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Acidification of lactoferrin-casein micelle complexes in skim milk

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ABSTRACT

Lactoferrin electrostatically bound to the casein micelles when added to milk, which caused the absolute zeta potential to decrease and the micelle size to increase. On acidification, the lactoferrin progressively dissociated from the micelles, which, at pH below ~5.0, caused the zeta potential of the casein micelles to be the same as those in milk without added lactoferrin. Acidification caused increased levels of casein to dissociate from the casein micelles at ~pH 5.0 as the level of added lactoferrin in the milk increased. Lactoferrin decreased the pH at which the milk gelled and caused the G′ and yield stress of the set gels to increase at low levels of added lactoferrin, decrease at intermediate levels of added lactoferrin and increase again at high levels of added lactoferrin. This unusual effect of lactoferrin on gelation was hypothesised to be due to combined effects of dissociated casein and the lower gelation pH.

1. Introduction

Lactoferrin is found at low levels (~0.02%) in bovine milk, but at higher levels in human milk (0.1%). Lactoferrin is a basic glycoprotein with molecular mass of about 80 kDa. The tertiary structure of lactoferrin provides two homologous domains each with an iron binding site, although in its natural state only about 15% of the available sites have ferric ions bound. The bound iron gives lactoferrin solutions a distinct pink colouration, whereas apo-lactoferrin solutions are colourless and holo-lactoferrin solutions are blood red. Lactoferrin is a basic protein with an isoelectric pH of about 8.7; thus, at the natural pH of milk, lactoferrin is positively charged. Lactoferrin is reported to have numerous biological functions including immuno-regulatory, anti-microbial, anti-viral, anti-fungal, anti-tumour, and anti-inflammatory properties (Farnaud & Evans, 2003; Jenssen & Hancock, 2009; Kanyshkova, Buneva, & Nevinsky, 2001; Lonnerdal, 2003; Shimazaki, 2000; Steijns & van Hooijdonk, 2000).

The casein proteins in skim milk are found in the form of casein micelles composed of the four casein gene products associated into the macromolecular micelle complexes via colloidal calcium phosphate linkages and hydrophobic interactions (Dalgleish, 2011; de Kruif & Holt, 2003; de Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, 1992; Horne, 1998, 2006; Huppertz et al., 2017; Walstra, 1990, 1999). κ-Casein is found at the surface of the casein micelles, with the para-κ-casein region associated with the micelle core and the glyco-macro-peptide region protruding from the surface as a hair, which provides considerable steric stability to the micelles (Dalgleish, 2011; Horne, 1998; Walstra, 1990). As the caseins are acidic proteins, at the natural pH of milk the casein micelles are negatively charged; however, this charge diminishes as the pH is reduced towards the caseins isoelectric point at about pH 4.6 (Anema & Klostermeyer, 1996; Schmidt & Poll, 1986).

In milk, most of the natural lactoferrin is bound to the casein micelles (Anema & de Kruif, 2011; Croguennec, Li, Phelebon, Garnier-Lambrouin, & Gésan-Guiziou, 2012). When further bovine lactoferrin is titrated into the skim milk, high levels, up to about 1.25% (w/v), can bind to the casein micelles (Anema & de Kruif, 2011, 2012, 2013; Croguennec et al., 2012). As lactoferrin is positively charged and the casein micelles are negatively charged at the natural pH of milk, the interaction between these was electrostatic in nature. The binding followed a Langmuir adsorption isotherm (Anema & de Kruif, 2011, 2012), and subsequent studies showed that the binding was predominantly at the surface of the casein micelles (Anema & de Kruif, 2013). The binding caused an increase in the size of the casein micelles as well as a reduction in the negative charge of the casein micelles, which is consistent with an electrostatic interaction (Anema & de Kruif, 2011, 2012).

On prolonged storage, the binding of lactoferrin to the casein micelles caused the casein micelle to dissociate releasing casein and lactoferrin to the serum, resulting in a decrease in the size of the casein micelles and the turbidity of the milk. These changes

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were dependent on the level of lactoferrin bound to the casein micelles and the temperature of storage of the complex after interaction. The disintegration of the casein micelles was more rapid at higher levels of added lactoferrin and at higher storage temperatures. At high lactoferrin levels and after long storage periods at moderate temperatures (20–40 °C), the milk had become distinctly transparent (Anema & de Kruif, 2011, 2012). Lactoferrin still bound to casein micelles cross-linked by transglutaminase, but the cross-linking prevented the disintegration of lactoferrin/casein micelle complex (Anema & de Kruif, 2012).

The interaction of lactoferrin with the casein micelles in milk changes the physical properties of the casein micelles such as their size, zeta potential and their colloidal integrity. As a consequence, this binding may affect the functional properties of the milk, and therefore this study examined the properties of the milk and the gels formed during the acidification of skim milk that had different levels of added lactoferrin. The changes in zeta potential, size, serum phase proteins and rheological properties were monitored during acidification.

2. Materials and methods

2.1. Lactoferrin and skim milk solutions

Lactoferrin (>90% purity; supplied by the Fonterra Cooperative Group, Auckland, New Zealand) was dissolved in water to give a solution containing 8.75% (w/w) lactoferrin, with the protein concentration determined using UV absorbance at 280 nm and known extinction coefficients (Castellino, Fish, & Mann, 1970; Shimazaki, 2000). Reconstituted skim milk of 18.2% (w/w) total solids was prepared from low heat skim milk powder (Fonterra Cooperative Group, Auckland, New Zealand) and water. A small amount of sodium azide (0.02%, w/v) was added to the lactoferrin and skim milk solutions and these solutions were stirred for about 6 h at ambient temperature (~20 °C) and then stored for a minimum of 12 h at 4 °C before use. The skim milk solution, lactoferrin solution and water were mixed to give solutions containing 10% skim milk solids and 0–4% added lactoferrin. These mixtures were allowed to react for 4 h at 4 °C before analysis.

2.2. Permeate collection

A control skim milk sample (no added lactoferrin) was adjusted to different pH in the range from 6.9 to 3.9 with 1 M NaOH and 1 M HCl. A sample of permeate from the milk at each pH was obtained by ultrafiltration using a 10 kDa membrane (Prep/Scale TFF 1 ft^2 cartridge, polyethersufone; Millipore Corporation, Bedford, MA, USA). The milk samples close to the isoelectric point of casein (~pH 4.6) coagulated during pH adjustment, and these were centrifuged at 4600 $\times g$ to deposit the casein and the supernatants were ultrafiltered to give a sample of permeate.

