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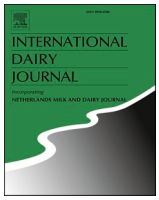

Research paper

κ-casein genotypes and minerals in raw milk and their impact on coagulation properties

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ABSTRACT

This study evaluated the impact of κ-casein (κ-CN) genotypes (AA, AB, BB, BE) and minerals (total, serum, and micellar) on coagulation properties of raw milk in 80 individual Holstein cows. The results showed that the influence of the ratio of κ-CN to total casein was significantly different between genotypes (BB > AB > BE > AA), but there was no significant influence on main milk ingredients (proteins, fat or minerals). Of all tested minerals, only total sodium was slightly lower in milk from cows with κ-CN genotype B. Rennet induced coagulation properties were statistically evaluated using ANCOVA, with pH as a covariate. The shortest rennet coagulation time (RCT) and the firmest curd were observed with the BE and BB genotypes. The impact of pH on RCT and the κ-CN percentages on curd firmness were strong, whereas the minerals showed only a rather small impact on RCT and the curd firmness.

1. Introduction

The composition of milk and the techno-functional properties of milk proteins (caseins and whey proteins, each of which have different genetic variants) are crucial factors that account for the manufacture, quality, yield, and nutritional properties of dairy products. These properties are affected by several factors related to the individual animal and its environment: breed, diet, lactation stage, health and hygiene, farm management, season, and milking system (Gai, Uniacke-Lowe, O'Regan, Faulkner, and Kelly (2021)).

Milk coagulation properties (MCPs) play a pivotal role in the efficiency and quality of cheese-making, making it essential to understand the factors influencing these properties. κ-CN, one of the key milk proteins, is a major determinant of MCPs due to its role in micelle stability and rennet-induced coagulation. Genetic variants of κ-CN have been shown to affect its relative content and, consequently, coagulation behaviour. Additionally, minerals such as calcium and phosphorus, distributed between the micellar and serum phases, are critical for gel formation and stability.

However, the effects of genetic variants and minerals as well as other factors like pH, potential post-translational modifications (PTMs) of caseins, and the applied milk-processing technologies on milk are not yet fully understood (Nadugala, Pagel, Raynes, Ranadheera, & Logan, 2022). Although the complex structure of casein micelles is still debated, there is common agreement on the "hairy micelle" model, which forms the basis of rennet-induced milk coagulation. κ-CN (169 amino acid residues) is cleaved from the casein micelle by the specific Phe^{105}-Met^{106} bond-dissociation triggered by chymosin/rennet. The N-terminal para-κ-CN (1–105) is the hydrophobic part that remains in the micelle, while the C-terminal caseinomacropeptide (106–169) is the part that is to some extent "lost" in the whey fraction during the cheese-making process (Holland & Boland, 2014). Numerous polymorphisms in the κ-CN gene have been identified, resulting in approximately 14 genotypes, with the A and B variants being the most common. These variants differ by two amino acids (136: Ile for Thr and 148: Ala for Asp) in the caseinomacropeptide region (Huppertz, Fox, & Kelly, 2018). For variant E, the difference to A is at the position 155 (Gly for Ser).

Caseins are flexible and unfolded proteins that consist of both hydrophobic and hydrophilic segments. Through casein-casein interactions, these individual caseins associate together and form a casein-micelle with nanoclusters of amorphous calcium phosphate (Holt, Carver, Ecroyd, & Thorn, 2013). Individual milk pH is mainly influenced

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by milk components (minerals, proteins, carbohydrates, fat) or lactation stages. Higher pH values may also indicate subclinical mastitis or intramammary infections. It has been observed that pH of milk and casein micelle size were positively correlated (in the range pH = 5.5–7.5, Sinaga, Bansal, & Bhandari, 2017). Additionally, pH of milk could destabilise the micellar integrity and a decrease of pH in milk causes the solubilisation of colloidal calcium phosphate from the micelles into the solution (Sinaga et al., 2017). Sodium, potassium and chloride are almost totally dissolved in the serum phase, as portions of calcium, phosphorus, magnesium and citrate are partitioned between micellar and serum fractions in bovine milks (Britten & Giroux, 2022; Runthala, Mbye, Ayyash, Xu, & Kamal-Eldin, 2023).

MCPs are conventionally measured using renneting meters and mechanical or optical devices that can record firmness over time. Typical MCP analyses are performed using different methods, such as the Formagraph (i.e. rennet coagulation time [RCT], curd firming time [k20], and curd firmness [a30]), rheology (measuring of the storage/loss modulus [G′, G″]), and near infrared (NIR) or mid-infrared reflectance spectroscopy. In this study, we applied multi-speckle diffusing-wave spectroscopy (MS-DWS) methodology. MS-DWS allows to analyse viscoelastic properties related to gel formation and network structure evolution in real-time, without mechanical contact with the milk sample and the Rheolaser Master can handle 6 samples in parallel. A good correlation between Rheology and DWS was found by Rohart, Michon, Confiac, and Bosc (2016) and Sandra, Cooper, Alexander, and Corredig (2011).

Minerals, particularly calcium, are well-known to play a crucial role in milk coagulation. Building on this understanding, we explored whether κ-CN genetics could influence mineral distribution, potentially explaining genotype-dependent differences in coagulation properties. This investigation was further motivated by intriguing findings that κ-CN BB-containing milks exhibit similar or even shorter RCT compared to AA milks, despite the higher relative concentration of κ-CN in the BB variant. (Bonfatti, Di Martino, Cecchinato, Degano, & Carnier, 2010; Guggisberg et al., 2024). According to conventional knowledge, a certain threshold (80%–90%) of cleaved κ-CN is necessary to coagulate micelles (McSweeney, 2007).

This study focuses on the influence of κ-CN genetic variants (AA, AB, BB, BE) and mineral composition on MCPs in raw milk. By addressing these factors, this research aims to contribute to the development of strategies for optimizing milk for cheese production. The findings are expected to benefit dairy farmers and cheesemakers by offering insights, allowing breeding programs and milk quality assessments to take coagulation efficiency and cheese yield into account. To achieve this, the MCPs of approximately 80 individual Holstein raw milks from one farm with a consistent feeding system were collected over several weeks, analysed for proteins, fat, minerals and MCPs, and classified by the main κ-CN variants. In addition, each raw milk sample was skimmed and separated by ultracentrifugation into a casein sediment and a whey serum fraction. Both fractions were analysed for key minerals to understand any possible influence on MCPs.

2. Materials and methods

2.1. Milk sample collection and gross composition

In this study, 87 milk samples from individual Holstein cows were sampled during the dry-feeding period (November to March). The milk was collected once from individual cows during morning milking and was immediately transported to the laboratory under controlled conditions (cooled at 4 °C). Upon arrival at the lab, the samples were prepared for analysis (e.g., aliquoting, ultracentrifugation). These preparatory steps were completed within 3–4 h of collection. Subsequent analysis in the lab for coagulation properties and gross composition were carried out on the same day. This approach ensured that the samples were analysed under conditions closely representative of their original state. Seven samples had to be eliminated before the statistical analysis due to infrequent genetic κ-CN variants (AE and EE, each <3) or high pH (7.19), somatic cell counts (SCC) > 300,000 counts mL^{-1} or very low sample volume. In total, 80 samples were included in the statistical evaluation (AA = 28, AB = 35, BB = 8, BE = 9). The samples were collected from healthy cows of one farm, receiving the same feeding regime, at lactation numbers ranging from 1 to 6.

The following parameters were determined by FT-IR to assess raw milk quality: fat, protein, and casein (MilkoScan™, Foss, Hamburg, Germany). Somatic cell counts were determined by Fossomatic™ (Foss, Hamburg, Germany). Mesophilic microbes were determined by microbiological standard methods (ISO 4833-1-2). The pH of the milk was measured with a pH transmitter 913 pH meter (Metrohm, Zofingen, Switzerland), and the pH meter was calibrated before use with either standards pH = 4 or 7 at 25 °C.

