Effect of alkalization and ultra-high-pressure homogenization on casein micelles in raw and pasteurized skim milk

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ABSTRACT

Mechanical and physicochemical treatments of milk induce structural modifications of the casein (CN) micelles, affecting their techno-functional properties in dairy processing. Here, we studied the effect of alkalization and ultra-high-pressure homogenization (UHPH) on CN micelles in raw skim milk (rSM) and pasteurized skim milk (pSM). The pH of both skim milks (approximately 6.7) was adjusted to 8.5 and 10.5 before UHPH at 100, 200, and 300 MPa. The structural changes of the CN micelles during the treatments were assessed using laser diffraction, transmission electron microscopy, and turbidity measurements. Finally, ultra-centrifugation ($70,000 \times g$ for 1 h at 20°C) was carried out to evaluate the protein's distribution between the supernatant (serum phase) and the pellet (colloidal phase) by gel electrophoresis and protein concentration measurement. Alkalization of both skim milks induced a significant reduction in turbidity, whereas an increase of the average particle size was observed, the effect being more severe in pSM than rSM. At alkaline pH, more proteins were recovered in the serum phase, which suggested that the CN underwent major rearrangements into nonsedimentable CN forms of various sizes, as confirmed by transmission electron microscopy. The amount of CN found in the serum phase at pH 8.5 also increased with the UHPH pressure. Although UHPH did not influence the average CN micelle size at pH 6.7 and 8.5, a pressure-dependent decrease was observed at pH 10.5 for both skim milks. The structural changes of the CN micelles observed in this study throughout the combination of alkalization and UHPH could be of interest for developing new dairy ingredients with improved functionality.

Key words: skim milk, casein micelle, alkalization, UHPH

INTRODUCTION

Casein micelles are self-associated colloids in milk, with sizes ranging from 50 to 600 nm in diameter and an average diameter of 150 nm (Fox and Brodtkorb, 2008). Casein micelles consist of 4 classes of phosphoproteins: α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN, which represent 80% of total proteins in bovine milk (Ahmad et al., 2009). The CN micelle's structure is stabilized by hydrophobic and electrostatic interactions, hydrogen bonding, and colloidal calcium phosphate (CCP; Vaia et al., 2006; Goulding et al., 2020). Changes in the physicochemical environment such as temperature, pH, ionic strength, and water activity lead to changes in CN micelle integrity throughout the disruption of various chemical interactions (Pelegrine and Gasparetto, 2005; Vaia et al., 2006; McMahon and Oommen, 2008; Madadlou et al., 2009a; Sinaga et al., 2017). Alkalization of milk has been associated with significant changes in CN micelle size (Vandijk, 1992; Ahmad et al., 2009). However, there are still divergences in the interpretations of the pH impact on CN micelle size in the alkaline range. The CN micelle dissociation has been attributed to several effects. The reduction of the hydrophobic interactions among the CN, an increase in electrostatic repulsions, and changes in the mineral balance between the colloidal and the aqueous phases involving calcium and phosphate have been reported (Vaia et al., 2006; Huppertz et al., 2008; Sinaga et al., 2017). However, other authors have shown that alkaline dissociated CN molecules rearranged into highly solvated nonsedimentable aggregates of heterogeneous sizes (Ahmad et al., 2009; Madadlou et al., 2009b).

As the structure of CN micelles is loosened and becomes more fragile at alkaline pH, the application of mechanical treatment may induce disruption of CN micelles and change their functional properties in milk. Recently, ultra-high-pressure homogenization (UHPH) has been used in the food industry to provide new products by protecting the sensory quality of sensitive components in food products and functional properties, and specific structural characteristics (Patrignani

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and Lanciotti, 2016). The UHPH technology combines the advantages of conventional homogenization such as emulsification and particle size reduction and the possibility of pasteurization with the use of higher pressure, up to 400 MPa (Dumay et al., 2013). In the dairy sector, UHPH has been investigated for inactivating enzymes, viruses, and bacteria and forming stable and homogeneous emulsions. In milk, UHPH treatment induces denaturation and aggregation of whey proteins (WP) and changes in the CN micellar structure and size (Hayes and Kelly, 2003; Sandra and Dalgleish, 2005; Chevalier-Lucia et al., 2011; Zamora and Guamis, 2015). For example, a CN micelle reduction of 5 to 33% has been reported after UHPH treatments (200–300 MPa; Hayes and Kelly, 2003; Chevalier-Lucia et al., 2011). The changes in the size of the CN micelles have been attributed to the partial removal of the κ -CN and α _s-CN at the micelle surface (Sandra and Dalgleish, 2005; Zamora and Guamis, 2015). However, to our knowledge, no study has been carried out on the effect of UHPH treatments on the CN micelle size and their structural association state at alkaline pH.