2.3. Zeta potential measurements

For the milk samples at the natural pH, the samples (20 μL) were dispersed in either 5 mL of permeate or 5 mL of calcium imidazole buffer (5 mM calcium chloride, 30 mM imidazole, 30 mM NaCl, pH 7.0) before zeta potential measurement. For the milk samples at different pH, the milk samples were slowly acidified with glucono-δ-lactone (GDL) at a 2.2% (w/v) level and held at 30 °C. The pH was monitored during acidification and when the pH was at the desired level, a subsample of the milk (20 μL) was placed in 5 mL of permeate (Section 2.2) that was at a similar pH. The zeta potential of the samples were measured using a Malvern Zetasizer Nano ZS instrument and the associated disposable folded capillary cells (Malvern Instruments, Malvern, Worcestershire, UK) using the methods reported previously (Anema & Klostermeyer, 1996).

2.4. Particle size measurements

The particle sizes in the milk samples were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments) and disposable plastic cuvettes. For the milk samples at the natural pH, the samples (20 μL) were dispersed in 5 mL of the calcium imidazole buffer (5 mM calcium chloride, 30 mM imidazole, 30 mM NaCl, pH 7.0) before particle sizing measurements at 20 °C. For measuring particle sizes during acidification, GDL (2.2%, w/w) was added to the milk sample, the sample gently mixed and then placed in the measuring cell of the instrument without dilution. The temperature of the cell was maintained at 30 °C, and the measurement position was 0.25 mm inside the front surface of the cell. The sizes were measured every 100 s until the milk gelled. The aggregation time was considered the point where the size started continuously increasing. The details and methodology of this sizing technique have been described previously (Anema & Li, 2003).

2.5. Centrifugation and electrophoresis

The milk samples had different levels of GDL added to attain pH ranging from the natural pH (~pH 6.5) to ~pH 4.0 after 3 h at 30 °C. The samples were then centrifuged (~27, 000 $\times g$, 25 °C, 1 h, in a bench centrifuge) and the supernatants were collected. The level and composition of protein in the original solutions and their respective supernatants were determined by microfluidic chip sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as has been described previously (Anema, 2009b).

2.6. Rheological measurements

The milk samples were slowly acidified using GDL (2.2%, w/w) at 30 °C to form acid gels. The rheological properties of the milk samples during and after acidification were determined using an AR2000 rheometer (TA Instruments UK, Cirencester, UK) equipped with a cone (4 cm, 4°) and plate geometry. The milk and GDL were mixed together and a subsample (~1.2 mL) was placed on the rheometer plate. The cone was lowered into position and the water trap/cover arrangement was placed over the samples to minimise evaporation.

The first measurements monitored the rheological properties of the sample during acidification at 30 °C, with the oscillation frequency set to 0.1 Hz and the strain set to less than 0.5%. The storage modulus (stiffness or G′) was measured every 30 s for a total of 3 h. After this gelation phase was completed, the rheological properties of the set gel were monitored as the temperature was reduced from 30 °C to 5 °C. Once the temperature was at 5 °C, the large deformation properties of the set gel were measured by progressively increasing the strain at a constant shear rate of 0.005 s^{-1} until the gel yielded. From this measurement, the yield stress and yield strain were obtained.

2.7. pH measurement during acidification

GDL was added to milk samples with 0 or 4% added lactoferrin. The samples were gently mixed and placed in a water bath set to 30 °C. The pH was monitored using a standard combination glass pH electrode and associated meter, with a two-point calibration (pH 4 and 7) carried out at the measurement temperature of 30 °C. The pH versus time curves were used to calculate the aggregation pH from the change in size with time from the particle sizing

experiments and the gelation pH from the change in storage modulus (G′) with time from the rheology experiments.

3. Results

3.1. Lactoferrin binding to casein micelles at the natural pH of milk

When lactoferrin was added to milk, it was found to bind to the casein micelles (Fig. 1A), and the binding followed a Langmuir adsorption isotherm, which is consistent with previous reports (Anema & de Kruif, 2011, 2012, 2013). When measured within about six hours of addition, the binding of lactoferrin caused an increase in casein micelle diameter (Fig. 1B), that was correlated with the level of added lactoferrin (Fig. 1D). The increase in size was due to the swelling of the casein micelles induced by the binding of the lactoferrin (Anema & de Kruif, 2011, 2012). Holding the sample for longer periods caused the disintegration of the micelles, although the size increase and disintegration could be reduced by holding the samples at lower temperatures (~4 °C) and could be eliminated by cross-linking the casein micelles before adding the lactoferrin (Anema & de Kruif, 2011, 2012).

The zeta potential will be discussed as absolute values and denoted as |zeta potential|, thus a lower |zeta potential| means that the zeta potential is closer to zero, whereas a higher |zeta potential| means that the zeta potential is further away from zero. The binding of lactoferrin to the casein micelles reduced the |zeta potential|. The lactoferrin has been shown to bind preferentially to the surface of the casein micelles, thus the decrease in |zeta potential| is presumably due to the positively charged lactoferrin titrating the charges on the surface of the negatively charged casein micelles, particularly the κ-casein (Fig. 1C). The effect was greater when the zeta potential was measured in calcium-imidazole buffer than in milk permeate, although the zeta potential in both buffers were correlated with the level of added lactoferrin (Fig. 1E). The ionic composition and ionic strengths of in the two dispersants were different, with a lower ionic strength and a less complex mineral composition for the calcium imidazole buffer than the milk

