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# Influence of ionic strength and temperature on mineral and protein partitioning of skim milk

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ABSTRACT

This study investigated the influence of increased ionic strength (*I*) induced by addition of NaCl (80–580 mM) to pasteurised skim milk minerals partitioning, casein micelle properties and β-casein partitioning as a function of temperatures (i.e., 4, 10, 25, and 55 °C). Increasing *I* influenced the partition of divalent minerals in serum phase, especially total and ionic calcium concentration in the serum phase, while it had no effect on monovalent mineral partitioning. pH significantly decreased with increasing *I* due to changes in mineral equilibria, and increased protein content in the milk serum phase, especially at low temperatures. The average particle size and net zeta-potential decreased with increasing *I* most pronounced at 25 and 55 °C. The results obtained in this study provide new insights into mineral and protein partitioning of milk as influenced by ionic strength relevant for control of mineral distribution and for the development of new dairy products.



## 1. Introduction

Milk is a biological fluid produced in the mammalian mammary glands, and supports the growth of infants. Milk has a complex composition as made of an emulsion of fat globules, proteins, carbohydrates (mainly lactose), and minerals (Chen, Leong, Kentish, Ashokkumar, & Martin, 2019). Minerals in milk constitute a small fraction of the total constituents (~9 g $L^{-1}$), with the most important being calcium, magnesium, sodium and potassium as cations, and phosphate, citrate and chloride as dominant anions (Gaucheron, 2005). Calcium (Ca) is considered to be the most important of the milk minerals and it has an important role in casein micelle stability and protein–protein interactions (Lewis, 2011; Sun et al., 2022), and it can influence the functional, textural, and rheological properties of dairy products due to Ca-dependent interactions between dairy proteins (Barone, O'Regan, Kelly, & O'Mahony, 2022; Cooke & McSweeney, 2017; Pawlos, Znamirowska-Piotrowska, Kowalczyk, & Zaguła, 2022; Thybo, Lillevang, Skibsted, & Ahrné, 2020).

Total Ca in bovine milk amounts to ~30 mM of which ~20 mM is associated with colloidal phase of milk, mainly to the casein micelles and is known as colloidal calcium phosphate, CCP, while ~10 mM is dispersed in the serum phase of milk as soluble calcium; either dissolved as $Ca^{2+}$ (~1–3 mM) or as Ca complexed with citrate, phosphate, serum casein monomers and other ligands in the milk serum phase (Jiang, Liu, de Zawadzki, & Skibsted, 2023). Milk is a biphasic system (comprised of colloidal and serum phases) with the CCP of the colloidal phase in equilibria with the aqueous phase with various salts and proteins (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). The highly dynamic mineral equilibria and salt speciation of milk can alter whey proteins and casein micelles stability, thereby affecting the process-ability of milk and the final functionality of dairy-based products (Bijl, van Valenberg, Huppertz, & van Hooijdonk, 2013; Zhao & Corredig, 2015).

The addition of sodium chloride (NaCl) to bovine milk is known to alter the structure and charge of casein micelles due to changes in the mineral balance between the serum and the colloidal phase of milk. Usually, the monovalent cation sodium ($Na^+$) acts as screening to the negative charged phosphoseryl residues and carboxyl groups of caseins and vice versa for chloride (shielding amino group or cationic amino acid residues), with the extent of such interactions being dependent on pH. At low or innate ionic

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strength of milk (e.g., ≤80 mM), dairy proteins are more soluble and electrostatically repeal each other, while for increased ionic strength obtained by addition of salt (e.g., NaCl), such electrostatic repulsions diminish with a potential formation of protein aggregation and/or precipitation (Broyard & Gaucheron, 2015). In addition, it has been observed that addition of NaCl in milk can decrease pH in conjunction with increased levels of Ca in the serum phase of milk (Huppertz, Fox, & Kelly, 2018).

It has been reported in literature that the technological functionality of casein micelles can be influenced by different concentrations of NaCl. For example, Awad (2007) showed that during cheese making, rennet coagulation time increased with increasing concentration of NaCl, with the formed gel having reduced firmness and higher moisture content. Furthermore, the addition of NaCl (up to 400 mM) shifted the maximum buffering capacity of milk from pH 5.2 without NaCl addition to 5.5 due to the solubilisation of micellar phosphate (Salaün, Mietton, & Gaucheron, 2005). Huppertz and Fox (2006) investigated the influence of NaCl to increase the ionic strength of concentrate milk on heat and ethanol stability, establishing that a range of ⩾425 or ⩾210 mmol $L^{-1}$ NaCl prominently influenced heat stability and less extensively ethanol stability. In terms of powder functionality, Sikand, Tong, and Walker (2013) showed that the rehydration properties of micellar casein powder produced using salted water as diafiltration media was dramatically improved compared with a reference powder. Temperatures also play a role in the speciation and distribution of minerals and casein (e.g., β-casein) in the milk phases (i.e., colloidal and serum) (Liu, Weeks, Dunstan, & Martin, 2013; Post, Arnold, Weiss, & Hinrichs, 2012) with cooling- and heating-induced reaction being largely reversible. Only high temperatures >90 °C and long-time heating treatment for >20 min induce irreversible salt partitioning. For example, cooling, a fundamental practice in the dairy industry, influences the solubility of CCP and its speciation with a two-stage solubilisation kinetic (Jiang et al., 2023; Koutina, Knudsen, Andersen, & Skibsted, 2014). Similarly to the solubilisation kinetics of CCP, also β-casein increases its solubility and is released in the serum phase at low temperatures such as in the 2–10 °C region (France, Bot, Kelly, Crowley, & O'Mahony, 2021; Jiang et al., 2023). In contrast, heating, increases the solubility of a large part of milk salts but not for inorganic Ca-phosphate and CCP (Nieuwenhuijse & Huppertz, 2022).

