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Impact of cheese milk cold storage on milk coagulation properties, calcium contents, and cheese yield

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ABSTRACT

This study evaluated the impact of storage conditions (time, 0–48 h; temperature, 4–16 °C) of raw cheese milk on coagulation properties using diffusion wave spectroscopy. Specifically, rennet coagulation time (RCT) and curd firmness were affected by storage time and temperature. At 4–8 °C, the RCT increased with the storage time 24–48 h compared with the control, while curd firmness decreased. Cheese yield decreased remarkably at 8 °C and 24 h. At 12 °C, only the RCT increased at 48 h, while curd firmness decreased remarkably. A different finding was discovered at 16 °C: after a small RCT increase at 24 h, a significant decrease at 48 h was found, even though the pH was constant. Conversely, curd firmness was not significantly affected. Parallel to this study, free Ca^{2+} was analysed during cold storage, and the results were compared with total calcium analysis in whey.

1. Introduction

The rennet coagulation of milk is a two-step process: the primary phase is the enzymatic hydrolysis of the κ-casein present on the surface of casein micelles, while the secondary phase involves the aggregation and gelation of the destabilised micelles (Britten & Giroux, 2022). Milk coagulation properties (MCPs) affect the success of the cheese-making process and are influenced by different parameters, such as pH, titratable acidity, temperature, calcium and protein contents, and breed. These parameters influence cheese yield and quality (Pretto et al., 2011) at both the laboratory and industry levels (Troch et al., 2017). Therefore, they are relevant to the cheese industry, especially in countries where large amounts of milk are transformed into cheese (e.g., Switzerland).

Bovine milk contains fat, lactose, and about 3.5% protein, which can be divided into two main groups: caseins (β-casein, α_{S1}-casein, α_{S2}-casein, and κ-casein) and whey proteins (mainly β-lactoglobulin and α-lactalbumin; Britten & Giroux, 2022). Casein micelles (particle size: ~50–600 nm; Fox & Brodkorb, 2008) are composed of casein molecules held together by colloidal calcium phosphate (CCP) and hydrophobic interactions and are dispersed in the serum phase (Lucey & Horne, 2018). One of the key elements in the casein micelle structure are the calcium phosphate nanoclusters, which represent the micellar calcium phosphate (De Kruif & Holt, 2003).

In the absence of calcium or other divalent cations, the self-association of caseins is believed to be formed through the interactions of the hydrophobic regions, with the hydrophilic/charged regions projecting into the solvent (water) and providing a combination of electrostatic and steric stabilisation of the associated complexes (Anema, 2021). The effect of low temperature (4–16 °C) on the association behaviour of caseins has been extensively investigated by different researchers (Li et al., 2019; Li, O'Mahony, Kelly, & Brodkorb, 2020; O'Connell, Grinberg, & de Kruif, 2003), particularly in the context of MCPs (Luiz et al., 2021; Maciel et al., 2015; Malacarne et al., 2006; Raynal & Remeuf, 2000). In addition, low-temperature microfiltration (e.g., at 4 °C) of skim milk leads to ideal whey rich in caseins, especially β-casein, whereas microfiltration at a high processing temperature (e.g., 50 °C) generates ideal whey containing essentially no β-casein, which proves the dissociation behaviour of β-casein at low temperatures (France, Bot, Kelly, Crowley, & O'Mahony, 2021).

At low temperatures (0–4 °C), β-casein exists as monomers (Anema, 2021). As caseins contain phosphoserine residues and other charged sites, such as carboxylate residues, they can bind to calcium ions and a range of other metal ions, depending on pH, temperature, and ionic strength (Dalgleish & Parker, 1980).

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There are different chemical forms of calcium in milk, and their content and distribution between the soluble and colloidal phases of milk can be influenced by factors such as the breeding/feeding system, stage of lactation, or health status of the cow; genetic variations in milk protein composition (Masotti, Cattaneo, Stuknyte, Pica, & De Noni, 2020); and casein mineralisation (Huppertz, Heck, Bijl, Poulsen, & Larsen, 2021; Malacarne et al., 2014). Regarding rennet coagulation, colloidal calcium, which is bound to casein micelles, is essential for milk gelation. Casein micelles are stabilised by the negative charge on their surface, which is influenced by the bound micellar and ionic calcium. Consequently, if there is more Ca^{2+} in milk, the negative charge of the micelle will decrease, and coagulation will be faster (Bauland et al., 2020). In addition, ionic calcium can act as a cross-linker (Masotti et al., 2020). The addition of $CaCl_2$ to cheese milk decreases RCT, increases curd firmness, and reduces fat losses and the porosity of the cheese matrix (Lucey & Fox, 1993).

The balance between diffusible and colloidal calcium is strongly linked to pH value, which is normally around 6.7 for fresh raw milk (Huppertz et al., 2021). Reducing the pH value leads to an increase in chymosin activity and diffusible ionic calcium, which has a positive influence on RCT (Tsioulpas, Lewis, & Grandison, 2007). It has also been discovered that κ-casein is located on the surface of casein micelles and is called a "hairy layer" (Britten & Giroux, 2022). The resulting steric and electrostatic repulsion prevents the aggregation of casein micelles and are responsible for their colloidal stability. During the cheese-making process, κ-casein is modified, leading to destabilisation through milk acidification, coagulant addition, or a combination of both (Bauland et al., 2020).

The calcium in cows' milk has been widely researched ever since Wright & Papish (1929) established that it is an important mineral in milk. However, experimental data regarding the effects of bound or ionic calcium on milk coagulation is less readily available (Gustavsson et al., 2014; Ketto et al., 2017).

In a recent review, Tunick (2020) summarised a collection of physical and chemical methods for minerals in milk and dairy products. Ca^{2+} ion concentration in milk can be determined with a calcium-selective electrode, as already investigated since 1983 by different research groups (Allen & Neville, 1983; Geerts, Bekhof, & Jenness, 1983). However, routine Ca^{2+} measurements are still not carried out on a quality assurance basis (Tsioulpas et al., 2007) because of the lack of a standardised reference method. The major advantage of directly measuring the Ca^{2+} activity in milk is the fact that the dynamic equilibrium between soluble and colloidal Ca^{2+} is not confounded by a sample-preparation step.