2.2. Analysis of the genetic variants of κ-CN by PCR or LC-MS

Most of the genetic κ-CN variants were already known based on information obtained from the farmer (PCR test). Seven milk samples were analysed separately using the previously developed in-house LC-MS method (Guggisberg et al., 2024) because their κ-casein genotypes were unknown from the initial farmer data, necessitating further analysis to complete the dataset. The LC-MS method used is based on a defined cleavage of the protein with the endoprotease AspN, which generates specific peptide fragments that encompass the natural mutations. By distinguishing these peptides based on the mutations, the κ-casein genotype can be accurately assigned.

2.3. Analysis of the relative milk protein contents (αS1-, αS2-, β- and κ-CN, α-Lactalbumin [α-LA] and β-Lactoglobulin [β-LG] by LabChip

The proteins in the milk samples were diluted, prepared and separated with a Protein 80 Kit (Agilent, Basel, Switzerland) using an automated electrophoresis system (2100 Bioanalyzer, Agilent, Basel, Switzerland) according to the in-house method (Guggisberg et al., 2024). Protein standards of α-, β-, κ-CN and β-LG, α-LA, and BSA (Sigma-Aldrich, MO, USA) were separated on the same chip and used to allocate the proteins of the analysed milk samples.

κ- CN fractions as a percentage of either total milk proteins (MP), total casein, or β- and κ- CN were calculated thereafter according to the following three formulas:

$$\kappa - \mathrm{CN}\ (MP) = \frac{\kappa - \mathrm{CN}}{\sum all\ milk\ proteins} \times 100\% \quad (1)$$

$$\kappa - \mathrm{CN}\ (casein) = \frac{\kappa - \mathrm{CN}}{\sum all\ caseins} \times 100\%. \quad (2)$$

$$\kappa - \mathrm{CN}\ (\beta - \mathrm{CN}\ and\ \kappa - \mathrm{CN}) = \frac{\kappa - \mathrm{CN}}{\beta - \mathrm{CN} + \kappa - \mathrm{CN}} \times 100\% \quad (3)$$

2.4. Total, serum, and milk protein-associated and micellar metals

The milk samples were separated the same day of collection into a serum phase and a protein sediment (pellet) by ultracentrifugation (100,000×g), according to a method recently described by Huppertz, Heck, Bijl, Poulsen, and Larsen (2021) in order to overcome changes in mineral equilibria. The pellet was separated from the supernatant, and an aliquot of the supernatant was subsequently filtered over a 10-kDa Amicon Ultra-15 centrifugal filter (Merck AG, Buchs, Switzerland). Total metals (Ca, Mg, Zn, P, Na, and K) were analysed after the storage at −18 °C by ICP-OES (Agilent, Ca, USA) according to ISO 15151 (2018), using reference solutions obtained by SCP Science (Quebec, CAN). Analysis was performed in the original milk samples, in the pellet, and in the "free" or serum minerals in the 10-kDa permeate.

Units for minerals were mmol kg^{-1} (Ca, Mg, P, Na, K, Zn). Milk protein-associated and casein-associated metals were then calculated according to formulas 4 and 5:

$$\text{Milk protein} - \text{associated metals } [\text{mmol kg}^{-1}] = \textit{Concentration in milk } [\textit{mmol kg}^{-1}] - \textit{concentration in } 10 - \textit{kDa permeate } [\textit{mmol kg}^{-1}] \quad (4)$$

$$\text{Casein} - \text{associated metals (micellar) } [\text{mmol 10 g}^{-1} \text{ casein}] = \frac{(\textit{Concentration in milk} - \textit{concentration in } 10 - \text{kDa } \textit{permeate})}{\textit{casein content}} [\text{mmol 10 g}^{-1} \text{ casein}] \quad (5)$$

(The unit for casein was g 100 g^{-1}.)

2.5. Rheological analysis of cheese milk after adding rennet by DWS

The rennet was prepared as previously described by Guggisberg et al. (2024). Briefly, 1.15 g rennet (900 IMCU g^{-1}) was diluted in 10 mL water. This solution was diluted 1:10 and 100 μL of the diluted rennet solution (104 IMCU mL^{-1}) was added to the cheese milk while homogenising (60 s, thereof 15 s vortex), prior to the DWS analysis with the Rheolaser Master (Formulaction, Toulouse, France). The gelation point (RCT, as determined by a proprietary algorithm of the software) and elasticity index (curd firmness [nm^{-2}]) were observed. Curd firmness was defined as the value of the elasticity index at 30 min (EI30) or at the double time of the gelation point (EIRCTx2) after the addition of rennet. The mean values of n = 2 are provided.

2.6. Statistical analysis

The effects of κ-CN genetic variants on the contents of individual milk samples from one farm (same feeding) were analysed by one-way ANOVA followed by Tukey-HSD post-hoc test using R (www.r-project.org, version: 4.3.2). The statistical significance of the influence of genetic variants of κ-casein on gelation point (RCT) and curd firmness (EI30 and EIRCTx2) was analysed by one-way ANCOVA after adjusting pH values as a covariate. The significance level was established at $p < 0.05$. Post hoc tests were done when differences were considered at $p < 0.05$ by estimated marginal means (emmeans) with Bonferroni adjustment using R and library (emmeans).

The relationships between the different protein concentrations, minerals, gross chemical composition (fat, protein, casein), and pH with MCP were analysed by Spearman's correlation procedure in R. For some parameters, a normal distribution could not be assumed. After grouping the complete dataset into one of two groups—rate of the RCT, that is, fast or slow (criteria: RCT = 20 min); or strength of the coagulated gel (curd), that is, firm or weak (criteria: EIRCTx2: 0.005 nm^{-2})—O-PLS-DA was performed using R and following the library ropls (Thévenot, Roux, Xu, Ezan, & Junot, 2015) to identify the top predictors for a given model. In this study, column standard scaling was used prior to modelling. Top predictors were sorted by their variable importance in projection (VIP), and a cut-off of VIP = 1.5 was chosen.

3. Results and discussion

3.1. Analysis of milk samples: milk composition, κ-casein genotypes and pH

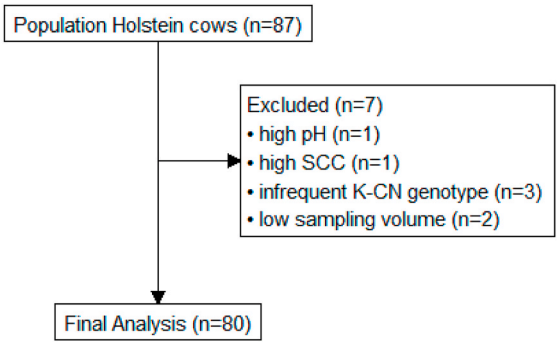
In our analysis, we argue that focusing on the CSN3 gene, which governs the expression of κ-CN, is crucial due to its established role in stabilizing, coagulating, and aggregating of casein micelles. Table 1 represents the components and properties of the fresh morning milk samples (n = 80) from one farm. Of the 87 milk samples collected, seven were excluded from the data-analysis (Fig. 1), and therefore n = 80 samples were used for statistical analysis. The κ-CN genotypes were either previously known and provided by the farmer (n = 73) or were analysed by LC-MS (n = 7). The following genotypes were found: AA (n = 28), AB (n = 35), BB (n = 8), and BE (n = 9). In their review article, Gai et al. (2021) stated that in most European breeds, the A variant of κ-CN is more frequent than the B variant, while E is the least common. BE and EE variants are rare in κ-CN.

Gross composition (fat, protein, casein) of individual milk samples and variation between samples were within the expected range. However, we observed a relatively high variation in free fatty acids (fFA)

Table 1
Important parameters found in the n = 80 individual Holstein milk samples (AA = 28, AB = 35, BB = 8, BE = 9).

Component	Mean (Median for somatic cell counts & mesophiles)	Minimum	Maximum
Somatic cell counts [1 × 10^3 counts mL^{-1}]	29	6	287
Mesophiles [1 × 10^3 CFU g^{-1}]	13.5	1.2	83
Fat [%, w/w]	4.5	2.5	5.8
pH [−]	6.73	6.62	6.87
Protein [%, w/w]	3.72	2.89	5.05
Casein [%, w/w]	2.91	2.24	4.00
Free fatty acids [mmol 10 kg^{-1}]	1.86	1.30	8.50
κ-casein (of total MP) [%]	13.05	8.70	19.10
κ-casein (of total casein) [%]	15.78	10.50	22.50
κ-casein (of β- and κ-CN) [%]	25.22	17.80	35.40
Zinc [mmol kg^{-1}]	0.060	0.031	0.106
Phosphorus [mmol kg^{-1}]	32.53	25.19	41.33
Sodium [mmol kg^{-1}]	12.95	8.70	20.66
Magnesium [mmol kg^{-1}]	4.43	3.29	5.76
Potassium [mmol kg^{-1}]	37.74	31.20	43.48
Calcium [mmol kg^{-1}]	30.66	25.95	42.66

pH values of individual milk samples were not standardized by HCl or NaOH. MP: milk proteins; CFU: colony forming unit.



