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Heat stability of sheep's skim milk: Aggregation and interaction of proteins

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ABSTRACT

Sheep's milk proteins are susceptible to heat-induced coagulation, but the protein interactions under high heat treatment have not been determined. Heat stability and protein interactions of sheep's skim milk (SSM) at pH 6.2–7.2 were examined at 140 °C. SSM had the longest heat coagulation time at pH 6.9, but became very unstable at higher or lower pH. Protein aggregates formed consisted mainly of whey proteins and κ-casein (κ-CN)-depleted casein micelles. Modification of SSM pH alters ionic calcium concentration, dissociation of caseins and electrostatic interactions, resulting in different extents of protein interactions. The extent of dissociation of κ-CN from casein micelles increased with increasing pH (from ~6.6 to 7.0) before and after heat treatment, contributing to κ-CN-depleted casein micelle aggregation. High ionic calcium concentrations, low levels of κ-CN on casein micelles and ready dissociation of κ-CN from casein micelles may be responsible for the low heat stability of sheep's milk.

1. Introduction

Sheep's milk has lower heat stability than cows' milk. Sheep's milk can be effectively pasteurised via either vat or high-temperature short-time treatment, but cannot be easily processed by UHT treatment. Unlike cows' milk, which can be stored for 6 months after UHT treatment without the addition of a stabiliser (Gaur, Schalk, & Anema, 2018), UHT treatment of sheep's milk can produce a large amount of sediment immediately after packaging (Martinez Alonso, Magdalena Vera, Mendez Donega, & Berterreche Alvarez, 2009). The sediment that is formed during UHT treatment is composed mainly of proteins (O'Connell & Fox, 2003; Singh, 2004). Therefore, to improve sheep's milk heat stability, it is important to understand how proteins in sheep's milk aggregate at high temperatures.

The heat stability of sheep's milk has not been investigated in depth. In their heat coagulation time (HCT)–pH profile, Fox and Hoynes (1976) showed that sheep's milk had a marked stability maximum at ~pH 6.8 (measured at 140 °C) but became very unstable at higher and lower pH values. Muir and Tamime (1993) also reported a similar HCT–pH profile for sheep's milk, but its heat stability was not further studied. A negative correlation between the heat stability and the non-protein nitrogen fraction of sheep's milk was reported in the study of Muir and Tamime (1993).

The heat-induced changes in cows' milk have been extensively investigated. It has been reported that the important factors affecting the colloidal stability of cows' milk are calcium ions and pH, which affect the attractive/repulsive forces between micelles, facilitate the aggregation of micelles, and possibly change the conformation of κ-casein (κ-CN) at the micelle surface (indirectly reducing steric repulsions) (Singh, 2004). Additionally, heat treatment of cows' milk significantly alters the serum-phase environment around the casein micelles (such as pH, soluble calcium ions and breakdown of lactose) and the casein micelles themselves (association of whey proteins, dephosphorylation and casein dissociation), and these changes contribute to the coagulation of milk proteins. For instance, heating cows' milk at 140 °C resulted in dissociation of κ-CN and the formation of κ-CN/whey protein complexes, thereby reducing the protective effects of the hairy layers of casein micelles (Singh & Latham, 1993). Increasing the pH of cows' milk before heating further increased the dissociation of κ-CN after heat treatment and thus the quantity of κ-CN/whey protein complexes in the serum phase (Anema, 2008; Singh & Latham, 1993), leading to the aggregation of unstable casein micelles.

However, it has been reported that the composition of the milk could have an impact on these heat-induced changes (Deeth &

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Lewis, 2016; Pan, Ye, Dave, Fraser, & Singh, 2022). The composition of sheep's milk is considerably different from that of cows' milk; for instance, the main fractions of sheep's milk versus cows' milk are 5.5% and 3.4% for protein content, 197.5 mg 100 g^{-1} and 112.0 mg 100 g^{-1} for calcium contents, 61.6% and 32.7% for β-casein (β-CN) (of total casein), 22.8% and 10.3% for α_{S2}-casein (α_{S2}-CN) (of total casein), 6.7% and 39.7% for α_{S1}-casein (α_{S1}-CN) (of total casein), and 8.9% and 11.6% for κ-CN (of total casein) (Balthazar et al., 2017; Li, Delger, Dave, Singh, & Ye, 2022); these differences could have an impact on the stability and the rate and extent of interactions of sheep's milk proteins during heating. Given the complexity of the reactions that occur during heat treatment, the result obtained from cows' milk cannot be directly extrapolated to that of sheep's milk. Therefore, these heat-induced changes in sheep's skim milk (SSM) were investigated in this study.

This study investigated the aggregation of proteins, the dissociation of caseins and the association of whey proteins in SSM at 140 °C and in the pH range 6.2–7.2. The HCT–pH profile of SSM was used as an indicator to select the pH and the heating time for further analysis. The ionic calcium concentration, the particle size distribution and the protein composition in unheated and heated samples were determined to develop an understanding of the differences observed in the HCT–pH profiles of SSM.

2. Materials and methods

2.1. Materials and pH adjustment

The raw sheep's milk was purchased from Fernglen Ltd (Masterton, New Zealand). A small amount of sodium azide (0.01%, w/w) was added to the raw milk as a preservative. The raw sheep's milk samples were skimmed at 3000 × *g* and 20 °C for 15 min using a bench centrifuge (Heraeus Multifuge X3R; Thermo Fisher Scientific Inc., Waltham, MA, USA). The composition of the SSM was analysed using a MilkoScan FT1 (Foss, Hillerød, Denmark) and is shown in Table 1. The pH of the SSM was adjusted to values between 6.2 and 7.2 by slowly adding 3 M HCl or 3 M NaOH to well-stirred solutions. The natural pH of the SSM was 6.59 ± 0.02. The milk samples were kept at ambient temperature for at least 2 h before the final pH reading and minor readjustment.

2.2. Heat stability testing

The HCT of the SSM was examined at 140 °C as a function of pH (6.2–7.2), as described by Davies and White (1966). A 2 mL subsample of pH-adjusted SSM was transferred into a glass vial (capacity, 8 mL; height, 63 mm; diameter, 17 mm) and heated at 140 °C with continuous rocking (8 cycles min^{-1}) in a thermostatically controlled oil bath. The HCT was defined as the time that elapsed between placing the vial in the oil bath and the first visible onset of coagulation. The heat-up time for the milk to reach 140 °C was around 2 min.

Table 1
Composition of sheep skim milk.

