



pH-dependent sedimentation and protein interactions in ultra-high-temperature-treated sheep skim milk

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ABSTRACT

Sheep milk is considered unstable to UHT processing, but the instability mechanism has not been investigated. This study assessed the effect of UHT treatment (140°C/5 s) and milk pH values from 6.6 to 7.0 on the physical properties of sheep skim milk (SSM), including heat coagulation time, particle size, sedimentation, ionic calcium level, and changes in protein composition. Significant amounts of sediment were found in UHT-treated SSM at the natural pH (~6.6) and pH 7.0, whereas lower amounts of sediment were observed at pH values of 6.7 to 6.9. The proteins in the sediment were mainly κ -casein (CN)-depleted casein micelles with low levels of whey proteins regardless of the pH. Both the pH and the ionic calcium level of the SSM at all pH values decreased after UHT treatment. The dissociation levels of κ -, β -, and α_{S2} -CN increased with increasing pH of the SSM before and after heating. The protein content, ionic calcium level, and dissociation level of κ -CN were higher in the SSM than values reported previously in cow skim milk. These differences may contribute to the high amounts of sediment in the UHT-treated SSM at natural pH (~6.6). Significantly higher levels of κ -, β -, and α_{S2} -CN were detected in the serum phase after heating the SSM at pH 7.0, suggesting that less κ -CN was attached to the casein micelles and that more internal structures of the casein micelles may have been exposed during heating. This could, in turn, have destabilized the casein micelles, resulting in the formation of protein aggregates and high amounts of sediment after UHT treatment of the SSM at pH 7.0.

Key words: particle size, casein micelle, whey protein, ionic calcium, protein composition

INTRODUCTION

Sheep milk is promoted as a good alternative to cow milk for humans because of its high nutritional content, including higher concentrations of proteins, fats, vitamins, and minerals, compared with cow milk (Park et al., 2017). Unlike the commonly seen cheeses and yogurt products made from sheep milk, sheep milk products in liquid form are not widely available in most markets and exist only in some small farms or rural areas such as mid-east Asian and Mediterranean basins (Tamang and Kailasapathy, 2010; Kapaj and Deci, 2017).

A combination of heating temperature and heating time is frequently used to treat milk to produce a product with a significantly extended shelf life. For sheep milk, it is difficult to produce liquid products with a long shelf life using commercial thermal treatments (such as UHT treatment) because of its low heat stability (Raynal and Remeuf, 1998). For instance, sheep milk coagulates under UHT processing conditions, leading to a high amount of sediment (mainly protein aggregates) during storage (Martinez Alonso et al., 2009). The instability of the milk can lead to sedimentation after UHT treatment, with aggregated material settling at the bottom of the container during storage. In general, the sediment consists of milk proteins, specifically caseins. Gaur et al. (2018) reported that all sediments were composed mostly of κ -CN-depleted casein micelles (~85%) with low levels of β -LG and α -LA compared with those in the bulk milk. Similar observations were also reported by Malmgren et al. (2017), who showed a predominant content of β - and α_S -CN but no whey proteins or κ -CN in the sediment after UHT processing of milk. The sedimentation has been attributed to the dissociation of κ -CN from the casein micelles during UHT processing, resulting in the aggregation of κ -CN-depleted micelles. However, as κ -CN-depleted casein micelles do not always aggregate to form a sediment, other factors must induce the aggregation (Gaur et al., 2018). Higher pH values of the milk could result in a lower level of ionic calcium and a higher charge on the casein micelles, which could prevent the aggregation of

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casein micelles that are depleted in κ -CN. In contrast, the decreased charge on the casein micelles and the higher levels of ionic calcium at low pH could induce aggregation of the casein micelles (Dumpler et al., 2020). In fact, no sheep milk products that have been subjected to UHT treatment without stabilizers can be preserved at room temperature for as long as UHT-treated cow milk with satisfactory organoleptic quality. Almost all UHT-treated cow milks can be stored for at least 6 mo without the addition of stabilizers, although they may form a layer of sediment during storage, which is usually thin and does not affect the quality of the milk (Gaur et al., 2018).

Several factors, including pH, ionic calcium level, protein concentration, and protein composition, are thought to affect the heat stability of milk during UHT processing. The negative effects of increased ionic calcium on the heat stability of milk have been widely reported (Crowley et al., 2014; Chen et al., 2015; Deeth, 2020; Dumpler et al., 2020). As the milk proteins usually aggregate via calcium bridging (Deeth, 2020), high ionic calcium is considered to be a primary factor in the low heat stability of milk. The pH of milk also plays an important role in stabilizing it during heating. Lewis et al. (2011) investigated the mechanism for sedimentation in UHT-treated cow milk using a centrifugation method and showed that the amount of sediment increased when the pH was lowered, regardless of an increased or constant level of ionic calcium. This clearly showed that there is a strong relationship between the amount of sediment and the ionic calcium level, and between the amount of sediment and the pH in UHT milk. Additionally, the dissociation of κ -CN from the casein micelles that occurs as a consequence of UHT treatment also contributes to the instability of milk (Anema, 2017). Extensive studies have shown that dissociation of κ -CN from the casein micelles in milk occurs during heating (McMahon, 1996; Hillbrick et al., 1999; Malmgren et al., 2017). Anema (2017) investigated the age gelation of reconstituted UHT-treated cow skim milk in the absence of proteolysis by plasmin and found that the gelled materials consisted mainly of κ -CN-depleted casein micelles with very low levels of associated whey proteins; κ -CN dissociated from the micelles to a significant extent during UHT processing but changed little on further storage. Therefore, it was suggested that sediment formation was initiated by the κ -CN-depleted casein micelles via calcium bridging (Anema, 2017). Furthermore, the heat stability of milk is known to be dependent on the protein concentration. Milks with higher protein content have lower heat stability than those with lower protein content (Crowley et al., 2015; Anema, 2017).