SCC: Somatic cell counts

Fig. 1. Samples that have to be eliminated due to different reasons.

(Table 1). The free fatty acids were generally 1.3 mmol 10 kg^{-1} or below, but occasionally increased slightly, with a maximum of 8.5 mmol 10 kg^{-1}. Previous studies have suggested that these fFA values were randomly distributed and more likely to be associated with factors such as lactation stage, milking system, and milk sampling handling rather than the individual cows or their genetic variants (Woodhouse & Kelton, 2023). We did not analyse lactose, given its negligible influence on MCP. Huppertz et al. (2021) did not find a significant variation in this parameter within 48 milk samples from Holstein-Friesian cows.

The mean pH value was 6.73 with an expected range (min. 6.62 and max. 6.87). The expected mean pH of bulk milk is: 6.70. Similar pH values were also found by Huppertz et al. (2021) in 48 individual milk samples (pH = 6.75 ± 0.05). Individual milk pH is mainly influenced by milk components or lactation stages. Higher pH values may also indicate subclinical mastitis or intramammary infections.

Fractions of κ-CN and minerals are shown in Table 1, and the values are consistent with the expected ranges. Some variation was observed between the samples, with relatively large variations in Na and Zn.

Table 2
Mineral contents found in the n = 80 individual milk 10-kDa permeates (AA = 28, AB = 35, BB = 8, BE = 9) and the calculated fraction of the milk protein-associated (colloidal) and the casein-associated mineral contents. Minerals in the pellet (after ultracentrifugation) is also provided.

Components	Mean	Minimum	Maximum
Zinc[a] (10 kDa-permeate) [mmol kg^{-1}]	< detection limit		
Phosphorus (10 kDa-permeate) [mmol kg^{-1}]	14.65	9.69	19.21
Sodium[b] (10 kDa-permeate) [mmol kg^{-1}]	13.81	10.00	21.31
Magnesium (10 kDa-permeate) [mmol kg^{-1}]	3.12	2.47	3.70
Potassium[b] (10 kDa-permeate) [mmol kg^{-1}]	38.70	31.46	45.52
Calcium (10 kDa-permeate) [mmol kg^{-1}]	9.26	4.57	13.10
Phosphorus (milk protein-associated) [mmol kg^{-1}]	17.8	12.11	28.09
Magnesium (milk protein-associated) [mmol kg^{-1}]	1.31	0.41	2.88
Calcium (milk protein-associated) [mmol kg^{-1}]	21.31	15.72	34.93
Phosphorus (casein-associated) [mmol 10 g^{-1} casein]	6.13	4.37	7.77
Magnesium (casein-associated) [mmol 10 g^{-1} casein]	0.442	0.168	0.726
Calcium (casein-associated) [mmol 10 g^{-1} casein]	7.34	5.61	8.99
Zinc (pellet after ultracentrifugation) [mmol kg^{-1}]	0.549	0.257	0.737
Phosphorus (pellet after ultracentrifugation) [mmol kg^{-1}]	175.2	133.4	206.7
Sodium (pellet after ultracentrifugation) [mmol kg^{-1}]	15.23	11.09	22.40
Magnesium (pellet after ultracentrifugation) [mmol kg^{-1}]	15.47	11.93	20.98
Potassium (pellet after ultracentrifugation) [mmol kg^{-1}]	44.39	36.83	52.17
Calcium (pellet after ultracentrifugation) [mmol kg^{-1}]	201.6	159.4	264.5

[a] Zinc concentration in 10-kDa permeate was very low or even below the limit of determination, for this reason, no protein-associated or casein-associated zinc was calculated.

[b] Sodium and potassium values in the 10-kDa permeate were similar to those in milk, meaning, that there is no protein- or casein-associated sodium and potassium.

3.2. Mineral composition after ultracentrifugation of the milk

Defatted milk was subjected to ultracentrifugation (100,000×*g*) to separate proteins from serum. The serum was then filtered with a 10-kDa filter to obtain a completely protein-free solution in which minerals were "free" from any caseins. Table 2 shows the "free ionic", "soluble" or serum minerals P, Mg, and Ca in the 10-kDa permeate. The calculated proportions of milk protein- or casein-associated (micellar) Ca, Mg, and P are provided (calculated after formulas 4 and 5).

As the Zn content of the 10-kDa permeate was at the quantification limit, and similar results were found for Na and K in both milk and the 10-kDa permeate, no calculated protein- or casein-associated content was calculated or shown for these three compounds in Table 2. Regarding Ca, 30.1% of the total Ca in milk was found in the 10-kDa permeate, a value similar with those published by Huppertz et al. (2021). Similarly, ~45% of P and ~70% of Mg were found in the 10-kDa permeate. Huppertz et al. (2021) reported the same amount of P; however, they found ~65% of Mg, a value lower than the 70% found in this study. Malacarne et al. (2014) found ~70% of Ca and ~52% of P associated with protein in 81 milk samples, which aligns well with the results of the present study (Table 2). The values for "soluble" Ca in the 10-kDa permeate were also similar to those found in whey (after the addition of rennet) in an earlier study by Guggisberg, Loosli, Blaser, Badertscher, and Schmidt (2022).

The casein-associated minerals Ca, P, and Mg are represented in Fig. 2, with significant variations observed between milk samples from individual cows. This variation is notable, as these casein-associated minerals were individually "standardised" by their own casein amount. This phenomenon could be due to the different potential bonding associations between casein-associated minerals, such as ionic bonds of Ca and Mg with amino acid residues or different forms of calcium phosphate nanoclusters, although the PTMs of the caseins may also play an important role.

We calculated a mean value of 7.34 mmol Ca per 10 g of casein and about 6.13 mmol P per 10 g of casein (ratio: 1 : 0.84). The value for Mg was much lower: around 0.442 mmol per 10 g of casein. By contrast, casein-associated Ca and P were highly correlated (Fig. 3, top), with a correlation coefficient of R = 0.69, indicating a constant casein-associated Ca/P ratio. The correlation coefficient of the Ca/P ratio in milk was similar (R = 0.78, Fig. 3, middle), and that of the 10-kDa

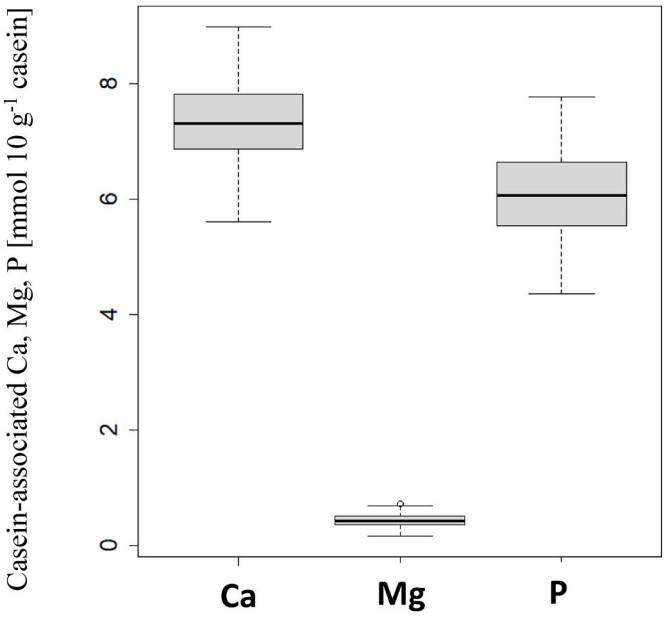


Fig. 2. Casein-associated minerals Ca, Mg and P from 80 Holstein cows. Values are expressed as mg of casein-associated Ca, Mg, P per g casein.