This study aimed to provide fundamental knowledge on: (i) the influence of ionic strength increased by the addition of NaCl and temperature on mineral equilibria of skim milk with an emphasis on Ca speciation, and (ii) the consequences for casein micelle properties and β-casein content in the serum phase partitioning from colloidal to serum phase. The knowledge provided is useful for those interested in temperature and NaCl-induced changes in the physicochemical properties of fresh skim milk for tailoring fresh dairy-based products or for controlling minerals content in the colloidal phase.

## 2. Materials and methods

### 2.1. Materials and sample preparation

Pasteurised commercial skim milk (72 °C for 15 s) having 0.1% (w/w) fat content was purchased locally (Arla Foods, Denmark) with macro-chemical composition determined using a Milkoscan™ FT2 (FOSS, Hillerød, Denmark) with the exception of protein content that was determined as described in section 2.4. The values for protein, fat, carbohydrate and citrates were 4.12, 0.085, 4.94 and 0.18 (%, w/w), respectively. Sodium chloride (NaCl) was added to milk aliquots (100 mL) at increasing concentrations of 0.50, 0.75, 1.00, 2.50, and 3.00% (w/w). Samples were equilibrated at different temperatures of 4.0, 10.0, 25.0, and 55.0 °C for 12 h under continuous stirring. For each sample, serum phase was produced using a Beckman Optima XE-90 ultracentrifuge (Beckman-Coulter, Brea, CA, USA) at 100,000 × *g* for 1 h and at the temperature used in this study of 4, 10, 25 and 55 °C. Skim milk without sodium chloride addition was used as a benchmark. The pH of samples was measured using an Intellical PHC805 pH meter (Hach company, CO, USA) calibrated using standard pH solution of 2.00, 4.00, 7.00, and 10.00 at the relevant temperature. The water used was of ultra-pure grade (Milli-Q Plus system, Millipore Corp, Bedford, MA, USA). All chemicals and reagents were of analytical grade and purchased from MilliporeSigma (Merck group, Burlington, MA, USA).

### 2.2. Mineral profile

Mineral profile of milk and serum phases was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using an Agilent 5100 ICP-OES (Agilent Technologies, CA, USA) according to the method previously described by Rasmussen, Suwal, van den Berg, Yazdi, and Ahrné (2020). The parameters analysed were Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na), and Phosphorus (P). Samples were treated before injection by acid digestion using nitric and hydrochloric acid in a Multiwave GO microwave system (Anton Paar, Graz, Austria). For each of the aforementioned elements, standard curves were prepared at specific wavelengths using a multi-element ICP standard solution. The minerals determined in milk without the addition of NaCl were 33.4, 7.02, 25.2, 42.5, 18.5, mM for Ca, Mg, P, K, and Na, respectively.

### 2.3. Ionic calcium and conductivity

Ionic calcium concentration ($[Ca^{2+}]$) of all samples (e.g., skim milk and respective serum phases) was determined using an ion-selective electrode (Metrohm AG, Herisau, Switzerland) as previously described by Barone, Moloney, O'Regan, Kelly, and O'Mahony (2020) and Crowley et al. (2014) with minor modifications. The system was calibrated with standard solutions made of $CaCl_2$, and ultra-pure water at different concentrations (0.50, 1.00, 3.00, 6.00, and 9.00 mM $CaCl_2$) The ionic strength of the standards solution was provided using imidazole (13.5 mM) and potassium chloride (67.5 mM) at different temperatures of 4, 10, 25, and 55 °C using a thermostatic water bath equipped with cooling unit (Grant Optima TC120, Grant Instruments, Shepreth, UK). The calibration curve was obtained from the linear relationship of the log of the $[Ca^{2+}]$ and the relative electrode potential difference (mV) based on the Nernst equation. Conductivity was measured using a Sension$^+$ EC71 GLP (Hach company, Iowa, USA), using three standard solutions of 0.00147, 1.00, and 12.88 mS $cm^{-1}$. Ionic strength of the sample was calculated from the conductivity value using inverse pseudo-linear Marion and Babcock approach and temperature compensation according to the method of Crowley et al. (2014).

### 2.4. Particle size distribution and zeta-potential

The particle size distribution using dynamic light scattering (DLS) and ζ-potential was measured using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK) at 25 °C. Samples added NaCl were diluted (1:100) with a solution made of imidazole and NaCl at pH 6.7 in line with the ionic strength of the sample. A refractive index value of 1.45 was used for protein, and the dispersant refractive index varied in agreement with the different [NaCl]. The ζ-potential of samples was measured at 25 °C for 120 s in automatic voltage mode and calculated using the Smoluchowski model.

