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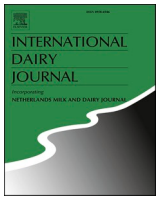
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Research paper

Influence of minor β-CN genotypes on coagulation properties and proteolytic pathways of bovine milk

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A B S T R A C T

Numerous studies have examined the effects of the two most common β-casein (β-CN) genetic variants, A1 and A2, on the physicochemical and functional properties of bovine milk. However, relatively minor variants of this protein such as B and I remain under-researched. This study collected milk with four β-CN genotypes, A1B, A2B, A1I, and A2I, across three collection occasions to investigate their functional and proteolytic properties. Milk of the B variant exhibited similar behaviour to A1 regarding rennet and acid coagulation properties, while I was similar to A2. Furthermore, the Glu_{121}-Arg_{122} and Arg_{122}-Gln_{123} bonds in the β-CN B variant were more sensitive to plasmin-derived proteolysis than Ser_{122} in other variants, leading to more peptides from cleavage at Arg_{122}-related sites. These findings support grouping genotypes based on the amino acid (AA) at position 67, as variants sharing the same AA at this position exhibit similar processability, while also highlighting the significant impact of AA at position 122 for proteolysis in milk or dairy products.

1. Introduction

The importance of milk protein genotypes for milk physicochemical and functional properties has been widely discussed. Since the introduction of 'A2 milk' to the dairy market, studies on the processing properties of milk containing β-CN genetic variants A1 and A2 have been published (Gai et al., 2021). Besides these two most common genetic variants, five other β-CN variants are also found in European cows, including A3, B, C, I and E (Sebastiani et al., 2020). The B variant has the same AA on position 67 as the A1 variant, as do variants B, C, F, G, so these are recognized as 'A1-type'; 'A2-type' variants include A3, E, D, I and H (Bodnár et al., 2018). Among these, the B and I variants are relatively more frequent than the others (Massella et al., 2017). In a study on Dutch Holstein Frisian cows, the frequency of these two variants were 0.020 and 0.192, respectively, while that of A1 was 0.283, of A2 was 0.504, and of A3 was 0.001 (Visker et al., 2011).

It was reported by Sebastiani et al. (2020) that the B variant was derived from the A1 variant of β-CN, and the I variant was derived from the A3 variant, which itself originated from the A2 variant. An evolutionary mapping of β-CN genetic variants is shown in Fig. 1. Primary structures of the B and I variants are different at position 67, 93 and 122, where the B variant has His_{67}, Met_{93}, Arg_{122}, and the I variant has Pro_{67}, Leu_{93} and Ser_{122} (Sebastiani et al., 2020), which may contribute to differences in milk characteristics.

Knowledge of the effect of β-CN B and I variants on milk composition and functional properties is limited, and studies have primarily compared them to A1 and A2 rather than to investigate themselves alone, or compare to each other in recent years (Daniloski et al., 2022; Gai et al., 2021). A positive correlation of the I variant and protein content (%) and protein yield was determined by Visker et al. (2011), who also reported a correlation between the I variant and casein yield and κ-CN contents, in comparison to the B, A1 and A2 variants. However, Vigolo et al,. (2022) reported higher protein, fat, and casein contents in milk containing the β-CN B variant compared to the A1 and A2 variants. (Bonfatti et al 2010a, 2010b) also reported a positive correlation between the B variant and β-CN content, and the B variant was reported to be linked to good rennet coagulation properties in milk from Holstein-Friesian and Jersey cows (Jensen et al., 2012), with shorter rennet coagulation time, faster curd-firming rate, and higher curd firmness compared to the other β-CN genetic variants, i.e., A1, A2, A3 and I, in milk from Danish Holstein cows (Poulsen et al., 2013). Darewicz and Dziuba (2007) compared the emulsifying properties of A1, A2 and B variants of β-CN, and reported that the B variant was less efficient as an emulsifier compared to the A2 variant.

