFG and contains a variety of lipids, proteins, and carbohydrate moieties (Zhao et al., 2019; Brink et al., 2020). The surface composition and the size of MFG can be altered by processing treatments (such as heat treatment and homogenization). The changes in the surface composition and the size of MFG can affect

their interactions with digestive enzymes within the gastrointestinal tract, thereby influencing the digestion of MFG (Thum et al., 2023).

Previous studies have shown that the surface structure of MFG released from the stomach can be affected by the processing treatment (such as heat treatment, homogenization, and their combination; Zhao et al., 2019; Pan et al., 2021b). Pan et al. (2021b) showed that the MFG of raw and pasteurized sheep milks released from the gastric phase remained almost unchanged at an early stage of gastric digestion, but appeared to coalesce and become larger at longer digestion times. However, the MFG of homogenized and pasteurized or heated sheep milks remained small and were well embedded within protein or peptide particles toward the end of gastric digestion when released to the small intestine. Therefore, the surface layer of the MFG released from the stomach could be different in differently treated milks, which might in turn have an effect on lipid digestion in the small intestinal phase.

The effect of processing treatments on the digestion of MFG by pancreatic lipase has been studied extensively (Ye et al., 2010; Islam et al., 2017; Liang et al., 2017; Zhao et al., 2019, 2022). The homogenization of cow milk has been found to increase the initial digestion rate of the MFG compared with raw cow milk but not to affect the final extent of lipid digestion; heat treatment of the homogenized milk decreased the initial lipid digestion rate and the final extent of lipid digestion (Liang et al., 2017; Zhao et al., 2019). The effect of homogenization on lipid digestion has been attributed to the reduced MFG size, the increased surface area of the MFG, and the reduced MFGM area on the MFG surface, which allows more pancreatic lipase to bind with the interface of MFG and to easily hydrolyze the core fat (Ye et al., 2010). The subsequent heat treatment might alter the interfacial structure of the MFG (such as the binding of denatured whey proteins with the MFG surface), thereby influencing the adsorption of lipase on to the surface of the MFG and thus the lipolysis rate (Liang et al., 2017). These studies confirm that homogenization- and heat-induced alterations in the size and the surface composition of MFG could

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affect lipid digestion in the small intestinal phase to different extents (Michalski, 2009; Zhao et al., 2019).

The lipid digestion of cow milk has attracted extensive research; however, in comparison, knowledge of the lipid digestion of sheep milk is quite limited. Sheep milk is of high nutritional value and has potential for the development of nutritional and functional milk products, attracting a growing number of consumers worldwide. Investigating the lipid digestion of sheep milk is essential to advance our understanding of the unique digestion characteristics of sheep milk lipids. As the composition differs between sheep milk and other types of milk, such as cow milk (Balthazar et al., 2017), this study will fill the knowledge gap and provide insight into how sheep milk lipid are digested. Pan et al. (2021a) investigated the *in vitro* lipid digestion characteristics of human, cow, and goat milks and found different lipolysis degrees and released fatty acid compositions among the different milks. This was attributed to different MFG sizes and fatty acid compositions (Pan et al., 2021a). Previous studies have shown that sheep milk has different MFG sizes and lipid compositions from cow, goat, and human milks (Balthazar et al., 2017; Roy et al., 2020), which probably leads to different lipid digestion behaviors of sheep milk. Therefore, in-depth identification of the lipid constituents in the milk lipidome during gastrointestinal digestion is necessary for a more detailed understanding of the lipid digestion behavior in sheep milk.

The present study aimed to provide a better understanding of how the lipid digestion behavior of sheep milk is affected by different processing treatments. It examined the effects of different heat treatments (pasteurization at 75°C/15 s and heating at 95°C/5 min) and homogenization (200/50 bar) on the lipid digestion behavior of sheep milk using a human gastric simulator for the gastric phase and a pH-stat for the small intestinal phase. The lipid composition, the lipolysis rate, and the lipolysis degree among the differently processed sheep milks during *in vitro* gastrointestinal digestion were compared; this would provide information for the formulation of sheep milk products.

MATERIALS AND METHODS

Only routine animal procedures (milking) were conducted in this study, so Institutional Animal Care and Use Committee approval was not required.

Sample Preparation and Chemicals

Fresh sheep milk was collected from 2 companies (Spring Sheep Milk Co. and Maui Milk Co. Ltd., Waikato, New Zealand). The fresh sheep milk was pasteurized at 75°C for 15 s. Homogenized milk was

obtained by homogenizing at 200/50 bar and 65°C in a 2-stage valve homogenizer in the Massey University pilot plant. In the experiments, the homogenized milk was pasteurized at 75°C for 15 s in a pilot-scale indirect UHT plant (Alfa-Laval) to make homogenized and pasteurized (**homo-past**) milk, and homogenized and heated (**homo-heat**) milk was obtained by heating the homogenized milk to 95°C in the UHT plant and then transferring to a water bath and holding for 5 min. After heat treatment, the milks were immediately cooled to 20°C in cold running water. The average fat globule sizes, which were determined using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK), were $d_{43} = 4.52 \pm 0.14 \mu\text{m}$ and $d_{43} = 0.62 \pm 0.07 \mu\text{m}$ for the unhomogenized sheep milk and the homogenized sheep milk, respectively.

Pancreatin from porcine pancreas (EC 232-468-9; catalogue no. P7545, Sigma Chemical Co., St. Louis, MO) had a trypsin activity of 6.1 ± 0.2 units/mg solid, as tested in preliminary experiments. All other chemicals were of analytical grade and were purchased from BDH Chemicals (BDH Ltd., Poole, UK) or Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. All solutions were prepared using Milli-Q water purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA).

Simulated intestinal fluid (**SIF**) was prepared at a $1.25 \times$ concentration according to the method described by Brodkorb et al. (2019). The SIF consisted of 6.8 mM KCl, 0.8 mM KH_2PO_4 , 123.4 mM NaCl, and 0.33 mM $\text{MgCl}_2(\text{H}_2\text{O})_6$, and its pH was adjusted to 7 using a 6 M HCl solution. $\text{CaCl}_2(\text{H}_2\text{O})_2$ was added into the SIF immediately before the digestion experiment to achieve a final concentration of 0.6 mM to avoid precipitation during storage at -20°C . The SIF was then supplemented with water to achieve a $1 \times$ concentration.

In Vitro Gastric Digestion

As this study is a follow-up to our previously published study on the gastric digestion of differently processed sheep milks, the chemicals for the gastric digestion are as described in our previous publication reported by Pan et al. (2021b). Simulated salivary fluid (**SSF**) and simulated gastric fluid (**SGF**) were prepared at $1.25 \times$ concentration according to the method described by Brodkorb et al. (2019) with slight modifications. The SSF was prepared based on the salt composition only, because milk contains no starch. The SGF (pH 1.5) did not include gastric lipase because this study focused on the lipid digestion in internal phase. The *in vitro* gastric digestion was performed using a human gastric simulator (**HGS**), as developed by Kong and Singh (2010). The gastric digestion procedure is as described

in our previous publication. The gastric emptying rate was 3.6 mL/min and the emptied digesta was removed from the bottom of the stomach chamber at 20-min intervals to accurately control the gastric emptying. The gastric digestion time was up to 240 min, and the digesta removed from the HGS at each time interval was filtered through a mesh with a pore size diameter of 1 mm for further analysis; the solid mass with size greater than 1 mm was put back into the HGS for further digestion. The gastric digesta emptied at 20 (**G20**) and 180 (**G180**) min were used for the *in vitro* small intestinal digestion.