Particularly, the influence of the equilibria of CCP and "free" ionic calcium during the cold storage of milk before the cheese-making process is often an unconsidered and unknown factor to the cheese maker, as collection at irregular intervals, cold storage on the farm, and mixing in large tanks at the cheese factory are now standard practice (Maciel et al., 2015).

Usually, raw milk is quickly cooled after milking and stored on the farm for 1–3 days at 4–8 °C to reduce transport costs. Milk is also stored in practice for periods of various lengths and cooled (4–18 °C) before the cheese-making process. Cold storage, compared with room temperature, improves the bacteriological quality of milk but modifies several of its properties (Malacarne et al., 2013). These changes, which may affect the cheese-making properties of milk, include small dissolution of CCP and β-casein, which is claimed to impair milk rennet coagulation by increasing RCT, reducing curd firmness, and lowering cheese yield (Maciel et al., 2015). The coagulation characteristics of cows' milk are more impaired by cold storage compared with goats' or ewes' milk (Raynal & Remeuf, 2000).

Cooling milk leads to the solubilisation of casein; the CCP dissolves, causing disruption of the casein micelles and protein surface (Maciel et al., 2015). Thus, the internal modification of the casein micelles might change. Consequently, rennet activity is slowed down, and clotting takes longer (Troch et al., 2017). This effect is, however, partially reversible after the milk is tempered to a renneting temperature of about 32 °C (Maciel et al., 2015; Raynal & Remeuf, 2000). According to Swiss law [Verordnung des EDI über die Hygiene bei der Milchproduktion (Regulation of the EDI concerning hygienic aspects of milk production) (916.351.021.1) Article 14, Paragraph 7 (2005). https://fedlex.data.admin.ch/filestore/fedlex.data.admin.ch/eli/cc/2005/824/20130101/de/pdf-a/fedlex-data-admin-ch-eli-cc-2005-824-20130101-de-pdf-a.pdf], milk intended for cheese production must be stored at a maximum temperature of 18 °C; however, if the storage temperature is higher than 8 °C, the milk must be processed within 24 h after the first milking.

MCPs can be analysed by several techniques/instruments. Although dynamic rheology is often used (as a reference) in research laboratories (not routinely), different instruments can be used to analyse several milk samples in parallel (control laboratories). The Formagraph (Foss, Hillerød, Denmark), which was previously used to analyse milk samples, has been replaced by systems using optical principles, such as the Optigraph (10 samples in parallel, AMS, Frépillon, France) and Rheolaser Master (6 samples in parallel, Formulaction, Toulouse, France), instead of a mechanical principle. The Optigraph is based on near-infrared spectroscopy, while Rheolaser Master is based on multi-speckle diffusing wave spectroscopy (MS-DWS). MS-DWS allows the study of viscoelastic properties related to gel formation and network structure evolution with time, without being in mechanical contact with the milk sample (Rohart, Michon, Confiac, & Bosc, 2016). In our study, we investigated this novel technology of MS-DWS, since a good correlation of diffusing wave spectroscopy (DWS) and rheology of milk gels was found by Rohart et al. (2016) and Sandra, Cooper, Alexander, and Corredig (2011).

With the ongoing centralisation of milk processing leading to longer storage times on-farm and to increased delivery distances, milk storage habits are changing. To understand the effects of these changes on cheese making, this study aimed to investigate the influence of storage time (0–48 h) and temperature (4–16 °C) on the MCPs of raw tank milk using the new MS-DWS technique and evaluated the resulting cheese yields. In addition, we evaluated the effect of storage conditions (0–48 h) of raw tank milk (4–16 °C) on (a) free ionic calcium content and (b) total calcium contents in the resulting cheese and whey.

2. Material and methods

2.1. Milk sample collection and gross composition

A sample of regional raw cows' milk (not representative of all of Switzerland) from one producer in Switzerland (~20 cows) was delivered to the laboratory shortly after morning milking once per week for four consecutive weeks in March 2021 (dry feeding, late winter season) and in April/May 2021(grass feeding, early summer season). The composition of the raw milk was determined by FT-IR based on the following parameters: fat, protein, lactose, solids nonfat, total solids, and freezing point (MilkoScan™ Mars, FOSS, Hamburg, Germany). The pH of the milk was measured with the pH transmitter pH2100e and an Ingold electrode (Mettler Toledo, Greifensee, Switzerland), and the pH probe was calibrated using standards of pH 4 and pH 7 at 25 °C (LLG, Meckenheim, Germany).

2.2. Design of the experiment

Every week, the fresh morning milk was stored cool for 48 h at different temperatures (4 °C, 8 °C, 12 °C, or 16 °C) in a controllable

refrigerator (Typ SB2/160, Weiss Umwelttechnik GmbH, Reiskirchen-Lindenstruth, Germany) with slight magnetic stirring to prevent excessive creaming. Rheological analyses (Rheolaser Master, Formulaction, Toulouse, France) were assessed at delivery and after 24 h and 48 h, and free calcium measurements were taken during the whole cold storage time (48 h).

2.3. Preparation of the rennet solution

The rennet used was natural (rennet powder with a minimum of 75% chymosin [IMUC: 1:1,000,000], Bichsel AG, Grosshöchstetten, Switzerland). The rennet powder had been previously diluted with distilled water (1.15 g powder in 10 mL Milli-Q water and 1:10 dilution). The rennet solution was used in such a way that the coagulation time was between 20 and 30 min for most of the tank milks, with 80 μL of rennet solution for 20 mL of milk.