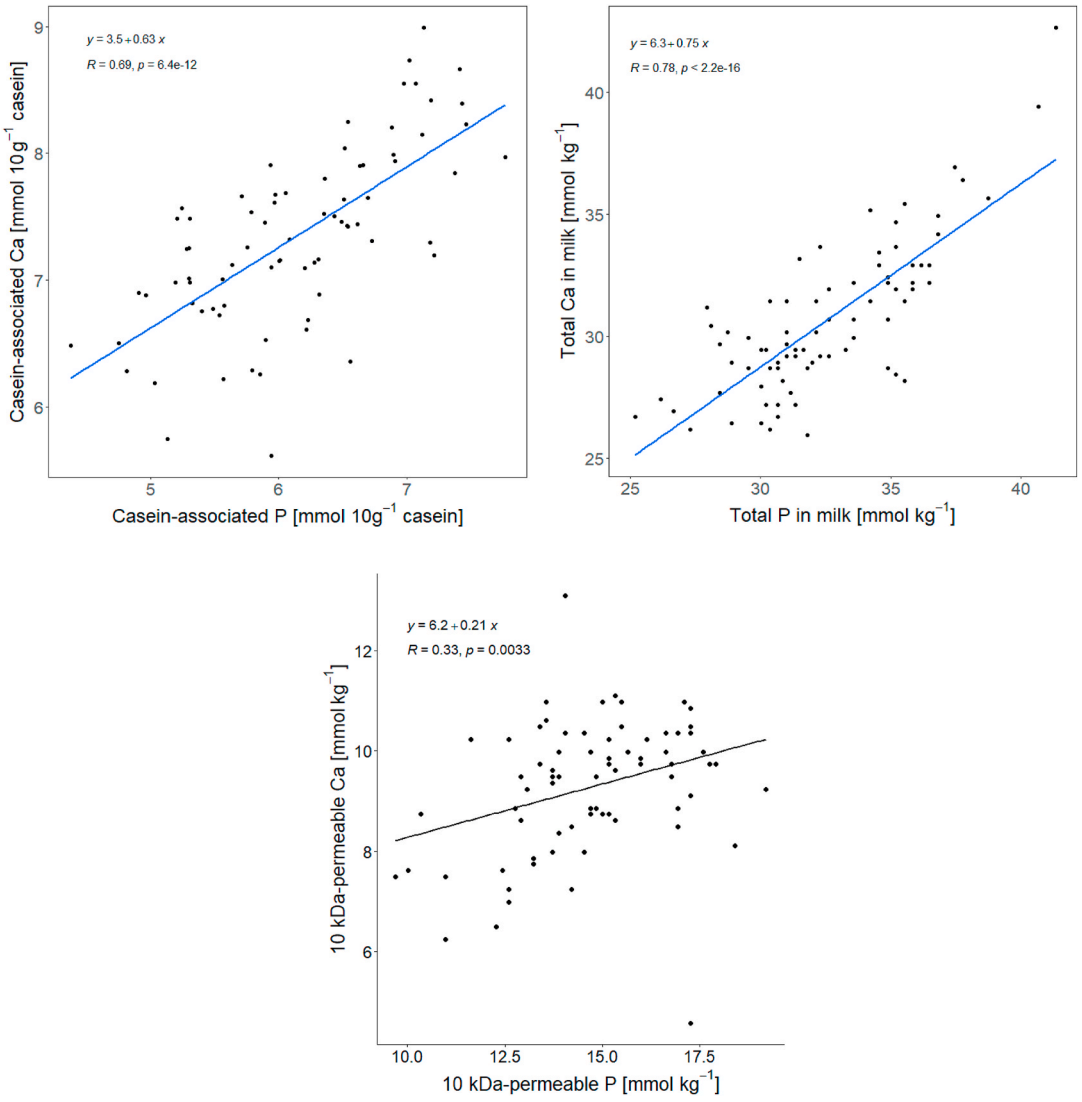


Fig. 3. Correlation between casein-associated (top), total (middle) and 10 kDa-permeable (bottom) P and Ca in milk samples from 80 milk samples (Holstein).

permeate was only 0.33 (Fig. 3, bottom).

For the strong correlation of Ca and P in milk or casein-associated fractions it is reasonable to assume that around 70% of the total Ca and around 61% of the total P were associated via calcium phosphate nanoclusters or other ionic bonds with amino acid residues to the casein micelles. Remarkable is the relatively constant casein-associated Ca/P ratio and the rather large variation between different cows. The correlation coefficient of the Ca/P ratio in 10-kDa permeate is in contrast only 0.33, meaning that Ca and P were not really correlated. In other words, the authors assume that not only calcium-phosphates, but also other combinations (e.g. calcium citrates, calcium chloride ….) might be possible in the 10-kDa permeate.

Similar findings were obtained by Huppertz et al. (2021), who analysed total minerals (Ca, Mg, P, Na, K) in 48 Holstein-Friesian cows, as

Table 3
Influence of κ-CN phenotype on fat, pH, protein, casein, κ-casein fractions, expressed in percentage of total milk protein, total casein, and β and κ-casein in milk samples (morning milk) from n = 80 Holstein cows.

κ-CN genotypes	Somatic cell counts [× 1000 counts mL^{-1}]	Mesophiles [× 1000 CFU mL^{-1}]	pH [−]	Fat [%, w/w]	Protein [%, w/w]	Casein [%, w/w]	κ-casein (MP) [%]	κ-casein (casein) [%]	κ-casein (β- and κ-CN) [%]
AA (n = 28)	52.8 ± 54.8	17.7 ± 14.0	6.73 ± 0.05	4.42 ± 0.75	3.74 ± 0.52	2.93 ± 0.41	11.83^{b} ± 2.14	14.37^{b} ± 2.57	23.54^{b} ± 3.97
AB (n = 35)	50.4 ± 57.8	24.6 ± 23.0	6.73 ± 0.06	4.67 ± 0.53	3.78 ± 0.45	2.95 ± 0.36	13.57^{a} ± 2.29	16.41^{a} ± 2.72	25.96^{ab} ± 3.91
BB (n = 8)	34.3 ± 27.4	20.0 ± 17.6	6.73 ± 0.03	4.39 ± 0.41	3.50 ± 0.30	2.72 ± 0.21	14.70^{a} ± 2.71	17.71^{a} ± 2.82	27.67^{a} ± 4.21
BE (n = 9)	18.3 ± 7.0	14.1 ± 9.2	6.73 ± 0.02	4.53 ± 0.29	3.58 ± 0.22	2.80 ± 0.17	13.33^{ab} ± 1.38	15.97^{ab} ± 1.65	25.43^{ab} ± 3.95
P-value	NS	NS	NS	NS	NS	NS	0.003	0.003	0.025

In columns, significant differences between means are shown by different superscript letters ($P < 0.05$).
MP: Milk protein; CFU: colony forming units.

well as in the 10-kDa permeate after ultracentrifugation at 100,000×g. The calculated values of micellar Ca and P showed a strong correlation (R = 0.89) in that study as well.

Table 2 presents pellet-associated minerals (Ca, P, Mg, Zn, Na, and K) after ultracentrifugation of milk. The correlation between pellet-associated Ca and P was high as well, with R = 0.79. Similar to the rather high ranges of variation of Zn and Na in milk, high ranges of Zn and Na were also found in the pellets.

3.3. Milk composition grouped by κ-casein genotypes

To study the milk components as a function of κ-CN genotypes, we performed a one-way ANOVA. For quality parameters, such as SCC and mesophiles, pH-value, all main components (protein, casein, fat), no significant differences between κ-casein genotypes were found (Table 3). It is remarkable that a low and narrow pH range was found for the BB and BE genotypes compared to genotypes AA and AB, as shown in Fig. 4a. This is remarkable because a low and narrow pH range is suggested with better udder health, that can positively influence milk quality and coagulation properties (Schaeren, 2007, pp. 16–21).