In Vitro Small Intestinal Digestion

The *in vitro* small intestinal digestion of the sheep milks was carried out using a pH-stat (SI Analytics Titroline 7000 Titrator, Xylem Inc., Rye Brook, NY). Before digestion, the digesta emptied from the HGS at 20 and 180 min of gastric digestion were adjusted to pH 7.5 using 1 M NaOH solution. The digestion procedure for the intestinal phase followed the method of Brodtkorb et al. (2019). An aliquot (25 mL) of the gastric digesta was mixed with prewarmed (37°C) SIF electrolyte (25 mL) to reach a ratio of 1:1, and the pH of the mixture was adjusted to 7.0. The digestion was conducted for 2 h, with the pH being maintained at 7.0 by automatic pH-stat titration with a 0.05 M NaOH solution. To better estimate the volume of NaOH consumed during the lipid digestion, simulated intestinal digestions of bovine skim milk containing 0% to 4% protein were carried out to build a standard curve. The volume of NaOH consumed because of the presence of SIF components and proteins was estimated using the standard curve. The volume of NaOH solution required to neutralize the pH of the digesta was recorded and was used to calculate the release of free fatty acids (**FFA**) during the lipid digestion using the following equation:

$$\frac{\mu\text{mol}_{\text{fatty acids}}}{\text{mL}_{\text{gastric digesta}}} = \frac{[V_{\text{NaOH for sample}}(t) - V_{\text{NaOH for blank}}(t)] \times C_{\text{NaOH}} \times 1,000}{V_{\text{gastric digesta}}}$$

where $V_{\text{NaOH}}(t)$ is the volume of NaOH solution added into the reaction mixture at digestion time t , C_{NaOH} is the molar concentration of the NaOH solution (0.05 M), and $V_{\text{gastric digesta}}$ is the volume of the gastric digesta used (25 mL).

A site-filling model described by Ye et al. (2013) was used to calculate the rate constant of FFA release, as follows:

$$\ln\left(\frac{C_{\text{max}} - C_t}{C_{\text{max}}}\right) = -k_1 t,$$

where C_t is the molar concentration of released FFA at time t (min), C_{max} is the molar concentration of released FFA at the end of the digestion (120 min), and k (min^{-1}) is the rate constant.

Lipidomics

The milk and digesta samples were thawed and well shaken for 1 min using a vortex mixer before aliquoting. A biphasic liquid–liquid extraction method was used for untargeted metabolomics, and the lower organic phase containing extracted lipids was obtained for a lipidomic profile using liquid chromatography coupled to mass spectrometry (**LCMS**). Briefly, 300 μL of milk sample and 800 μL of prechilled (-20°C) chloroform:methanol (50:50, vol/vol) were added into a 2-mL Eppendorf tube and vortex mixed for 30 s; the tube was then placed in a freezer (-20°C) to precipitate proteins. After 60 min, this mixture was mixed with 400 μL of water, agitated for 30 s, and centrifuged at 11,000 rev/min and 4°C for 10 min using an Eppendorf centrifuge 5427 R (Eppendorf AG, Germany). A 200- μL aliquot of the lower organic layer was evaporated to dryness under a stream of nitrogen and stored at -80°C before analysis. Pooled quality control (**QC**) samples for the differently processed sheep milks were prepared by combining 50 μL of the lower organic phase from each sample of raw, pasteurized, homo-past, or homo-heat sheep milk in a new tube. The pooled QC samples were well-mixed and dried under a stream of nitrogen, and these dried samples were stored at -80°C until analysis.

The lipid extracts were analyzed using a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography system coupled to a Shimadzu LCMS-9030 mass spectrometer. A 2-mL sample was injected into a Waters CSH-C18 column (2.1 mm \times 100 mm, 1.7 μm particle size) and the temperature of the column oven was 60°C . The chromatographic conditions were as follows: total run time, 15 min; flow rate, 400 $\mu\text{L}/\text{min}$; solvent A, 10 mM ammonium formate and a mixture of water, acetonitrile, and isopropanol in a ratio of 5:3:2 (vol/vol/v); solvent B, 10 mM ammonium formate and a mixture of water, acetonitrile, and isopropanol in a ratio of 1:9:90 (vol/vol/v). The solvent gradient program was as follows: 10% to 45% solvent B (0–2.7 min), 45% to 53% solvent B (2.7–2.8 min), 53% to 65% solvent B (2.8–9.0 min), 65% to 89% solvent B (9.0–9.1 min), 89% to 92% solvent B (9.1–11.0 min), and 92% to 100% solvent B (11.0–11.1 min); the sample was held for 0.8 min (11.1–11.9 min) before being returned to the starting conditions of 10% solvent B in 0.1 min (11.9–12.0 min); before injection of the next sample, the column was re-equilibrated under the starting conditions for 15 min (Abshirini et al., 2021). Mass spectrometry analy-

sis was performed in positive ion mode. The following mass spectrometer conditions were used: gas temperature, 150°C; nebulizing gas flow rate, 2.0 L/min; heater gas flow rate, 10 L/min; interface temperature, 300°C; drying gas flow rate, 10 L/min; desolvation line temperature, 250°C; heater block temperature, 400°C; source voltage, +4.0 kV; sheath gas flow rate, 10 L/min. Spectra were obtained over the range 250 to 1,250 m/z , and the data-independent acquisition data were collected in 20 m/z windows from 300 to 1,100 m/z . High-purity nitrogen was used for the drying and collision gases.

Data Processing

Data processing of the untargeted LCMS lipidomics data were performed using the untargeted data processing software package MSDIAL (v. 4.90; <http://prime.psc.riken.jp/compms/msdial/main.html>), which contains the LipidBlast database internally (v. 2022, <https://fiehnlab.ucdavis.edu/projects/LipidBlast>; Tsugawa et al., 2015). The data-independent acquisition spectra were used to identify the aligned peaks. The lipidomic features were searched against the built-in lipid library in silico-generated lipid fragmentation spectra. The locally weighted scatterplot smoother regression analysis and the pooled QC samples were used to correct per-feature run-order and normalize the resultant peak intensity table. Features within the pooled QC samples with an average QC-to-blank sample ratio of less than 5 and a CV of 30% were removed.

FFA Analysis

The individual FFA released from all lipids (including FFA, monoglycerides [MG], diglycerides [DG], and triglycerides [TG]) in the small intestinal digesta were extracted using the Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 30 mL of the digesta was mixed with 15 mL of chloroform:methanol (1:2, vol/vol) and then vortexed for 30 s. The mixture was subsequently centrifuged at $3,500 \times g$ for 5 min using a bench centrifuge Heraeus Multifuge X3R (Thermo Fisher Scientific Inc., Waltham, MA) for complete separation. After collecting the organic phase, the water phase was re-extracted twice following the same procedure. The organic phases of 3 extractions were pooled together and dried using an evaporator (Thermo Fisher Scientific, Rockford, IL). The dried lipids were dissolved by adding 4 mL of methanol. The lipid extracts from triplicate samples were pooled together for analysis.

