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Partitioning of the milk fat globule membrane between buttermilk and butter serum is determined by the thermal behaviour of the fat globules

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ABSTRACT

Isolation of the industrially interesting milk fat globule membrane (MFGM) components from dairy streams is challenging, and a full exploitation of their benefits can be gained by better understanding of the behaviour of the fat globule membrane fraction during milk fat processing. In this study, microfiltration of the cream before churning and the comprehensive compositional analysis of the process streams revealed new insights on MFGM partitioning during the phase inversion in butter making. After removal of the smallest fat globules by microfiltration, a reduced phospholipid content was reflected in the buttermilk, but not in the butter serum. Regardless of the cream washing, buttermilk and butter serum lipids were different in PL-to-fat ratio, phospholipid composition, degree of unsaturation and melting behaviour. We suggest that partitioning of the MFGM fraction between butter and buttermilk is a direct result of the physico-chemical properties of the fat globules, partly related to the globule size.

1. Introduction

Milk is an oil-in-water (O/W) emulsion of fat, proteins, lactose and minerals. Fat is present in the form of globules ranging in size between 0.2 and 15 µm (Walstra, 1983). The globules are stabilised by the milk fat globule membrane (MFGM), consisting of proteins and polar lipids, including glycerophospholipids and sphingolipids (Dewettink et al., 2008). MFGM lipids and membrane-associated proteins have attracted considerable attention due to their reported health benefits. The glycerophospholipid components of MFGM, such as phosphatidyl choline and phosphatidyl serine, have been reported to improve cognitive functions, lower cellular stress reactions, inhibit inflammation, regulate membrane fluidity especially in lymphocytes and prevent liver diseases (Küllenberg, Taylor, Schneider, & Massing, 2012). Milk sphingomyelin (SM) has also proven to efficiently prevent cholesterol absorption in the intestine (Noh & Koo, 2004) promote brain development (Oshida et al., 2003) and have anticarcinogenic effects (Lemonnier et al., 2003; Schmelz, Sullards, Dillehay, & Merrill, 2000). The latter have been attributed also to certain MFGM proteins (Ito et al., 1993; Spitsberg & Gorewit, 1997, 2002). In addition, the MFGM-associated glycoproteins have shown to reduce the risk of autoimmune diseases (Guggenmos et al., 2004; Stefferl et al., 2000) and *Helicobacter pylori* infection (Hirmo, Kelm, & Iwersen, 1998; Wang, Hirmo, Millen, & Wadstrom, 2001). Overall, there is an increased interest to enrich these components from dairy streams. However, the composition of the commercial MFGM preparations is heterogeneous and may lead to different nutritional value between the products (Brink et al., 2020).

It is possible to enrich MFGM fractions in the processing of high-fat products such as butter. In the butter making process, the O/W cream emulsion containing 25–46% fat is broken during mechanical destabilization, so-called churning, and subsequently, the lipids are reorganised forming the butter mass (Wrede & Buchheim, 1994). While phase inversion is induced by beating air in the cooled cream during churning, the serum proteins and fat globules assemble on the surface of air bubbles. Butter granules are formed when the fat globules come into close contact and aggregate because of the partial disintegration of the MFGM layer (King, 1953; Wrede & Buchheim, 1994). Part of the liquid fat is squeezed out from the

fat globules enclosing the crystalline fat network, and at this stage, the aqueous phase, namely, the buttermilk, can be separated. Complete phase inversion is accomplished by further mixing during which excess moisture is pressed out, and the remaining water (16–20%) forms finely dispersed droplets inside the butter mass (Juriaanse & Heertje, 1988). Upon butter melting, butteroil and the aqueous phase, called butter serum, can be recovered.

Prior to churning, cream is usually thermally treated (ripened) to partially crystallise milk fat and optimise butter consistency (Juriaanse & Heertje, 1988; King, 1953). By changing the thermal treatment of the ripening, different polymorphic triacylglycerol (TAG) crystal forms can be induced, affecting the final structure of butter (Buldo, Kirkensgaard, & Wiking, 2013; Rønholt, Kirkensgaard, Pedersen, Mortensen, & Knudsen, 2012). Polymorphic crystal structures having lamellar structures of double (2L) and triple (3L) chain length are present in the crystallised milk fat (Lopez, Lesieur, Bourgaux, Keller, & Ollivon, 2001; Lopez et al., 2002). The cross-sectional packing of the longitudinal organisations determines the crystal stability: α, β’ or β; β being the most stable and having the highest melting point (Sato, 2001). Typical milk fat melting curve consists of the low-melting fraction (LMF), medium-melting fraction (MMF) and high-melting fraction (HMF). LMF corresponds to the melting of the unstable 3L structures having thickness of 71.3 Å below about 13 °C (Lopez, Lesieur, Bourgaux, Keller, & Ollivon, 2001; Lopez et al., 2002). MMF indicates the melting of the metastable crystal structures of 3L (65 Å) + 2L (46.5 Å) between 13 and 21 °C. HMF refers to the progressive melting of 2L species having thickness about 40–41.6 Å. It begins around 21 °C and continues until the final melting of milk TAG below 39 °C. Milk fat has lower melting temperature than would be expected from its fatty acid (FA) composition, which is a result of the non-random esterification of FA in TAG reflecting the complex regulation of de novo FA synthesis, desaturation and uptake of long chain FA, and further TAG synthesis, in mammary gland (Leskinen et al., 2016; Leskinen et al., 2019; Leskinen, Ventto, Kairenius, Shingfield, & Vilkki, 2019; Parodi, 1981, 2004; Timmen & Patton, 1989; Tzompa-Sosa, van Valenberg, van Aken, & Bovenhuis, 2016).

Despite the reported differences in phospholipid concentration and composition, the aqueous streams in the butter making process, buttermilk and butter serum, are regarded as rather similar MFGM

fragment-rich materials. There exist reports indicating that SM is enriched in butter serum (Bourlieu et al., 2018; Britten, Lamothe, & Robitaille, 2008; Lopez, Blot, Briard-Bion, Cirié, & Graulet, 2017) and phosphatidyl ethanolamine (PE) in buttermilk (Bourlieu et al., 2018; Lopez, Blot, Briard-Bion, Cirié, & Graulet, 2017). Lopez Blot et al. (2017) suggested that the rigid SM-rich domains had higher affinity toward fat phase and therefore enriched in butter serum. The same authors have reported the enrichment of unsaturated FA in the phospholipids of buttermilk and saturated FA butter serum phospholipids. This was expected to result from the preferential retention of saturated FA containing glycerophospholipids in butter during churning. Still, the factors determining the lipid distribution in buttermilk and butter remain as open questions.

Separation of the valuable MFGM components from the complex dairy matrices is difficult. Removal of major milk proteins by cream washing prior to butter making has been proposed to increase the yield of MFGM in the buttermilk and butter serum dry matter (Jukkola et al., 2019; Lamothe, Robitaille, St-Gelais, & Britten, 2008; Morin, Britten, Jiménez-Flores, & Pouliot, 2007). However, cream washing by microfiltration was reported to reduce also cream MFGM content, that was accompanied by a corresponding increase in small lipid droplets and membrane fragments in the wash solution (Jukkola et al., 2019; Lamothe et al., 2008). When the distribution of MFGM components in the buttermilk and butter serum was investigated, the buttermilk MFGM content was observed to be lower upon cream washing (Lamothe et al., 2008). However, the opposite was reported for butter serum: slightly higher phospholipid content in the butter serum obtained from the washed cream compared to non-washed cream. An explanation for this observation remains elusive. Differences in the phospholipid composition of buttermilk and butter serum were also reported in the same study but no further insights were offered. Better understanding on the behaviour of the MFGM lipids during butter making is of utmost importance to fully enable the potential health benefits of milk lipids.

