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Impact of genetic κ-casein variants (A, B, E) on chymosin-induced milk coagulation properties: Application of a new LC–MS-based genotyping method

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ABSTRACT

This study evaluated the impact of κ-casein (κ-CN) protein phenotype (AA (n = 18), AB (n = 18), AE (n = 5), BB (n = 3)) in the raw milk of 44 individual Holstein cows from a single farm on coagulation properties using diffusion wave spectroscopy. κ-CN variants were analyzed using a newly developed LC–MS method in individual milk samples, and relative milk protein fractions were quantified by LabChip-analysis. κ-CN fractions expressed in the percentage of total milk protein, total casein, and β- and κ-CN were then calculated. The gross composition of the individual milk samples and variation between individual cows was analyzed and was rather large, as expected. Traits were analyzed using an ANCOVA model with pH as a covariate.

Our results showed that mean percentages of κ-CN fractions increased for κ-CN phenotypes in the order AA < AE < AB < BB. Rennet coagulation time was slightly affected, but curd firmness was significantly affected by κ-CN phenotype (BB > AE > AB > AA) when controlling for pH.

1. Introduction

After the addition of rennet, milk coagulation properties are mainly determined by rheological properties. The rennet coagulation of milk is divided in two-steps: the primary step is an enzymatic hydrolysis of the κ-casein (κ-CN (amino acid sequence: 1–169), cleavage between: Phe^{105}-Met^{106}) that is present on the surface of casein micelles, while the second step involves the aggregation and gelation of destabilized micelles (Britten & Giroux, 2022). Milk coagulation is a fundamental step in cheesemaking but substantial variations in milk coagulation properties have been found among individual bovine milk. Therefore, they are highly relevant to the cheese industry because around 47% of the milk produced in Switzerland was converted to cheese in 2022 (Schweizer Milchwirtschaft in Zahlen, 2021/2022).

Some progress has already been realized in understanding the major milk proteins, giving some physical insights into the behavior of the caseins (α_{S1}-, α_{S2}-, β- and κ-CN), the casein interactions and micelles, and the casein-mineral interactions (Huppertz, Heck, Bijl, Poulsen, & Larsen, 2021). Notable differences in nonsedimentable casein (after ultracentrifugation of milk) were observed (κ-CN phenotypes: BB > AB > AA). Higher contents of nonsedimentable caseins with more glycosylated κ-CN could be due to increased repulsion between the micelles, resulting in the formation of a less cohesive sediment during centrifugation (Huppertz et al., 2021).

It is well-established that the 4 main caseins in milk form casein micelles (particle size: ~50–600 nm; Fox & Brodkorb, 2008) and the cohesion results from mainly hydrophobic interactions or micellar calcium phosphate (MCP). Casein micelles are dispersed in the serum phase (Lucey & Horne, 2018) and one main element in the structure of these casein micelles is considered to be the calcium phosphate nanoclusters (De Kruif & Holt, 2003). Among the caseins, κ-CN is the least phosphorylated and the only glycosylated casein (Britten & Giroux, 2022) that stands out ("hairy layer") from the surface area of the micelle (Walstra, 1999) and that plays a relevant role in the development of the casein micelle size (Bijl, Huppertz, van Valenberg, & Holt, 2014). A negative relationship between the proportion of κ-CN to total casein and the casein micelle size was suggested. In addition, an influence of the casein micelle dynamics is believed to be connected to the κ-CN

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glycosylation level. The κ-CN variants A and E tended to correlate with larger casein micelles, the more glycosylated κ-CN B correlated with smaller casein micelles (Bijl, de Vries, van Valenberg, Huppertz, & Van Hooijdonk, 2014). The same authors also found that the average casein micelle size varied considerably between milk samples from individual cows.

Milk samples with a low average casein micelle size were related to the B variant of κ-CN and a higher relative concentration of glycosylated κ-CN. Day, Williams, Otter, and Augustin (2015) found similar results: the relative amount of κ-CN to total casein was higher in the group with smaller casein micelles, and the nonglycosylated and glycosylated κ-CN contents were higher in milk samples with smaller casein micelles (mainly with κ-CN AB and BB phenotypes).

In addition, casein micelles contain large proportions of calcium, phosphate and other ions. Bijl, Huppertz, van Valenberg, and Holt (2019) presented a quantitative model and calculations about mineral distributions from individual Holstein-Friesians cows. A rather large variation between individual milk samples was found.

In the milk of 48 Holstein-Friesian cows, Huppertz et al. (2021) found a difference of >2-fold of the mineralization of the casein fraction, which was expressed in mmol protein-associated calcium 10 g^{-1} casein. The reasons for this were unknown and could not be correlated to casein composition and/or genetic variants of casein. Genetic variants of milk proteins, especially caseins, have been identified as a cause of differences in coagulation properties, cheese yield and quality (Nadugala, Pagel, Raynes, & Ranadheera, 2022).