The total amount of fatty acids (TFA) was analyzed using the method described by Zhu et al. (2013) with slight modifications. Briefly, 200 μ L of the lipid ex-

tract was transferred into a 10-mL screw-cap glass tube and then mixed with 0.5 mL of internal standard (100 mg nonadecanoic acid/mL heptane), 0.7 mL of 10 M NaOH, and 5 mL of methanol. The tube was incubated in a water bath at 55°C for 1.5 h with handshaking for 5 s every 20 min. It was cooled to room temperature by immersion in tap water after incubation, and 0.58 mL of 12 M H₂SO₄ was added to the tube. The tube was well-mixed and incubated in a water bath for another 1.5 h at 55°C, with vigorous handshaking for 5 s every 20 min. The sample was then centrifuged at $3,500 \times g$ for 10 min at 20°C. The heptane layer containing fatty acid methyl esters was transferred into a 350 μ L glass insert fitted into an autosampler vial. The vial was capped and stored at -18°C before gas chromatographic analysis.

The ester form of fatty acids (EFA), including MG, DG, and TG forms, of the lipid extracts was determined using the sodium methoxide transesterification method. Briefly, 200 μ L of the lipid extract was mixed with 0.5 mL of internal standard and 0.5 mL of sodium methoxide (1% in methanol) in a glass tube. The capped tube was then incubated in a 50°C water bath for 60 min with handshaking for 5 s every 20 min. The sample was cooled to room temperature and then mixed with 5 mL of 2% glacial acetic acid solution using a vortex mixer. After 10 min, it was centrifuged at $1,000 \times g$ for 10 min at 20°C. The upper heptane layer was transferred into a 350 μ L glass insert fitted into an autosampler vial and stored at -18°C before gas chromatographic analysis. Using this method, which excluded all FFA from the lipid extracts, the FFA content was obtained by subtracting the EFA content from the TFA content of the same extract. All analyses were done in triplicate.

The amount of each individual FFA was calculated as

$$\text{FFA}_i = \text{TFA}_i - \text{EFA}_i,$$

where FFA_i is the amount (mol) of the individual FFA, TFA_i is the total amount (mol) of the individual fatty acid, and EFA_i is the amount (mol) of the ester form of the individual fatty acid.

Gas Chromatographic Analysis of Fatty Acid Methyl Esters

The compositions of the fatty acids extracted were determined by gas chromatography using an Agilent 7890 system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA) and a capillary column (Supelco Park, Bellefonte, PA). The helium carrier gas flow rate was 20 cm/s and the column head pressure was 76 kPa. The oven temperature program was as follows: level 1, 180°C held for 5 min;

level 2, 210°C at 1°C/min and then held for 25 min. The injector and detector temperatures were set at 270°C. The fatty acids were represented as the percentage of the total fatty acid weight within each sample.

Statistical Analysis

The peak intensity of each individual fatty acid was converted to a relative proportion of the total lipids. A 2-way ANOVA test followed by multiple comparisons were used to verify differences in the abundances of the fatty acids in sheep milk and its digesta. These analyses were carried out using GraphPad Prism v. 8.4.0 software (GraphPad Software). The lipidomics data were transformed by generalized log-transformation and autoscaling to correct for heteroscedasticity, to reduce the skewness of the data, and to reduce mask effects. Principal component analysis (PCA) identified differential lipid metabolites among the differently treated sheep milks. The heatmap was clustered by Euclidean distance and Ward's minimum variance method. The PCA and the heatmap analysis were produced using MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>).

RESULTS AND DISCUSSION

Comparison of Lipidomes of Differently Processed Sheep Milks

Effect of Processing Treatments on The Lipidome of Sheep Milk. In the milk samples, 126 lipids from 2 lipid classes were identified, i.e., 125 TG and one phosphatidylcholine (PC). Differences in the lipidomic composition after different processing treatments of the sheep milk are shown in Figure 1. Four Types of sheep milk were used, each type included 3 replicates. In total, therefore, the full data sets for 12 sheep milk samples were included in the PCA. The PCA of the lipidomics data provided evidence of compositional differences among the differently treated sheep milks. Notably, the unprocessed (raw sheep milk) and the most processed (homo-heat sheep milk) samples were on opposite sides of the component 1 axis (principal component 1), with the other (pasteurized and homo-past) sheep milks sitting more in the center of principal component 1 (Figure 1A). There appeared to be minor differences among the differently processed sheep milks but these were not statistically significant ($P > 0.05$ from ANOVA of intertypes comparison). The heatmap showed the top 60 lipids of the differently processed sheep milks. The raw and the processed (i.e., pasteurized, homo-past, and homo-heat) sheep milks were grouped into 2 different clusters; 37 lipids were more abundant in the raw sheep milk than in the

processed sheep milks, and ~95% of these lipids were TG with a carbon number less than 50. In contrast, the abundances of the remaining 23 lipids were higher in the pasteurized, homo-past, and homo-heat sheep milks than in the raw sheep milk; ~65% of these lipids were TG with a carbon number greater than 50. Previous studies showed that heat treatment of milk promoted the hydrolysis of TG and produced more DG and FFA (Xu et al., 2020), and that a higher intensity of heat treatment could increase the hydrolysis of the TG (Zhang et al., 2022). This could have been due to the hydrolysis of the TG by heat-resistant lipases from contaminating bacteria and heat damage to the MFGM that enhanced the exposure of TG to lipase and aggravated the hydrolysis of the TG (Jukkola et al., 2018, Zhang et al., 2022). Therefore, the lipid compositions of the differently processed sheep milks could have been slightly altered by the heat-induced hydrolysis of the TG.

Comparison of Lipidomes of The Gastric Digesta. Lipidomics analysis detected 150 lipid species from 6 different lipid classes (i.e., 131 TG, 6 PC, 5 DG, 4 lysophosphatidylcholines [LPC], 3 sphingomyelins [SM], and one phosphatidylethanolamine [PE]). Figure 2 shows the differences in the lipidomes of the gastric digesta obtained from the differently processed sheep milks and at the different emptying times of 20 and 180 min. Three Types of sheep milk (including raw, homo-past and homo-heat sheep milk) were used, each type included 2 samples obtained from 20 and 180 min of gastric digestion, and each time point included 3 replicates. In total, therefore, the full data sets for 18 sheep milk samples were included in the PCA. The G20 and the G180 were fully separated from each other in the raw and homo-past sheep milks but were partially separated in the homo-heat sheep milk (Figure 2A), suggesting that the lipid composition of the gastric digesta was different at the different emptying times. The heatmap shows the 65 significantly ($P < 0.05$ from an ANOVA of intertypes comparison) different lipids of the gastric digesta (Figure 2B). In the raw sheep milk, the G20 contained higher abundances of 56 lipids (43 TG and 13 polar lipids) but lower abundances of 7 lipids (all TG) than the G180; the homo-past sheep milk showed lower abundances of 43 lipids (all TG) but higher abundances of the remaining 22 lipids (13 polar lipids, 7 TG, and 2 DG) in the G20 than in the G180; the homo-heat sheep milk showed lower levels of 43 lipids (all TG) and higher levels of 7 lipids (all TG) in the G20 than in the G180. These results suggested that the gastric digestion time had a significant effect on the lipid composition of the gastric digesta, which could in turn have influenced the lipid digestion in the small intestinal phase.