The aim of this study was to investigate the distribution of MFGM in the buttermilk and butter serum produced from cream and microfiltered cream, which was deficient in the smallest fat globules. By

thoroughly comparing the composition and thermal behaviour of the lipids in the cream washing and butter making process streams, the MFGM partitioning during butter making was elucidated.

2. Materials and methods

2.1. Materials

Bovine cream (40.9 ± 0.7% fat) was collected and pasteurized (75 °C, 20 s) in a dairy unit one day prior to each experiment (Valio Ltd., Jyväskylä, Finland). The creams were stored at 5 °C and processed the following day. The lipid standards (1,2-dinonadecanoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine, tritridecanoin and tridecanoic acid methyl ester) were purchased from Larodan, Sweden. Supelclean™ LC-Si SPE Tubes (500 mg, 3 mL) were purchased from Sigma-Aldrich (MO, USA). All reagents used in analyses were analytical grade.

2.2. Cream washing by microfiltration

Filtration was carried out as described previously (Jukkola et al., 2019) using a uniform trans-membrane pressure (UTP) filtration system (Tetra Alcross®, Tetra Pak, Denmark) equipped with multichannel tubular ceramic membranes, with 1.4 µm pore size and 0.24 m^2 filtration surface area (Membralox®, Pall Corporation, France). Ten kilograms of cream were diluted (1:10) with deionised water and preheated to 50 °C. The filtration of the diluted cream was continued until the mass of the retentate corresponded to the amount of the initial cream. The procedure was repeated three times. The temperature was kept at 50–55 °C and the pressure at 0.6 bar UTP during the filtrations. Samples from the retentate and permeate were collected after each cycle and stored at 4 °C until analysis. The collected permeates were lyophilised for further analysis and the creams were tested as liquid suspensions. Three replicate filtration experiments were carried out in the intervals of two weeks.

2.3. Butter making

Microfiltration retentates and non-washed creams were churned into butter. Prior to churning (Elba 30, Elecrem, France), the creams were ripened following the temperature program: 36 h at 5 °C, 2 h at 19 °C and cooling to 12 °C. The churning temperature was 12.0 °C. After buttermilk separation, the butter mass was worked into butter by mixing gently, 30 s after which the residual buttermilk was removed and combined to the primary buttermilk. The working step was repeated once.

Butter serum was obtained by melting butter (40 g) 1 h at 60 °C and subsequently centrifuging (15 min, 2000 × *g*). The aqueous phase at the bottom was collected and re-centrifuged (10 min, 1500 × *g*) to remove the oil traces. Buttermilk and butter washing solutions were lyophilised for further analysis. Creams and butter serum were analysed as liquid material.

2.4. Chemical composition

2.4.1. Lipid extraction

Lipids from cream, buttermilk and butter serum were extracted with modified method of Blight and Dyer (1959). Cream and butter serum (250 µL) were diluted to 1 mL using 0.9% NaCl and 4 mL of dichloromethane/methanol (2:1) was added. The suspensions were vortexed for 15 s and shaken vigorously at ambient temperature for 30 min. After centrifugation at 1500 × *g* for 5 min, the lower phase was collected in a clean tube and the upper layer was re-extracted with 2 mL of dichloromethane, as described above. The extracts were combined and evaporated to dryness under nitrogen stream. The buttermilk and permeate lipids were extracted similarly from 50 mg of lyophilised powder, which was suspended in 1 mL 0.9% NaCl prior to the extraction.

2.4.2. Solid phase extraction

For solid phase extraction, a slightly modified analysis of that described by Jukkola et al. (2019) was used. Briefly, the lipid samples were supplemented with internal standard lipids prior to solid phase extraction. Phospholipid standards used included 1,2-dinonadecanoyl-*sn*-glycero-3-phosphatidylcholine and 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine. The triacylglycerol standard was tritridecanoin. The lipid samples were dissolved in 0.25 mL dichloromethane/methanol (2:1) and applied to the columns, which were preconditioned with 2 mL hexane. To elute the neutral lipids, 2 mL hexane/diethylether (4:1) and 2 mL of hexane/diethylether (1:1) were used. The polar lipids were eluted with 2 mL of methanol and 2 mL of dichloromethane/methanol/H_2O (3:5:2). The extracts were evaporated to dryness under N_2 and stored frozen for further analysis.

2.4.3. Thin layer chromatography

We used an adapted TLC method to which a second dimension was added for better separation of dairy phospholipids. In this method**,** polar lipids were dissolved in 0.2 mL dichloromethane/methanol and applied to TLC-plates. The plates were let to develop in a chamber containing dichloromethane/methanol/ammonium hydroxide (65:25:4) for 1 h for the separation of sphingomyelin and anionic phospholipids: phosphatidyl serine (PS) and phosphatidyl inositol (PI), from phosphatidyl choline (PC) and PE. The plates were turned 90° and eluted again with dichloromethane/methanol/H_2O (65:25:4) for 1 h, during which the anionic phospholipids were separated from SM. The plates were stained by spraying 0.001% Rhodamine 6G in water and observed under UV-light. The bands containing the lipids were scraped off the plate into clean tubes and flushed with N_2. Due to the low amounts of anionic phospholipids in the samples, the spots of PS and PI were combined to ensure that the detection limit for all FA was reached. Because of a lack of internal standard, a series of SM, PS and PI standards of varying concentrations were applied to the individual TLC plates and processed similarly as the corresponding samples.

2.4.4. Methylation and gas chromatography

Methylation of total polar lipids. Polar lipids directly from SPE were methylated with 0.5 mL of boron trifluoride (14%) in MeOH by incubating for 90 min in a boiling water bath as described by Jukkola et al. (2019). After cooling, the samples were supplemented with 1.5 mL distilled water.

Methylation of glycerophospholipid classes and neutral lipids. The individual polar lipid classes that were scraped off the TLC plate (except sphingomyelin, see below), and neutral lipids directly from SPE, were saponified with 1 mL of 3.7 M NaOH in 49% methanol by incubating for 30 min in a boiling water bath according to Suutari and Laakso (1990). Prior to saponification, the samples and standards containing anionic phospholipids were supplemented with a tridecanoic acid methyl ester standard. After cooling to room temperature, 4 mL of 3.3 M HCl in 48% methanol was added and the methylation reaction was let to occur at 80 °C for 30 min and cooled to room temperature.

Methylation of sphingomyelins. The sphingomyelins were extracted from the TLC silica with 3 mL dichloromethane/methanol (2:1) by shaking 30 min after which the silica was pelleted by centrifugation (1500 × *g*, 5 min) and re-extracted similarly. The solvent was evaporated under N_2 stream. The sphingomyelin FA were methylated with 1 mL of methanol/32% HCl (5:1) by incubating 5 h at 75 °C. Prior to methylation, the samples and standards containing sphingomyelins were supplemented with the tridecanoic acid methyl ester standard. After cooling, 1 mL of deionised water was added.

