Effect of reverse osmosis and ultra-high-pressure homogenization on the composition and microstructure of sweet buttermilk

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ABSTRACT

Buttermilk (BM), the by-product of butter making, is similar to skim milk (SM) composition. However, it is currently undervalued in dairy processing because it is responsible for texture defects (e.g., crumbliness, decreased firmness) in cheese and yogurt. One possible way of improving the incorporation of BM into dairy products is by the use of technological pretreatments such as membrane filtration and homogenization. The study aimed at characterizing the effect of pre-concentration by reverse osmosis (RO) and single-pass ultra-high-pressure homogenization (UHPH) on the composition and microstructure of sweet BM to modify its techno-functional properties (e.g., protein gel formation, syneresis, firmness). The BM and RO BM were treated at 0, 15, 150, and 300 MPa. Pressure-treated and control BM and RO BM were ultracentrifuged to fractionate them into the following 3 fractions: a supernatant soluble fraction (top layer), a colloidal fraction consisting of a cloudy layer (middle layer), and a high-density pellet (bottom layer). Compositional changes in the soluble fraction [lipid, phospholipid (PL), protein, and salt], as well as its protein profile by PAGE analysis, were determined. Modifications in particle size distribution upon UHPH were monitored by laser diffraction in the presence and absence of sodium citrate to dissociate the casein (CN) micelles. Microstructural changes in pressure-treated and non-pressure-treated BM and RO BM particles were monitored by confocal laser scanning microscopy. Particle size analysis showed that UHPH treatment significantly decreased the size of the milk fat globule membrane fragments in BM and RO BM. Also, pressure treatment at 300 MPa led to a significant increase in the recovery of total lipids, CN, calcium, and phosphate in the BM soluble fraction (top layer) following ultracentrifugation. However, PL were primarily concentrated in the pellet cloud (middle layer), located above the pellet in BM concentrated by RO. In contrast, PL were evenly distributed between soluble and colloidal phases of BM. This study provides insight into the modifications of sweet BM constituents induced by RO and UHPH from a compositional and structural perspective.

Key words: sweet buttermilk, ultra-high-pressure homogenization, reverse osmosis, casein, phospholipids

INTRODUCTION

Buttermilk (BM) is the by-product of butter manufacturing. During the cream churning process, the milk fat globules are destabilized due to agitation and air incorporation, leading to a phase inversion and the release of milk fat globule membrane (MFGM) fragments into the BM aqueous phase (Bourlieu et al., 2018). As a result, the volume of butter and BM arise to approximately equal proportions based on the churning of 40% cream (Morin et al., 2007). Thus, from the latest butter production statistics, approximately 122,000 t and 973,000 t of BM were produced annually in Canada (Statista, 2022) and in the United States (USDA, 2022), respectively.

Buttermilk and skim milk (SM) exhibit similar compositional characteristics in terms of nonfat milk solids [e.g., lactose, caseins, whey proteins (WP), and salts; Corredig and Dalgleish, 1997]. However, they differ with regard to phospholipid (PL) content, with BM containing up to 10 times more PL than SM (Britten et al., 2008). Phospholipids have been shown to have beneficial effects on humans, such as a decrease in total- and low-density lipoprotein cholesterol concentrations upon daily BM supplementation (Conway et al., 2013).

Beyond their beneficial health effects, PL are responsible for the good emulsifying properties of BM, which find many applications in food formulations (e.g., bakery goods, snacks, and chocolate; Vanderghem et al., 2010; Barukčić et al., 2019). However, these PL are known to impair cheese manufacturing due to reduced gel formation, weaker rennet gel, lower contraction capacity of the gel, and an increase in moisture content and yield...
Considering that both RO and UHPH induce important changes in the soluble-colloidal equilibria and microstructure of milk, pre-treating sweet BM with these technologies can potentially modify the equilibria of its constituents and improve its use in cheesemaking. Therefore, this study aimed to examine the effect of combining RO and UHPH on sweet BM composition and microstructure. The BM and RO BM, with their respective soluble fractions, were characterized after UHPH treatment (0, 15, 150, and 300 MPa).

MATERIALS AND METHODS

Materials

No human or animal subjects were used, so this study did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board. Raw cream (approximately 40% fat) was obtained from a local dairy plant (Quebec, QC, Canada). Sodium citrate dihydrate, ethyl ether, petroleum ether, chloroform, and methanol were all provided by Fisher Chemical. Sodium azide, ammonium hydroxide, HCl, and phenolphthalein were obtained from Thermo Fisher Scientific. Nitric acid was purchased from Sigma-Aldrich. Ammonium thiocyanate and iron(III) chloride hexahydrate were supplied by Sigma-Aldrich, 45-µm syringe filters by Chromatographic Specialties Inc., and grade 1 and grade 5 qualitative filter paper (12.5 mm) by Whatman. All of the following were purchased from BioRad: Mini-PROTEAN TGX Stain-Free Gels (12%, 15-well comb, 15 µL), Laemmli sample buffer, Precision Plus Protein All Blue Standards, and 10 × Tris/Glycine/SDS buffer. Fast Green FCF, Nile Red, and 2-mercaptoethanol were supplied by Sigma-Aldrich.