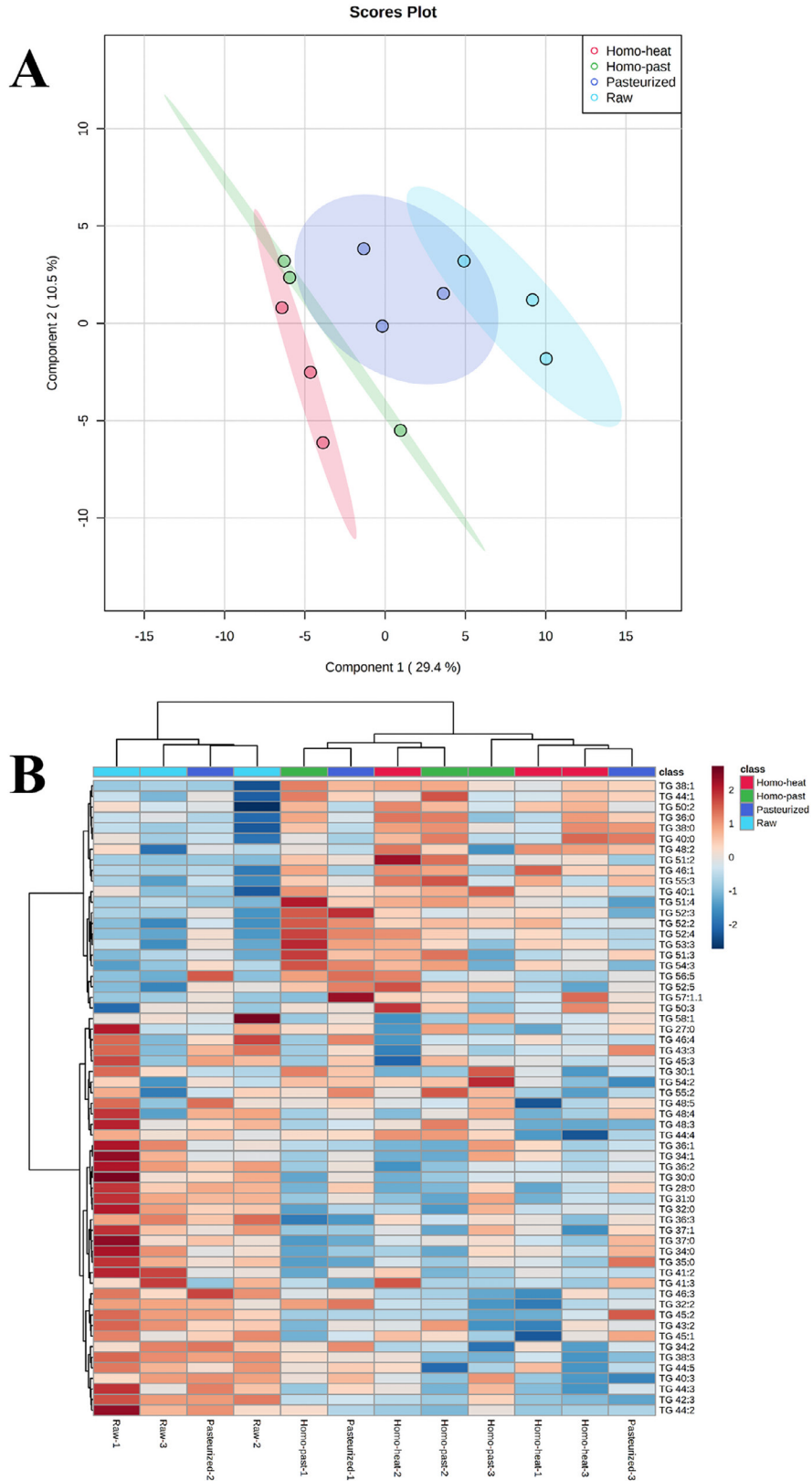


Figure 1. Lipidome differences among raw, pasteurized, homogenized, and pasteurized (homo-past), and homogenized and heated (homo-heat) sheep milks. (A) Principal component analysis and (B) heatmap of all lipids in the differently processed sheep milks. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable). TG = triglycerides.

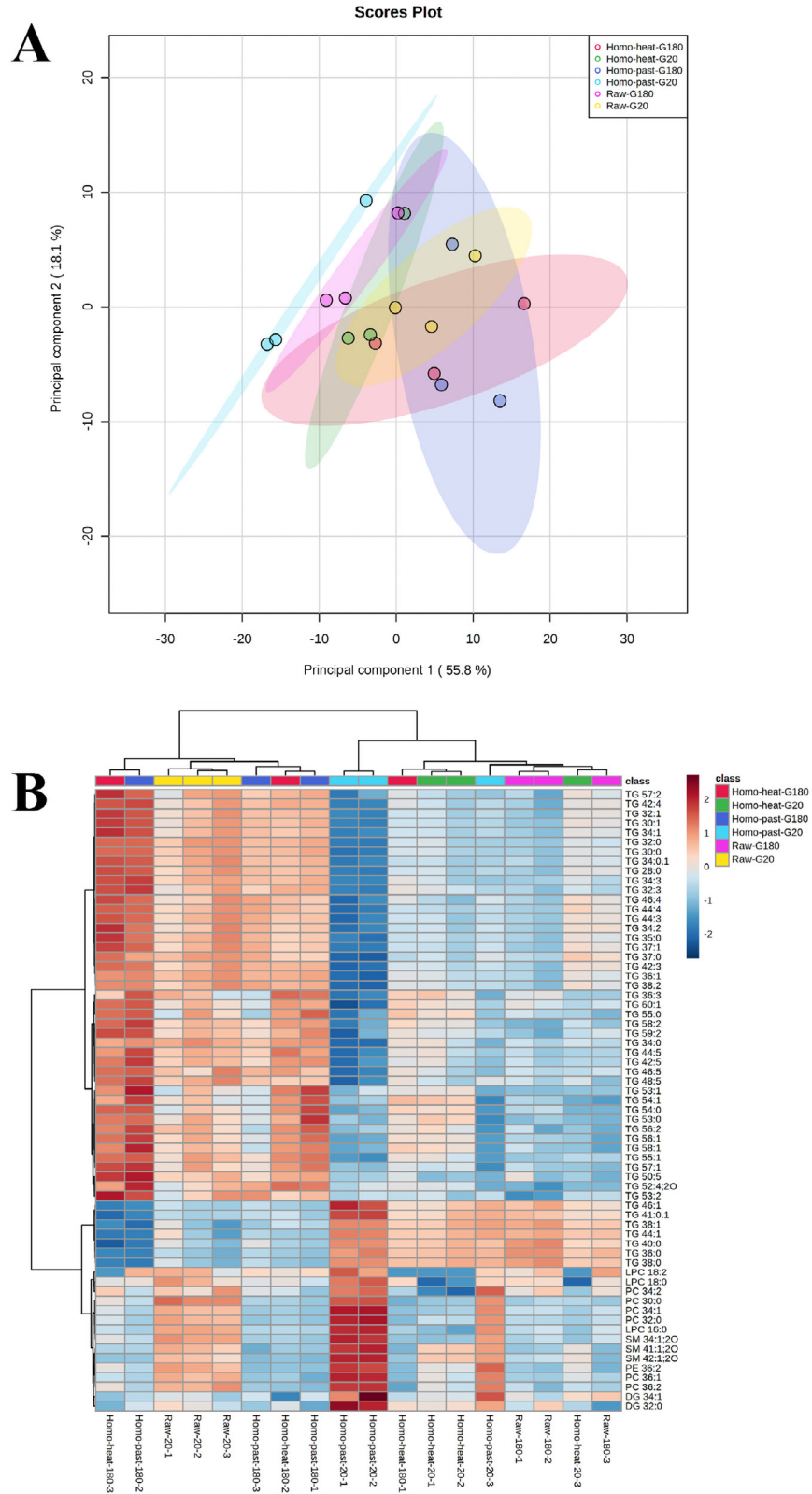


Figure 2. Lipidome differences of the gastric digesta emptied at 20 and 180 min among raw, homogenized and pasteurized (homo-past), and homogenized and heated (homo-heat) sheep milks. (A) Principal component analysis and (B) heatmap of all lipids in the gastric digesta. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable). PC = phosphatidylcholine; TG = triglycerides.