2.4. Rheological tests and separation of renneted cheese milk into cheese and whey

Rheological tests were performed using Rheolaser Master (Formulaction, Toulouse, France) based on DWS, a dynamic light-scattering technique. Due to the Brownian motion of the scatterers in a product, the speckle image changes as a function of time and provides information about the viscoelastic properties of the product. From the decorrelation curve (Brownian motion of particles), it is possible to compute the "mean square displacement" (nm^2)using the Rheolaser Master software, of which the inverse was taken to define the curd firmness (nm^{-2}).

The milk was divided into five or six samples of 20 mL each and then preheated to 32 °C for 15 min. Afterwards, samples of the preheated milk were mixed with the rennet solution for 60 s. During the enzymatic phase of coagulation, the clotting enzyme split κ-casein at the Phe_{105}—Met_{106} bond into para-κ-casein and the macropeptide. Gel formation was monitored at 32 °C for a total of 60 min after adding the rennet solution. From the data obtained by Rheolaser Master, gel point (RCT, measured at 10^{-3} nm^{-2}) and elasticity index (curd firmness, nm^{-2}) were observed. Curd firmness was defined as the value of the elasticity index at 40 min.

Twenty millilitres of curd were centrifuged (10,000×*g*) at 20 °C for 10 min using a Sigma 4-16 KS centrifuge (Sigma, Osterode am Harz, Germany). Whey was decanted from the cheese to determine the calcium content in each part.

2.5. Analysis of calcium (total calcium and free ionic calcium)

Total calcium in the milk samples was extracted following the International Dairy Federation method (ISO/IDF, 2007) through the decomposition of the samples with nitric acid at normal pressure. The calcium content was then analysed in the plasma of a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent, Switzerland) by comparing the samples with standard solutions. The total calcium of the fresh milk was measured for each batch on the day of delivery. Calcium in whey and cheese was measured after centrifugation of the curd.

The absolute calcium content in whey or cheese, as well as the yield of the whey or cheese, was calculated from the calcium analysis results (eqs. (1)–(3)).

$$\text{Calcium}_{\text{milk}}(\text{absolute})(\text{mg}) = \frac{\text{Calcium total in sample } (\frac{\text{mg}}{\text{kg}})}{1000} * \text{original milk weight (g)} \quad (1)$$

$$\text{Calcium}_{\text{cheese}}(\text{absolute})(\text{mg}) = \frac{\text{Calcium total in sample}(\frac{\text{mg}}{\text{kg}})}{1000} * \text{cheese yield (g)} \quad (2)$$

$$\text{Calcium}_{\text{whey}}(\text{absolute})(\text{mg}) = \frac{\text{Calcium total in sample } (\frac{\text{mg}}{\text{kg}})}{1000} * \text{cheese whey (g)} \quad (3)$$

The relative calcium contents of whey and cheese were calculated from the amount of total calcium in milk and the absolute calcium contents in whey or cheese (eqs. (4) and (5)).

$$\text{Relative calcium content in whey} = \frac{\text{absolute calcium in whey (mg)}}{\text{absolute calcium}_{\text{milk}}(\text{mg})} * 100\% \quad (4)$$

$$\text{Relative calcium content in cheese} = \frac{\text{absolute calcium in cheese (mg)}}{\text{absolute calcium}_{\text{milk}}(\text{mg})} * 100\% \quad (5)$$

From the relative calcium contents in cheese and whey, the proportion of calcium (from cheese/whey) was calculated and found to be around 3 (eq. (6)).

$$\text{Proportion of calcium (cheese/whey)} = \frac{\text{relative amount of calcium in cheese (\%)}}{\text{relative amount of calcium in whey (\%)}} \quad (6)$$

Over the chilled storage period, the ionic calcium concentration in milk was measured using a combined calcium-ion-selective electrode (Metrohm, Zofingen, Switzerland), which was connected to a pH metre (913, Metrohm, Zofingen, Switzerland). The electrode was calibrated at each temperature (4–16 °C) before milk measurement, with seven calcium standards selected from the range of 0.1–10 mmol L^{-1} at the given temperatures of 4 °C, 8 °C, 12 °C, and 16 °C. The ionic strength of the standards was adjusted to 150 mmol L^{-1} with KCl. Samples were equilibrated with the probe for 2 min prior to taking measurements. The calibration curve was derived from the −log of Ca^{2+} ion concentration (mmol L^{-1}) and the electrode relative potential difference (mV), which was linear. The free ionic calcium concentration was calculated from the regression equation derived from the calibration curve. Measurements with the electrode were recorded each minute for 48 h in the refrigerator at 4 °C, 8 °C, 12 °C, and 16 °C. To control whether the electrode gave a stable electrical output (mV), the calibration values were considered, while a 10-fold increase in concentration (e.g., 0.1 mM compared with 1 mM) should lead to an increase of about 29 mV (Lewis, 2011).

2.6. Statistical analysis

The whole experiment was repeated using two different milk preparations for each temperature: 4 °C, 8 °C, 12 °C or 16 °C. Each morning, the milk sample was analysed immediately after milking/delivery. A *t*-test (Microsoft Excel 2016) was performed to prove the differences in milk composition between the morning milk from late winter and the early summer season. ANOVA was carried out using the R program (4.1.1) to confirm the significance of the effects of different storage temperatures at 24 h and 48 h on the RCT of the milk (at time = 0). The significance level was established at $p < 0.05$. Tukey's HSD post-hoc test was conducted when differences were considered at $p < 0.05$.

3. Results and discussion

3.1. Mineral composition and pH of the milk samples

Table 1 presents the contents of the morning milk samples at delivery. All values were in the expected range. Means and standard deviations did not show any striking values or wide variations. However, a small variation was found between the weekly tank milks, even from the same producer. The broadest relative standard deviation was found for fat, whereas the narrowest relative standard deviation was found for the freezing point (Table 1). The *t*-test between the early summer and late winter milk samples showed a significant effect only for fat content, which was slightly lower at the beginning of the summer season. The change from hay in winter to grazing in summer or the slightly later stage of lactation might have been responsible for this effect. However, this effect was found not to be relevant to this study because the renneting properties of milk were mainly regulated by the protein fraction and not influenced by the fat fraction. Unhomogenised milk fat globules do not play an active role in gel formation, acting only as an inert filler in curd formation (Maciel et al., 2015).