Methods

Production of BM. Figure 1 shows the processing steps of BM and RO BM. Briefly, raw cream was pasteurized (Chalinox/Hydro-Québec CFI-25, Quebec, Canada) at 85°C for 30 s and matured overnight at 10°C. The following day, the matured cream was churned (75 rpm, at approximately 12°C) using a pilot plant scale butter churn with a capacity of 8 to 15 L (Qualtech Equipment). The released BM was then heat-treated for 10 min up to 40°C, followed by cream separation using a Westfalia cream separator (LWA-205, DeLaval). Skimmed BM was aliquoted into 2 batches, one 20-L batch used for RO and one 5-L batch for unconcentrated BM experiments.
**RO.** Twenty liters of skimmed BM were concentrated by RO using a pilot filtration unit with a capacity of 20 L (Model 1812 Lab Unit, Filtration Engineering Company Inc.), equipped with a 0.32 m² spiral-wound membrane. The membrane consisted of polyamide with a 99% average NaCl rejection (Model AG1812C-34D, General Electric Co.). Filtration was carried out at 1.72 MPa outlet pressure at 27°C. The concentration was controlled by measuring the Brix degree (PAL-1 Pocket Refractometer, ATAGO), which increased from an initial value of approximately 9.2% to approximately 20.7%. The resulting RO BM concentrates were concentrated approximately 2.2-fold.

**UHPH.** Buttermilk and RO BM were aliquoted into 4 one-liter batches. One batch of each (BM and RO BM) was used as a control. The remaining 3 batches of BM and RO BM were used for homogenization (1 pass) at 15 MPa, 150 MPa, or 300 MPa, respectively, using a UHPH system (Nano Debee Model 45-4). Samples were rapidly cooled at 10°C by the cooling system of the apparatus located directly at the outlet of the homogenization chamber. Afterward, sodium azide was added to each batch at a concentration of 0.02% (wt/vol) to protect against microbial spoilage.

**Ultracentrifugation.** All samples, with or without the addition of sodium citrate to dissolve the CN micelles (2 and 4% for BM and RO BM, respectively), were ultracentrifuged at 70,000 × g at 20°C for 1 h to separate the soluble and colloidal phases (Optima XE Ultracentrifuge, Beckman). The upper lipid layer was carefully removed with a spatula and discarded, and the remaining 3 phases were separated accordingly (Figure 2). The soluble supernatant (SN, top layer) was first removed with a pipette and weighed, followed by the pellet cloud (middle layer) located above the pellet. The pellet (bottom layer) was resuspended with Milli-Q water to the original sample volume, and properly mixed using an agitator. Regarding samples added with sodium citrate, only the soluble SN was used for PAGE and particle size and size distribution. All samples were either stored at 4°C until further microstructural analyses or frozen until further compositional analyses.
Compositional and Structural Analyses

**Compositional Analyses.** All compositional analyses were carried out on the BM and RO BM (0, 15, 150, and 300 MPa) soluble phase recovered following ultracentrifugation. Total (non-centrifuged) BM and RO BM samples were analyzed as control groups. For PL quantification, CN pellet and pellet cloud were analyzed additionally. Frozen samples were thawed at 4°C overnight 1 d before analysis. All analyses were carried out in triplicates.

**Nitrogen Distribution.** The total nitrogen, NPN, and non-CN nitrogen contents were quantified using the Kjeldahl method according to AOAC International 991.20, 991.21, and 998.05, respectively (AOAC, 2005). The true protein was determined as (total nitrogen − NPN) × 6.38. The CN was calculated as (total nitrogen − non-CN nitrogen) × 6.38 and the WP as (non-CN nitrogen − NPN) × 6.38.

**TS and Ash Content.** The TS were determined according to AOAC International 991.20, 991.21, and 998.05, respectively (AOAC, 2005). The weight of the TS was noted. The ash content was determined according to AOAC International 925.09 (AOAC, 2005). The TS samples were calcined in the same crucible on a heating plate and placed in an ash oven (Lindberg, Lindberg Blue box) at 550°C for 16 h. The ash weight was noted.

**Salts.** The specific salts composition was measured according to the International Dairy Federation method IDF 229:2018, with slight modifications (IDF, 2018). The ashes were rehydrated in 1 mL of 25% (vol/vol) nitric acid and diluted up to 50 mL with Milli-Q water. Samples were passed through a 0.45-µm syringe filter before analysis by Inductively Coupled Plasma Atomic Emission Spectrometry (Model 5110, Agilent Technologies). The calcium, magnesium, potassium, sodium, and phosphate contents were noted (ppm) and converted into mmol/L.

**Total Lipids.** The total lipid content of samples was analyzed by the Mojonnier method according to AOAC International 925.32 (AOAC, 2005).

**PL.** The PL were quantified according to the method of Stewart (Stewart, 1980). The BM and RO BM samples were diluted in distilled water in a ratio of 1:4 and 1:8, respectively. One mL of the diluted sample was mixed with 2 mL of chloroform-methanol solution (2:1). The samples were agitated for 1 min and centrifuged at 2,500 × g for 10 min at 20°C. The lower chloroform phase was removed with a syringe and transferred to a tube, and samples were stored under a fume hood overnight to evaporate the chloroform. The following day, 2 mL of Stewart reagent (0.1 M ammonium ferrothiocyanate) and 2 mL of chloroform were added. The samples were agitated for 3 min and centrifuged at 2,500 × g for 10 min at 20°C. The lower chloroform phase was removed with a syringe and transferred to a Multiskan Spectrum (Thermo Labsystems) and analyzed with a Fluoroskan Ascent FL (Thermo Fisher Scientific). The optical density was measured at 485 nm. The PL concentration of the samples was calculated by comparison of the measured 485-nm optical density with a standard curve using phosphatidylcholine.