In the homo-past sheep milk, the lower levels of TG but the higher levels of DG and polar lipids in the G20 than in the G180 may have been a consequence of the homogenization of the sheep milk, which changed the surface structure of the MFG (Teng et al., 2020). Homogenization of sheep milk reduces the MFG size and increases the surface area of the MFG, which were covered with proteins (mainly casein micelles; Teng et al., 2020). As a result, an increasing number of MFG were incorporated into the protein matrix through casein-casein interactions at an early stage of digestion, because pepsin cleaves the κ -casein of casein micelles and results in coagulation of the micelles (Pan et al., 2021b; Roy et al., 2021; Li et al., 2022). The homogenized MFG that were incorporated into the protein matrix may have been less accessible for the enzymes. With increasing digestion time, the pH of the gastric mixture decreased and the pepsin activity gradually increased; thus the proteins on the surface of the MFG could have been hydrolyzed to a greater extent by pepsin (Pan et al., 2021b). Consequently, the entrapped homogenized MFG could have been released from the protein matrix to the liquid phase at a long digestion time, increasing the level of TG in the G180. This was evidenced by the result that the G20 of the raw sheep milk and the G180 of the homo-past and homo-heat sheep milks were clustered together (Figure 2B), indicating that the lipid compositions of these 3 gastric digesta samples were similar. Additionally, a proportion of small native MFG that were not influenced by homogenization may have been present in the liquid phase in the stomach. A previous study on the lipid composition of different-sized MFG showed that, in raw sheep milk, the smaller MFG contained significantly higher levels of DG, polar lipids, and some TG than the larger MFG (Mesilati-Stahy et al., 2011). The species of lipids (13 polar lipids, 7 TG, and 2 DG, Figure 2B) that were abundant in the homo-past sheep milk were aligned with those abundant in the small MFG of raw sheep milk reported by Pan et al. (submitted manuscript). Small native MFG are less likely to be incorporated into the curds because of their relatively intact interfacial composition. Further, the small native MFG in the liquid phase could be reduced with increasing digestion time because of gastric emptying. Therefore, the differences in lipid composition between the G20 and the G180 of the homo-past sheep milk can be attributed to the different amounts of small native MFG being retained in the liquid phase (Figure 2B).

For the homo-heat sheep milk, no differences in polar lipids and DG between the G20 and the G180 were found, suggesting that most small MFG may have been incorporated into the protein matrix. The intense heat treatment (95°C/5 min) of sheep milk could result in

greater denaturation of the whey proteins (Pan et al., 2022) and greater association of denatured whey proteins with the surface of small native MFG, compared with pasteurization (Ye et al., 2004). The small native MFG could be coated with a mixture of denatured whey proteins and caseins, thereby being incorporated into the protein matrix through casein-whey protein or whey protein-whey protein interactions during digestion and then reducing the differences in polar lipids between the G20 and the G180.

When the lipid compositions of the G20 among the differently processed sheep milks were compared, the raw, homo-past, and homo-heat sheep milks were separated from each other (Figure 2A). This suggested that the lipid compositions of the G20 were significantly different among the raw, homo-past, and homo-heat sheep milks. For the G20, the raw sheep milk had higher abundances of 43 lipids (all TG) than the homogenized (homo-past and homo-heat) sheep milks, and the abundances of polar lipids were highest in the homo-past sheep milk and lowest in the homo-heat sheep milk (Figure 2B). As discussed above, large proportions of the homogenized MFG could have been incorporated within the protein matrix, with only the small native MFG remaining in the liquid phase at an early stage of gastric digestion, which could contribute to the lower abundances of TG but the higher abundances of polar lipids in the G20 of the homogenized sheep milks than the raw sheep milk. The homo-heat sheep milk may have incorporated most of the small native MFG into the protein matrix because of the interactions between denatured whey protein/casein-coated MFG and the protein matrix. However, for the G180, the raw and homogenized (homo-past and homo-heat) sheep milks were separated but the homo-past and homo-heat sheep milks appeared to overlap with each other (Figure 2A). The G180 showed lower abundances of 43 TG and higher abundances of 7 TG in raw sheep milk than in homogenized sheep milk (Figure 2B), which could be attributed to the release of more homogenized MFG from the protein matrix at a late stage of gastric digestion, compared with the raw sheep milk. No differences in the polar lipids and DG of the G180 between the raw and homogenized sheep milks were found (Figure 2B). This could have been a consequence of gradually emptied small MFG in both the raw and homogenized sheep milks during gastric digestion, leading to the low abundance of polar lipids in the G180 for both the raw and homogenized sheep milks.

Comparison of Lipidomes of The Intestinal Digesta. Lipidomics analysis detected 150 lipid species from 6 different lipid classes (i.e., 131 TG, 6 PC, 5 DG, 4 LPC, 3 SM, and one PE). The abundance of these lipids was very low as most of the lipid had been

hydrolyzed by the lipase after 2 h of small intestinal digestion. The differences in the lipidomic compositions of the intestinal digesta after 120 min of digestion of the G20 (**G20-Int**) and the G180 (**G180-Int**) among the differently processed sheep milks are shown in Figure 3. The PCA shows that the G20-Int of the raw sheep milk and the other digesta appeared to be separated, and that the other groups tended to overlap with each other. This indicated that the lipid composition of the G20-Int of the raw sheep milk was different from those of the other small intestinal digesta samples. The heatmap shows the 11 significantly ($P < 0.05$ from an ANOVA of intertypes comparison) different lipids in the small intestinal digesta of the differently processed sheep milks. The heatmap clearly shows that the G20-Int of the raw sheep milk was more abundant in 9 lipids (7 TG and 2 DG) but less abundant in 2 lipids (one SM and one LPC) than the other small intestinal digesta. By comparison with the other digesta samples, the G20 of the raw sheep milk had the highest fat content, less surface area of the MFG available for the enzyme to contact, and a relatively intact MFGM because of the low pepsin activity at an early stage of gastric digestion (Pan et al., 2021b). In contrast, the surface proteins of the G180 in all types of sheep milk could have been hydrolyzed by gastric pepsin to a greater extent because of the decreased pH and the increased pepsin activity at a longer digestion time. Additionally, the MFG of homogenized sheep milk have a larger surface area for enzymes and bile to interact with compared with those of raw sheep milk, possibly leading to a faster lipolysis rate of the MFG in both the G20 and the G180. Therefore, the G20 of the homogenized sheep milks and the G180 of all types of sheep milk showed differences in the abundances of TG compared with the G20 of the raw sheep milk in the small intestinal phase.

Comparison of Lipolysis Rates During Small Intestinal Digestion

The amounts of FFA released after small intestinal digestion of the G20 and the G180 of the raw, pasteurized, homo-past, and homo-heat sheep milks are presented in Figures 4A and 4B. For all digesta samples, the amount of FFA released increased markedly at the beginning of digestion, followed by a progressive slowing down, leading to a pseudoplateau after 10 min. For the G20-Int, the amount of total FFA released was highest in the raw sheep milk after 120 min of intestinal digestion, followed by the pasteurized, homo-heat, and homo-past sheep milks (Figure 4A). For the G180-Int, the amount of total FFA released followed the order: homo-past > homo-heat > pasteurized > raw after 120 min of intestinal digestion. These results are in line

with the fat contents of gastric digesta reported in our previous study (Pan et al., 2021b), which showed the same order in the fat contents of the G20 and the G180 of differently treated sheep milks.

