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The influence of alkaline earth metal equilibria on the rheological properties of rennet-induced skim milk gels

Darren R. Cooke · Paul L. H. McSweeney

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Abstract The mineral equilibria of milk can have a major influence on the properties of rennet milk gels. The influence of increased Mg, Ca, and Sr concentrations on milk and rennet milk gels was investigated. Reconstituted skim milk was supplemented with MgCl_2 , CaCl_2 , or SrCl_2 at levels from 2.5–10 mmol.L^{-1} and adjusted to pH 6.6. Dynamic rheological properties of the renneted milks at 32 °C were investigated during 6-h time sweeps and by frequency sweeps. Whey was separated from rennet gels by centrifugation at the gelation point and also 6 h after rennet addition. The concentrations of Mg, Ca, Sr, and inorganic P in milks and wheys were determined. Levels of casein-bound Mg, Ca, and Sr increased with the addition of their respective chloride salt. The percentage of CN-bound inorganic P increased with the increasing concentration of each divalent cation. Milk gelation time decreased with the increasing concentration of each divalent cation salt. Milks containing 10 mmol.L^{-1} of CaCl_2 or 10 mmol.L^{-1} of SrCl_2 exhibited buffering capacities significantly higher ($P<0.05$) than the 10 mmol.L^{-1} of MgCl_2 and control milks. An increase in G' and a decrease in $\tan \delta$ were observed in milk gels supplemented with the divalent cation salts. Milk gels supplemented with either CaCl_2 or SrCl_2 had superior rennet gel properties compared with MgCl_2 -supplemented milk gels (especially at low frequencies). This study provides information on the link between the mineral fortification of milk and modulation of milk gel properties and potentially for the mineral fortification of cheese.

Keywords Rennet-induced gel · Mineral equilibria · Rheology · Alkaline earth metals

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1 Introduction

In milk, calcium exists in a dynamic equilibrium between soluble and insoluble forms, namely insoluble casein-bound calcium phosphate nanoclusters (CCP) and soluble Ca salts of citrates and phosphates and free Ca^{2+} ions. Supplementing milk with mineral salts can change this equilibrium (Mekmene et al. 2009; Philippe et al. 2003; Udabage et al. 2001). In particular, the addition of Ca^{2+} can lead to the formation of excess calcium phosphate salts in the aqueous phase and subsequent precipitation to the micellar phase forming CCP nanoclusters and also the binding of individual Ca^{2+} ions to the charged groups on caseins, all of which increase the attractive forces within the casein micelle. CCP nanoclusters are ~2.5-nm diameter granules of insoluble Ca phosphate complexes that also contain Mg and citrate in their structure (McGann et al. 1983a, b; Srinivasan and Lucey 2002). These nanoclusters are involved in ionic attraction with numerous phosphoserine centers on casein molecules and represent an important structural component of the casein micelle (Horne 1998). The addition of salts affects Ca^{2+} activity, CCP concentration, the proportion of casein in the micelle, and the ionic strength of the milk (Udabage et al. 2001), all of which can influence rennet gel properties. Addition of Ca to cheese milk is common practice for many cheese varieties to enhance renneting and yield (Lucey and Fox 1993; Wolfschoon-Pombo 1997), but the level of addition is important. McMahon et al. (1984) reported a minimum in rennet coagulation time (RCT) with a 0.05-mol.L^{-1} CaCl_2 addition to skim milk but a dramatic increase in RCT at 0.4 mol.L^{-1} .

Rennet-induced gelation is the primary step in the manufacture of most cheese varieties. The addition of the enzyme chymosin cleaves the Phe₁₀₅–Met₁₀₆ bond of κ -CN which destabilizes the casein micelle leading to the Ca^{2+} -mediated aggregation of *para*-casein micelles ultimately forming a three-dimensional gel network of partly fused *para*-micelles. Addition or removal of Ca does not influence the enzymatic phase if the pH is kept constant, but the aggregation phase is dependent on Ca concentration (Van Hooydonk et al. 1986; Zoon et al. 1988). A certain CCP level is required for proper renneting properties as partial removal of CCP content by EDTA increases RCT (Choi et al. 2007; Udabage et al. 2001). Ca addition to milk can lead to an increased strength of rennet gels (Sandra et al. 2012; Udabage et al. 2001; Zoon et al. 1988).

Cooke and McSweeney (2013) observed that the addition of SrCl_2 at the salting stage of Cheddar cheese manufacture caused significant alterations to its rheological properties, with Sr exhibiting a behavior similar to that previously reported for Ca addition. In the same study, the addition of MgCl_2 at salting had no effect on the rheological properties of cheese. The present study investigates if similar alterations in rheological properties occur in rennet-induced milk gels supplemented with these salts and with CaCl_2 .

Rennet-induced milk gels are viscoelastic and can be characterized using dynamic small amplitude oscillatory rheometry (DSAOR). The relationship between the DSAOR and aging of gels has been investigated by numerous authors in prolonged gelation experiments to monitor changes due to factors such as proteolysis (Esteves et al. 2002; Srinivasan and Lucey 2002) and the modification of Ca equilibrium (Choi et al. 2007; Zoon et al. 1988). In the present study, the mineral equilibria at the gelation

point and late aging of rennet milk gels supplemented with salts of divalent cations of alkaline earth metals were determined in order to investigate the effects of changes in mineral equilibria during gel aging on their rheological properties. pH and temperature were kept constant to investigate the effects on rennet gels caused solely by the addition of divalent salt cations.

2 Materials and methods

2.1 Milk sample preparation

Low-heat skim milk powder was reconstituted (10% w/w) in deionized water for 1 h at room temperature. Sodium azide (0.02%) was added at the time of reconstitution to prevent bacterial growth. MgCl_2 , CaCl_2 , and SrCl_2 were prepared as 1-mol.L^{-1} stock solutions and added to the milks along with deionized water to give final concentrations of 2.5 mmol.L^{-1} , 5.0 mmol.L^{-1} , or 10 mmol.L^{-1} of each salt in respective milks. The total volume added was 1% v/v; deionized water was added to the control to compensate for the dilution of the experimental milks. The milks and salt solutions were mixed for 1 h at room temperature. These milks were adjusted to pH 6.6 using 2 N NaOH and held overnight at 4 °C. The pH of the milks was readjusted to 6.6 if necessary. Three independent batches of each milk type were prepared for analyses. One sample from each batch was used for each analysis.