The existing literature on the effects of minor β-CN genotypes on milk characteristics are mostly over 10 years old, and the conclusions are

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conflicting. In addition, to the best of authors' knowledge, the effect of these minor genotypes on proteolysis in milk has not been studied. To expand the current limited knowledge on any possible effects of the B and I variants on milk physicochemical, functional and enzymatic properties, milk containing four different β-CN genotypes, A1B, A1I, A2B, A2I were investigated in this study. In addition, to determine whether grouping minor genetic variants into main genetic variants by their identical AA at position 67 may lead to their loss of uniqueness, comparison between minor genotypes and previously published results of main genotypes, as well as between A1A1, A2A2 and 'A1-type', 'A2-type', were also carried out. In summary, this study provides a comprehensive investigation of the significance of these minor β-CN genotypes.

2. Materials and methods

2.1. Milk samples

Whole bovine milk was obtained from Teagasc Food Research Centre, Moorepark (Cork, Ireland); each cow in the study was sampled on three occasions. All the milk samples were from spring-calving Holstein cows (varying calving dates from the end of January to March), in mid-lactation.

Genetic variants of milk proteins were determined at Nestlé Research, Lausanne, Switzerland, using ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS; Fuerer et al., 2020, Supplemental Table S1). Milk samples for the study on physicochemical and functional properties were obtained from 20 cows with four different β-CN genotypes; five milk samples had the A1B genotype, five samples were the A2B genotype, five were the A1I genotype and five were the A2I genotype. Each individual fresh milk sample was stored at 4 °C, and 0.05 % w/v sodium azide was added to inhibit the growth of microorganisms. This sample was used for determination of gross composition, freezing point, pH, ionic concentration, and fat globule size, as well as for rennet coagulation and acid coagulation analyses.

A portion of the raw milk was skimmed at 3500 rpm at 4 °C for 30 min using an Avanti® J-E centrifuge (Beckman Coulter, Inc. Palo Alto, California, U.S.), and filtered through glass wool to remove fat residues. This skimmed milk was used for casein micelle size, polydispersity index (PDI) and zeta potential measurements, as described by Gai et al. (2023).

Milk samples for the study on enzymatic properties were obtained on a separate occasion from 16 cows with four different β-CN genotypes; four milk samples had the A1B genotype, six samples were the A2B genotype, three were the A1I genotype and three were the A2I genotype (Supplemental Table S2). These samples were skimmed as described above, pooled, and frozen at −80 °C for later analyses.

In order to compare some key functional properties of milk samples with the minor genotypes investigated in the current study, data from Gai et al. (2023) were used for the three main genotypes, i.e., A1A1, A1A2 and A2A2. In the study of Gai et al. (2023), milk samples were collected on 3 separate occasions from the same group of 15 spring-calving Holstein cows (5 cows per genotype, calving between the end of January to March), in mid-lactation.

2.2. Measurement of rennet- and acid-induced coagulation

Rennet-induced coagulation properties and acid-induced coagulation properties of individual raw milk samples were determined by a Discovery Series Hybrid Rheometer (TA Instruments, Waters Corp., New Castle, Delaware, U.S.), and an AR-G2 rheometer (TA Instruments, Waters Corp., New Castle, Delaware, U.S.), respectively, as described by O'Connell et al. (2006) with minor modifications. Throughout this paper, the terms "rennet coagulation property" "rennet coagulation profile", "rennet coagulation time" are used as concise expressions for "rennet-induced milk coagulation", and the same is applied to "acid--induced milk coagulation". Coagulation properties were assessed by measuring rennet coagulation time (RCT), acid coagulation time (ACT), gel strength (G′), and gel firming rate (ΔG'), calculated as the slope of G′ over time during rennet and acid coagulation. Details of both methods were described previously by Gai et al. (2023). These analyses were carried out once on each individual milk sample from the three collections.

2.3. Enzymatic properties

Plasmin activity of individual defrost skim milk samples (as described in section 2.1) was determined using the coumarin peptide method (Richardson and Pearce, 1981), as described by Gai et al. (2025). Plasmin activity (nmol mL^{-1} AMC min^{-1}) in milk with four β-CN genotypes, A1B (0.098 ± 0.055), A2B (0.099 ± 0.027), A1I (0.095 ± 0.039), A2I (0.105 ± 0.064) did not significantly differ ($p > 0.05$).