The three major natural genetic variants of κ-CN A, B, and E differ in the three amino acids at positions 136 (position 136 was not further investigated in the present study), 148 and 155 (protein without signal peptide). Variant A has the amino acid aspartic acid (Asp) at position 148, while variant B has alanine (Ala) at the same position. The two variants A and E differ by serine (Ser: variant A) or glycine (Gly: variant E) at position 155. Both positions are in the caseinomacropeptide (CMP) of the κ-CN, the peptide that is split off from the κ-CN during the rennet treatment of milk and remains in the whey. With a lower frequency, there are also other natural variants that share mutations with the variants A, B, and E studied here; these are not considered and are treated as well as A, B, and E.

The qualitative determination of the genetic variant can be done in different ways. The most common method is genetic determination using specific primers and PCR by sequencing DNA. This determination is made either directly in blood or in the root cells of tail hairs. This determination is relatively fast, but blood must be taken from the animals, or at the very least, there must be direct access to the animals. Another method is the determination by two-dimensional gel electrophoresis (Jensen, Holland, Poulsen, & Larsen, 2012). The intact milk proteins are separated by isoelectric focusing, which is followed by denaturing gel electrophoresis, whereby the different variants of the proteins form specific points. After the prior identification of these points, they can be assigned to individual variants of the proteins. This method is relatively laborious and has the disadvantage that eukaryotic proteins are mostly post-translationally modified (*e.g.*, glycosylations and phosphorylations) and, thus, manifest in a combinatorial multitude of forms in the 2D gel. The distinction between the different genetic and post-translational variants is not always clear. The determination of genetic variants is also sometimes done directly by liquid chromatography with UV detection (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Visser, Slangen, & Rollema, 1991), LC–MS (Day et al., 2015; Frederiksen et al., 2011, especially for β-CN see: De Poi et al., 2020), a combination of LC–MS and UV detection (Miranda, Bianchi, Krupova, Trossat, & Martin, 2020), or capillary zone electrophoresis (Heck et al., 2008). Chromatography has been optimized to separate the different variants of the intact proteins and assign them accordingly. This analysis is relatively fast, but as with two-dimensional gel electrophoresis, the large number of genetic and post-translational variants can make unambiguous assignment difficult.

Few studies have investigated the effect of casein genetic variants on cheese quality, and most of these have focused on κ-CN because of its significant effect on coagulation rate and curd firmness. The κ-CN BB and AB phenotypes correlate with more fat-entrapment and water retention in cheese (Di Gregorio et al., 2017). κ-CN was found to be highly effective (Jensen et al., 2012; Zicarelli et al., 2021), but the genetic variants of the other caseins (α_{S1}- and β-CN) and the major whey protein β-lactoglobulin (β-Lg) might also contribute to milk coagulation properties.

In the present work, liquid chromatography coupled to mass spectrometry (LC–MS) was used to develop a relatively rapid but unambiguous determination of the different κ-CN variants (A, B and E) in milk. The determination is purely qualitative and not quantitative. Less common genetic κ-CN variants, such as J, C, F and others which are sub-variants of the main variants A, B and E, were not determined using this LC–MS method, respectively were assigned to the three main variants.

This means that it is not necessary to have access to the blood or tail hairs of the animals because it is possible to determine the κ-CN variants directly in individual milk. The method is based on the measurement of the amino acids of the two regions that distinguish the A, B, and E variants. The first treatment with chymosin releases the CMP of κ-CN (κ-CN, cleavage between: Phe^{105}–Met^{106}) which harbors the differences of the three genetic variants. CMP is soluble in whey, while the caseins and fat that interfere with the following analytical steps remain as cheese. The remaining whey is subsequently digested by the endoproteinase Glu-C. This proteinase cleaves all proteins at the C-terminal of glutamic acid residues, producing the peptides DSPE (single letter code for the peptide Asp–Ser–Pro–Glu: variants A and E, positions: 148–151), ASPE (single letter code for the peptide Ala–Ser–Pro–Glu: variant B, positions: 148–151), SPPE (single letter code for the peptide Ser–Pro–Pro–Glu: variants A and B, positions: 155–158), and GPPE (single letter code for the peptide Gly–Pro–Pro–Glu: variant E, positions: 155–158). Because the cows get one variant from each of the two parents, these can be homozygous and heterozygous; thus, the Glu-C treatment of the milk can result in different combinations of the above peptides (Table 1). The four resulting peptides are subsequently separated by liquid chromatography, ionized, and selectively measured by mass spectrometry. The known multitude of phosphorylation and glycosylation modifications of κ-CN were reduced to the only phosphorylation of the peptides DSPE and ASPE (Table 1), distinguishing A from B. All other post-translational modifications were no longer relevant for the identification of the κ-CN genetic variants.