It is well known that sheep milk has higher concentrations of proteins and minerals than cow milk and that their protein compositions differ (Balthazar et al., 2017). A recent study showed that a marked increase in casein micelle size and aggregation of the casein micelles were observed in sheep skim milk (SSM) heated at 85 to 90°C (Pan et al., 2022). However, these changes occurred to a significantly lower extent in cow milk than in sheep milk heated in the same temperature range (80–90°C) in the study of Raynal and Remeuf (1998), which might be attributed to the higher protein content, different casein composition, or both, in sheep milk. Therefore, sheep milk can be expected to react differently under severe heat treatment. Only a few studies indicating the mechanism behind the high sedimentation in UHT-treated sheep milk have been published. Fox and Hoynes (1976) reported that sheep milk showed lower heat stability (measured at 140°C using oil bath methods) than cow milk, which could be attributed to a lower level of κ -CN in sheep milk than in cow milk. However, the protein interactions in sheep milk during heating in a UHT plant were not illustrated. Therefore, the objective of the present study was to investigate the sedimentation and protein interactions in UHT-treated SSM at different pH values.

MATERIALS AND METHODS

Institutional animal care and use committee approval was not required for this study because only routine animal procedures (milking) were conducted.

Materials and pH Adjustment

Fresh sheep milk was purchased from Fernglen Ltd. (Masterton, New Zealand). A small amount of sodium azide (0.01%) was added to the raw milk as a preservative. The raw sheep milk samples were skimmed using a milk skimmer. The composition of the SSM was analyzed using a MilkoScan FT1 (FOSS) and is shown in Table 1. The pH of the SSM was adjusted to values between 6.2 and 7.2 by slowly adding 2 M HCl (food grade) or 2 M NaOH (food grade) to well-stirred SSM.

Table 1. Composition of sheep skim milk (SSM); mean \pm SD

Component	g/100 mL of SSM
Fat	0.27 \pm 0.02
Lactose	5.04 \pm 0.09
Protein	5.94 \pm 0.01
TS	12.27 \pm 0.12

The milk samples were kept at ambient temperature for 2 h before the final pH reading and minor readjustment. The natural pH of the SSM was $\sim 6.62 \pm 0.02$.

UHT Treatment

Skimmed sheep milk at natural pH (~ 6.6) and pH values from 6.7 to 7.0 were heated at 140°C for 5 s in an indirect UHT plant (pilot plant, Massey University, Palmerston North, New Zealand). After heat treatment, these milk samples were immediately cooled to room temperature and packaged in aseptic milk bottles (capacity 2,000 mL). The UHT-treated SSM was kept at room temperature for 6 h before further analysis.

For the justification of pH selection for UHT treatment, the heat coagulation time (HCT) of the SSM was determined at different pH values before UHT treatment to ensure that SSM in a certain pH range could withstand UHT treatment without causing severe fouling in the UHT plant. The HCT of the SSM as a function of pH (6.2–7.2) was examined at 140°C , as described by Davies and White (1966). Preliminary results showed that the HCT was highest at pH 6.8 and decreased when the pH value was lower or higher than pH 6.8 (data not shown). The HCT of the SSM at pH 6.2 and 6.4 were lower than 120 s, whereas the SSM at other pH values had HCT values >200 s. In our previous experiments, the required heat-up time for SSM to reach 140°C was ~ 120 s, which meant that the SSM at pH 6.2 and 6.4 coagulated before reaching 140°C . The SSM at natural pH (~ 6.6) and pH values from 6.7 to 7.2 had longer HCT than the heat-up time. Therefore, SSM at natural pH (~ 6.6) and pH 6.7 to 7.0 were selected for further investigation on the UHT plant.

pH and Ionic Calcium Level

Unheated and heated SSM samples at different pH values were prewarmed in a water bath at 20°C for 1 h to equilibrate the temperature. The pH was then measured using a pH 700 Benchtop Meter (Oakton Instruments). The ionic calcium level in these milk samples was determined using an Orion calcium-selective electrode (9720BNWP; Thermo Fisher Scientific Inc.) coupled with the pH 700 pH Benchtop Meter. Calibration was conducted using standard (0.5–5 mM) CaCl_2 in 80 mM KCl solution. The millivolt value of all milk samples was measured and recorded by dipping the electrode into the milk sample. The recorded millivolt value was converted to the ionic calcium level using a calibration curve obtained from the standard CaCl_2 -KCl solution.

Size Distribution of SSM

The particle size distributions of the SSM before and after UHT treatment were determined using a MasterSizer 2000S (Malvern Instruments). The refractive index of the dispersant (reverse osmosis water) was set to 1.33 and the refractive index of the skim milk was set to 1.50. The particle absorption index was set to 0.001. The SSM was shaken well before measurement. The milk sample was added to the dispersion unit until an obscuration between 10 and 20% had been reached. Each sample was measured in triplicate at 20°C . The results are shown as the average value of 3 measurements.

Separation of Milk Protein Fractions

Unheated and heated SSM were centrifuged to obtain different fractions of soluble protein. The large aggregates formed during heating were centrifuged at $3,000 \times g$ for 10 min at 20°C , and the weight of wet sediment obtained after centrifugation was measured and analyzed by reversed-phase HPLC. To determine the amount of sediment, the liquid phase of centrifuged milk was decanted into a beaker, and the centrifuge tube containing sediments was placed on a paper towel for 1 min to drain off the excess liquid. The container, including any sediment, was weighed and recorded.

Protein particles <100 nm were obtained by ultracentrifuging the milk samples at $48,800 \times g$ for 26 min at 20°C using a Sorval WX 80+ Ultracentrifuge (Thermo Fisher Scientific Inc.). This centrifugation condition has been proven to efficiently remove colloidal stable casein micelles while retaining soluble proteins (<20 nm) and submicellar particles (20–100 nm, mainly consisting of κ -CN/whey protein complexes with comparably small amounts of calcium-sensitive caseins) in the supernatant (Dumpler et al., 2017). After ultracentrifugation, the resultant supernatants, defined as the serum phase in this study, were analyzed by reversed-phase HPLC.