**Protein Profiles.** The protein profiles of BM and RO BM soluble and colloidal phases and those of the soluble phases fortified with sodium citrate were analyzed by SDS-PAGE under non-reducing and reducing (50 µL of 2-mercaptoethanol and 950 µL of Laemmlli buffer) conditions. The BM and RO BM samples were diluted in distilled water (1:49 and 1:124, respectively). A 25-µL volume of each dilution was mixed with 25 µL of the sample buffer. The solutions were loaded onto precast 12% acrylamide gels and Precision Plus Protein All Blue Standards with molecular weight standards between 10 and 250 kDa were used as a molecular weight standard.
marker. The analyses were done in a Mini-PROTEAN Tetra Cell (BioRad) at a constant voltage of 120 V for 1 h. After, the gels were stained with Coomassie blue solution (BioRad) for 1 h and de-stained overnight with a methanol-acetic acid-distilled water mixture (1:1:8). The gels were scanned the following day using a ChemiDocTM MP imaging system (BioRad).

Microstructural Analyses

**Particle Size and Size Distribution.** Analyses of particle size and size distribution of BM and RO BM samples (0, 15, 150, and 300 MPa) with and without the addition of sodium citrate were conducted using a laser light diffraction particle size analyzer (Malvern Mastersizer 3000, Malvern Instruments Ltd.) equipped with a dispersion unit (Hydro) adjusted at a speed of 1,500 rpm. An absorption index of 0.001, a real refractive index of 1.460 for BM and a refractive index for water of 1.333 were used. Samples were diluted in distilled water until an obscuration of approximately 5% in the diffractometer cell was obtained. Volume moment mean diameter (D$_{4,3}$), surface area mean diameter (D$_{3,2}$), and size distribution were noted. Each sample was measured in triplicate.

**Confocal Laser Scanning Microscopy.** The microstructure of BM and RO BM (0, 15, 150, and 300 MPa) was investigated based on published methods (Gallier et al., 2010; Lopez and Ménard, 2011; Marco-Molés et al., 2012; Giarratano et al., 2020) with some adaptations, using confocal laser scanning microscopy (CLSM). A volume of 100 µL of BM or RO BM was mixed with 6 µL of 1% Fast Green FCF (proteins) and 2 µL of 1% Nile Red (PL). After a 20-min waiting period, on a hot plate at 40°C, 20 µL of each sample was equately mixed with 20 µL of 3% low-melting agarose. A plate with double-sided tape was prepared on the hot plate. Ten microliters of each sample solution was placed on the double-side tape, and a coverslip was gently placed on top. Imaging was performed on a Leica SP8 confocal microscope (Leica Microsystems Inc.) equipped with a 40×/0.85 PL-apochromat objective. The sample was excited with 488- and 638-nm solid-state lasers, and the light was sequentially collected at 550 to 590 nm and 655 to 755 nm.

Statistical Analysis

All the experiments and measurements were carried out in triplicate, and the mean values were considered for comparisons at a 95% confidence level ($P < 0.05$). The obtained data were analyzed for significant differences between the BM and RO BM and between pressure treatments by 2-way ANOVA using Bonferroni multiple comparisons procedure (GraphPad Prism 9.2.0). Phospholipid analyses were also conducted on total (non-centrifuged) fractions comparing the BM and RO BM using 2-way ANOVA.

RESULTS AND DISCUSSION

**Overall Composition of BM and RO BM**

The overall composition of BM, RO BM, and of their ultracentrifugation SN are reported in Table 1. The BM composition obtained aligns with previously conducted studies (Mistry et al., 1996; Morin et al., 2006; Britten et al., 2008). The RO concentration of BM by a 2-fold factor led to an increase of all components by approximately a similar factor.

<table>
<thead>
<tr>
<th>Item</th>
<th>TS (g/100 g)</th>
<th>Ash (g/100 g)</th>
<th>Total lipids (g/100 g)</th>
<th>Phospholipids (g/100 g)</th>
<th>True protein (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10.20 ± 0.12$^A$</td>
<td>0.72 ± 0.03$^A$</td>
<td>0.73 ± 0.03$^A$</td>
<td>0.12 ± 0.02$^A$</td>
<td>3.06 ± 0.14$^A$</td>
</tr>
<tr>
<td>SN 0</td>
<td>6.83 ± 0.14$^a$</td>
<td>0.51 ± 0.05$^a$</td>
<td>0.38 ± 0.04$^{a,b}$</td>
<td>0.05 ± 0.02$^b$</td>
<td>0.73 ± 0.09$^a$</td>
</tr>
<tr>
<td>SN 15</td>
<td>6.76 ± 0.15$^a$</td>
<td>0.53 ± 0.01$^a$</td>
<td>0.32 ± 0.02$^a$</td>
<td>0.06 ± 0.02$^a$</td>
<td>0.71 ± 0.01$^a$</td>
</tr>
<tr>
<td>SN 150</td>
<td>6.90 ± 0.16$^a$</td>
<td>0.52 ± 0.01$^a$</td>
<td>0.34 ± 0.02$^{a,b}$</td>
<td>0.07 ± 0.01$^a$</td>
<td>0.78 ± 0.10$^a$</td>
</tr>
<tr>
<td>SN 300</td>
<td>7.17 ± 0.24$^a$</td>
<td>0.48 ± 0.08$^a$</td>
<td>0.42 ± 0.03$^a$</td>
<td>0.08 ± 0.01$^a$</td>
<td>0.88 ± 0.08$^a$</td>
</tr>
</tbody>
</table>