The present work aimed to explore the combined effects of UHPH treatment on skim milk (SM) in the alkaline pH range and the result on CN's structural modifications and change in protein associations and distribution between the colloidal and serum phases. The combination of alkalization and UHPH could be an efficient way to control the CN micelle size to develop dairy ingredients with new techno-functional properties.

MATERIALS AND METHODS

Materials

Fresh raw whole milk was sourced from a local distributor (Quebec, QC, Canada) and split into 2 batches. One batch was kept raw, and the other half was pasteurized at 72°C for 20 s (Chalinox/Hydro-Québec CFI-25). Raw and pasteurized milk were then skimmed using a Westfalia cream separator (LWA-205, DeLaval) to obtain raw skim milk (rSM) and pasteurized skim milk (pSM). The SM composition was determined using LactoScope FTIR milk analyzer (Table 1; Delta Instruments). Sodium azide (0.02%; wt/vol) was added to prevent microbial growth.

Methods

The detailed flowchart of the experimental design is provided in Figure 1.

pH Adjustment

The initial pH value of rSM and pSM was 6.7 ± 0.05 . Sodium hydroxide (NaOH, Sigma-Aldrich) 1.0 M was used to adjust the milk's pH to 8.5 ± 0.05 and 10.5 ± 0.05 . Samples were then stored at 4°C for 24 h to allow pH equilibration. The pH was then readjusted to the targeted pH (pH 8.5 and 10.5) before UHPH treatments. This pH range was chosen based on the work of Liu and Guo (2008) and Sinaga et al. (2017).

UHPH of Milk

For each replicate, the pH-adjusted rSM and pSM were homogenized using one stage UHPH (Nano De-bee, model 45–4, Bee International) at 100, 200, and 300 MPa. The SM samples were kept at 4°C in the inlet reservoir before being forced by the intensifying pump through the UHPH emulsifying cell consisting in a narrow zirconia nozzle (0.20 mm diameter, model Z8, Bee International), connected to 12 interaction chambers that increase the cavitation, shear, and turbulence time. The Nano DeBee allowed a reverse flow setup. In this configuration, the fluid was forced to flow back from the interacting chambers toward the nozzle, generating a high shear impact between the opposing streams before exiting through the outlet port located just before the nozzle. The samples were subjected to rapid cooling by a heat exchanger with circulating water at 10°C, located at the immediate outlet port to minimize any increase in temperature within the processing chamber. Nonhomogenized rSM and pSM were used as control samples.

Ultracentrifugation

After UHPH, the samples were ultracentrifuged at $70,000 \times g$ for 1 h at 20°C using the Optima XPN-90 ultracentrifuge (Beckman Coulter). The pellets and supernatants were collected and kept frozen until further analyses.

Table 1. Composition of raw (rSM) and pasteurized (pSM) skim milk¹

Item (%)	rSM	pSM
TS	8.69 ± 0.31	9.13 ± 0.31
Lipids	0.12 ± 0.01	0.13 ± 0.02
Proteins	4.28 ± 0.1	4.63 ± 0.05
Lactose	4.61 ± 0.1	4.63 ± 0.05

¹Mean $\pm$ SD (n = 3).

Figure 1. Schematic representation of the experimental design. UHPH = ultra-high-pressure homogenization.

Characterization and Composition

Particle Size Analysis. Mean particle diameter and particle size distribution (PSD) were measured with a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments) and the analysis was carried out at 23°C (Hayes and Kelly, 2003). Refrac-

tive indexes were set at 1.39 and 1.33 for CN micelles and water, respectively (Alexander et al., 2002). The sample particle characteristics are reported by the volume-weighted mean diameter ($D_{4,3}$), the surface-weighted mean diameter ($D_{3,2}$), and the span value. Each replicate was run 3 times, with 3 measurements per run.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was carried out on control and homogenized rSM and pSM at different pH values (pH 6.7, 8.5, and 10.5) to examine changes in milk microstructure. Samples were prepared as described by Marciniak et al. (2018). Samples were diluted 10-fold and prepared by successively placing a droplet of the diluted sample and 3% uranium acetate on 200 mesh nickel grids covered with a Formvar film and air-dried. A Jeol JEM-1230 TEM device operating at 80 kV was used for imaging. A Gatan US1000SP1 ultrascan camera (Gatan Inc.) was used for image capture, and the images were analyzed using Gatan 2.11 software.