Table 2 shows the pH values of the milk at delivery and during the storage period at the different storage temperatures. All pH values were in the expected range of 6.62–6.74 and did not change much during cold storage. The pH values tended to be slightly higher after the storage period of 48 h, increasing from 6.69 to 6.71 (after 24 h) and 6.73 (after 48 h). ANOVA did not show a significant difference during the cold storage, but a paired *t*-test between pH (0 h) and pH (48 h) showed a *p*-value <0.05. The values prove that no lactic acid fermentation was initiated during the entire storage period (0 h–48 h), but as the milk system is buffered, bacterial growth cannot be excluded. Malacarne et al. (2013) and Schmutz and Puhan (1981) reported an increase in pH value during cold storage. This effect could be related to micellar calcium dissociation, as suggested by Schmutz and Puhan (1981).

3.2. Coagulation of cheese milk (RCT and curd firmness)

The renneting properties of the milk samples were studied using Rheolaser Master at delivery and after 24 h and 48 h of storage (at 4–16 °C). Fig. 1 illustrates changes in the gelation points of the milk samples in relation to the storage time, as means [n = 2 (late winter season and early summer season)] and standard deviations. At storage temperatures of 4 °C and 8 °C, the gelation points steadily increased at 24 h and 48 h and were significantly different for each period. At 12 °C, the gelation points at delivery and after 24 h were similar, but the gelation point was significantly higher after 48 h. A completely different picture was observed after storage at 16 °C (Fig. 1, right). After a small but significant increase at 24 h, the gelation point dropped significantly at 48 h.

These results indicate that during storage, complex equilibrium states with calcium and other ions were established, which proved not to be completely reversible when a temperature of 32 °C for 15 min before rennet addition was selected. The effects of cooling were only partly reversible after the milk was returned to the renneting temperature, which is consistent with the findings of

Table 1
Values of milk composition at delivery, analysed by Fourier transform infrared or atomic absorption spectroscopy.[a]

Storage condition	Fat (%)	Protein (%)	Lactose (%)	Solids non-fat (%)	Total solids (%)	Freezing point (°C)	Calcium (mg kg^{-1})
4 °C (winter/summer season) (n = 2)	4.17 ± 0.32	3.54 ± 0.04	4.83 ± 0.04	9.00 ± 0.05	13.14 ± 0.42	−0.53 ± 0.005	1090 ± 57
8 °C (winter/summer season) (n = 2)	4.19 ± 0.17	3.62 ± 0.13	4.82 ± 0.07	9.07 ± 0.11	13.26 ± 0.10	−0.52 ± 0.001	1060 ± 0
12 °C (winter/summer season) (n = 2)	4.08 ± 0.17	3.66 ± 0.13	4.72 ± 0.01	9.03 ± 0.13	13.10 ± 0.05	−0.52 ± 0.001	1080 ± 28
16 °C (winter/summer season) (n = 2)	4.21 ± 0.16	3.59 ± 0.06	4.83 ± 0.01	9.04 ± 0.08	13.28 ± 0.06	−0.53 ± 0.002	1070 ± 0
Mean (n = 8)	4.16	3.60	4.80	9.03	13.19	−0.525	1075
Standard deviation (n = 8)	0.16	0.08	0.05	0.07	0.17	0.002	25
P-value [t-test (winter/summer)]	0.002*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Abbreviation: n.s., not significant. All components were analysed by Fourier transform infrared except calcium that was analysed by atomic absorption spectroscopy.

Table 2
pH values of milk at delivery and during storage at different temperatures.[a]

Storage condition	pH			Delta pH	
	At delivery	After 24 h storage	After 48 h storage	24 h−0 h	48 h−0 h
Winter season					
4 °C	6.72	6.73	6.78	0.01	0.06
8 °C	6.62	6.63	6.61	0.01	−0.01
12 °C	6.71	6.78	6.76	0.07	0.05
16 °C	6.62	6.63	6.67	0.01	0.05
Summer season					
4 °C	6.74	6.74	6.77	0	0.03
8 °C	6.71	6.73	6.78	0.02	0.07
12 °C	6.71	6.73	6.71	0.02	0
16 °C	6.72	6.70	6.79	−0.02	0.07
Mean (n = 8)	6.69	6.71	6.73	0.015	0.04
Standard deviation (n = 8)	0.04	0.05	0.06	0.024	0.029
P-value [t-test (winter/summer)]	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Abbreviation: n.s., not significant; a paired t-test between pH (0 h) and pH (48 h) showed a P-value <0.05.

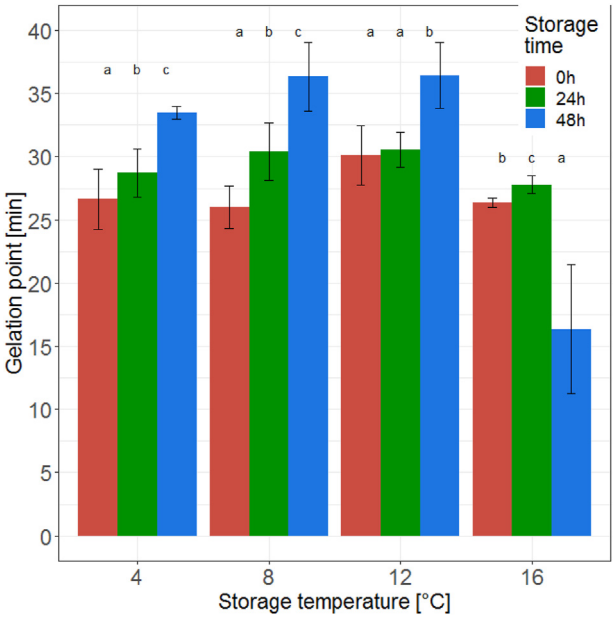

Fig. 1. Mean and standard deviation of the rennet coagulation time in relation to the storage time (■, 0 h; ■, 24 h; ■, 48 h) and temperature. Different lowercase letters show a significant difference ($p < 0.05$) between the different storage times within the same storage temperature set.