2.2 Chemical composition

The total Ca and Mg contents of the milks and wheys were determined by atomic absorption spectroscopy (Varian SpectraAA-100, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) according to IDF (2007), and their Sr content was analyzed based on the same method for Ca determination according to IDF (2007) except with a wavelength of 460.7 nm and a lamp current of 10 mA. The total inorganic phosphorus content of the milks and whey was determined using the colorimetric method of Allen (1940).

2.3 Dynamic small amplitude oscillatory rheometry

Milk samples were heated to 32 °C and maintained at this temperature for 30 min. An aliquot (100 μL) of diluted chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands) (1:10 dilution) was added to 25 g of reconstituted skim milk and stirred using a glass rod for 1 min at 32 °C. This milk was then transferred to a controlled shear stress rheometer equipped with a Peltier concentric cylinder system and a conical rotor (diameter 28 mm \times length 42 mm; model AR-G2; TA instruments, Waters LLC, Leatherhead, Surrey, UK). Approximately 5 min elapsed between rennet addition and the commencement of oscillation on the rheometer. A 6-h time sweep was performed on samples which were oscillated at a frequency of 0.1 Hz and 0.05% strain. This was found to be within the linear viscoelastic range for these milk gels. After the time sweep, a frequency sweep was performed maintaining a strain of 0.05% and increasing the frequency from 0.001 to 1.0 Hz. Parameters recorded were the storage modulus (G')

and $\tan \delta$, which is the ratio of viscous to elastic properties (G''/G'). Gelation time was taken as the time at which $G' \geq 1$.

2.4 Separation of curds and whey

Milk samples were heated to 32 °C and maintained at this temperature for 30 min. An aliquot (80 μ L) of diluted chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands) (1:10 dilution) was added to 20 g of reconstituted skim milk at 32 °C. Rennet gels were cut at their gelation times which were predetermined from dynamic small amplitude oscillatory rheology results. After a heal time of 5 min, cut curds were centrifuged at 1,700g for 3 min. The supernatant (whey) and the pellet were collected. The moisture contents of pellets were determined by oven drying at 103 °C for 12 h and calculating the difference between pellet mass before and after drying. This mass difference was expressed as the percentage of CN-bound water. Rennet gels were also made and allowed to stand for 6 h after rennet addition before being cut and subjected to the same treatment as above.

2.5 Buffering capacity

The buffering capacity of the milk samples was determined at 25 °C based the work of Lucey et al. (1993). An automated pH titration system (Metrohm AG 907 Titrando, Herisau, Switzerland) was used for acid-base titrations. The pH electrode was calibrated with the following pH buffers: 4.0, 7.0, and 9.0. The calibration slope of the pH electrode was maintained $\geq 98\%$. Milk samples (40 ml) were titrated from the initial pH of ~ 6.6 to 3.0 with 0.5 N HCl and back-titrated to pH 9.0 with 0.5 N NaOH. Titrants were added in 0.1-ml increments at 30-s intervals to allow for the equilibration of titrant and milk. The change in pH (dpH), resulting from the incremental addition of acid or base, and the volume of titrant used in the titration were recorded by the titrator software and exported to a Microsoft Excel spreadsheet. Buffering indices (dB/dpH) were calculated according to Van Slyke (1922). Buffering curves were prepared by plotting buffering index as a function of pH. The change in the total volume of sample due to the addition of acid or alkali during the titration was taken into account in the buffering index calculations. Microsoft Excel was used to calculate the area under the buffering curves. The curves were integrated between the pH limits of 5.8 to 4.1, based on the work of Hassan et al. (2004). The difference in area between the acidification and alkalization buffering curves was then calculated. The magnitude of this area is thought to be directly related to the CCP content of milk (Lucey et al. 1993); so in this study, the magnitude of this area is considered to be related to the insoluble divalent cation phosphate level. Buffering capacity of milks was reported as buffering area.

2.6 Statistical analysis

ANOVA was carried out using the PASW Statistics version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD post hoc test. The level of significance was determined at $P < 0.05$.

3 Results and discussion

3.1 Buffering capacity of supplemented milks from acid-base titration

The buffering capacity of the control and supplemented milks are shown in Table 1. All milks displayed a buffering peak \sim pH 5 during titration with 0.5 N HCl and an absence of this peak during the back titration with 0.5 N NaOH (results not shown), which is in agreement with Lucey et al. (1993). With the addition of 2.5 mmol.L⁻¹ of all salts, there were no significant differences ($P>0.05$) in buffering area between supplemented milks and the control. At 5.0- and 10-mmol.L⁻¹ addition levels, the CaCl₂ and SrCl₂ milks had a significantly greater ($P<0.05$) buffering area compared to the control milk. The MgCl₂-supplemented milks did not differ significantly ($P>0.05$) from the control milk at any addition level. Both the 10-mmol.L⁻¹ CaCl₂ and 10-mmol.L⁻¹ SrCl₂ milks had significantly higher buffering areas ($P<0.05$) than the 10-mmol.L⁻¹ MgCl₂ milk. The buffering peak at \sim pH 5 is attributed to the solubilization of Ca phosphate nanoclusters and liberation of phosphate ions therefrom that combine with H⁺, resulting in buffering (Lucey et al. 1993). Thus, increased buffering around this pH value is indicative of a higher CCP level in milk. The higher buffering area observed for the 10-mmol.L⁻¹ CaCl₂ and SrCl₂ milks may be due to the increased formation of nanoclusters.