Protein Composition Analysis

Milk and the supernatants obtained from centrifuged milk samples were analyzed by reversed-phase HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 μm XB-C18 RP, Phenomenex) to determine the protein composition, as described by Bobe et al. (1998). The quantities of whey proteins and caseins in the supernatants were determined by comparing the relative peak area of the supernatant fraction in the heated SSM with that in the original unheated SSM. All peak areas of these chromatograms were obtained using peak integration algorithm LabSolutions software (Shimadzu Corp.).

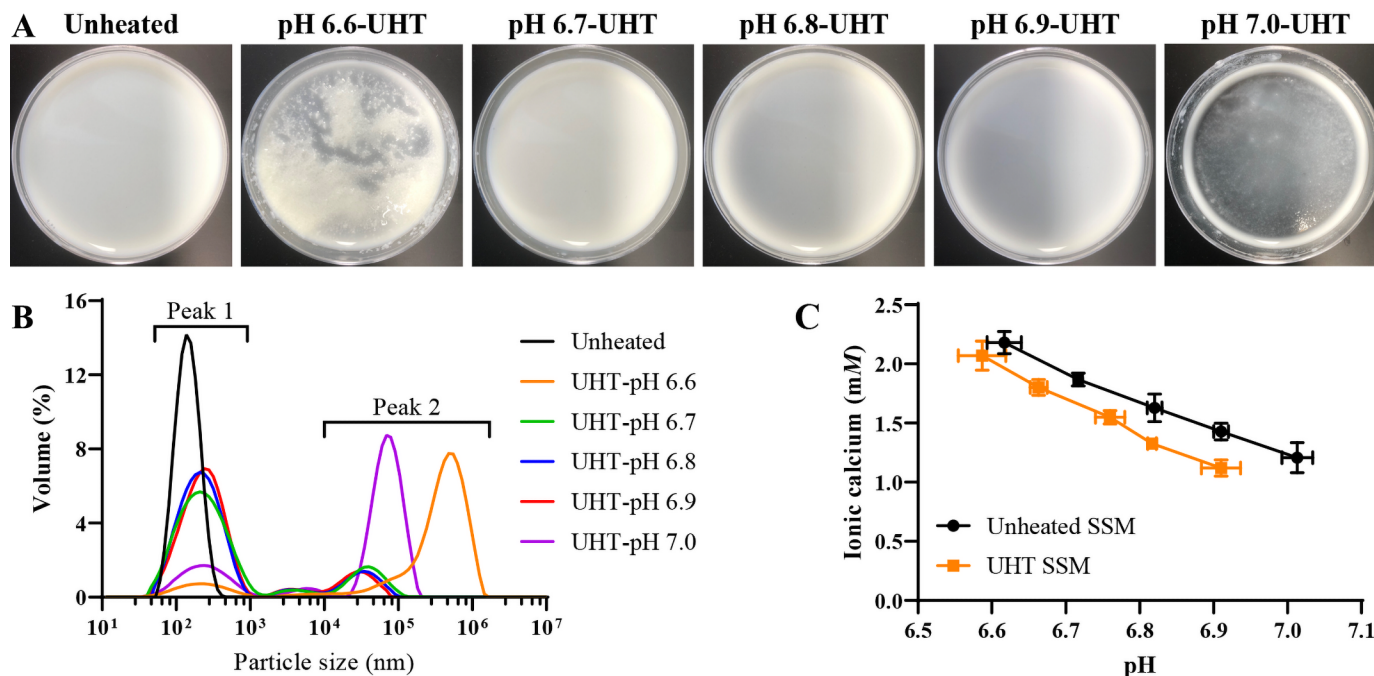


Figure 1. (A) Appearance of unheated sheep skin milk (SSM) at natural pH (~6.6) and UHT-treated SSM at different pH values; (B) particle size distributions of unheated SSM and UHT-treated SSM at different pH values; (C) ionic calcium concentration as a function of the pH profile in SSM before and after UHT treatment. Error bars represent standard deviations.

Statistical Analysis

All experiments reported were repeated 3 times using freshly collected sheep milk samples, and the results are given as the mean \pm standard deviation. GraphPad Prism 8.4.0 was used to plot the data (GraphPad Software). At a significance level of $P < 0.05$, 1-way and 2-way ANOVA, as well as Tukey's multiple comparison test, were used in the statistical analysis.

RESULTS AND DISCUSSION

Stability of UHT-Treated SSM

Heat-Induced Protein Aggregation in SSM.

Figure 1A shows the macrostructures of the SSM at different pH values before and after UHT treatment. Large amounts of coagulated material were found immediately in the SSM at the natural pH (~6.6) after the UHT treatment. However, there was no visible coagulation in the UHT-treated SSM at pH 6.7, 6.8, or 6.9. The UHT-treated SSM at pH 7.0 had visible coagulation but those coagulates appeared much smaller than that formed in the UHT-treated SSM at the natural pH (~6.6).

Particle Size Distribution of SSM. Figure 1B shows the particle size distributions of SSM samples at different pH values before and after UHT treatment.