Component	Composition
Protein (g 100 mL^{-1})	6.68 ± 0.40
Casein (g 100 mL^{-1})	5.12 ± 0.33
α_{S2}-casein (% of total casein)	16.03 ± 1.51
α_{S1}-casein (% of total casein)	41.91 ± 0.59
β-casein (% of total casein)	34.91 ± 1.02
κ-casein (% of total casein)	7.14 ± 0.95
Fat (g 100 mL^{-1})	0.62 ± 0.27
Lactose (g 100 mL^{-1})	4.82 ± 0.10
Total solids (g 100 mL^{-1})	13.29 ± 0.63
Casein/protein (%)	76.51 ± 0.51

2.3. Heat treatment

SSM at pHs 6.8, 6.9 and 7.0 was heated at 140 °C for 120, 150, 200, 300 and 500 s using the same method as described above. After heat treatment, these milk samples were immediately immersed in cold running water and cooled to room temperature. The heated milk samples were kept at room temperature for 6 h before further determination.

2.4. pH and ionic calcium concentration

Unheated and heated SSM samples at different pH values were preheated in a water bath at 20 °C for 1 h to equilibrate the temperature. The pHs of these samples were then determined using a pH 700 Benchtop Meter (Oakton Instruments, Vernon Hills, IL, USA). The concentration of ionic calcium in these milk samples was determined as described in Li, Ye, and Singh (2019), using an Orion calcium-selective electrode (9720BNWP, Thermo Fisher Scientific Inc., Beverly, MA, USA) coupled with the pH 700 pH/mV Benchtop Meter. Calibration was conducted using standard (0.5–5 mM) $CaCl_2$ in 80 mM $CaCl_2$–KCl solution. The millivolt value was measured and recorded by dipping the electrode into the milk samples. The recorded millivolt value was converted to the ionic calcium concentration using the calibration curve obtained from the standard $CaCl_2$–KCl solution.

2.5. Separation of milk protein fractions

Unheated and heated SSM samples were centrifuged to obtain different fractions of soluble protein, as described by Dumpler, Wohlschläger, and Kulozik (2017). After centrifugation, the protein composition of the resultant supernatants was analysed by RP-HPLC. The fractions obtained are summarised below.

Fraction A: large aggregates (>3 μm) sedimented at 3000 × *g* for 10 min at 20 °C using a 50 mL centrifuge tube (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China) and a bench centrifuge Heraeus Multifuge X3R coupled with a swing bucket rotor (TX-750; Thermo Fisher Scientific Inc.).

Fraction B: colloidally stable micelles (100–1000 nm) sedimented at 48,800 × *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 all casein micelles while κ-CN/whey protein complexes and soluble proteins remain in the supernatant (defined as the serum phase) (Dumpler et al., 2017). The level of individual proteins associated with the sedimented casein micelles under 48,800 × *g* for 26 min was calculated by subtracting the level of protein in the supernatant of fraction B from that of fraction A.

Fraction C: submicellar particles (20–100 nm) sedimented at 70,000 × *g* for 60 min. The particles in this size range consist mainly of κ-CN/whey protein complexes with comparably small amounts of calcium-sensitive caseins (Dumpler et al., 2017; Morand, Guyomarc'h, Pezennec, & Famelart, 2011). Therefore, the submicellar particles obtained are referred to as κ-CN/whey protein complexes in the following sections.

Fraction D: soluble proteins (<20 nm).

2.6. Particle size analysis

Particle size measurements of SSM heated at different conditions were performed on a MasterSizer 2000S (Malvern Instruments GmbH, Herrenberg, Germany), as described by Pan, Ye,

Dave, Fraser, and Singh (2023). Briefly, the milk samples were shaken well and added to the dispersion unit to obtain an obscuration of 10–20%. The refractive index of the dispersant (water) and the skim milk was set to 1.33 and 1.50, respectively. Each sample was measured in triplicate at 20 °C and the average value of 3 measurements was used.

2.7. Protein composition analysis

Milk and the supernatants obtained from centrifuged milk samples were analysed by RP-HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 μm XB-C18 RP; Phenomenex, Torrance, CA, USA) to determine the protein composition, as described by Bobe, Beitz, Freeman, and Lindberg (1998). As the intensive heating results in different extent of glycosylation of proteins, this could overlap the individual peaks in the chromatogram. Previous studies showed that the measurement of peak height is a better method than the measurement of peak area especially if peaks are poorly resolved (Dyson & Smith, 1998; Grant & Clarke, 1971; Kadjo, Dasgupta, Su, Liu, & Kraiczek, 2017); it is less affected by asymmetry and overlap (Meyer, 1995; Snyder, 1972). In this study, therefore, the quantity of whey proteins and caseins in the ultracentrifugal supernatants was determined by comparing the relative peak height of the supernatant fraction in the heated SSM with that in the original unheated SSM. All peak heights of these chromatograms were performed using peak integration algorithm LabSolutions software (Shimadzu Corporation, Kyoto, Japan).

2.8. Statistical analysis

All experiments reported were repeated three times using freshly collected sheep's milk samples, and the results are shown as the mean ± standard deviation. Although there were some variations between individual milks, the same trends and relationships as reported here have been found for all samples examined to date. Graphs and analysis of variance tests were produced using GraphPad 8.4.0 (GraphPad Software).

3. Results and discussion

3.1. Heat coagulation time

The HCT of the SSM as a function of pH from pH 6.2 to pH 7.2 is shown in Fig. 1. The HCT increased as the pH of the SSM was increased, reaching a maximum at pH 6.9. However, a further increase in the pH led to a decrease in the HCT. Similar trend for HCT-pH profile of sheep's milk has been reported but the pH value for maximum HCT shows a difference compared with a previous report, which showed that sheep's milk had the longest HCT at pH 6.8 and that the HCT decreased when the pH value was higher or lower than pH 6.8 (Fox & Hoynes, 1976). Variations in pH values for maximum HCT of cows' milk has also been found. The study conducted by Fox and Hoynes (1976) showed that the maximum HCT of cows' milk varied with pH and was maximum at around pH 6.6. Another study by O'Connell and Fox (2000) reported a similar trend in the maximum HCT with pH at 6.6 for cows' milk. The maximum HCT of milk has been found to be influenced by several factors such as milk composition and heat treatment conditions (Mclean, Graham, Ponzoni, & Mckenzie, 1987). Therefore, pH values for maximum HCT between various studies could be different. However, the maximum HCT (~10 min) of the SSM observed in this study was markedly lower than that of cows' skim milk reported previously (Fox & Hoynes, 1976); that is, the maximum HCT of cows' milk was ~20–30 min (measured at 140 °C) at ~pH 6.7. The lower heat stability of sheep's milk has been attributed to its higher protein content and lower level of κ-CN than cows' milk (Fox & Hoynes, 1976; Raynal-Ljutovac, Park, Gaucheron, & Bouhallab, 2007). Other possible factors, such as different pH-induced changes in the surface charge of the casein micelles, electrostatic repulsion and ionic calcium activity prior to heat treatment have also been reported to affect milk protein interactions (such as aggregation of casein micelles and dissociation of caseins) during heating. However, how these parameters result in the low heat stability of sheep's milk has not been clarified. Based on the HCT–pH profile, the samples with pH 6.8–7.0 and heating times of 120–500 s were selected to further investigate the heat coagulation in SSM, and the results are shown in the following sections.