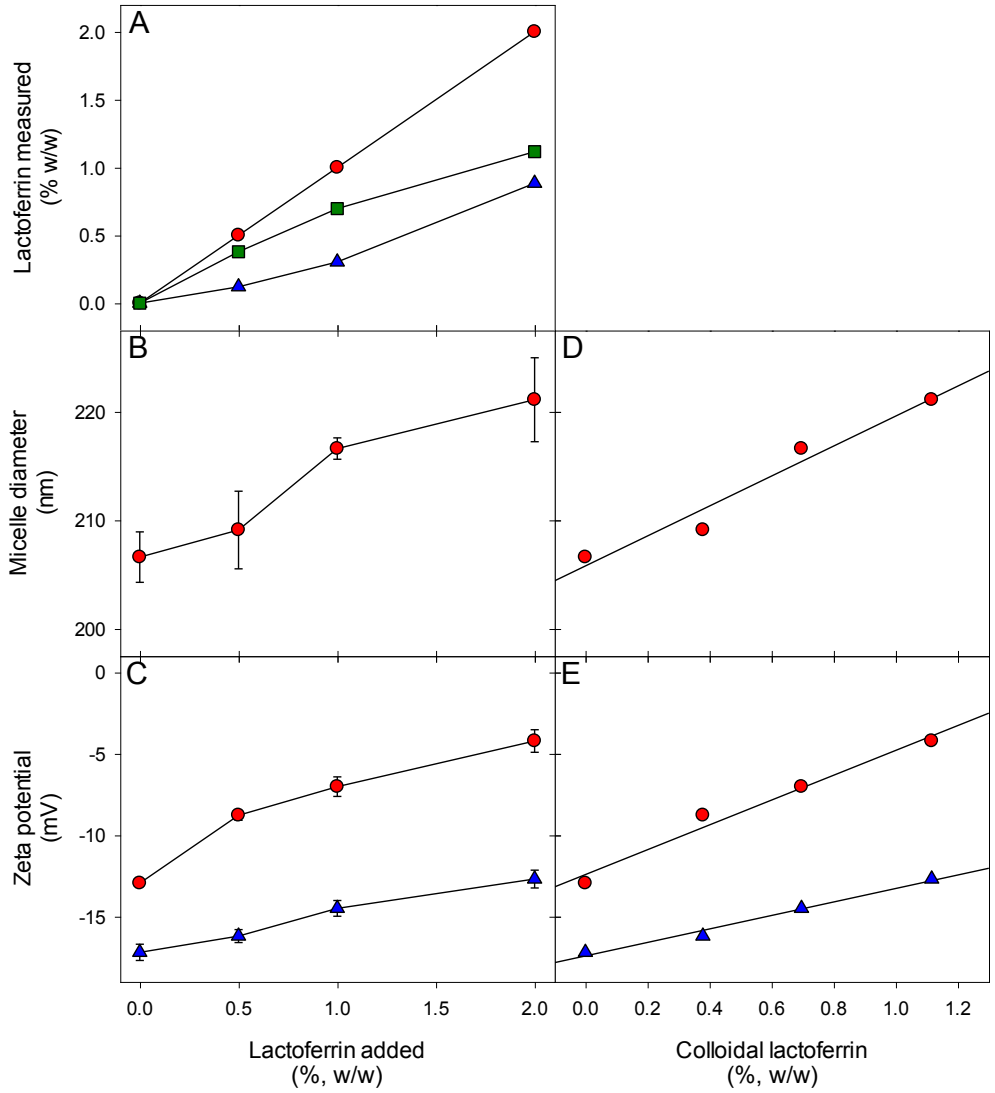

Fig. 1. Panel A: level of added lactoferrin to milk (●), level of lactoferrin in the serum phase (▲) and level of lactoferrin bound to the casein micelles (■). Panel B: casein micelle sizes for skim milk samples with different levels of added lactoferrin. Error bars represent the standard deviations of three repeated measurements. Panel C: zeta potential of casein micelles in skim milk with different levels of added lactoferrin as measured in Ca-imidazole buffer (●) or milk permeate (▲). Error bars represent the standard deviations of five repeated measurements. Panel D: relationship between the casein micelle size and level of colloidal lactoferrin. Panel E: relationship between the zeta potential, as measured in Ca-imidazole buffer (●) or milk permeate (▲), and level of colloidal lactoferrin.

permeate (Holt, 1985), which probably accounts for the differences in |zeta potential| between these buffer systems.

3.2. Zeta potential during acidification

The milk was slowly acidified with GDL (2.2%, w/w) and subsamples were taken as the pH declined. These were dispersed in milk permeate taken at a similar pH and the zeta potentials were determined (Fig. 2). For each sample, the |zeta potential| progressively declined with pH; however, at pH above ~5.0, the |zeta potential| was lower as the level of added lactoferrin increased. The zeta potential curves began to merge at pH below about 6.0 and at pH 5.0 or lower all curves were similar regardless of the level of added lactoferrin. Interestingly, the iso-electric point where the zeta potential was zero was at about pH 4.2, significantly lower than the pH 4.6 reported in the literature for the isoelectric point of casein in milk. This lower experimental iso-electric point has been reported previously (Guyomarc'h, Renan, Chatriot, Gamerre, & Famelart, 2007).

3.3. Size changes during acidification

The size changes of the casein micelles during the acidification of the milk with added GDL was monitored. The rate of acidification of the milk was unaffected by the addition of lactoferrin indicating that the added lactoferrin had little buffering effect in milk (Fig. 3A). As the buffering of milk is predominantly due to the milk salts (especially citrate, serum phosphate and colloidal phosphate) and the casein proteins (Holt, 1985; Lucey, Hauth, Gorry, & Fox, 1993), it is not surprising that lactoferrin has no effect on the acidification of the milk by GDL. As the initial particle sizes were dependent on the level of added lactoferrin (Fig. 1B, D), the change in size during acidification when compared with the initial size for each sample was monitored (Fig. 3A). As the pH of the milk declined, the size of the casein micelles initially decreased, and then after a certain time/pH was reached (the aggregation time/aggregation pH), the size dramatically increased as the casein micelles aggregated. The aggregation time increased and aggregation pH decreased markedly as the level of added lactoferrin was increased (Fig. 3B). For the milk sample without lactoferrin, the aggregation time was about 16 min, which corresponded to an aggregation pH of 5.37. However, when 2.0% of lactoferrin was added to the milk before acidification, the aggregation time increased to about 50 min, which corresponded to an aggregation pH of 4.92.

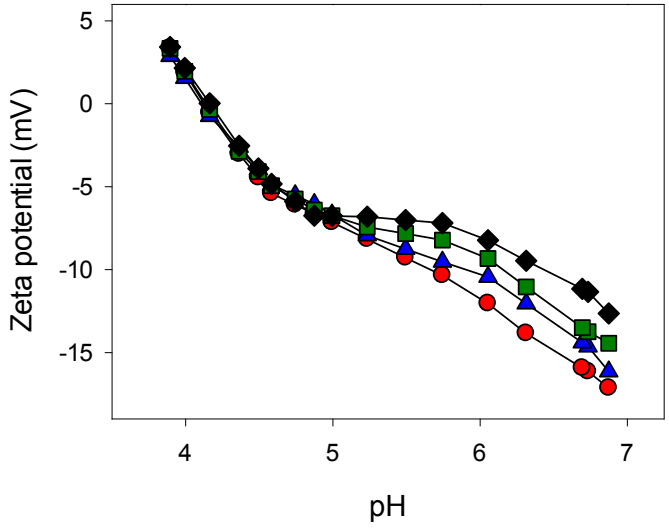

Fig. 2. Zeta potential with pH for casein micelles in milk with 0% (●), 0.5% (▲) 1% (■) and 2% (◆) added lactoferrin; the results represent the average of five repeated measurements.