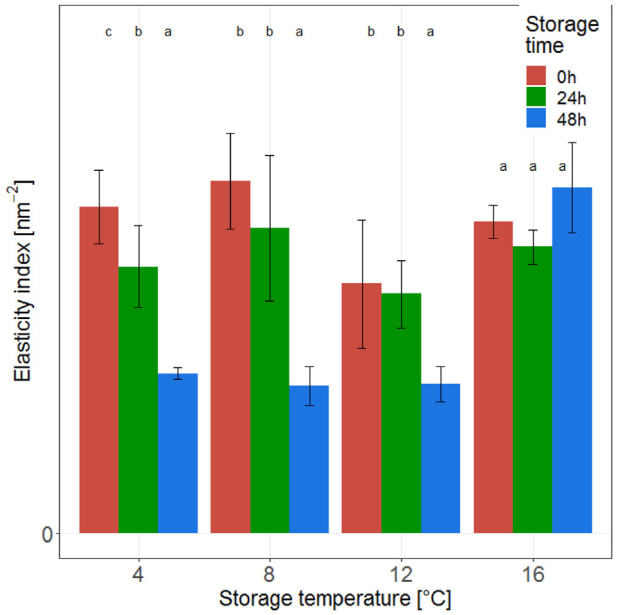

Fig. 2. Mean and standard deviation of the elasticity index in relation to the storage time (■, 0 h; ■, 24 h; ■, 48 h) and storage temperature. Different lowercase letters show a significant difference ($p < 0.05$) between the different storage times within the same storage temperature set.

Raynal and Remeuf (2000) and Troch et al. (2017). The calcium in milk is in a dynamic equilibrium between the colloidal and aqueous phases (Masotti et al., 2020). This equilibrium is strongly dependent on pH and temperature, and the mineral equilibria between the colloidal and aqueous phases of milk, the calcium and phosphate equilibria in particular, play an important role in maintaining the stability of casein micelles and, generally, the physical properties of milk and milk products.

Fig. 2 presents the curd firmness (defined as elasticity index at 40 min) results. At storage temperatures of 4–12 °C, the elasticity index dropped significantly after 48 h of storage. Conversely, at a storage temperature of 16 °C, the elasticity index did not change significantly but tended to increase after 48 h of storage as the gelation point dropped significantly. The reason for the significant drop in the gelation point of the milk sample stored for 48 h at 16 °C could be the growing microflora, even if the pH did not decrease significantly (Table 2). This observation could be due to the buffer capacity of milk.

In the Malacarne et al. (2013) study, when milk was stored at 13–15 °C for 24 h, the number of lipolytic, proteolytic, psychrotrophic, and mesophilic lactic acid bacteria significantly increased, with no major changes in rennet coagulation parameters (measured by the Formagraph). Conversely, Malacarne et al. (2013) found that milk storage at 4–6 °C and 8–12 °C significantly impaired the rennet coagulation properties after 12–48 h of storage and demonstrated increased clotting time and reduced curd firmness. Raynal and Remeuf (2000) found similar results analysed by the Formagraph, with increased coagulation times and lower firmness for cows' milk stored at 4 °C for 24 h (RCT +20%) and 48 h (RCT +29%). Cold storage at > 4 °C was not performed in that study.

Over the past few years, several studies have focused on the effects of cold storage conditions on the chemical and physico-chemical characteristics, as well as processing properties, of milk, but only some studies have considered raw milk (Malacarne et al., 2013). In general, cold storage of raw milk results in the dissociation of casein, mainly β-casein, and inorganic calcium from the casein

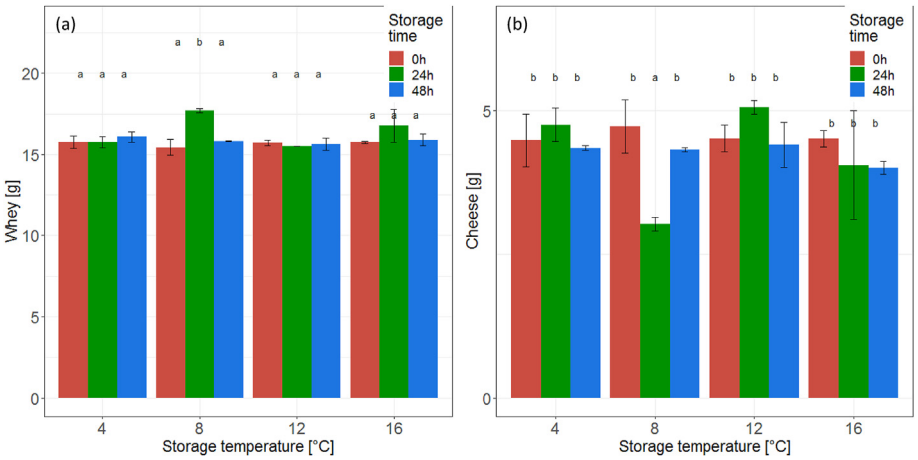

Fig. 3. Whey yield (a) and cheese yield (b) (from 20 mL of milk) in relation to the storage time (■, 0 h; ■, 24 h; ■, 48 h) and storage temperature. Different lowercase letters show a significant difference ($p < 0.05$) between the different storage times within the same storage temperature set.

micelles, as well as impaired rennet coagulation properties and cheese yield (Maciel et al., 2015). Furthermore, increased activities of the plasmin, proteases, and lipases of growing microflora (mainly psychrotrophic bacteria) have been reported (Malacarne et al., 2013).

