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Changes in milk fat globules and membrane lipids under the shear fields of microfiltration and centrifugation

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Abstract

This study compared the efficiency of centrifugal and microfiltration separation of milk fat globules (MFG) from bovine cream and the changes that take place in the corresponding lipid membranes (MFGM). Creams were washed with water (1:10) and subjected to either centrifugation or microfiltration to fractionate proteins and other non-fat milk components. Protein analyses of the obtained fractions were carried out by gel electrophoresis. Lipid extraction and thin layer chromatography were also employed to separate lipid types and the amount of polar lipids were determined by gas chromatography. The effect of flow conditions on MFG's colloidal properties and MFGM components was evaluated based on estimates of the average rate of energy dissipation in microfiltration and centrifugation processes. Both were equally effective in removing the protein fraction (93% yield) as well as non-fat dry matter (~100% removal). Microfiltration reduced the mean particle size by 0.3 μm, whereas the opposite was observed for centrifugal separation (average size increase by 0.8 μm). The latter process also induced a more significant reduction in the electrostatic charges (zeta potential) of the colloids in the cream, which relates to the changes in the milk fat globule surface composition and the release of MFGM components. The dissociated polar lipids amounted to 24% and 20% upon centrifugation and microfiltration, respectively. Overall, the results suggest that MFG and MFGM are partially damaged under the shear forces typical of centrifugal and microfiltration separation. A high separation efficiency, with minimal fat globule damage and high MFGM yield is possible by adopting microfiltration under carefully optimized conditions.

Keywords: milk fat globules, microfiltration, centrifugation, milk fat globule membrane (MFGM), shear

1 Introduction

The polar lipids of milk provide nutrients that support the growth, health and development of neonates and infants [1-3]. The lipid composition in bovine milk resembles more to that of human milk compared to plant-derived sources, which differ in chemical, physical and structural properties, leading to different digestion pathways [4-6]. The polar lipids in milk consist mainly of phospho- and sphingolipids that are located at the milk fat globule membrane (MFGM) [7,8], which protects the fat globules, e.g., prevents coalescence and enzymatic degradation. In the course of butter making, the polar lipid membrane is detached from the surface of the fat globules and accumulates in the aqueous phases, such as buttermilk and butter serum. Related compositions can be concentrated and incorporated in infant formulas. However, any losses of polar lipid membranes, which typically occurs upon heating, agitation, pumping and homogenization, will limit their otherwise potential nutritional value [9-12]. The separation of polar lipids from dairy fractions has been pursued by using different approaches but their efficiency and limited isolation yield remain challenging, mainly because process-induced depletion and the presence of impurities in the respective complex systems [13-15].

Recent studies have examined process-induced damages on MFGM upon separation [16-18]. For instance, Holzmuller et al. [17] reported centrifugal shear forces to cause a release in MFGM components. Moreover, it has been noted that major losses, especially in components located in the outer layers of the MFGM, occur upon increasing the number of centrifugal processing cycles. In addition, cream washing may selectively remove MFGM components from the interface and decrease stability [16,19,20]. The extent of MFGM rupture is expected to depend on the specific conditions applied during handling and processing. For instance, Brans et

al. [21] have proposed that compared to traditional centrifugal separation, less MFGM damage may occur during low-shear microfiltration. However, if applied for extended time, microfiltration can still be detrimental to MFGM, as evidenced by the changes in the size of milk fat globules [22].

Despite its importance, and to the best of our knowledge, the effect of microfiltration on MFGM damage and the fate of polar lipids have not been assessed so far. Therefore, this study examines the extent of MFGM rupture under the influence of shear and processing time during microfiltration and centrifugation, two of the most typical operations used in dairy separation.

2 Experimental

2.1 Materials

Bovine cream (fat 40.9±0.7%, protein 2.11±0.04%, dry matter 46.3±0.7%, pH 6.75±0.05) was collected and pasteurized (75 °C and 20 s) in a dairy unit (Valio Ltd. Jyväskylä, Finland). The samples were fresh and no more than one day under storage at 5 °C elapsed prior to each experiment. All reagents used were analytical grade.

2.2 Microfiltration

Filtrations were carried out with a uniform trans-membrane pressure (UTP) filtration system (Tetra Alcross®, Tetra Pak, Denmark). A multichannel tubular ceramic membrane was adopted (1.4 μm pore size and 0.24 m^2 filtration surface area, Membralox®, Pall Corporation, France). The filtration system was cleaned before and after each experiment according to [23]. Briefly, a three-step washing procedure was adopted, including base-acid-base steps at

temperatures 75 °C, 50 °C, 75 °C, respectively. Finally, the systems was rinsed and pure water flux was determined at the end of the washing procedure.

A dilution-concentration procedure was employed in three cycles to study the effect of processing on milk fat globules (MFG). In particular, 10 kg of cream was diluted (1:10 ratio) with deionized water and heated to 50 °C in a water bath. The filtration system was heated to 50 °C with deionized water and drained prior addition of the diluted cream into the system. A 15-minute start-up stabilization time was allowed by circulating retentate and permeate at the beginning of each cycle before collecting the permeate. Then, diluted cream (100 kg) was filtered until the mass of the retentate reached the initial amount of cream (10 kg). The resultant retentate was collected and the procedure was repeated for three cycles.

The microfiltration temperature was kept at 50–55 °C throughout the experiments and the filtration was operated at 0.6 bar UTP. Samples were withdrawn after each cycle as retentate and permeate and stored at 8 °C before analysis. Three replicate filtrations were performed for each experimental condition.

2.3 Centrifugal separation

Centrifugal separation was performed in three washing cycles similarly to microfiltration. Briefly, 10 kg of cream was diluted with deionized water (1:10 ratio) and separated into cream and skim fractions using disk stack centrifugal separator (Frau CN2S, Italy) equipped with 51 disks (outer radius 54.5mm, height 51mm, angle 52°, inner radius 15mm). Three separation cycles were concluded and samples were collected after each one. The temperature was kept between 50 and 55 °C during the whole process. The experiments were performed in triplicate.