3.2 Rennet gelation time of supplemented milks

Rennet gelation time decreased with the increasing concentration of all salts (Table 2). A reduction in RCT of milk due to CaCl₂ addition has also been reported by numerous authors (McMahon et al. 1984; Sandra et al. 2012; Udabage et al. 2001; Van Hooydonk et al. 1986; Zoon et al. 1988). Both CaCl₂ and SrCl₂ decreased gelation time to a greater extent than MgCl₂ at most addition levels investigated. The reduction in gelation time was similar ($P>0.05$) between CaCl₂ and SrCl₂ at both 2.5- and 10-mmol.L⁻¹ addition levels. The difference in gelation times between the salts, specifically between MgCl₂ and the other two, may be due to the formation of nanoclusters in the micelles of the CaCl₂ and SrCl₂ milks, but not in the MgCl₂ milk. Van Hooydonk et al. (1986) also observed that CaCl₂ reduced gelation time more

Table 1 Differences in buffering area between acid and alkaline titration curves for milks supplemented with chloride salts of Mg, Ca, and Sr

Salt addition (mmol.L ⁻¹)	Area (mm ²)
Control (0)	19.1 \pm 0.5 ^d
MgCl ₂ 2.5	19.2 \pm 0.1 ^d
MgCl ₂ 5.0	20.3 \pm 0.5 ^{bcd}
MgCl ₂ 10	20.7 \pm 0.1 ^{bcd}
CaCl ₂ 2.5	19.5 \pm 0.8 ^{cd}
CaCl ₂ 5.0	21.6 \pm 1.4 ^{abc}
CaCl ₂ 10	22.3 \pm 0.7 ^a
SrCl ₂ 2.5	20.2 \pm 0.5 ^{bcd}
SrCl ₂ 5.0	21.8 \pm 0.8 ^{ab}
SrCl ₂ 10	22.9 \pm 0.8 ^a

Buffering area were studied between pH limits of 5.8 to 4.1
Means ($n=3$) within a column without a common lowercase superscript letter are significantly different ($P<0.05$)

Table 2 Effects of added divalent cation salts on rheological properties of rennet-induced gels

Salt addition (mmol.L ⁻¹)	Gelation time (min)	G'_{6h} (Pa)	Loss tangent	Slope of log G' vs log frequency
Control (0)	36.3±0.3 ^a	84.3±1.8 ^h	0.51±0.00 ^a	0.26±0.00 ^a
MgCl ₂ 2.5	25.4±1.0 ^b	109.6±0.1 ^g	0.49±0.01 ^b	0.25±0.00 ^b
MgCl ₂ 5.0	20.3±0.0 ^{cd}	116.4±1.2 ^c	0.49±0.01 ^b	0.25±0.00 ^b
MgCl ₂ 10	15.6±1.2 ^f	130.6±0.2 ^c	0.47±0.00 ^c	0.24±0.00 ^c
CaCl ₂ 2.5	19.8±0.2 ^{cd}	112.7±0.4 ^f	0.47±0.00 ^c	0.24±0.00 ^c
CaCl ₂ 5.0	17.6±0.7 ^e	120.5±0.5 ^d	0.45±0.00 ^d	0.23±0.00 ^d
CaCl ₂ 10	11.9±0.9 ^g	137.0±0.4 ^a	0.41±0.01 ^f	0.22±0.00 ^f
SrCl ₂ 2.5	21.1±0.5 ^c	108.7±1.1 ^g	0.47±0.00 ^c	0.24±0.00 ^c
SrCl ₂ 5.0	19.3±0.4 ^d	121.2±0.6 ^d	0.44±0.00 ^e	0.23±0.00 ^e
SrCl ₂ 10	13.0±0.2 ^g	134.1±0.5 ^b	0.38±0.00 ^g	0.21±0.00 ^f

Gelation time defined as the point when gels had a storage modulus ≥ 1 Pa. 5 min added to these values to compensate for time between rennet addition and the commencement of oscillation, G'_{6h} measured ~6 h after rennet addition, *Loss tangent* measured 6 h after rennet addition at a frequency of 0.001 Hz

Means ($n=3$) within a column without a common lowercase superscript letter are significantly different ($P<0.05$)

than MgCl₂ at the same molar addition level (3 mmol.L⁻¹) and suggested that the total activity of divalent cations may be less important than interactions between the micelle and insoluble salt complexes in the context of rennet coagulation.

3.3 Mineral partition between curds and whey

Addition of the divalent cation salts to the milks lead to an increase in the level of CN-bound cationic component of these salts (Figs. 1, 2, and 3). Binding of these cations to caseins may occur via monoester phosphate groups on serine and threonine residues, carboxylic groups of aspartic acid and glutamic acid (Dickson and Perkins 1971), phenolic, sulfhydryl, and imidazole groups (Gaucheron et al. 1997) and/or as insoluble divalent cation phosphate nanocluster complexes. After ~6 h of aging, an increase in soluble Ca, Mg, and Sr was observed in a number of the milk gels. This decrease in casein-associated minerals coincided with a slight but significant decrease ($P<0.05$) in

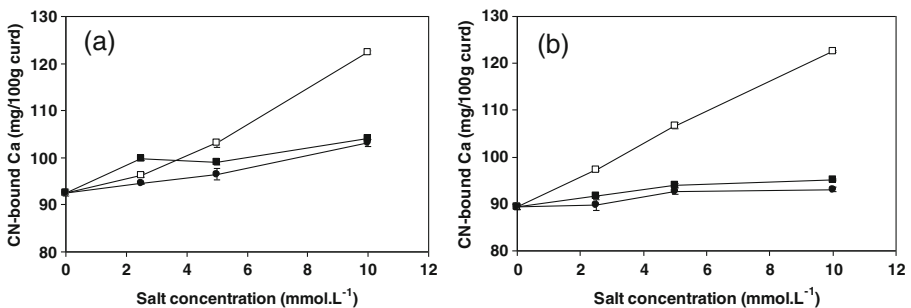


Fig. 1 CN-bound Ca in rennet-induced milk gels supplemented with MgCl₂ (closed square), CaCl₂ (open square), or SrCl₂ (closed circle) at the **a** gelation point and **b** 6 h after rennet addition. Error bars ± 1 standard deviation ($n=3$)

