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Comparative lipidomics analysis of different-sized fat globules in sheep and cow milks

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ABSTRACT

The effect of milk fat globule (MFG) size and species (sheep versus cow) on the lipid and protein compositions of sheep and cow milks was studied. The MFGs in raw cow and sheep milks were separated into six significantly different-sized (1.5–5.5 μm) groups by a gravity-based separation method, and their fatty acids, their lipidomes and the protein compositions of their MFG membranes were determined. The proportions of polar lipids increased but glycoproteins decreased with decreasing MFG size in both sheep milk and cow milk; the fatty acid composition showed few differences among the MFG groups. The average size of each MFG group was comparable between sheep milk and cow milk. Sheep milk contained higher proportions of short-chain fatty acids, medium-chain fatty acids and sphingomyelin than cow milk in all MFG groups. The proportion of glycoproteins was higher in cow MFG membrane than in sheep MFG membrane. The results suggested that the lipid and protein compositions were markedly species and size dependent.

1. Introduction

Milk fat globules (MFGs) are secreted in a diverse range of sizes (0.2–15 μm) depending on the animal species (Argov et al., 2008), the lactation stage (Mesilati-Stahy and Argov-Argaman, 2014), the season (Briard et al., 2003; Li et al., 2022) and animal nutrition (Lopez et al., 2008). There can be significant differences in the compositions of the core lipids and the milk fat globule membrane (MFGM) between small and large MFGs (Briard et al., 2003; Lopez et al., 2011; Mesilati-Stahy et al., 2011; Michalski et al., 2003). The differences in the lipid compositions of different-sized MFGs in cow milk have been well researched. For instance, small cow MFGs contained more medium-chain and polyunsaturated fatty acids than large cow MFGs (Lopez et al., 2011; Mesilati-Stahy et al., 2011); higher concentrations of unsaturated fatty acids (Walter et al., 2020b) and higher phosphatidylcholine to phosphatidylethanolamine ratios (Walter et al., 2020a) were found in small MFGs an in large MFGs for cow milk. Some studies have also reported the relationship between the size of the native MFGs and the protein composition of the MFGM. Lu et al. (2016) analysed the MFGM protein compositions of two different-sized cow MFG fractions (obtained using a centrifugation method) and showed that lactadherin, lactoferrin/lactotransferrin, fatty-acid-binding protein, cluster of differentiation 14 and mucins 1/4/15 were enriched in larger MFGs (7.6 ± 0.9 μm) compared with smaller MFGs (3.3 ± 1.2 μm). However, these differences between different-sized MFGs have not been studied in sheep milk.

Cow, buffalo, goat and sheep milks are among the most consumed animal milks in the world. Some studies have compared the overall lipid compositions of sheep milk and cow milk. For instance, Pietrzak-Fiećko and Kamelska-Sadowska (2020) compared the lipid contents of milks from different mammalian species and showed that sheep milk had the highest fat content, whereas cow milk had the highest cholesterol concentration. Teng et al. (2020) determined the fatty acid compositions of

Abbreviations: MFG, milk fat globule; MFGM, milk fat globule membrane; SDS, sodium dodecyl sulphate; LC–MS, liquid chromatography coupled to mass spectrometry; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; LOWESS, locally weighted scatterplot smoother; QC, quality control; ANOVA, one-way analysis of variance; PCA, Principal component analysis; MCF, more medium-chain fatty acid; LCFA, long-chain fatty acid; TG, triglyceride; DG, diglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; Cer, ceramide; SE, sterol ester; ST, sterol; CN, carbon numbers; LMW, low molecular weight; MMW, medium molecular weight; HMW, high molecular weight; MUC 1, mucin 1; XO/XDH, xanthine oxidase/xanthine dehydrogenase; β-LG, β-lactoglobulin; α-LA, α-lactalbumin; CLA, conjugated linoleic acid; PAS, periodic acid Schiff.

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sheep milk and cow milk and found that sheep milk contained a higher percentage of unsaturated fatty acids but a lower percentage of saturated fatty acids than cow milk.

Previous studies on the composition of MFGs and the MFGM as a function of different MFG sizes have focused exclusively on cow milk (Lu et al., 2016; Mesilati-Stahy et al., 2011); the compositional variations in MFG size fractions between sheep milk and cow milk have yet to be elucidated. Although the overall differences in the milk fat between different species have been studied, the dominant view of milk fat overlooks the influence of MFG size on the compositional differences between species. A clear understanding of the differences in the compositions of the milks from different mammalian species and the compositions of different-sized MFGs could encourage the consumption of different milk sources, not just for nutritional reasons, but also as a targeted supplement to improve human development and health (Thum et al., 2023). Raz et al. (2023) investigated the effect of MFG size on the metabolic fingerprint of *B. subtilis* and found that *B. subtilis* incubated with small MFGs showed a higher concentration of metabolites and an enhanced growth of *B. subtilis* and large MFGs reduced the concentration of metabolites. Additionally, MFGs varying in size have different surface area-to-volume ratios, which can make them more accessible to digestive enzymes, such as lipases. The composition of MFGs, such as the types of fatty acids and phospholipids, can affect how efficiently they are broken down and metabolized in the gastrointestinal tract. Therefore, determining the lipid and protein compositions of different-sized MFGs could provide more information for the development of new products with different technological properties and an understanding of their potential impact on digestion and postprandial metabolism (Secor, 2009; Diez et al., 2007). For example, the dairy drinks with small MFGs can be designed for athletes and older people to fast digest and absorb to build muscle.

This study aimed to understand the size-dependent lipid and protein compositions of the MFGs in sheep milk, and the compositional variations between sheep milk and cow milk. Different-sized MFGs of sheep milk and cow milk were separated according to a gravity-based separation method, resulting in six MFG size groups. The protein and lipid compositions of each MFG group in sheep milk and cow milk were analysed and compared.

2. Materials and methods

2.1. Milk collection

Bulk raw cow milk and sheep milk in mid lactation were collected from a central tank at Massey University No. 4 dairy farm (Palmerston North, New Zealand) and Fernglen Farm Limited (Masterton, New Zealand) respectively at 4 °C and sent to the laboratory using chill boxes and stored at 4 °C cool room before use (used within 2 h on arrival). The milking season for cow and sheep is from August 2021 to May 2022 and April 2021. Mid-season was defined as 101–220 DIM for cow (330 DIM in total) and 71–140 DIM for sheep (220 DIM in total). The herd consisted of 614 cows (mostly Friesian-Jersey cross-breed) and ~1000 sheep (mostly Lacaune-East Friesian cross-breed). Cows and sheep of all ages were milked once daily. The compositions of the milks were analysed using a MilkoScan FT1 (Foss Electric, Hillerød, Denmark) and are shown in Supplementary Table 1. The milk collection was replicated three times on different days.

2.2. MFG separation

Different-sized MFGs were separated using a gravity-based separation method described by Ma and Barbano (2000) with slight modifications. A 60 mL milk sample was separated into a syringe (60 mL capacity) without the needle instead of a cylindrical plastic column. The syringe with the milk sample was held vertically (tip side down) at 17 °C for 20 h in a climate chamber ICH110eco (Memmert GmbH + Co. KG, Schwabach, Germany). The skim milk was divided into five fractions and was drained from the bottom of the syringe by gently pushing the plunger. The different MFG fractions from top to bottom were defined as F1 to F5. Fraction F5 was 5 mL, and the other fractions (F1–F4) were 10 mL each. The cream layer at the top of the milk was also collected.