| RO BM |                 |              |                        |                         |                        |
| Total | 19.62 ± 0.89$^B$ | 1.30 ± 0.10$^B$ | 1.37 ± 0.06$^B$ | 0.31 ± 0.00$^B$ | 5.68 ± 0.26$^B$ |
| SN 0  | 14.89 ± 1.35$^a$ | 0.96 ± 0.07$^a$ | 0.82 ± 0.17$^a$ | 0.10 ± 0.01$^a$ | 1.43 ± 0.16$^a$ |
| SN 15 | 14.66 ± 1.70$^a$ | 0.91 ± 0.03$^a$ | 0.77 ± 0.15$^a$ | 0.11 ± 0.02$^a$ | 1.49 ± 0.17$^a$ |
| SN 150| 16.46 ± 1.46$^a$ | 0.99 ± 0.09$^a$ | 0.97 ± 0.15$^a$ | 0.13 ± 0.02$^a$ | 1.56 ± 0.18$^a$ |
| SN 300| 13.88 ± 0.59$^a$ | 0.87 ± 0.08$^a$ | 0.96 ± 0.10$^a$ | 0.15 ± 0.02$^a$ | 1.78 ± 0.17$^a$ |

$^{a,b}$Significant difference ($P < 0.05$) is indicated column-wise for BM and RO BM independently.

$^A,B$Significant difference ($P < 0.05$) between total BM and total RO BM is indicated column-wise with different capital letters.

$^1$Values are means ± SD (n = 3).
Compositional Modifications in SN

**Protein and NPN.** The protein and NPN distribution between BM and RO BM SN are presented in Table 2. The content of WP increased from approximately 0.33 g/100 g in SN from BM to 1.07 g/100 g in SN from RO BM, whereas the CN content of SN from both samples remained unchanged (0.44–0.56 g/100 g; mean values of all BM SN and RO BM SN, respectively). During RO, the CN micelles in the colloidal phase were more mineralized because of the increased presence of soluble calcium, magnesium, and phosphorus (Le Graet and Brulé, 1982). We hypothesized that the higher mineral recovery upon RO concentration (Dussault-Chouinard et al., 2019), especially the calcium in the colloidal fraction, helped in maintaining the CN micelles integrity upon pressure application. The increase in viscosity of the RO BM due to concentration could also have negatively affected the efficiency of the UHPH. Concentration by RO followed by UHPH might also have induced reassociation of partially UHPH dissociated CN into lower-density complexes recovered above CN micellar pellet. In fact, reassociation of the dissociated CN micelles following UHPH treatment in the serum phase of concentrated micellar CN added with WP has been reported (Sørensen et al., 2014). However, for the CN, the soluble WP content did not change significantly in RO BM but only slightly decreased upon pressure application of 15 MPa, indicating WP-CN association in the colloidal phase. Similar results were reported in milk CN concentrate containing a high ratio of WP at pH 6.7 (Sørensen et al., 2014).

**Milk Salts.** Concentration by RO increased significantly ($P < 0.05$) the salt (calcium, potassium, magnesium, sodium, and phosphate) content (Table 3). However, the calcium content in the soluble fractions did not increase to the same degree due to the reduced calcium solubility upon water removal (Liu et al., 2012). Similar results were obtained by Dussault-Chouinard et al. (2019) when concentrating SM by RO. These authors observed a shift of the calcium equilibrium from the soluble to the colloidal phase, with calcium being more associated with the CN micelles (Dussault-Chouinard et al., 2019).

Whereas no significant difference in calcium content of BM was observed between SN 0, 15, and 150 MPa, significantly higher amounts of calcium were found in samples treated at 300 MPa (11.05 ± 0.33 mmol/L) compared with 15 MPa (9.89 ± 0.41 mmol/L; $P =$ 0.01). The calcium content in the soluble fractions did not increase to the same degree due to the reduced calcium solubility upon water removal (Liu et al., 2012). Similar results were obtained by Dussault-Chouinard et al. (2019) when concentrating SM by RO. These authors observed a shift of the calcium equilibrium from the soluble to the colloidal phase, with calcium being more associated with the CN micelles (Dussault-Chouinard et al., 2019).

<table>
<thead>
<tr>
<th>Item</th>
<th>True protein (g/100 g)</th>
<th>NPN (g/100 g)</th>
<th>Whey protein (g/100 g)</th>
<th>Casein (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM SN 0</td>
<td>0.73 ± 0.09 $^{a}$</td>
<td>0.15 ± 0.01 $^{a}$</td>
<td>0.37 ± 0.07 $^{a}$</td>
<td>0.36 ± 0.04 $^{a}$</td>
</tr>
<tr>
<td>BM SN 15</td>
<td>0.71 ± 0.01 $^{a}$</td>
<td>0.15 ± 0.01 $^{a}$</td>
<td>0.36 ± 0.04 $^{a}$</td>
<td>0.35 ± 0.03 $^{a}$</td>
</tr>
<tr>
<td>BM SN 150</td>
<td>0.78 ± 0.10 $^{a}$</td>
<td>0.15 ± 0.02 $^{a}$</td>
<td>0.35 ± 0.04 $^{a}$</td>
<td>0.43 ± 0.10 $^{a}$</td>
</tr>
<tr>
<td>BM SN 300</td>
<td>0.88 ± 0.08 $^{a}$</td>
<td>0.16 ± 0.01 $^{a}$</td>
<td>0.25 ± 0.06 $^{a}$</td>
<td>0.63 ± 0.03 $^{a}$</td>
</tr>
<tr>
<td>RO BM SN 0</td>
<td>1.43 ± 0.16 $^{a}$</td>
<td>0.23 ± 0.03 $^{a}$</td>
<td>1.29 ± 0.08 $^{a}$</td>
<td>0.37 ± 0.13 $^{a}$</td>
</tr>
<tr>
<td>RO BM SN 15</td>
<td>1.49 ± 0.17 $^{a}$</td>
<td>0.22 ± 0.03 $^{a}$</td>
<td>0.98 ± 0.06 $^{b}$</td>
<td>0.81 ± 0.15 $^{a}$</td>
</tr>
<tr>
<td>RO BM SN 150</td>
<td>1.56 ± 0.18 $^{a}$</td>
<td>0.23 ± 0.01 $^{a}$</td>
<td>1.02 ± 0.05 $^{b}$</td>
<td>0.58 ± 0.14 $^{a}$</td>
</tr>
<tr>
<td>RO BM SN 300</td>
<td>1.78 ± 0.17 $^{a}$</td>
<td>0.25 ± 0.04 $^{a}$</td>
<td>0.98 ± 0.08 $^{b}$</td>
<td>0.46 ± 0.14 $^{a}$</td>
</tr>
</tbody>
</table>