To assess potential differences in their susceptibility to plasmin-driven proteolysis, pooled skim milk samples were digested with a high concentration of purified plasmin (25 μL mL^{-1}, Sigma-Aldrich, Merck Group, Burlington, Massachusetts, USA) at 37 °C for up to 24 h. This approach was used to override natural variations in plasmin activity, and allow proteolysis to be measured within hours instead of days, as described by Gai et al. (2025). Skim milk samples without added plasmin were incubated under the same conditions as controls. All samples, collected at various time points during incubation, were immediately frozen at −80 °C to terminate proteolysis and later thawed for analysis using urea-polyacrylamide gel electrophoresis (urea-PAGE) and peptide profiling.

2.4. Urea-PAGE analysis

Individual skim milk samples, with or without additional plasmin,

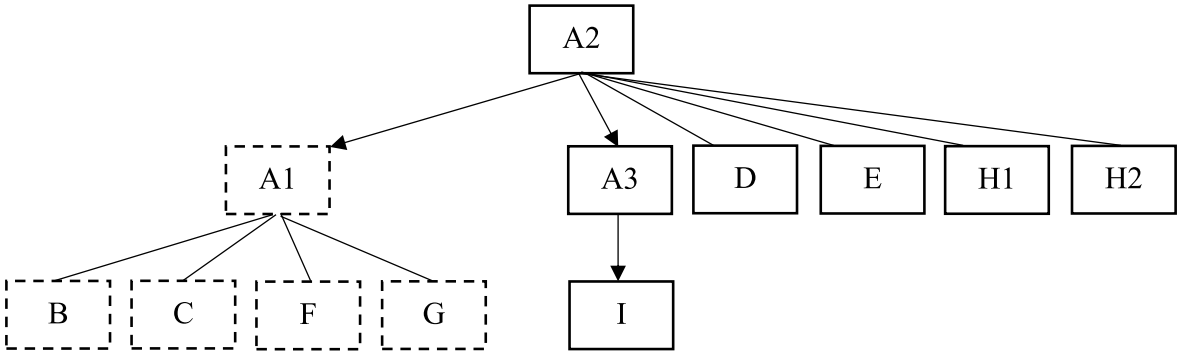


Fig. 1. Evolutionary mapping of β-CN genetic variants. Arrows indicate the direction of mutation. Solid-outline blocks represent variants with proline (Pro) at amino acid position 67, while dashed-outline blocks represent variants with histidine (His) at the same position.

were analyzed using urea-polyacrylamide gel electrophoresis (urea-PAGE). Gels were prepared following a modified method of Shalabi & Fox (1987), based on Andrews (1983), using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts, UK). Coomassie Brilliant Blue G250 staining was performed as described by Blakesley and Boezi (1977). Milk samples were diluted 1:1 with sample buffer (Andrews, 1983), and 5 μL was loaded onto the gels.

2.5. Peptide profiling

The pH 4.6-soluble fraction of milk, both from samples incubated without added plasmin and those incubated with exogenous plasmin for 24 h, was prepared by acidification, as described by Gai et al. (2025). The profiles of peptides in milk samples were determined using a reversed-phase ultra-performance liquid chromatography (RP-UPLC) H-Class Core System (Waters Corp, Milford, MA, USA), monitored by a Waters Acquity UPLC TUV Detector, operated by Empower 3 software (Waters Corp, Milford, MA, USA) (Gai et al., 2025).

Peptides from proteolysis were further analyzed using ultra-performance liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) with a Vanquish Flex UPLC coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) at Nestlé Research, Lausanne, Switzerland, as detailed by Gai et al. (2025). Analyses were conducted on pH 4.6-soluble fractions of milk before and after 7-day incubation, as well as after 24-h incubation with plasmin. Each sample was analyzed in triplicate. Peptide identification was performed using PEAKS Studio (Ma et al., 2003) and peptide distributions across four β-CN genotypes were compared, with a specific focus on β-CN-derived peptides and cleavage sites.

2.6. Statistical analyses

To determine if there were any differences between the four β-CN genotypes, statistical analyses were carried out on all the parameters measured. Prior to statistical analysis, all data sets were tested for normality using the Shapiro-Wilk test at a significance level, α, of 0.05.

Outliers were identified in data of acid coagulation properties using boxplots based on the interquartile range (IQR) method and were excluded from further statistical analysis. Data were analyzed using one-way repeated measures ANOVA, with the three sampling points treated as repeated measures. Each genotype included five measurements per sampling ($n = 5$). Tukey's range test was used to compare mean values between samples at a significance level, α, of 0.05. Experimental results are expressed as mean ± standard deviation. All statistical analyses were performed using SPSS (IBM, Version 26).