However, to the best of our knowledge, there is no documented method on the direct analysis of peptides in renneted milk by LC–MS to determine the genetic κ-CN variants A, B and E.

Therefore, the aim of the present study was to investigate κ-CN variants of individual raw milk samples from Holstein cows (from one farm and the same feeding) on milk coagulation properties and establish whether the differences found are related to genetic κ-CN variants (A, B and E) and κ-CN proportions.

2. Materials and methods

2.1. Milk sample collection and gross composition

Milk samples (n = 54) from individual Holstein cows from one farm (Agroscope, Posieux) were sampled in the morning,

Table 1
The peptides DSPE, ASPE, SPPE, and GPPE formed at the end of the milk sample treatment. The natural phosphorylation of serine (DSPE and ASPE) was considered for MS analysis. Retention time and *m/z* (compared to the synthetic tetra-peptides) defined the identification of these peptides. The combination of the found peptides defined the genetic variants of κ-CN.

Peptides				
Three letter code:	Asp−Ser*−Pro−Glu	Ala−Ser*−Pro−Glu	Ser−Pro−Pro−Glu	Gly−Pro−Pro−Glu
Single letter code:	DS*PE	AS*PE	SPPE	GPPE
Position within the protein:	148−151	148−151	155−158	155−158
Retention time [min]	1.5	1.6	4.0	4.6
m/z	527.135	483.146	429.196	399.186
κ-CN variant A	x		x	
κ-CN variant B		x	x	
κ-CN variant E	x			x
κ-CN genotype AA	x		x	
κ-CN genotype AB	x	x	x	
κ-CN genotype AE	x		x	x
κ-CN genotype BB		x	x	
κ-CN genotype BE	x	x	x	x
κ-CN genotype EE	x			x

* Phosphorylation.

transported chilled directly to the lab, and analyzed for coagulation properties the same day.

The cows were fed the same way with corn-silage (30%), hay (38%), dried clover (Rumiluz) (14%), potato (14%), and mash (4%). The cows were in the lactation stages 1–8 with a mean of 3.0. The average milk yield was 28 kg d^{-1} ± 8.4 kg d^{-1}.

The following parameters were determined by FT-IR to assess the raw milk composition: somatic cell count, fat, protein, and casein (MilkoScan™ Mars, FOSS, Hamburg, Germany). The pH of the milk was measured with a pH transmitter pH2100e instrument equipped with 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).

Because of either high somatic cell counts (>300,000 counts mL^{-1}), low volume of sample, or minor phenotypes, for example, κ-CN phenotypes (BE, EE, $n < 3$), 10 samples had to be excluded from this study, meaning that 18 AA, 18 AB, 5 AE, and 3 BB samples were remaining ($n = 44$) and analyzed in this study. Clear differences in the frequency of the identified genetic variants were evident. β-CN variants were generally A^1A^2 or A^1A^1, but not A^2A^2 (information from the farmer, data not shown), while genotypes of α_{S1}-CN and β-Lg were not known.

Extreme pH values of the milk were corrected either with 0.1 M HCl or 1 M NaOH to reach a pH range between pH ≥ 6.61 and pH ≤ 6.77.

2.2. Analysis of the phenotypes of κ-casein by LC−MS

For the determination of κ-CN variants, milk was skimmed and casein proteins were renneted simultaneously, where CMP was remaining in the whey. For this purpose, 1 mL of milk was mixed with 70 μL of chymosin solution (1 mg mL^{-1} chymosin (Naturen Plus 1400 NB, Chr. Hansen, Hørsholm, Denmark, IMCU: 1400) in deionized water) in a 1.5 mL Eppendorf tube and incubated for 30 min at 32 °C in a thermomixer at 300 rpm. After vortexing for 5 s, followed by centrifugation for 15 min at 14,000 rpm (20,000 *g*) at 4 °C, 200 μL of the supernatant were heated to 95 °C at 600 rpm for 5 min in a thermomixer and then immediately cooled on ice. Then, 10 μL of the solution were mixed in a new 1.5 mL Eppendorf tube with 20 μL Glu-C Reaction Buffer 2× and 10 μL Endoproteinase Glu-C (100 ng μL^{-1}), both from Bioconcept (Bioconcept, Allschwil, Switzerland), and incubated in a thermomixer for 16 h at 37 °C and 300 rpm. After this, the solution was diluted with 160 μL of deionized water, and 100 μL were transferred to an HPLC tube. Next, 10 μL were injected and separated by liquid chromatography (3000 RS, Thermo Fisher, MA, USA) using a C18 RP column (Acquity HSS T3, 1.8 μm, Waters, MA, USA) and a 0.3 mL min^{-1} gradient from mobile phase A (water containing 0.1% formic acid) to mobile phase B (acetonitrile containing 0.1% formic acid) of 5–95% B within 20 min. The eluting peptides were analyzed in a mass spectrometer (maXis 4G, Bruker, MA, USA), here coupled with an electrospray in positive mode (3400 V capillary voltage, 500 V end plate offset, 200 °C, 4 L min^{-1} dry gas), by taking full MS scans between 75 and 1500 *m/z* at a 2 Hz measurement rate. The specific masses of the four target peptides were subsequently isolated electronically from the full scans. Peaks corresponding to both the expected mass and expected retention time (both parameters were previously determined with synthetic peptides, Synpeptide, Shanghai, China) were considered for analysis (Fig. 1). Two of the four peptides used in the method— DSPE and ASPE—are mostly phosphorylated at the serine, and accordingly, this phosphorylation (DS*PE and AS*PE with * indicating the phosphorylation) was considered in the analysis.