The RCT increase after cold storage of the milk at 4–12 °C may be a consequence of the release of caseins, calcium, magnesium, phosphate, and citrate from casein micelles; slightly higher pH values; and consistently slightly lower rennet activity. This is consistent with the Malacarne et al. (2014) study, where lower levels of casein mineralisation showed worse rennet coagulation properties. Additionally, up to 30% of β-casein dissociates from casein micelles on cooling, primarily due to the reduction of hydrophobic interactions (Huppertz, Fox, & Kelly, 2018). Conversely, at 16 °C and after 24 h of storage, enzymatic and microbial degradation might occur during cold storage due to the growth of psychrotrophic bacteria, depending on the initial number of bacteria after milking (Panthi, Jordan, Kelly, & Sheehan, 2017). Some proteinases may hydrolyse α_S- and β-caseins and influence the accessibility of rennet to κ-casein, thereby influencing the coagulation properties.

3.3. Recovery of whey/cheese

Fig. 3 shows the distribution of renneted milk (20 mL) into whey and curded cheese. All samples started from exactly 20 mL of milk. Sixty minutes after rennet addition, the renneted milk was centrifuged. The resulting amounts of whey and curded cheese were weighted, and the significantly lowest cheese yield was found after 24 h of storage at 8 °C. The reason for this gap was assumed to be the different total or free calcium contents in these cheese samples. A similar but not significant reduction in curded cheese yield was found at 16 °C storage temperature for 24 h and 48 h storage time. However, a slightly but not significantly higher cheese yield was found after storage at 4 °C or 12 °C for 24 h.

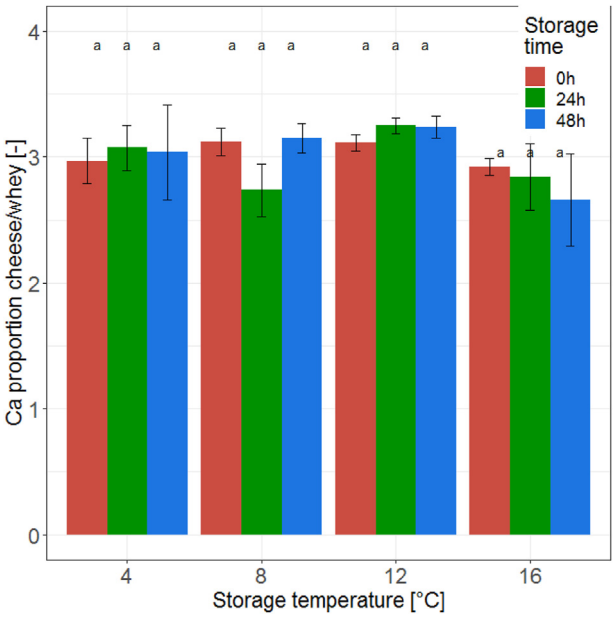

Fig. 4. Mean and standard deviation of total calcium proportion of cheese to whey in relation to the storage time (■, 0 h; ■, 24 h; ■, 48 h) and storage temperature. Different lowercase letters show a significant difference ($p < 0.05$) between the different storage times within the same storage temperature set.

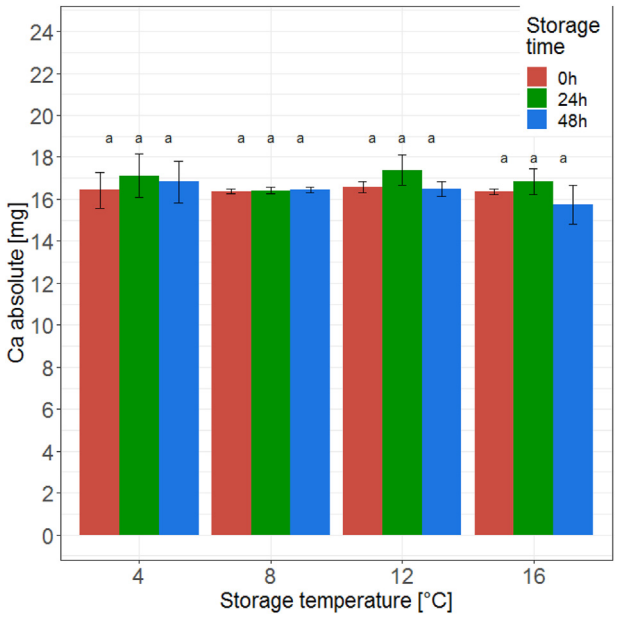

Fig. 5. Mean and standard deviation of absolute calcium transferred to the cheese in relation to the storage time (■, 0 h; ■, 24 h; ■, 48 h) and storage temperature. Different lowercase letters show a significant difference ($p < 0.05$) between the different storage times within the same storage temperature set.

3.4. Total calcium in cheese and whey

A similar picture was seen for the proportion of the relative calcium contents between cheese and whey (Fig. 4). A factor of around 3 was calculated in the present study (eq. (6)) for all treatments, meaning that three times more calcium was found in cheese compared with whey. A factor below 3 was found after storage at 8 °C for 24 h and 16 °C for 24 h and 48 h. All other values were around 3 or slightly above. These results reveal that at a storage temperature of 8 °C for 24 h and 16 °C for 48 h, less calcium was transferred into the cheese matrix. To prove this hypothesis, the total amount of calcium transferred from the milk into the curded cheese was calculated. Fig. 5 shows the results of these calculations.

The transferred calcium amount (around 16–17 mg of total calcium per cheese) was quite stable and not significantly different for any treatment. The above hypothesis proved to be wrong, as there was no significant difference in the storage time and temperature of the 16–17 mg of total calcium transferred into all cheese samples. A higher or lower yield did not change the absolute total calcium amount transferred from the cheese milk into the cheese. A lower cheese yield was compensated for by a higher calcium concentration, and the reason for the differences in cheese yield could not be explained by the calcium concentration. Obviously, the calcium and casein matrix was a constant factor in the different cheeses, but presumably, the structures (or texture) of the different cheeses were different. It was assumed that the water content was lower in the cheese produced at 8 °C storage temperature and 24 h storage time.

Malacarne et al. (2006) compared Italian Brown and Italian Friesian herd milk and found a significant difference in cheese yield at 24 h after production, but no significant difference in the calcium content of the cooked whey. Other contents of the whey, such as protein, fat, and phosphorus, played a more important role. When cheese yield and casein content were correlated, a correlation coefficient of 0.88 was found. The higher casein content in Italian Brown was interconnected with the higher calcium content in Italian Brown, compared with Italian Friesian with lower casein and calcium contents.