Fundamental parameters were compared between milk between A1A1, A1A2, A2A2, A1B, A2B, A1I, A2I using the same statistical method; the data used for parameters measured in A1A1, A1A2 and A2A2 milk were previously published by Gai et al. (2023).

3. Result and discussion

3.1. Rennet coagulation properties

Differences between β-CN genotypes in terms of milk protein and fat contents, as well as casein micelle size, fat globule size, PDI and zeta potential, were compared. There was no obvious correlation of either B or I genetic variant of β-CN with fat content and no significant differences were determined for milk protein content between all four genotypes ($p > 0.05$, data not shown). The B variant was associated with larger casein micelle size than the I variant, when they were heterozygous and paired with the same allele, either the A1 or A2 variant (i.e., casein micelle size in A1B, A2B, A1I and A2I milk were $172.55^{b} \pm 19.98$ nm, $159.12^{ab} \pm 15.57$ nm, $155.38^{a} \pm 7.03$ nm and $154.60^{a} \pm 11.39$ nm, respectively, $p < 0.05$). However, the relationship between β-CN allelic variants A1, A2, B and I and casein micelle size is unclear, as κ-CN genotype also influences casein micelle size (Di Gregorio et al., 2017).

No statistically significant differences in PDI, zeta potential or fat globule size were determined between milk of the four β-CN genotypes ($p > 0.05$, data not shown). Thus, any possible effects of such parameters on milk rennet coagulation properties can be ignored in this study.

The rennet coagulation profiles of raw milk samples with (a) three main β-CN genotypes (A1A1, A1A2 and A2A2), and (b) four minor β-CN genotypes (A1B, A2B, A1I and A2I) are shown in Fig. 2, plotted using the average values of individual measurements with the same genotype from three collection occasions. The profiles of three main genotypes were discussed in previously published study (Gai et al., 2023); although A2A2 milk had lower G' (gel firmness) throughout renneting compared to the other two genotypes, differences in RCT (min) and G′ at different time points between three genotypes were not significant ($p > 0.05$).

The four minor genotypes exhibited similar coagulation profiles.