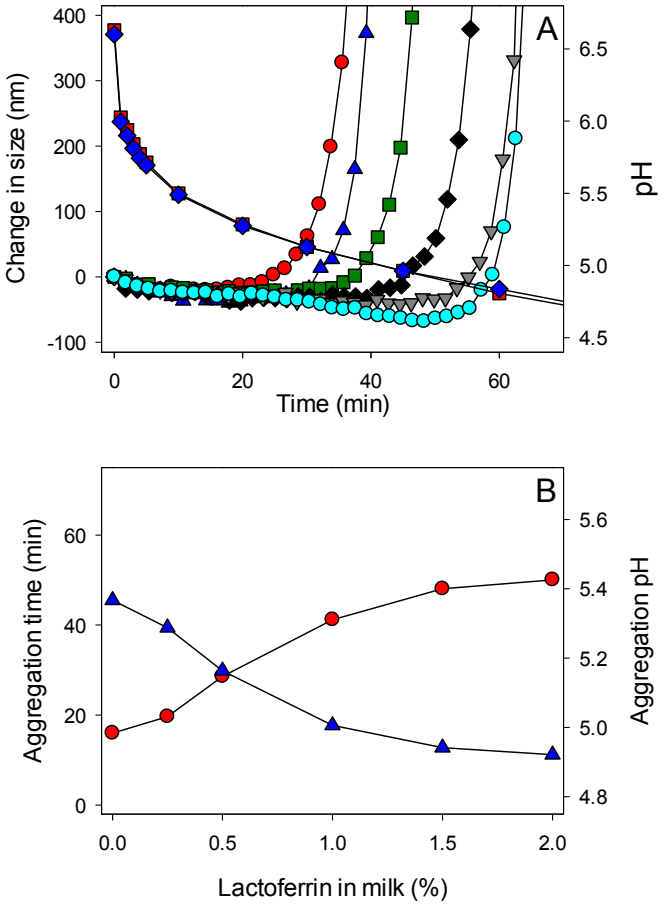

Fig. 3. A: Change in casein micelle size and pH for milk samples with 2.2% w.w-1 added GDL. The milk samples had 0% (●, ■), 0.25% (▲), 0.5% (■), 1.0% (◆), 1.5% (▼) or 2.0% (●, ◆) added lactoferrin. B: Aggregation time (●) and aggregation pH (▲) for skim milk samples with different levels of added lactoferrin.

3.4. Dissociation of casein-lactoferrin during acidification

Subsamples of milk were taken during acidification and the level of serum protein was determined. The electrophoresis traces of the milk and the serums for the samples with 0, 1 and 2% added lactoferrin at different pH are shown in Fig. 4A–C respectively. From the electrophoresis traces, the level of lactoferrin (Fig. 4D), casein (Fig. 4E) and α-lactalbumin and β-lactoglobulin combined (Fig. 4F) in the serum was determined. All of the available α-lactalbumin and β-lactoglobulin was in the serum phase regardless of the pH or the level of added lactoferrin (Fig. 4A–C, F). For the caseins, low levels were in the serum at pH 6.7 and at pH below 4.75 regardless of the level of added lactoferrin. At intermediate pH between 5.75 and 4.75, some casein was transferred to the serum phase, with the level increasing as the level of added lactoferrin in the milk was increased. The maximum dissociation was observed in the sample with 2.0% added lactoferrin and at pH 5.0 (Fig. 4A–C, E).

For the samples with added lactoferrin, the level of lactoferrin in the serum generally increased as the pH declined, with almost all added lactoferrin in the serum at pH 4.5 or below. A slightly unusual behaviour was observed at pH between 5.5 and 4.75, where the level of serum phase lactoferrin remained constant or slightly