2.4 Analyses

2.4.1 Composition. Dry matter content was analysed gravimetrically by drying the samples overnight at 105 °C [24]. Total fat content was analysed according to the extraction method of Röse-Gottlieb (ISO 1211, IDF 1:2010). The Kjeldahl method was used to determine the total nitrogen and a conversion factor 6.38 was applied to calculate the protein content in the sample, according to the standards (ISO 8968-1:2014, IDF 20-1:2014).

2.4.2 Protein analysis by gel electrophoresis. Proteins in the unprocessed cream, microfiltered cream and separated cream were analysed by reducing tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The cream samples were diluted in water to a protein concentration ~40 µg/mL and mixed (3:1) with tricine sample buffer (0.1 M Tris–HCl buffer pH 6.8, 20% glycerol, 4% SDS, 10% beta-mercaptoethanol, 0.02% bromophenol blue). The proteins were denaturated at 100 °C for 5 min. After cooling down to room temperature, the samples were centrifuged and loaded in a Criterion 4–20% TGX gel (Bio-Rad Laboratories Inc., US). The amount of protein deposited per well was 10 µg. The running buffer contained trizma base (3.03 g/L), glycine (14.42 g/L) and SDS (1.0 g/L). The electrophoretic migration for the gel was performed at 200 V and 100 mA over 65 min. The proteins were stained with Coomassie blue over 60 min with stirring. After staining, the gel was destained with a solution containing 40% ethanol and 10% acetic acid for 15 min and then overnight with a solution containing 5% ethanol and 7.5% acetic acid. After washing, the gel was stored in deionized water and scanned using a Gel Doc EZ Imager (Bio-Rad Laboratories Inc., US). The molecular mass of proteins present in the different bands was determined from the migration of molecular mass markers (All Blue Precision Plus Protein standards; Bio-Rad Laboratories Inc., US) and compared with other reports [4,14,15,25,26].

2.4.3 Lipid extraction. Lipids from the permeate and skim fractions were extracted by lyophilizing 15–30 mL of the sample and weighting the dry material (~20 mg) into 10 mL Kimax® tubes with PTFE-faced rubber lined caps. The powder was dissolved in 1 mL of 0.9% NaCl, followed by 4 mL dichloromethane-methanol (2:1) addition. The tubes were vortexed for 15 s and shaken vigorously (300 rpm) for 30 min at room temperature. Then, the phase boundary was sharpened by centrifugation (1600 g for 5 min), and the organic layer at the bottom was transferred to a clean Kimax® tube and evaporated under N_2 stream. The aqueous layer was re-extracted with dichloromethane-methanol (20:1) and the organic layer was combined with the first extract. The extraction of the lipids from the cream fractions was carried out correspondingly, except 100 µL of sample volume was applied and supplemented with 900 µL of 0.9% NaCl.

2.4.4 Lipid class separation. Thin layer chromatography (TLC) was employed to separate lipid classes. The extracted lipid samples from permeate and skim fractions were supplemented with 10 µL of the standard lipid containing 1,2-Dinonadecanoyl-L-α-glycerophosphorylcholine (Larodan, Sweden) 11.4 mg/mL dissolved in chloroform and 200 µL of dichloromethane-methanol (100:1). Then, the samples were applied to the TLC plate (Silicagel 60, glass plates, 20x20, Merck, Germany) and let to develop in the chamber containing elution solution petroleum ether-diethylether-acetic acid (80:30:1) for 50 min. The plate was sprayed with 0.001% Rhodamine 6G aqueous solution and the fat containing spots were visualized under UV light and scraped off the plate. The polar lipids were dissociated off the silica by extraction with dichloromethane-methanol-H_2O (8:4:3) as described above and stored frozen in N_2 atmosphere until further processing.

146 The amount of the cream samples required for polar lipid analysis exceeded the capacity
147 of TLC-based lipid class separation procedure. Therefore, solid phase extraction (SPE) was
148 applied. The lipid samples were supplemented with 10 µL of the standard lipid containing 1,2-
149 Dinonadecanoyl-L-α-glycerophosphorylcholine 9.98 mg/m dissolved in 200 µL of
150 dichloromethane-methanol (2:1). Then, the samples were applied to the SPE tubes (3 mL
151 Supelclean™ LC-SI SPE Tubes, Supelco, PA, USA), preconditioned with hexane.
152 The neutral lipids were eluted with 2 mL hexane-diethylether (4:1) and 2 mL hexane-
153 diethylether (1:1). The polar lipids were eluted with 2 mL methanol and 2 mL dichloromethane-
154 methanol-H_2O (3:5:2). The eluted fractions were evaporated under N_2 stream and stored frozen
155 in N_2 atmosphere until further processing.
156 ***2.4.5 Determination of polar lipid concentration.*** The amount of polar lipid class was
157 analysed by gas chromatography (GC) after converting the fatty acids to methyl esters. Fatty
158 acids from the polar lipid fractions were methylated by adding 500 µL of boron trifluoride in
159 14% methanol (Sigma-Aldrich, USA) into polar lipid samples. After flushing with N_2, the tubes
160 were incubated for 90 min in the boiling water bath. Then, 600 µL of hexane-
161 methyltertbutylether (1:1) and 1.5 mL of deionized water was added into the cooled samples and
162 shaken vigorously (300 rpm). The aqueous phase was removed and the organic phase was
163 washed with 3 mL of 0.01% (w/w) NaOH. To sharpen the phase boundary, the tubes were
164 centrifuged for 15 min (1600 g). The organic phase was dried with anhydrous sodium sulphate
165 and transferred to the GC vial. To concentrate the samples, the solvent was evaporated under
166 nitrogen stream and the methyl ester residue was dissolved in 100 µL of hexane.
167 The methyl esters were separated on Zebron ZB-FAME column (60 m x 250 µm x 0.2
168 µm) in an Agilent 7890 A GC system. The temperature in the oven was raised gradually to 280

169 °C and the gas flow in the detector was 350 mL/min, 30 mL/min and 35 mL/min for air, H_2 and
170 N_2, respectively. Split ratio 20:1 was adopted. The concentration of the polar lipids was
171 calculated by comparing the peak areas of the sample methyl esters to the peak area of the
172 methyl ester of the internal standard lipid.