Turbidity. Turbidity of rSM and pSM samples treated by alkalization and UHPH and their respective controls were monitored using a Hach Model 2100AN IS turbidimeter (Hach Company). The instrument was calibrated using formazin primary standards (Hach Company), ranging between 20 and 7,500 nephelometric turbidity units. Samples (30 mL) of each solution were analyzed in a clean sample cell.

Protein Content. Analysis of the protein content in the supernatant of nonhomogenized and UHPH-treated rSM and pSM at the different pH was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad) based on the well-known reaction of proteins with alkaline copper tartrate solution and Folin-Ciocalteu reagent (Lowry et al., 1951). Samples were diluted 5-fold before analysis and the absorbance read at 750 nm with an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies Inc.). Bovine serum albumin (Bio-Rad) was used as a protein standard to generate a linear calibration curve with concentrations ranging from 0 to 2,000 µg/mL. Two measures of the optical density were carried out for each sample.

Determination of Protein Distribution Between the Serum and Colloidal Phases. Protein profiles of control, UHPH-treated milk (rSM and pSM), and their respective supernatants were determined by SDS-PAGE under reducing conditions, using 12% polyacrylamide gels (TGX Stain-Free, Bio-Rad). Samples were diluted 6-fold with deionized water. Twenty microliters of diluted samples was combined with 1 µL of β-mercaptoethanol and 19 µL of 2× Laemmli buffer (Bio-Rad). Samples were then heated at 100°C for 5 min, and 15 µL was loaded into the gel. Five microliters of molecular weight marker (Precision plus pre-stained, Bio-Rad) was loaded in the first well. Running buffer was prepared with 100 mL of 10× Tris-glycine SDS buffer (Bio-Rad) and 900 mL of deionized water. Electrophoresis was carried out at 15 mA per gel. Gels were stained for 60 min with a solution of Coomassie Brilliant Blue R-250 [10% acetic acid, 40% ethanol, and

50% water (vol/vol)] and unstained with a solution of 10% acetic acid, 10% methanol, and 80% water (vol/vol). Images of the gel were captured with the Chemi-Doc MP Imaging System (Bio-Rad).

Statistical Analysis

All experiments were carried out in triplicate. A factorial analysis carried out to assess the simple effects and interactions between pH value (6.7, 8.5, and 10.5), UHPH level (0, 100, 200, and 300 MPa), and the pasteurization of milk (rSM or pSM) by ANOVA. Tukey's test was then carried out for multiple mean comparisons using Statistical Analysis System (SAS) University Edition, SAS Studio 3.5 software (SAS Institute Inc.). The analyses were carried out at a significance level of $P < 0.05$.

RESULTS

Effect of pH and UHPH on Milk Properties

The effects of alkaline pH and UHPH on the PSD were monitored by laser diffraction for rSM and pSM. Figure 2 shows the results of the effect of UHPH pressures on the PSD of rSM and pSM at different pH values.

At pH 6.7, both nonhomogenized rSM and pSM controls (0 MPa) show similar PSD, characterized by the main population ranging from approximately 0.04 to about 1.0 µm and a tiny population above 1.0 µm (Figure 2A). When UHPH pressure was increased, a narrowing of the width of the principal peak was observed and the effect was more extreme on pSM than rSM (Table 2). Furthermore, the larger particles observed above 1 µm in the PSD of both nonhomogenized SM samples disappeared as the pressure increased to 200 MPa.

The UHPH treatment also modified the SM PSD profiles at pH 8.5 (Figure 2B). Contrary to pH 6.7, UHPH did not affect the span index for the rSM, whereas UHPH treatments at 300 MPa of pSM significantly ($P < 0.001$) decreased the span index values. In addition, increasing the pH from 6.7 to 8.5 resulted in significantly larger sized particles in the nonhomogenized rSM ($P = 0.022$) and pSM ($P = 0.0169$) compared with pH 6.7 (Table 2). The particle size profiles for both nonhomogenized SM samples further increased as pH was adjusted to 10.5 (Figure 2C). However, after UHPH treatment, the size profiles shifted progressively back toward original values, indicating a strong pressure effect on the particle size at pH 10.5. However, a significant decrease in the span value was observed

Figure 2. Particle size distribution for raw skim milk (rSM; plain line) and pasteurized skim milk (pSM; stippled line) at pH 6.7 (A), 8.5 (B), and 10.5 (C) for control (blue), 100 MPa (green), 200 MPa (red), and 300 MPa (orange) samples.