2.3. Determination of MFG size

The MFG sizes of the whole milk, the cream and the five fractions of skim milk were determined using a Malvern MasterSizer 2000 (Malvern Instruments Ltd., Malvern, UK). All milk samples were diluted in a solution containing 2% sodium dodecyl sulphate (SDS) and 20 mM EDTA, pH 6.7, to dissociate the casein micelles. Each sample was measured in triplicate.

2.4. Fatty acid analysis

The total lipids were extracted from the milk samples using the B&D method (Bligh and Dyer, 1959). Briefly, the lipids were extracted by mixing 5 mL of milk sample with 5 mL of chloroform:methanol (1:2, v/v) and the organic phases at the bottom were collected. The extraction procedure was repeated twice, and the extracted organic phases were pooled. After evaporation of the solvent by nitrogen flow in an evaporator (Thermo Fisher Scientific, IL, USA), 2 mL of methanol was added to dissolve the lipids. The total amounts of individual fatty acids were analysed using a protocol developed by Zhu et al. (2013). Briefly, 200 μL of the extract was transferred to a 10 mL screw-cap glass tube. After the solvent had been evaporated using the evaporator, 0.5 mL of nonadecanoic acid (C19:0; CAS No. 646-30-0; molecular weight = 298.5) in heptane (1 mg/mL) as internal standard, 0.7 mL of 10 *M* NaOH and 5 mL of methanol were added. The well-sealed tubes were incubated in a water bath at 55 °C for 1.5 h. The tubes were then allowed to cool to room temperature and 0.58 mL of 12 *M* H_2SO_4 was added. The tubes were well mixed by manually shaking and were incubated again in the water bath at 55 °C for 1.5 h. They were then cooled to room temperature and centrifuged at 3500×*g* for 10 min at 20 °C. The heptane layer was transferred to a 350 μL glass insert fitted in an autosampler vial and stored at −18 °C before gas chromatographic analysis.

The fatty acid composition was determined using an Agilent 7890 system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was initially held at 180 °C for 5 min, then programmed to 210 °C at a rate of 1 °C/min and held at 210 °C for 25 min. The temperatures of both the flame ionization detector and the injector were set at 270 °C; Helium at 20 cm/s was used as the carrier gas and the column head pressure was 76 kPa. Peak identification was based on the relative retention times of the internal standard. The amount of each fatty acid was calculated by comparing its peak area with that of the internal standard, and the total amount of fatty acids was obtained by summing the calculated amount of each fatty acid. The fatty acids were recorded as the percentage of total fatty acids (w/w) within each sample.

2.5. Lipidomics of different-sized MFGs

The milk was thawed and thoroughly shaken by hand to mix (60 s) prior to aliquoting. The extraction method was a biphasic liquid–liquid extraction, as used in the laboratory for untargeted metabolomics. The lower organic phase containing lipids was measured by liquid chromatography coupled to mass spectrometry (LC–MS). Briefly, 300 μL of milk was mixed with 800 μL of prechilled (−20 °C) chloroform:methanol (50:50, v/v), agitated for 30 s and placed in a −20 °C freezer for 60 min to allow protein precipitation; this was followed by the addition of 400 μL of water, vortex-mixing for 30 s and centrifugation at 11,000 rev/min and 4 °C for 10 min in an Eppendorf Centrifuge 5427 R (Eppendorf AG, Hamburg, Germany). A 200 μL aliquot of the lower organic layer was removed and evaporated to dryness under a stream of nitrogen. Pooled

lipid quality control (QC) samples for sheep milk or cow milk were prepared by combining 50 μL of the lower organic phase from each sample of sheep milk or cow milk in a new tube. The pooled QC samples were well mixed and dried under a stream of nitrogen. These dried samples were stored at −80 °C until analysis.

The lipid extracts were analysed using a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography® system coupled to a Shimadzu LC−MS-9030 mass spectrometer. A 2 mL sample was injected on to a Waters CSH-C18 column (2.1 mm × 100 mm, 1.7 μm particle size) and the column oven was set to 60 °C. The chromatographic conditions were as follows: total run time, 15 min; flow rate, 400 μL/min; solvent A, 10 m*M* ammonium formate and a mixture of water, acetonitrile and isopropanol in a ratio of 5:3:2 (v/v/v); solvent B, 10 m*M* ammonium formate and a mixture of water, acetonitrile and isopropanol in a ratio of 1:9:90 (v/v/v). The solvent gradient programme was as follows: 10–45% solvent B (0–2.7 min), 45–53% solvent B (2.7–2.8 min), 53–65% solvent B (2.8–9.0 min), 65–89% solvent B (9.0–9.1 min), 89–92% solvent B (9.1–11.0 min) and 92–100% solvent B (11.0–11.1 min), and held for 0.8 min (11.1–11.9 min) before returning 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 analysis 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 250–1250 *m/z* and the data-independent acquisition data were collected in 20 *m/z* windows from 300 to 1100 *m/z*. High-purity nitrogen was used for the drying and collision gases.

2.6. Isolation of MFGM material

The MFGM material of the sheep milk and the cow milk was isolated from milk samples containing different-sized MFGs using a centrifugation method described by Ye et al. (2002) with slight modifications. Phosphate-buffered saline (PBS; containing 0.137 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4 and 1.8 m*M* KH_2PO_4, pH 6.8) was used as the washing solution in this study instead of simulated milk ultrafiltrate. Briefly, the milk samples containing different-sized MFGs were centrifuged at 15,000×*g* for 20 min at 20 °C using a temperature-controlled ultracentrifuge Avanti JXN-26 (Beckman Coulter, Brea, CA, USA) and the top layer (cream) was collected using a spatula. The cream was washed with PBS to remove proteins that did not associate with the MFGM. The cream was suspended in 10 vol of PBS and allowed to stand for 1 h at room temperature. The top layer of this mixture was collected after it had been centrifuged at 15,000×*g* for 20 min at 20 °C. The washing step in PBS was done twice. The washed cream was stored at 4 °C before further analysis.

2.7. MFGM protein composition analysis

The protein composition of the washed cream was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration of each washed cream was measured using Kjeldahl method (AOAC 991.20.II). The washed cream was mixed with different amount of sample buffer based on their protein concentration, containing 0.5 *M* Tris-HCl, 10% glycerol, 10% (w/v) SDS (20 mL), 0.01% bromophenol blue and 100 m*M* dithiothreitol, to reach a protein concentration of 1 mg/mL. The mixture was vortexed and heated at 70 °C for 10 min in a temperature-controlled water bath.

Before loading on to the gel, the mixture was centrifuged at 11,000×*g* for 5 min to remove the fat. A 10 μL aliquot of the mixture was loaded into each well of a 4–15% polyacrylamide gel. The same amount of molecular weight standard protein solution (Bio-Rad Laboratories, Hercules, CA, USA) was also added to the well for identifying the MFGM proteins. The protein bands were fixed by 5% glutaraldehyde solution (Sigma-Aldrich, Poole, UK) and stained with Coomassie brilliant blue G-250 (0.03 g in 100 mL of 10% acetic acid solution) for 30 min. Visualization of the gels was performed using a Molecular Dynamics Model PD-SI computing laser densitometer (Molecular Dynamics Inc., Sunnyvale, CA, USA), and the result was analysed using Image Lab software version 6.1 (Bio-Rad Laboratories).

2.8. Data processing

Data processing of the untargeted LC–MS lipidomics data was performed using the untargeted data processing software package MS-DIAL (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 (LOWESS) regression analysis and the pooled QC samples were used to correct 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 coefficient of variation of 30% were removed. 6 size groups and whole milk were used and 3 batches of milk were included. In total, therefore, the full datasets for 21 sheep milk samples and 21 cow milk samples were included in the lipidomic analysis.