173 ***2.4.6 Particle sizing and zeta potential.*** Particle size distribution was measured for creams
174 before and after the processes with a Mastersizer 2000 (Malvern Instruments, Malvern, UK)
175 based on light scattering according to [27] and [23]. A refractive index of 1.458 was adopted for
176 the dispersed phase and the samples were analysed in triplicate.

177 The zeta potential of the milk fat globules was measured on the following day after
178 processing using an electrophoretic mobility instrument (Zetasizer, Nano series ZS90, Malvern
179 Instruments, Malvern, UK) according to Michalski et al. [23]. The samples were diluted 500-fold
180 in 10mM saline (pH 6.8) and measured in a Malvern Dip Cell at 20 °C. Three readings were
181 collected for each individual sample and the measurements were run in triplicate.

182 ***2.4.7 Shear analysis.*** The effect of flow conditions on milk fat globules was evaluated
183 based on estimates for the average energy dissipation rate $\langle\varepsilon\rangle$. The highest shear forces in disk-
184 stack centrifuges are typically attributed to the feed zone wherein the suspension is accelerated to
185 high rotational velocities prior to its injection to the stack [28,29]. The local energy dissipation
186 rate can vary considerably depending on the feed zone geometry [28,29]. In this study, a direct
187 suspension feed mechanism into the disk-stack was employed resulting in the inlet tube
188 essentially comprising the feed zone. Therefore, the separation processes shared a similarity in
189 that they took place under high Reynolds number flow ($Re \approx 2\times10^4$ in microfiltration;
190 axial/tangential $Re \approx 4\times10^3$ and 9×10^4 in centrifuge inlet) within an axisymmetric geometry.

For centrifugal separation, $\langle\varepsilon\rangle$ was approximated by the power P per unit mass m needed to accelerate a fluid volume of a size equivalent to the inlet region V_{in} from rest to the wall tangential velocity ωR_{in} during a residence time $T_{\mathrm{in}} = V_{\mathrm{in}}/Q$:

$$\langle\varepsilon\rangle = \frac{P}{m} = \frac{\frac{1}{2}Q\rho\omega^2 R_{\mathrm{in}}^2}{\rho V_{\mathrm{in}}} = \frac{\omega^2 R_{\mathrm{in}}^2}{2T_{\mathrm{in}}}, \quad (1)$$

where Q is the volumetric flow rate, ρ is the density, R_{in} is the inlet channel radius and ω is the angular velocity [30]. For microfiltration, $\langle\varepsilon\rangle$ was estimated by assuming a simple axial turbulent duct flow that neglects the filtrate flux over the channel wall [31,32]. Utilizing a force balance for a microfiltration channel, the wall shear stress was expressed using the measured axial pressure drop (ΔP) by $\tau_w = d\Delta P/(4L)$, where d is the channel diameter and L is its length [31,32]. The mean energy dissipation rate was determined from:

$$\langle\varepsilon\rangle = \frac{2f\langle v\rangle^3}{d}, \quad (2)$$

where $f = 2\tau_w/(\rho\langle v\rangle^2)$ is the Fanning friction factor and $\langle v\rangle$ is the measured mean axial flow velocity. To evaluate how the turbulent flow conditions affect particle flocculation and coalescence, the size of the smallest turbulent eddies (Kolmogorov microscale) and their characteristic response time (Kolmogorov timescale) were calculated according to Eqs. (3) and (4) by using the continuous phase kinematic viscosity (ν):

$$\eta = \left(\frac{\nu^3}{\langle\varepsilon\rangle}\right)^{1/4} \quad (3)$$

and

$$\tau_\eta = \left(\frac{\nu}{\langle\varepsilon\rangle}\right)^{1/2}. \quad (4)$$

Furthermore, the Stokes number

$$\mathrm{St} = \frac{\tau_\eta}{\tau_\mathrm{p}} \quad (5)$$

was derived using the particle response time, $\tau_p = D^2\rho_\mathrm{p}/(18\mu)$, where D and ρ_p are the particle diameter and density, respectively, and μ is the continuous phase dynamic viscosity [33].

3 Results and discussion

3.1 Process efficiency

Filtration performance was evaluated based on the permeate flux and permeation of non-fat solids. The permeate flux decreased with increasing filtration cycles, as explained by concentration polarization. Moreover, the concentration of the feed increased at the end of each filtration cycle, making the filtration more arduous (Fig. 1). Nevertheless, the permeate flux remained much higher compared to that previously reported for cream microfiltration [22]. Evidently, the dilution of cream with water decreased the concentration and increased the permeate flux. Note: it should be noted that the current setup used for cream microfiltration was not optimal in terms of water consumption and process efficiency but it was chosen to reproduce the conditions sued in the centrifugal separation process (i.e. three washing and separation cycles combined with drainage instead of diafiltration to achieve valid comparison).