### 2.5. Protein content and β-casein quantification

Nitrogen content of the samples (e.g., serum phase and milk) was determined by Dumas using the NRapid Max Exceed (Elementar, Frankfurt, Germany). The nitrogen content was converted to protein content calculated per 100 g of product (%, w/w) with a conversion factor of 6.38. Quantification of β-casein of serum phase and milk was performed using reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent series 1200 system, Agilent Technologies) using the method described by Bot, Crowley, and O'Mahony (2020). A reverse phase C18 column (Zorbax C18, 4.6 × 250 mm 5 mm, Agilent Technologies) was used. The elution gradient was eluent A (10.0%, w/v, HPLC-grade acetonitrile, 89.9% ultrapure water and 0.10% trifluoroacetic acid) and B (89.9% HPLC grade acetonitrile, 10.0% ultrapure water and 0.10% trifluoroacetic acid). The injected volume was 40 mL and detection was carried out at 214 nm. A calibration curve for β-casein was established using 7 points, ranging from 0.070 to 1.5 mg $mL^{-1}$ using analytical standards having a purity level of ≥95%.

### 2.6. Statistical data analysis

Samples were prepared three times independently, and all analyses were performed in triplicate for each independent experiment. The data generated was subjected to one-way analysis of variance (ANOVA) using R i386 version 3.3.1 (R foundation for statistical computing, Vienna, Austria). A Tukey's paired-comparison post-hoc test was used to determine statistically significant differences ($p$ <0.05) between mean values for different samples, at the 95% confidence level. Results are expressed as mean value ± standard deviation, and statistically significant differences are identified in tables using super-script letters, unless otherwise stated.

## 3. Results and discussion

### 3.1. Influence of ionic strength and temperature on minerals profile and ionic calcium

The mineral concentrations of skim milk serum phase as a function of increased ionic strength ($I$) induced by the addition of sodium chloride at temperatures ranging from 5 to 55 °C is displayed in Table 1. As expected, the content of the monovalent sodium (Na) of the produced serum phase increased as a function of increased $I$, due to the addition of sodium chloride [NaCl] without a significant effect of temperatures on Na content (Table 1). The increased $I$ have not influenced the potassium (K) concentration in the serum phase which showed to be in equilibrium between the two phases of skim milk. Indeed, K concentration of the serum phase ranged from 39.7 to 45.2 mM at all conditions studied, which was not significantly different from milk (~42.5 mM) with such a range of [K] being in line with the literature (Gaucheron, 2001).

Magnesium (Mg) is known to have similar properties to calcium (Ca) in terms of interactions with dairy proteins (Oh & Deeth, 2017), and both concentrations increased in the serum phase with increasing $I$. In detail, Mg content increased slightly but significantly ($p$ <0.05) in the serum phase in the $I$ range from 250 to 593 mM, especially at 4 °C. Although a large proportion of Mg (2/3 of the total) is in the serum phase of milk, the rest of it is associated to colloidal calcium phosphate (CCP), thereby its slight increase in serum phase at colder temperatures is due to the increased CCP solubility as discussed forward (Cashman, 2011). The increase in total Ca in the serum phase was not linearly dependent on the increasing $I$ provided by NaCl but approached a steady value for each temperature at high NaCl concentrations (i.e., ≥507 mM). Such Ca concentration at high Na concentration decreases with increasing temperature. Such property is in agreement with an ion-exchange mechanism (eq. (1)) previously outlined by Gaucheron (2005).

$$\mathrm{Ca-casein} + 2\mathrm{Na}^{+} \rightleftharpoons \mathrm{Na_2-casein} + \mathrm{Ca}^{2+} \quad (1)$$

The equilibrium constant of such exchange reaction (eq. (1)) decreases with increasing temperature corresponding to $\Delta H^{0}_{exch}$ <0 (Jiang, Liu, de Zawadzki, & Skibsted, 2021; Liu, Jiang, Ahrné, & Skibsted, 2022). This is confirmed by the Ca content in serum phase that increased considerably at 4 °C when compared with 55 °C, with an percentage increase of 40.1 and 32.9% at 80 and 593 mM of $I$, respectively. In contrast to the total Ca content in the serum phase, ionic calcium ([$Ca^{2+}$]) levels depend linearly on increased $I$ (Fig. 1). This linearity adds further support to an ion-exchange mechanism with only partial exchange of calcium in the caseins. The content of phosphorus (P) in milk serum increased significantly ($p$ <0.05) from 80 to 593 mM of $I$ at all temperatures studied, with the exception of the samples produced at 55 °C. In detail, P content increasing percentage was 15.0, 20.2, 49.6% at 4, 10 and 25 °C, respectively, while at 55 °C, P content was largely unchanged, similar to what was observed for Ca at 55 °C. The content of P in the serum phase showed the same partitioning kinetic in the serum phase as Ca as previously observed by Jiang et al. (2023) for fresh milk not added with sodium chloride, with such increase of P content is mainly ascribable to (a) an extent dissolution of CCP due to increased $I$, which reduces the activity coefficient of ions (Willey, 2004), and secondary (b) ion-exchange from carboxyl group and free phosphoserine group of casein (Gaucheron, 2005).