2.9. Statistical analysis

The peak intensity of each individual fatty acid was converted to a relative proportion of the total lipids. A two-way analysis of variance (ANOVA) test followed by multiple comparisons was used to verify differences in the abundances of the fatty acids in sheep milk and cow milk. 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 auto-scaling to correct for heteroscedasticity, to reduce the skewness of the data and to mask effects. Principal component analysis (PCA) identified differential lipid molecules between different-sized MFG groups and between sheep milk and cow milk. 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).

3. Results and discussion

3.1. MFG size

The particle size distributions of the whole milk, the cream and fractions F1–F5 obtained from cow milk and sheep milk are shown in Fig. 1A and B, respectively. The cream of both cow milk and sheep milk showed two peaks including a peak at size larger than 17 μm and a peak at size smaller than 17 μm, whereas only a single peak (<17 μm) was observed in the other milk samples. The average fat globule size ($D_{4,3}$, volume-weighted mean diameter) of the whole milk, the cream and fractions F1–F5 is shown in Fig. 1C. For both sheep milk and cow milk, the fat globule size was highest in the cream, followed by the whole milk and fractions F1, F2, F3, F4 and F5. Statistical analysis showed that there were significant ($P < 0.05$) differences in the MFG size between each of the size groups, and that there were no significant ($P > 0.05$) differences in the fat globule size in each corresponding fraction between sheep milk and cow milk. This finding for the average MFG sizes of sheep whole milk and cow whole milk does not agree with previously reported findings that sheep milk has a smaller fat globule size than cow milk (Crowley, Kelly, Lucey and O'Mahony, 2017; Roy et al., 2021). It has been proposed that maternal physiology, breed, herd nested within a

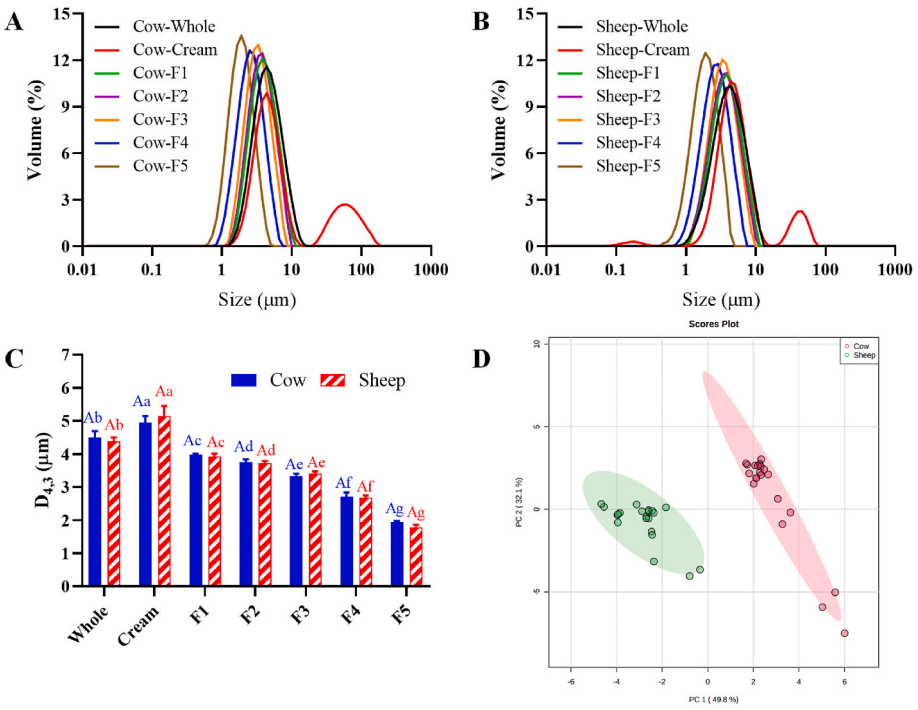

Fig. 1. Particle size distributions of the whole milk, cream and fractions F1–F5 obtained from (A) cow milk and (B) sheep milk. (C) Fat globule sizes of the whole milk, cream and fractions F1–F5 obtained from cow milk (blue bars with no pattern) and sheep milk (red bars with lines). Different capital letters represent significant ($P < 0.05$) differences between cow milk and sheep milk. Different lower-case letters represent significant ($P < 0.05$) differences among the fat globule groups. (D) Principal component analysis of the differences in the fatty acid compositions of sheep milk and cow milk.

herd, season and diet can result in MFG size variations within species (Thum et al., 2023). Therefore, the difference in the MFG sizes of sheep whole milk and cow whole milk between this and previous studies may be due to the different milk sources used.

3.2. Fatty acid profile of different-sized MFGs

The fatty acid compositions of the different-sized MFG groups were determined by gas chromatography. Table 1 presents an overview of the major fatty acid composition; unknown minor fatty acids were grouped and are presented as "Others". The methodology employed here for measuring fatty acids may have inherent challenges in precisely characterizing certain fatty acid profiles, potentially leading to an underrepresentation or misrepresentation of the fatty acid composition. As there are certain proportions (~5%) of unidentified fatty presented, it contains the sum of all the slight errors in measuring individual fatty acids. Six fatty acids (C10:0, C12:0, C14:0, C16:0, C18:0 and C18:1) in the whole milk, cream and fractions F1–F4 accounted for over ~ 76% and ~78% of the total fatty acids for sheep milk and cow milk respectively; these fatty acids were lower in fraction F5 of the sheep milk (~73%) and the cow milk (~75%). Fraction F5 of sheep milk had the lowest proportions of C4:0 and C18:3 and the highest proportion of C16:0 compared with the other MFG groups. However, cow milk showed a different pattern; fraction F5 had the lowest proportions of C4:0, C6:0 and C8:0 and the other major fatty acids did not show significant changes among the MFG groups. Both sheep milk and cow milk showed an increasing trend for the "Others" unknown fatty acids (included 26 unidentified fatty acids) as the MFG size decreased. The significant interactions ($P < 0.05$) in the impacts of species and MFG sizes were only found for C4:0 and C18:3 and not for other fatty acids. This indicated that the MFGs in the sheep milk and the cow milk had similar fatty acid composition patterns in the different fractions and that the MFG size had little impact on the composition of the major fatty acids.

The statistical differences in the fatty acid composition found between MFG groups for cow and sheep milk (Table 1) indicated that the composition of the major fatty acids was not influenced by the MFG size in both sheep milk and cow milk. This is different from previous findings reported by Mesilati-Stahy et al. (2011) and Lu et al. (2016), who showed that smaller MFGs contained more unsaturated fatty acids (such as C18:1, C18:2 and C18:3) than larger MFGs but no significant differences were reported for C4:0, C6:0 and C8:0. The reason for the differences between the results presented here and in previous studies is unclear. Mesilati-Stahy and Argov-Argaman (2014) explained that the relationship between size and lipid composition of the bovine MFG is affected by lactation stage. However, the milk source used in this study was collected at mid-lactation stage for both sheep and cows. Therefore, lactation would not be the reason for the discrepancy between current study and previous studies. One possible reason for the discrepancies could be that GC methodology used by Mesilati-Stahy and Agrov-Argaman (2014) did not allow for SCFAs analysis. Another possible reason for the variation could be the differences in breed, diet, feeding, and season, as these factors have been proven to affect the fatty acid composition of milk (Mohsin et al., 2019).