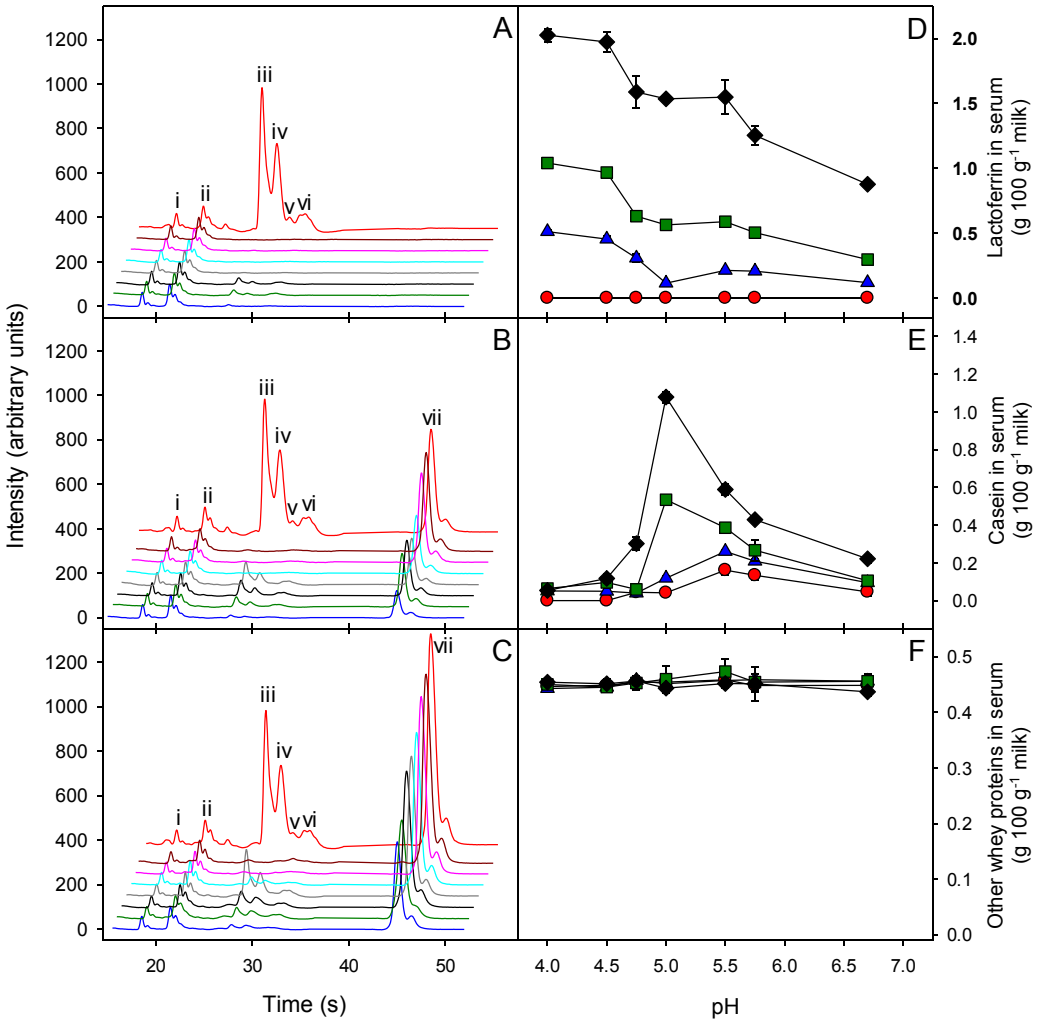

Fig. 4. Electrophoretic traces of milk and serum samples from skim milk with 0% (A), 1% (B) and 2% (C) added lactoferrin. The traces from top to bottom are milk (red) and serum obtained from the milk at ~pH 6.70, 5.75, 5.50, 5.00, 4.75, 4.50 and 4.00. The peaks are α-lactalbumin (i), β-lactoglobulin (ii), β-casein (iii), α_{S1}-casein (iv), α_{S2}-casein (v), κ-casein (vi) and lactoferrin (vii). Lactoferrin (D), casein (E) and other whey proteins (F) in serum phase for samples with 0% (●), 0.5% (▲) 1% (■) and 2% (◆) added lactoferrin. Error bars represent standard deviations of triplicate measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decreased at the region where the casein was dissociated from the casein micelles. This suggests that slightly higher levels of lactoferrin bound to the casein micelles in this pH range where the caseins were dissociating. A possible explanation for this increased binding may be the swelling of the casein micelles at these pH values. In previous studies, it was shown that the lactoferrin bound predominantly to the surface of the casein micelles, whereas the lysozyme bound to the interior of the casein micelles (Anema & de

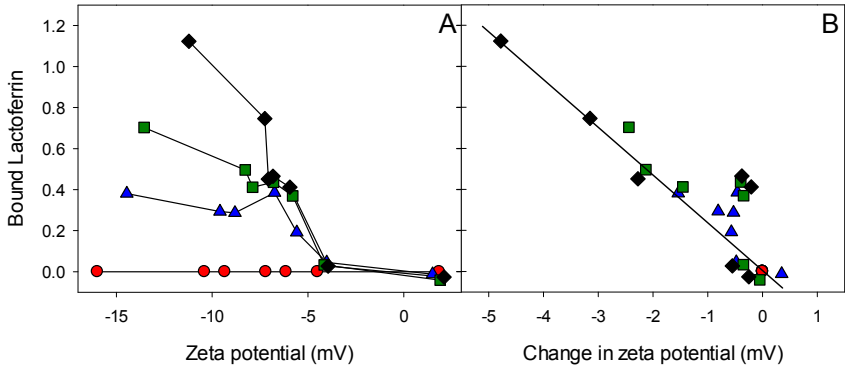

Fig. 5. Relationship between bound lactoferrin and zeta potential (A) or the difference in zeta potential between the sample without added lactoferrin and the sample with added lactoferrin at each pH (B). The samples had 0% (●), 0.5% (▲) 1% (■) and 2% (◆) added lactoferrin.