Table 2. Particle size parameters of raw (rSM) and pasteurized (pSM) skim milk¹

pH value	Pressure (MPa)	D _{4,3} ² (nm)		D _{3,2} ³ (nm)		Span	
		rSM	pSM	rSM	pSM	rSM	pSM
6.7	0	196.3 ± 32.2 ^a	183.0 ± 13.2 ^a	79.0 ± 7.7 ^{ab}	73.6 ± 1.7 ^a	2.89 ± 0.11 ^{bc}	2.93 ± 0.20 ^c
	100	171.0 ± 6.6 ^a	170.3 ± 7.5 ^a	75.06 ± 1.0 ^a	76.0 ± 0.0 ^a	2.82 ± 0.06 ^{ab}	2.77 ± 0.12 ^c
	200	160.0 ± 5.0 ^a	172.0 ± 8.7 ^a	73.1 ± 1.1 ^a	93.0 ± 11.7 ^{ab}	2.75 ± 0.08 ^{ab}	2.22 ± 0.24 ^{ab}
	300	155.7 ± 16.9 ^a	172.3 ± 4.7 ^a	79.9 ± 19.1 ^{ab}	104.3 ± 3.1 ^c	2.48 ± 0.39 ^a	1.91 ± 0.02 ^a
8.5	0	280.3 ± 28.6 ^{bc}	259.0 ± 33.8 ^{bc}	104.7 ± 14.2 ^{bc}	100.4 ± 13.6 ^c	2.93 ± 0.12 ^{bc}	2.88 ± 0.08 ^c
	100	233.0 ± 19.2 ^{ab}	226.7 ± 29.5 ^{bc}	94.5 ± 9.1 ^{abc}	97.7 ± 15.1 ^{bc}	2.89 ± 0.02 ^{bc}	2.74 ± 0.08 ^c
	200	222.3 ± 10.4 ^{ab}	197.3 ± 14.6 ^{ab}	91.4 ± 2.9 ^{abc}	94.1 ± 6.3 ^{ab}	2.83 ± 0.07 ^{bc}	2.52 ± 0.06 ^{bc}
	300	208.0 ± 2.0 ^{ab}	197.0 ± 14.7 ^{ab}	83.86 ± 5.9 ^{abc}	112.8 ± 11.6 ^d	2.96 ± 0.14 ^{bc}	2.03 ± 0.08 ^a
10.5	0	734.0 ± 151.2 ^e	701.0 ± 154.4 ^e	291.7 ± 37.3 ^f	280.3 ± 33.2 ^f	3.19 ± 0.63 ^c	3.35 ± 0.58 ^d
	100	469.3 ± 55.8 ^d	433.7 ± 35.4 ^d	217.0 ± 13.1 ^e	202.7 ± 3.8 ^e	2.80 ± 0.24 ^{ab}	2.74 ± 0.28 ^c
	200	322.3 ± 21.2 ^c	277.7 ± 16.7 ^c	141.7 ± 4.9 ^d	135.0 ± 1.7 ^d	2.82 ± 0.26 ^{ab}	2.53 ± 0.12 ^{bc}
	300	226.7 ± 3.2 ^{ab}	210.7 ± 13.3 ^{bc}	97.4 ± 2.9 ^{bc}	93.8 ± 3.7 ^{ab}	2.74 ± 0.05 ^{ab}	2.67 ± 0.09 ^{bc}

^{a-f}Mean values in the same column with different superscript lowercase letters are significantly different ($P < 0.05$).

¹Means ± SD (n = 3).

²D_{4,3}: volume-weighted mean diameter.

³D_{3,2}: surface-weighted mean diameter.

for rSM and pSM for all the various levels of pressure tested using UHPH at pH 10.5.

Figure 3 presents TEM images of rSM and pSM at 0 and 300 MPa showing typical milk microstructure obtained at the different pH. At pH 6.7, although the presence of dense and compact micellar structures of different sizes was observed, no significant changes were detected after UHPH at 300 MPa for rSM and pSM. After alkalization at pH 8.5 and 10.5, the presence of larger CN structures was noted. However, these larger aggregated CN forms were disrupted when treated at 300 MPa, resulting in smaller CN particles.