Extraction of the methyl esters and GC analysis. The methyl esters were extracted from the aqueous suspensions to the organic phase by adding 1 mL of hexane/MTBE (1:1) and shaken vigorously (300 rpm) for 10 min at room temperature. The aqueous phase was removed, and the organic phase was washed with 2 mL of 0.01 % (w/w) NaOH. To sharpen the phase boundary, the tubes were centrifuged for 20 min (1600 × *g*). The organic phase was dried with anhydrous sodium sulphate and transferred to the GC vial. To concentrate the samples, the solvent was evaporated under N_2 stream and the methyl ester residue was dissolved in 100 µL of hexane. Agilent 7890 A GC system was used for the separation of the methyl esters in a Zebron ZB-FAME column (60 m × 250 µm × 0,2 µm). The temperature gradient in the oven was: 70 °C (held 4 min), 70 to 110 °C at 8 °C min^{-1} (held 0 min), 110 to 190 °C at 3 °C min^{-1} (hold 5 min), 190 to 280 °C at 6 °C min^{-1} (hold 3 min). The detector the gas flow was 350, 35 and 30 mL min^{-1} for air, N_2, and H_2,

respectively. A split ratio was 20:1. The peak areas of fatty acids in the standard lipids were used as a reference in calculation of the concentration of the sample lipids. The degree of unsaturation (DUS) was calculated by the equation (1), where n = 6.

$$DUS = \frac{\sum_{i=1}^{n} i(amount\ of\ FA\ having\ i\ double\ bonds)(mg\ g^{-1})}{total\ amount\ of\ FA\ (mg\ g^{-1})} \quad (1)$$

2.4.5. Protein composition

Protein composition of the non-washed and washed creams, and buttermilks and butter serums obtained from these creams were visualised on SDS-PAGE (Criterion 4–20% TGX gel, Bio-Rad Laboratories Inc., USA) to see the efficiency of cream washing on protein removal and subsequent effect on protein composition in buttermilk and butter serum. Sample volume was adjusted to be the same in each material to be able to see the changes in individual protein intensity before and after microfiltration of the cream (the protein load on the gel was varied).

2.5. Thermal behaviour

Thermal analyses were conducted on a Mettler Toledo DSC 3+ differential scanning calorimetry in 40 µL aluminium pans (1/3 ME-51119870) which were hermetically sealed. An empty 40 µL aluminium pan was used as a reference.

2.5.1. Cream ripening mimicking program

Isothermal heating at 60 °C for 10 min; followed by a gradient from 60 to 5 °C at a rate of 5 °C min^{-1}; isothermal heating at 5 °C for 10 min followed by a gradient from 5 to 19 °C, at a rate of 1 °C min^{-1}; isothermal heating at 19 °C for 15 min; followed by a gradient from 19 to 0 °C, at a rate of 0.2 °C min^{-1}. The material in this experiment was lyophilised microfiltration retentate (washed cream) and the corresponding buttermilk. The sample amount was 15–20 mg, average graph of the three parallel samples is shown.

244

245 *2.5.2. Thermal behaviour of neutral lipids*

246 Isothermal heating at 65 °C for 10 min; cooling from 65 °C to –40 °C (1 °C min^{-1}; heating from –40 °C
247 to 65 °C (3 °C min^{-1}). Sample amount was 4–10 mg fat from the SPE extraction (w/o supplementation of the
248 lipid standard). Average graph of the two parallel samples is shown.

249

250 *2.6. Statistical analysis*

251

252 Statistical analysis was done using ANOVA one-way analysis and Tukey's multiple comparison test.

253

254 **3. Results and discussion**

255

256 *3.1. Effect of microfiltration on the protein composition of buttermilk and butter serum*

257

258 In cream microfiltration, dry matter including proteins, lactose and minerals, can be separated from
259 the fat globules (Jukkola et al., 2019), which can be further processed for suitable applications, e.g., to
260 produce buttermilk enriched with MFGM and butter. In our study, three rounds of microfiltration were
261 sufficient to remove majority of the serum proteins from buttermilk and butter serum (caseins, β-
262 lactoglobulin and α-lactalbumin, Fig. 1). Consequently, MFGM proteins were enriched, including xanthine
263 oxidase/dehydrogenase, butyrophilin; and periodic acid Schiff 6/7. The low serum protein content
264 facilitated the isolation of the MFGM fractions from the buttermilk and butter serum enabling desired
265 applications.

266 A polar lipid (PL) concentration of 1.6 mg g^{-1} (wet weight) was determined in the non-washed
267 cream but after the microfiltration the concentration was reduced to 1.0 mg g^{-1}, indicating that 34% of PL
268 was removed by cream washing (Table 1). The detailed stream composition upon cream washing indicated
269 a significant reduction in PL concentration also in buttermilk. The buttermilk from the non-washed cream

contained 0.89 mg g^{-1} PL, whereas the buttermilk from the washed cream contained no more than 0.20 mg g^{-1}. Interestingly, the removal of the PL by microfiltration was reflected only in the buttermilk PL, but not in butter serum PL (Fig. 2). In fact, the concentration of PL in butter serum rather increased from 8.2 mg g^{-1} (butter serum from non-washed cream) to 9.3 mg g^{-1} (butter serum from the washed cream). This fact raised interest to further investigate the distribution of the lipids in buttermilk, butter serum and permeate and to better understand the behaviour of MFGM during cream washing and churning.

3.2. Lipid composition in the butter making process streams

The lipids in the cream, buttermilk, butter serum and microfiltration permeate were examined in terms of the PL-to-fat ratio (fat = PL + neutral lipids) to elucidate the potential structure of the lipids in the respective streams. A high PL-to-fat would indicate the presence of very small lipid droplets or membrane fragments. Owing to the smaller surface-to-volume ratio of the large fat globules, the creams contained very low ratios of PL, 0.39 and 0.30% in the non-washed and washed creams, respectively (Table 1). In contrast, the permeated lipids consisted of 8.1% PL-to-fat. The buttermilk from non-washed cream or washed cream contained 5.8 or 3.8 % PL-to-fat, respectively. The recovery of a large amount of neutral lipids in the permeate and buttermilk pointed to the possibility that the MFGM in these streams was not only in the form of membrane fragments, as reported previously for buttermilk (Britten et al., 2008; Lopez et al., 2017; Morin, Jiménez-Flores & Pouliot, 2007) but also, at least partly, as intact small fat globules. In contrast, based on the PL-to-fat ratio, the lipid structures in the butter serum were totally different compared with buttermilk. In the butter serum obtained from the non-washed cream, the PL proportion per fat was 26% and slightly lower (22%) in the one from the washed cream, indicating very small lipid droplets or different emulsion-forming lipid structures comprising MFGM fragments, for instance vesicles. Bovine milk contains also exosomes, which are extracellular vesicles consisting of proteins, polar lipids and RNA, and their functions are related to cell signalling and modulation of immunology (Blans et al., 2017; Johnstone, Adam, Hammond, Orr & Turbide, 1987; Reinhardt, Lippolis, Nonnecke, & Sacco, 2012).

Presumably, due to their small size (50–200), the exosomes were at least partly fractionated in permeate, but data on their distribution between buttermilk and butter serum from non-washed cream can't be provided by this study.

3.3. Crystallisation behaviour of buttermilk and cream fat during DSC program mimicking cream ripening

Usually, cream is thermally treated before churning to induce the formation of stable fat crystals. To separately observe the behaviour of the lipid droplets in buttermilk and in cream during the ripening program, the lyophilised materials were analysed by DSC, which was programmed mimicking the cream ripening profile. This analysis was possible only for the washed materials, because the non-washed buttermilk contained too little lipids per dry matter to observe the thermal transitions by DSC.