$^{a,b}$Significant difference indicated column-wise for BM and RO BM independently. Different letters indicate significant difference ($P < 0.05$).

Values are means ± SD (n = 3).
The marked decrease in soluble calcium observed in the SN 300 treatment (11.05 ± 0.33 mmol/L) is significant compared with the 15 MPa samples (15.25 ± 0.29 mmol/L; P < 0.05). To determine whether the effect of UHPH on the repartition of the PLs could be attributed to the 3 main fractions in the BM, they were primarily concentrated in the lower-density pellet cloud for the RO samples. This interesting shift of the PL from the soluble phase to the pellet cloud suggested that the concentration of BM by RO induced significant changes within the MFGM material and their interactions with other BM components. The higher lipid content of the SN treated at 300 MPa compared with the 15 MPa samples was somewhat unexpected because the PL content did not significantly change among the other pressure treatments. The increase in total lipids likely occurred from other lipid classes, probably tiny fat globules that could not be skimmed, which might have interacted with proteins or the MFGM (Gauvin et al., 2018a).

**PL.** As seen in Figure 3, the distribution of the PL in RO BM differed from the BM. Although the PL were more evenly distributed between the 3 main fractions in the BM, they were primarily concentrated in the lower-density pellet cloud for the RO samples. This interesting shift of the PL from the soluble phase to the pellet cloud suggested that the concentration of BM by RO induced significant changes within the MFGM material and their interactions with other BM components.

The effect of UHPH on the repartition of the PLs between the soluble (SN) and colloidal fractions (pellet and pellet cloud) after ultracentrifugation (70,000 × g at 20°C for 1 h) was then monitored. The PL content in the pellet was relatively low in the BM and RO BM, which was expected because this fraction is known to contain mainly CN micelles (Gauvin et al., 2018a). Surprisingly, UHPH did not induce any significant change in the PL contents of the 3 phases for the BM and RO BM (Table 4). Fauquant et al. (2014) reported that the homogenization of BM at 80 MPa induced the formation of small vesicles consisting of polar lipids with membrane proteins from the disrupted MFGM fragments (Fauquant et al., 2014). Using much higher-pressure treatment, their association with the CN in the RO BM could not be ruled out as the amount of soluble CN decreased considerably compared with normal BM. The marked decrease in soluble calcium observed in the

### Table 3. Milk salts composition (in mmol/L) of total buttermilk (BM), total reverse osmosis (RO) BM, and their respective supernatants (SN) treated at various pressure levels (0, 15, 150, and 300 MPa)

<table>
<thead>
<tr>
<th>Item</th>
<th>Calcium</th>
<th>Potassium</th>
<th>Magnesium</th>
<th>Sodium</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25.39 ± 0.73A</td>
<td>34.51 ± 1.74A</td>
<td>4.03 ± 0.32A</td>
<td>21.75 ± 2.08A</td>
<td>30.55 ± 1.20A</td>
</tr>
<tr>
<td>SN 0</td>
<td>10.08 ± 0.15A, b</td>
<td>29.94 ± 0.74A</td>
<td>2.82 ± 0.14A</td>
<td>20.44 ± 1.16A</td>
<td>15.77 ± 0.67b</td>
</tr>
<tr>
<td>SN 15</td>
<td>9.89 ± 0.41A</td>
<td>28.19 ± 1.68A</td>
<td>2.70 ± 0.11A</td>
<td>20.16 ± 2.64A</td>
<td>15.25 ± 0.29A</td>
</tr>
<tr>
<td>SN 150</td>
<td>10.02 ± 0.11A</td>
<td>29.70 ± 0.30A</td>
<td>2.82 ± 0.14A</td>
<td>19.71 ± 0.63A</td>
<td>16.11 ± 0.17A</td>
</tr>
<tr>
<td>SN 300</td>
<td>11.05 ± 0.33B</td>
<td>30.33 ± 1.72A</td>
<td>2.87 ± 0.13A</td>
<td>18.60 ± 0.92A</td>
<td>16.88 ± 0.53A</td>
</tr>
<tr>
<td>RO BM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48.60 ± 4.21B</td>
<td>67.15 ± 3.62B</td>
<td>7.50 ± 0.33B</td>
<td>35.04 ± 1.02B</td>
<td>57.35 ± 3.39B</td>
</tr>
<tr>
<td>SN 0</td>
<td>17.07 ± 1.10A</td>
<td>63.41 ± 4.34A</td>
<td>5.56 ± 0.31A</td>
<td>34.27 ± 1.33A</td>
<td>29.65 ± 1.86A</td>
</tr>
<tr>
<td>SN 15</td>
<td>17.81 ± 1.23A</td>
<td>62.85 ± 3.83A</td>
<td>5.44 ± 0.41A</td>
<td>34.02 ± 3.48A</td>
<td>31.50 ± 2.74A</td>
</tr>
<tr>
<td>SN 150</td>
<td>18.76 ± 1.78A</td>
<td>67.06 ± 4.23A</td>
<td>5.72 ± 0.45A</td>
<td>34.40 ± 2.06A</td>
<td>32.44 ± 3.17A</td>
</tr>
<tr>
<td>SN 300</td>
<td>18.11 ± 1.64A</td>
<td>64.58 ± 5.59A</td>
<td>5.61 ± 0.39A</td>
<td>34.47 ± 2.48A</td>
<td>30.58 ± 2.52A</td>
</tr>
</tbody>
</table>