The effects of alkaline pH and UHPH treatments on the particle size parameters of rSM and pSM are shown in Table 2. Statistical analysis revealed a significant effect of the interaction between pH and pressure ($P < 0.0001$) for the volume-weighted mean diameter (D_{4,3}; Supplemental Figure S1; <https://doi.org/10.17632/g6zj5dfrm3.2>; Brisson, 2022). While alkalization of the nonhomogenized samples induced a significant increase of the D_{4,3}, the effect of UHPH treatment differs from one pH to the other. A more significant reduction of the particle size was observed at pH 10.5 compared with pH 8.5 and 6.7. The D_{4,3} decreased very significantly ($P < 0.001$) for rSM and pSM while increasing the pressure from 0 to 300 MPa.

Statistical analysis revealed a highly significant interaction between pH and pressure ($P < 0.0001$) and a significant effect on the pasteurization treatment and pressure ($P = 0.0487$) of the surface-weighted mean diameter (D_{3,2}; Supplemental Figure S1). Whereas no effect of UHPH on the D_{4,3} was observed at pH 6.7 for both SM types, a significant reduction of the D_{3,2}

of the CN micelles was noted for the pSM treated at 300 MPa. In contrast, the UHPH treatment did not affect the D_{3,2} for rSM at pH 6.7. However, only the pSM samples were affected by the UHPH at pH 8.5. Furthermore, maximal D_{3,2} values were reached at pH 10.5 for control rSM and pSM. However, as the UHPH pressure increased, a significant decrease of D_{3,2} was noted for both SM types.

Effect of Treatments on Turbidity

The turbidity of protein solutions was used to assess their level of aggregation, particle density, and solubility. The turbidity of SM samples was measured as a function of pH and UHPH treatment, as shown in Figure 4. For the most part, the turbidity of rSM and pSM decreased drastically as the pH increased from 6.7 to pH 10.5. As the UHPH pressure was increased, the turbidity of rSM at pH 6.7 and 8.5 decreased slightly but remained constant at pH 10.5. Likewise, the turbidity of pSM at pH 6.7 also decreased as the UHPH pressure increased; however, at alkaline pH UHPH had no effect. A significant triple interaction was observed between pasteurization treatment, pH, and pressure ($P = 0.0130$) on the turbidity values. For example, the turbidity of nonhomogenized rSM (0 MPa) samples decreased as the pH increased. Over the same range of pH, lower turbidity values were recorded for pSM. Comparable results were observed at 100 MPa, except for rSM at pH 8.5, which showed significantly lower turbidity than at 0 MPa. At 200 and 300 MPa, the effect of pasteurization was only seen for pH 6.7 and 8.5. A similar result was noted at pH 8.5 and 200 MPa,

with a significant difference ($P = 0.0259$) between rSM and pSM. Finally, at 300 MPa, the turbidity decreased, but no significant differences were seen between pH 8.5 and 10.5.

Effect of pH and UHPH on Protein Distribution

To understand the effect of alkaline pH and UHPH on the protein distribution within the serum and col-

Figure 3. Microstructure images of raw skim milk (rSM; A) and pasteurized skim milk (pSM; B) for control and 300 MPa samples at different pH values observed by transmission electron microscopy at 4K.

Figure 4. Turbidity of raw skim milk (rSM; black bars) and pasteurized skim milk (pSM; white bars), at pH 6.7, 8.5, and 10.5 for control, 100, 200, and 300 MPa. Bars represent mean values of triplicates, and error bars indicate SD for each treatment. NTU = nephelometric turbidity unit.

loidal phase, the treated rSM and pSM were fractionated by ultracentrifugation, as described above, and we measured the protein content of each serum fraction (supernatant). Figure 5 shows SDS-PAGE analysis of the repartition of milk proteins in the control and pressure-treated rSM and pSM following ultracentrifugation in the serum fraction at different pH values. The SDS-PAGE analysis shows differences in the partition of the CN after ultracentrifugation. The distribution of CN in the supernatants varied according to the pH and UHPH treatments and the pasteurization. For rSM at pH 6.7 (Figure 5A), only small amounts of the 3 types of CN (α_s -CN, β -CN, and κ -CN) were recovered in the serum phase, independent of the UHPH pressure. In contrast, very faint CN bands were observed in the supernatant for the UHPH-treated pSM samples, compared with the nonhomogenized control, with β -CN and κ -CN almost undetectable for the UHPH samples. However, these slight modification in CN distribution at initial pH between the colloidal and serum phases were not significant based on protein content, as shown in Figure 6 (for both rSM and pSM at pH 6.7). In comparison, at pH 8.5 as the UHPH pressure was increased (Figure 5B and 5E), α_s -CN, β -CN, and κ -CN band intensity in the supernatant increased, which correlated with a significant increase in total protein concentration in rSM and pSM supernatants. Last, the pH adjustment of pSM resulted in a significant increase of protein content into the serum fractions (Figure 6),

especially at pH 10.5. However, no effect was observed on those samples after using UHPH treatment.