DSC curves of milk fat crystallisation and especially melting are complex because of the wide range of different fatty acids and broad distribution of TAG, which form polymorphic groups with various lamellar structures, and their endotherms partly overlap (Lopez et al., 2001). The crystallisation profiles of the washed cream and the buttermilk produced from the washed cream were quite similar and typical for milk fat during fast cooling from 60 to 5 °C; the washed cream fat had crystallisation peaks at 9.8 and 15.6 °C and buttermilk at 8.9 and 16.6 °C (Fig. 3A). However, great differences were observed when the materials were heated to 19 °C to melt the unstable crystals (Fig. 3B): the washed cream fat had a wide endotherm between 5 and 12 °C, which reflects the melting of the most unstable crystal structures. Additionally, a clear endotherm with a melting point at 16.1 °C, indicating the melting of the metastable crystal structures could be recognised. The buttermilk fat had a wide melting range starting from 5 °C until 13 °C, indicating the melting of the most unstable structures. However, the peak around 16 °C was virtually absent and only a very small endotherm at 17.3 °C was observed, indicating the absence of the metastable crystal forms in the buttermilk. The most interesting observation was the formation of stable crystals monitored in the buttermilk and washed cream during cooling from 19 °C and below, which may be relevant to the

crystallisation during churning: The stable crystals formed in the washed cream at 13.2 °C, but in the buttermilk it occurred only at 10.5 °C, which was clearly below the churning temperature (Fig. 3C). Thus, our study proves that the buttermilk lipids were not fully crystallised during churning. The small amount of crystalline fat in those incompletely crystallised fat globules could not possibly disrupt the globule membrane and the globules remained intact in the aqueous phase. This is in line with the previous studies (Lopez et al., 2001; Michalski, Ollivon, Briard, Leconte & Lopez, 2004), in which higher supercooling and a displacement of crystallisation temperature towards lower temperatures were discovered, when the fat globule size decreased.

3.4. Melting behaviour of the neutral lipids in the butter making process streams

The neutral and polar lipids of the non-washed and washed cream, microfiltration permeate, buttermilk and butter serum produced from the washed cream were isolated, and the melting behaviour of the neutral lipids, consisting primarily of TAG, was evaluated to verify our hypothesis that the partitioning of the fat globules between the streams was driven by the physical properties of the lipids. The analysis showed that the melting behaviour of the neutral lipids in cream, washed cream, permeate and buttermilk was typical of the milk fat in each fraction. The melting point of the major endotherm (MMF) in buttermilk was 15.5 °C (non-washed cream) and 15.6 °C (washed cream), which are close to that of microfiltration permeate, 15.9 °C (Fig. 4A,B). In the creams, the melting point was slightly higher for the non-washed cream, 16.2 °C or for the washed cream, 16.4 °C. The observed melting behaviour in the above-mentioned materials suggest that the FA composition in these streams corresponded to the non-fractionated milk fat.

The melting profile of butter serum neutral lipids was very different from typical milk fat. Two endothermic peaks could be recognised. The first at a melting point of 14.1 °C (non-washed cream) and 14.3 °C (washed cream). The second large peak at a melting point of 47.4 °C (non-washed cream) and 45.1 °C (washed cream), which are much higher than those of the native milk fat. The second peak indicates the presence of the crystals with higher melting point than those of typical milk fat. This kind of melting

348 behaviour in the butter serum lipids shows that there was a fraction of TAG with high melting point
349 concentrated in butter serum. It is possible that during cream ripening, the TAG, which crystallised first,
350 were concentrated on the fat globule surface and, possibly, associated with the MFGM. These structures
351 were then reorganised to hydrophilic particles during butter melting and ended up in the butter serum.
352 This membrane-associated, high-melting butterfat fraction was described by Jenness and Palmer (1945)
353 and characterised further for FA composition by Patton and Keeney (1958). However, in the recent
354 literature the specific properties of butter serum neutral lipids are mostly ignored.
355
356 *3.5. Fatty acid composition of the neutral lipids in the butter making process streams*
357
358 To further support our findings from the thermal behaviour, the neutral lipids of buttermilk and
359 butter serum, creams and microfiltration permeate were analysed for FA composition. The results revealed
360 clear differences between the FA compositions in buttermilk and butter serum: The FA in butter serum
361 neutral lipids were significantly enriched with saturated FA, especially C16:0 and C18:0, but contained less
362 medium chain FA (C8:0, C10:0, C12:0) and unsaturated FA compared with buttermilks (Table 2). Again, the
363 buttermilks and permeate neutral lipids resembled each other in FA composition. The neutral lipid FA
364 composition in the cream represents bulk milk fat composition and is for the most part similar to the
365 neutral lipid FA composition of buttermilk but different from the butter serum. The neutral lipids in these
366 streams consist >98% of TAG (minor amounts of diacylglycerols and free fatty acids were also present, data
367 not shown). The melting point of TAG depends on the FA composition and position of the FA in the glycerol
368 backbone (Breitschuh & Windhab, 1998; Sato, Ueno, & Yano, 1999). The high concentration of long chain
369 saturated FA (C16:0 and C18:0) and low concentration of short and medium chain FA and unsaturated FA
370 provide support for the high melting temperature in butter serum TAG. Even if the short chain FA are
371 saturated, their presence lowers the TAG melting point, especially when esterified with C16:0, as suggested
372 by Tzompa-Sosa et al., 2016. The difference in the physical properties of the lipids in the above-mentioned
373 streams was visible also during the analysis: after drying neutral lipids at 30 °C, butter serum lipids were

white and solid but the buttermilk, permeate and cream lipids remained liquid. Since the FA composition was different from bulk milk fat, we ruled out that the neutral lipids in butter serum was the result of contamination from the oil phase upon butter serum separation. Thus, there is indication that the saturated neutral lipid portion was originally integrated in the crystalline fat structures, which then were concentrated in butter serum upon butter melting.

3.6. Phospholipid composition in the butter making process streams

The phospholipid composition in the buttermilks and butter serums was compared to determine any differences in the membrane lipid structures of the fat globules, which have different physical properties. Cream phospholipid compositions were also evaluated to gain insights on the effect of cream washing on the composition of the membrane lipids. The lipids that were selected in the analysis were those that are most relevant to phospholipids in milk fat, namely, PC, PE and SM. The anionic phospholipids, PS and PI were not separated from each other and therefore they are referred thereafter as 'anionic phospholipids'.

Comparison of the phospholipid composition of the buttermilk and butter serum from non-washed and washed creams indicated that the cream washing process did not affect phospholipid composition. However, the phospholipid composition in the buttermilk and butter serum was different, which has been observed previously (Bourlieu et al., 2018; Britten et al., 2008; Lopez et al., 2017; Rombaut, Van Camp, & Dewettinck, 2006). Our study shows the enrichment of SM in the butter serum fraction (29.8 and 30.4% for the butter serums from non-washed and washed creams, respectively), and a depletion in anionic phospholipids (7.1 and 5.4%, respectively) compared to the buttermilks (22.5 and 18.3% SM and 11.6 and 10.6% anionic phospholipids in the buttermilk from non-washed and washed cream, respectively) (Fig. 5). The proportion of PE was higher in buttermilks (34.7 and 34.7%) compared to butter serums (27.8 and 29.2%) but the results were not statistically significant.