The ratio of Ca:P in the serum phase was higher at low temperatures than at high temperatures. Values for Ca:P were 0.76 and 0.78 at 80 mM of $I$ at 4 and 10 °C, respectively, and gradually increased with increasing $I$ with final values of 0.87 and 0.82 at 593 mM of $I$ at 4 and 10 °C, respectively. In contrast, at higher temperatures, the Ca:P ratio was lower than at lower temperatures but gradually increased with increasing $I$ (from 0.74 to 0.78 and from 0.52 to 0.73 at 80 and 593 mM of $I$ for 25 and 55 °C, respectively).

It has been established that the addition of NaCl does not primarily influence the partitioning of monovalent minerals in milk phases. Salt addition, will only have some effect at higher temperatures due to increased salt diffusion coefficient (Gaucheron, 2001). However, the addition of NaCl, and thus increased ionic strength, can alter the partitioning and balance between milk phases of Ca followed by P and Mg. Specifically for Ca, NaCl induces ion-exchange phenomena between $Na^{+}$ and $Ca^{2+}$ ions at the phosphoseryl residues of the casein molecules (eq. (1)) (Gaucheron, 2001; Nieuwenhuijse & Huppertz, 2022), thereby increasing Ca content in the serum phase. Low temperature also further increased total Ca in the serum phases due to the increasing solubility of colloidal calcium phosphate (CCP). Indeed, P also increased along with Ca, which is ascribable to increased CCP solubility, as the Ca:P ratio determined in the present study was in line with previous reports for different calcium phosphate salts (Nieuwenhuijse & Huppertz, 2022; Raynaud, Champion, Bernache-Assollant, & Thomas, 2002).

Ionic calcium ($Ca^{2+}$) is known to influence dairy protein physicochemical properties, especially during the processing of dairy-based products (Barone, Yazdi, Lillevang, & Ahrné, 2021). The ionic calcium concentration ([$Ca^{2+}$]) of skim milk and respective serum phase as a function of ionic strength ($I$) is displayed in Fig. 1. The [$Ca^{2+}$] of skim milk and respective serum phase samples increased, almost linear (lowest $R^2$ was 0.978), as a function of increasing $I$. This increase in [$Ca^{2+}$] was also found to be

**Table 1**
Mineral concentration (mM) of serum phase produced from skim milk at different temperatures (i.e., 4, 10, 25, 55 °C) as a function of increasing ionic strength (mM) obtained by addition of sodium chloride.[a]