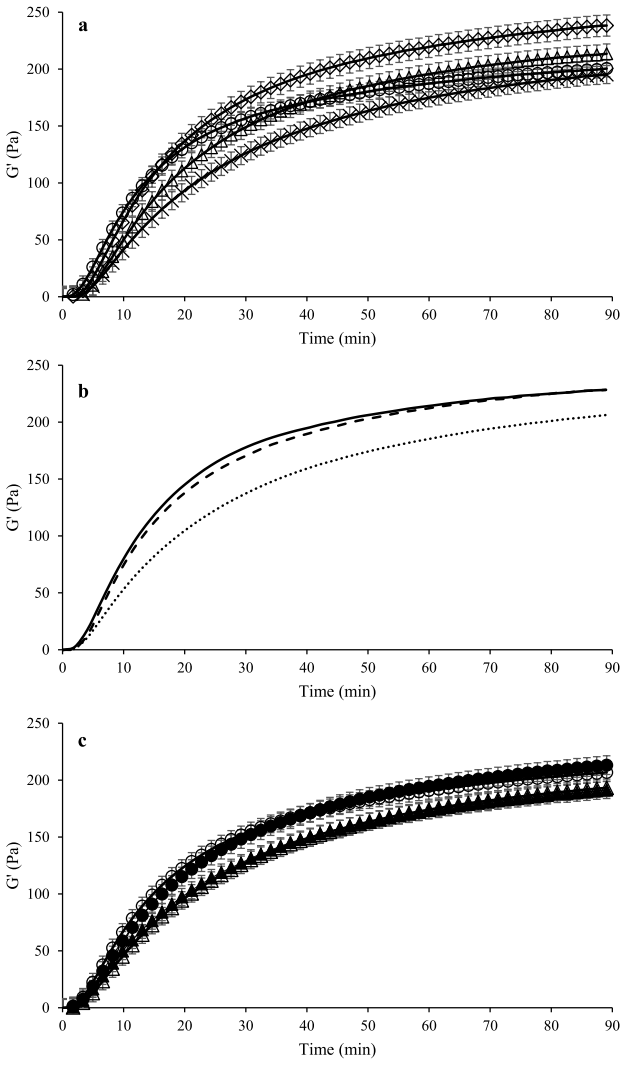


Fig. 2. Changes in storage modulus (G′) as a function of incubation time during rennet coagulation. Milk samples with four minor β-CN genotypes is presented in (a) as A1B (○), A2B (◇), A1I (△) and A2I (×). Data shown are average values of data from three collections, $n = 5$. Error bars represent standard error. Milk samples with three main β-CN genotypes are presented in (b) as A1A1 (continuous line), A1A2 (dashed line) and A2A2 (dotted line), which were replotted using data published in Gai et al. (2023). Comparison between A1A1 and "A1-type", and A2A2 and "A2-type" is shown in (c) as A1A1 (●), A2A2 (▲), A1A1+A1B (○) and A2A2+A2I (△). Data shown are from three collections, $n = 5$. Error bars represent standard error.

Among them, A1B milk showed the fastest increase in G′ during the first 1000 s, whereas A2B milk had the highest gel-strength development thereafter. The gel-firming rate (ΔG'), determined from coagulation profiles based on the average values of individual measurements (Fig. 2), was lowest for A2I (0.109), followed by A1I (0.135). In contrast, A1B and A2B milk displayed similar values. This finding is in line with the study of Poulsen et al. (2013), who reported shorter rennet coagulation time and higher curd firming rate associated with the B variant in comparison to the I variant of β-CN for milk from Danish Holstein and Jersey cows. Curd firmness in Simmental cow milk containing the B variant was higher than for the I variant (Bonfatti et al 2010b), which was not seen in the current study.

Although Bonfatti et al. (2010a, 2010b) suggested that the improved rennet coagulation properties of milk from the B variant may be attributed to its higher protein, fat, and calcium contents, the primary structure of β-CN also plays a role, no significant differences were determined in these parameters between genotypes in the current study. His_{67} and Arg_{122} are two positively charged residues, making the repulsion forces between micelles with the B variants weaker, due to lower negative charge (Vigolo et al., 2022), which may promote coagulation.

When comparing all the 7 genotypes, A1B milk had the shortest rennet coagulation time (2.02 ± 1.05 min) while A2A2 milk had the longest (3.52 ± 2.24 min); the gel firmness of A2B coagulum was the highest at different renneting times, and the gel firming rate of A1B was the highest, but the difference between milk with 7 β-CN genotypes was not significant ($p > 0.05$, data not shown).

As mentioned in Section 1, the B variant belongs to the 'A1-type', while the I variant falls under the 'A2-type'. Therefore, rennet coagulation profiles were compared among A1A1, A2A2, and their respective grouped types, A1-type and A2-type (Fig. 2c). The coagulation profiles within each pair were broadly similar; for example, A1A1 milk was broadly comparable to the 'A1-type' (A1A1 and A1B), as was A2A2 milk to the 'A2-type' (A2A2 and A2I).

In summary, the influence of β-CN genotype on milk rennet coagulation properties is not statistically significant, and minor genotypes B and I have similar rennet coagulation properties to the more common genotypes A1 and A2, respectively.

3.2. Acid coagulation properties

After deleting outlier data, the acid coagulation curves of A1I and A2I milk were below the other two types of milk (Fig. 3b), implying a lower gel firmness over coagulation. In comparison to acid coagulation profiles of A1A1, A1A2 and A2A2 milk (Fig. 3a–Gai et al., 2023), profiles of A1I and A2I milk were similar to those of A1A2 and A2A2 milk, respectively, and those of milk samples with β-CN A1 and B variant (A1A1, A1B and A2B) showed higher gel firmness compared to that of milk with the A2 and I variants (Fig. 3a and b). G′ values of the minor β-CN genotypes were all lower than those of A1A1 milk at all times; statistically significant differences were determined for G' (Pa) at 1 h between 7 genotypes ($p < 0.05$, the full set of data not shown), where those of A2I, A1I and A2A2 were the lowest, with the values being 9.83 ± 4.91, 11.45 ± 3.89, 11.17 ± 5.18, respectively, and those of A1A1 the highest, i.e., 23.90 ± 17.82 Pa. Regarding the gel firming rate (slope ΔG'), A2I (0.008) and A2A2 (0.009) were similar, and lower than the four genotypes A1I (0.011), A1A2 (0.012), A1B (0.012) and A2B (0.015), which had a similar value, while A1A1 milk had the highest firming rate (0.024), Fig. 3.