Significance difference (P < 0.05) is indicated column-wise for BM and RO BM independently. Significant difference (P < 0.05) between total BM and total RO BM is indicated column-wise with different capital letters.

Values are means ± SD (n = 3).
RO BM combined with the increase in the main salts in the serum phase could also have fostered the formation of higher-density MFGM particles. Further investigations would be needed to provide more insight into the associative behavior of PL in concentrated BM.

**Protein Profiles of BM and RO BM, and Their Respective Fractions.** The protein profiles of total BM and RO BM, and their respective fractions (SN, pellet, and pellet cloud), including SN with added sodium citrate (2% for BM, 4% for RO BM), were analyzed using SDS-PAGE under non-reducing and reducing conditions to get insight into the type of interactions involved between the proteins. Addition of sodium citrate aimed at dissolving existing CN. As seen in Figure 4, under non-reducing conditions, the band intensity of the main WP, α-LA and β-LG, slightly decreased for the control BM, with increasing pressure up to 300 MPa, likely due to pressure-induced denaturation and aggregation (Lopez and Ménard, 1996). This effect was not observed for the RO BM as the band intensities of the WP remained the same irrespective of the pressure applied. As discussed previously, cream pasteurization before churning already creates denatured β-LG-CN-complexes (Hickey et al., 2017), probably reducing the effects of subsequent UHPH treatment, especially in the RO BM samples. The likely increase in temperature during UHPH attributable to turbulence, cavitation, and shear stress has been reported to be approximately 17.6 to 18.5°C per tranche of 100 MPa (Hayes and Kelly, 2003, Thiebaud et al., 2003), which, in our conditions (maximum of 300 MPa), would be insufficient to induce further denaturation and aggregation of the proteins. For both the BM and RO BM, no MFGM proteins (e.g., butyrophilin, periodic acid Schiff 6/7) were detectable in the SN, indicating that they interacted with other milk proteins. Upon sodium citrate addition, which disrupted the CN by chelating the colloidal calcium, some MFGM proteins became visible, suggesting they were interacting non-covalently with the CN micelles. Upon concentration of BM, MFGM proteins were more present in the pellet cloud, supporting our assumption that the pellet cloud contains a significant amount of MFGM material.

Figure 5 shows the SDS-PAGE gels under reducing conditions. Comparing the results obtained with the non-reducing condition, we observed the presence of specific proteins upon 2-mercaptoethanol addition. Not

![Figure 3. Phospholipid distribution in buttermilk (BM) and reverse osmosis (RO) BM at various pressure levels (0, 15, 150, and 300 MPa).](image)

![Figure 3. Phospholipid distribution in buttermilk (BM) and reverse osmosis (RO) BM at various pressure levels (0, 15, 150, and 300 MPa).](image)

<table>
<thead>
<tr>
<th>Item</th>
<th>Total</th>
<th>Supernatant</th>
<th>Pellet</th>
<th>Pellet cloud</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM 0</td>
<td>0.12 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>0.15 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>0.08 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>300</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RO BM 0</td>
<td>0.31 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>0.12 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>0.13 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>300</td>
<td>0.15 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant difference (P < 0.05) is indicated column-wise for each compound, BM, and RO BM independently.
<sup>b</sup>Significant difference (P < 0.05) between total BM and total RO BM is indicated column-wise with different capital letters.

Values are means ± SD (n = 3).
only the main CN bands (αs-CN, β-CN, and κ-CN) at around 25 kDa, but milk proteins such as immunoglobulins, BSA, and periodic acid Schiff 6/7 became more present under reducing conditions. Moreover, β-LG and α-LA were present in all lanes independently of the pressure applied. Thus, in all samples, the WP interacted with other proteins in the form of disulfide-linked aggregates. Because we found no differences upon the different pressure treatments, we concluded that these interactions occurred principally during pasteurization of the cream (85°C/30 s) before churning.