2.3. The analysis of the protein content (α_{S1}- and α_{S2}-, β- and κ-casein, α-lactalbumin and β-lactoglobulin) was performed by LabChip

The proteins in the milk samples were separated with a Protein 80 Kit (Agilent, CA, US) using an automated electrophoresis system (2100 Bioanalyzer, Agilent, CA, US), and electrophoresis was performed according to the description given by the manufacturer. Briefly, milk samples were diluted 1:7 with urea solution (7 M Urea in phosphate buffered saline (PBS)) and well mixed using a vortex. To 4 μL of the sample, 2 μL of denaturation solution (200 μL sample buffer provided by the Protein 80 kit, mixed with 7 μL of dithiothreitol (DTT, 1 M)) were mixed and briefly centrifuged (300 *g*, 15 s). Samples and the protein ladder (provided by the kit) were denatured (95 °C, 5 min), cooled at ambient temperature, again centrifuged (300 *g*, 15 s), and completed with 84 μL of water. The samples were either immediately analyzed or kept at 4 °C in the dark. Protein standards of α-, β-, κ-CN, and β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and BSA (Sigma−Aldrich, C6780, C6905, C0406, L0130, L6010, A4503, MO, USA) were separated on the same chip and used to attribute the proteins of the unknown milk samples.

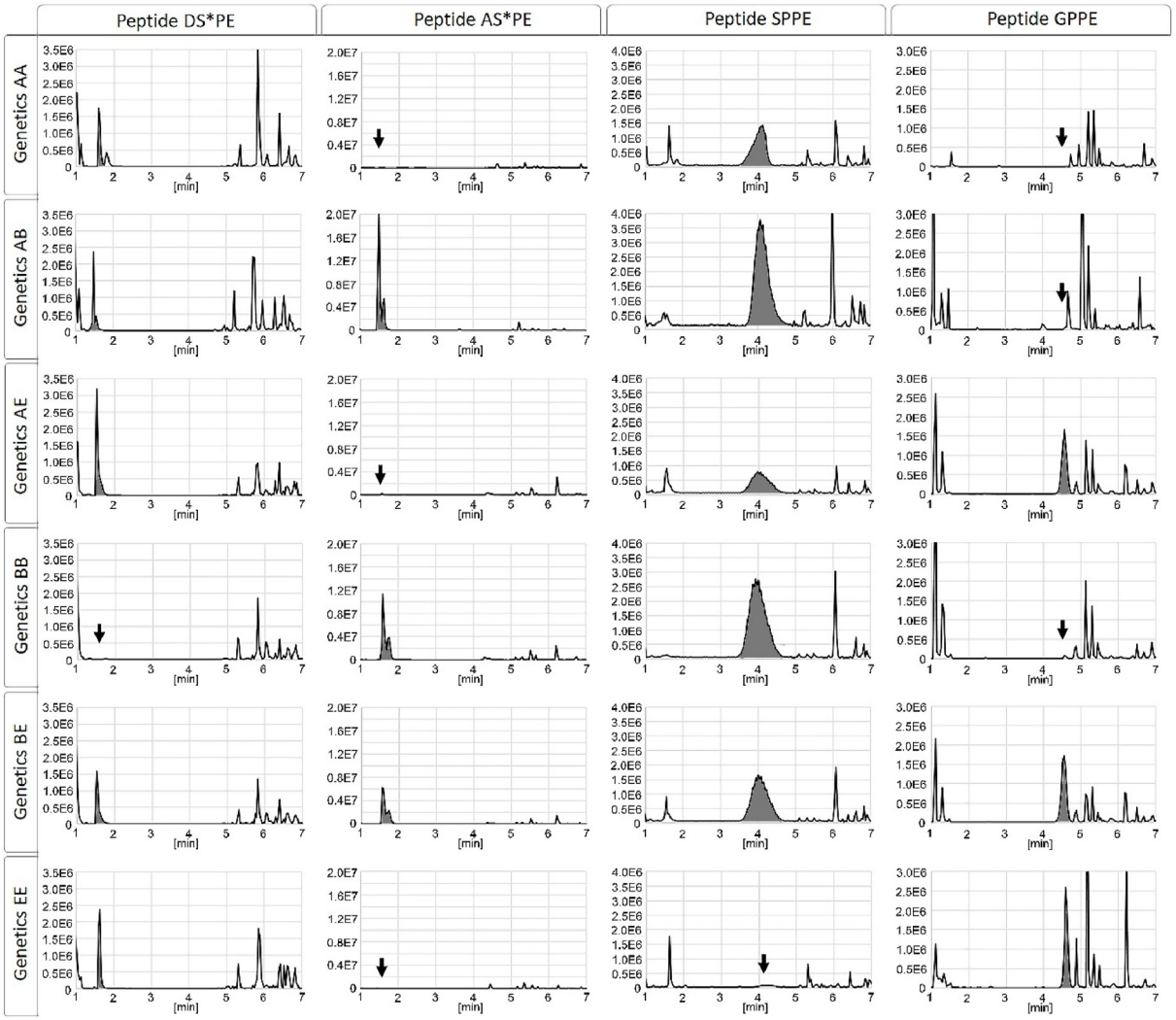

Fig. 1. Extracted ion chromatograms for milk samples with different κ-CN genetic characteristics (one genetic disposition *per* row). The columns each show the extracted ion chromatograms *per* peptide observed with the retention time on the x-axis and the abundance on the y-axis. These data were extracted according to Table 1 with an accuracy of ±0.5 *m/z*. The peaks corresponding to the expected retention time (also according to Table 1) were colored gray. An arrow marks the absence of the corresponding peak. Each genetic combination is characterized by a different combination of the peptides found, as detailed in Table 1.

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:

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

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

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

2.4. Preparation of the rennet solution for rheological analysis of milk

The rennet was freshly prepared every week according the study of Guggisberg, Loosli, Blaser, Badertscher, and Schmidt (2023) and 80 μL of rennet solution for 20 mL of milk was applied. No $CaCl_2$ was added.

2.5. Rheological analysis of renneted cheese milk

Rheological tests were performed using a Rheolaser Master (Formulaction, Toulouse, France) based on diffusing-wave spectroscopy (DWS), which was applied as published elsewhere (Guggisberg et al., 2023)