It should be noted that the markedly increased "Others" unknown fatty acids in the smallest MFG fraction (Table 1) have not previously been reported. The results for these unknown fatty acids must be interpreted with caution because the raw milk samples may have contained some indigenous bacteria that could contain some odd- and branched-chain fatty acids (such as iso C14:0, iso C15:0, anteiso C15:0, iso C16:0, iso C17:0 and anteiso C17:0) in the bacterial membrane lipids (Vlaeminck et al., 2006). These indigenous bacteria in milk may have precipitated to the bottom during separation, leading to an increased proportion of unknown fatty acids. This indicates that elevated fatty acid composition derived from bacteria is expected to be enriched in the small MFG fraction. However, the bacterial content was not clarified in the study. The bacterial content in each fraction should be further studied to verify if the existence of bacteria affected the fatty acid composition.

Sheep milk contained overall higher proportions of C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C18:2, conjugated linoleic acid and C18:3

Table 1
Fatty acid compositions (g/100 g fat) of different-sized milk fat globule groups from sheep milk and cow milk[a].

Fatty acid	Species	Whole milk	Cream	F1	F2	F3	F4	F5	*P* value (groups)	*P* value (species)
C4:0	Sheep	2.57 ± 0.16	2.28 ± 0.05	2.45 ± 0.06	2.48 ± 0.02	2.41 ± 0.01	2.48 ± 0.04	2.29 ± 0.06	0.0018	ns
	Cow	2.55 ± 0.21	2.56 ± 0.14	2.64 ± 0.13	2.59 ± 0.09	2.59 ± 0.08	2.20 ± 0.40	2.11 ± 0.12	0.0248	
C6:0	Sheep	2.74 ± 0.12	2.58 ± 0.19	2.67 ± 0.16	2.66 ± 0.13	2.65 ± 0.13	2.65 ± 0.14	2.45 ± 0.14	ns	<0.0001
	Cow	2.14 ± 0.12	2.18 ± 0.04	2.19 ± 0.03	2.19 ± 0.04	2.17 ± 0.04	2.01 ± 0.14	1.77 ± 0.10	0.0002	
C8:0	Sheep	2.84 ± 0.24	2.73 ± 0.29	2.77 ± 0.22	2.74 ± 0.20	2.78 ± 0.18	2.83 ± 0.17	2.64 ± 0.23	ns	<0.0001
	Cow	1.37 ± 0.04	1.40 ± 0.04	1.39 ± 0.01	1.40 ± 0.02	1.42 ± 0.07	1.33 ± 0.01	1.19 ± 0.06	<0.0001	
C10:0	Sheep	8.90 ± 1.08	8.92 ± 1.44	8.75 ± 0.99	8.70 ± 0.95	8.85 ± 0.89	9.27 ± 0.81	9.10 ± 1.02	ns	<0.0001
	Cow	3.38 ± 0.19	3.33 ± 0.06	3.26 ± 0.12	3.31 ± 0.12	3.31 ± 0.14	3.30 ± 0.09	3.17 ± 0.12	ns	
C10:1	Sheep	0.28 ± 0.02	0.35 ± 0.18	0.28 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.34 ± 0.02	0.37 ± 0.02	ns	ns
	Cow	0.27 ± 0.01	0.32 ± 0.07	0.29 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.32 ± 0.04	0.26 ± 0.01	ns	
C12:0	Sheep	5.01 ± 0.77	4.88 ± 0.77	4.84 ± 0.66	4.83 ± 0.64	5.07 ± 0.56	5.38 ± 0.57	5.40 ± 0.74	ns	<0.0001
	Cow	3.61 ± 0.16	3.66 ± 0.27	3.63 ± 0.18	3.68 ± 0.16	3.83 ± 0.41	3.66 ± 0.14	3.43 ± 0.07	ns	
C14:0	Sheep	11.13 ± 0.88	10.94 ± 0.75	10.88 ± 0.62	10.86 ± 0.68	11.06 ± 0.55	11.48 ± 0.51	11.40 ± 0.77	ns	0.0221
	Cow	11.52 ± 0.37	11.50 ± 0.52	11.61 ± 0.44	11.75 ± 0.35	11.73 ± 0.42	11.66 ± 0.29	10.89 ± 0.19	ns	
C14:1	Sheep	0.21 ± 0.05	0.22 ± 0.03	0.20 ± 0.02	0.25 ± 0.07	0.28 ± 0.06	0.25 ± 0.01	0.28 ± 0.01	ns	<0.0001
	Cow	0.80 ± 0.05	0.79 ± 0.06	0.82 ± 0.06	0.84 ± 0.05	0.86 ± 0.05	0.87 ± 0.05	0.81 ± 0.05	ns	
C15:0	Sheep	1.31 ± 0.09	1.28 ± 0.05	1.28 ± 0.04	1.28 ± 0.05	1.28 ± 0.06	1.28 ± 0.04	1.23 ± 0.08	ns	<0.0001
	Cow	1.09 ± 0.06	1.11 ± 0.10	1.09 ± 0.10	1.12 ± 0.08	1.13 ± 0.09	1.14 ± 0.12	1.05 ± 0.05	ns	
C16:0	Sheep	23.72 ± 1.13	23.45 ± 0.79	23.38 ± 0.59	23.18 ± 0.80	23.49 ± 0.61	24.19 ± 0.54	24.23 ± 0.91	ns	<0.0001
	Cow	27.60 ± 1.24	27.80 ± 1.54	27.17 ± 1.39	27.49 ± 1.31	27.41 ± 1.40	27.60 ± 1.20	25.86 ± 0.44	ns	
C16:1	Sheep	1.27 ± 0.16	1.41 ± 0.01	1.42 ± 0.04	1.57 ± 0.18	1.56 ± 0.22	1.34 ± 0.11	1.45 ± 0.25	ns	<0.0001
	Cow	1.68 ± 0.23	1.60 ± 0.10	1.79 ± 0.07	1.79 ± 0.08	1.83 ± 0.13	1.82 ± 0.10	1.55 ± 0.05	ns	
C17:0	Sheep	0.65 ± 0.03	0.71 ± 0.10	0.65 ± 0.05	0.63 ± 0.04	0.62 ± 0.05	0.61 ± 0.05	0.57 ± 0.05	ns	<0.0001
	Cow	0.84 ± 0.04	0.85 ± 0.03	0.84 ± 0.05	0.84 ± 0.05	0.81 ± 0.05	0.80 ± 0.05	0.79 ± 0.01	ns	
C18:0	Sheep	7.87 ± 1.51	8.13 ± 1.59	7.64 ± 1.55	7.30 ± 1.47	6.95 ± 1.44	6.30 ± 1.30	5.57 ± 1.14	ns	<0.0001
	Cow	10.71 ± 0.83	10.95 ± 0.58	10.50 ± 0.70	10.30 ± 0.68	9.87 ± 0.70	9.57 ± 0.77	9.58 ± 0.24	ns	
C18:1	Sheep	20.37 ± 1.98	20.69 ± 2.03	20.61 ± 1.94	20.20 ± 1.91	19.78 ± 1.86	19.16 ± 1.76	17.48 ± 1.60	ns	<0.0001
	Cow	23.29 ± 2.18	23.73 ± 1.78	23.62 ± 1.80	23.38 ± 2.03	22.89 ± 2.02	22.60 ± 2.16	22.26 ± 0.81	ns	
C18:2	Sheep	2.49 ± 0.24	2.44 ± 0.29	2.53 ± 0.31	2.59 ± 0.37	2.49 ± 0.35	2.46 ± 0.33	2.24 ± 0.34	ns	<0.0001
	Cow	1.51 ± 0.15	1.66 ± 0.22	1.62 ± 0.09	1.53 ± 0.09	1.61 ± 0.18	1.56 ± 0.12	1.67 ± 0.22	ns	
CLA	Sheep	1.77 ± 0.32	1.79 ± 0.32	1.73 ± 0.27	1.75 ± 0.24	1.75 ± 0.23	1.74 ± 0.28	1.78 ± 0.27	ns	<0.0001
	Cow	1.03 ± 0.09	1.03 ± 0.04	1.08 ± 0.06	1.08 ± 0.07	1.09 ± 0.09	1.11 ± 0.11	1.29 ± 0.16	ns	
C18:3	Sheep	1.81 ± 0.14	1.90 ± 0.15	1.87 ± 0.05	2.05 ± 0.19	1.98 ± 0.23	1.71 ± 0.15	1.51 ± 0.17	0.0232	<0.0001
	Cow	0.79 ± 0.06	0.82 ± 0.08	0.86 ± 0.11	0.77 ± 0.03	0.82 ± 0.11	0.72 ± 0.11	0.80 ± 0.08	ns	
Others	Sheep	5.05 ± 0.98	5.30 ± 0.70	6.04 ± 0.21	6.64 ± 0.49	6.69 ± 0.26	6.53 ± 0.59	10.01 ± 2.05	0.0004	ns
	Cow	5.82 ± 1.34	4.71 ± 0.36	5.58 ± 0.44	5.65 ± 0.88	6.31 ± 0.71	7.74 ± 1.71	11.51 ± 2.29	0.0003	