This result showed that the release of FFA was approximately complete after 10 min of intestinal digestion in all samples, regardless of the fat content or the structure of the MFG in the gastric digesta. However, the rate of FFA release during intestinal digestion showed differences among the differently processed sheep milks. The rate of FFA release is shown in Figure 4C. The FFA in raw sheep milk were released at a significantly ($P < 0.001$) slower rate in the G20 than in the G180. One possible explanation is that the fat content of the G180 was lower than that of the G20, resulting in a higher ratio of enzyme to fat in the G180 than in the G20 and thus a faster release rate of fatty acids in the G180. Another possible explanation is that the MFG of the G180 in the raw sheep milk could have contained less MFGM protein and could have been more vulnerable to pancreatic lipase than those of the G20. Previous studies showed that the degree of hydrolysis of MFGM proteins by pepsin increased with increasing digestion time because of the lowered pH and the increased pepsin activity (Ye et al., 2011; Wang et al., 2019). It is therefore likely that the MFGM proteins (such as butyrophilin, PAS 6/7, and mucins) could have been digested to a greater extent at 180 min than at 20 min of gastric digestion. As a consequence, MFG with less MFGM in the G180 showed a faster fatty acid digestion rate than those in the G20. In contrast, the G20 of the homo-past and homo-heat milks showed significantly ($P < 0.01$) higher rates of FFA release than the G180. Our previous study showed that most of the MFG of homo-past and homo-heat sheep milks were released in their intact status to the small intestine at 20 min of gastric digestion, whereas they flocculated via protein-peptide or peptide-peptide interactions at 180 min of gastric digestion (Pan et al., 2021b). The protein or peptide coating on the surfaces of the MFG in homogenized sheep milk might reduce the surface area of the MFG with which pancreatic lipase can interact. This could slow down the hydrolysis of the MFG by pancreatic lipase during small intestinal digestion, leading to a lower release rate of fatty acids in the G180 than in the G20.

For the G20, the homo-heat milk showed the highest digestion rate of the MFG, followed by the homo-past, pasteurized, and raw milks (Figure 4C). The release rate of FFA was significantly ($P < 0.01$) higher in the homogenized milks than in the unhomogenized milks (Figure 4C). These findings are consistent with previous findings for cow milk, which showed that homogenized and heat-processed cow milk had a higher

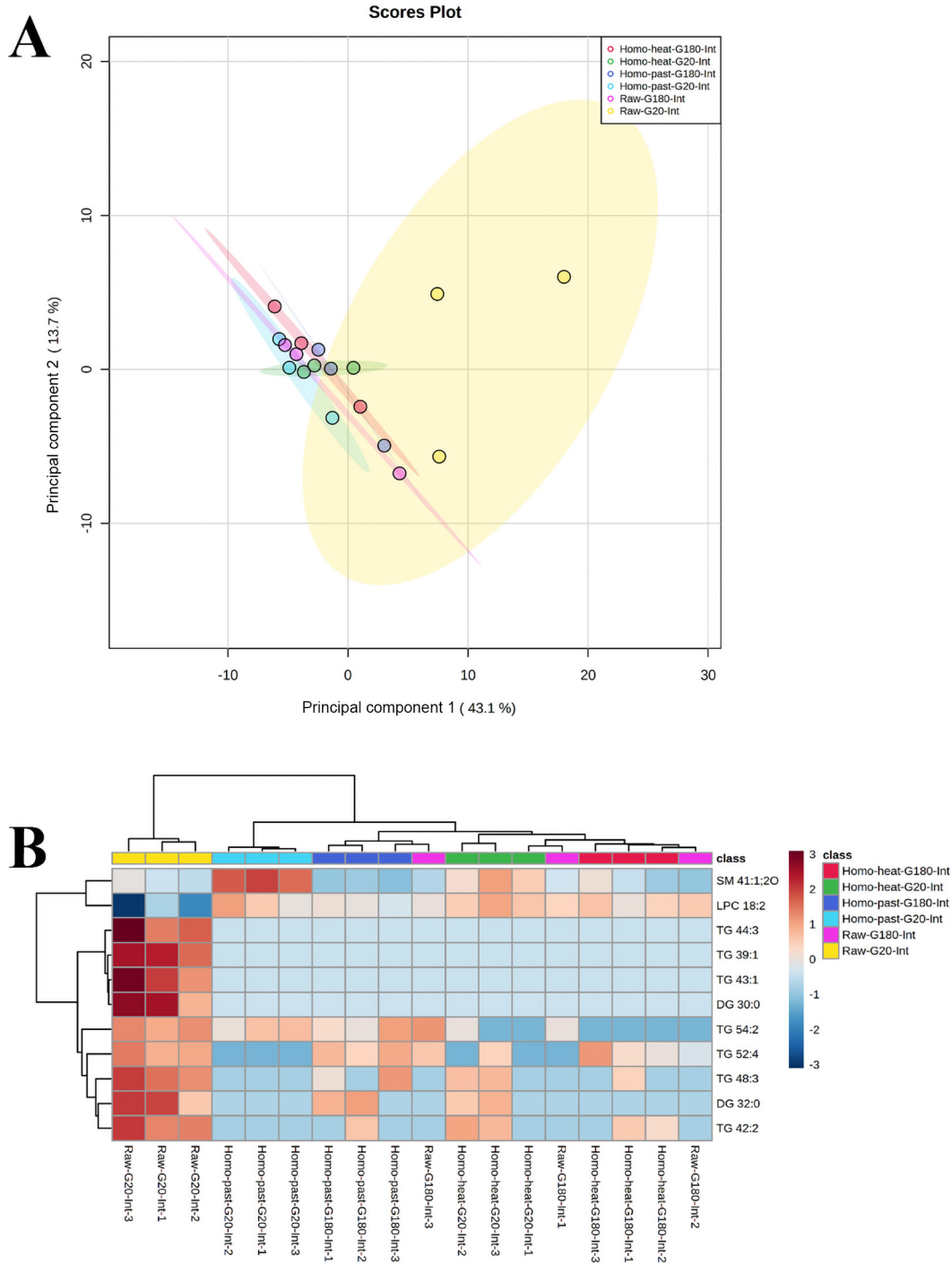


Figure 3. Lipidome differences of the intestinal digesta after 120 min of digestion of the gastric digesta emptied at 20 and 180 min, among the raw, homogenized and pasteurized (homo-past), and homogenized and heated (homo-heat) sheep milks. (A) Principal component analysis and (B) heatmap of lipids in the intestinal digesta. Red shades = homo-heat-G180-Int; green shades = homo-heat-G20-Int; indigo shades = homo-past-G180-Int; cyan shades = homo-past-G20-Int; magenta shades = raw-G180-Int; yellow shades = raw-G20-Int. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable). PC = phosphatidylcholine; TG = triglycerides.

digestion rate of MFG at the initial stage compared with raw milk (Ye et al., 2010; Liang et al., 2017; Zhao et al., 2019). Homogenized milk has a higher surface

area of MFG than raw milk, improving the interactions of MFG with pepsin, lipase, and bile salts during digestion. More proteins (mainly caseins) would attach