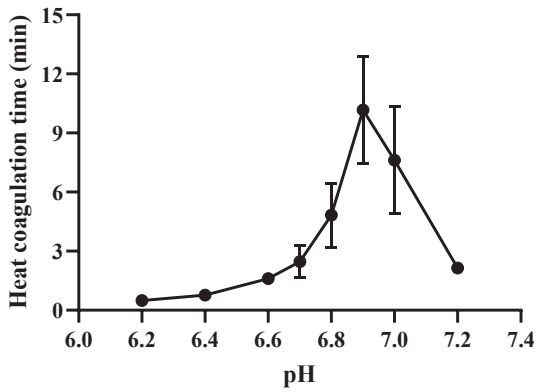

Fig. 1. Heat coagulation time–pH profile of sheep skim milk at 140 °C.

3.2. Particle size

The average particle size (D [4, 3]) of the SSM before and after heat treatment was determined and is shown in Fig. 2A. After heat treatment, the particle size of the SSM at all pH values increased with an increase in the heating time. This may have been due to the association of whey proteins with casein micelles and protein aggregation during heating (Pan et al., 2022). However, the changes in particle size of the heated SSM occurred to different extents and showed significant (P <0.0001) differences among the different pH values. For example, at the heating time of 300 s, the particle size of the SSM at pH 6.9 (~0.90 μm) was significantly (P <0.0001) lower than those at pH 6.8 (~9.13 μm) and pH 7.0 (~13.81 μm). These results suggested that the proteins of the SSM were relatively more stable at 140 °C and pH 6.9, and were more susceptible to aggregation during heating and larger protein particles were formed at pH 6.8 and pH 7.0.

3.3. pH and ionic calcium concentration

The ionic calcium concentration–pH profile of unheated SSM is shown in Fig. 2B. The ionic calcium concentration decreased with increasing pH, which was in line with results found previously for cows' milk (Lewis, 2011). Previous studies on cows' skim milk have shown that there is a strong negative correlation between the ionic calcium concentration and the pH of cows' milk (Gaur et al., 2018; Ho et al., 2018), and the ionic calcium concentration has been reported to be an important factor that alters the heat stability of milk (Deeth & Lewis, 2017).

Lewis, Grandison, Lin, and Tsioulpas (2011) showed that there is a sharp boundary between the instability of milk that produces large amounts of sediment and the stability of milk that creates small amounts of sediment; in which a small shift in ionic calcium concentration at the boundary could convert a stable milk to an

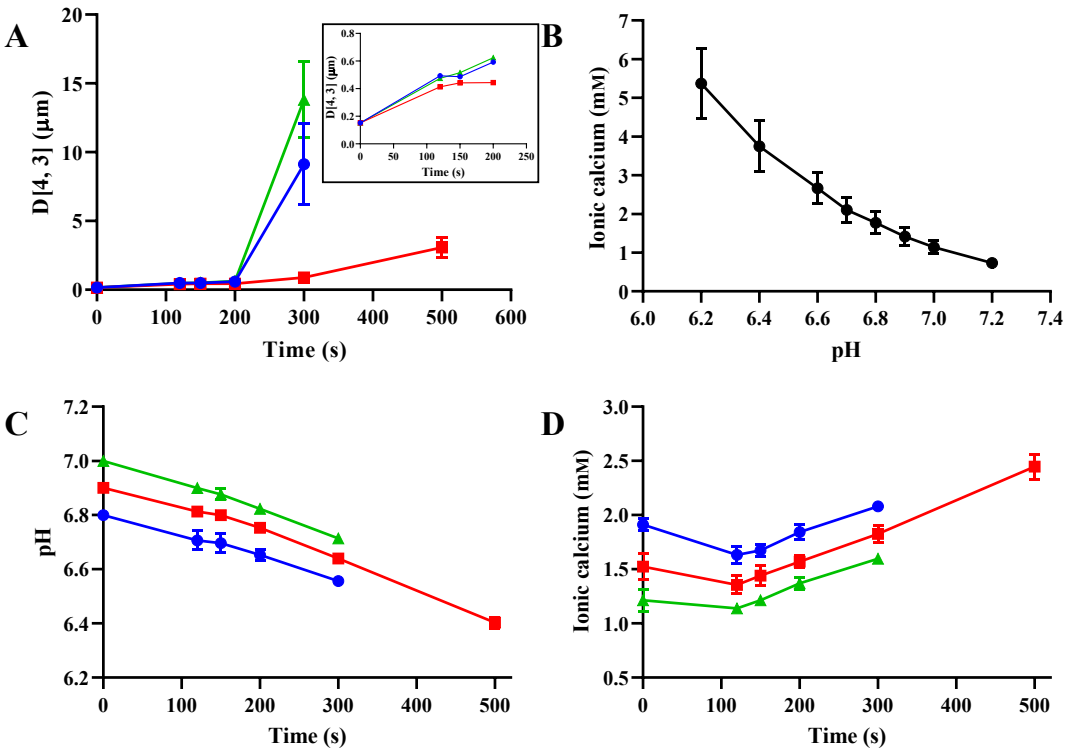

Fig. 2. Changes in particle size of heated sheep skim milk (A), ionic calcium−pH profile of unheated sheep skim milk (B), and pH (C) and ionic calcium concentration (D) of heated sheep skim milk at 140 °C as a function of time. pH 6.8 (●), pH 6.9 (■) and pH 7.0 (▲).

unstable milk or vice versa. In addition, Dumpler, Huppertz, and Kulozik (2020) reported that the low heat stability at the acidic pHs of milk can be attributed to salt-induced coagulation because the colloidal stability of casein micelles might be lowered by the reduced micellar surface charge, the reduced electrostatic repulsion and the collapse of the hairy layer because of charge neutralisation.

The ionic calcium concentration of the SSM (~2.7 mM) at the natural pH (~6.6) in the present study was higher than that of cows' milk (1.8−2.3 mM) at the natural pH, as reported previously by Lewis (2011), which is in agreement with previous reports that sheep's milk has a higher concentration of ionic calcium than cows' milk (Li et al., 2022; Lin, 2002; Silanikove, Shapiro, & Shamay, 2003). Therefore, the lower heat stability of the SSM at pH <6.9 was assumed to be due to the charge neutralisation that was induced by the lowered pH and the increased ionic calcium concentration. In contrast, the destabilisation of casein micelles at the more basic pHs of the milk was correlated with the heat-induced dissociation of κ-CN from the casein micelles, which results in an increased tendency towards aggregation (Singh, 2004).

The changes in pH of the SSM after heating are shown in Fig. 2C. The pH after heating decreased in all samples and the decrease rate of the pH was similar among samples with different pHs when the heating time was less than 300 s. This is in agreement with earlier studies, which found a decreasing trend in the milk pH as the heating time was increased (Fox, 1981). The decline in pH of the SSM after heat treatment may have been due to the production of organic acids from lactose decomposition, precipitation of tertiary calcium phosphate with the concomitant release of H^+ ions and the heat-induced dephosphorylation of casein with the subsequent precipitation of the released phosphate as tertiary calcium phosphate (Fox, 1981; Pyne & McHenry, 1955).