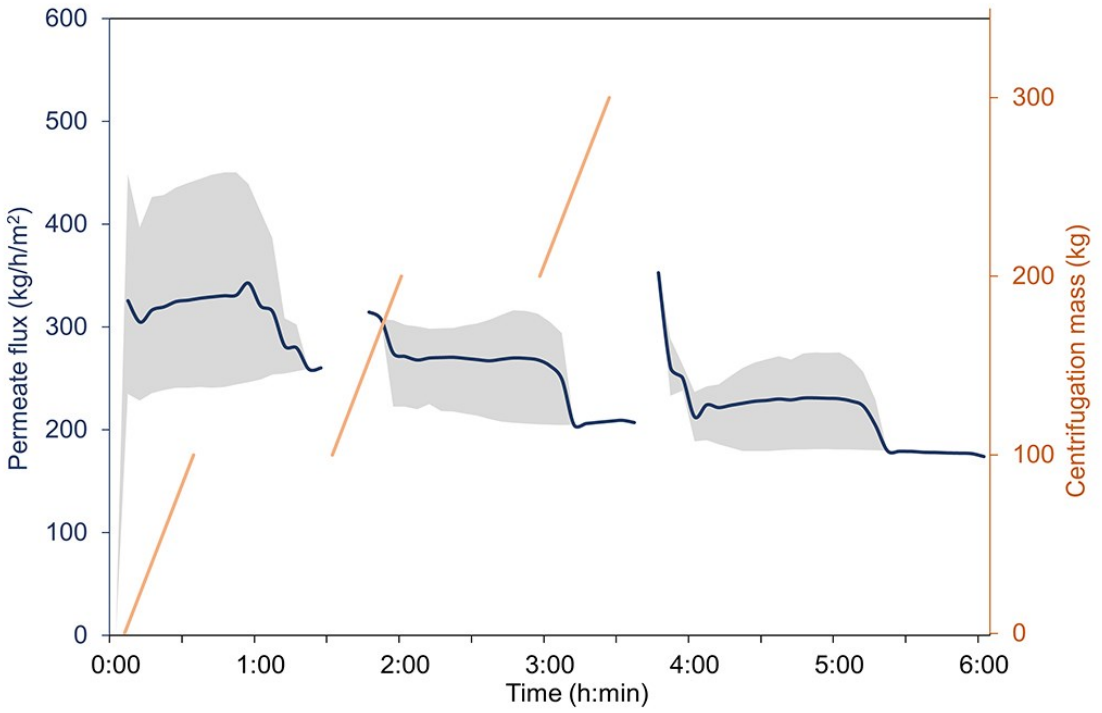


Figure 1. Effect of processing time or cycles during microfiltration and centrifugal separations using a three-cycle cream washing. Microfiltration performance is expressed in terms of average permeate flux (left axis) for the three repetitions and the area indicated in grey corresponds to the standard deviation around the averaged profile. Centrifugal separation is plotted in terms of separation mass, right axis

The flux was not measured from the centrifugal separation process. However, the processing time for each separation cycle remained constant, indicating no reduction in process efficiency between the separation cycles (Fig. 1). The average total processing time for microfiltration was ~5.5h, whereas it was 3.5h for the centrifugation process. Hence, microfiltration involved a longer exposure to shear compared to centrifugal separation, leading to possible damages in the fat globules and MFGM.

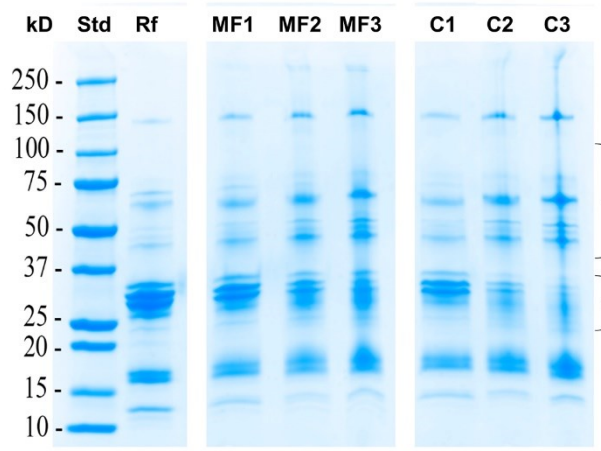
240 The potential microbial spoilage was monitored by pH measurements upon processing and
241 no reduction in pH was observed in any of the experiments (data not shown).

242 **3.2 Composition**

243 ***3.2.1 Chemical composition.*** The effect of processing on cream composition and MFGM
244 integrity was evaluated via protein, dry matter and lipid analyses. Despite the differences in
245 separation methods and processing times, similar cream compositions were obtained in the
246 products. Both microfiltration and centrifugal separation reduced 85%, 92% and 93% of the
247 cream protein after cycles 1, 2 and 3, respectively. The fat content of the resultant cream varied
248 between 35% and 38%. The permeate and the skim fractions contained no detectable dry matter
249 nor protein after cycles 2 and 3, indicating that everything was separated, except for the proteins
250 that were possibly bound to the fat globules. The amount of protein remaining in the resultant
251 cream was 0.17% and 0.14% after microfiltration and centrifugal separation, respectively.

252 ***3.2.2 Protein profiling.*** An electrophoretic analysis was performed to (i) identify the
253 residual proteins in the creams; (ii) to visualize the presence of membrane associated proteins
254 and, (iii) to compare the relative amount of MFGM proteins and milk serum proteins. The results
255 showed a reduction in caseins with increasing processing cycles (Fig 2). Moreover, the processed
256 creams had more intense bands for high-molecular-weight proteins compared to the unprocessed
257 one. The intensities of the bands between 40 and 80 kD, identified as MFGM proteins, increased
258 with increasing processing cycles indicating that they were concentrated by the washing instead
259 of being released from the MFGM surface. Similarly, bands corresponding to xanthine oxidase
260 (XO) were intensified with the processing cycles for both microfiltered and separated cream,
261 indicating that XO was not released from MFGM. Contrarily, Holzmuller et al., [17] reported a
262 decrease in XO upon cream washing and centrifugal separation explained by high shear,

coalescence and incorporation of air upon processing. High-molecular-weight protein MUC1/Myocin was identified in the microfiltered cream samples but less in cream or separated cream samples.