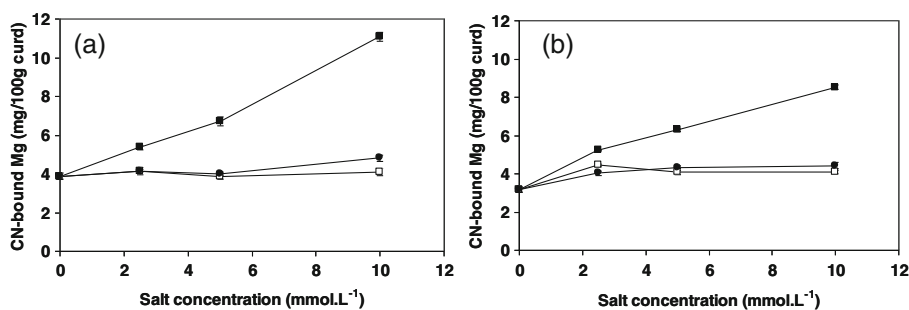


Fig. 2 CN-bound Mg in rennet-induced milk gels supplemented with MgCl₂ (closed square), CaCl₂ (open square), or SrCl₂ (closed circle) at the **a** gelation point and **b** 6 h after rennet addition. Error bars ± 1 standard deviation ($n=3$)

CN-bound water in all gels during aging (Table 3). The contraction of the *para*-casein network during aging creates pores which fill with unbound moisture (whey). The decrease in water associated with casein over time could lead to the solubilization of minerals due to increased water content in gel pores and permit the internal rearrangements of caseins in *para*-casein micelles. It should be noted that CN-bound water decreased independently of the addition of divalent cation salts. Addition of the divalent cation salts increased the percentage of CN-bound inorganic phosphorus (P_i) values at all addition levels compared to the control gel (Fig. 4). The level of CN-bound P_i decreased by late aging only in gels with a 10-mmol.L⁻¹ concentration of any of the three divalent cation salts and also in the gel made from milk containing 5.0-mmol.L⁻¹ SrCl₂. Van Hooydonk et al. (1986) suggested that complexes of divalent cations and phosphates may bind to caseins when divalent cations are added to milk. All of the P_i associated with the micelles is thought to be present in the CCP (Zoon et al. 1988).

3.3.1 Effect of CaCl₂ addition on mineral partition

The amount of CN-bound Ca increased with level of the addition of CaCl₂ (Fig. 1). The percentage of CN-bound Ca in the CaCl₂-supplemented gels did not deviate from the control by more than 1% (Table 3). Similarly, Philippe et al. (2005) observed that the percentage of Ca associated remains constant with increased Ca addition to casein

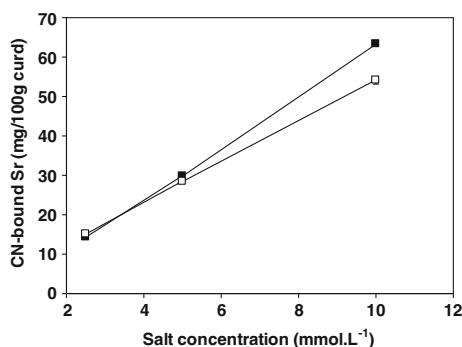


Fig. 3 CN-bound Sr in rennet-induced milk gels supplemented with SrCl₂ at the gelation point (closed square) and 6 h after rennet addition (open square). Error bars ± 1 standard deviation ($n=3$)

Table 3 Percentages of CN-bound Ca, Mg, Sr, and H₂O in rennet-induced gels supplemented with chloride salts of divalent metal ions at the gelation point and 6 h after rennet addition

Salt addition (mmol.L ⁻¹)	Percentage of CN-bound Ca		Percentage of CN-bound Mg		Percentage of CN-bound Sr		Percentage of CN-bound H ₂ O	
	Gelation point	6 h after rennet addition	Gelation point	6 h after rennet addition	Gelation point	6 h after rennet addition	Gelation point	6 h after rennet addition
Control (0)	68.6±0.5 ^{deA}	67.9±0.2 ^{dA}	32.8±1.4 ^{bcA}	27.8±1.1 ^{cdB}	—	—	82.1±0.2 ^{aA}	80.1±0.2 ^{aB}
MgCl ₂ 2.5	72.8±0.3 ^{ba}	69.7±0.1 ^{cB}	30.2±0.6 ^{cdA}	29.4±0.8 ^{cA}	—	—	82.6±0.4 ^{aA}	80.1±0.2 ^{aB}
MgCl ₂ 5.0	73.2±0.3 ^{ba}	71.0±0.3 ^{bb}	28.4±1.0 ^{dA}	26.4±0.3 ^{dB}	—	—	82.7±0.5 ^{aA}	79.7±0.4 ^{aB}
MgCl ₂ 10	76.6±0.2 ^{aA}	71.9±0.2 ^{aB}	29.9±0.6 ^{dA}	23.6±0.3 ^{eB}	—	—	81.8±0.4 ^{aA}	79.8±0.3 ^{aB}
CaCl ₂ 2.5	68.0±0.3 ^{fa}	67.6±0.1 ^{dA}	34.6±0.6 ^{bb}	37.7±0.5 ^{aA}	—	—	81.9±0.2 ^{aA}	79.8±0.2 ^{aB}
CaCl ₂ 5.0	67.9±0.5 ^{fa}	68.2±0.4 ^{dA}	33.7±1.2 ^{ba}	34.6±1.4 ^{ba}	—	—	82.3±0.3 ^{aA}	79.9±0.2 ^{aB}
CaCl ₂ 10	68.9±0.2 ^{deA}	68.2±0.6 ^{dA}	34.4±1.2 ^{ba}	34.4±0.9 ^{ba}	—	—	82.2±0.5 ^{aB}	80.2±0.2 ^{aB}
SrCl ₂ 2.5	69.7±0.1 ^{cdA}	68.2±0.8 ^{dB}	34.1±1.6 ^{ba}	33.9±1.0 ^{ba}	62.8±1.0 ^{aA}	60.8±0.5 ^{aB}	82.6±0.4 ^{aA}	80.4±0.4 ^{aB}
SrCl ₂ 5.0	70.1±0.9 ^{1cA}	69.9±0.5 ^{cA}	33.8±0.9 ^{ba}	35.9±1.0 ^{baA}	63.1±0.6 ^{aA}	58.1±0.4 ^{bb}	82.2±0.5 ^{aA}	80.2±0.3 ^{aB}
SrCl ₂ 10	73.5±0.5 ^{ba}	69.9±0.4 ^{cB}	40.2±1.5 ^{aA}	37.3±1.4 ^{aA}	63.6±0.5 ^{aA}	57.0±1.0 ^{bb}	82.5±0.5 ^{aA}	80.0±0.2 ^{aB}