Although Ketto et al (2017) reported that weaker acid gels were associated with larger casein micelle size, this does not seem to be the case in this study, since the gel firmness in milk containing a β-CN B variant was higher than for the I variant, while its casein micelle sizes were larger. The better acid coagulation properties of the B variant are possibly due to the higher pI of this variant of β-casein (Jensen et al., 2012), which may have led to β-CN B coagulating at a higher pH

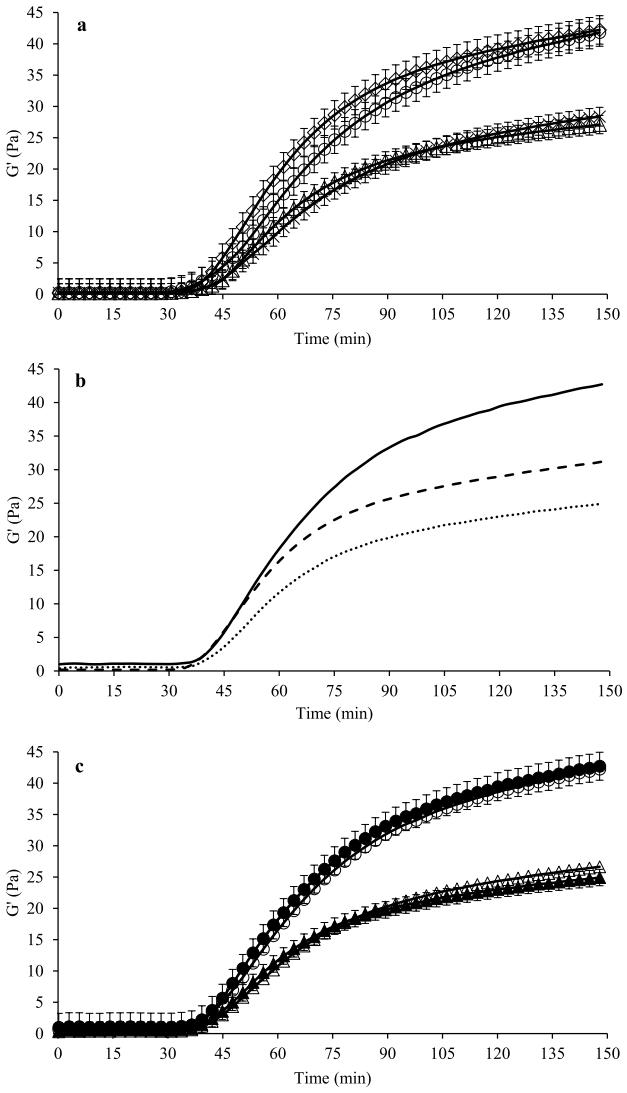


Fig. 3. Changes in storage modulus (G′) as a function of incubation time during acid coagulation. Milk samples with four minor β-CN genotypes is presented in (a) as A1B (○), A2B (⋄), A1I (△) and A2I (×). Data shown are average values of data from three collections, n = 5. Error bars represent standard error. Milk samples with three main β-CN genotypes are presented in (b) as A1A1 (continuous line), A1A2 (dashed line) and A2A2 (dotted line), which were replotted using data published in Gai et al. (2023). Comparison between A1A1 and "A1-type", and A2A2 and "A2-type" is shown in (c) as A1A1 (●), A2A2 (▲), A1A1+A1B (○) and A2A2+A2I (△). Data shown are from three collections, n = 5. Error bars represent standard error.

compared to the I variant.

As shown in Fig. 3c, the acid coagulation profile of 'A1-type' milk was similar to that of A1A1 milk, that of 'A2-type' milk was similar to A2A2 milk. Thus, in summary, the acid coagulation properties of milk with the β-CN B variant are similar to that of the A1 variant, while those of the I variant are similar to those of the A2 variant.