| Ion | T (°C) | Ionic strength (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 80 | 165 | 208 | 250 | 507 | 593 |
| Calcium | 4 | 19.2 ± 2.28$^{a}$ | 22.3 ± 3.04$^{b}$ | 23.2 ± 3.26$^{c}$ | 24.0 ± 1.51$^{d}$ | 24.8 ± 1.16$^{d}$ | 25.2 ± 1.54$^{e}$ |
| | 10 | 17.0 ± 2.93$^{a}$ | 18.7 ± 3.29$^{b}$ | 18.6 ± 3.01$^{b}$ | 19.0 ± 3.56$^{c}$ | 20.3 ± 4.34$^{d}$ | 21.4 ± 5.90$^{e}$ |
| | 25 | 12.1 ± 0.57$^{a}$ | 13.4 ± 4.89$^{b}$ | 14.5 ± 2.21$^{c}$ | 15.5 ± 4.96$^{d}$ | 18.0 ± 1.63$^{e}$ | 19.0 ± 1.01$^{f}$ |
| | 55 | 11.5 ± 1.61$^{a}$ | 12.2 ± 1.16$^{b}$ | 13.8 ± 1.93$^{c}$ | 14.3 ± 2.04$^{d}$ | 15.0 ± 1.62$^{e}$ | 16.9 ± 2.10$^{f}$ |
| Phosphorus | 4 | 25.3 ± 0.51$^{a}$ | 26.2 ± 0.64$^{b}$ | 27.7 ± 2.46$^{c}$ | 28.2 ± 2.60$^{d}$ | 30.5 ± 0.66$^{e}$ | 29.1 ± 1.22$^{f}$ |
| | 10 | 21.7 ± 1.70$^{a}$ | 23.7 ± 1.91$^{b}$ | 23.5 ± 1.51$^{ab}$ | 23.3 ± 3.01$^{ab}$ | 23.6 ± 2.85$^{b}$ | 26.1 ± 3.27$^{c}$ |
| | 25 | 16.3 ± 2.35$^{a}$ | 18.7 ± 2.72$^{b}$ | 19.5 ± 3.51$^{c}$ | 22.9 ± 1.46$^{d}$ | 24.0 ± 4.07$^{e}$ | 24.4 ± 1.92$^{e}$ |
| | 55 | 22.2 ± 1.25$^{b}$ | 21.3 ± 1.70$^{a}$ | 22.5 ± 2.33$^{c}$ | 21.2 ± 2.04$^{a}$ | 22.0 ± 0.43$^{b}$ | 23.2 ± 0.83$^{d}$ |
| Magnesium | 4 | 5.10 ± 1.01$^{a}$ | 5.47 ± 1.27$^{b}$ | 5.24 ± 1.21$^{c}$ | 5.15 ± 1.49$^{d}$ | 5.72 ± 1.29$^{e}$ | 5.36 ± 2.10$^{f}$ |
| | 10 | 4.27 ± 1.33$^{b}$ | 4.63 ± 1.36$^{d}$ | 4.54 ± 1.30$^{c}$ | 3.87 ± 1.49$^{a}$ | 4.55 ± 0.64$^{c}$ | 4.87 ± 1.75$^{e}$ |
| | 25 | 4.87 ± 0.21$^{d}$ | 3.92 ± 1.42$^{b}$ | 3.88 ± 2.24$^{a}$ | 4.17 ± 1.19$^{c}$ | 5.08 ± 0.27$^{e}$ | 5.10 ± 1.05$^{e}$ |
| | 55 | 4.88 ± 0.54$^{a}$ | 4.87 ± 1.16$^{ab}$ | 4.96 ± 1.42$^{bc}$ | 4.97 ± 1.13$^{ab}$ | 5.07 ± 0.66$^{c}$ | 5.32 ± 1.77$^{d}$ |
| Potassium | 4 | 44.3 ± 2.64$^{cd}$ | 44.7 ± 1.97ec | 42.4 ± 3.44$^{ab}$ | 42.1 ± 1.00$^{abc}$ | 44.4 ± 1.48$^{d}$ | 41.2 ± 2.49$^{a}$ |
| | 10 | 41.8 ± 2.63$^{ab}$ | 43.2 ± 1.22$^{cd}$ | 42.7 ± 2.84$^{ab}$ | 42.8 ± 2.27$^{bc}$ | 41.3 ± 2.64$^{a}$ | 43.8 ± 1.27$^{d}$ |
| | 25 | 41.2 ± 2.92$^{b}$ | 43.5 ± 1.72$^{c}$ | 40.5 ± 1.57$^{b}$ | 43.7 ± 2.78$^{c}$ | 39.7 ± 1.63$^{a}$ | 39.9 ± 2.13$^{a}$ |
| | 55 | 43.0 ± 2.38$^{ab}$ | 41.6 ± 2.67$^{a}$ | 42.5 ± 3.87$^{ab}$ | 43.0 ± 4.36$^{bc}$ | 42.4 ± 3.42$^{ab}$ | 45.2 ± 3.29$^{d}$ |
| Sodium | 4 | 18.7 ± 2.37$^{a}$ | 105 ± 5.21$^{b}$ | 141 ± 9.09$^{c}$ | 182 ± 16.6$^{d}$ | 447 ± 10.3$^{e}$ | 491 ± 28.2$^{f}$ |
| | 10 | 16.2 ± 1.65$^{a}$ | 100 ± 2.71$^{b}$ | 138 ± 13.5$^{c}$ | 180 ± 17.6$^{d}$ | 402 ± 15.9$^{e}$ | 511 ± 43.6$^{f}$ |
| | 25 | 18.3 ± 2.06$^{a}$ | 98.6 ± 6.42$^{b}$ | 141 ± 10.9$^{c}$ | 155 ± 15.7$^{d}$ | 430 ± 18.2$^{e}$ | 493 ± 40.9$^{f}$ |
| | 55 | 19.0 ± 2.49$^{a}$ | 102 ± 8.04$^{b}$ | 143 ± 8.21$^{c}$ | 187 ± 14.3$^{d}$ | 438 ± 17.5$^{e}$ | 540 ± 46.1$^{f}$ |

[a] Values followed by different superscript letters in the same row are significantly different ($p$ <0.05).

significantly higher ($p$ <0.05) at low temperatures (i.e., 4 and 10 °C) than at high temperatures (i.e., 55 °C), while samples produced at 25 °C all had intermediate [$Ca^{2+}$] levels. In detail, the initial [$Ca^{2+}$] (i.e., 80 mM of $I$) were 1.25, 1.81, 2.93 and 2.62 mM for milk samples, while respective serum phase had 1.41, 2.18, 2.73, 2.91 mM at 4, 10, 25 and 55 °C, respectively. At high $I$ (i.e., 593 mM), [$Ca^{2+}$] levels were 8.41, 7.62, 5.19 and 3.75 mM for milk samples, with the respective serum phase having higher [$Ca^{2+}$] (8.80, 8.82, 5.79, 4.53 mM at 4, 10, 25 and 55 °C, respectively). The difference between the two extreme ionic strengths investigated (i.e., 80 and 593 mM) expressed as $\Delta_{[Ca^{2+}]}$ were 5.79, 4.69, 3.38 and 2.50 for milk samples and 5.89, 5.48, 3.61 and 3.10 for respective serum phase produced at 4, 10, 25, 55 °C, respectively. Interestingly, the proportion of [$Ca^{2+}$] out of the total Ca determined in serum phase increased as a function of decreasing temperature and increasing $I$, meaning that an increased proportion of CCP solubilised from the colloidal to serum phase. The ratio between $Ca^{2+}$ and total Ca decreases with increasing temperature in agreement with endothermic calcium binding to the caseins, citrate, phosphate as ligand in milk serum corresponding to $\Delta H^{0}_{complex}$ >0 for the reaction between Ca:Ligand as is well documented for both phosphates and citrates (Liu et al., 2022).