Means (*n*=3) within a column without a common lowercase superscript letter are significantly different (*P*<0.05)Means (*n*=3) within a row for the same parameter without a common uppercase superscript letter are significantly different (*P*<0.05)

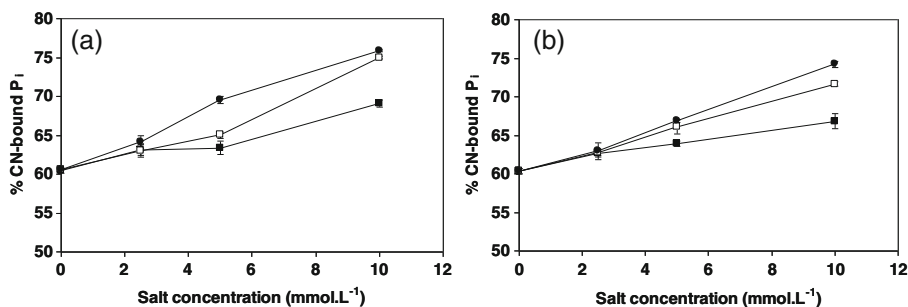


Fig. 4 Percentage of CN-bound inorganic phosphorous (P_i) in rennet-induced milk gels supplemented with $MgCl_2$ (closed square), $CaCl_2$ (open square), or $SrCl_2$ (closed circle) at the **a** gelation point and **b** 6 h after rennet addition. Error bars ± 1 standard deviation ($n=3$)

micelle suspensions, which indicates that added Ca precipitates to the CN-bound phase in order to maintain an equilibrium ratio due to the aqueous phase being saturated with Ca salts. Philippe et al. (2003) reported that 20% of the added Ca remained soluble regardless of the $CaCl_2$ addition level (up to $13.5 \text{ mmol.kg}^{-1}$). The percentage of CN-bound P_i increased with increasing $CaCl_2$ concentration (Fig. 4). Co-precipitation of the added Ca^{2+} with phosphate has been observed in numerous studies (Philippe et al. 2003, 2005; Udabage et al. 2000; Van Hooydonk et al. 1986). The co-precipitation of the added Ca with P_i from the aqueous phase strongly indicates nanocluster formation. Zoon et al. (1988) found that the more calcium a sample contained at a certain phosphate concentration, the higher the amount of CCP was present. However, new CCP nanoclusters formed at unreacted phosphoserine centers after Ca^{2+} addition to milk is thought to differ from the natural form of CCP (Philippe et al. 2003). $CaCl_2$ addition had no impact on CN-bound Mg levels at the gelation point (Table 3 and Fig. 2) at all concentrations studied.

3.3.2 Effect of $MgCl_2$ addition on mineral partition

The amount of CN-bound Mg increased with the level of addition of $MgCl_2$ (Fig. 2). The percentage of CN-bound Mg decreased in the control milk gel after 6 h (Table 3). The lower association of Mg with the casein network compared to Ca is likely due to the fact that Mg phosphate salts are not at saturation in milk unlike Ca phosphates (Philippe et al. 2005). The percentage of CN-bound Mg in the control milk gel was $\sim 33\%$ at the gelation point which is close to values previously reported for bovine milk ($\sim 35\%$) (Gaucheron 2005). This value decreased to $\sim 27\%$ after ~ 6 h of aging. The percentage of CN-bound Mg was similar ($P>0.05$) at the gelation point and after 6 h ($\sim 30\%$) for the 2.5-mmol.L^{-1} $MgCl_2$ gel. Van Hooydonk et al. (1986) reported that $\sim 30\%$ of the added Mg was CN-bound after adding 3 mmol.L^{-1} of $MgCl_2$ to milk. However, the percentage of CN-bound Mg decreased in the 5.0-mmol.L^{-1} $MgCl_2$ milk gel and to a greater extent in the 10-mmol.L^{-1} $MgCl_2$ milk gel after aging (Table 3). The decrease in CN-bound Mg during aging may be explained by the decrease in the percentage of CN-bound water (Table 3) leading to an increased free water in the network, causing an increased solubility of any CN-bound Mg salt complexes formed. $MgCl_2$ addition appears to cause more displacement of soluble Ca to the CN-bound

phase than SrCl_2 addition (Table 3). Addition of MgCl_2 at all levels increased the percentage of CN-bound Ca, with the 10-mmol.L^{-1} MgCl_2 gel having $\sim 8\%$ higher CN-bound Ca than the control milk gel. Van Hooydonk et al. (1986) observed an increase in CN-bound Ca and Ca^{2+} activity due to MgCl_2 addition and attributed this to the added Mg^{2+} exchanging with Ca^{2+} from soluble citrate complexes. MgCl_2 may also cause a displacement of non-nanocluster CN-bound Ca as the caseins have a higher affinity for Mg binding (Dickson and Perkins 1971). This would increase ionic Ca^{2+} in aqueous phase and, coupled with the possible displacement of Ca from soluble Ca salt complexes, this may explain the increase in CN-bound Ca in MgCl_2 milks. Zhang and Aoki (1995) reported that Mg phosphate alone has no cross linking ability, but increasing the Mg concentration ($2.5\text{--}10\text{ mmol.L}^{-1}$) when forming artificial casein micelles leads to an increased CN-bound Ca and inorganic phosphate and could thereby indirectly promote calcium crosslinking of caseins. The increases in CN-bound Ca and P_i at all addition levels of MgCl_2 in the present study suggest that levels of CCP nanoclusters may increase in these milks but not sufficiently to change the buffering capacity (Table 1). Previous studies have reported that a greater increase in CN-bound inorganic phosphate is observed in casein systems due to CaCl_2 compared to MgCl_2 added at the same levels (Philippe et al. 2005; Van Hooydonk et al. 1986). This is in agreement with the 5.0- and 10-mmol.L^{-1} levels of both salts in the present study.