The changes in the ionic calcium concentration of the SSM after heating are shown in Fig. 2D. The ionic calcium concentration decreased at the initial stage of heating, but increased upon prolonged heating. The heat treatment of cows' milk has been reported to reduce the ionic calcium concentration because of the precipitation of calcium phosphate during thermal processing (Chandrapala, McKinnon, Augustin, & Udabage, 2010; Geerts, Bekhof, & Scherjon, 1983). However, the decrease in ionic calcium concentration after heating is a reversible process, and some or all of the calcium ion activity can be recovered upon cooling (Singh, 2004). During heating, calcium and soluble phosphate transfer from the serum to the micelles, which can reduce the negative charges on the micelles and decrease electrostatic repulsions.

Sheep's milk has a higher content of casein micelles compared with cows' milk, which can lead to more extensive interactions between micelles, probably resulting in the formation of greater amount of aggregates. The decreased ionic calcium concentration in the heated SSM is in accordance with the observation in cows' milk, in which the calcium and phosphate are transferred to the colloidal phase upon heating (De La Fuente, Olano, & Juárez, 2002; Singh, 2004; Zhang & Aoki, 1996). Therefore, it is possible that the part of calcium ions irreversibly bind to the newly formed micelle aggregates, leading to a decrease in ionic calcium concentration after heat treatment. However, the precipitation of calcium phosphate could lead to a re-equilibration between HPO_4^{2-} and $H_2PO_4^-$ / PO_4^{3-} in the serum phase, producing more H^+ ions; this probably resulted in a decrease in pH when the buffering capacity of soluble phosphates was exceeded after heat treatment (Chandrapala et al., 2010). Therefore, the increasing trend of the ionic calcium concentration in the SSM at the longer heating times was attributed to the further decrease in pH, which could in turn increase the

concentration of ionic calcium (Chen, Grandison, & Lewis, 2015). Additionally, the dephosphorylation of the caseins upon heating at 140 °C could reduce the binding of ionic calcium to caseins, which could also contribute to the increase in the ionic calcium concentration (Fox, 1981; Singh, 2004). The ionic calcium concentration might increase with increasing heating time, the SSM might have formed protein aggregates when the ionic calcium concentration reached a critical level, thus increasing the particle size, as shown in Fig. 2A.

3.4. Heat-induced protein aggregation

The changes in the relative amounts of non-sedimentable proteins in the SSM after centrifugation at 3000 × *g* for 10 min are presented in Fig. 3. At all pHs, there was a decreasing trend for all caseins and whey proteins as the heating time increased, but κ-CN decreased to a lesser extent than the other proteins. This indicated that heat treatment of the milk resulted in the formation of large protein aggregates, which were composed mainly of whey proteins and casein micelles that were depleted in κ-CN, as observed in a previous report (Pan et al., 2022). When the heating time exceeded 200 s, the levels of non-sedimentable β-CN, α_{S2}-CN, α_{S1}-CN and β-LG in the SSM at pH 6.9 decreased at a slower rate compared with those at pH 6.8 and pH 7.0, whereas their κ-CN and α-lactalbumin (α-LA) counterparts showed few differences among the pH values. The results suggested that the casein micelles of the SSM were more stable at pH 6.9 than at pH 6.8 and pH 7.0 during heating, which is in line with the particle size results (Fig. 2A).

3.5. Protein dissociation from casein micelles

The levels of individual proteins in the supernatant obtained by centrifuging unheated SSM at 48,800 × *g* for 26 min are shown in Fig. 4; the supernatant obtained with this centrifugation condition is termed the serum phase in the following description. The level of κ-CN was found to increase significantly (P <0.05) with increasing pH compared with that at the natural pH (~6.6), whereas the other serum-phase caseins changed little (P >0.05). This indicated that