Table 3
Continuous measuring of ionic calcium with calcium ion-selective electrode in milk from early summer season.

Temperature	Ionic calcium (mmol L^{-1})				
	1 h	6 h	12 h	24 h	48 h
4 °C	3.20	3.44	3.38	3.29	3.15
8 °C	2.74	2.81	2.79	2.69	2.52
12 °C	2.88	2.98	2.93	2.81	2.70
16 °C	3.04	3.14	3.06	2.87	3.19

3.5. Free ionic calcium in milk samples during storage

Measurement of changes in the levels of calcium and phosphate, as well as magnesium and citrate, associated with casein micelles when the temperature changes is not an easy task (Anema, 2021). Calcium phosphate solubility increases on cooling and re-equilibrates with the serum. Concurrently, β-casein dissociates into monomers from micelles (0–4 °C; Anema, 2021).

Fig. 6 presents a series of charts showing the potential of the calcium ion-selective electrode during the cold storage of the milk samples. At storage temperatures of 4 °C, 8 °C, and 12 °C, a similar picture was found. After a short period of equilibration to the specified temperature, the potential was reduced for some hours (Ca^{2+} concentration increased) but increased afterwards (Ca^{2+} concentration decreased). A completely different process was found for the storage temperature of 16 °C. The negative potential decreased for some hours (Ca^{2+} concentration increased) and increased again for about 30 h (Ca^{2+} concentration decreased). Afterwards, the potential decreased (Ca^{2+} concentration increased).

Generally, the ion-selective electrode indicated an increase in free ionic calcium in the first 6 h of storage. Free ionic calcium concentrations were calculated from their temperature-dependent calibration curves (Table 3). Considering the changes in pH values (Table 2) and free ionic calcium concentrations (Table 3), the significant RCT changes during cold storage could not be fully explained. Therefore, it was assumed that the dissociation of casein micelles might play an important role in RCT prolongation during cold storage (4–12 °C).

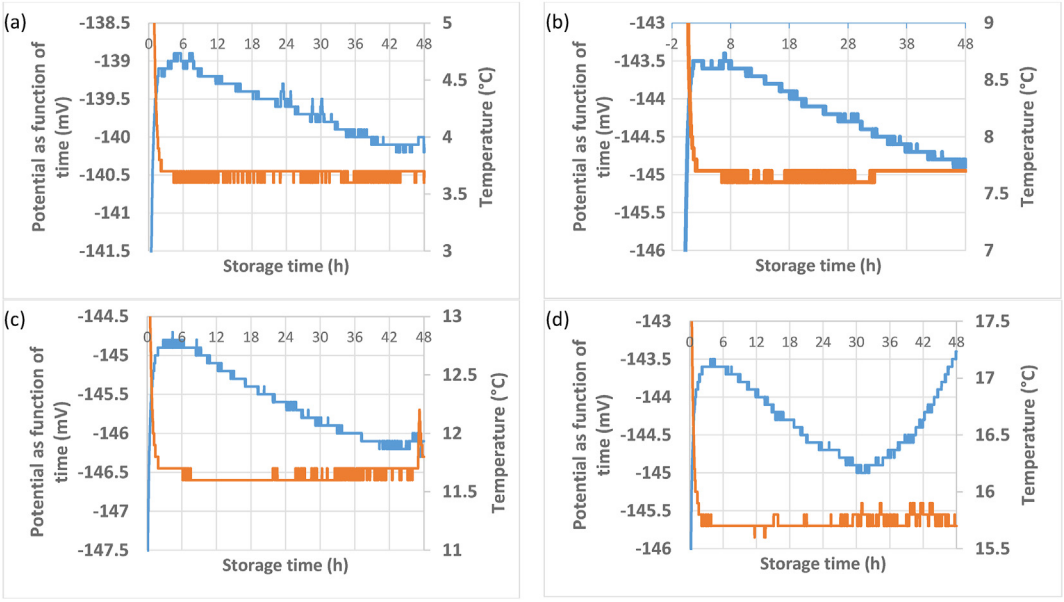

Fig. 6. Potential of the Ca ion selective electrode during storage of milk from early summer at (a) 4 °C, (b) 8 °C, (c) 12 °C and (d) 16 °C storage, milk from early summer season. Blue line corresponds to potential (mV), orange line corresponds to temperature (°C).

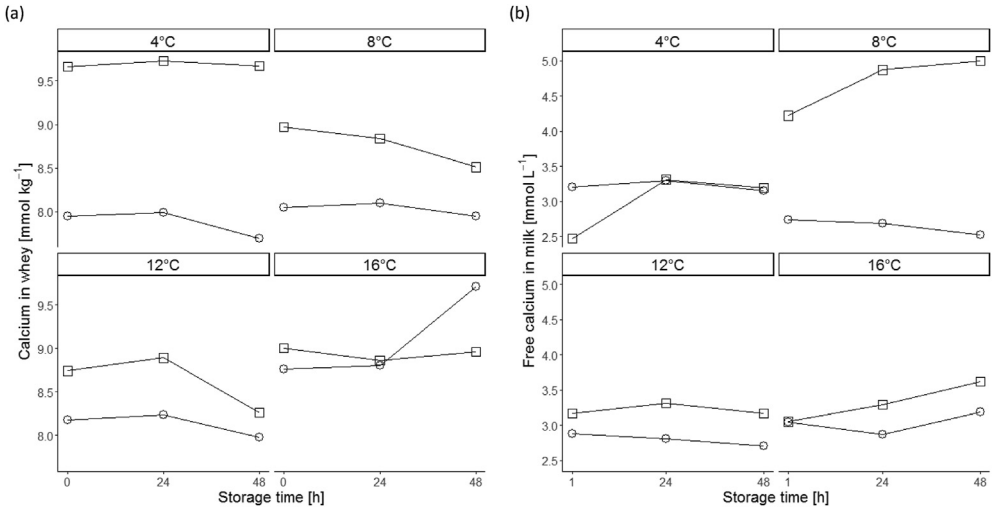

Fig. 7. Total calcium content in whey (a) during 48 h of storage time and different storage temperatures (4–16 °C) compared with the free ionic calcium content in milk (b) measured by an ion-selective electrode: ○, late winter season; □, early summer season.