DISCUSSION

This study investigated the effect of alkalization and UHPH treatment of SM on the integrity and size of CN micelles. Both rSM and pSM were tested to see the influence of pasteurization treatment on the sensitivity of CN micelles to alkalization and UHPH treatments. The results show the alkaline pH and UHPH pressure have a significant influence on the structure of the CN micelles and their repartition between the serum and colloidal phases, which could have an extensive effect on their functionality during dairy processing.

Effect of pH and UHPH on PSD and Parameters

At the initial pH of SM (6.7), 2 populations were observed. These results agreed with the literature on SM PSD. Zamora et al. (2007) also reported a principal population at 0.2 μm , corresponding to CN micelles, and a minor one around 3.8 μm corresponding to remaining fat globules that could not be separated with the cream separator. In addition, because CN micelles are the most abundant colloidal particles in SM, any modifications of the particle size parameters can be associated with change in conformation and structure of the micelle (Fox and Brodtkorb, 2008; Chen et al.,

2019). At pH 6.7, we observed a very slight decrease in the volume-weighted mean diameter ($D_{4,3}$) after UHPH treatment, which agreed with the results obtained by Hayes and Kelly (2003) and Lodaite et al. (2009), who reported a CN micelle size reduction of about 5% in the UHPH range (>200 MPa). In contrast, a larger size reduction has been observed, up to 33%, for phosphocasein dispersion (Chevalier-Lucia et al., 2011). Hayes and Kelly (2003) observed a decrease in the particle size of pSM, from 183.5 nm (0 MPa) to 160.2 nm (300 MPa). This study obtained a comparable but insignificant decrease from 196.3 and 155.7 nm for pSM using similar UHPH pressure. This slight decrease in the particle size parameters may be due to a partial dissociation of the CN micelle surface, and the size reduction of the remaining milk fat globules under UHPH (Sandra and Dalgleish, 2005). The absence of the larger population observed for higher pressure (Figure 2A) has been attributed to the disruption of the milk fat globules into submicronic and more homogeneous fat globules (Zamora et al., 2012). It resulted in the sharpening of the principal peak associated with the CN micelles (Figure 2), as a function of pressure, that correlated with a decrease of span values which reflect higher homogeneity of the CN micelle size distribution (Tan and Nakajima, 2005). At initial pH (6.7), the majority (95%) of the CN is incorporated into micellar form sta-

bilized by CCP (Chen et al., 2019; Zouari et al., 2020). Various molecular forces are essential to maintain the structure and stability of CN micelles, which depend on a balance between electrostatic repulsions and hydrophobic interactions. In addition, CCP crosslinks the CN molecules and neutralizes the negatively charged phosphoserine groups, allowing hydrophobic interactions between CN (Broyard and Gaucheron, 2015). Thus, the slight decrease in particle size observed after UHPH could be related to the disruption of hydrophobic and ionic interactions throughout the high pressure, cavitation, turbulence, and shear effects (Bouaouina et al., 2006). Furthermore, it has been reported that in raw milk, κ -CN, and to a lesser extent α_s -CN, present on the surface of the native CN micelle are readily affected by UHPH and get partially solubilized, resulting in a decrease in the CN micelle size (Sandra and Dalgleish, 2005; Regnault et al., 2006). However, as observed by SDS-PAGE, we did not detect the solubilization of either κ -CN and α_s -CN after UHPH. Instead, whereas no dissolution was observed in rSM, a decrease in nonsedimentable CN recovered in the serum phase was noted for pSM after ultracentrifugation. The exact reasons for our discrepancies with those authors are not known.