In addition to the phospholipid composition, the FA composition in the studied phospholipids was different when buttermilk and butter serum were compared (Table 3). As a general conclusion, the long chain unsaturated FA were enriched in buttermilks whereas in the butter serum phospholipids the long chain saturated FA were enriched. The degree of unsaturation (DUS), a parameter by which the fluidity of lipids based on solely the content of unsaturated FA can be roughly estimated, was defined here as average ratio of double bonds per fatty acid molecule in each lipid class. Based on our results on FA composition, DUS was calculated in all the studied lipid classes. An interesting observation is the consistently higher DUS in all the buttermilk phospholipids compared to butter serum (Table 3). This indicates the more fluid nature of the MFGM structures in that fraction and is in line with the DUS of neutral lipids (Table 2). During cream ripening, the crystalline shell has been reported to form on the surface of the fat globule (Juriaanse & Heertje, 1988). Thus, the MFGM phospholipids have certainly an important role in the crystallization.

Associated with this, it is not surprising that SM, which consist almost exclusively of long chain saturated FA (C22:0, C23:0 and 24:0) and having high crystallisation temperature, tended to concentrate in butter rather than buttermilk. Instead, PE and anionic phospholipids have high DUS, and therefore it is reasonable to expect that these phospholipids are enriched in buttermilk and microfiltration permeate. Even if the statistically significant PC enrichment in butter serum could not be confirmed as reported earlier (Bourlieu et al., 2018; Rombaut et al., 2006), the higher DUS (0.65 and 0.68 in the buttermilk from the non-washed and washed cream, respectively) compared with that of butter serum (0.54 and 0.53, respectively) is highlighted in this particular phospholipid. The results suggest that buttermilk and butter serum differ also in the physical properties of MFGM lipids. This is explained either from the fluidity of the fat globules of different size and/or the partitioning of the globules based on their thermal behaviour.

The curvature of the membrane is affected by the phospholipid composition. PC, having a large head group, can easily accommodate saturated FAs and tends to assemble in lamellar structures (Cullis & De Kruijff, 1979). Lamellar structures are better suited to accommodate large fat globules having smoother surfaces than those of the small fat globules, which have high curvature. This is possibly a reason for our finding of the concentration of the more saturated forms of PC in butter serum. SM associates with

cholesterol and forms rigid liquid-ordered domains called lipid rafts, mostly on the outer leaflet of the MFGM (Lopez et al., 2011). As rafts are less fluid compared with the glycerophospholipid forming liquid-disordered matrix, it was not surprising that SM was enriched in the MFGM parts, which were concentrated in the butter phase, and further in butter serum. PE has small head group and is typically present in curved membrane structures; this lipid accommodates preferably unsaturated FA, favouring a negatively curved membrane assembly (Cullis & De Kruijff, 1979). Thus, it is suitable that lipids in the strongly negatively curved inner leaflet of the outer bilayer of small lipid droplets are the reason for their enrichment in buttermilk. Enrichment of PE in buttermilk has been reported also earlier (Lopez et al., 2017). From the results of our study can be detected, that the PE/PC ratio in the PL fraction was > 1 for the buttermilk and microfiltration permeate, which were assumed to contain small fat globules. The PE/PC ratio was lower <1 for those fractions originating from large fat globules (cream, butter serum). These observations are in line with the earlier studies (Lopez et al., 2011) where the proportions of phospholipids in small and large fat globules were evaluated.

Our results indicate that microfiltration induced some damage to the fat globule membrane given that the permeated phospholipids were enriched in PC, which has been suggested to be loosely integrated in the outer layers of MFGM (Zheng, Jiménez-Flores, & Everett, 2014). In addition to intact small fat globules, the microfiltration permeate was assumed to contain some fragmented MFGM originating from the large fat globules, which are more susceptible to shear stress than small fat globules (Wiking, Björck, & Nielsen, 2003). Somewhat disintegrated MFGM fragments most probably are fractionated in buttermilk, as identified previously via TEM imaging of buttermilk (Lambert et al., 2016; Morin et al., 2007). However, the procedure of MFGM isolation by high speed centrifugation used in these studies may selectively concentrate MFGM fragments, whereas small lipid droplets may have resisted sedimentation, which may have led to the conclusion that the fragments were highly enriched in buttermilk. Our results support the hypothesis that in the butter making process, the small fat globules dominate buttermilk over those of MFGM.

4. Conclusions

Phase inversion dynamics is expected to be critical in the partitioning of MFGM in butter making. Rather than a full disintegration and release in the buttermilk, to a great extent, the membrane material is entrapped in butter mass because of the non-complete detachment of the membrane from the neutral fat. Thus, only a minor portion of MFGM material remains in buttermilk. These are the fluid, small fat globules that do not participate in the crystalline butter mass formation. Instead, the crystalline MFGM fractions (possibly associated with the very-high-melting TAG fraction), which participate in the formation of the crystalline network in butter, are released in butter serum upon butter melting. What kind of structures they form during the melting process should be examined by imaging and other direct enquiries. The balance of MFGM components between buttermilk and butter serum can be influenced by adjusting the churning temperature and/or the cream ripening program. Differences in lipid structure may lead to variations in the properties of the buttermilk and butter serum based on MFGM, which have a direct impact as far as the technological functionality and nutritional value.

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Table 1

Polar lipid content and percentage of fat in the butter making process streams from the non-washed and washed cream. [a]

Lipid	Cream		MF permeate	Buttermilk		Butter serum	
	Non-washed	Washed		Non-washed	Washed	Non-washed	Washed
Polar lipid content (mg g^{-1})	1.6 ± 0.2	1.0 ± 0.2	0.025 ± 0.002	0.89 ± 0.16	0.20 ± 0.06	8.2 ± 0.2	9.3 ± 0.5
Polar lipid per fat (%)	0.39 ± 0.04	0.29 ± 0.05	8.1 ± 1.4	5.8 ± 1.0	3.8 ± 0.9	26 ± 3	22 ± 2

[a] Fat = polar lipids + neutral lipids.

Table 2

Fatty acid composition (%) and degree of unsaturation (DUS) of neutral lipids in the butter making process streams from the non-washed and washed cream. [a]

Fatty acid	Cream		Microfiltration permeate	Buttermilk		Butter serum	
	Non-washed	Washed		Non-washed	Washed	Non-washed	Washed
C4:0	1.6 ± 0.2	1.1 ± 0.0	0.7 ± 0.1	1.0 ± 0.1	2.4 ± 0.4	0.6 ± 0.1	0.7 ± 0.1
C6:0	2.8 ± 0.3	1.8 ± 0.1	1.2 ± 0.1	1.9 ± 0.5	3.3 ± 0.5	0.9 ± 0.1	1.0 ± 0.1
C8:0	2.3 ± 0.2	1.4 ± 0.1	1.0 ± 0.1	1.5 ± 0.4	2.3 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
C10:0	5.6 ± 0.4	3.5 ± 0.2	2.9 ± 0.2	3.4 ± 0.7	4.8 ± 0.5	2.0 ± 0.3	1.9 ± 0.3
C12:0	5.5 ± 0.1	4.4 ± 0.2	3.5 ± 0.2	4.1 ± 0.6	5.0 ± 0.5	3.0 ± 0.4	2.8 ± 0.4
C14:0	14.4 ± 0.1	14.8 ± 0.5	12.6 ± 0.4	13.8 ± 1.0	13.9 ± 0.3	13.0 ± 0.8	12.2 ± 0.8
C14:1	1.7 ± 0.0	1.5 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.6± 0.1	0.9 ± 0.1	0.9 ± 0.1
C15:0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
C16:0	32.2 ± 0.3	35.4 ± 0.0	35.7 ± 0.4	35.2 ± 0.8	30.5 ± 1.3	41.9 ± 1.2	40.4 ± 1.2
C16:1	1.6 ± 0.0	1.7 ± 0.0	1.4 ± 0.0	1.7 ± 0.1	1.6 ± 0.0	1.0 ± 0.1	1.1 ± 0.1
C18:0	8.2 ± 0.1	7.9 ± 0.4	11.7 ±0.3	10.5 ± 0.8	9.2 ± 0.7	16.3 ± 1.0	16.2 ± 1.0
C18:1	19.9 ± 0.7	21.2 ± 0.6	23.2 ± 0.8	20.7 ± 2.5	20.3 ± 0.3	15.9 ± 0.4	18.1 ± 0.4
C18:2	1.3 ± 0.1	1.9 ± 0.0	1.8 ± 0.3	1.8 ± 0.2	1.8 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
Other	1.9 ± 0.2	2.1 ± 0.1	2.0 ± 0.5	1.8 ± 0.4	2.3 ± 0.6	1.6 ± 0.2	1.6 ± 0.2
DUS	0.27 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.22 ± 0.01	0.25 ± 0.01