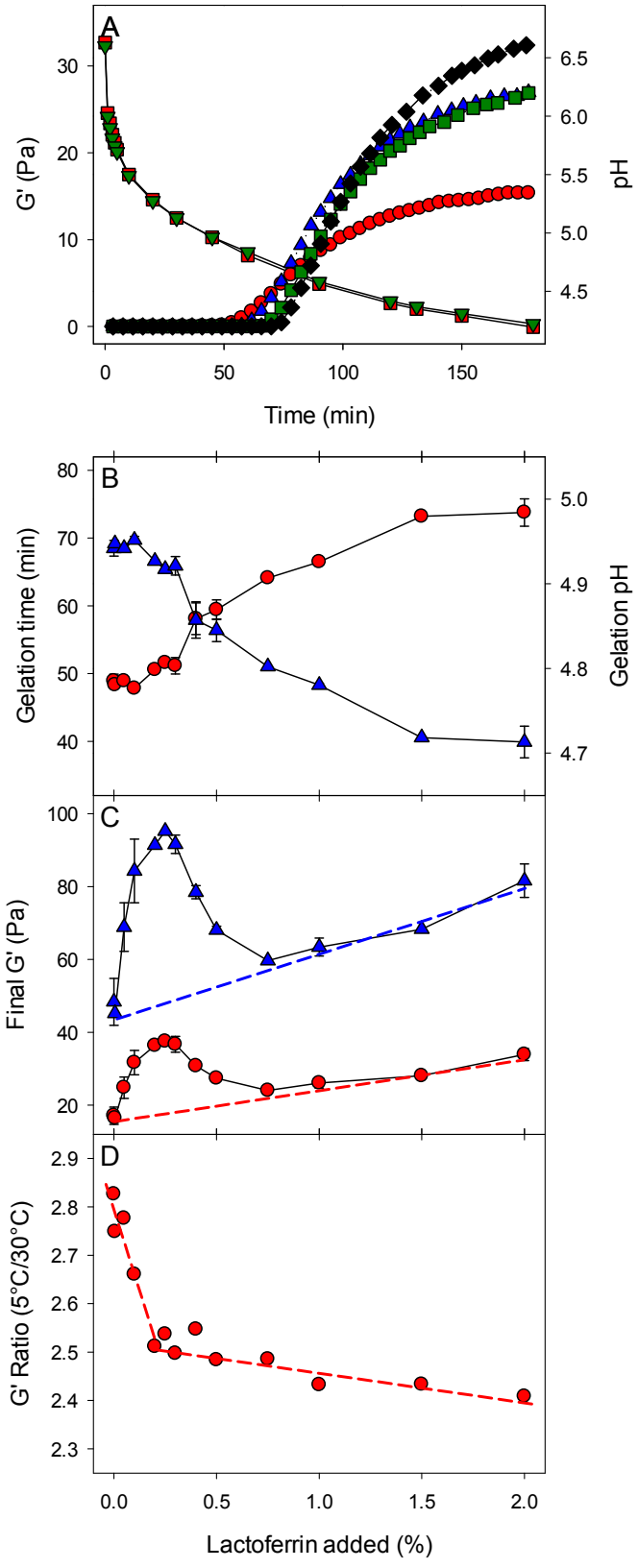

Fig. 6. Panel A: change in storage modulus (G′) and pH for milk samples with 2.2% w.w^{-1} added GDL. The milk samples had 0% (●, ■), 0.5% (▲), 1.0% (■), or 2% (◆, ▼) added lactoferrin. B: Gelation time (●) and gelation pH (▲) for skim milk samples with different levels of added lactoferrin. C: Final storage modulus (G′) at 30 °C (●) or 5 °C (▲) for skim milk samples with different levels of added lactoferrin. Error bars represent standard deviations from at least three repeated measurements. D: Ratio of G′ at 5 °C to G′ at 30 °C.

Kruif, 2013). It was believed that the larger lactoferrin (~80 kDa) could not penetrate beyond the casein micelle surface, whereas the smaller lysozyme (~14 kDa) could penetrate to the interior of the micelles. As the milk pH is decreased, the casein micelles can swell and allow some casein to dissociate, and this may allow the lactoferrin to penetrate further into the casein micelles, increasing the level bound to the micelles and decreasing the level in the serum phase.

A plot of bound lactoferrin against the zeta potential for the samples that were acidified, showed that, for the samples with 0.5, 1.0 or 2.0% added lactoferrin, as the |zeta potential| decreased, the level of bound lactoferrin also decreased so that at a |zeta potential| of about −7 mV or lower, all samples had the same level of bound lactoferrin regardless of the level of lactoferrin added to the milk. At a |zeta potential| of −4 or lower, there was virtually no lactoferrin bound to the casein micelles (Fig. 5A). A linear relationship was observed between the bound lactoferrin and the change in zeta potential (the difference in zeta potential between the sample without added lactoferrin and the sample with added lactoferrin). A few points were sitting above the straight line, and these were the samples where some casein dissociated from the casein micelles, and a slightly higher level of bound lactoferrin was observed.

3.5. Acid gelation during acidification

The milk samples with different levels of added lactoferrin were slowly acidified at 30 °C by the addition of GDL (2.2%, w/w) and the rheological properties during acidification were monitored. All samples showed typical gelation curves with the sample initially in a liquid state with a low G′ until, after a certain time, the sample gelled and the G′ progressively increased on further acidification (Fig. 6A). The point at which G′ reached 1 Pa was considered the gelation point and the time/pH at this point was the gelation time/pH. The G′ after 3 h was considered the final G′.

On addition of lactoferrin to the milk, the gelation time increased markedly from about 50 min for the sample with no added lactoferrin to over 70 min for the sample with 2% added lactoferrin. This corresponds to a decreasing gelation pH from about pH 4.95 for the sample with no added lactoferrin to pH 4.7 for the sample with 2% added lactoferrin (Fig. 6B). When the

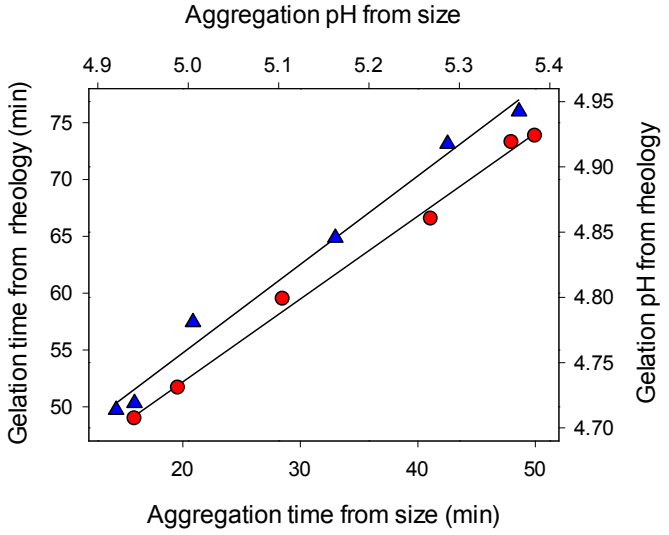

Fig. 7. Relationship between aggregation time from size measurements and gelation time for rheology (●), or aggregation pH from size measurements and gelation pH from rheology (▲).

aggregation time and aggregation pH from the size measurements (Fig. 3B) were compared with the gelation time and gelation pH from the rheology measurements (Fig. 6B) a linear relationship was obtained (Fig. 7); however, the size measurement technique detected gelation earlier and at a higher pH than that obtained by rheology.