3.3.3 Effect of SrCl_2 addition on mineral partition

The amount of CN-bound Sr increased with the level of addition of SrCl_2 (Fig. 3). The percentage of CN-bound Sr at the gelation point was $\sim 63\%$ in all SrCl_2 -supplemented milks (Table 3). This value decreased in these milks during aging with the value of the 10-mmol.L^{-1} SrCl_2 milk gel decreasing to the greatest extent. There was a lower percentage association of Sr with casein ($\sim 63\%$) compared to Ca ($\sim 68\%$) in the control milk gel. The percentage of CN-bound P_i increased with the increasing SrCl_2 concentration (Fig. 4). Of all the divalent cation salts, SrCl_2 at 5.0 and 10 mmol.L^{-1} increased CN-bound P_i to the greatest extent. Insoluble Sr phosphate complexes have the ability to crosslink caseins and form aggregates, but this crosslinking ability is lower than for Ca phosphates (Zhang and Aoki 1995). The addition of 5.0 and 10 mmol.L^{-1} of SrCl_2 caused a significant increase ($P < 0.05$) in CN-bound Ca compared to the control milk gel. Increased CN binding of Ca due to the presence of Sr has been reported by Zhang and Aoki (1995). The percentage of CN-bound Mg was much higher in the 10-mmol.L^{-1} SrCl_2 milk gel compared to the control milk gel at both the gelation point and 6 h after rennet addition. It is likely that Sr phosphate nanoclusters formed in the SrCl_2 milks. These Sr nanoclusters may have a different stoichiometric ratio of ions compared to conventional Ca-based CCP nanoclusters (Cooke and McSweeney 2013) and may have slightly different properties as a result. In the 10-mmol.L^{-1} CaCl_2 milk gel, the level of CN-bound Ca remained constant between the gelation point and during aging, whereas the CN-bound Mg and Sr decreased in the 10-mmol.L^{-1} MgCl_2 and SrCl_2 milks, respectively, during this time period (Figs. 1, 2, and 3). This decrease in CN-bound Mg or Sr led to a concurrent decrease in Ca in the respective milks 6 h after rennet addition, indicating that their ability to promote Ca binding decreased. It is also possible that the Sr phosphate nanoclusters are less stable during aging compared with Ca phosphate nanoclusters.

3.4 Dynamic rheological properties as a function of renneting time

The G' values as a function of time for rennet-induced gels are shown in Fig. 5. Gel properties were studied for 6 h after rennet addition. It is evident that the addition of $MgCl_2$, $CaCl_2$, and $SrCl_2$ at all concentrations caused an increase in gel firming rate. The slow increase in the G' above 10 Pa probably reflects the ongoing fusion of micelles and other structural rearrangements which results in an increase in the contact area between aggregated particles (Lucey 2002; Mellema et al. 2002), increasing the

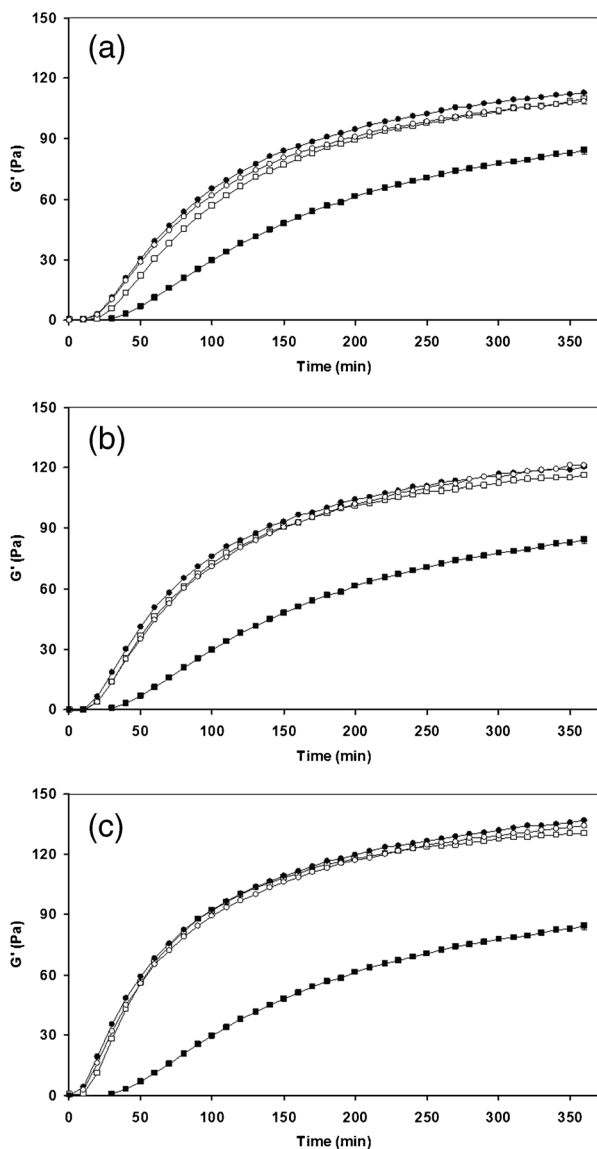


Fig. 5 Storage modulus (G') as a function of time for rennet-induced milk gels produced from control milk (closed square) and milks supplemented with $MgCl_2$ (open square), $CaCl_2$ (closed circle), or $SrCl_2$ (open circle) at concentrations of **a** 2.5 mmol.L⁻¹, **b** 5.0 mmol.L⁻¹, and **c** 10 mmol.L⁻¹. Error bars ± 1 standard deviation ($n=3$)

number of bonds in the network. Supplemented milk gels had much higher G' values 6 h after rennet addition (G'_{6h}) compared to the control (Table 2). Increasing the concentration of all salts from 2.5 to 10 mmol.L⁻¹ also increased G'_{6h} . At an addition level of 2.5 mmol.L⁻¹, the SrCl₂ and MgCl₂ milk gels had similar ($P>0.05$) G'_{6h} values, whereas at 5.0- and 10-mmol.L⁻¹ addition levels, the values for SrCl₂ were higher ($P<0.05$) than those of gels with MgCl₂ added. The CaCl₂-supplemented milk gels had a higher G'_{6h} than MgCl₂ milk gels at all addition levels. Zoon et al. (1988) reported that G' was ~10% higher in milk gels supplemented with 6-mmol.L⁻¹ added CaCl₂ after long aging times. Udabage et al. (2001) observed an increase in G' after ~3 h of aging when CaCl₂ was increased from 5 to 10 mmol.kg⁻¹. Differences in G' between both CaCl₂ and SrCl₂ gels compared with MgCl₂ gels suggest that a higher nanocluster concentration in the former milks had a major effect. Udabage et al. (2001) observed an increase in G' of rennet milk gels with an increase in CCP content up to ~130% of that in the original micelles. Conversely, Choi et al. (2007) found that the removal of CCP from milk at constant pH led to a decrease in G' . In native casein micelles, it is estimated that ~10% of phosphoserine clusters are unreacted, i.e., not involved in stabilizing nanoclusters (Holt 2004). An increased number of individual nanoclusters within the casein micelles of the CaCl₂ or SrCl₂ milks (assuming their spatial distribution remains similar to those in native micelles) would presumably limit the amount of fusion between contacting micelles. However, partial micellar fusion should increase the number of sites where nanocluster can form. Reduced micellar fusion leads to an increased strand thickness and strength and therefore more resistance to endogenous stresses, giving the gel a more elastic-like character and less tendency to rearrange (Mellema et al. 2002). Therefore, an increase in casein-casein affinity without increased particle fusion may explain the increased gel strength.