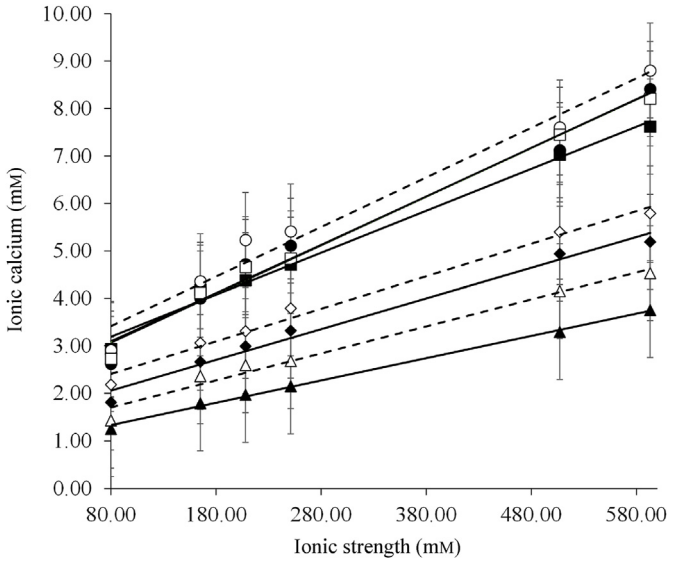


**Fig. 1.** Concentration of ionic calcium (mM) as a function of increased ionic strength (as NaCl) addition level for skim milk (—) and respective serum phase (···) at 55 °C (▲), 25 °C (◆), 10 °C (■), and 4 °C (●). Lines represent linear regression and hollow marks represent serum phase samples.

### 3.2. pH monitoring

The pH changes of milk and respective serum phase as a function of $I$ and temperature are displayed in Fig. 2. Samples generated at 55 °C had significantly lower ($p$ <0.05) pH than those produced at 4, 10 and 25 °C. The onset pH for the milk sample at 55 °C was 6.46 and steadily decreasing to pH 6.19 as for 593 mM of $I$. In contrast, other samples (i.e., milk and serum phase) produced at different temperatures had an onset pH of 6.69 and final pH ranging from

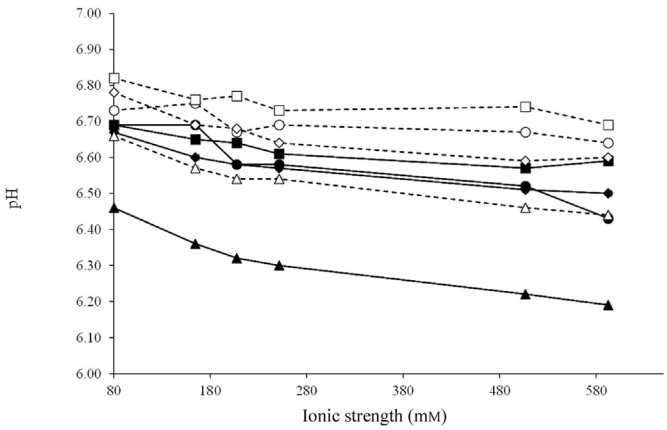


**Fig. 2.** Changes in pH as a function of ionic strength sourced as sodium chloride (NaCl) for skim milk (—) and respective serum phase (···) at 55 °C (▲), 25 °C (◆), 10 °C (■), and 4 °C (●). Hollow marks represent serum phase samples.

6.70 to 6.43 at 593 mM of *I*. This finding is in agreement with the solubility chemistry of CCP of milk that at high temperatures has lower solubility, forming calcium phosphate with release of hydrogen ions (eq. (2)) (Barone et al., 2021; Jiang et al., 2023; Lewis, 2011).

In the present study, the combination of temperatures and increasing *I* was found significantly to influence [$Ca^{2+}$] and pH of milk and respective serum phase. It is established that an increase in ionic strength by NaCl addition increases [$Ca^{2+}$] due to a decrease in the ion activity coefficients of calcium and phosphate both in the colloidal and serum phase of milk along with ion-exchange effect between $Ca^{2+}$ and $Na^{+}$ at single casein level (as previously outlined), causing an increase in the solubility and dissociation of calcium phosphate (Huppertz & Fox, 2006; Nieuwenhuijse & Huppertz, 2022). In addition, increasing temperature affected the solubility of calcium phosphate with high temperatures such as 55 °C lowered solubility, resulting in low [$Ca^{2+}$] and thereby decreased pH as seen in the present study and in agreement with eq. (2) (Lewis, 2011):

$$3Ca^{2+} + 2HPO_4^{2-} \leftrightarrow Ca_3(PO_4)_2\downarrow + 2H^{+} \quad (2)$$