In a similar study by Walsh et al. (1998), also no significant influence of κ-CN genetic variants on casein content or gross milk composition was observed. However, the κ-CN genotypes significantly influenced the relative concentration of κ-CN (Table 3) as a percentage of the total milk proteins, caseins, or β- and κ-CN (BB > AB ~ BE > AA) for 80 individual Holstein cow milk samples, analysed by an in-house LabChip method. These results were in agreement with other findings recently reported by Guggisberg et al. (2024), who used the same technique in 44 Holstein milk samples and found the order BB > AB > AE > AA. Similar results (BB > AB > AA) were found as well earlier in the relative content of κ-CN by Bonfatti, Chiarot, and Carnier (2014) in a large cohort of 2015 individual milk samples from Simmental cows. In that study, the κ-CN contents were analysed by RP-HPLC, where glycosylated and non-glycosylated κ-CN forms were also differentiated. Glycosylation levels can vary, starting low and increasing from early to mid-lactation, before decreasing again in late lactation (Bonfatti et al., 2014). These modifications could be critical for micelle formation and rennet-induced coagulation.

A higher protein and a higher κ-CN content were reported for κ-CN variant B compared to κ-CN variant A by Bonfatti et al. (2010), which is in agreement with our results. As a consequence, sites of post-translational modifications (PTMs), such as glycosylation and phosphorylation, are more abundant in κ-CN variant B. PTMs might be essential for the stability of casein micelles, putatively providing sites for strong ionic interaction between peptide chains by linking them via PO_3^{2-} and Ca^{2+}. Although κ-CN does not contain phosphoserine clusters and plays a minimal role in calcium binding, partial phosphorylation at Ser149 and Ser127 has been reported by Mercier, Brignon, and Ribadeau-Dumas (1973).

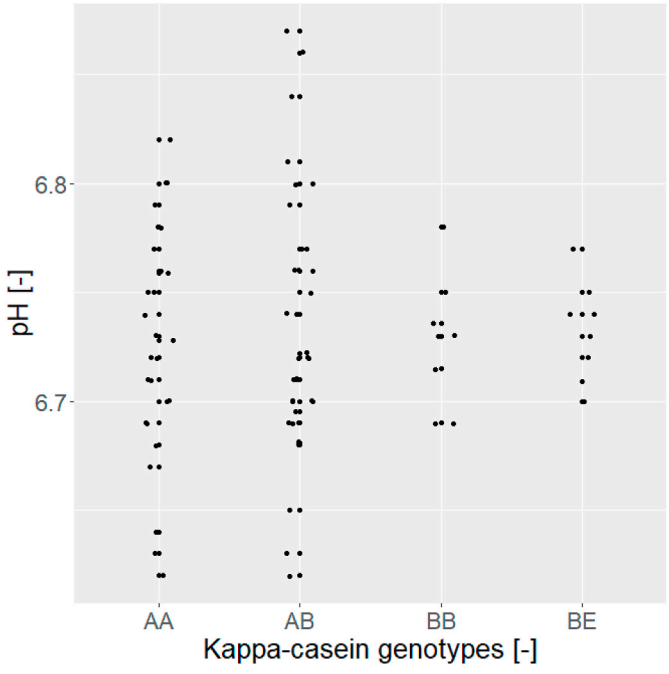


Fig. 4a. pH values depending on the κ-casein genotypes.

Ketto et al. (2017) found slightly different results for 99 Norwegian red cattle milk samples analysed by a capillary electrophoresis method. κ-CN affected the relative concentration of κ-CN-1P in the following order: BB > AB > AA > BE. However, these results were not significantly different among the κ-CN genotypes in that study.

Additionally, regarding minerals in milk (Table 4), for Na a weak but significant difference ($F(3,76) = 2.913$, $p = 0.04$) for the 4 κ-CN genotypes was observed. After Tukey's HSD test, the significance was no longer visible. Again, a low and narrow Na range was found (Fig. 4b) for the BB and BE genotypes compared to genotypes AA and AB. A supposed relationship between Na content and SCC was noted (Fig. 5), with Na levels positively correlated with higher SCC for most of the samples (R = 0.38). Four samples, however, showed higher levels between 200 and 300 × 10^3 counts mL^{-1}. Giannuzzi et al. (2024) recently observed in a study with 1013 individual Holstein-Friesian cows a significant positive association between SCC and Na, S, and Fe levels in milk. An early sign of inflammation in the mammary gland is believed to be associated with an increase in SCC, which increases the permeability of the blood–milk barrier, triggering the transfer of molecules, including Na, into milk. Cendron, Franzoi, Paneasa, De Marchi, and Cassandro (2021) found lower SCC values for κ-CN BB genotype compared to AA and AB in a large study with 5316 Holstein-Friesian cows. We observed a similar trend (Table 3).

The (calculated) milk protein-associated and casein-associated minerals Ca, Mg, and P were not significantly different among the four κ-CN genotypes, nor were the pellet-associated minerals (Ca, Mg, P, Zn, Na, and K) (Table 4). These results are in agreement with another study by Huppertz et al. (2021) in 48 Holstein-Friesian cows, in which differences in casein "mineralisation" were also not significantly different among the AA, AB, AE, and BB genotypic groups.

In our study we analysed 80 cows compared to 48 cows in the mentioned study and the results were in agreement. This allows the assumption that there is most probably no direct influence of κ-CN genotype to the amount of minerals in the individual milk or in the associated casein micelles.

Another aspect not considered in the present study was the particle size dependency of casein micelles classified by the κ-CN genotype.

3.4. Rheological data

The rheological parameters RCT, EI30, and EIRCTx2 were measured using the DWS technique and analysed for all 80 individual milk samples. After classification into the genotypes AA, AB, BB, and BE, the rheological data were analysed using an ANCOVA model with pH as a covariate, as shown in Table 5. The ANCOVA model was chosen, as it is well recognised that pH is influencing the MCP's. To prove this assumption, RCT was shown in a scatter plot with pH to analyse the correlation (Fig. 6); therefore, pH was used as a covariate in the ANCOVA model. Many previous studies have also reported a positive correlation between pH and RCT (Ikonen, Morri, Tyriseva, Ruottinen, & Ojala, 2004; Cassandro et al., 2008; Britten et al., 2022). In general, reducing milk pH increases both the activity of chymosin and the amount of diffusible ionic calcium, which has a positive impact on the different phases of coagulation (Malacarne et al., 2014).

We found a slightly faster coagulation for the BB genotype, while BE was significantly different (Fig. 7a) from AA and AB (BE < BB < AB <

Table 4
Influence of κ-CN phenotype on total minerals in milk samples (morning milk) and casein-associated forms from n = 80 Holstein cows.

κ-CN genotypes	Zn [mmol kg^{-1} milk[b]]	P [mmol kg^{-1} milk[b]]	Na [mmol kg^{-1} milk[b]]	Mg [mmol kg^{-1} milk[b]]	K [mmol kg^{-1} milk[b]]	Ca [mmol kg^{-1} milk[b]]	P [mmol 10 g^{-1} casein[c]]	Mg [mmol 10 g^{-1} casein[c]]	Ca [mmol 10 g^{-1} casein[c]]
AA (n = 28)	0.059 ± 0.018	32.22 ± 3.33	13.93 ± 2.98	04.44 ± 0.53	38.17 ± 2.55	30.40 ± 3.08	6.09 ± 0.76	0.46 ± 0.12	7.32 ± 0.65
AB (n = 35)	0.060 ± 0.013	33.02 ± 3.29	12.81 ± 2.59	04.50 ± 0.53	37.47 ± 2.40	31.05 ± 3.49	6.18 ± 0.80	0.45 ± 0.11	7.36 ± 0.76
BB (n = 8)	0.064 ± 0.018	32.41 ± 2.56	11.63 ± 1.83	04.14 ± 0.28	37.63 ± 2.74	29.72 ± 1.97	6.16 ± 0.75	0.41 ± 0.08	7.49 ± 0.75
BE (n = 9)	0.060 ± 0.018	31.64 ± 3.45	11.60 ± 1.09	04.39 ± 0.34	37.51 ± 3.15	30.77 ± 2.48	5.92 ± 0.72	0.38 ± 0.08	7.13 ± 0.65
P-value	ns	ns	0.04[a]	ns	ns	ns	ns	ns	ns

In columns, significant differences between means are shown by different superscript letters (P < 0.05).
[a] After the Tukey (HSD) test the p-value of the means of total Na was no longer significantly different for the 4 κ-CN genotypes.
[b] Milk samples (morning milk).
[c] Casein-associated forms.