In addition, an increase in span values was observed as the pH increased, particularly at pH 10.5. The results clearly showed that CN micelles are highly af-

Figure 5. SDS-PAGE profile of control and ultra-high-pressure homogenization (UHPH)-treated raw milk (A, B, and C) and pasteurized milk (D, E, and F) at pH 6.7 (A and D), 8.5 (B and E), and 10.5 (C and F) and their respective supernatant (S) recovered after ultracentrifugation at $70,000 \times g$ for 1 h at 20°C. MWC = molecular weight control (kDa). Lf = lactoferrin.

fects by the alkaline pH and show an increase in the heterogeneity of the CN particle size. Madadlou et al. (2009b) stated that alkalization at a pH up to 12.0 induces the individual caseins to gain negative charges, thus increasing the net global negative charge. They attribute this phenomenon to complete deprotonation of the carboxyl groups, combined with a loss of positive charge on the histidine and lysine residues and an increase of the negative charges on the phosphoserine residues. Their deprotonation converted them from single to double negatively charged units, leading to strong electrostatic repulsions (Post et al., 2012). These reactions may have contributed to the distancing of the individual CN polypeptide chains from one another, resulting in a looser and more expanded CN micelle structure. Nevertheless, the attractive electrostatic forces and hydrophobic interactions within the CN micelles could still be sufficient to maintain the micellar cohesion and as such play an essential role in their self-assembly at higher pH. According to Liu and Guo (2008), the hydroxyl ions, introduced by NaOH addition, increase the electrical conductivity and thus the number of hydrogen bonds between serum and colloidal phases, likely causing breakage of some hydrogen and hydrophobic bonds between the CN polypeptidic chains and eventually weakening the micelle structure (Liu and Guo, 2008). Moreover, hydrophobic interactions among the CN have been shown to have little dependence on pH, suggesting that electrostatic inter-

actions play an essential role in the pH-dependent behavior of CN micelles (Anema, 1998). Contrary to our observations, Vaia et al. (2006) and Sinaga et al. (2017) found that CN micelles were disrupted at alkaline pH between pH 8.0 to 11.0. These authors suggested that the CN micelle disintegration was due to reduced cohesive interactions between the hydrophobic areas on the different CN. They also proposed that decreased ionic calcium and phosphate concentrations increase the serum phase's solvent quality, which maintains the cohesive interactions. Furthermore, the presence of attractive and repulsive interactions between the proteins may lead to CN dissociation (Vaia et al., 2006; Sinaga et al., 2017). However, Ahmad et al. (2009) showed that these solubilized dissociated CN molecules reorganized into soluble aggregates of increasing sizes, which agrees with our laser diffraction observations.

Although more studies are still needed to decipher the exact mechanisms that CN micelles undergo during alkalization, our TEM images clearly showed the presence of numerous micron size particles at pH 10.5, which provides further evidence of the formation of larger self-associating CN forms at alkaline pH.

Our results also showed a significant interaction between pH and UHPH pressure on the reduction of CN micelle size. The CN micelle structure is thought to be weakened at higher pH values, which is likely only supported by hydrophobic interactions. In this loosened state, the casein micelles are likely more prone to

Figure 6. Protein content (mg/mL) in milk supernatants as a function of ultra-high-pressure homogenization (UHPH), pH, and pasteurization treatment. Bars represent mean values of triplicates, and error bars indicate SD for each treatment. rSM = raw skim milk; pSM = pasteurized skim milk. Bars with lowercase letters (a–e) are significantly different ($P < 0.05$) within the same pasteurization treatment. Bars with uppercase letters (A, B) are significantly different ($P < 0.05$) within the same pH $\times$ pressure treatments.

disruption by UHPH. Similar to our study, Madadlou et al. (2009a) also reported that sonication induced a global particle size reduction of loosened and fragilized CN micelles at alkaline pH, through cavitating forces, which could act similarly to the cavitating forces generated by UHPH.

Effect of pH and UHPH on Casein Micelle Structure and Protein Distribution Between the Serum and Colloidal Phase

Turbidity measurements and gel electrophoresis profiles to assess the effect of pH and UHPH on protein structure and distribution were assessed after the partition of SM serum and colloidal micellar phases through ultracentrifugation. As previously mentioned, turbidity depends on the particle's size, concentration, and light-scattering properties. In SM, CN micelles are the primary light-scattering particles, and changes in their turbidity will reflect mainly structural modifications to the micelles (Madadlou et al., 2009b). For the most part, our results showed a significant decrease in the turbidity of rSM and pSM as pH increased from 6.7 to pH 10.5. Changes in turbidity with UHPH treatment depended on the pasteurization of SM and pH. For example, UHPH treatment reduced the turbidity for rSM at pH 6.7 and 8.5 and for pSM at pH 6.7 only. At pH 6.7, the significant decrease of rSM turbidity at 200 and 300 MPa may be due to partial disintegration of the CN micelle surface and possible collapse of the protruding κ -CN glycosylated part on the surface of the CN micelles. At that pH, the CN micelles are at their most compact structure, leading to the highest scattering factor of particle, hence higher turbidity. On the other hand, at higher pH values, the self-aggregating loose and expanded CN structures probably contain more voids than their micellar more compact arrangement, which may reduce the light beam scattering and the associated measured turbidity. Moreover, the CN size increase is concomitant with a smaller specific surface area available for light scattering, leading to a decrease in the observed turbidity of CN solutions with increasing pH in the alkaline range (Liu and Guo, 2008; Madadlou et al., 2009b).