From the data obtained by Rheolaser Master, the gelation point (rennet coagulation time (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 the double time of the gelation point (EI2GP) or at 30 min (EI30) after adding the rennet. Mean values of n = 5 or 6 are provided.

2.6. Statistical analysis

The effects of κ-CN phenotypes on the milk composition (somatic cell counts, fat, pH, protein, casein, β-CN fraction compared milk protein, casein, and β-CN contents) were analyzed by one-way ANOVA followed by Tukey-HSD post-hoc test using R (http://www.r-project.org, version: 4.3.1).

The statistical significance of the influence of phenotypes of κ-CN on gelation point (RCT) and curd firmness (EI30 and EI2GP) was analyzed by one-way ANCOVA (covariate = pH to adjust for differences in pH). 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).

3. Results and discussion

3.1. Milk composition

Table 2 represents the components of the fresh morning milk samples from the individual Holstein cows (n = 44) at delivery. The average protein and casein contents were in the expected range. Fat content was partly high but also in the range of this farm in a longer view (data not shown). Samples with high somatic cell counts (>300,000 [cells mL^{-1}]) were excluded from the current study; therefore, the remaining median for somatic cell counts was 38,000 (cells mL^{-1}). The values for pH were in the range of pH ≥ 6.61 to pH ≤ 6.77; otherwise, the milk samples were corrected either with 0.1 M HCl or 1 M NaOH. The average pH was 6.7, which is in line with the expected pH values for bulk milk. The mean κ-CN fraction of milk proteins was around 13%, 15% as a fraction of total caseins, or 25% as a fraction of β- and κ-CN.

3.2. κ-Casein protein phenotypes analyzed by LC–MS

To study the κ-CN composition without the need for individual genetic testing, an LC–MS method was developed and tested using synthetic peptides. The treatment of the milk was developed with one milk each with the properties AA, AB, AE, BB, BE, and EE, which was determined by genotyping.

Subsequently, the method was validated in a previous study (data not shown) with a total of 138 single milk samples for which the κ-CN genetics were already known by genetic analysis (AA: 61, AB: 51, AE: 9, BB: 11, BE: 5, and EE: 1 sample). The samples were collected from animals (different herds, different villages), and the number of different genotypes roughly reflects their distribution in Switzerland. All samples were correctly identified with the new method, and no false results were obtained.