[a] Fatty acids having concentration higher than 1% (in the non-washed cream) are shown; data are the average of three samples ± standard deviation.

Table 3

Fatty acid composition (%) and degree of unsaturation (DUS) of phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, anionic phospholipids and sphingomyelin) in the butter making process streams from the non-washed and washed cream. [a]

Fatty acid	Cream		Microfiltration permeate	Buttermilk		Butter serum	
	Non-washed	Washed		Non-washed	Washed	Non-washed	Washed
Phosphatidyl choline							
C14:0	6.4 ± 0.1	6.8 ± 0.2	6.7 ± 0.4	7.0 ± 0.1	6.1 ± 0.4	9.7 ± 0.0	8.8 ± 0.2
C16:0	36.6 ± 0.1	33.2 ± 1.9	36.6 ± 1.6	33.2 ± 0.9	29.4 ± 0.4	39.4 ± 1.1	36.7 ± 0.8
C16:1	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.1	1.7 ± 0.0	2.0 ± 0.1	1.4 ± 0.0	1.4 ± 0.0
C18:0	9.9 ± 0.4	11.5 ± 0.9	10.0 ± 1.4	7.0 ± 0.2	7.0 ± 0.0	9.5 ± 0.3	10.1 ± 0.3
C18:1	35.0 ± 1.0	35.0 ± 1.0	35.3 ± 1.9	36.6 ± 0.3	42.4 ± 0.5	29.4 ± 1.0	31.6 ± 0.3
C18:2	6.9 ± 0.0	7.2 ± 0.3	5.6 ± 1.1	9.1 ± 0.2	8.6 ± 0.3	6.4 ± 0.1	6.5 ± 0.3
Other	3.9 ± 0.5	4.9 ± 0.2	4.5 ± 0.7	4.8 ± 0.1	4.4 ± 0.2	4.1 ± 0.2	4.9 ± 0.2
DUS	0.61 ± 0.02	0.59 ± 0.00	0.52 ± 0.03	0.68 ± 0.02	0.70 ± 0.02	0.52 ± 0.04	0.54 ± 0.02
Phosphatidyl ethanolamine							
C16:0	12.1 ± 0.2	12.5 ± 1.0	13.1 ± 1.6	8.9 ± 0.5	8.0 ± 0.1	12.1 ± 0.2	12.3 ± 0.3
C16:1	1.4 ± 0.1	1.4 ± 0.1	0.9 ± 0.6	1.4 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	1.5 ± 0.0
C18:0	10.2 ± 0.6	9.6 ± 0.1	11.3 ± 0.7	8.9 ± 0.3	7.8 ± 0.3	11.8 ± 0.1	11.5 ± 0.0
C18:1	60.7 ± 0.1	59.3 ± 0.4	60.6 ± 3.2	60.7 ± 2.1	65.3 ± 0.4	52.2 ± 0.1	53.6 ± 0.8
C18:2	12.7 ± 0.3	12.7 ± 0.3	9.5 ± 1.9	13.7 ± 0.0	11.3 ± 0.3	14.0 ± 0.2	13.7 ± 0.2
Other	2.9 ± 0.4	4.7 ± 0.5	4.6 ± 0.7	6.4 ± 0.4	5.8 ± 0.6	8.3 ± 0.1	7.2 ± 0.1
DUS	0.90 ± 0.02	0.91 ± 0.01	0.87 ± 0.03	0.98 ± 0.01	1.02 ± 0.01	0.94 ± 0.01	0.92 ± 0.01
Anionic phospholipids							
C16:0	5.9 ± 0.3	5.6 ± 0.0	7.0 ± 0.9	5.1 ± 0.4	5.3 ± 0.3	8.3 ± 0.9	10.3 ± 0.9
C18:0	35.4 ± 0.3	35.6 ± 0.2	33.4 ± 0.3	30.5 ± 0.3	29.1 ± 2.1	30.3 ± 1.2	30.6 ± 1.0
C18:1	45.9 ± 0.8	44.7 ± 0.0	49.8 ± 1.9	46.4 ± 0.0	49.9 ± 1.3	41.0 ± 0.1	41.2 ± 0.2
C18:2	8.0 ± 0.9	9.6 ± 0.1	5.7 ± 0.9	10.4 ± 0.1	9.0 ± 0.8	10.0 ± 0.2	8.4 ± 0.1
Other	4.8 ± 0.5	4.5 ± 1.0	3.1 ± 0.6	7.7 ± 2.0	6.7 ± 1.3	10.5 ± 0.5	9.6 ± 0.4
DUS	0.72 ± 0.01	0.65 ± 0.04	0.68 ± 0.04	0.79 ± 0.04	0.77 ± 0.03	0.67 ± 0.09	0.63 ± 0.07
Sphingomyelin							
C16:0	17.1 ± 0.2	16.9 ± 1.7	20.0 ± 0.2	15.8 ± 1.1	13.9 ± 1.5	18.7 ± 1.8	15.6 ± 1.1
C18:0	6.7 ± 3.5	0.9 ± 0.7	5.2 ± 1.4	3.2 ± 1.6	8.7 ± 2.3	6.7 ± 3.5	3.5 ± 0.9
C22:0	20.4 ± 3.7	21.5 ± 1.4	20.5 ± 0.6	21.0 ± 1.6	16.7 ± 1.7	20.2 ± 2.8	23.6 ± 0.3
C23:0	18.8 ± 2.9	22.6 ± 1.4	21.3 ± 1.0	21.9 ± 0.4	17.6 ± 1.9	20.9 ± 3.3	24.9 ± 0.4
C22:4	1.1 ± 0.1	3.1 ± 0.1	1.4 ± 1.0	1.6 ± 1.3	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C24:0	16.7 ± 1.4	18.5 ± 0.6	17.5 ± 1.5	17.5 ± 1.2	13.3 ± 1.5	17.1 ± 2.9	20.4 ± 0.6
C24:1	6.1 ± 1.5	6.6 ± 0.3	5.1 ± 0.1	6.8 ± 1.4	5.2 ± 0.4	2.5 ± 2.1	3.0 ± 2.6
Other	13.1 ± 1.4	9.7 ± 0.5	8.9 ± 1.0	12.1 ± 1.3	24.1 ± 0	13.7 ± 1.7	8.7 ± 0.6
DUS	0.29 ± 0.04	0.27 ± 0.02	0.41 ± 0.03	0.22 ± 0.07	0.19 ± 0.02	0.13 ± 0.02	0.16 ± 0.01

[a] Fatty acids having concentration higher than 1% (in the non-washed cream) are shown; data are the average of three samples ± standard deviation.