3.3. Susceptibility of casein to plasmin-derived proteolysis

In milk samples with different β-CN genotypes, the proteolysis of β-CN and α_{S1}-CN was not visible in urea-PAGE during incubation over 24 h without the addition of plasmin; proteolysis following the addition of plasmin was broadly similar in four milk samples (Fig. 4).

The B variant is different to the rest of variants included in this study

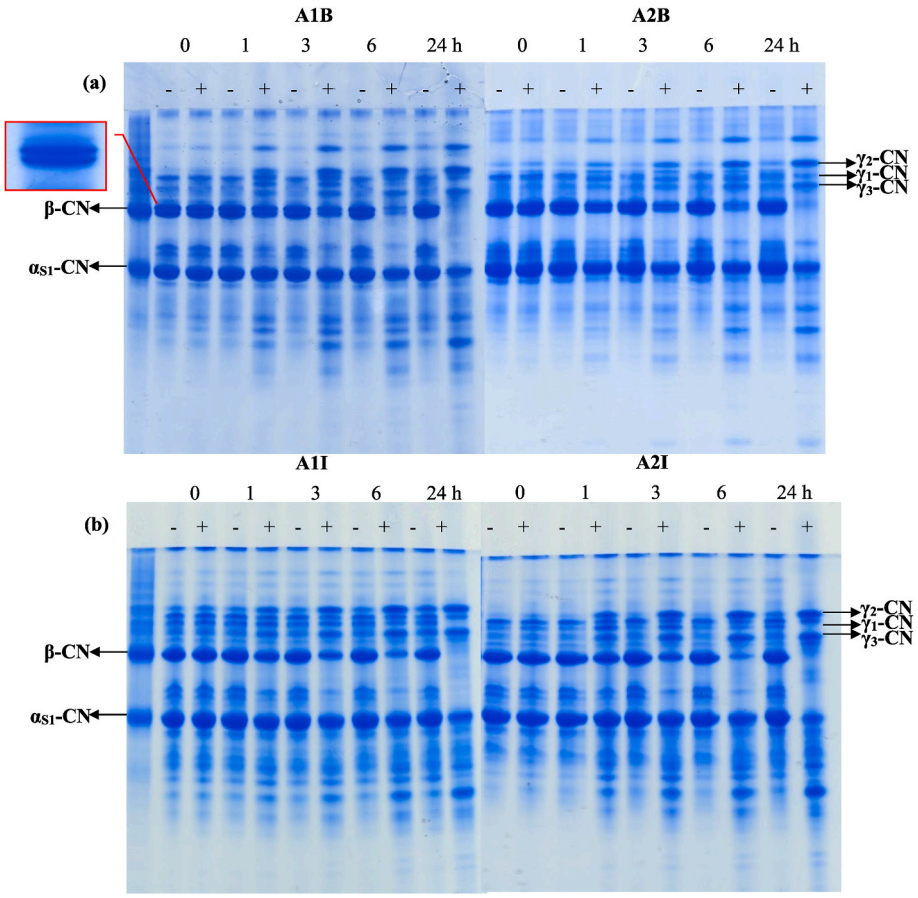


Fig. 4. Urea-PAGE electrophoretograms of pooled (a) A1B and A2B milk, (b) A1I and A2I milk during incubation at 37 °C without (−) or with plasmin for different time periods. A sodium caseinate standard is shown in the first Lane. The double β-CN band in milk containing β-CN B is highlighted.

(A1, A2 and I) at AA position 122, where there is an Arg instead of Ser, which was consistently noted to give a doublet of β-CN bands in urea-PAGE gels. Kemp et al, (1975) investigated the rate of phosphorylation of β-CN by SMP-dependent protein kinase, and reported that β-CN B was the most active substrate, with a 70-fold faster phosphorylation rate than A1, A2, A3 or C β-CN. The electrophoretic