For unheated SSM, only one peak in the particle size range from 0.02 to 0.30 μm was observed. Two major peaks were observed in UHT-treated SSM: the left-hand peak (peak 1) ranged from 0.02 to 1 μm and the right-hand peak (peak 2) ranged from ~10 to 2,000 μm . After UHT treatment, peak 2 of the SSM at the natural pH (~6.6) appeared at the rightmost position and peak 1 was significantly smaller, indicating that the proportion of small protein particles was reduced and the protein had formed large aggregates (ranging from ~10 to 1,445 μm), which is in agreement with the changes in appearance observed in Figure 1A. The UHT-treated SSM at pH 7.0 showed a similar pattern to the UHT-treated SSM at natural pH (~6.6) but with less peak-shifting (the large aggregate particle size ranged from ~20 to 180 μm). The UHT-treated SSM samples at pH 6.7 to 6.9 had similar particle size distributions after UHT treatment, showing that peak 1 was increased and that peak 2 (large aggregates ranging from ~10 to 100 μm) was reduced compared with the UHT-treated SSM samples at the natural pH (~6.6) and pH 7.0. The results of the particle size distribution are in agreement with the visual appearances presented in Figure 1A.

Ionic Calcium Level-pH Profile in SSM. The ionic calcium level-pH profiles of the SSM before and after UHT treatment are shown in Figure 1C. The highest ionic calcium level (~2.2 mM) of the SSM was

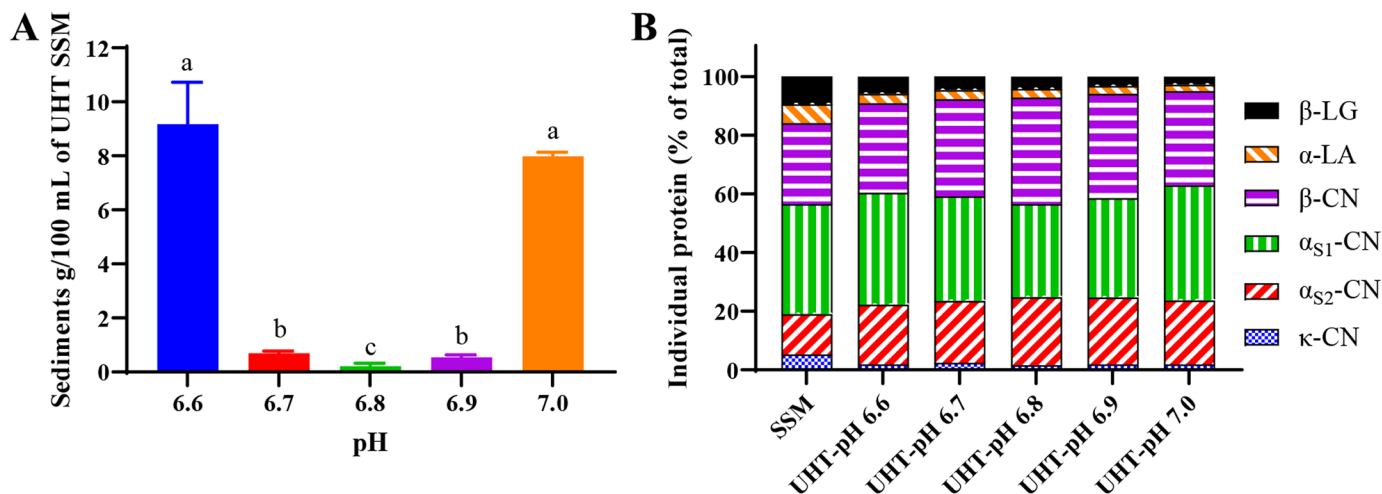


Figure 2. (A) Weights of sediments obtained from UHT-treated sheep skim milk (SSM) after centrifugation at $3,000 \times g$ and 20°C for 10 min; and (B) percentages of β -LG, α -LA, β -CN, α_{S1} -CN, α_{S2} -CN, and κ -CN in SSM and sediments of UHT-treated SSM at natural pH (~ 6.6) and pH 6.7–7.0. Different lowercase letters (a–c) above the columns in panel A indicate significant differences ($P < 0.05$). Error bars represent standard deviations.

found at the natural pH (~ 6.6). After adjustment of the pH, the ionic calcium level decreased with increasing pH, with levels of ~ 1.8 , 1.6 , 1.4 , and 1.2 mM for pH 6.7, 6.8, 6.9, and 7.0, respectively. There was a negative linear relationship between ionic calcium level and pH for SSM, as found for cow milk (Lewis et al., 2011; Gaur et al., 2018). Previous studies have shown that ionic calcium can be complexed with inorganic or organic phosphate with increasing pH because of reduced solubility of calcium phosphate, thereby decreasing the ionic calcium level in cow milk (Vaia et al., 2006; Ho et al., 2018). The complexation of ionic calcium with phosphate could be accompanied by the release of casein-bound calcium from micelles because of the re-equilibrium between soluble and colloidal calcium and the increased negative charge of proteins, consequently weakening the internal micellar structure and affecting the stability of casein micelles during heating (Horne, 2016).

After UHT treatment of the SSM at pH ranging between 6.6 and 7.0, the ionic calcium levels decreased by ~ 4 to 7% (Figure 1C). This was similar to previous studies on cow milk, which showed that the UHT treatment of cow milk decreased the ionic calcium level by 5% (Chen et al., 2015). However, the pH of the SSM decreased by around 0.03 to 0.1 units in the pH range 6.6 to 7.0 after UHT treatment, which differed from reports on cow milk noting that UHT treatment has little effect on its pH (Chen et al., 2015; Deeth and Lewis, 2016). There is little information on the reduction in the pH of UHT milk. Pyne and McHenry (1955) and Van Boekel et al. (1989) presumed that heat-induced acidity of the

milk in the temperature range from 100 to 130°C was due to the thermal decomposition of lactose, displacement of the calcium phosphate equilibrium, and the liberation of phosphate from the casein micelles with subsequent precipitation of the released phosphate as tertiary calcium phosphate. As the lactose contents of SSM (Table 1) and cow milk (Balthazar et al., 2017) are similar, the breakdown of lactose should not be the main reason for the pH decrease in UHT-treated SSM. Belec and Jenness (1962) investigated the dephosphorization of caseins in skim milk heated at 140°C and showed that a higher protein content increased the rate of dephosphorization. This indicates that the higher protein content of SSM might produce more phosphate and thus result in more precipitation of tertiary calcium phosphate during UHT treatment, leading to a decrease in its pH.