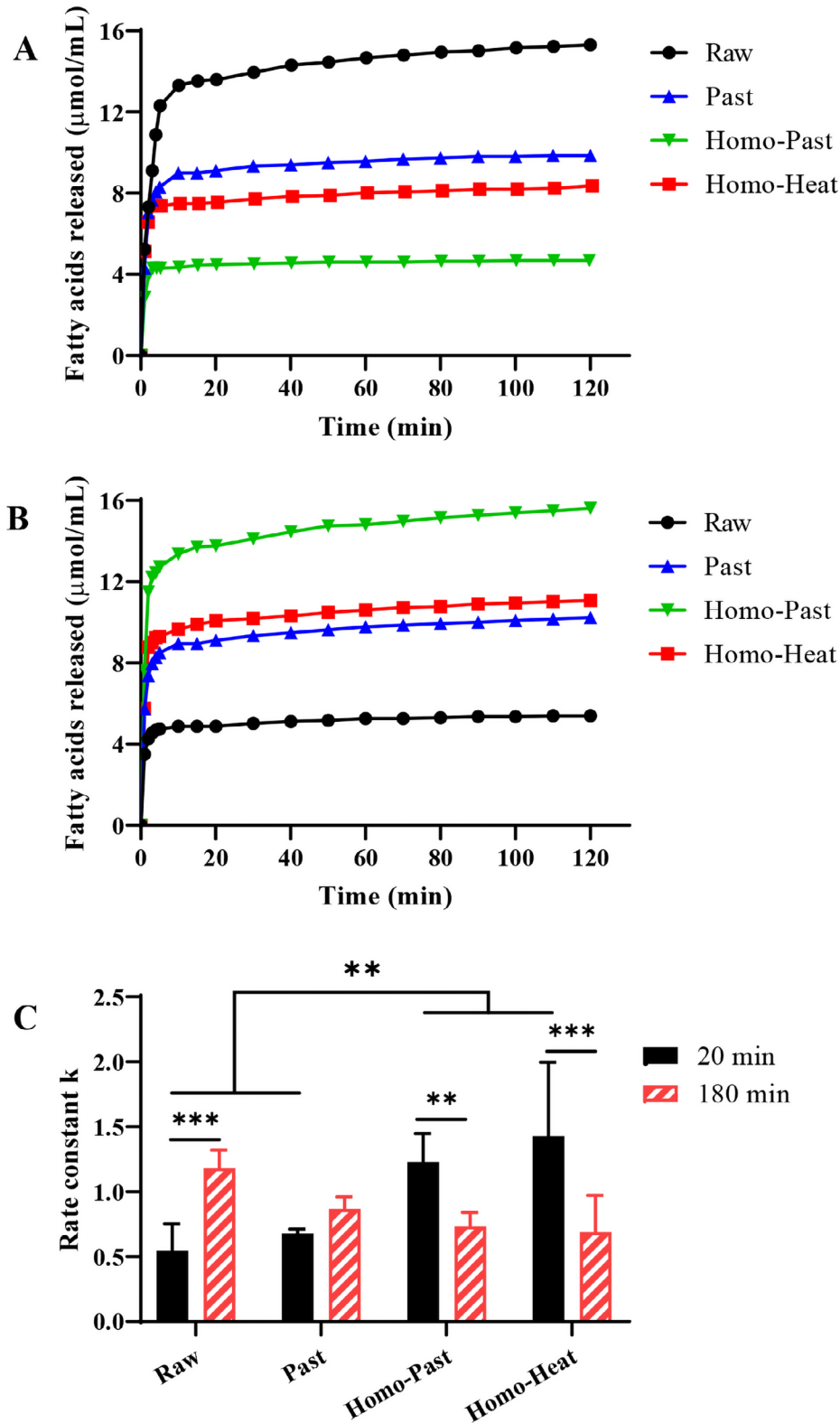


Figure 4. Total fatty acids released from the gastric digesta emptied at (A) 20 and (B) 180 min from differently processed sheep milks after 120 min of in vitro intestinal digestion for raw milk, pasteurized (past) milk, homogenized and pasteurized (homo-past) milk, and homogenized and heated (homo-heat) milk. (C) The rate constant k of fatty acids released from the gastric digesta emptied at 20 (black without pattern) and 180 (red with lines) min of differently processed sheep milks after 120 min of in vitro intestinal digestion. Significant difference levels: $**P < 0.01$; $***P < 0.001$. Error bars show the standard deviation.

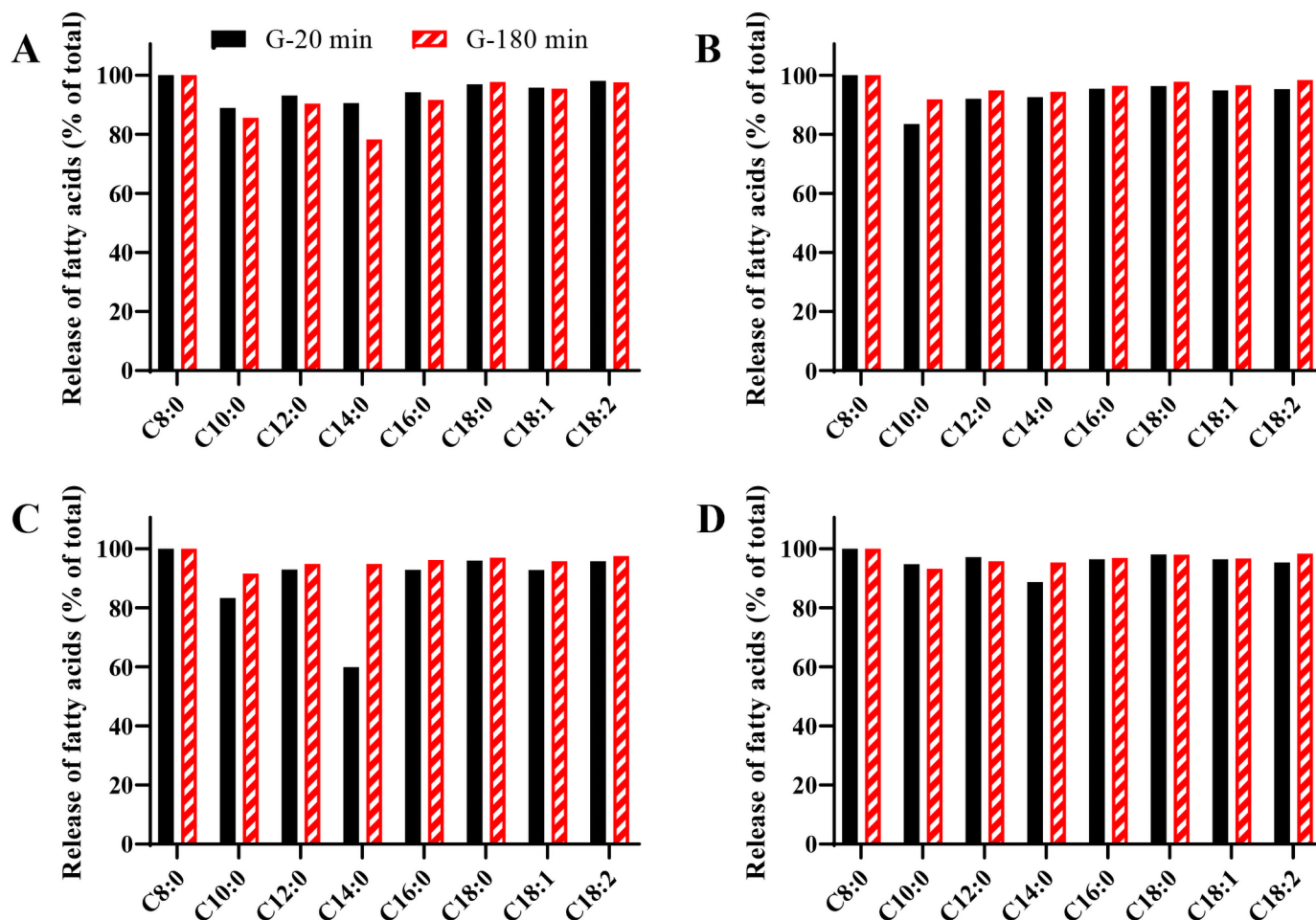


Figure 5. Individual fatty acids released after 2 h of small intestinal digestion of the gastric digesta emptied at 20 min (G20, black bars) and 180 min (G180, red bars with right-slanting lines) of raw (A), pasteurized (B), homogenized and pasteurized (C) and homogenized and heated (D) sheep milks.