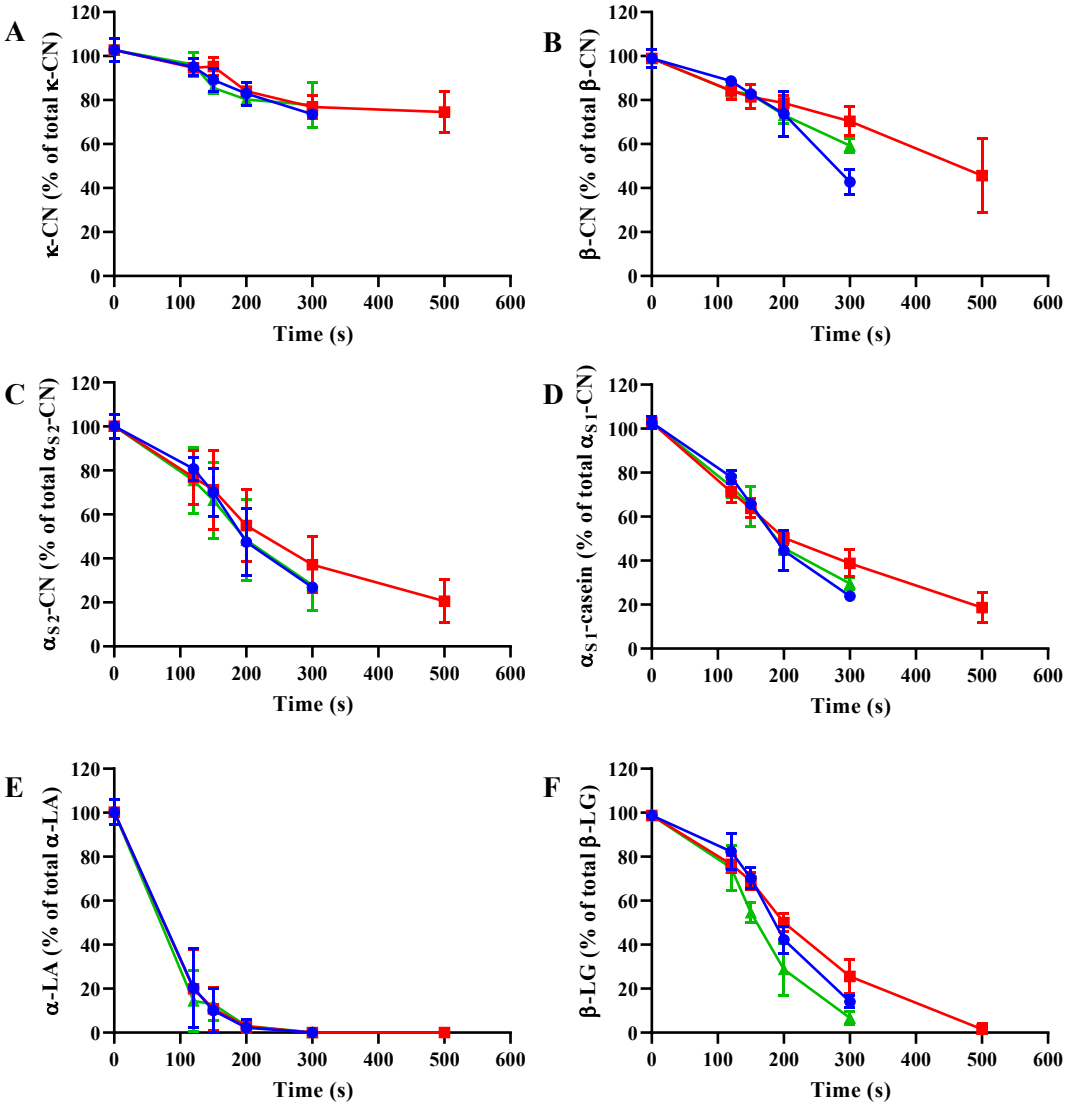

Fig. 3. Changes in non-sedimentable (A) κ-casein (κ-CN), (B) β-casein (β-CN), (C) α_{S2}-casein (α_{S2}-CN), (D) α_{S1}-casein (α_{S1}-CN), (E) α-lactalbumin (α-LA) and (F) β-lactoglobulin (β-LG) in sheep skim milk at pH 6.8 (●), pH 6.9 (■) and pH 7.0 (▲), centrifuged at 3000 × *g* for 10 min and heated at 140 °C for different times.

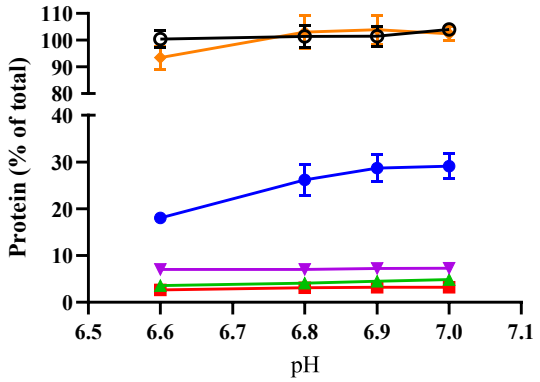

Fig. 4. Changes in the levels of non-sedimentable κ-casein (κ-CN) (●), β-casein (β-CN) (▼), α_{S2}-casein (α_{S2}-CN) (■), α_{S1}-casein (α_{S1}-CN) (▲), α-lactalbumin (α-LA) (◆) and β-lactoglobulin (β-LG) (○) in unheated sheep skim milk at different pH values and centrifuged at 48,800 × *g* for 26 min.

increasing the pH of the unheated SSM resulted in the dissociation of κ-CN from the casein micelles before heat treatment. This is different from previous findings in cows' milk, which showed that increasing the pH of unheated cows' skim milk from 6.3 to 7.1 did not induce dissociation of κ-CN from the casein micelles (Anema & Klostermeyer, 1997; Anema & Li, 2000). It is possible that the connection between κ-CN and the rest of the casein micelle could be more susceptible to pH changes in sheep's milk than in cows' milk. The different protein compositions and total solids between sheep's milk and cows' milk (Balthazar et al., 2017) may be responsible for the different dissociation behaviour of κ-CN as induced by pH changes. The level of soluble κ-CN in the serum phase of unheated SSM (~18.1% of total κ-CN, Fig. 4) at the natural pH was higher than that of unheated cows' skim milk (~7–10% of total κ-CN) at the natural pH (Anema, 1998, 2008; Dumpler et al., 2017; Li et al., 2019). This may suggest less κ-CN on the casein micelles of sheep's milk than of cows' milk. As the remarkable stability of casein micelles relies on the κ-CN at the surface of the micelle (Anema, 2018; Dalgleish, 1992; Huppertz, Fox, & Kelly,

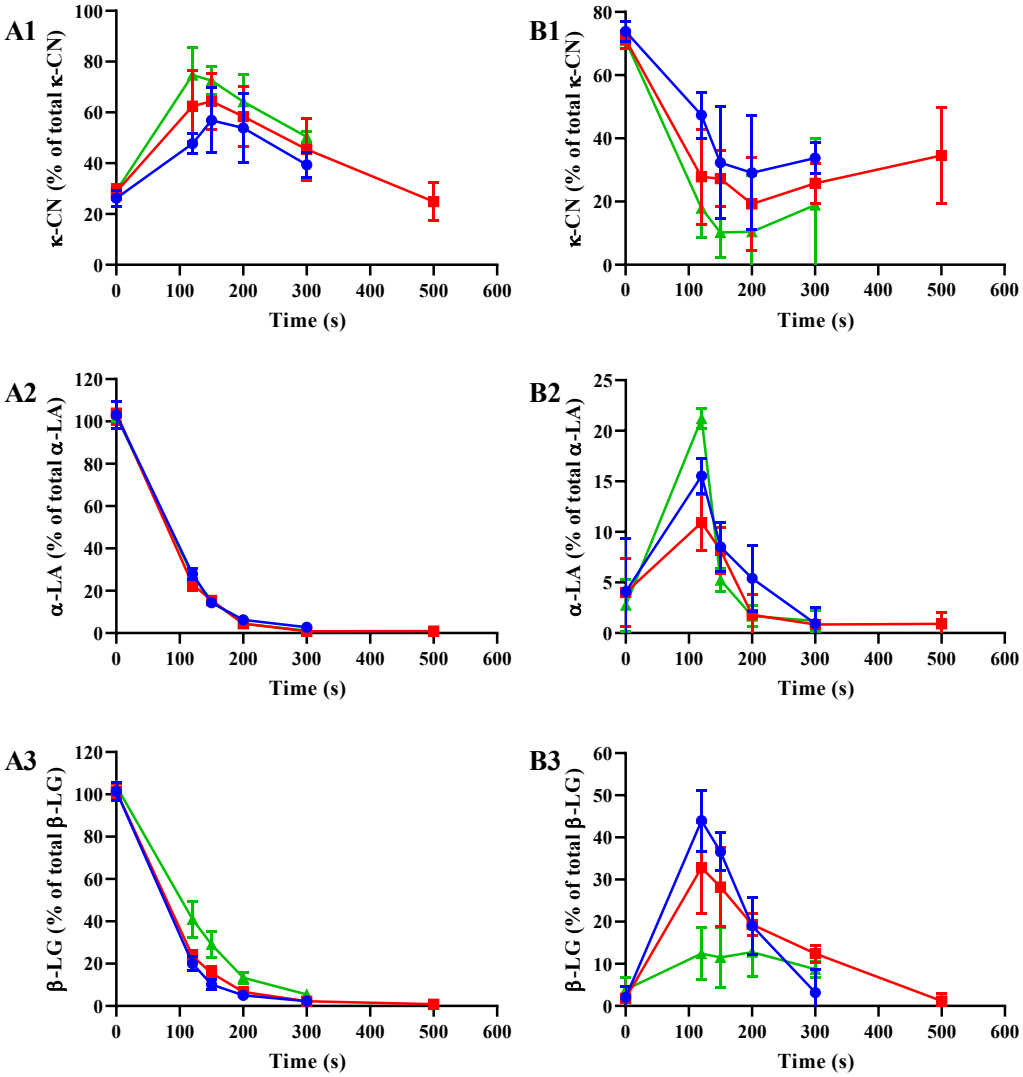

Fig. 5. Changes in non-sedimentable individual proteins in heated (140 °C) sheep skim milk pH 6.8 (●), pH 6.9 (■), and pH 7.0 (▲) centrifuged at 48,800 × *g* for 26 min (A) and changes in individual proteins in the colloidal-stable micelles of heated (140 °C) sheep skim milk (B): A1 and B1, κ-casein (κ-CN); A2 and B2, α-lactalbumin (α-LA); A3 and B3, β-lactoglobulin (β-LG).