Characterization of Sediments

Weight of Sediments of UHT-Treated SSM. The weights of the sediments obtained from centrifuging the SSM at $3,000 \times g$ for 10 min are shown in Figure 2A. The UHT-treated SSM at natural pH (~ 6.6) had the greatest sediment weight, and there was a significant decrease ($P < 0.0001$) in sediment weights as the pH increased to pH 6.8. However, upon further increase in the pH of the SSM, the sediment weights increased significantly ($P < 0.0001$). There was a remarkable increase in sediment weight for the SSM at pH 7.0. The results suggested that SSM at natural pH (~ 6.6) and pH 7.0 formed a large amount of aggregates dur-

ing UHT treatment, and that the amount of aggregate could be reduced by adjusting the pH of the SSM to a range from pH 6.7 to 6.9.

Previous studies on the UHT treatment of cow milk demonstrated that sedimentation increased when the pH was lowered, the ionic calcium level was increased, or both (Lewis et al., 2011; Gaur et al., 2018). The results of this study showed a similar pattern when the pH of the SSM was increased from the natural pH (~6.6) to pH 6.7, which decreased the ionic calcium level from ~2.2 to 1.8 mM (Figure 1C), leading to a marked decrease in sedimentation (Figure 2A). Coagulation of proteins has been reported to occur during UHT processing when the pH of cow milk is lower than ~6.5, and this is believed to be due to high ionic calcium levels in the milk (Belicieu et al., 2012; Deeth and Lewis, 2015). The present study showed that the ionic calcium level in the SSM at the natural pH (~6.6) was higher than 2.0 mM and could cause instability of the caseins in the UHT milk, which is in agreement with previous results on cow milk (Lewis et al., 2011). However, a further increase in the pH from 6.9 to 7.0 significantly ($P < 0.01$) decreased the ionic calcium level to ~1.2 mM (Figure 1C) and markedly increased the sedimentation (Figure 2A). This is different from the previous findings for cow milk, which showed no increase in sedimentation when the pH was further increased or the ionic calcium level was decreased (Lewis et al., 2011; Gaur et al., 2018). It is unclear why sheep milk produced significant amounts of sediment at a more basic pH. A possible explanation is that the protein content (5.9%, Table 1) and the ionic calcium level (1.2 mM) at pH 7.0 of the SSM were higher than those (3.3% for protein content, <0.7 mM for ionic calcium level at pH ~7.0) of cow milk, as reported previously (Gaur et al., 2018). The higher protein content in the SSM could increase the probability of the proteins coming in contact with each other, and the ionic calcium level of the SSM at pH 7 might still be high enough for the formation of large protein aggregates during heating (Singh, 2004). An alternative explanation is that the casein composition of sheep milk is different from that of cow milk, which may affect protein interactions during UHT treatment (Amigo et al., 2000; Li et al., 2022). The protein interactions are further discussed in the following sections.

These results suggested a boundary between UHT-treated SSM samples that produced high amounts of sediment and those that produced very low amounts of sediment. A small alteration in pH at the boundary region could convert SSM from those that generate low amounts of sediment to those that generate high amounts of sediment or vice versa. This is generally in agreement with previous reports on cow milk, which showed the presence of a sharp boundary between sta-

bility (pH >6.55 or ionic calcium level <2.0 mM) and instability (pH <6.55 or ionic calcium level >2.0 mM) toward sedimentation (Lewis et al., 2011). It is possible that the attractive force and electrostatic repulsion of SSM casein micelles at pH 6.7 to 6.9 reaches a critical level that prevents the aggregation of casein micelles as the pH adjustment, heat-induced deposition of calcium phosphate onto the micelles, and association of whey proteins with casein micelles can alter the surface charge or provide steric repulsion for micelles. When the attractive and repulsive forces exceed the critical level (possibly at pH <6.7 or >6.9), protein aggregation would occur (Dumpler et al., 2020).

Protein Composition of Sediments. The protein composition of sediments obtained from UHT-treated SSM is shown in Figure 2B. The protein composition of the SSM was ~78.8% α_S -CN (α_{S1} - + α_{S2} -CN) and β -CN, ~5.2% κ -CN, and ~16.0% whey proteins (α -LA + β -LG). After UHT treatment, the sediment of the SSM at all pH values showed similar protein composition, with higher concentrations of α_S -CN and β -CN (89.0–93.1% combined) and lower concentrations of α -LA, β -LG (5.0–9.1% combined), and κ -CN (1.6–2.5% κ -CN) compared with the unheated SSM. These results suggested that the sediments of the UHT-treated SSM were composed mainly of α_S -CN and β -CN with low levels of κ -CN and whey proteins, regardless of pH value, although significant differences in the amounts of sediment were observed between samples with different pHs (Figure 2A). The protein composition of sediment is in line with previous reports for cow milk, which showed that the sediment formed from UHT-treated cow milk was composed of κ -CN-depleted casein micelles with low concentrations of denatured whey proteins (Malmgren et al., 2017; Gaur et al., 2018). κ -Casein at the surface of casein micelles is thought to limit their self-association, contributing to their remarkable stability (De Kruif et al., 2012). Therefore, casein micelles depleted in κ -CN could be less stable when the SSM pH is lower or the ionic calcium level is higher than a critical level, resulting in casein micelle aggregation and sedimentation (Anema, 2019).