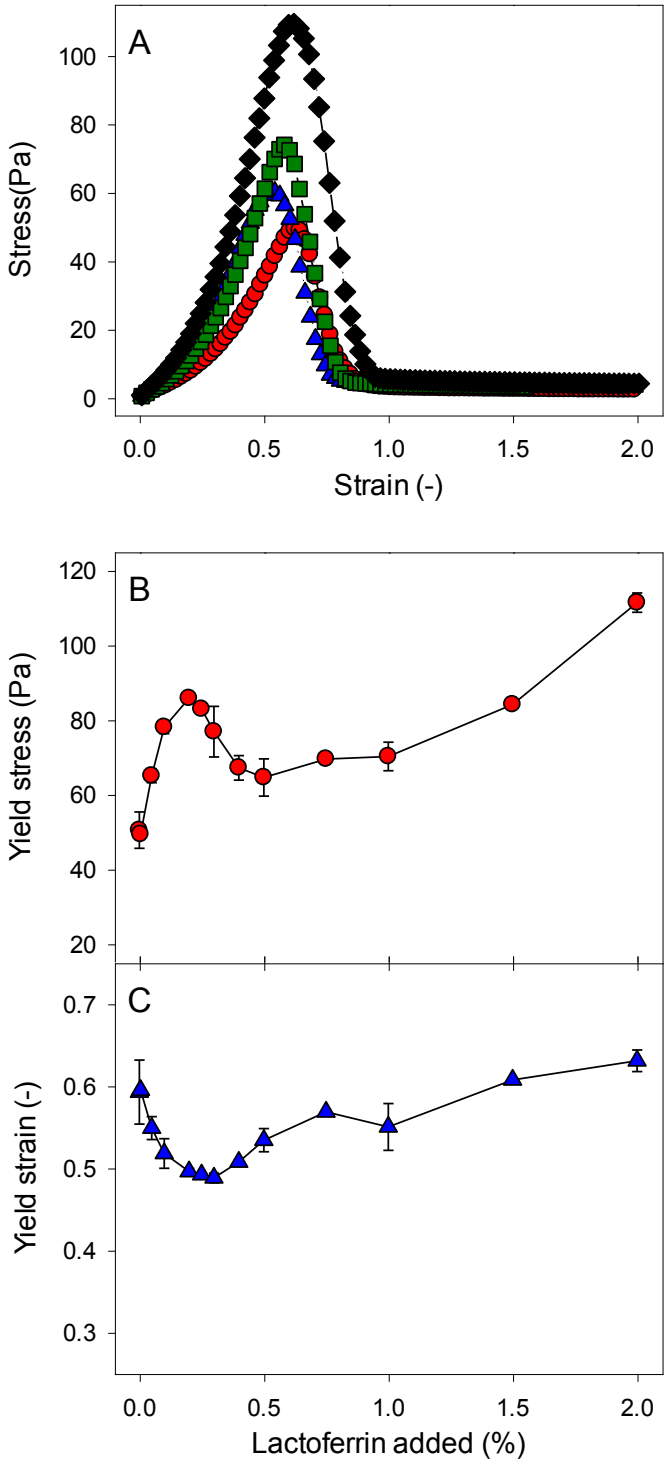

Fig. 8. Panel A: change in stress with increasing strain for acid gels prepared from milk with 0% (●), 0.5% (▲), 1.0% (■), or 2% (◆) added lactoferrin. Panel B: yield stress for gels prepared from skim milk samples with different levels of added lactoferrin. Panel C: yield strain for gels prepared from skim milk samples with different levels of added lactoferrin. Error bars represent standard deviations from at least three repeated measurements.

The final G′ of the acid gels showed a very unusual behaviour as the level of added lactoferrin increased. The final G′ for the gels from the milk with no added lactoferrin was about 17 Pa, and when low levels of lactoferrin were added to the milk, the final G′ of the gels increased to a maximum of about 37 Pa at 0.25% added lactoferrin. At higher addition levels the G′ decreased to about 24 Pa at 0.75% added lactoferrin before increasing again when further lactoferrin was added (Fig. 6C). The experiment was repeated several times at points around the local maximum at 0.25% added lactoferrin to ensure that this was a real effect.

After 3 h of acidification a gel had formed and the temperature was slowly reduced to 5 °C and the G′ was measured at this lower temperature. The same general behaviour of the G′ with added lactoferrin was observed at 5 °C as was observed at 30 °C however the G′ was substantially higher to 5 °C than at 30 °C (Fig. 6C). When the ratio of the G′ at 5 °C to that at 30 °C was compared (Fig. 6D), the ratio was about 2.8 for the gel from the sample with no added lactoferrin, and this ratio decreased markedly to about 2.5 with added lactoferrin up to about 0.25%. The ratio decreased slightly for the samples with higher levels of added lactoferrin, with the ratio being about 2.4 for the gel from the sample with 2% added lactoferrin. The markedly higher final G′ at 5 °C than at 30 °C has been reported previously (Anema, 2008; Anema, Lee, Lowe, & Klostermeyer, 2004; Bikker, Anema, Li, & Hill, 2000; Graveland-Bikker & Anema, 2003) and this is thought to be due to the loosening of the casein network structure and an increase in particle size within the gel network at lower temperatures as a consequence of the reduced strength of the hydrophobic interactions. This results in an increase in inter-particle bonds within the casein network structure resulting in an increased final G′ at the lower temperatures (Anema, 2008; van Vliet, Roefs, Zoon, & Walstra, 1989).

The yield properties of the gels at 5 °C were determined by increasing the strain at a constant rate until the gel broke. As the strain increased, the stress increased to a maximum and then decreased (Fig. 8A). The maximum stress was considered the yield stress and the strain at this maximum stress was the yield strain. As with the final G′, as the level of added lactoferrin in the milk increased, the yield stress of the acid gels increased to a maximum at about 0.2% added lactoferrin, then decreased to a minimum when about 0.5% lactoferrin was added before increasing again at higher addition levels. As the added lactoferrin level in the milk increased, the yield strain of the acid gels decreased to a minimum at about 0.3% added lactoferrin and then increased at higher addition levels.

4. Discussion

Consistent with previous studies (Anema, 2018; Anema & de Kruif, 2011, 2012; Croguennec et al., 2012), lactoferrin was found to bind to the casein micelles, and the binding caused an increase in size and a decrease in the |zeta potential| of the casein micelles (Fig. 1). The increase in size is not specifically due to the binding of the lactoferrin to the casein micelles but rather a loosening of the casein micelle structure causing a swelling and concomitant increase in size (Anema & de Kruif, 2011, 2012). The decrease in the |zeta potential| is expected for an electrostatic interaction where charge neutralization occurs on binding. Studies have shown that lactoferrin bound to the surface of the casein micelles, thus neutralizing the surface charges and reducing the |zeta potential|, whereas the smaller basic protein lysozyme bound to the interior of the casein micelles and had no effect on the zeta potential of the casein micelles (Anema & de Kruif, 2011, 2013). Interestingly a smaller decrease in |zeta potential| was observed when measured in permeate than in Ca-imidazole buffer, possibly due to the lower

ionic strength of the Ca-imidazole buffer or through specific ion effects from the complex mixture of minerals in the permeate (Fig. 1).

After binding the lactoferrin and the subsequent reduction in pH, the |zeta potential| of the casein micelles in milk and those in the milk with added lactoferrin progressively merged as the pH declined so that at pH below ~5.0 there was no measurable difference between control samples and those with added lactoferrin (Fig. 2). This was accompanied by a reduction in the level of lactoferrin bound to the casein micelles as the pH declined (Fig. 4). As the pH is decreased, the casein proteins become less negatively charged until the isoelectric point is reached whereas the positive charge of lactoferrin increases (Anema & de Kruif, 2016). As a consequence of the reduced negative charge on the casein micelles and the higher net positive charge on lactoferrin, less lactoferrin would electrostatically bind to the casein micelles and the excess that was bound at the higher pH will be dissociated to the serum phase as the pH declines.