The marker peptides used were selected to include the region of natural amino acid mutation and to avoid, as far as possible, any known post-translational modifications. This reduced the multitude of different posttranslational modifications (PTMs), such as glycosylation, to one unique peptide each.

The advantage of the new peptide-based identification of genetic variants therefore lies precisely in the fact that a specific peptide indicates the phenotypes, whether the peptide is present or not. In contrast, with the previous mass spectrometry-based methods, where the intact protein is measured, the data must first be deconvoluted to combine the different charge states, and then, there are still many different peaks left that contain the respective PTMs. The measurement of only 4 peptides for the determination of the κ-CN phenotypes also allows a rapid transfer of the method to other systems. In case of doubt, the required peptides can be synthesized and injected into a new system.

However, the sole focus on 4 specific peptides is also the disadvantage of the new method. It is only possible to determine the phenotypes and does not provide information about the different glycosylations and phosphorylations, which may influence the behavior of the κ-CN. In addition, the simultaneous determination of genotypes of other proteins is not possible, but this was not the goal in the development of this new method.

Table 2
Compositional properties of the milk samples (morning milk) from n = 44 Holstein cows.

Component	Mean (median for somatic cell counts)	Minimum	Maximum
Somatic cell counts [counts mL^{-1}]	38000 (median)	7000	210000
Fat [%, w/w]	4.48	2.96	6.99
pH [−][a]	6.69	6.61	6.77
Protein [%, w/w]	3.66	2.82	4.35
Casein [%, w/w]	2.86	2.19	3.44
κ-CN (of total MP) [%]	12.82	9.30	16.60
κ-CN (of total casein) [%]	15.43	11.10	20.30
κ-CN (of β- and κ-casein) [%]	25.24	19.60	31.4

[a] After correction with either 0.1 M HCl or 1 M NaOH.
MP, milk protein.

3.3. Milk composition as a function of κ-casein genotype A, B and E

The κ-CN phenotype significantly affected the composition of the κ-CN contents as a fraction of the total milk proteins (κ-CN (MP)), caseins (κ-CN (casein)), or β- and κ-CN (κ-CN (β- and κ-CN)) in cheese milk (Table 3). The phenotype BB had the highest content of κ-CN compared with the phenotypes AA, AB, and AE, whereas somatic cell counts, fat, pH, protein, and casein were not significantly affected by the κ-CN genotype. These findings are in agreement with results from HPLC from Bonfatti, Chiarot, and Carnier (2014), where κ-CN genotype BB was associated with increased total κ-CN compared with genotype AA. Many factors affecting the κ-CN glycosylation are still unknown, and the role is often not clear (Bonfatti et al., 2014), but an association of κ-CN phenotype BB and small casein micelles has been suggested.

3.4. Rheological data as a function of κ-casein phenotype

Because rheological data during coagulation are known to be influenced by pH (Britten & Giroux, 2022), pH was taken as a covariate into an ANCOVA model. Rheological data such as gelation point (GP) or elasticity indexes at 30 min (EI30) or at the double

Table 3
Influence of κ-CN phenotype on fat, pH, protein, casein and κ-CN fractions, expressed in percentage of total milk protein, total casein, and β and κ-CN in milk samples (morning milk) from n = 44 Holstein cows.

Genetics	Somatic cell counts [$\times 10^3$ counts mL^{-1}]	Fat [%, w/w]	pH [−][1]	Protein [%, w/w]	Casein [%, w/w]	κ-CN (MP) [%]	κ-CN (casein) [%]	κ-CN (β- and κ-CN) [%]
AA (n = 18)	45.7 ± 33.2	4.53 ± 0.92	6.69 ± 0.02	3.81 ± 0.30	2.99 ± 0.23	12.58[a] ± 1.30	15.09[a] ± 1.69	24.94[a] ± 2.82
AB (n = 18)	51.5 ± 44.6	4.40 ± 0.86	6.69 ± 0.05	3.53 ± 0.48	2.76 ± 0.38	12.82[a] ± 1.41	15.43[a] ± 1.62	25.16[a] ± 2.51
AE (n = 5)	78.0 ± 80.7	4.73 ± 1.15	6.67 ± 0.04	3.53 ± 0.48	2.76 ± 0.42	11.52[a] ± 1.45	14.02[a] ± 1.77	23.8[a] ± 2.88
BB (n = 3)	46.3 ± 25.5	4.24 ± 0.68	6.68 ± 0.03	3.76 ± 0.19	2.94 ± 0.17	16.33[b] ± 0.31	19.77[b] ± 0.50	31.10[b] ± 0.26
p-value	NS	NS	NS	NS	NS	0.0001	0.0002	0.001

[1] Extreme pH values of the milk were corrected either with 0.1 M HCl or 1 M NaOH to reach a pH range between pH ≥ 6.61 and pH ≤ 6.77.
MP, milk protein. Values without a common superscript are significantly different at p < 0.05.