Figure legends

Fig. 1. Protein composition in the buttermilk and butter serum obtained from non-washed or washed cream. Lanes are: A, buttermilk, cream non-washed; B, buttermilk, cream washed; C, butter serum, cream non-washed; D, butter serum, cream washed. Abbreviations: XO/XDH, xanthine oxidase/dehydrogenase; BTN, butyrophilin; PAS 6/7, periodic acid Schiff 6/7; B-LG, β-lactoglobulin; A-LA, α-lactalbumin.

Fig. 2. The distribution of the recovered polar lipids (PL) in the buttermilk (■), butter serum (■) and microfiltration permeate (□) showing the loss of PL in buttermilk, when the cream was washed by microfiltration. Values are calculated by using the mean concentration of PL (mg g^{-1}) in the streams and multiplied by the volume of the stream, SD < 5%.

Fig. 3. Crystallisation behaviour of the fat globules from washed cream (——) and from the buttermilk produced from the washed cream (**-----**): A, cooling from 60 to 5 °C at a rate of 5 °C min^{-1}; B, heating from 5 to 19 °C, at a rate of 1 °C min^{-1}; C, cooling from 19 to 5 °C at a rate of 0.2 °C min^{-1}.

Fig. 4. The melting curves of the neutral lipids in butter making process streams (——, cream; —·-, MF permeate; **-----**, buttermilk; ·····, butter serum): A, non-washed cream; B, washed cream showing also microfiltration (MF) permeate. DSC program: Isothermal heating at 65 °C for 10 min; cooling from 65 to –40 °C (1 °C min^{-1}); heating from –40 °C to 65 °C (3°C min^{-1}).

Fig. 5. Phospholipid composition of the streams of butter making process: ■, phosphatidyl choline; ■, phosphatidyl ethanolamine; □, anionic phospholipids; ◪, sphingomyelin. Buttermilk and butter

serum were produced from the non-washed (non-W) and washed cream (W). Microfiltration (MF) permeate is only produced from the washed cream. The data are average of 2 technical replicates of three samples.