2018), the lower level of κ-CN attached to the casein micelles and the vulnerable connection of κ-CN with casein micelles in the SSM may contribute to the low heat stability of sheep's milk and thus prompt the aggregation of the casein micelles during high heat treatment.

Fig. 5 shows the changes in the levels of κ-CN, α-LA and β-LG in the serum phase obtained from the SSM at pH 6.8–7.0 after centrifugation at 48,800 × *g* for 26 min and in the colloidal-stable micelle phase of the SSM after heat treatment. The level of serum-phase κ-CN in the SSM at all pHs increased in the first 150 s of heating, followed by a decrease with a longer heating time. The level of serum-phase κ-CN was highest in the SSM at pH 7.0 and lowest in the SSM at pH 6.8 throughout the heating period (Fig. 5A1). This indicated that the dissociation of κ-CN occurred during the early stage of heating and that raising the pH of the SSM increased the dissociation level of κ-CN. The greater level of dissociation of κ-CN at higher pH has been attributed to the enhanced electrostatic repulsion between the individual sub-micelles at a more alkaline pH (Anema & Klostermeyer, 1997; Anema & Li, 2000; Dumpler, 2018; Pan et al., 2023). In contrast, the level of κ-CN in the colloidally stable micelle phase showed a decreasing trend when the heating time was less than 200 s, followed by an increasing trend with further heating (Fig. 5B1). This suggested that a longer heating time led to the reassociation of κ-CN with the casein micelles probably because of a heat-induced decrease in pH (Deeth & Lewis, 2016; Singh, 2004).

Serum-phase α-LA and β-LG decreased markedly in the pH range 6.8–7.0 and ~95% of the α-LA and β-LG were lost in the serum phase when the heating time exceeded 200 s (Fig. 5A2 and A3). The loss of serum-phase α-LA and β-LG could be attributed to either the formation of large whey protein aggregates or the association of denatured whey proteins with the casein micelles (Pan et al., 2022). In contrast, whey proteins in micelle phase showed a different pattern; that is, α-LA and β-LG increased during the first 120 s and then decreased upon further heating (Fig. 5B2 and B3). This indicated that a proportion of whey proteins were associated with the casein micelles initially, and those casein micelles with associated whey proteins formed sufficiently large aggregates to sediment on ultracentrifugation when the heating time was further extended. These results match previous studies of Pan et al. (2022) and Singh (2004), who reported that the aggregation of casein micelles in SSM could be attributed to the bridging effect of the whey protein on the surface of the casein micelles. Further studies are required to verify the bridging effect of whey proteins that are coated on the surface of casein micelles.

Interestingly, by comparing the whey proteins between the serum phase (Fig. 5A2 and A3) and the micelle phase (Fig. 5B2 and B3), not all the serum-phase whey proteins associated with the casein micelles after heat treatment, but the unassociated whey proteins were still centrifuged down by ultracentrifugation. This confirmed that parts of denatured whey proteins might form large aggregates by themselves during heating and that the whey protein aggregates were sufficiently large to sediment on ultracentrifugation. This finding is consistent with other research on cows' milk, which showed that the sedimentable whey protein is either associated with casein micelles or in whey protein aggregates that are sufficiently large to sediment on ultracentrifugation (Crowley et al., 2015; Oldfield, Singh, Taylor, & Pearce, 2000).

Although the SSM at pH 6.9 had intermediate values for ionic calcium concentration, the proportion of κ-CN on the casein micelles and the level of dissociation of κ-CN from the casein micelles compared with the SSMs at pH 6.8 and pH 7.0, the SSM at pH 6.9 still showed the maximum heat stability (Fig. 1). Previous studies have stated that the heat instability at more acidic pHs of milk could be due to the bridging effect of the high concentration of ionic calcium among the casein micelles and that the heat instability at more basic pHs of milk could be due to the greater dissociation of κ-CN (Dumpler, 2018; Singh, 2004). The maximum heat stability of the SSM at pH 6.9 might be linked to the level of ionic calcium in the serum phase and the level of dissociation of κ-CN that alters the steric and electrostatic interactions and maintain the stability of casein micelles.

3.6. Formation of κ-CN/whey protein complexes

The levels of κ-CN, α-LA and β-LG in the κ-CN/whey protein complexes are shown in Fig. 6. The levels of κ-CN, α-LA and β-LG increased significantly ($P < 0.05$) at all pH values when the SSM was

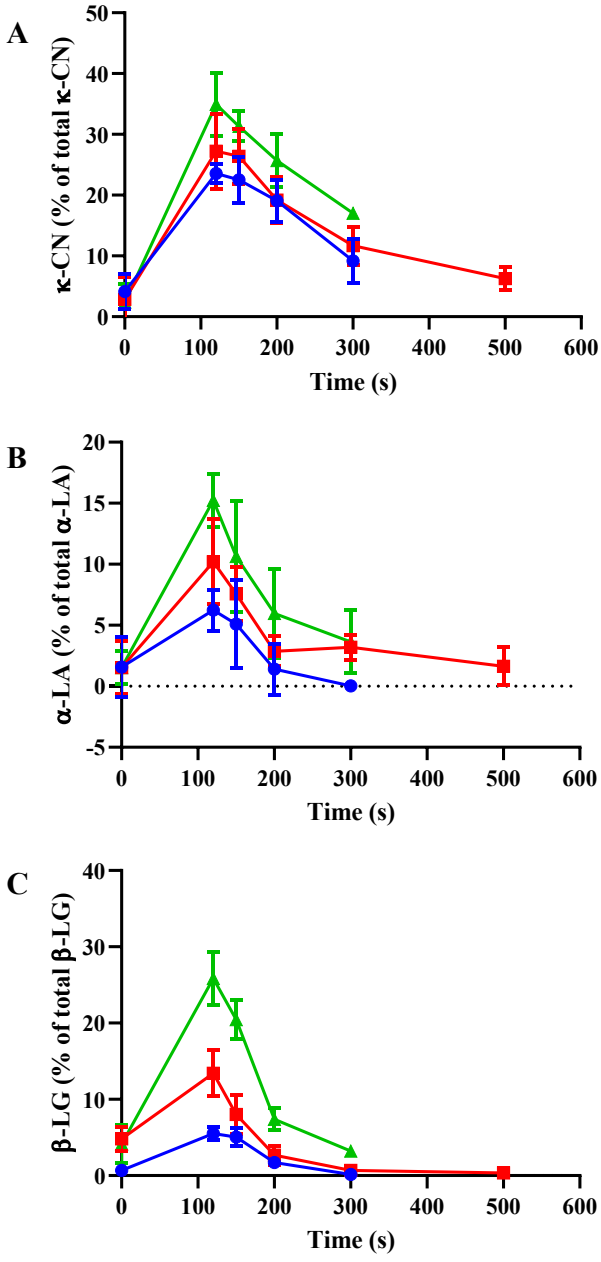

Fig. 6. Changes in the levels of κ-casein (κ-CN), α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) in κ-CN/whey protein complexes of sheep skim milk at pH 6.8 (●), pH 6.9 (■), and pH 7.0 (▲) and heated at 140 °C for different times.