to the surface of the MFG after the homogenization of milk, which could increase the affinity of MFG to pepsin and pancreatic lipase compared with raw milk (Zhao et al., 2019). Additionally, it has been reported that caseins are less resistant to hydrolysis by pepsin than some MFGM proteins (especially glycoproteins;

Table 1. Individual fatty acids released after 2 h of small intestinal digestion of the gastric digesta emptied at 20 min (G20) and 180 min (G180) of raw, pasteurized, homogenized and pasteurized (Homo-past), and homogenized and heated (Homo-heat) sheep milk

Item	Raw	Pasteurized	Homo-past	Homo-heat
Total fatty acids (mg)				
G20	18.461	11.961	10.002	14.142
G180	7.064	18.572	27.074	23.405
Free fatty acids (mg)				
G20	17.424	11.301	9.073	13.523
G180	6.536	17.893	25.938	22.625
Total free fatty acids released (% of total fatty acids)				
G20	94.4	94.5	90.7	95.6
G180	92.5	96.3	95.8	96.7

Le et al., 2012) and that MFGM phospholipids could inhibit lipid hydrolysis by pancreatin (Ye et al., 2010). The MFG of homogenized milk contained less MFGM but more caseins on the surface than those of unhomogenized milk, which could accelerate the release of FFA in homogenized milk. These different rates of FFA release might also be affected by the digestive content of the gastric digesta, as the ratio of enzymes to digestive substrates was different among the samples. Further studies may be required to investigate the effect of the concentration of digestive substrates on the lipolysis rate during the small intestinal digestion of differently processed milks.

Comparison of Lipolysis Degrees During Small Intestinal Digestion

The lipolysis degree during digestion was expressed as the percentage of released FFA (in mg) versus the

total fatty acids present in the digesta after 2 h of small intestinal digestion. The percentages of individual fatty acids and total FFA released from the G20 and the G180 of all the sheep milks after 2 h of intestinal digestion are shown in Figure 5 and Table 1, respectively. After 2 h of intestinal digestion, all sheep milk samples showed a similar pattern of FFA release. For instance, a large proportion of the lipids in the G20 of the raw sheep milk was digested, with a range from ~89% to 98% of the FFA being released (Figure 5). The percentage of released fatty acids reached over ~85% for all types of fatty acids in both the G20 and the G180 and little differences in the individual fatty acids between the G20 and the G180 were found regardless of the processing treatment (Figure 5). This indicated that the release of fatty acids was almost complete within 20 min for all types of sheep milk, which is in line with the result shown in Figure 4. For the G180 of the raw sheep milk, a similar lipolysis degree was observed, with 92.5% of the total FFA being released, compared with ~94.4% for the G20 (Table 1). However, the amount of total fatty acids released was different among the differently processed sheep milks (Table 1), which was associated with the fat content of the gastric digesta; our previous study reported that the fat content of digesta followed the order: raw > pasteurized > homo-heat > homo-past in the G20 and homo-past > homo-heat > pasteurized > raw in the G180 (Pan et al., 2021b).

The present study showed little difference in the lipolysis degree among the differently processed sheep milks (Table 1). This is different from previous results for cow milk reported by Zhao et al. (2019) who found that homogenization and heat processing of cow milk resulted in a higher degree of lipolysis after 2 h of simulated small intestinal digestion. The differences between this study and the previous study may be attributed to the increased concentration of lipase activity adapted from the INFOGEST protocol (Brodkorb et al., 2019). The INFOGEST protocol recommended adding porcine pancreatin suspension into the digestion mixture to achieve a trypsin activity of 100 U/mL, which was converted to a concentration of 20 mg/mL for porcine pancreatin in this study. The concentration of pancreatin was much higher than that reported previously by Zhao et al. (2019) who used a porcine pancreatin concentration of 1.6 mg/mL. The higher enzyme concentration may have narrowed the gap in the lipolysis degree among the differently processed sheep milks. This can be confirmed in future work by testing the effect of different concentrations of enzymes on the lipolysis degree of differently processed milks. Additionally, the ratio of enzyme to substrate could be different between this study and previous studies,

which could also contribute to the differences in the lipolysis degree.

The lipolysis degree (90%–97%) of the differently processed sheep milks presented here is much higher than the expected theoretical maximum value (~67%; Corstens et al., 2018). The specific chemical compositions of sheep MFG may contribute to their high lipolysis degree during digestion. Previous studies showed that sheep milk had higher concentrations of short-chain and medium-chain fatty acids than cow, goat, and human milks (Balthazar et al., 2017). Therefore, these fatty acids can quickly disperse into the aqueous phase after hydrolysis by lipase, reducing the inhibitory effect of interface saturation on the surface of the MFG and thus promoting the digestion of the MFG. Moreover, porcine pancreatin could contain bile-salt-stimulated lipase and pancreatic-lipase-related protein-2 (Salhi et al., 2020), which can nonspecifically hydrolyze various substrates, such as TG, DG, MG, cholesteryl esters, and the esters of fat-soluble vitamins, and thus could drive the hydrolysis toward completion (Kim Ha and Lindsay, 1993; Li et al., 2007; Salhi et al., 2021). For instance, Salhi et al. (2021) showed that gastric lipase and pancreatic lipase hydrolyzed around 2-thirds of the total ester bonds in milk, producing MG and FFA during digestion, and that adding bile-salt-stimulated lipase resulted in the hydrolysis of MG. The concerted action of these 3 lipases could result in the complete digestion of milk TG, generating free glycerol and FFA as the end products and not *sn*-2 MG and FFA. Therefore, the higher concentration of porcine pancreatin used in the current study probably had higher concentrations of bile-salt-stimulated lipase and pancreatic-lipase-related protein-2, which would contribute to the greater extent of lipolysis of sheep MFG.

CONCLUSIONS

This study investigated the effect of heat treatment and homogenization on sheep milk lipid digestion during simulated gastrointestinal digestion. The results revealed that heat treatment (pasteurization at 75°C/15 s and heating at 95°C/5 min) led to differences in the abundance of TG in sheep milk. Analysis of the lipid composition in the gastric digesta indicated that heat treatment and homogenization affected the release of fat during gastric digestion. During the intestinal phase, milk fat lipolysis was nearly complete within 10 min for all types of sheep milk, with the processing treatment influencing the rate of lipolysis. Homogenization significantly increased the lipolysis rate without altering the degree of lipolysis during digestion. The findings highlighted the impact of differences in MFG

size and surface structures on interactions with enzymes and lipid composition during digestion. However, no significant differences in lipid composition or degree of lipolysis were observed in the small intestinal digesta among differently processed sheep milk. This study enhances the understanding of how heat treatment and homogenization influence lipid digestion under simulated gastrointestinal conditions.

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