Microstructural Changes in BM Following RO and UHPH

Particle Size Distribution. Particle size distribution within the different BM and RO BM samples was analyzed by laser diffraction. In some samples, sodium citrate was added at a concentration of 2% for BM and 4% for RO BM, to completely dissociate the CN micelles before particle size measurements. The changes in the D_{4,3} (volume-weighted mean diameter) and D_{3,2} (surface-weighted mean diameter) values are represented in Table 5. Comparing BM and RO BM, the control (0 MPa) D_{4,3} and D_{3,2} were significantly lower for RO BM compared with BM (P < 0.0001 and P = 0.02, respectively). The D_{4,3} values significantly decreased with increasing pressure for the BM and RO BM, especially between 0 MPa and 300 MPa in the BM (P < 0.0001) and the RO BM (P < 0.0001), respectively. The D_{3,2} values also significantly decreased for the BM and RO BM with increasing UHPH pressure, but to a lower extent than for the D_{4,3} values. Again, the D_{3,2} value for the control (0 MPa) was significantly lower after concentrating the BM by RO (P = 0.02). Both the D_{4,3} and D_{3,2} values decreased, easing pressure, with increasing pressure for samples where sodium citrate was added to dissociate the CN micelles. Although the control (0

Figure 4. SDS PAGE under non-reducing conditions with 12% acrylamide gels of (a) buttermilk (BM) 0 and 15 MPa, (b) BM 150 and 300 MPa, and (c) reverse osmosis (RO) BM 0 and 15 MPa, and (d) RO BM 150 and 300 MPa. MWM = molecular weight markers; SN = supernatant; SC = sodium citrate; P = pellet, PC = pellet cloud.
MPa) and 15 MPa samples in the presence of sodium citrate showed similar values, applying higher pressure decreased the D_{3,2} significantly, especially when comparing 0 MPa to 300 MPa (with \( P < 0.0001 \) for both the BM and RO BM). In prior studies, pressure treatment at 200 MPa decreased the CN micelle size, whereas an increase was observed at higher-pressure levels (350 MPa; Hayes and Kelly, 2003; Sandra and Dalgleish, 2005). The particle size decrease was explained by partial CN micelle dissociation (5 to 30%) 

![Figure 5](image)

**Figure 5.** SDS PAGE under reducing conditions using 2-mercaptoethanol and 12% acrylamide gels of (a) buttermilk (BM) 0 and 15 MPa, (b) BM 150 and 300 MPa, and (c) reverse osmosis (RO) BM 0 and 15 MPa, and (d) RO BM 150 and 300 MPa. PAS 6/7 = periodic acid Schiff 6/7; MWM = molecular weight markers; SN = supernatant; SC = sodium citrate; P = pellet; PC = pellet cloud.

### Table 5.

<table>
<thead>
<tr>
<th>Sodium citrate</th>
<th>MPa</th>
<th>0</th>
<th>15</th>
<th>150</th>
<th>300</th>
<th>0</th>
<th>15</th>
<th>150</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D_{4,3}</td>
<td></td>
<td></td>
<td></td>
<td>D_{3,2}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>0.51 ± 0.00^a</td>
<td>0.47 ± 0.00^b</td>
<td>0.34 ± 0.00</td>
<td>0.27 ± 0.00^b</td>
<td>0.42 ± 0.00</td>
<td>0.37 ± 0.01</td>
<td>0.28 ± 0.00</td>
<td>0.18 ± 0.02</td>
<td>0.25 ± 0.00</td>
</tr>
<tr>
<td>RO BM</td>
<td>0.31 ± 0.00^b</td>
<td>0.31 ± 0.00^c,b</td>
<td>0.26 ± 0.00</td>
<td>0.10 ± 0.00</td>
<td>0.28 ± 0.00</td>
<td>0.28 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>0.68 ± 0.00^a</td>
<td>0.65 ± 0.00</td>
<td>0.52 ± 0.00</td>
<td>0.37 ± 0.00</td>
<td>0.62 ± 0.00</td>
<td>0.61 ± 0.00</td>
<td>0.41 ± 0.00</td>
<td>0.31 ± 0.00</td>
<td>0.31 ± 0.00</td>
</tr>
<tr>
<td>RO BM</td>
<td>0.45 ± 0.00^b</td>
<td>0.44 ± 0.00^a</td>
<td>0.38 ± 0.00</td>
<td>0.27 ± 0.00</td>
<td>0.42 ± 0.00</td>
<td>0.42 ± 0.00</td>
<td>0.28 ± 0.00</td>
<td>0.23 ± 0.00</td>
<td>0.23 ± 0.00</td>
</tr>
</tbody>
</table>

*Significant difference indicated row-wise for samples with and without sodium citrate and for D_{4,3} and D_{3,2} independently. Different letters indicate significant difference (\( P < 0.05 \)).

Sodium citrate additions were 2% for BM and 4% for RO BM. Values are means ± SD (n = 3)
observed for milk (Hayes and Kelly, 2003; Sandra and Dalglish, 2005; Chevalier-Lucia et al., 2011; Mohan et al., 2016). Instead, we observed a constant decrease in particle size from 15 up to 300 MPa in the presence of sodium citrate. Based on the general decrease in D₄,₃ and D₃,₂ values upon CN micelle dissociation with sodium citrate, we concluded that UHPH severely affected the MFGM material because the only remaining particles in suspension following CN micelle dissociation were the MFGM fragments and vesicles.