### 3.3. Particle size and ζ-potential

The average particle size (APS) and ζ-potential (ζ-pot) as a function of increasing *I* and temperatures of milk is displayed in Figs. 3 and 4, respectively. Initial values (i.e., at 80 mM of *I*) for APS and ζ-pot were in line with previously published reports for casein micelles (de Kruif & Huppertz, 2012; de Kruif & Tuinier, 2002). At high *I* (i.e., 593 mM) milk sample progressively reduced both APS and ζ-pot at all the temperatures studied. Changes in both APS and ζ-pot were more significant ($p$ <0.05) at 55 °C than the other temperatures used in this study, with values for APD decreasing from 185 to 158 nm and for ζ-pot from −16.8 to −12.7 mV. Changes for sample generated using other temperatures (i.e., 4, 10 and 25 °C) and from 80 to 593 mM of *I*, decreased on average of ~10 nm and ~2.50 mV for APD and net ζ-pot, respectively.

Reduction in ζ-pot induced by change *I* by NaCl addition is due to changes in ionic strength of the serum phase influencing casein micelles. Indeed, the addition of NaCl increases the [$Ca^{2+}$] level in the serum phase of milk, which is known to decrease ζ-pot of casein micelles (Barone et al., 2021; Huppertz & Fox, 2006). Additionally, it is well established that exists an interrelationship between the pH

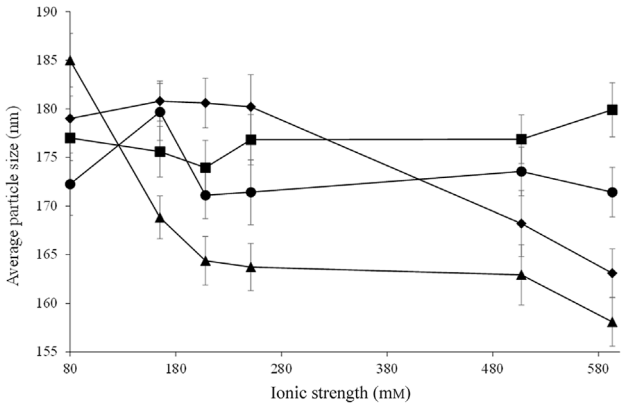


**Fig. 3.** Average particle size diameter as a function of ionic strength (mM) for skim milk at 55 °C (▲), 25 °C (◆), 10 °C (■), and 4 °C (●).

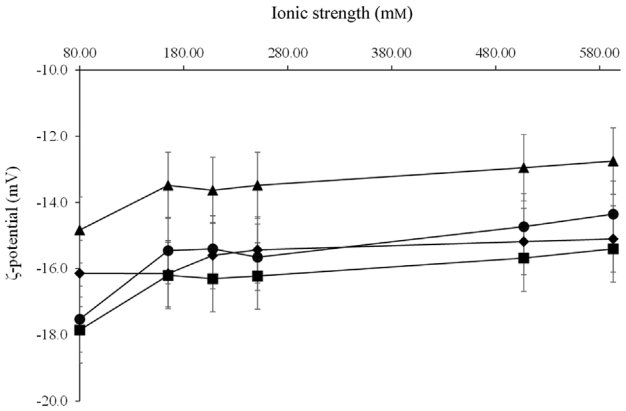


**Fig. 4.** Zeta potential as a function of ionic strength (mM) for skim milk at 55 °C (▲), 25 °C (◆), 10 °C (■), and 4 °C (●).

and [$Ca^{2+}$] in milk, and an increase in ionic strength levels as induced by NaCl addition further extend the exchange at casein level of $Ca^{2+}$ ions with $Na^{+}$ ions and thereby decrease the pH. This is due to the altered speciation and equilibria of CCP and calcium phosphate from casein micelles to serum phase, resulting in pH variation (Fig. 2) (Nieuwenhuijse & Huppertz, 2022; Zhao & Corredig, 2015). This was more evident for high temperature (i.e., 55 °C) in which the high total content of $Na^{+}$ (see Table 1) induced more significant variation in ζ-pot and, less extensively in APS, due to the lower pH as a consequence of calcium phosphate formation in agreement with eq. (2) when compared with the other temperature used in the study.

### 3.4. Total protein and β-casein content

Total protein and β-casein content of the produced serum phase from skim milk as a function of ionic strength and temperatures are both displayed in Table 2. Low temperatures (i.e., 4 and 10 °C) significantly increase ($p$ <0.05) protein content partitioned from the colloidal to serum phase of milk when compared with other temperatures (i.e., 25 and 55 °C). This was observed to be more pronounced at 4 °C, as the protein content increased from an initial value (i.e., 80 mM of *I*) of 1.90% to 2.13% (w/v) at 593 mM of I, which counted for ~53% of the total protein content of milk, and more specifically ~14.7% of partitioned net protein content from the colloidal phase. For samples produced at 10, 25 and 55 °C, the net protein content, partitioned from the colloidal phase at high *I* (i.e., 593 mM) were 9.22, 5.48 and 1.92% (w/v). Increasing *I* of milk, induced caseins to be depleted in the serum phase through modifications in the salt distribution between aqueous and micellar phases as previously outlined by Gaucheron (2005) and Zhao and Corredig (2015) with such being more pronounced at low temperatures (i.e., 4 and 10 °C). In more detail, the depletion of caseins in the serum was also positively correlated with the increasing content of β-casein in the serum phase (Table 2), with its partitioning mechanism and kinetics strongly influenced by temperature (especially at temperature ≤10 °C) (Jiang et al., 2023). Interestingly, β-casein concentration in the serum phase increased linearly, in a similar fashion to what was observed for total protein content, meaning that high *I* levels induced its partitioning in the serum phase, however, its relative proportion out of total casein was observed to domain by temperature rather than *I*, with values of 17.5, 10.2, 4.48 and 1.05% at 593 mM of *I* at 4, 10, 25 and 55 °C, respectively.