Abbreviations: CLA, conjugated linoleic acid; ns, no significant difference.
P value (groups) shows differences among the different milk fat globule groups. *P* value (species) shows differences between sheep milk and cow milk. The group and species variations were analysed using one-way analysis of variance.
[a] Means ± standard deviations.

and lower proportions of C14:1, C16:0, C16:1, C17:0, C18:0 and C18:1 than cow milk, but no significant ($P > 0.05$) differences were found for C4:0, C10:1 and the "Others" unknown fatty acids (Table 1). This is in agreement with previously reported results that the fatty acid content of sheep milk has comparable C4:0, more medium-chain fatty acids (MCFAs, including carbon numbers between 6 and 15) but fewer long-chain fatty acids (LCFAs, including carbon numbers larger than 16) than cow milk (Balthazar et al., 2017).

To investigate the difference in the distribution of fatty acids between sheep milk and cow milk, PCA was performed and the results are shown in Fig. 1D. The fatty acid compositions of sheep milk and cow milk were completely separated. The clustering highlights that the lipid compositions of the different-sized MFG groups were more similar to each other within the milks from the same species than to the milks from different species. According to the fatty acid composition shown in Table 1, sheep milk contained more MCFAs than cow milk in all the MFG groups, in accordance with previous studies (Ramos and Juarez, 2011; Teng et al., 2020). These differences in the MCFAs may have contributed to the complete separation between sheep milk and cow milk in the PCA plot (Fig. 1D).

3.3. Lipid composition of different-sized MFGs

The total lipid composition for each major lipid class of sheep milk and cow milk was calculated by summing the peak areas and creating a sum normalized concentration; the results are shown in Fig. 2. Lipidomics analysis detected 231 lipid species from six different lipid classes that included triglyceride (TG), ceramide, diglyceride (DG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), sterol ester and sterol. TGs (167) were the predominant lipids, followed by ceramide (34), DGs (11), PC (9), PE (5) and SM (3) for both sheep milk and cow milk. The relative proportions of DG and ceramide decreased with decreasing MFG size, whereas TG, PC, PE and SM showed an increasing trend as the MFG size decreased. This suggested that small MFGs contain higher proportions of TG and polar lipids (including PC, PE and SM) but lower proportions of DG and ceramide than large MFGs. The result is in line with previously reported findings for cow milk by Leonie Walter et al. (2020a,b), which showed that cow milk with abundant small MFGs contained a higher relative abundance of total PC compared to that with abundant large MFGs. The detailed differences in TGs between groups are covered in subsequent sections.