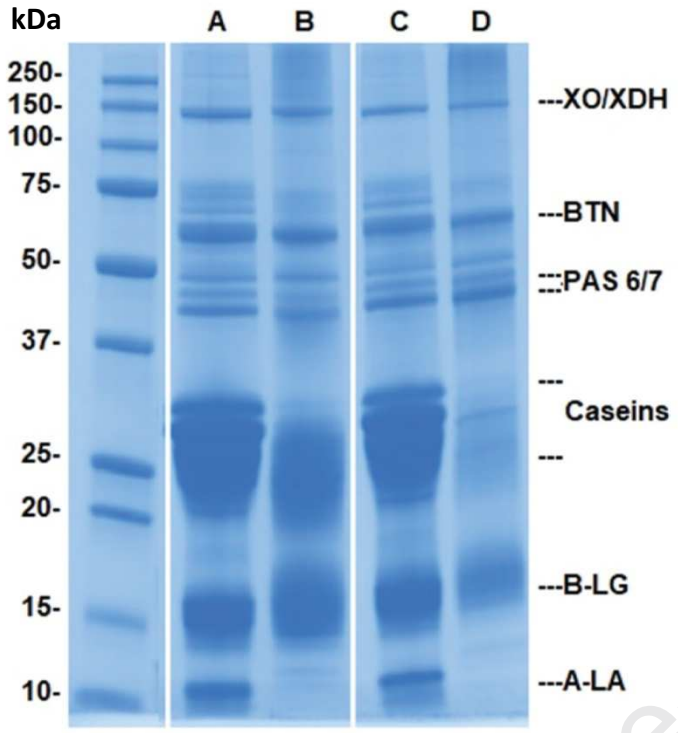

Figure 1

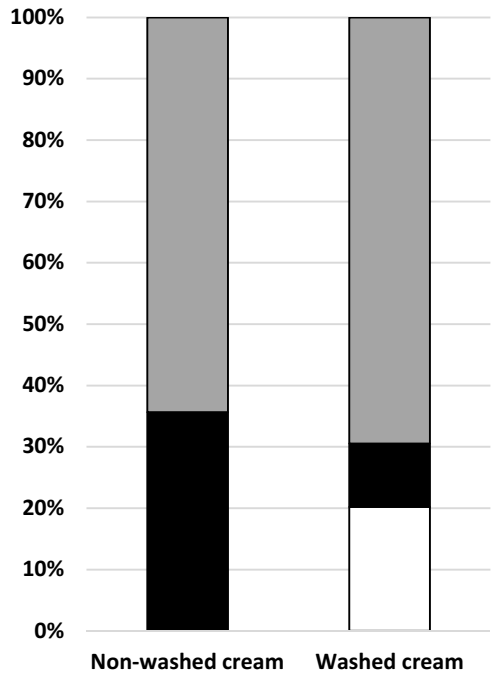

Figure 2

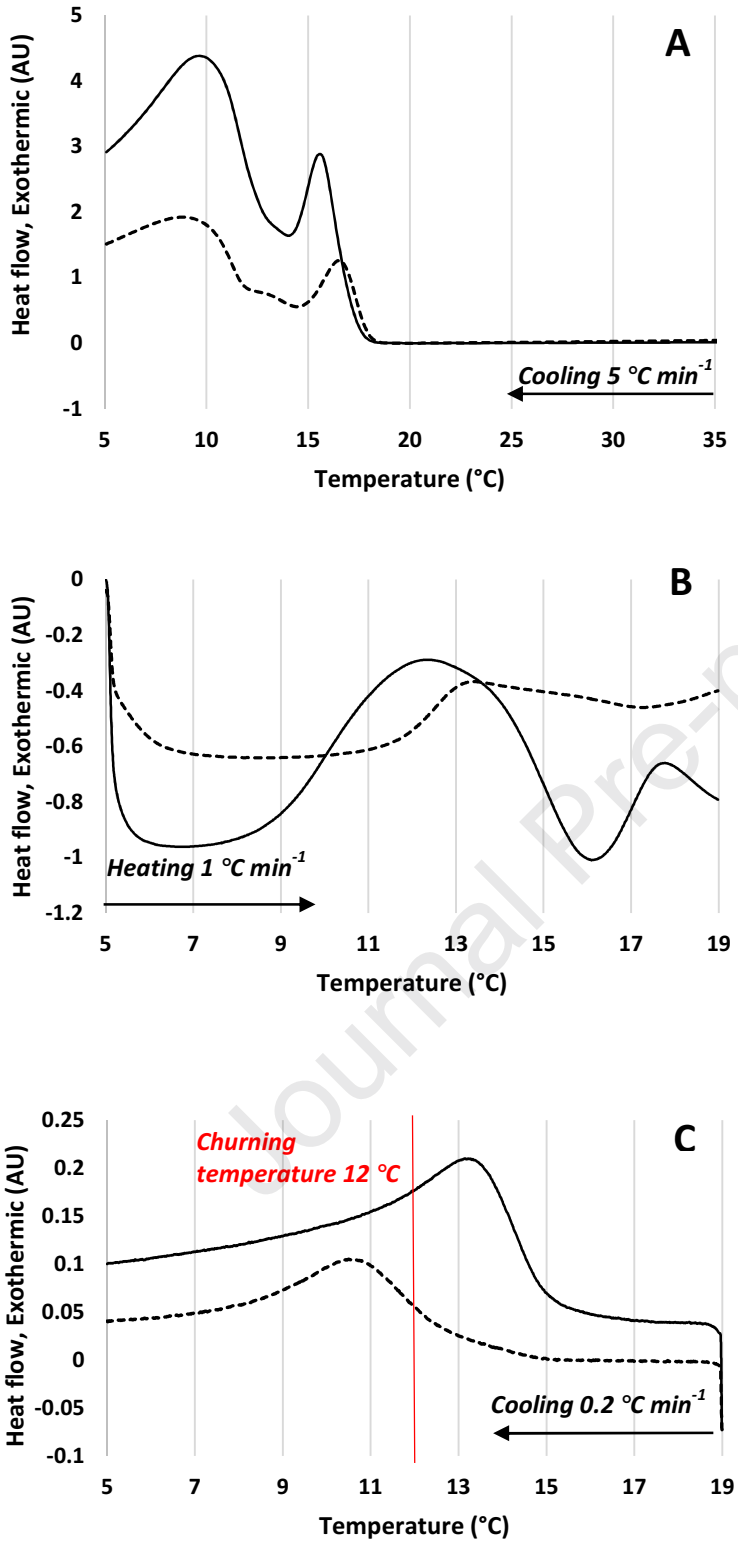

Figure 3

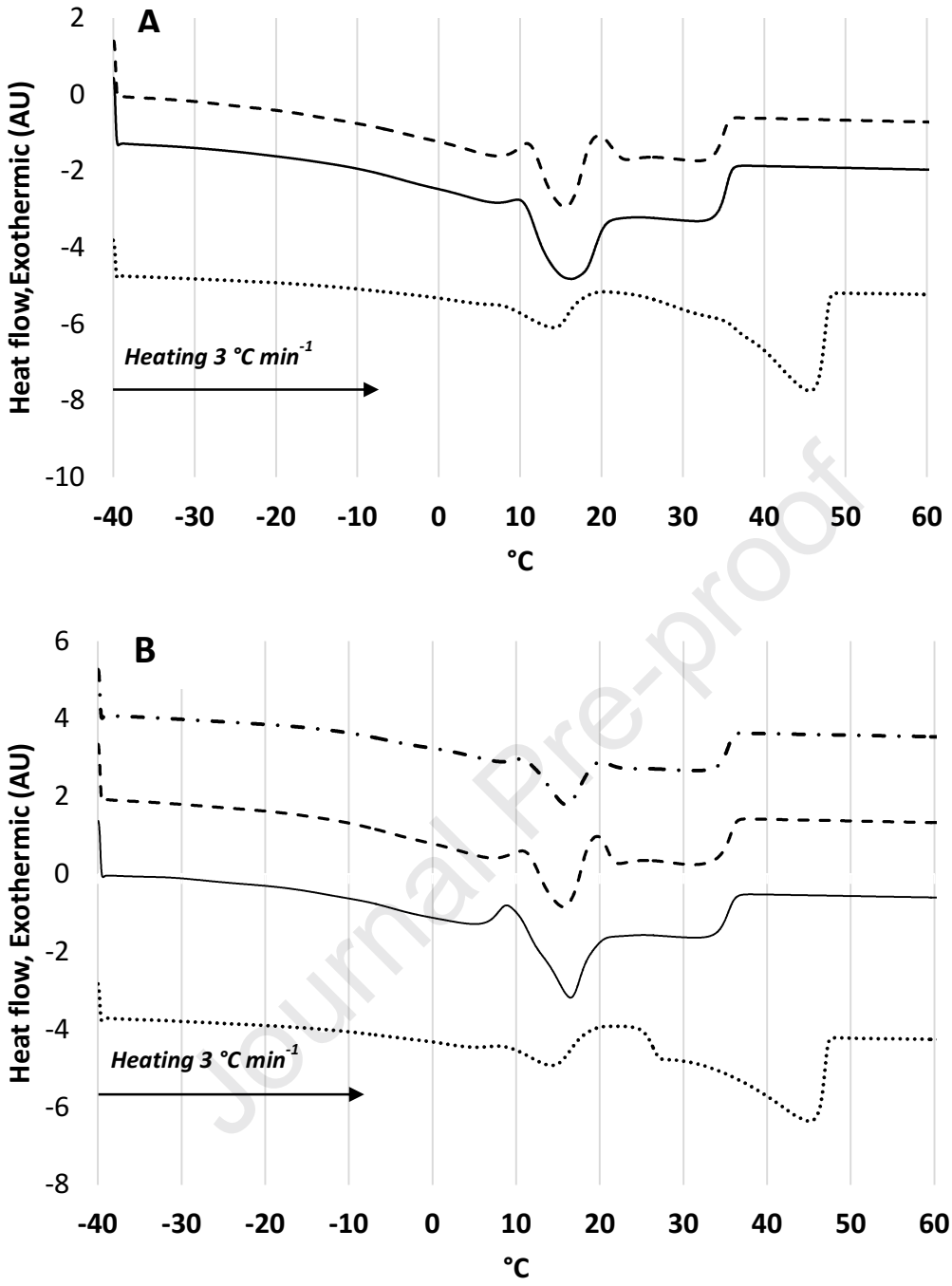

Figure 4

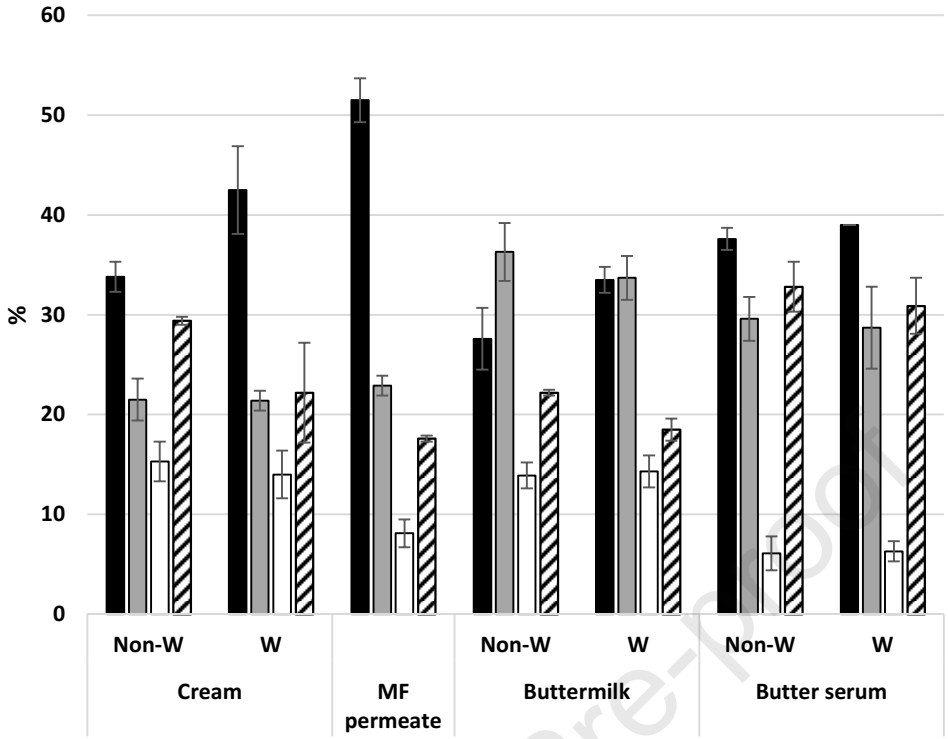

Figure 5