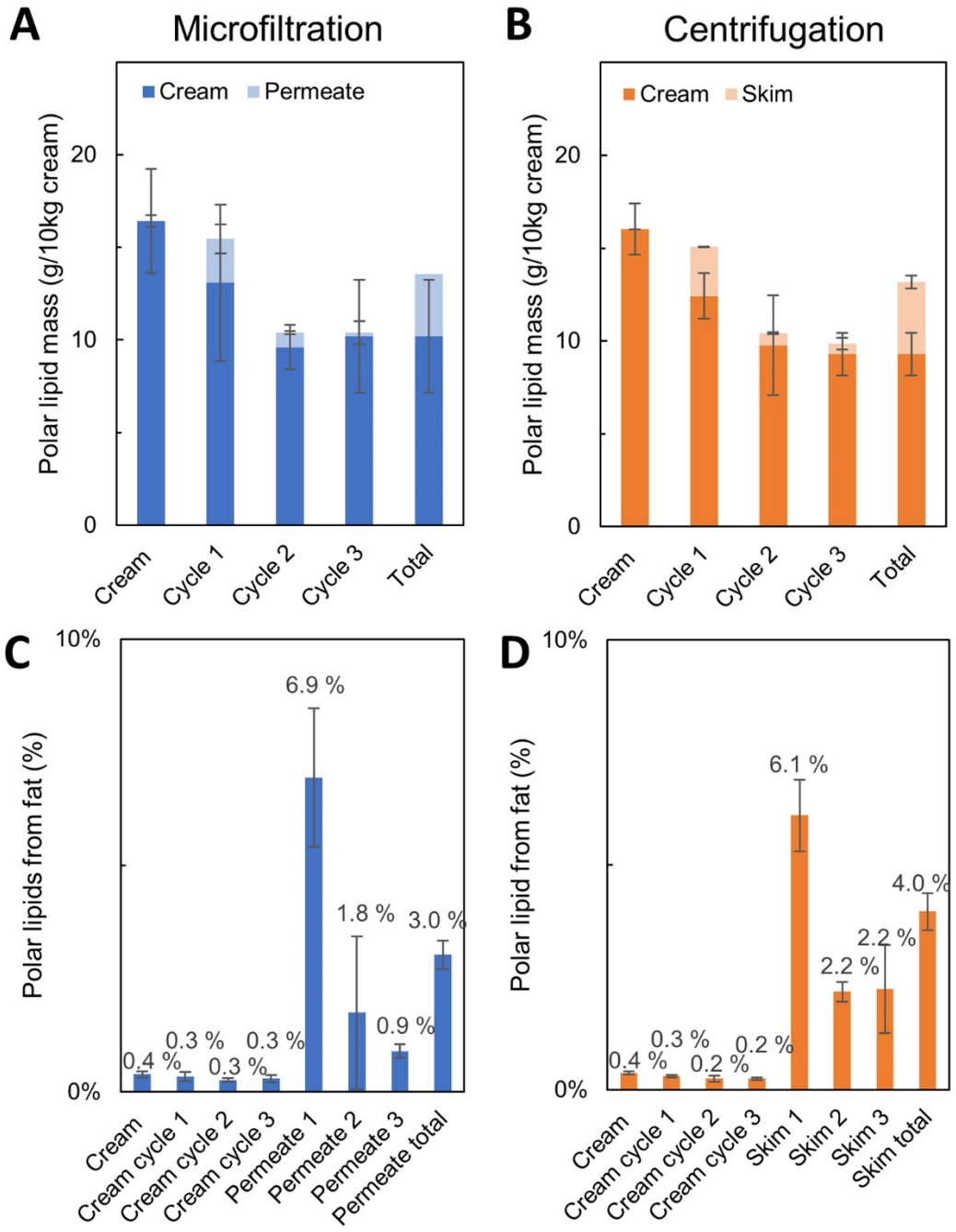
	Rf	MF1	MF2	MF3	C1	C2	C3
Muc1/Myocin	0.2	1.1	1.6	2.1	0.8	0.1	0.2
XO	1.5	2.9	5.4	6.6	3.7	7.1	8.5
LP, LF, CD36	2.0	2.3	0.3	1.6	1.0	1.3	4.1
BTN	4.5	7.1	8.6	9.1	6.8	12.7	12.9
Adipo.	0.5	1.4	1.7	2.9	0.7	2.2	3.6
PAS6/7	2.3	4.6	10.0	8.4	4.9	8.6	9.4
CNs	61	51	39	34	49	28	18
B-Lg	20	26	31	35	29	37	39
PP3							
aLa	7.2	4.1	2.9	0.4	4.5	3.1	3.8

Figure 2. SDS-PAGE protein profiling of processed creams (left) and their relative quantities derived from the band intensities (right). Std = molecular weight standard, Rf = cream, MF = microfiltered cream, C = centrifugally separated cream. Numbers 1–3 indicate the numbers of washing cycles. The protein concentration was constant between each lane (~10 µg). Muc1 = mucin 1, XO = xanthine oxidase, LP = lactoperoxidase, LF = lactoferrin, CD36 = cluster of differentiation 36, Adipo. = adipophilin, PAS6/7 = periodic acid and shiff 6/7, CNs = caseins, B-Lg = betalactoglobuline, PP3 = proteose peptone 3, aLa = alpha-lactalbumin.

It is worth noting that a "tailing" of the bands was observed in cycle 3 cream samples (Fig. 2), which might have affected the intensity measurements. This was most likely caused by the residual lipids in the samples after cycle 3 that were not diluted because of their very low protein concentration.