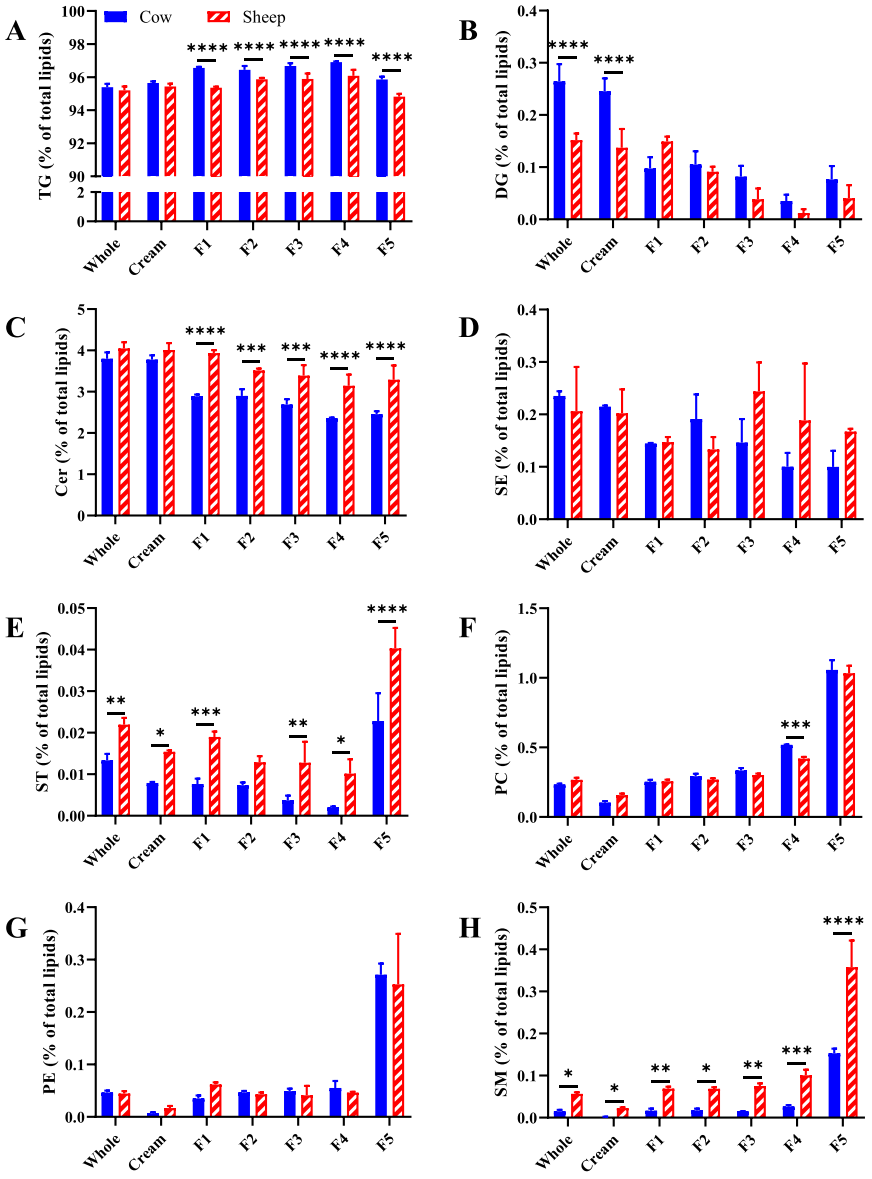

Fig. 2. (A) Triglyceride (TG), (B) diglyceride (DG), (C) ceramide (Cer), (D) sterol ester (SE), (E) sterol (ST), (F) phosphatidylcholine (PC), (G) phosphatidylethanolamine (PE) and (H) sphingomyelin (SM) contents in different-sized milk fat globules of cow milk (blue bars with no pattern) and sheep milk (red bars with lines). Level of significance: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Sheep milk had significantly ($P < 0.05$) lower proportions of TG but significantly ($P < 0.01$) higher proportions of ceramide than cow milk in fractions F1–F5 (Fig. 2A and C). Sheep milk contained a significantly ($P < 0.01$) lower proportion of DGs than cow milk in the whole milk and the cream, whereas no differences were found for fractions F1–F5 (Fig. 2B). The proportion of sterol was significantly higher in sheep milk than in cow milk (Fig. 2E), whereas there was no significant ($P > 0.05$) difference in sterol ester between sheep milk and cow milk (Fig. 2D). The result differs from previously reported results by Pietrzak-Fiećko and Kamelska-Sadowska (2020) and Agyare and Liang (2021), who showed that the concentration of cholesterol of cow milk fat and sheep milk fat were not significantly different based on cholesterol/total fat. The disparity between current and previous studies could be attributed to different methodologies and milk sources. For polar lipids, there were no significant ($P > 0.05$) differences in the PC and PE contents between sheep milk and cow milk (Fig. 2F and G). However, sheep milk had a significantly higher ($P < 0.01$) proportion of SM in all milk fractions (Fig. 2H) and significantly higher ($P < 0.001$) total polar lipids (= PC + PE + SM) in whole, cream and F1 (data not shown) than cow milk. The effect of the species × sizes on the lipid composition was significant ($P < 0.05$) for TG, DG, ceramide, SE, sterol, PC and SM but not significant ($P > 0.05$) for PE. This indicates that MFG sizes had different impacts on the lipid composition of MFGs from the milks of the different species.

The polar lipid composition for both sheep milk and cow milk revealed that the concentrations of PC, PE and SM increased with decreasing MFG size (Fig. 2F–H), indicating that the smaller MFGs were coated with more polar lipids. The results are consistent with that reported by Lopez et al. (2011), who compared the concentrations of polar lipids in whole (~4.2 μm), small (~1.6 μm) and large (~6.5 μm) MFGs and found that small MFGs contained the highest concentration of polar lipids (8.91 mg/g fat), followed by whole MFGs (6.25 mg/g fat) and large MFGs (2.72 mg/g fat); however, the results of the current study

differ to the previous findings for cow milk reported by Mesilati-Stahy et al. (2011), who showed few differences in the levels of PE and PC in different-sized MFG groups. Variations in polar lipids between studies can possibly be attributed to the different analytical methods or milk sources used, such as milk from different breeds, stage of lactation, seasonal variations and conditions of feeding (Ménard et al., 2010; Tai et al., 2022). With respect to the polar lipid species, the results are in accordance with previous literature for milk; that is, PE, PC and SM are the major polar lipids characterized for milk samples (Et-Thakafy, Guyomarc'h, & Lopez, 2017; Lu et al., 2016; Ménard et al., 2010; Mesilati-Stahy et al., 2011).

When the compositions of the polar lipids were compared, there were significant ($P < 0.001$) differences in their total concentrations (total polar lipids = PC + PE + SM, data not shown for total polar lipids) between sheep milk and cow milk in the whole milk (0.285 versus 0.351%), the cream (0.108 versus 0.187%) and fraction F1 (0.291 versus 0.367%), but not in the MFG fractions with smaller size (F2–F5) ($P > 0.05$). The relative concentration of SM in all MFG groups was significantly ($P < 0.05$) higher in sheep milk than in cow milk (Fig. 2H), whereas no significant ($P > 0.05$) differences were observed for PC and PE in most of the MFG groups (Fig. 2F and G). This is in line with a previous study, which showed that sheep milk contained a significantly ($P < 0.001$) higher proportion of SM but comparable proportions of PC and PE when compared with cow milk (Agyare and Liang, 2021; Et-Thakafy et al., 2017). The SM content has been associated with different MFGM features such as structures and melting temperatures, which probably influence the interfacial properties of the MFGM and thus the functional properties and the digestion behaviour of the fat globules as lipid digestion is an interfacial process where the MFGM play an important role in aiding digestion (Et-Thakafy et al., 2017; Tai et al., 2022).

Several studies have reported the phospholipid composition of different-sized MFGs in cow milk. Mesilati-Stahy et al. (2011) showed that large MFGs contain high levels of PE and PC but a similar level of SM compared with small MFGs. In contrast, Lu et al. (2016) compared the lipid compositions of two different-sized MFG groups (7.6 ± 0.9 versus 3.3 ± 1.2 μm) in cow milk and showed that small MFGs had higher proportions of PE and PC and a similar proportion of SM compared with large MFGs. The reason for the differences between these studies is unclear. It has been suggested that the lipid composition of milk could be affected by physiological characteristics, such as weight, somatic cell count, pregnancy, day in milk, parity and milk production traits, including milk yield, fat yield, protein content, fat content and the ratio of fat to protein, on the individual animal level (Ceciliani et al., 2021). Therefore, these differences in the lipid composition among different studies could be due to the different milk sources used.

The difference in the lipidomes between sheep milk and cow milk is shown in Fig. 3. The PCA of the lipidomics data provided evidence of the considerable differences between the lipidomes of sheep milk MFGs and cow milk MFGs (Fig. 3A). The PCA highlights that 47.7% of the variance within the samples was in principal component 1, which clearly separated the species. A further 23.9% of the variance is explained in principal component 2, which separated the MFG groups with different sizes. The heatmap showed 169 significantly different lipids (FDR *P*-value <0.05 from a *t*-test of interspecies comparison) between sheep milk and cow milk (Fig. 3B). The sheep milk and the cow milk were clearly grouped into two different clusters. The abundances of 51 lipids were higher in cow milk than in sheep milk, in which TG (~74%) was the largest group followed by ceramide (~16%) and DG (~8%). Sheep milk had higher abundances of the remaining 118 lipids than cow milk, with TG, ceramide, polar lipids (including PC, PE and SM) and DG accounting for ~ 75, 14, 7 and 3% respectively.

3.3.1. *Lipidomes of different-sized MFGs in sheep milk*

The differences in the lipidomes among the different fractions of sheep milk are shown in Fig. 4A. The PCA plot shows that the whole milk and cream samples overlapped markedly, and that the other fractions were well separated from each other (Fig. 4A1). The 134 different lipids (*P*-value <0.05 from an ANOVA of inter-fraction comparison) are shown in the heatmap (Fig. 4A2). Overall, 83 lipids were higher in the whole sheep milk, the cream and fractions F1 and F2, in which TG (~69%) was the predominant lipid, followed by ceramide (~22%) and DG (~9%). In contrast, the other 51 lipids were higher in the other smaller MFG fractions (F3–F5), in which TGs (~61%) and polar lipids (~31%, including PC, PE and SM) were the predominant groups.