Characterization of Serum Proteins: Protein Content of SSM Serum Phase

Figure 3 shows the protein contents of the serum phases obtained by centrifuging the SSM samples at $48,000 \times g$ for 26 min before and after UHT treatment. The protein contents of the serum samples of unheated SSM did not show significant differences ($P > 0.05$) with different pH values. In contrast, the serum-phase protein content of the UHT-treated SSM increased with increasing pH, and the UHT-treated SSM at pH 6.9

and pH 7.0 had significantly ($P < 0.05$) higher serum protein content than the UHT-treated SSM at natural pH (~6.6). This is in agreement with previous results for cow milk, which showed that the concentration of protein in the serum phase increased as the pH of cow skim milk was increased after heat treatment in the pH range of 6.3 to 7.1 (Anema and Klostermeyer, 1997). Therefore, the increased serum-phase protein content in the UHT-treated SSM might be attributed to the increasing dissociation of caseins from the casein micelles during heating (Anema and Klostermeyer, 1997).

The serum-phase protein contents of the SSM at the natural pH (~6.6) and pH 6.7 to 6.8 decreased significantly ($P < 0.05$) after UHT treatment, whereas those of the UHT-treated SSM at pH 6.9 to 7.0 were not significantly different ($P > 0.05$) from those of the unheated SSM (Figure 3). The decreased protein contents of the UHT-treated SSM serums could be attributed to the cosedimentation of denatured whey proteins with casein micelles on ultracentrifugation (Anema, 2020). In contrast, an increasing proportion of denatured whey proteins would remain soluble at higher pH values. More κ -CN would dissociate from the casein micelles during heating milk at higher pHs and still complex with the denatured whey proteins, and these denatured whey proteins complexed with κ -CN would remain soluble in the serum phase (Anema and Klostermeyer, 1997) and thus increase the protein content of the SSM serum after UHT treatment. This probably narrows the differences in the protein contents of the serums between unheated and UHT-treated SSM at pH 6.9 and 7.0. The detailed changes in the individual proteins of the serum are discussed in the following sections.

Dissociation of Caseins from Casein Micelles

The changes in individual proteins of the supernatants obtained from the centrifugation of the SSM at $48,800 \times g$ for 26 min were quantitatively analyzed before and after UHT treatment and are shown in Figure 4. For both unheated and UHT-treated SSM, increasing the pH of the SSM significantly increased the levels of serum-phase κ -, β -, and α_{S2} -CN. This indicated that increasing the pH of the SSM resulted in the dissociation of κ -, β -, and α_{S2} -CN from the casein micelles into the serum phase in all SSM samples.

The percentages of serum-phase κ -CN, β -CN, and α_{S2} -CN were significantly higher in UHT-treated SSM than in untreated SSM at all pH values. However, the percentage of α_{S1} -CN in the serum phase did not show significant differences between unheated and UHT-treated SSM regardless of pH values (Figure 4C). These results indicated that UHT treatment of SSM resulted in the dissociation of κ -, β -, and α_{S2} -CN from the casein

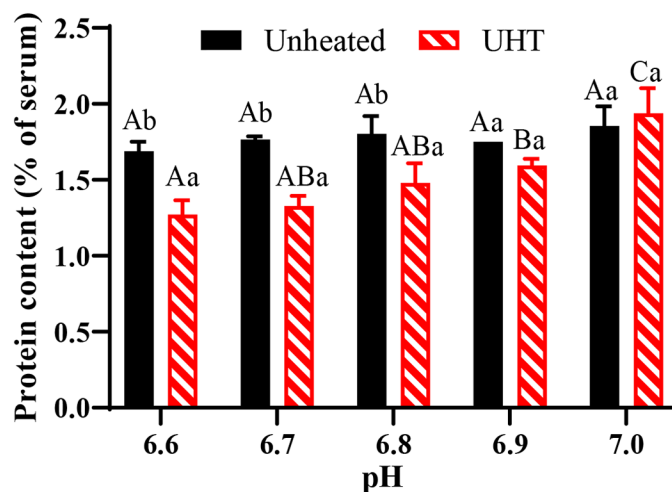


Figure 3. Protein contents of the supernatant in unheated (solid black bars) and UHT-treated (red striped) sheep skim milks (SSM) obtained from centrifugation at $48,800 \times g$ and 20°C for 26 min. Different uppercase (A–C) and lowercase (a, b) letters represent significant differences ($P < 0.05$) among different pH values and between unheated and UHT-treated SSM, respectively. Error bars represent standard deviations.

micelles into the serum phase at all pHs but had little effect on the dissociation of α_{S1} -CN.

There is no detailed information on compositional changes in the individual caseins of heated sheep milk. The results observed in the current study are generally in agreement with previous reports on cow skim milk, which showed that the dissociation levels of α_{S1} -CN, β -CN, and κ -CN increased with increasing pH (from pH 6.5 to 6.9) in the temperature range 20 to 120°C (Anema and Klostermeyer, 1997; Anema, 1998). Additionally, caseins are more negatively charged at alkaline pH, which would enhance the electrostatic repulsion between the individual submicelles (Sinaga et al., 2017). The enhanced repulsive forces could lead to a looser casein micelle structure, which could contribute to the easy dissociation of caseins from the micelles (Liu and Guo, 2008; Madadlou et al., 2009). Therefore, the greater dissociation of α_{S1} -CN, β -CN, and κ -CN at higher pH could have been due to the enhanced electrostatic repulsion between the individual caseins at alkaline pH.