279 The results indicate that the cream became more concentrated with high-molecular-
280 weight proteins while the amount of caseins decreased. The relative amount of membrane-
281 associated proteins, such as XO, butyrophilin (BTN), cluster of differentiation 36 (CD36),
282 adipophilin and PAS 6/7 increased with increasing washing cycles for both processes, suggesting
283 that the proteins remained in the MFGM. Moreover, myocin/mucin 1 was visualized in
284 microfiltered creams with increasing intensity within washing cycles. Such concentration was
285 not observed in centrifugal separation, indicating that some loosely attached proteins might have
286 been released. Similar results for microfiltration were reported by Steffen et al. [34].
287 The centrifugal process resulted in a steeper decrease in caseins after the three washing
288 cycles compared to microfiltration. However, incorporation of a-La was more prominent in
289 centrifugal separation. While the method adopted here has limitations in protein quantification,
290 nonetheless, it gives a good indication of the protein distribution and supports the findings from
291 the other analyses discussed herein.
292 Steffen et al., [34] recently elucidated the effect of pasteurization on protein adsorption
293 upon cream microfiltration. As expected, casein and beta-lactoglobulin (B-Lg) were found to
294 adsorb on MFGM when pasteurization was done prior to microfiltration. In contrast, no casein or
295 B-Lg were present in MFGM material if pasteurization was applied after microfiltration. Hence,
296 it is reasonable to propose that the shear applied during microfiltration did not necessarily cause
297 protein adsorption since caseins and B-Lg were already adsorbed before filtration (and
298 centrifugal separation). Nevertheless, the process was able to remove most of the caseins.
299 Overall, pasteurizing the cream after filtration is expected to maximize the separation efficiency.
300 ***3.3.3 Polar lipid composition.*** The mass balance of polar lipids was carried out for both
301 microfiltration and centrifugation to further estimate the effect of shear on MFGM and fat

302 globule disruption. The initial creams in microfiltration and centrifugal separation contained
303 1.58±0.27 mg/g (16.43±2.81 g) and 1.54±0.13 mg/g (16.02±1.38 g) polar lipids, respectively,
304 which are in good agreement with those reported previously [35] and [36]. The microfiltration
305 process yielded 13.1 g, 9.6 g and 8.5 g polar lipids in the cream after washing cycle 1, 2 and 3,
306 respectively (Fig. 3A). Clearly, the smallest fat globules (diameter < 1.4 μm) permeated during
307 the first cycle, causing a decrease in the polar lipid mass. Reduction in the polar lipid mass was
308 less prominent after cycle 2 and only 2% of the cream polar lipids permeated after cycle 3.
309 The centrifugal separation process yielded 12.4 g, 9.8 g and 9.3 g polar lipid after cycle 1,
310 2 and 3, respectively; very similar results compared to those from the microfiltration process.
311 However, more polar lipids permeated especially after cycle 3 (6%) during centrifugation (Fig.
312 3B). Therefore, the total polar lipid permeation was slightly higher in centrifugal separation
313 compared to that of microfiltration.



314

315 **Figure 3.** Polar lipid mass balance for cream after microfiltration (A) and centrifugation (B)

316 processes, and relative amounts of polar lipids from fat in fractions from microfiltration (C) and

317 centrifugation (D). Percentages in A and B indicate the permeation of polar lipids in each three

318 washing cycles and in cumulative total.

Rombaut et al. [36] reported partitioning of the smallest fat globules upon skimming of milk to significantly decrease the content of milk polar lipids. In particular, raw milk was reported to contain 0.04% polar lipids, which contributes less than 1% of the total fat. Skim milk contained 0.1% fat (less than 1 μm in size combining 25% of fat globule numbers) from where polar lipids were reported to represent 19% [36]. Hence, small fat globules comprise large surface area (relatively more polar lipids) and, therefore, among them, up to half of the original milk polar lipids may have permeated [37].

To estimate whether the permeated fat globules were intact or damaged, the ratio of polar lipid-to-fat was examined. After cycle 1, microfiltration and centrifugal separation induced 0.9% and 1.0% permeation of fat, from where 7% and 6% comprised of polar lipids, respectively (Fig. 3 C,D). Presumably, this is due to permeation of small fat globules enriched with polar lipids. Theoretically, filtration with 1.4μm membrane cut-off removes 5% and 32% of the volume and numbers of milk fat globules (globules smaller than 1.4μm), respectively [37]. If the initial skimming of milk is taken into account, then microfiltration of cream is expected to remove fat globules of sizes between 1μm and 1.4μm. This yields a ~7% reduction in the number of fat globules. Hence, it may be argued that the loss of small fat globules contributed to the loss of polar lipid material upon the first washing cycle. The ratio of polar lipid-to-fat decreased after cycle 2 and 3 indicating a decrease in small fat globule permeation and possible leakages of the larger milk fat globules. Noticeable, the surface active proteins also permeated at this stage, leaving the affected fat globules with no surface coverage and making them prone to coalescence. The ratio of polar lipid to fat in microfiltration permeate decreased linearly with increasing washing cycles (Fig. 3C). Contrarily, in the skim fraction the ratio remained the same

after cycle 2 and 3 (Fig. 3D), indicating that more polar lipids may have been disintegrated from the native fat globules upon centrifugal separation compared to that of microfiltration. A loss of total polar lipid material in the mass balance calculations is noted (Fig. 3), owing to the variation among raw materials, methods and analysis.

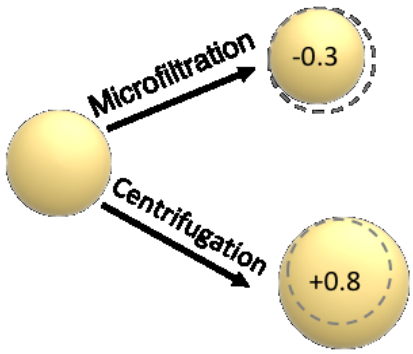
It has been noted in previous reports that an increased number of washing cycles in centrifugal separation cause depletion of MFGM polar lipids [18,35]. The loss in polar lipid material may cause an imbalance in the original ratio between individual polar lipids and loss of certain polar lipids. The polar lipids arrange on MFGM surfaces into ordered lipid domains and liquid disordered phase, depending on their conformation and properties. The ordered domains usually comprise of sphingomyelin and cholesterol and have higher resistance to rupture [18,38,39]. Previously, Zheng et al. [18] indicated the desorption of PE and PC upon centrifugal separation, given that these lipids are loosely bound. Hence, it can be expected that both, microfiltration and centrifugation, induced changes in the MFGM, e.g., both released some of the loosely attached polar lipids. The effect of microfiltration on individual polar lipid desorption remains a subject for elucidation.