Fig. 4A3 shows the fractional variation in the TG composition. The TGs were divided into low molecular weight (LMW; CN24–CN36, CN is the total carbon number of the three fatty acids in the TG), medium molecular weight (MMW; CN37–CN49) and high molecular weight (HMW; CN50–CN62) TGs, as reported by Pacheco-Pappenheim et al. (2021). The LMW TGs decreased with decreasing MFG size; the MMW TGs increased as the MFG size decreased; the HMW TGs remained roughly unchanged with the MFG size. Statistical analysis showed that the small MFGs (fractions F3–F5) in sheep milk contained significantly ($P < 0.05$) lower proportions of LMW TGs but significantly ($P < 0.05$) higher proportions of MMW TGs than the large MFGs (whole milk, cream and fractions F1 and F2). These differences in the abundance of TGs and polar lipids drove the different-sized MFG groups to separate from each other.

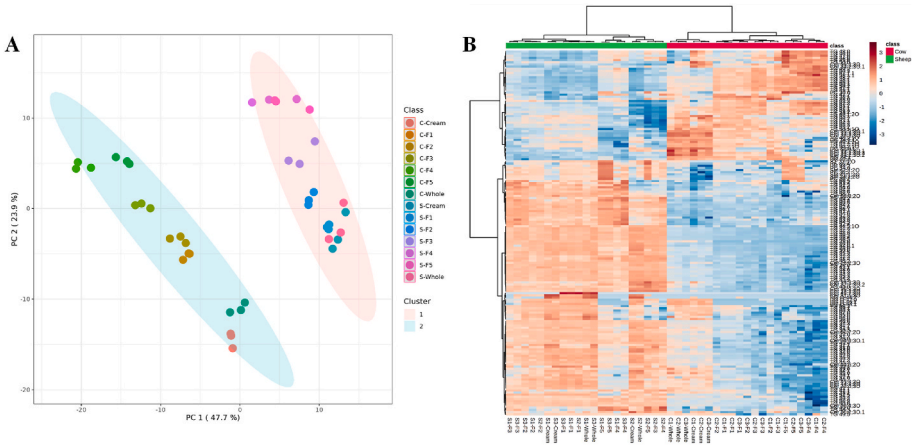

Fig. 3. Differences in lipidomes of different-sized milk fat globule groups in sheep milk (S) and cow milk (C). (A) Principal component analysis and (B) heatmap of lipids in sheep milk and cow milk samples. The heatmap colours reflect the abundance of milk lipids (mean-centred and divided by the standard deviation of each variable).

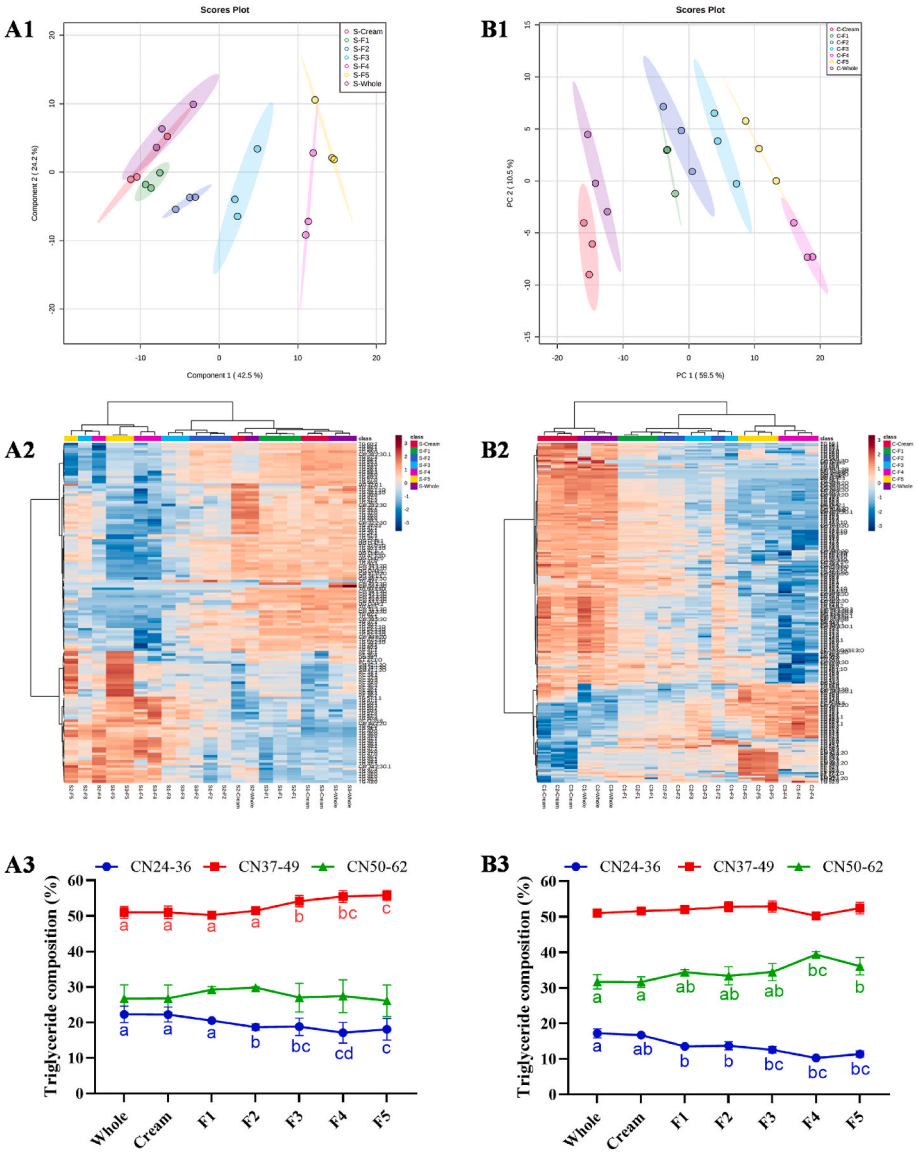

Fig. 4. Differences in lipidomes of different-sized milk fat globule groups in (A) sheep milk and (B) cow milk. (A1 and B1) Principal component analysis, (A2 and B2) heatmap and (A3 and B3) fractional variation of milk fat triglycerides. The heatmap colours reflect the abundance of milk lipids (mean-centred and divided by the standard deviation of each variable). Low molecular weight [carbon numbers (CN) 24–36], medium molecular weight (CN37–CN49) and high molecular weight (CN50–CN62) triglyceride groups.

3.3.2. *Lipidomes of different-sized MFGs in cow milk*

The lipidome results of the different-sized MFG groups in cow milk are shown in Fig. 4B. The PCA plot shows that each fraction was distributed separately from the left side (larger MFGs) to the right side (smaller MFGs) in cow milk; similar to sheep milk (Fig. 4A1), the cream of cow milk was close to the whole milk (Fig. 4B1). The heatmap shows the 199 different lipids (P-value <0.05 from an ANOVA of inter-fraction comparison) in all milk fractions (Fig. 4B2); 141 lipids were identified as less abundant lipids in small-sized MFG groups (including fractions F4 and F5), in which TG, ceramide and DG accounted for ~ 75, 18 and 7% respectively. The other 58 lipids (~64% TGs and ~29% polar lipids) were the most abundant classes in fractions F4 and F5, compared with whole milk, cream and fractions F1–F3. The lipidome results for sheep milk (Fig. 4A) and cow milk (Fig. 4B) suggested that smaller MFGs contain more polar lipids. This is in agreement with the results reported by Mesilati-Stahy and Argov-Argaman (2014), who showed that the concentration of LCFA was higher in smaller MFGs than in bigger MFGs.