**Table 2**
Concentration of total protein and β-casein (%, w/v) in serum phase of milk at 4, 10, 25 and 55 °C as a function of increasing ionic strength.[a]

| Component | T (°C) | Ionic strength (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 80 | 165 | 208 | 251 | 507 | 593 |
| Total protein content | 4 | 1.90 ± 0.08$^{a}$ | 2.21 ± 0.33$^{bc}$ | 2.10 ± 0.14$^{b}$ | 1.92 ± 0.13$^{a}$ | 2.11 ± 0.08$^{bc}$ | 2.23 ± 0.02$^{c}$ |
| | 10 | 1.43 ± 0.06$^{a}$ | 1.52 ± 0.21$^{ab}$ | 1.47 ± 0.22$^{ab}$ | 1.41 ± 0.38$^{ab}$ | 1.59 ± 0.36$^{b}$ | 1.68 ± 0.44$^{b}$ |
| | 25 | 1.04 ± 0.03$^{a}$ | 1.10 ± 0.13$^{ab}$ | 1.24 ± 0.10$^{b}$ | 1.15 ± 0.11$^{b}$ | 1.16 ± 0.07$^{b}$ | 1.21 ± 0.06$^{b}$ |
| | 55 | 0.88 ± 0.10$^{a}$ | 0.96 ± 0.05$^{ab}$ | 0.99 ± 0.04$^{ab}$ | 1.02 ± 0.03$^{ab}$ | 1.01 ± 0.05$^{ab}$ | 1.18 ± 0.02$^{b}$ |
| β-Casein content | 4 | 0.262 ± 0.018$^{a}$ | 0.268 ± 0.030$^{a}$ | 0.266 ± 0.015$^{a}$ | 0.269 ± 0.018$^{b}$ | 0.270 ± 0.021$^{b}$ | 0.282 ± 0.017$^{b}$ |
| | 10 | 0.164 ± 0.007$^{a}$ | 0.167 ± 0.004$^{ab}$ | 0.173 ± 0.006$^{bc}$ | 0.176 ± 0.009$^{c}$ | 0.179 ± 0.010$^{cd}$ | 0.183 ± 0.011$^{d}$ |
| | 25 | 0.009 ± 0.001$^{a}$ | 0.011 ± 0.001$^{ab}$ | 0.011 ± 0.001$^{ab}$ | 0.013 ± 0.001$^{b}$ | 0.012 ± 0.002$^{b}$ | 0.013 ± 0.001$^{b}$ |
| | 55 | ND | ND | ND | ND | ND | ND |

[a] Values followed by different superscript letters in the same row are significantly different ($p$ <0.05); ND = not detected.

## 4. Conclusions

The increased ionic strength due to addition of sodium chloride to milk was found to induce changes the mineral equilibria and physicochemical properties of colloidal and serum phase of skim milk, and these changes are temperature dependent. Mineral equilibria of milk can be modified with more minerals being released into the serum phase, especially the divalent metal ions, by addition of NaCl. Total calcium content along with phosphorus increased in the serum phase of milk with the increasing ionic strength levels. Low temperatures further increased ionic calcium concentration and protein content in the serum phase, with β-casein increasing linearly along with the total protein content. High temperatures and ionic strength reduced average particle size and pH of milk, which is due to calcium phosphate release into the serum phase along with the high sodium content. The results obtained in this study provide fundamental insights into the influence of ionic strength on milk minerals distribution and physicochemical properties relevant for controlling mineral balance in milk and/or tailored dairy-based products having medium salinity (e.g., Ayran, cheese-like dips, etc.). Changes in moderate ionic strength can be used to increase mineral content of serum phase of milk, and more specifically, serum calcium increased with increasing ionic strength and decreased with increasing temperature.

## Author contribution statement

**Giovanni Barone**: Conceptualization, data curation, supervision, investigation, methodology, writing – original draft. **Federica Cirrincione**: Data curation, formal analysis, investigation, methodology, writing – review & editing. **Yuan Jiang**: Formal analysis, data curation. **Valentin Rauh**: writing – review & editing. **Søren K. Lillevang**: writing – review & editing. **Maria Fiorenza Caboni**: writing – review & editing. **Leif H. Skibsted**: Supervision, Data curation, investigation, writing – review & editing. **Lilia Ahrné**: Conceptualization, data curation, supervision, funding acquisition, investigation, methodology, writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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