3.3 Estimation of shear-induced damage

3.3.1 Particle size and zeta potential. The shear-induced damage was estimated based on particle size and zeta potential measurements. The particle size was expected to increase due to permeation of the smallest fat globules and casein micelles [23]. However, microfiltration decreased the mean fat globule size slightly after the first cycle (Table 1) after which the particle size remained constant. Thus, the actual reduction in particle size was presumably much stronger, indicating possible rupture of the milk fat globules. However, the particle size did not decrease after cycle 2 and 3 suggesting that such size reduction did not dependent on the shear or

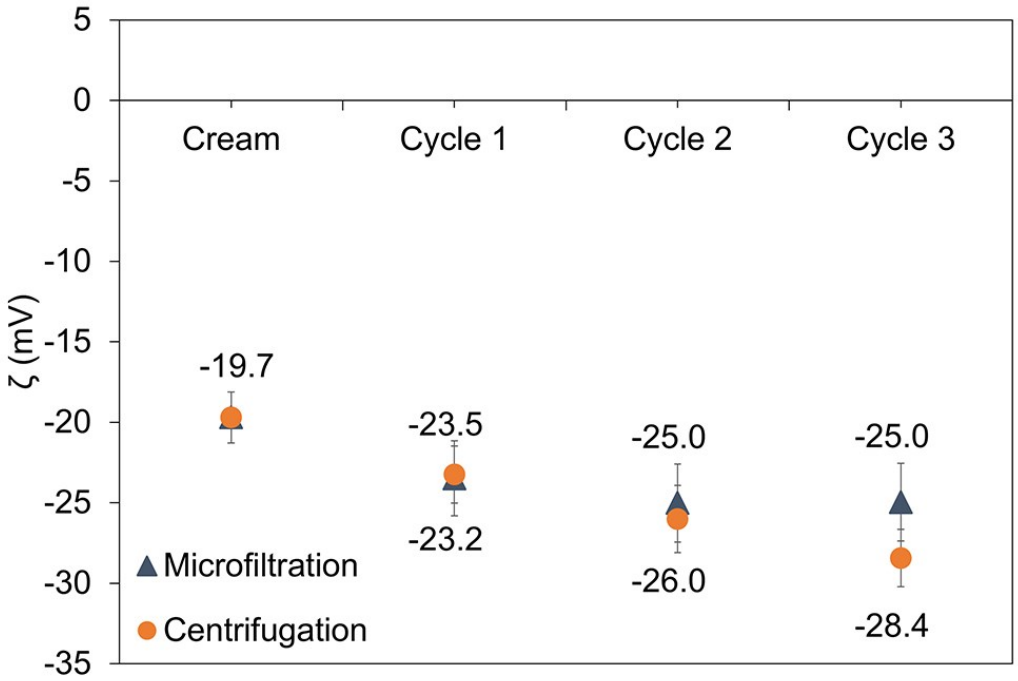
365 shearing time. Bullon et al., [40] reported a reduction in fat droplet size after 30 minutes
366 microfiltration, after which the size remained constant. This was explained by the breaking of the
367 emulsion flocs due to shearing forces during the first minutes of the filtration. In contrast to
368 microfiltration, centrifugal separation increased the mean fat globule size after each separation
369 cycle, due to separation of the smallest particles and possible process-induced damage and
370 coalescence. Similar results were reported, for instance by Kathriarachchi et al., [41] and
371 Holzmuller et al., [17], who found an increase in milk fat globule size and coalescence with
372 increasing separation cycles upon cream washing. An open question remains whether these
373 effects caused a difference in cream composition and disruption of milk fat globule membrane
374 components.
375
376 **Table 1.** The effect of microfiltration and centrifugal separation processing cycles on milk fat
377 globule particle size.

Sample	Particle size µm, D [4. 3]
Cream	4.1±0.12
Microfiltered Cream cycle 1	3.84±0.10
Microfiltered Cream cycle 2	3.78±0.07
Microfiltered Cream cycle 3	3.77±0.05
Centrifuged Cream cycle 1	4.26±0.03
Centrifuged Cream cycle 2	4.49±0.10
Centrifuged Cream cycle 3	4.87±0.14



378

379 The surface charge of the milk fat globules were estimated by means of electrophoretic
380 mobility and zeta potential measurements. The zeta potential of the fat globules decreased upon
381 process for both separation methods (Fig. 4). The decrease in zeta potential was more
382 pronounced in centrifugation after cycles 2 and 3 compared to that of microfiltration, which
383 reached a plateau after cycle 1. The results for zeta potential coincided with those of particle size,
384 indicating that the given processing modified the surface of the milk fat globules and possibly
385 released MFGM components due to shear stress and change in the equilibria.



386

387 **Figure 4**. The effect of cream washing cycles (1-3) upon microfiltration (triangle symbols) and
388 centrifugal separation (circle symbols) on milk fat globule surface charge as measured by the
389 zeta potential.

390

391 A more negative zeta potential of milk fat globule upon microfiltration was
392 reported previously by Jukkola et al., [23] and was explained by a decrease in mineral

composition. Furthermore, Bourlieu et al. [42] reported an accumulation of free fatty acids on the MFGM that made the zeta potential more negative (and increased the electrostatic repulsion). In addition, incorporation of air and washing were shown to release MFGM components and lower the zeta potential [16]. Various factors can be considered to influence the reduced zeta potential. After cycle 1, in both processes, the lower mineral concentration decreased the counterion concentration, which made the fat globules more negatively charged (Fig. 4). After cycles 2 and 3, all the minerals were permeated. Hence, the zeta potential remained constant in the microfiltration process. The lower zeta potential in centrifuged cream after cycles 2 and 3 are explained by the formation of free fatty acids, coalesced fat droplets and possible release of MFGM components.