Fig. 4B3 shows the fractional variation in TG composition. The LMW TGs decreased with decreasing MFG size; the MMW TGs remained roughly unchanged with decreasing MFG size; the HMW TGs increased as the MFG size decreased. Statistical analysis showed that the smaller MFG groups (fractions F4 and F5) contained significantly ($P < 0.05$) more HMW TGs but significantly ($P < 0.05$) fewer LMW TGs than the larger MFG groups (whole milk, cream and fractions F1 F2, and F3). Similar to the findings for sheep milk (Fig. 4A), these differences in the TGs and polar lipids between the MFG groups drove the small MFGs to be different from the large MFGs. This is consisted with previous studies on other species that the content of polar lipid was natively correlated to MFG size in goat milk (Argov-Argaman et al., 2021) and cow milks (Mesilati-Stahy and Argov-Argaman 2014). In comparison with sheep milk, cow milk contained significantly ($P < 0.05$) lower proportions of LMW TGs in all MFG groups, which is in line with the fatty acid composition result that cow milk contained fewer MCFAs than sheep milk (Table 1).

3.4. *Protein composition of MFGM*

Fig. 5A shows the SDS-PAGE profile of the MFGM proteins in the

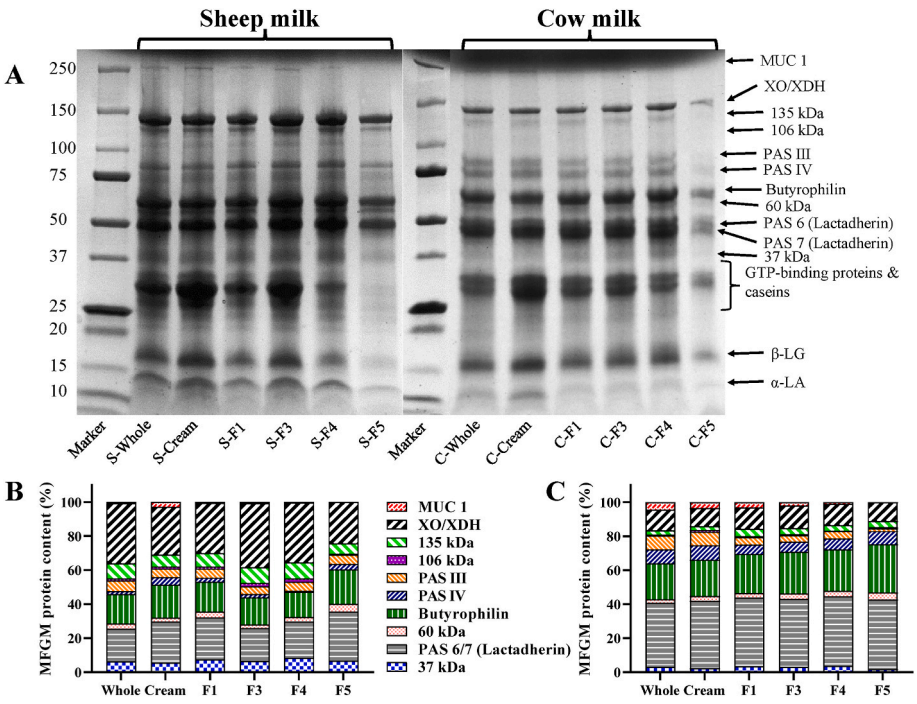

Fig. 5. (A) SDS-PAGE profile of milk fat globule membrane (MFGM) isolated from different-sized fat globules of sheep milk and cow milk. The molecular weight unit for the numbers shown in the left of SDS-PAGE gel is kDa. The protein compositions of (B) sheep MFGM and (C) cow MFGM. MUC 1, mucin 1; XO/XDH, xanthine oxidase/xanthine dehydrogenase; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

different-sized MFG groups. Sheep milk and cow milk had similar MFGM protein compositions. The only difference was a single band at periodic acid Schiff (PAS) 6 and PAS 7 (PAS 6/7 are also known as lactadherin) positions in sheep milk, whereas cow milk had two bands. This is in agreement with previous results reported by Cebo and Martin (2012), who compared the MFGM proteins in cow milk and non-cow milk using SDS-PAGE and mass spectrometry analysis and identified two bands for cow milk lactadherin but a single band for sheep milk lactadherin.

The detailed MFGM protein (molecular weight ≥37 kDa as a whole) composition of sheep milk and cow milk is shown in Fig. 5B and C respectively. Xanthine dehydrogenase/oxidase, PAS 6/7 (lactadherin) and butyrophilin were the major MFGM proteins in both sheep milk and cow milk. The results are generally in agreement with previous reports of Thum et al. (2023), who stated that butyrophilin, xanthine dehydrogenase/oxidase, lactadherin and adipophilin are enriched in cow MFGM and sheep MFGM. However, the compositions of the individual MFGM proteins were different between sheep milk and cow milk. Sheep MFGM contained higher proportions of xanthine dehydrogenase/oxidase and bands at 135, 106 and 37 kDa but lower proportions of mucin 1 (MUC 1), PAS IV (also known as cluster of differentiation 36), butyrophilin and PAS 6/7 than cow MFGM. This indicated that sheep milk contained more non-glycosylated proteins (including xanthine dehydrogenase/oxidase and adipophilin) but fewer glycoproteins (including MUC 1, PAS III and PAS IV) than cow milk. For the different-sized MFGs, both sheep milk and cow milk showed similar patterns, in which smaller MFGs contained lower proportions of MUC 1, PAS III and PAS IV but higher proportions of proteins at 135, 106 and 60 kDa, butyrophilin and PAS 6/7 than larger MFGs. This suggested that smaller MFGs have less glycoproteins than larger MFGs. The differences in the MFGM protein composition among the MFG groups can be attributed to the different affinities of the MFGM for proteins as the size-dependent fatty acid and polar lipid composition could lead to different surface polarities among the MFG fractions (Lu et al., 2016). MFGM is primarily composed of amphiphilic lipids, including phospholipids and glycolipids, with varying hydrophobic and hydrophilic characteristics. MFGM proteins exhibit different affinities for these lipids, leading to selective binding (depending on the structure and charge of properties of both the proteins and lipids) and partitioning (depending on the lipid composition and surface polarity of different MFG fractions). As a result, the lipid composition of different MFG fractions can create variations in MFGM protein composition.

4. Conclusions

This study showed that gravity-based separation effectively separated milk into six significantly different-sized MFG groups. The lipid and protein compositions of the different-sized MFG groups in sheep milk and cow milk were compared. The MFG size had little impact on the composition of the fatty acids in both sheep milk and cow milk, whereas smaller MFGs had higher proportions of polar lipids (including PC, PE and SM) but lower proportions of LMW TGs than larger MFGs in both milks. The TG composition showed that the MMW TGs of sheep milk and the HMW TGs of cow milk increased with decreasing MFG size. More glycoproteins were observed in large MFGs than in small MFGs.

The lipid and protein compositions were significantly different between sheep milk and cow milk. The MFGs of sheep milk had higher proportions of short-chain fatty acids, MCFAs, SM and LMW TGs than those of cow milk in all size groups. Sheep milk contained more non-glycosylated proteins but fewer glycoproteins in the MFGM than cow milk. These differences might potentially affect the functional properties and digestion behaviours of the MFGs of sheep milk and cow milk.

CRediT authorship contribution statement

Zheng Pan: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **Aiqian Ye:** Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. **Karl Fraser:** Supervision, Formal analysis, Writing – review & editing. **Siqi Li:** Writing – review & editing. **Anant Dave:** Supervision, Writing – review & editing. **Harjinder Singh:** Supervision, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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.crfs.2023.100655.

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