heated for 120 s, followed by a decrease with longer heating. The levels of κ-CN, α-LA and β-LG in the SSM were highest at pH 7.0 and lowest at pH 6.8, and followed the same order throughout the period of heating. This indicated that increasing amounts of κ-CN/whey protein complexes formed in the SSM at the early stage of heating; these newly formed complexes might either reassociate with the casein micelles or form larger κ-CN/whey protein complex aggregates upon further heating; raising the pH of the SSM increased the proportion of κ-CN/whey protein complexes in the serum phase. Previous studies have shown that the most denatured β-LG is observed in the serum phase and is associated with κ-CN when the pH of the milk is higher than pH 6.8; in contrast, at pH lower than 6.8, the denatured β-LG is present on the surface of casein micelles (Deeth & Lewis, 2017; Kudo, 1980; Singh & Latham, 1993). These findings suggested that the preferential association of whey proteins with the casein micelles at lower pH may have been due to the higher level of κ-CN at the surface of the casein micelles, whereas more whey proteins in the serum phase at higher pH could be attributed to the increased amount of dissociated κ-CN in the serum phase during heating.

Based on the results for colloidally-stable micelle phase (Fig. 5B1–B3), only κ-CN showed an increasing trend on prolonged heating; the levels of α-LA and β-LG decreased. We can infer that the newly formed κ-CN/whey protein complexes aggregated with either other complexes or whey proteins instead of reassociating with the casein micelles; only a small proportion of dissociated κ-CN that was not complexed with whey proteins might reassociate with the casein micelles at a longer heating time. It is therefore likely that the complexation of κ-CN with denatured whey proteins in the serum phase sensitised the casein micelles to the destabilising effect of heat-induced calcium phosphate precipitation or the exposure of hydrophobic groups and promoting the aggregation of casein micelles.

4. Conclusions

This study examined the heat stability of SSM at 140 °C and discussed the protein interactions in SSM at pHs 6.8–7.0 at different heating times ranging from 0 to 500 s. The results showed that SSM had maximum heat stability at pH 6.9 and became increasingly unstable at higher and lower pH values, and confirmed that sheep's milk has lower heat stability than cows' milk, by comparison with the HCT–pH profile reported previously for cows' milk. The aggregates formed in the SSM during heating were composed mainly of κ-CN-depleted casein micelles and whey proteins. The occurrence of maximum heat stability at pH 6.9 was likely due to the relatively low ionic calcium concentration and low κ-CN dissociation, which keep the casein micelle relatively stable.

In comparison with previously reported results on cows' milk, lower proportions of κ-CN in the casein micelles and higher ionic calcium concentrations were observed in the SSM, and the κ-CN of the SSM dissociated from the casein micelles to a greater extent under the same heating conditions. These differences observed in SSM could contribute to the easy aggregation of casein micelles. These findings suggest that the casein micelles with a low level of κ-CN, the vulnerable connection of κ-CN with the casein micelles and the high ionic calcium concentration may be responsible for the lower heat stability of sheep's milk than of cows' milk.

Declaration of competing interest

None.

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References

Anema, S. G. (1998). Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120 °C. *Journal of Agricultural and Food Chemistry, 46*, 2299–2305.

Anema, S. G. (2008). On heating milk, the dissociation of κ-casein from the casein micelles can precede interactions with the denatured whey proteins. *Journal of Dairy Research, 75*, 415–421.

Anema, S. G. (2018). Age gelation, sedimentation, and creaming in UHT milk: A review. *Comprehensive Reviews in Food Science and Food Safety, 18*, 140–166.

Anema, S. G., & Klostermeyer, H. (1997). Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100 °C. *Journal of Agricultural and Food Chemistry, 45*, 1108–1115.

Anema, S. G., & Li, Y. (2000). Further studies on the heat-induced, pH-dependent dissociation of casein from the micelles in reconstituted skim milk. *LWT – Food Science and Technology, 33*, 335–343.

Balthazar, C. F., Pimentel, T. C., Ferrão, L. L., Almada, C. N., Santillo, A., Albenzio, M., et al. (2017). Sheep milk: Physicochemical characteristics and relevance for functional food development. *Comprehensive Reviews in Food Science and Food Safety, 16*, 247–262.

Bobe, G., Beitz, D. C., Freeman, A. E., & Lindberg, G. L. (1998). Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 46*, 458–463.

Chandrapala, J., McKinnon, I., Augustin, M. A., & Udabage, P. (2010). The influence of milk composition on pH and calcium activity measured in situ during heat treatment of reconstituted skim milk. *Journal of Dairy Research, 77*, 257–264.

Chen, B., Grandison, A. S., & Lewis, M. J. (2015). Effect of seasonal variation on some physical properties and heat stability of milk subjected to ultra-high temperature and in-container sterilisation. *Food Chemistry, 181*, 227–234.

Crowley, S. V., Boudin, M., Chen, B., Gazi, I., Huppertz, T., Kelly, A. L., et al. (2015). Stability of milk protein concentrate suspensions to in-container sterilisation heating conditions. *International Dairy Journal, 50*, 45–49.

Dalgleish, D. G. (1992). Sedimentation of casein micelles during the storage of ultra-high temperature milk products—a calculation. *Journal of Dairy Science, 75*, 371–379.

Davies, D. T., & White, J. C. D. (1966). The stability of milk protein to heat: I. Subjective measurement of heat stability of milk. *Journal of Dairy Research, 33*, 67–81.

De La Fuente, M. A., Olano, A., & Juárez, M. (2002). Mineral balance in milk heated using microwave energy. *Journal of Agricultural and Food Chemistry, 50*, 2274–2277.

Deeth, H., & Lewis, M. (2016). Protein stability in sterilised milk and milk products. In P. L. H. McSweeney, & J. A. O'Mahony (Eds.), *Advanced dairy chemistry. Vol. 1B. Proteins: Applied aspects* (4th ed., pp. 247–286). New York, NY, USA: Springer.