3.5 Dynamic rheological properties as a function of frequency

Figure 6 displays G' values as a function of frequency for rennet gels. The G' values increased with the increasing frequency in agreement with Zoon et al. (1988) as bonds in the matrix have less time to relax if the timescale of the applied stress is shorter. The G' values of gels at all addition levels of MgCl₂, CaCl₂, and SrCl₂ were higher than the control milk gel at all frequencies analyzed. For 2.5-mmol.L⁻¹ addition levels, the G' values of MgCl₂, CaCl₂, and SrCl₂ were similar ($P>0.05$) at all frequencies. However, at addition levels of 5.0 mmol.L⁻¹, the G' value of the MgCl₂ milk gel was lower than both the CaCl₂ and SrCl₂ milk gels, and this difference increased further at 10 mmol.L⁻¹, especially at low frequencies (<0.01 Hz). Zoon et al. (1988) observed that lower levels of CCP in skim milk can lead to lower G' values as a function of frequency in rennet-induced gels, whereas higher CCP has the opposite effect. These results indicate that the addition of CaCl₂ and SrCl₂ to milks at 5.0–10 mmol.L⁻¹ led to rennet gels with increased elastic properties and rigidity than the gels made from the respective MgCl₂-supplemented milks.

Tan δ as a function of frequency in rennet gels is shown in Fig. 7. Tan δ values generally decreased with the increasing frequency in agreement with Zoon et al. (1988). At low frequencies (below 0.1 Hz), all supplemented milk gels had lower tan δ values than the control milk gel. At 2.5-mmol.L⁻¹ addition levels, the tan δ at 0.001 Hz was lower in both the CaCl₂ and SrCl₂ milk gels compared to the MgCl₂ gel. This difference became more marked at 5.0- and 10-mmol.L⁻¹ supplementation levels. At

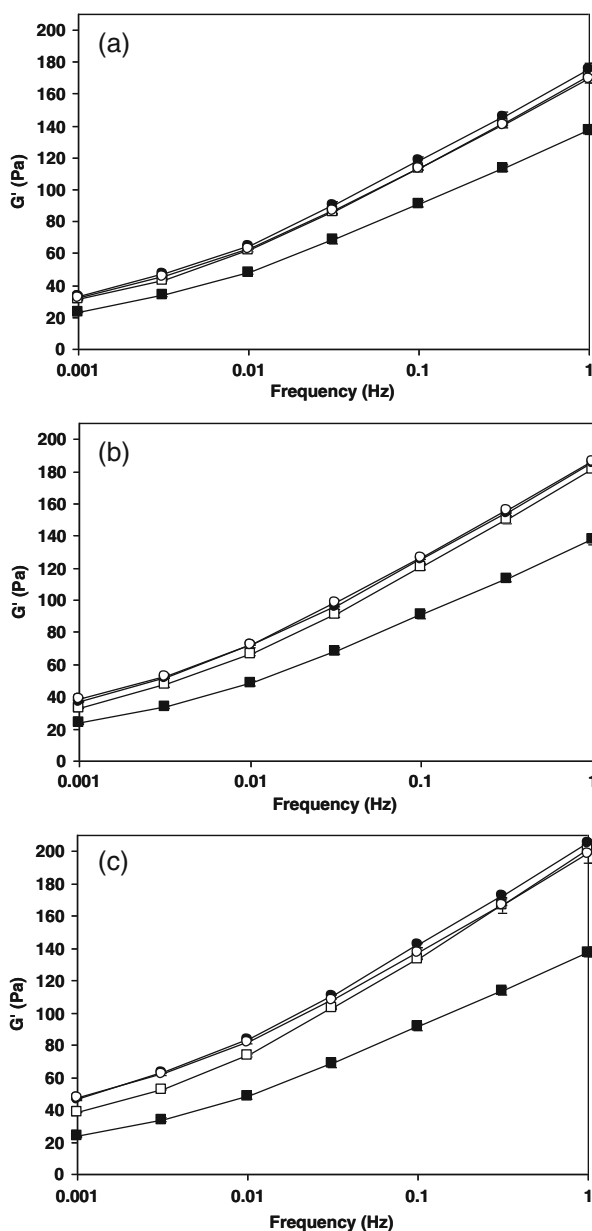


Fig. 6 Storage modulus (G') as a function of frequency for rennet-induced milk gels produced from control milk (closed square) and milks supplemented with $MgCl_2$ (open square), $CaCl_2$ (closed circle), or $SrCl_2$ (open circle) at concentrations of **a** 2.5 mmol.L⁻¹, **b** 5.0 mmol.L⁻¹, and **c** 10 mmol.L⁻¹. Frequency sweep was carried out 6 h after rennet addition. Error bars ± 1 standard deviation ($n=3$)

0.1 Hz, the $\tan \delta$ values of all the $MgCl_2$ gels and the control gel were similar ($P>0.05$); however, the gels made from milk containing 5.0 or 10 mmol.L⁻¹ of $CaCl_2$ and $SrCl_2$ were lower than both the equivalent gels containing $MgCl_2$ and the control gel. At low frequencies (below 0.01), $\tan \delta$ values of 10 mmol.L⁻¹ of $SrCl_2$ milk gels were lower