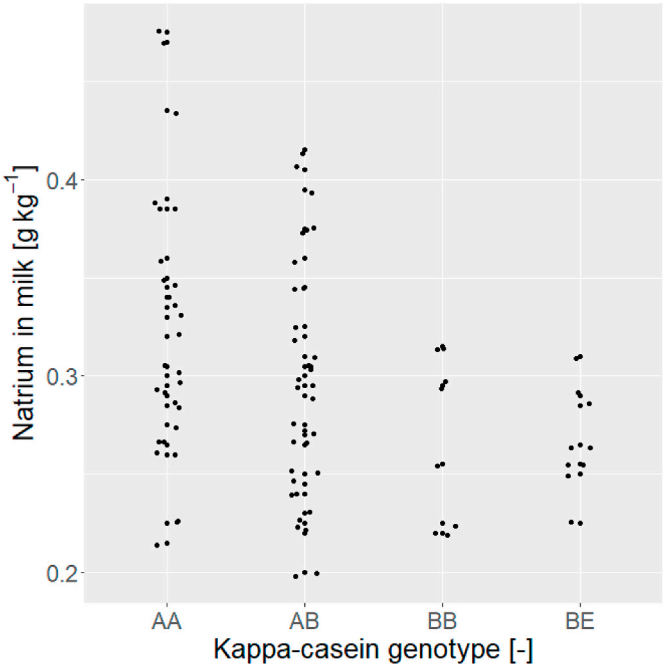


Fig. 4b. Na contents depending on the κ-casein genotypes.

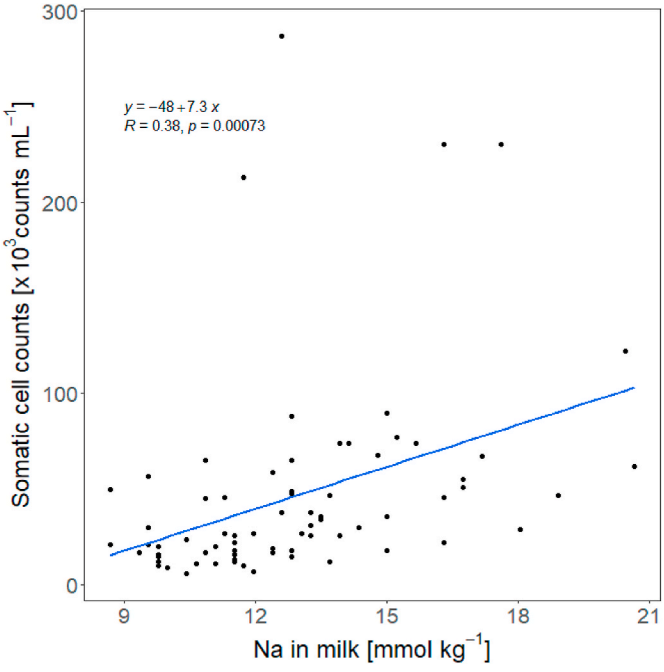


Fig. 5. Correlation between Na and somatic cell counts (R = 0.38).

AA). The highest values for EI30 were found for BB and were significantly different from AA (BB > BE > AB > AA). A similar trend was observed for EIRCTx2 (Fig. 7b), where BB was significantly firmer compared to AA (BB > BE > AB > AA). A reason for these findings could be that more relative κ-CN in B variants could lead to faster aggregating of the casein-micelles, probably due to a higher substrate to enzyme ratio (κ-CN: rennet), and a firmer coagulum due to more hydrophobic interactions in the coagulum.

Another interpretation for these findings could be that a higher percentage of κ-CN in B variants could lead to a stronger coagulum due to more calcium-phosphate interactions and due to starting from smaller casein micelles. Smaller casein micelles (not measured in this study) could lead to a firmer coagulum due to building a denser coagulum, with slightly less water inside the casein micelles (Glantz et al., 2010; Kelly, O'Mahony, & Tobin, 2024). The literature generally shows a smaller casein micelle size for the κ-CN BB genotype than for AA (Huppertz et al., 2021; Nadugala et al., 2024). The casein micelle size from individual cows is known to influence the functional properties of milk and can vary from smaller (154 nm) to larger (230 nm) micelles (Bijl, de Vries, van Valenberg, Huppertz, & Van Hooijdonk, 2014; De Kruif & Huppertz, 2012). Genetic factors, along with feeding regimes, season, calcium and citrate content, and pH, might influence the average micelle size (Nadugala, Pagel, Raynes, & Ranadheera, 2022). The more glycosylated κ-CN B variant was found to correlate with smaller casein micelles, resulting in shorter gelation times and firmer curds, while the κ-casein A and E tend to form larger casein micelles (Bijl et al., 2014; Day, Williams, Otter, & Augustin, 2015; Di Gregorio et al., 2017).

Cendron et al. (2021) showed a similar order of κ-CN genotypes and

Table 5
Influence of κ-casein phenotype on rheological properties (gel point, EI30, EIRCTx2) in milk samples (morning milk) from n = 80 Holstein cows.

Genetics	RCT [min]	EI30 [nm^{-2}]	EIRCTx2 [nm^{-2}]
AA (n = 28)	20.33 ± 3.23 $^{(a)}$	2.69e^{-3} ± 1.41e^{-3} $^{(b)}$	4.13e^{-3} ± 1.51e^{-3} $^{(b)}$
AB (n = 35)	19.97 ± 4.86 $^{(a)}$	3.95e^{-3} ± 2.50e^{-3} $^{(a)}$	5.11e^{-3} ± 2.20e^{-3} $^{(ab)}$
BB (n = 8)	17.29 ± 2.66 $^{(ab)}$	5.52e^{-3} ± 2.75e^{-3} $^{(a)}$	6.53e^{-3} ± 2.34e^{-3} $^{(a)}$
BE (n = 9)	16.77 ± 1.83 $^{(b)}$	4.51e^{-3} ± 1.30e^{-3} $^{(a)}$	5.29e^{-3} ± 8.65e^{-4} $^{(ab)}$
P-value[a]	0.02	0.001	0.006

In columns, significant differences between means are shown by different superscript letters (P < 0.05).
[a] ANCOVA, with variable (pH) as a covariate. EI30, elasticity index after 30 min. EI2GP, elasticity index at 2x gelation point.