The content of free ionic calcium was different for every milk at the beginning of the experiment because each experiment started with a different raw milk. Tsiouplas et al. (2007) found a range of 1 mmol L^{-1} to 5.29 mmol L^{-1} free ionic calcium, with a mean of 1.88 mmol L^{-1}, when analysing the milk of 235 cows. Our results were in the same range at the beginning of the analysis.

In another study, the amount of soluble calcium increased steadily by 10% in cows' milk after 48 h of storage at 4 °C, as found after ultracentrifugation with a fluorometric method (Raynal & Remeuf, 2000). The release of calcium into the soluble phase during the cold storage of cows' milk through the dissolution of CCP and the dissociation of β-casein has been previously reported (Raynal & Remeuf, 2000). In the current study, the increase in soluble calcium was parallel to the increase in soluble casein during the storage time of 48 h. This continuous increase in soluble calcium during cold storage (48 h), measured by this indirect method, seems to give a different picture compared with when analysed online with an ion-selective electrode in a continuous manner. The reason for this discrepancy is unknown. As far as the authors know, this is the first time that free ionic calcium content has been measured by an ion-selective electrode over 48 h directly in raw milk during cold storage.

3.6. Comparison of free ionic calcium in milk with total calcium in whey

To prove the behaviour of the free ionic calcium, measured by the ion-selective electrode, the measured total calcium contents in whey at time-points 0 h, 24 h, and 48 h (Fig. 7a) were compared with the corresponding free ionic calcium values (Fig. 7b). Total calcium values in whey were supposed to be closely related to free ionic calcium values because all the caseins in whey were eliminated by coagulation. The total calcium values in whey (Fig. 7a) were, as expected, consistently higher than the corresponding free ionic calcium values (Fig. 7b) calculated from the ion-selective electrode.

According to the literature, caseins may interact with electrodes (suppression effect by caseins). Silanikove, Shapiro, and Sjamay (2003) noted that most of the results of free ionic calcium in milk in the literature are underestimated. This statement is difficult to prove, as different ion-selective electrodes are on the market and no reference method for free ionic calcium is available. Most researchers have directly measured Ca^{2+} in milk without subjecting it to any treatment, which could potentially alter the ionic environment and affect the final result (Lin, Lewis, & Grandison, 2006; Tsioulpas et al., 2007). In raw milk, two-thirds of the total calcium is in the micellar/colloidal phase and is bound to the casein micelles. About one-third is soluble calcium found in the serum phase and is either ionic/free calcium Ca^{2+} (~7% of total calcium) or associated as calcium citrate (~23% of total calcium) or phosphates (~2% of total calcium; Deeth & Lewis, 2015; Oh & Deeth, 2017). These equilibrium states are temperature dependent.

Fig. 7a shows that total calcium contents in whey at 4–12 °C decreased from 24 h to 48 h but increased at 16 °C in the same time range. A similar behaviour was found for the free ionic calcium at a lower content level (Fig. 7b). Between 24 h and 48 h and at 4–12 °C, the free ionic calcium decreased (except for one outlier at 8 °C), while it increased at 16 °C. These results prove the close relationship between the total calcium content in whey (analysed by a microwave plasma atomic emission spectrometer) and the free ionic calcium content in milk (analysed by an ion-selective electrode) at the corresponding time-points and temperatures. These results reveal that the dissociation of caseins and the release of free ionic calcium ended after some hours of storage (~3–12 h), and the free calcium content decreased again, eventually triggered by phosphate and citrate balances. It was only at 16 °C that a further release of calcium (after 30 h) was found, and we believe that more free ionic calcium was released by enzymatic/proteolytic degradation of caseins with undetermined microbes (probably psychrotrophs). Luiz et al. (2021) also suggested that psychrotrophic proteases could partially degrade β- and κ-caseins. Moreover, psychrothrophic proteases are associated with technological concerns, such as changes in clotting time (Luiz et al., 2021).

4. Conclusion

The aim of this study was to provide further knowledge of the effect of cold storage of raw milk before the cheese-making process on MCPs and calcium balances. Cold storage of raw milk can alter MCPs, as complex balances of mainly casein dissociation, calcium, and other ions could be altered during storage. The results indicate that the influence of MCPs and cheese yield is likely to depend on storage time and storage temperature. The subsequent heating and equilibrating of the stored milk before coagulation (15 min at 32 °C) seemed not to recover all the modifications of the cold storage of the raw milk. At lower

temperatures (4–8 °C), similar effects on MCPs were found. At a higher storage temperature of 16 °C, the MCPs changed the most after 24 h; the RCT was significantly shorter. The cheese yield was significantly lower at a storage time of 24 h and a temperature of 8 °C, even though the total calcium transferred to all cheese samples was almost constant.

The results of the online development of the free ionic calcium content during cold storage were proven by comparison with the corresponding total calcium content in whey. A good agreement was found between 24 h and 48 h of storage of the raw milk. The RCT decrease at 16 °C after ~30 h of storage time was parallel to an increase in free calcium ions (analysed by the ion-selective electrode), which is in line with theoretical considerations, as casein micelle destabilisation increases with increased free calcium ions. However, the interpretation of these results was not easy because multiple effects could be involved (casein solubilisation, calcium balances with phosphates/citrates, uncontrolled degradation of casein micelles by activities of endogenous enzymes, and growth of undetermined bacteria).

Based on this illustrated experimental design, it is recommended to store raw milk at around 12 °C for a maximum period of 24 h before the cheese-making process, as these conditions alter the MCPs the least, which is in accordance with Swiss law.

Declaration of competing interest

None.

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