The analysis of the distribution of proteins within the serum and colloidal fractions of SM was carried out by SDS-PAGE. The results showed only some slight effect of UHPH and pH treatments on rSM and pSM samples. As expected, for both control SM (pH 6.7, 0 MPa), ultracentrifugation induced the sedimentation of CN micelles within the pellet (colloidal fraction), whereas the supernatant (serum fraction) was mainly composed of the WP such as β -LG (15 kDa) and α -LA (14 kDa; Ahmad et al., 2009; Anema et al., 2014). However, gel

electrophoresis showed a slight loss of β -LG and α -LA for pSM into the colloidal fraction regardless of the pressure applied. β -LG, and to a lesser extent α -LA, are well known to undergo thermal denaturation and aggregation in the serum phase before associating with κ -CN at the surface of the micelles via intermolecular disulfide interactions during thermal treatment (Corredig and Dalgleish, 1996, 1999; Anema and Li, 2003). As β -LG and α -LA sedimented with the CN micelles in the pellet, a lesser number of proteins were observed in the supernatant for pSM compared with rSM. However, as reported by Rynne et al. (2004), the extent of WP denaturation is relatively low (less than 3%) for HTST pasteurized milk at 72°C, and this would explain the relatively low WP loss in the CN micelles for the pSM, which was insignificant in terms of total protein loss. On the other hand, following alkalization of both SM, SDS-PAGE analysis showed a partial recovery of nonsedimentable CN species in the supernatant at pH 8.5, whereas a complete recovery was observed at pH 10.5. The results were expected and in concordance with the observations of Ahmad et al. (2009). These authors suggest that the dissociation of the CN micelles in the alkaline pH range led to their reorganization into highly hydrated CN particles of various sizes, which can be seen in the increase in average size ($D_{4,3}$ and $D_{3,2}$) and span values, but also in our TEM images (Figure 3; Ahmad et al., 2009). Moreover, as the CN were no longer sedimentable after ultracentrifugation, the loss of WP observed at pH 6.7 for pSM was reverted entirely. All the WP were recuperated in the serum, confirming their association with the CN during pasteurization as mentioned above. However, we observed for the first time that the CN distribution between the serum and colloidal fraction was dependent on both pH and UHPH as the protein content in the serum phase increased sharply (Figure 6) with the UHPH pressure, suggesting a strong synergistic effect. Adjusting the pH of SM at alkaline pH induces a significant increase in the protein content in the serum phase, attributable to the solubilization of all the CN species as observed by SDS-PAGE.

Overall, the use of UHPH on SM adjusted at alkaline pH affects the protein concentration differently in the serum phases, attributed principally to nonsedimentable CN (Zouari et al., 2020). Intense CN bands were observed in the SDS-PAGE supernatant of SM control samples at higher pH values, especially at pH 10.5. It has been proposed that CN micelles progressively start swelling at alkaline pH, inducing their dissociation followed by their reorganization into smaller highly hydrated particles and aggregates (Ahmad et al., 2009). The lower density of these more hydrated CN molecular forms prevents them from sedimenting in the pellet

after ultracentrifugation (Ahmad et al., 2009). The lack of effect observed for UHPH-treated samples at pH 10.5 showed that such drastic alkalization itself is enough to disperse all CN into the serum phase. However, we showed that complete disruption of the CN micelle could be obtained at lower pH (8.5) when combined with UHPH treatment (200 and 300 MPa).

CONCLUSIONS

This study investigated the effect of alkalization and UHPH treatment on CN micelle structural and organizational state in rSM and pSM. The important disruption of CN micelles in SM by a combination of pH and UHPH is seen here for the first time. Our results showed that alkalization induces higher CN particle size throughout micelle swelling and their rearrangement into different increasing size forms, whereas UHPH treatment helps dissociate these fragile loose CN structures. As a result, complete redispersion of the CN micelles could be achieved at lower alkaline pH by using a UHPH treatment. Overall, this study showed the strong potential of using alkalization and UHPH to change the CN micelle structure synergistically, and modify their techno-functional properties and behavior when incorporated in dairy products.

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