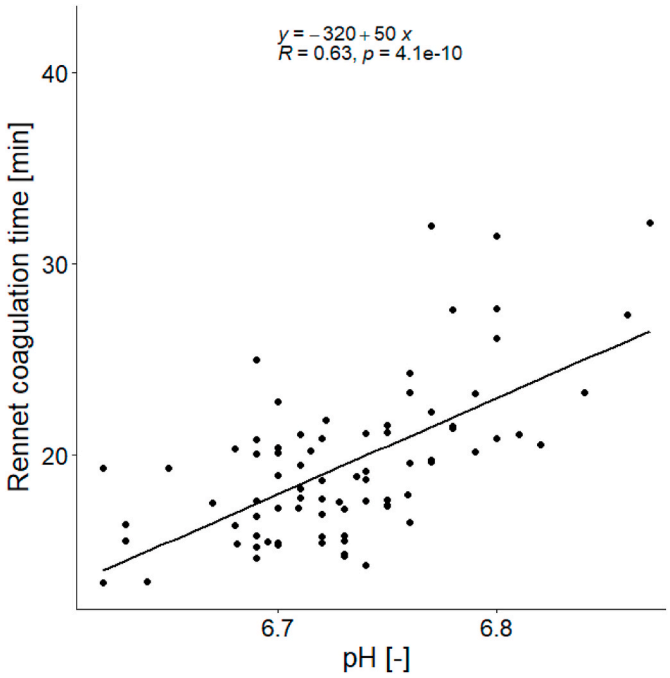
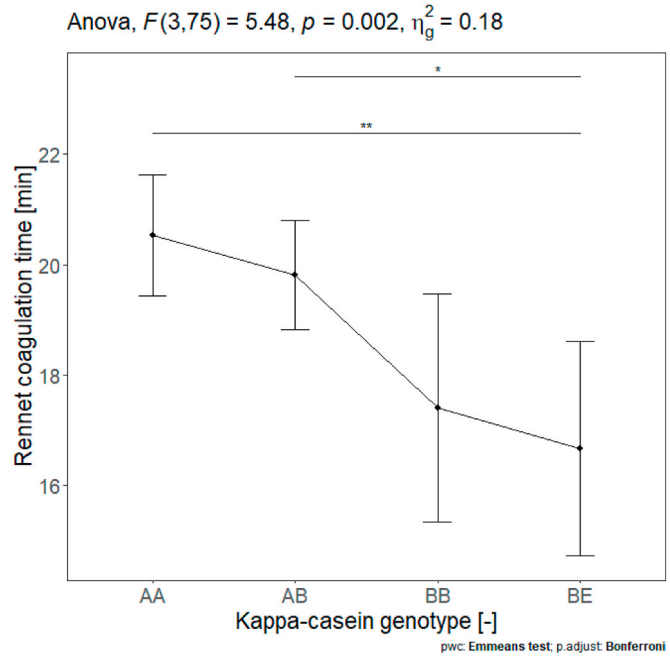
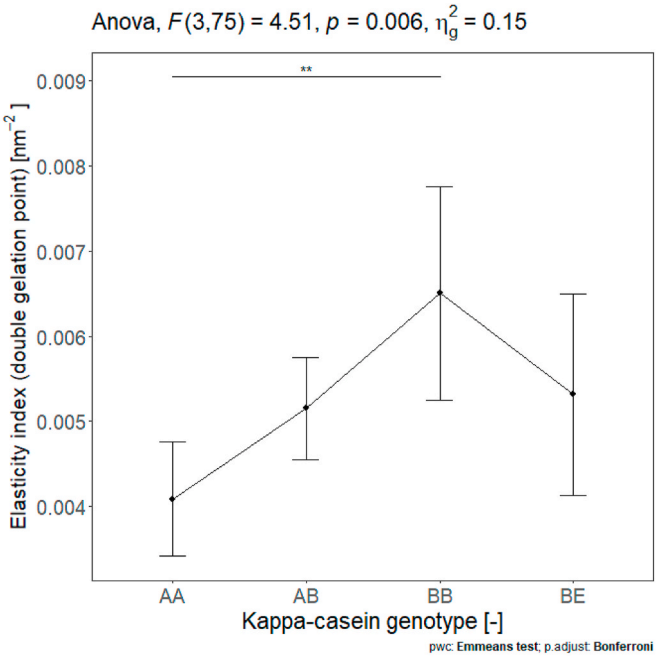


Fig. 6. Correlation between pH and rennet coagulation time (RCT).



pwc: pair-wise comparison

Fig. 7a. Results of ANCOVA –analysis of RCT and κ-casein genotypes, with pH as a covariate.



pwc: pair-wise comparison

Fig. 7b. Results of ANCOVA –analysis of elasticity index (double gelation point) and κ-casein genotypes, with pH as a covariate.

RCT (BB < BE < AB < EE < AE < AA) and curd firmness (BB > AB > BE > EE > AE > AA) in a study involving around 5000 Friesian-Holstein cows. Interestingly, that study found no significant difference in RCT between β-CN genotypes A1A1, A1A2, and A2A2. Furthermore, Comin et al. (2008) suggested that the κ-CN genotypes have a stronger correlation with milk coagulation properties than β-CN genotypes.

However, in their review on genetics and MCRs, Bittante, Penasa, and Cecchinato (2012) showed that the 6 principal milk proteins of cattle are encoded by highly polymorphic genes, with up to 47 identified variants. The effects of variants in the gene encoding β-CN were up to now much less studied than the effects of κ-CN variants. Bisutti et al. (2022) recently found in a study with 1133 Holstein Friesian cows that β-CN A2A2 genotype alters the milk protein profile and slightly worsens coagulation properties compared to A1A1 and A1A2. In our present study, most of the milk samples came from the A1A1 and A1A2 genotypes, but not all of the 80 samples were genotyped for β-CN.

The effects of variants in the gene encoding α_{S1}-CN have rarely been studied, likely due to the small number of minor alleles and small effect on MCP, as found in a study by Joudu et al. (2007) performed with 118 Estonian cattle. The differences between genotypes were mostly not significant, probably due to the high standard error resulting from the comparatively small number of animals representing each genotype. However, recent findings about α_{S1}-κ-CN genotype BCAA, BBBB, and BBAA have provided an updated perspective (Olsen et al., 2023). Recently, Olsen et al. (2023) pooled individual milk samples according to their α_{S1}- and κ-CN composite genotypes. The comparison of the α_{S1}- and κ-CN genotypes (with similar pH at the time of rennet addition) showed shorter renneting times for BBBB compared to BBAA. However, the authors also showed that α_{S1}-κ-CN genotype BCAA was even faster than BBBB. Unfortunately, the α_{S1}-κ-CN genotype BCBB was not available in that study. This demonstrates that genotypes other than κ-CN, such as α_{S1}-CN genotype could also have a considerable influence on MCPs. On the other hand, α_{S1}-κ-CN genotype BBAA was significantly higher in casein content than BCAA, suggesting that the faster RCT could be attributed to this parameter or another factor, such as casein micelle size, which was supposed to be responsible for the different RCTs. Ketto et al. (2017) found that milk with α_{S1}-CN BC genotype contained smaller casein micelles compared with α_{S1}-CN BB. In our study, the α_{S1}-CN genotype was unknown for individual milk samples.

In a meta-analysis of several studies, Bittante et al. (2012) found that RCT was influenced by κ-CN genotypes in the order BB < AB < AE < AA, and curd firmness in the order BB > AB > AA > AE. Although whey proteins are not involved in gelation, data from 10 studies revealed a moderate effect of β-Lg genotype on RCT and other MCRs. The authors of this review did not specify a reason for this surprising effect. They, however, suggested that the impact of the β-Lg genotype on gelation,

previously thought to be insignificant, needs further study. As described in several reports, genotypes of milk proteins might influence both the amount and proportion of different proteins in milk, thereby affecting the dimensions of the casein micelles. However, a comparison of the results of different studies revealed that important parameters, such as rennet type and amount, milk temperature (32°C–35°C), and milk sample age (fresh, cooled, sometimes preserved/unpreserved and analysed up to 5 days later) varied between studies.

3.5. Correlation between coagulation properties and milk components

The selected correlation coefficients between the coagulation properties and the milk components are presented in Table 6. As expected, pH was significantly associated with the RCT, with a trend for pH found only for EI30 and EIRCTx2. Total Ca and P in the permeate were weakly negatively correlated with the RCT, while Na in the pellet was weakly positively correlated with the RCT. A weak negative correlation was also found between the relative κ-CN fraction and the RCT. Relative κ-CN fractions were all significantly correlated with EI30 and EIRCTx2. This is an interesting finding, because more rel. κ-CN leads to a firmer coagulum and more rel. κ-CN leads to a lower RCT. The interpretation of these results might be connected to the structure of the casein micelles and the mean size of the casein micelles. An interpretation of these results was already mentioned in the previous chapter.

All other correlations were not significant, and a few trends were found: protein, casein, and minerals (Ca, P, Zn) were slightly correlated with EIRCTx2 (Table 6).

Total Ca, Mg, and P, along with their casein-associated forms, were also shown to be important for MCPs. In a similar study of MCPs, milk protein-associated minerals were categorised into optimal, suboptimal, poor, and non-coagulating with 81 milk samples by Malacarne et al. (2014). The highest colloidal mineral contents (Ca, Mg, and P) were found in the "optimal" milk samples in that study, while the mean Na content was lowest. Stocco et al. (2021) confirmed the positive effect of high Ca content on coagulation patterns, with only a mild effect of the Na on MCPs. In our study, we observed similar trends: milk with higher Ca content in milk exhibited shorter coagulation times and firmer curds, while casein-associated and milk protein-associated Ca slightly correlated with EI30 and EIRCTx2 (Table 6). Total Na and pellet-associated Na showed a weak negative correlation with EI30.

Table 6
Correlation coefficients between coagulation properties and milk components and milk parameters (Spearman correlation).