Deeth, H., & Lewis, M. J. (2017). *High temperature processing of milk and milk products*. Hoboken, NJ, USA: John Wiley & Sons.

Dumpler, J. (2018). *Heat stability of concentrated milk systems: Kinetics of the dissociation and aggregation in high heated concentrated milk systems*. Wiesbaden, Germany: Springer Spektrum.

Dumpler, J., Huppertz, T., & Kulozik, U. (2020). Heat stability of milk and concentrated milk: Past, present, and future research objectives. *Journal of Dairy Science, 103*, 10986–11007.

Dumpler, J., Wohlschläger, H., & Kulozik, U. (2017). Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. *Dairy Science & Technology, 96*, 807–826.

Dyson, N. A., & Smith, R. M. (1998). *Chromatographic integration methods*. Cambridge, UK: Royal Society of Chemistry, Information Services.

Fox, P. F. (1981). Heat-induced changes in milk preceding coagulation. *Journal of Dairy Science, 64*, 2127–2137.

Fox, P. F., & Hoynes, M. C. T. (1976). Heat stability characteristics of ovine, caprine and equine milks. *Journal of Dairy Research, 43*, 433–442.

Gaur, V., Schalk, J., & Anema, S. G. (2018). Sedimentation in UHT milk. *International Dairy Journal, 78*, 92–102.

Geerts, J., Bekhof, J., & Scherjon, J. (1983). Determination of calcium ion activities in milk with an ion-selective electrode. A linear relationship between the logarithm of time and the recovery of the calcium ion activity after heat treatment. *Netherlands Milk and Dairy Journal, 37*, 197–211.

Grant, D. W., & Clarke, A. (1971). Systematic study of the quantitative effects of instrument control on analytical precision in flame ionization gas chromatography. *Analytical Chemistry, 43*, 1951–1957.
Ho, Q. T., Murphy, K. M., Drapala, K. P., O'Callaghan, T. F., Fenelon, M. A., O'Mahony, J. A., et al. (2018). Effect of pH and heat treatment on viscosity and heat coagulation properties of milk protein concentrate. *International Dairy Journal, 85*, 219–224.
Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada (Ed.), *Proteins in food processing* (2nd ed., pp. 49–92). Sawston, UK: Woodhead Publishing.
Kadjo, A. F., Dasgupta, P. K., Su, J., Liu, S., & Kraiczek, K. G. (2017). Width based quantitation of chromatographic peaks: Principles and principal characteristics. *Analytical Chemistry, 89*, 3884–3892.
Kudo, S. (1980). The heat stability of milk: Formation of soluble proteins and protein-depleted micelles at elevated temperatures. *New Zealand Journal of Dairy Science and Technology, 15*, 255–263.
Lewis, M. J. (2011). The measurement and significance of ionic calcium in milk – A review. *International Journal of Dairy Technology, 64*, 1–13.
Lewis, M., Grandison, A., Lin, M. J., & Tsioulpas, A. (2011). Ionic calcium and pH as predictors of stability of milk to UHT processing. *Milchwissenschaft, 66*, 197–200.
Li, S., Delger, M., Dave, A., Singh, H., & Ye, A. (2022). Seasonal variations in the composition and physicochemical characteristics of sheep and goat milks. *Foods, 11*. Article 1737.
Lin, M.-J. (2002). *Measurement of ionic calcium and its role in milk stability* (PhD Thesis). Reading, UK: University of Reading.
Li, S., Ye, A., & Singh, H. (2019). Seasonal variations in composition, properties, and heat-induced changes in bovine milk in a seasonal calving system. *Journal of Dairy Science, 102*, 7747–7759.
Martinez Alonso, S., Magdalena Vera, J., Mendez Donega, J., & Berterreche Alvarez, J. (2009). *Method for UHT treatment of milk with a high protein content*. Patent PCT/ES2007/000109. Zamora, Spain: S. L. Leche Gaza.
Mclean, D. M., Graham, E. R. B., Ponzoni, R. W., & Mckenzie, H. A. (1987). Effects of milk protein genetic variants and composition on heat stability of milk. *Journal of Dairy Research, 54*, 219–235.
Meyer, V. R. (1995). Error sources in the determination of chromatographic peak size ratios. *Advances in Chromatography, 35*, 383–416.
Morand, M., Guyomarc'h, F., Pezennec, S., & Famelart, M.-H. (2011). On how κ-casein affects the interactions between the heat-induced whey protein/κ-casein complexes and the casein micelles during the acid gelation of skim milk. *International Dairy Journal, 21*, 670–678.
Muir, D., & Tamime, A. (1993). Ovine milk: 3. Effect of seasonal variations on the properties of set and stirred yoghurts. *Milchwissenschaft, 48*, 509–513.
O'Connell, J. E., & Fox, P. F. (2000). The two-stage coagulation of milk proteins in the minimum of the heat coagulation time-ph profile of milk: Effect of casein micelle size. *Journal of Dairy Science, 83*, 378–386.
O'Connell, J. E., & Fox, P. F. (2003). Heat-induced coagulation of milk. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Vol. 1. Proteins* (3rd ed., pp. 879–945). New York, NY, USA: Springer.
Oldfield, D. J., Singh, H., Taylor, M. W., & Pearce, K. N. (2000). Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. *International Dairy Journal, 10*, 509–518.
Pan, Z., Ye, A., Dave, A., Fraser, K., & Singh, H. (2022). Kinetics of heat-induced interactions among whey proteins and casein micelles in sheep skim milk and aggregation of the casein micelles. *Journal of Dairy Science, 105*, 3871–3882.
Pan, Z., Ye, A., Dave, A., Fraser, K., & Singh, H. (2023). pH-dependent sedimentation and protein interactions in ultra-high-temperature-treated sheep skim milk. *Journal of Dairy Science, 106*(3), 1626–1637.
Pyne, G. T., & McHenry, K. A. (1955). The heat coagulation of milk. *Journal of Dairy Research, 22*, 60–68.
Raynal-Ljutovac, K., Park, Y. W., Gaucheron, F., & Bouhallab, S. (2007). Heat stability and enzymatic modifications of goat and sheep milk. *Small Ruminant Research, 68*, 207–220.
Silanikove, N., Shapiro, F., & Shamay, A. (2003). Use of an ion-selective electrode to determine free Ca ion concentration in the milk of various mammals. *Journal of Dairy Research, 70*, 241–243.
Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology, 57*, 111–119.
Singh, H., & Latham, J. M. (1993). Heat stability of milk: Aggregation and dissociation of protein at ultra-high temperatures. *International Dairy Journal, 3*, 225–237.
Snyder, L. R. J. (1972). A rapid approach to selecting the best experimental conditions for high-speed liquid column chromatography. Part I—estimating initial sample resolution and the final resolution required by a given problem. *Journal of Chromatographic Science, 10*, 200–212.
Zhang, Z. P., & Aoki, T. (1996). Behaviour of calcium and phosphate in bovine casein micelles. *International Dairy Journal, 6*, 769–780.