timepoint of the gelation point (EI2GP) were analyzed and are presented in Table 4. GP was not significantly influenced by the κ-CN phenotype. One reason for this result could be the low sample number of the κ-CN phenotypes AE and BB and the rather large variance between the individual milk samples. A second reason might be the fact that four samples were found to be poorly coagulating in our study (GP > 35 min), all among the κ-CN genotypes AA (n = 2) and AB (n = 2). These four milk samples were not excluded, but they increased the standard deviation of the GP from the κ-CN phenotypes AA and AB considerably. Poor or non-coagulation milk is not a consistently defined term but is usually linked in several studies with a time point or a minimal firmness level during coagulation (Frederiksen et al., 2011). In the analysis of some larger studies, noncoagulating samples were eliminated to obtain more homogeneous results (*e.g.*, Vallas et al., 2012).

Noncoagulating milk is usually defined as a milk that does not form a gel within a permitted time limit of cheese production. It has been established that the B-variants of β-CN, κ-CN and β-Lg are the preferred variants for milk coagulation and the cheese-making process compared with β-CN A1/A2, κ-CN A, and β-Lg A (Poulsen et al., 2013). Most studies observed better coagulation properties in milk containing κ-CN variant B compared with the other variants, which correlates with a higher amount of κ-CN as a proportion of the casein fraction (Jensen et al., 2012).

Frederiksen et al. (2011) also studied the phenomenon of "poorly/noncoagulating" bovine milk (no coagulum within 45 min) in a herd of 53 Danish Holstein-Friesian cows; they found that the poor coagulation was not caused by a single quarter and was only detected in samples with κ-CN phenotype AA and contained larger casein micelles and a lower fraction of κ-CN relative to the total casein. Frederiksen et al. (2011) also stated that other factors were associated with noncoagulating milks, such as a combination of κ-CN phenotype AA and the presence of more phosphorylated variants of α_{S1}- and α_{S2}-CN. In another study by Gustavsson et al. (2014b), an association between poor/noncoagulation properties and the β-κ-CN phenotypes (A^1A^2/AE and A^2A^2/AA) in 400 Swedish Red was found; noncoagulating milk was suggested to have lower ionic and total calcium content as well as lower relative concentrations of β-Lg (Gustavsson et al. 2014a). In a study with Estonian dairy breeds, Joudu et al. (2009) found that noncoagulated milk originates mainly from κ-CN phenotype AA. A larger study by Poulsen et al. (2013) showed that poor coagulation was associated with β-CN variants A^2 and I in Scandinavian dairy breeds.

By using an ANCOVA model with pH as a covariate to adjust for slight differences in pH, there was a slight trend for an increase in the elasticity index (EI30) and a statistically significant difference in EI2GP between the groups, $F(3,33) = 3.677$, $p = 0.022$ (Fig. 2). In the current study, a significant increase of EI2GP for the BB phenotype compared with the other groups was found after Bonferroni adjustment ($p = 0.05$). The results must be considered with the low numbers of κ-CN phenotypes AE and BB. The low sample size for the κ-CN phenotypes AE and BB was representative of this specific herd used in the present study.

Table 4
Influence of κ-CN phenotype on rheological properties (gelation point, EI30, EI2GP) in milk samples (morning milk) from n = 44 Holstein cows.

Genetics	GP [min]	EI30 [nm^{-2}]	EI2GP [nm^{-2}][1]
AA (n = 18)	26.16 ± 6.36	$1.93e^{-3} \pm 1.07e^{-3}$	$3.50e^{-3} \pm 1.75e^{-3}$ (a)
AB (n = 18)	25.25 ± 7.28	$2.40e^{-3} \pm 1.95e^{-3}$	$4.23e^{-3} \pm 1.18e^{-3}$ (ab)
AE (n = 5)	21.46 ± 2.16	$2.65e^{-3} \pm 1.03e^{-3}$	$4.90e^{-3} \pm 1.90e^{-3}$ (ab)
BB (n = 3)	24.28 ± 6.59	$3.58e^{-3} \pm 2.01e^{-3}$	$6.10e^{-3} \pm 2.40e^{-5}$ (b)
p-value[1]	NS	0.061, NS	0.022

[1] ANCOVA, with variable (pH) as a covariate. GP, gelation point. EI30, elasticity index after 30 min. EI2GP, elasticity index at 2× gelation point.
Values without a common superscript are significantly different at p < 0.05.