In order to evaluate the effect of shear on milk fat globules upon microfiltration and centrifugal separation, the flow conditions of both process need to be estimated. Moreover, contradictory information has been reported about shear-induced damage on milk fat globules caused by processing. In particular, microfiltration has been documented to cause less damage to shear-sensitive milk components compared to centrifugal separation [21,43]. Nevertheless, long shear exposure in microfiltration has been shown to decrease the mean fat droplet size in cream MF [22] and several studies reported a decrease in droplet size of an oil-in-water emulsion upon microfiltration [40,44,45], suggesting that microfiltration can disrupt native milk fat globules. Moreover, membrane surface properties and droplet size/pore diameter ratio was reported to play an important role in the passage of oil droplets during filtration and droplet breaking phenomena [45,46]. In addition, hydrophobicity of the filtration membrane influences the filtration of an O/W emulsion: hydrophobic membranes are prone to partially break an emulsion, whereas hydrophilic membranes are able to retain the properties of the emulsion [40]. Centrifugal

separation was reported to cause high shear and disruption of milk fat globules and MFGM in various studies [17,18,41]. For instance, membrane protein-based Periodic acid and Shiff (PAS) 6/7 was found to diminish by 80% after the first washing cycle in cream separation process [17]; it is well known that proteins and phospholipids may be released from MFGM surface upon high shear, causing fat globule coalescence [18,35]. Such partitioning could contribute to the lowering of zeta potential in the present study.

3.3.2 Influence of flow conditions on milk fat globules. Based on the sigma model [47], the particle diameter with 50% separation efficiency is ca. 800 nm in the centrifugal separation, which suggests that the increase in mean particle size was not likely caused by an inadequate separation efficiency but by coalescence and flocculation promoted by the flow conditions [48-50]. To compare the potential for droplet breakage during centrifugal separation and microfiltration, the average energy dissipation rate $\langle\varepsilon\rangle$ for both processes was estimated according to equations 1 and 2, respectively. For centrifugal separation, $\langle\varepsilon\rangle$ resulted ≈ 60 W/kg (Eq. 1), corresponding to a turbulent eddy size of $\eta \approx 13$ μm (Eq. 3) and response time $\tau_\eta \approx 140$ μs (Eq. 4). For microfiltration, the average energy dissipation rate $\langle\varepsilon\rangle \approx 1200$ W/kg was found much higher than for centrifugation, implying a smaller eddy size $\eta \approx 6$ μm and a faster response time $\tau_\eta \approx 30$ μs. The large difference in $\langle\varepsilon\rangle$ comparing microfiltration and centrifugal separation is partly anticipated if one considers the dimensions of the channels, flow characteristics and the effect of axial rotation on turbulent flow, for example, in reducing the friction factor and inducing flow laminarization [51-53].

Although the collision rate can be enhanced by turbulence, coalescence was not observed in microfiltration. Moreover, the particle pair relative velocity differences in collisions do not likely contribute to an increased orthokinetic coalescence due to the small value of the Stokes

number (St << 1, Eq. 5), i.e. the particles follow streamlines under both flow conditions [54]. In turn, droplet breakup and deflocculation are more pronounced in microfiltration due to its larger $\langle\varepsilon\rangle$ as the droplets are subject to larger hydrodynamic stresses that scale according to $\langle\varepsilon\rangle^{1/2}$ [55-57]. Conversely, the larger turbulent eddy size of centrifugal separation could be less effective in disintegrating flocs, while the particle size polydispersity could increase flocculation and collision rate by differential settling at the stack [48].

Hence, unlike previously assumed, the data implies that the shear stress during centrifugation is much lower compared to that for microfiltration. However, the flow conditions of the centrifugal separation cause fat globules to flocculate and coalesce whereas microfiltration promotes deflocculation. The authors note that the pilot systems used here may differ significantly from the systems used in industrial scale. For instance, the pilot microfiltration system was operated at high crossflow velocities, much higher compared to those used in the industrial plants, possibly affecting the outcome of the shearing. This together with the use of diafiltration remains to be elucidated in the course of scale-up.

Overall, the experimental data supports our finding for microfiltration, which decreases milk fat globule particle size owing to shearing and deflocculation phenomena. Conversely, centrifugal separation increases the particle size as a result of flocculation and coalescence, owing to the streamlined geometry of the centrifuge.

Conclusions

The separation efficiency of centrifugal and microfiltration separation were compared as far as cream separation efficiency and MFGM yield. Both processes yield significant protein

461 removal, up to 93%, which is thus far, to the best of our knowledge, the highest reported for
462 cream microfiltration.

463 Despite the efficiency of the processes with regard to protein separation, significant
464 changes are observed in the milk fat globules and MFGM. Based on particle sizing, centrifugal
465 separation favours flocculation/coalescence, as also supported by its estimated lower average
466 energy dissipation rate. Some polar lipid material is released from the MFGM, which is more
467 evident in the centrifugation process. The release of polar lipids increase with the number of
468 processing cycles, indicating that the components are loosely bound and sensitive to turbulent
469 flow conditions and to changes in the serum equilibria. Such effects play a role in the formation
470 of free fatty acids as well as in the physical properties of the cream and the (reduced) MFGM
471 yield.

472 Overall, microfiltration and centrifugation produce MFGM-enriched cream that is
473 separated from milk serum proteins. The MFGM-enriched cream can be considered in butter
474 making, to transform buttermilk side streams into high-value ingredients for novel MFGM-
475 enriched foods, such as infant formulas. Compared to centrifugation, microfiltration offers the
476 benefit of a reduced water consumption and controlled selectivity, if optimized and combined
477 with diafiltration.

478 More efforts are needed to optimize the microfiltration process in order to minimize the
479 processing time, energy consumption, loss of small fat globules and desorption of the MFGM
480 components. The exploitation of the MFGM-enriched buttermilk is a subject that will be
481 addressed in a future communication.

482

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