Milk components and parameters	RCT [min][a]	EI30 [nm^{-2}][a]	EIRCTx2 [nm^{-2}][a]
pH [−]	0.56***	−0.40	−0.23
Protein [%, w/w]	NS	NS	0.28
Casein [%, w/w]	NS	NS	0.28
Ca (total) [g kg^{-1}]	−0.22	0.31	0.36
Ca (casein-associated) [mmol 10 g^{-1} casein]	NS	0.22	0.22
Ca (milk protein-associated) [mmol kg^{-1}]	NS	0.22	0.33
Zn (total) [mmol kg^{-1}]	NS	NS	0.33
P (total) [mmol kg^{-1}]	NS	0.25	0.34
P (10-kDa-permeate) [mmol kg^{-1}]	−0.24	0.21	0.21
Na (total) [mmol kg^{-1}]	NS	−0.24	NS
Na (pellet-associated) [mmol kg^{-1}]	0.23	−0.24	NS
κ-casein (MP) [%]	−0.24	0.46*	0.49**
κ-casein (casein) [%]	−0.23	0.44*	0.50**
κ-casein (β- and κ-CN) [%]	−0.22	0.41	0.48**

Numbers in table indicates the coefficients of correlation: NS, not significant, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.
RCT: rennet coagulation time, EI30: elasticity index after 30 min. EIRCTx2: elasticity index at 2x gelation point.
[a] Spearman correlation was used, as some parameters were not normally distributed.

3.6. Multivariate analysis of coagulation properties and key predictors

Multivariate analysis was conducted by classifying the entire dataset into two groups: rate of RCT, that is, fast (n = 48) or slow (n = 32); and EIRCTx2, that is, firm (n = 36) or weak (n = 44). An O-PLS-DA (Fig. 8) compared the two groups with either fast versus slow RCT or firm versus weak EIRCTx2 by investigating the main effects of discrimination. Model 1 (Fig. 8, left) for fast and slow RCT generated a predictive component with $R^2(X) = 0.248$, explaining only 25% of the variance along the x-axis. $R^2(Y)$ and $Q^2(Y)$ are indices for the model's quality in discriminating between the two groups (fast and slow). $R^2(Y)$ reached 0.571, and $Q^2(Y)$ reached 0.425, indicating a moderate model. Similar values were found for the second model (Fig. 8, right), with $R^2(X) = 0.349$, which explained only 35% of the variance along the x-axis. $R^2(Y)$ reached 0.401, and $Q^2(Y)$ reached 0.233.

The top predictors were found by their VIP values > 1.5 for model 1: RCT, pH, Na in the pellet, κ-casein (casein), and P in the permeate.

Na in the pellet was probably not as relevant to the RCT, as Na did not play an important role in coagulation, but rather indirectly, as lower Na levels (κ-CN genotypes BB and BE) were associated with narrower pH values (κ-CN genotypes BB and BE).

However, more P in the permeate might be associated with generally more non-micellar P that was able to bind together with Ca between positions in casein micelles and might therefore reduce RCT and might slightly increase EIRCTx2 (see also Table 6 for confirmation). Non-micellar P could be a limiting factor in the rennet-induced coagulation. McMahon, Brown, Richardson, and Ernstrom (1984) already found a decrease of RCT by addition of small amounts of phosphate 30 min prior to enzyme addition.

The top predictors for "fast" or "slow" RCT were pH-value and κ-CN ratio (see also Table 6 for confirmation).

For the second model, the following predictors had VIP values > 1.5: κ-casein (MP), κ-casein (casein), κ-casein (β- and κ-CN), RCT, total P and Ca. Protein, casein, and pH had VIP values < 1.5. These multivariate analysis results were consistent with the outcomes of the correlation analysis. The duration of RCT (Model 1) can be modelled with pH as the most important parameter, followed by the relative κ-CN content, Na in the pellet, and P in the permeate. These parameters were also found in the correlation analysis, with pH being the most significant. The firmness of the coagulated gel (Model 2) can be modelled using relative κ-CN content, and total Ca and P. These results were also consistent with the correlation analysis, where κ-CN content, total Ca, and P correlated with EIRCTx2. The VIP value in Model 2 was <1.5 for total Zn, which had a correlation of R = 0.33 (Table 6). Zn is only a minor mineral element; thus, its influence on MCPs may be limited due to its low concentration.

By contrast, Na and P may be important minerals, requesting a stronger focus in the future. The Na content seems to be influenced by genetics, as well as by extrinsic factors, such as SCC or subclinical infections, and it might indirectly influence the pH value of the milk, although no correlation between pH and Na or SCC was found in this study.

4. Conclusion

The study confirmed that milk from animals with the κ-CN BB genotype, corresponding to the CSN3 gene, exhibited a shorter RCT, firmer gel, and a higher proportion of κ-CN compared to the AA and AB genotypes. The BE genotype was comparable to the BB genotype in terms of RCT but had gel firmness comparable to the AB genotype. Furthermore, total, serum, or micellar minerals (Ca, Mg, and P) did not show any significant relationship with the four different κ-CN genotypes. It remains unclear whether the interaction between genotypes and MCPs is determined only by the protein fraction content or by the distinct three-dimensional conformation and physical-chemical properties of casein genotypes. Correlations and multivariate analyses revealed that high pH adversely affected RCTs. Thus, we recommend measuring the pH during

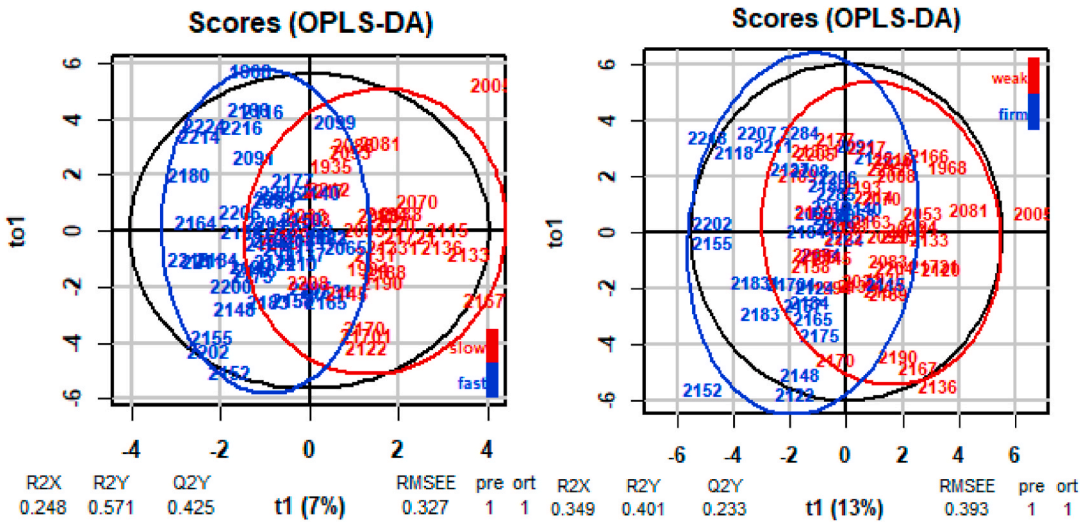


Fig. 8. Score plot for the orthogonal least squares-discrimination analysis (O-PLS-DA). 80 individual milk samples were classified in "fast" and "slow" RCT (left) and in "hard" or "weak" EIRCTx2 (right).

or after milking at the farm as a relatively simple way to separate milks with noticeable high pH values from cheese milk, which could help improve the efficiency of the cheese-making process.

Prioritising these research findings could expand the current knowledge in breeding programs and guide the development of high-quality raw milk with higher relative κ-CN proportions, enhancing the efficiency of raw-milk cheese production. Improved MCPs not only enhance cheese yield and quality but also contribute to the sustainability of dairy production. Efficient coagulation requires fewer resources, such as rennet and energy, and reduces processing time, which in turn minimizes waste and resource consumption. Following future trends in selecting genotypes that benefit the cheese industry will be essential. Selecting milk based on the genetic variants of individual cows and controlling pH values directly during or after milking may provide a straightforward strategy to optimise MCPs.

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CRediT authorship contribution statement

Dominik Guggisberg: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Doris Zbinden:** Formal analysis, Data curation. **Nicolas Fehér:** Writing – review & editing, Resources, Conceptualization. **Lukas Eggerschwiler:** Writing – review & editing, Resources, Conceptualization. **Andreas Bosshart:** Formal analysis. **Reto Portmann:** Writing – review & editing, Formal analysis, Conceptualization. **Lotti Egger:** Writing – review & editing, Formal analysis, Conceptualization. **Marlyse Raemy:** Formal analysis. **Remo S. Schmidt:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that there is no financial interest that could have influenced the work in this paper.

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