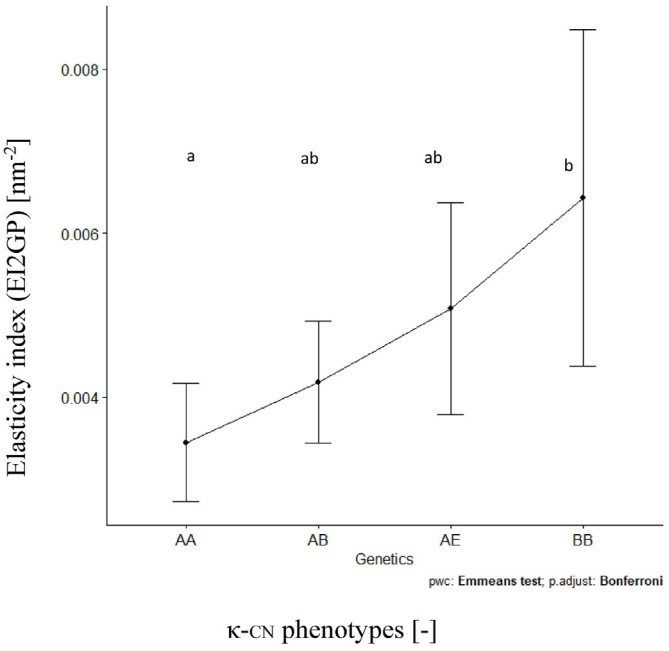

Fig. 2. Mean and standard error of the elasticity index [nm^{-2}] for the different κ-CN phenotypes at the double of the gelation point (EI2GP). Different lowercase letters in the same row show a significant difference ($p < 0.05$) between the different κ-CN phenotypes, after an ANCOVA test ($F(3,33) = 3.68$, $p = 0.022$).

Similar results were also found by Bonfatti, Di Martino, Cecchinato, Degano, and Carnier (2010), where κ-CN B was associated with shorter RCT and greater firmness compared with κ-CN A haplotypes in Simmental cows. A weak correlation was also found between EI2GP and κ-CN (casein) fraction ($r = 0.50$, $p = 0.068$) in the present study. Hallén, Allmere, Näslund, Andrén, and Lundén (2007) also found a positive association of total protein concentration with curd firmness but no association with RCT.

Most of the reported studies observed "better" coagulation properties in milk containing the κ-CN B allele compared with other variants (Di Gregorio et al., 2017; Jensen et al., 2012), which correlated with a higher amount of total κ-CN as a proportion of the casein protein fraction (Jensen et al., 2012). Because κ-CN is an crucial protein in renneting, focus has been on the variants of κ-CN and rheological properties during cheese manufacturing. Hallén et al. (2007), Hallén, Wedholm, Andrén, & Lundén (2008) also found higher G'_{max} (storage modulus), shorter RCT, and higher curd firming rate for cow milk containing κ-CN BB phenotype than the AA phenotype. Differences in rheological analyses and micelle stability were presumably associated with the micelle size and degree of phosphorylation/glycosylation of κ-CN (Jensen et al., 2012). The size of casein micelles is inversely related to κ-CN content and coagulation properties, where smaller casein micelles are associated with better coagulation (Frederiksen et al., 2011).

4. Conclusion

The aim of the present study was to provide further knowledge of the effects of κ-CN variants (A, B and E) on κ-CN fractions and milk coagulation properties from individual raw milk samples from one farm (same feeding). The analysis of κ-CN variants was directly

performed on amino acids in the milk samples using a newly developed LC–MS method. As the peptides and therefore the combinations of mass and retention time were unique in milk samples, this method was preferred over a less sensitive MS/MS method with longer scan times. In the future, this type of mass spectrometry-based analysis could also be extended to the determination of other natural variants of κ-CN, but also of other casein. Of course, the method would also be particularly interesting for minor proteins (milk fat globules membrane proteins are about 1000 times less concentrated than casein proteins), which are usually no longer detectable with a measurement of intact milk proteins.

The κ-CN phenotypes had no direct effect on casein concentrations or on any main ingredient of milk but were important for κ-CN fractions and firmness during the coagulation step. With the pivotal role of κ-CN in the primary phase of rennet-induced coagulation, these relative κ-CN fractions likely play a fundamental role of enzymatic hydrolysis, and it was suggested that this process is highly connected to the κ-CN phenotypes. However, the complex first steps in the cheese-making process are not only correlated to the κ-CN phenotypes and pH, but they are also linked to other factors, such as other casein and whey protein phenotypes, Ca-equilibria, phosphorylation of caseins and glycosylation of κ-CN, casein micelle size, proportion of κ-CN to total casein, and, most likely, to further factors unknown at the time.

It will be interesting to survey future trends in breeding and in the mixture and distribution of cows at the milk-producer level and to gain better insights into how a higher proportion of κ-CN phenotype BB could influence the cheese-making process and cheese quality. Selective breeding to increase the fraction of κ-CN phenotype BB might be advantageous to the cheese industry in the future.

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CRediT authorship contribution statement

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

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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