Although there are extensive reports on the pH-dependent dissociation of micellar casein in heated cow milk (Kudo, 1980; Singh and Fox, 1985; Singh and Creamer, 1991a,b; Anema et al., 1993; Anema and Stanley, 1998; Anema and Li, 2000), comparisons with the present study are limited because these previous studies examined the dissociation behavior in cow milk mainly at lower heating temperature ($<120^\circ\text{C}$). Only a few publications have reported the dissociation behavior of micellar caseins in cow milk after UHT treatment,

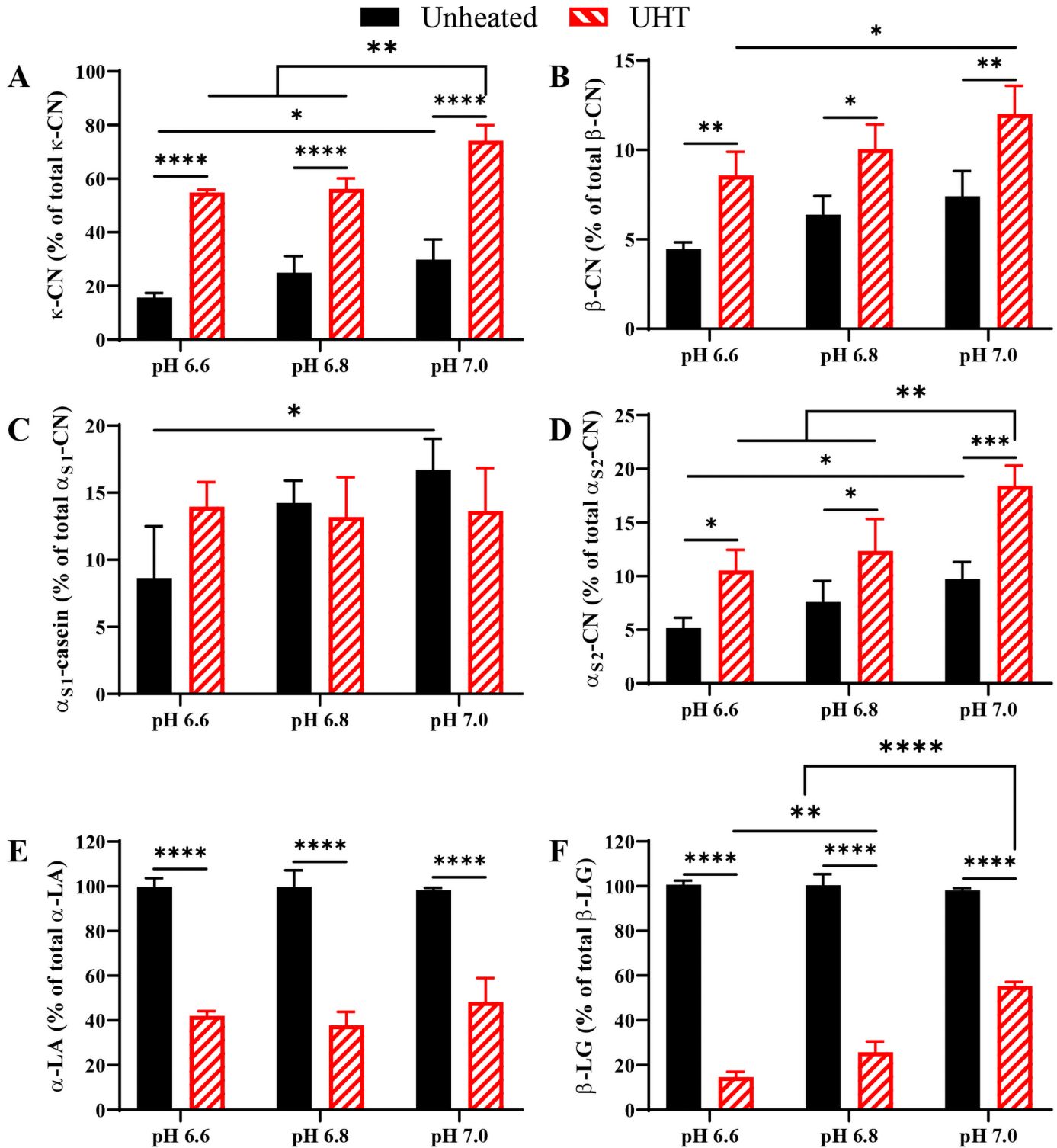


Figure 4. Levels of individual proteins (κ -CN, β -CN, α_{S1} -CN, α_{S2} -CN, α -LA, β -LG) in supernatants obtained by centrifuging unheated (solid black bars) and UHT-treated (red striped) sheep skin milks at $48,800 \times g$ and 20°C for 26 min. Error bars represent standard deviations. *, **, ***, and **** represent P -values < 0.05, 0.01, 0.001, and 0.0001, respectively.

but these reports focused on the dissociation behavior of the caseins in cow milk at the natural pH. Li et al. (2019) showed that ~30% of κ -CN dissociated from the casein micelles after heating cow skim milk at 140°C for 5 s. Liu et al. (2019) reported that ~31% of κ -CN, ~1.1% of β -CN, ~12% of α_{S2} -CN, and ~6% of α_{S1} -CN dissociated from the casein micelles in cow milk after indirect UHT treatment at 141°C for 2 s. Akkerman et al. (2021) showed that UHT treatment (141°C/4 s) of cow skim milk at the natural pH resulted in the dissociation of ~9% of κ -CN, ~15% of β -CN, and ~5% of α_{S1} -CN from the micelles. In the current study, ~55% κ -CN (of total κ -CN) in UHT-treated SSM at natural pH (~6.6) was present in the serum phase, indicating that ~39% κ -CN (by subtracting ~16% κ -CN in the serum of unheated SSM at natural pH) was dissociated from casein micelles after UHT treatment (Figure 4A). In comparison with the previous studies cited above, the heat-induced dissociation of κ -CN in UHT SSM at natural pH presented here is much higher than that in UHT-treated cow milk. It has been proven that a higher concentration of total solids increases the extent of dissociation of κ -CN (Singh and Creamer, 1991b; Anema, 1998). Therefore, the greater dissociation of κ -CN in the UHT-treated SSM can probably be attributed to the higher total solids and protein contents in sheep milk.