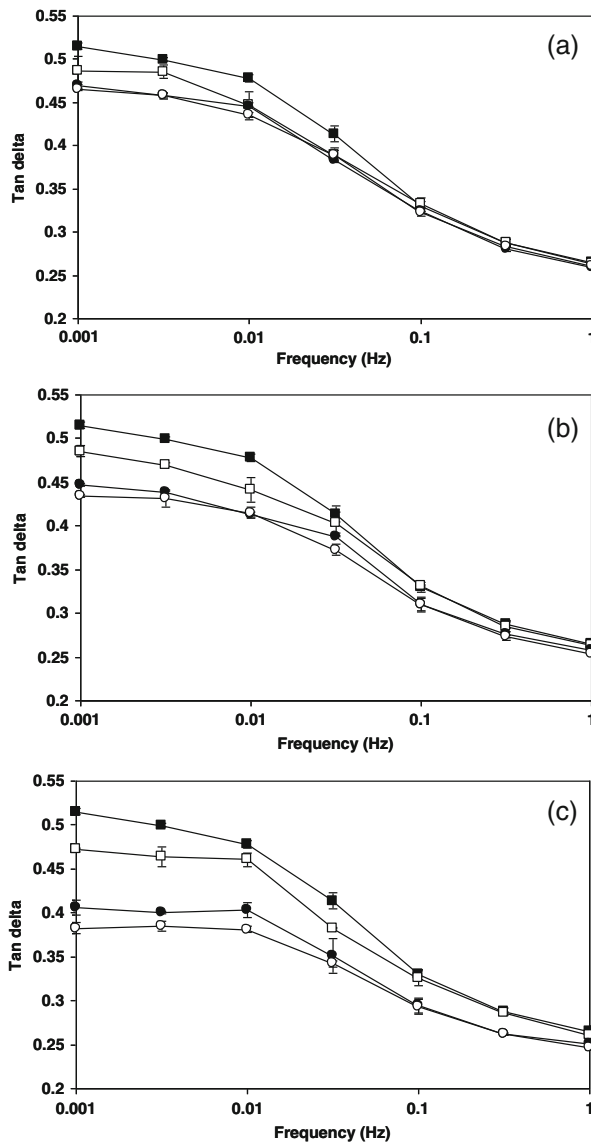


Fig. 7 Loss tangent ($\tan \delta$) as a function of frequency for rennet-induced milk gels produced from control milk (closed square) and milks supplemented with MgCl_2 (open square), CaCl_2 (closed circle), or SrCl_2 (open circle) at concentrations of **a** 2.5 mmol.L^{-1} , **b** 5.0 mmol.L^{-1} , and **c** 10 mmol.L^{-1} . Frequency sweep was carried out 6 h after rennet addition. Error bars ± 1 standard deviation ($n=3$)

($P<0.05$) than the corresponding CaCl_2 gels. The susceptibility of rennet milk gels to syneresis has been associated with a high value (>0.4) for $\tan \delta$ at long time scales (Van Vliet et al. 1991), which is related to increased network rearrangements after gel formation. The control milk gel had a $\tan \delta$ value of ~ 0.51 when measured at 0.001 Hz. Addition of MgCl_2 , CaCl_2 , and SrCl_2 significantly decreased ($P<0.05$) this value, with CaCl_2 and SrCl_2 addition having a much greater effect than MgCl_2 at all

concentrations added. Mellema et al. (2002) observed that the higher the percentage of Ca in micelles, the lower the value of $\tan \delta$ measured at 0.001 Hz. This indicates that the divalent cations may have reinforced the internal structure of the casein micelles in the supplemented milks and produced gels with increased resistance to syneresis. As partial particle fusion is related to the liquid-like or viscous behavior of primary particles in rennet milk gels (Mellema et al. 2002), it can be concluded that a higher $\tan \delta$ can indicate an increased particle fusion in rennet-induced gels. Presumably, an increased rigidity of the internal structure of micelles caused by increased nanocluster levels would retard micellar fusion. Zoon et al. (1988) found that rennet milk gels displayed higher $\tan \delta$ values with lower CCP, whereas gels with a higher CCP had lower $\tan \delta$ values especially at low frequencies.

For all rennet-induced gels, plotting $\log G'$ versus \log frequency curves gave straight lines ($r^2 > 0.98$ in all cases; results not shown). The power law exponent for the relationship between G' and frequency in rennet milk gels is in the range ~ 0.23 to 0.25 (Lucey 2002). The mean value of the power law exponent for the control rennet gel in the present study was ~ 0.26 , which is in close agreement with previously reported values for skim milk rennet gels (Esteves et al. 2002; Mishra et al. 2005; Srinivasan and Lucey 2002). Addition of divalent cations decreased the value of this parameter (Table 2), indicating a more solid-like character in these gels. CaCl_2 and SrCl_2 addition had a much greater impact than MgCl_2 especially at 10-mmol.L^{-1} addition levels where values of the CaCl_2 and SrCl_2 gels were ~ 0.21 compared to ~ 0.24 for the MgCl_2 gel.

4 Conclusions

This study demonstrated that the mineral equilibria of rennet milk gels have a major effect on gel properties during aging. It was evident that both the CaCl_2 - and SrCl_2 -supplemented milks had superior gel properties compared to the MgCl_2 -supplemented milks and the control milk. This was most likely due to the increased level of nanoclusters in the micelles of milks supplemented with CaCl_2 and SrCl_2 . The inability of added MgCl_2 to induce rennet gel properties similar to those of CaCl_2 - and SrCl_2 -supplemented gels was likely a consequence of the higher solubility of Mg phosphate salts compared to Ca and Sr phosphates (Zhang and Aoki 1995), preventing Mg phosphate nanoclusters from forming. As CN-bound levels of certain minerals decreased during aging, it is possible that a change in the dimensions or density of nanoclusters during gel aging may facilitate rearrangements. Ultimately, the increased nanocluster formation induced by the solubilities of various salts is a characteristic mechanism of casein micelles in milk to prevent tissue calcification, through their rheomorphic structure and phosphoserine centers (Holt 2004). This study provides information for future studies on the mineral fortification of milk, manipulation of mineral equilibria, and modulation of the rheological properties of milk gels and potentially the manufacture of cheese with milk supplemented with these mineral salts.

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