Interestingly, the binding of lactoferrin to the casein micelles caused significant levels of casein to dissociate from the casein micelles as the pH decreased, reaching a maximum at about pH 5.0, after which the level decreased again as the pH approached the isoelectric point (Fig. 4). Previous studies have shown that casein can dissociate from the casein micelles at ~pH 5.0, and the level that dissociated increased dramatically as the temperature decreased (Dalgleish & Law, 1988; Law, 1996; Singh, Roberts, Munro, & Teo, 1996). As the pH of milk declines, the colloidal calcium phosphate is progressively solubilised and at ~pH 5.0 only a small proportion of the original calcium phosphate is still associated with the casein micelles (Dalgleish & Law, 1989; Law, 1996; Singh et al., 1996). In addition, the charges on the casein proteins are reduced so that charge repulsions are diminished. At higher temperatures (>20−30 °C) the hydrophobic interactions are sufficient to maintain colloidal structure, whereas at lower temperatures (below ~20 °C and particularly below ~10 °C) the hydrophobic interactions are weakened and caseins are dissociated from the casein micelles (Dalgleish & Law, 1988; Law, 1996; Singh et al., 1996).

It appears that the binding of lactoferrin causes the dissociation to occur to a greater extent at higher temperatures (Fig. 4). It is known that binding lactoferrin to the casein micelles at the natural pH will ultimately cause the micelles to disintegrate (Anema & de Kruif, 2011, 2012), thus lactoferrin must reduce the ability of the colloidal calcium phosphate and/or hydrophobic interactions in maintaining the colloidal structure, and this results in increased dissociation of casein at ~pH 5.0.

On acidification, the aggregation point measured by size changes was detected earlier and at higher pH than the gelation point measured by rheology regardless of the level of added lactoferrin (Figs. 3, 6 and 7). This is because the size measurement is detecting the initial aggregation of the particles, which occurs significantly earlier and at a higher pH than the formation of the three dimensional gel network detected by rheology. The binding of lactoferrin to the casein micelles caused the micelles to aggregate, as measured by size changes (Fig. 3) or to gel, as measured by rheology (Fig. 6) at lower pH than the natural casein micelles in milk. At 2% added lactoferrin, the aggregation/gelation pH was 0.2 pH units lower than the milk with no added lactoferrin regardless of whether measured by size or rheology. Even though low levels of lactoferrin were bound to the caseins at pH below 5.0, this low level prevents the casein from aggregating until the pH is much lower. As higher levels of lactoferrin were bound at the higher addition levels even at pH below 5 (Fig. 4), the level of bound lactoferrin may need to be below a certain level before the casein aggregates, and this is at a lower pH as the level of added lactoferrin increases.

A complex effect of added lactoferrin on the final G′, yield stress and yield strain was observed with a local maximum in final G′ (Fig. 6) and yield stress (Fig. 8) at about 0.25% added lactoferrin, followed by a local minimum in G′ and yield stress at about 0.5−0.75% added lactoferrin after which both G′ and yield stress increased at higher lactoferrin levels. The yield strain showed a local minimum at about 0.25% added lactoferrin, with the yield strain increasing at higher addition levels (Fig. 8). It is not immediately apparent why such a complex gelation pattern is observed. Increasing levels of added lactoferrin causes increased dissociation of casein as the pH declines prior to final aggregation and gelation (Fig. 4), and the gelation occurs at a lower pH at as the level of added lactoferrin is increased (Figs. 3 and 6).

It is possible that there are two overlapping effects as a consequence of the lower gelation pH with increasing lactoferrin levels and the increased dissociation of casein at about pH 5.0 at increasing lactoferrin levels. The lower gelation pH appears to results in a progressive increase in final G'; however, at certain lactoferrin levels, a partial dissociation of the casein micelles can result in a markedly higher final G′ than non-dissociated casein micelles (very low lactoferrin levels) or substantially dissociated casein micelles (higher lactoferrin levels). These two overlapping effects could produce the observed maximum in the final G′ and yield stresses at increasing added lactoferrin levels.

In heated milks, it has been shown that the removal of some colloidal calcium phosphate from the casein micelles before heat treatment and subsequent acidification to form gels resulted in a decrease in the final G′ of the gels, whereas removal of higher levels of calcium phosphate increased the final G′ of the gels (Anema, 2009a). In another study, it was shown that, for yoghurts prepared from heated milks, the addition of citrate to the milk reduced the colloidal calcium phosphate level, and at low levels this resulted in an increase in the final G′ and yield stress of the gels, whereas at higher levels, a decrease in both the final G′ and yield stress was observed (Ozcan-Yilsay, Lee, Horne, & Lucey, 2007). Both the removal of colloidal calcium phosphate and the addition of citrate results in the partial dissociation of the casein micelles, and this clearly impacts the final G′ and yield stresses of the gels formed.

5. Conclusions

Lactoferrin electrostatically bound to the casein micelles in milk, reducing the casein micelle |zeta potential| and increasing their size. On decreasing the pH, the lactoferrin progressively dissociated from the casein micelles, and, as a consequence, the zeta potential-pH curves for native casein micelles in milk and those with bound lactoferrin merged as the pH declined and were indistinguishable at pH below about 5.0. The binding of the lactoferrin to the casein micelles caused increased dissociation of casein from the micelles as the pH was reduced, reaching a maximum dissociation at about pH 5.0, and at lower pH the casein aggregated as the milk gelled at the isoelectric point of the casein (~pH 4.6). The binding of lactoferrin caused the milk to gel at a lower pH than the native casein micelles. The final G′ and the yield stresses of the set gels showed an unusual behaviour with added lactoferrin as G′ and yield stress of the set gels increased when low levels of lactoferrin were added to the milk before acidification, decreased from a local maximum when intermediate levels of lactoferrin were added to the milk before acidification and increased again when high levels of lactoferrin were added to the milk before acidification. This unusual effect of lactoferrin on gelation was hypothesised to be due to combined effects of dissociated casein causing a marked increase in G'/yield stress of the set gel if gelation occurred at a higher pH, overlaid with the lower gelation pH causing a small increase in G′/yield stress.

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