As the remarkable stability of the casein micelles relies on κ -CN at the surface of the micelle, the removal of κ -CN from the micelles can induce their aggregation via calcium bridging when the ionic calcium concentration exceeds a critical level (Dalglish, 1992; Anema, 2019; Huppertz et al., 2018). Hence, the greater dissociation levels of κ -CN observed in UHT-treated SSM than in UHT-treated cow skim milk might reduce the protective effects of the κ -CN hairy layers on the casein micelles, leading to increased aggregation of the casein micelles in SSM during UHT treatment. Of note, significantly ($P < 0.05$) higher levels of κ -CN dissociated from the casein micelles after UHT treatment for the SSM at pH 7.0 than for the UHT-treated SSM at natural pH (~6.6) and pH 6.8 (Figure 4A). The higher proportions of β - and α_{S2} -CN dissociated from the casein micelles (Figures 4B and 4D) in the UHT-treated SSM at pH 7.0 would expose more internal structure of the casein micelles so that other proteins could interact with them during heating, contributing to the aggregation of the micelles. It could be hypothesized that the high amount of sediment in the UHT-treated SSM at pH 7.0 (Figure 2A) was induced by the greater dissociation of caseins and thus more interactions between the casein micelles. The different appearances and sizes of the aggregates of the UHT-treated SSM at natural pH (~6.6) and pH 7.0 (Figures 1A and 1B) might be explained by the dif-

ferent aggregation pathways (mainly calcium-bridging micelles because of a higher concentration of ionic calcium for natural pH ~6.6 and more interactions among micelles because of casein dissociation for pH 7.0) among the casein micelles. More studies are needed to verify whether the aggregates in UHT-treated SSM are formed in different ways at different pH values.

Interactions of Whey Proteins with Caseins and Casein Micelles

The contents of both β -LG and α -LA in the serum of the UHT-treated SSM decreased significantly ($P < 0.0001$) at all pH values compared with unheated SSM (Figures 4E and 4F), indicating that a proportion of the serum-phase β -LG and α -LA was denatured and precipitated with the casein micelles on ultracentrifugation, probably by associating with the casein micelles (Pan et al., 2022), by forming large whey protein aggregates, or both (Deeth and Lewis, 2017). This confirmed that the decreased protein content of the serum after UHT treatment (Figure 3) could be attributed to the precipitation of the denatured whey proteins on ultracentrifugation.

The contents of serum α -LA and β -LG remained unchanged in the unheated SSM, despite the changes in pH (Figures 4E and 4F). For the UHT-treated SSM, the level of serum-phase α -LA remained nearly unchanged with increasing pH, indicating that increasing the pH of the SSM had little effect on the denaturation and interaction of serum-phase α -LA during UHT treatment. In contrast, the serum-phase β -LG of UHT-treated SSM showed an increasing trend as the pH was increased. Similar results have been reported for cow milk, which showed that the level of whey protein/ κ -CN complexes was found to increase in heated cow skim milk when its pH was increased to a range pH 6.3 to 7.3 (Kudo, 1980; Singh and Fox, 1985; Singh and Latham, 1993). It has been shown that pH changes in milk (pH 6.0–9.0) have little effect on the denaturation rate of α -LA and β -LG (Hillier and Lyster, 1979); therefore, in this study, similar proportions of α -LA and β -LG would be expected to be denatured at all pH values and still complexed with κ -CN (Anema and Klostermeyer, 1997). However, as an increased proportion of κ -CN dissociated from the casein micelles at higher pH (Figure 4A), the denatured whey proteins complexed with dissociated κ -CN would also remain soluble in the serum.

It is not clear whether the whey proteins of the complexes in UHT-treated milk samples are composed mainly of β -LG or β -LG plus α -LA. The present study showed that only the serum-phase β -LG levels increased with increasing pH in the UHT-treated SSM, which does not support the results reported by Anema

and Klostermeyer (1997), who showed that increasing the pH (pH 6.3–7.2) of cow skim milk increased the levels of both α -LA and β -LG in the serum phase after heating at 80 to 90°C for 15 min. The differences between sheep milk and cow milk might be due to the different heating methods used or to the different structural properties of the whey proteins. Previous studies reported that the dissociation of κ -CN preceded the denaturation of β -LG and that the denatured whey proteins favored interactions with serum-phase κ -CN (Anema, 2008). Additionally, β -LG was denatured and associated with the casein micelles at a faster rate than α -LA in both sheep skim milk and cow skim milk heated at 80 to 130°C (Oldfield et al., 1998; Pan et al., 2022). It is possible that a large amount of denatured β -LG complexed with the dissociated κ -CN during the initial stages of UHT treatment before α -LA began to denature to any significant extent. In the meantime, as β -LG complexed with κ -CN became less accessible for the interaction with denatured α -LA, the later-denatured α -LA might be able to associate with other proteins (remaining unassociated with denatured β -LG, caseins, or casein micelles) only via sulfhydryl–disulfide or hydrophobic interactions. Moreover, at higher pH of the SSM, a greater amount of dissociated κ -CN might result in higher levels of complexation between β -LG and κ -CN in the serum phase, leading to less β -LG being available for α -LA to interact with (Anema, 2008). The denatured α -LA thus presumably interacted with other proteins mainly via hydrophobic bonds and formed large aggregates during heating. Further investigations are required to verify this hypothesis.

CONCLUSIONS

This study confirmed that the instability of sheep milk during UHT treatment was pH dependent. The amount of sediments for UHT-treated SSM was significantly higher at the natural pH (~6.6) and pH 7.0 than at pH 6.7–6.9. The sediment was composed mainly of κ -CN-depleted casein micelles with very low levels of whey proteins, regardless of the SSM pH. The high sedimentation in the UHT-treated SSM at the natural pH (~6.6) could probably be attributed to the high ionic calcium level and the significant dissociation of κ -CN from the casein micelles. The high amount of sedimentation formed at pH 7.0 might be due to the greater extent of dissociation of κ -, β -, and α_{S2} -CN from the casein micelles, which leads to the reduced protection by the κ -CN hairy layer and the exposure of the internal structure of the casein micelles, resulting in the aggregation of casein micelles.

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