Figure 6 shows the volume-based size distribution of the BM and RO BM samples. We noticed a bimodal distribution with the main peak at about 0.4 µm ranging between 0.1 and 1.0 µm, which includes the CN micelles (Fox and Brodkorb, 2008), small MFGM fragments (0.1–4.0 µm), and PL vesicles (100–300 nm; Fauquant et al., 2014). A smaller population was observed above 1.0 µm, with the second peak at approximately 4.89 µm. As reported by Fauquant et al. (2014), the latter represents other BM compounds, most likely remaining lipid droplets and large MFGM fragments (Fauquant et al., 2014). The particle size distribution for BM and RO BM remained similar for 0 and 15 MPa. However, a shift toward smaller particle sizes was observed at 150 and 300 MPa accompanied by an increase in volume density. A higher particle volume density was observed for BM compared with RO BM following dissociation of the CN micelles with sodium citrate (Figures 6b and d). Also, UHPH induced a decrease in particle volume density, which differs between the BM and RO BM samples. It started at 300 MPa for BM, whereas for RO this decrease started at 150 MPa. The increase in the first peak with a simultaneous decrease in the second peak corresponds to the enhanced presence of smaller particles (first peak) and fewer existing aggregates (second peak). Similar re-

![Figure 6. Particle size distribution of (a) buttermilk (BM), (b) BM + 2% sodium citrate, (c) reverse osmosis (RO) BM and (d) RO BM + 4% sodium citrate at various pressure levels (0, 15, 150, and 300 MPa). Black plain line = 0 MPa; black stippled line = 15 MPa; gray solid line = 150 MPa; gray stippled line = 300 MPa.](image-url)
Results were reported by Amador-Espejo et al. (2014) for whole milk homogenized at 200 MPa. However, these authors observed at 300 MPa a decrease in the first peak with an increase in the second peak related to fat aggregation (Amador-Espejo et al., 2014). The same study found higher D4,3 and D3,2 values at 300 MPa compared with 200 MPa, also related to the presence of fat aggregates. In our study, fat was not present in a large amount due to the creaming of the BM. Therefore, the main effect of UHPH observed on BM and RO BM was a decrease in the size of the MFGM material, probably due to the high turbulence, shear, and cavitation induced in the homogenization chamber (Thiebaud et al., 2003) resulting in small MFGM fragments and the possible formation of PL vesicles, as reported by Fauquant et al. (2014).

**Microstructural Changes in BM.** Figures 7 and 8 show the images of the distribution of the fluorescently labeled proteins and PL in BM and RO BM, respectively, as obtained by CLSM. Buttermilk samples (Figure 7) showed a clear pressure effect on the protein structure. At 0 MPa, the microstructure of the sample was dense, and particles seemed more prominent than in the pressure-treated samples. With increasing pressure from 0 to 300 MPa, the particles became much smaller with a fine, more homogeneous protein distribution in aggregates associated together into a filamentous manner. The PL distribution at 0 MPa aligns with the dense protein structure. The PL particles were more prominent and seemed to aggregate, but they progressively decreased in size with increasing pressure. At 150 and 300 MPa, the PL were almost not visible.

![Figure 7](image_url). Confocal laser scanning microscopy images at 40× magnification of buttermilk at various pressure levels (0, 15, 150, and 300 MPa), divided into proteins (Fast Green FCF) and phospholipids (Nile Red), and merged into one image. Scale bar = 10 µm.
anymore. Treating the concentrated BM with UHPH seemed to affect its microstructure significantly (Figure 8). Similar to the BM, increasing UHPH pressure led to modifications in the protein structure with interactions and aggregations observed upon higher UHPH. The PL seem to have even smaller particle sizes than those present in the BM samples, in line with previous particle size and size distribution results. The initial lower particle size in RO BM samples compared with BM might be related to modifications in the mineral composition of the serum and potential compositional change related to the concentration by RO on MFGM fragments integrity. The observed decrease in MFGM fragment size is visible in CLSM images, with the PL being so small that they are barely visible. A more homogeneous structure is observed with increasing homogenization pressure. However, in the CLSM image at 300 MPa, proteins seem to aggregate to a certain degree. As mentioned previously, denatured WP-CN complexes may start interacting with other MFGM fragments upon higher-pressure treatments (Sandra and Dalgleish, 2005). Shear, turbulence, and cavitation (Thiebaud et al., 2003) are probably responsible for MFGM material disruption, with the PL becoming less visible.

**CONCLUSIONS**

Our data evidence that both RO and UHPH have an effect on sweet BM constituents and their association state or microstructure. The RO concentration of BM modified the distribution of the MFGM PL between...
the 3 main centrifugal phases recovered, namely SN, pellet cloud, and pellet. Our results also showed that UHPH treatment affected the SN composition of BM following ultracentrifugation in terms of lipid, CN, calcium and phosphate contents. Ultra-high-pressure homogenization application further decreased the particle size distribution in BM and RO BM. The CLSM images confirmed the finer and more homogeneous particle size distribution with increasing pressure. This work evaluated the effect of UHPH and RO on sweet BM composition and microstructure and observed interesting differences. More research is needed to determine if these differences affect the techno-functional performance (e.g., rennet gel formation, crumbliness) of UHPH-treated sweet BM and RO BM when incorporated in dairy products, particularly cheese.

ACKNOWLEDGMENTS

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC, Ottawa; grant number CRDPJ/537396-2018), the Quebec Consortium for Industrial Bioprocess Research and Innovation (Quebec, Canada), and Novalait Inc (Quebec, Canada). The authors thank Diane Gagnon and Pascal Lavoie (Department of Food Sciences, Université Laval, Quebec, Canada) for their technical support. The authors also thank Alexandre Bastien (Institute of Integrative Biology and Systems, Université Laval) and Patrick Leclerc (Faculty of Forestry, Geography and Geomatics, Université Laval) for their technical support and assistance with the CLSM and inductively coupled plasma spectrometry, respectively. The authors have not stated any conflicts of interest.

REFERENCES


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