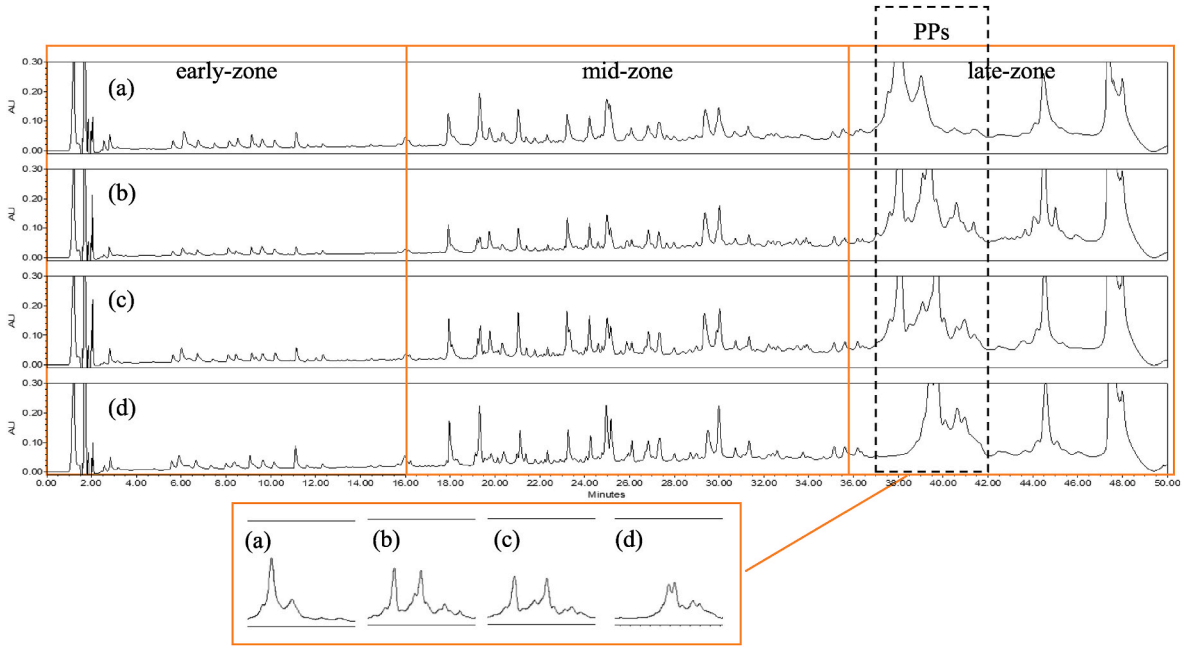


Fig. 5. UPLC chromatograms of pooled milk with β-CN (a) A1B, (b) A2B, (c) A1I, (d) A2I genotypes incubated at 37 °C with plasmin for 24 h. The proteose peptone zone is zoomed in to show the full-scaled peaks.

mobility of proteins in alkaline urea-PAGE gel is directly related to phosphorylation extent, as proteins separate according to differences in the net charge/mass ratios (Liu et al., 2019). Thus, the double band in urea-PAGE was probably due to differences in the degree of phosphorylation between the B variant and other genetic variants. However, Ser_{122} was not detected to be a phosphorylation site in peptide mapping obtained from mass spectrometry, and the reason for double β-CN bands requires further investigation.

3.4. Peptide profiling and identification

The distribution of peptides detected on UPLC profiles in incubated milk samples was divided into three groups by retention time, 0–16 min (early-zone), 16–36 min (mid-zone), and 36–50 min (late-zone), as described by Gai et al. (2025). Peptides eluting in the region 38–42 min, previously identified as proteose peptones (PPs) (Scollard et al., 2000), were also compared. Visually, peaks in 0–16 min were similar among the four genotypes (Fig. 5); in the region 16–36 min, peaks in the A2B milk had the lowest intensity, while those in the A2I milk had the highest intensity; the peak at 38 min, which was most intense in A1B, A2B and A1I milk, was not present in A2I milk (Fig. 5d). The region containing proteose peptones (PPs) at 37–42 min is zoomed in a small insert; a clear shift of PP peaks was observed between A1B (a) and A2I milk (d).

To compare the peptides derived from β-CN between two genetic variants, B and I only, peptide profiles of β-CN in A1B and A1I milk incubated for 24 h with plasmin were compared (Fig. 6); the ten most intense β-CN-derived peptide peak are labelled. In general, more peaks with lower areas were determined for β-CN I compared to B.

Among the ten peptides with the highest intensity, six were common, i.e., their peaks were found in both β-CN B and I variants (Tables 1–2).

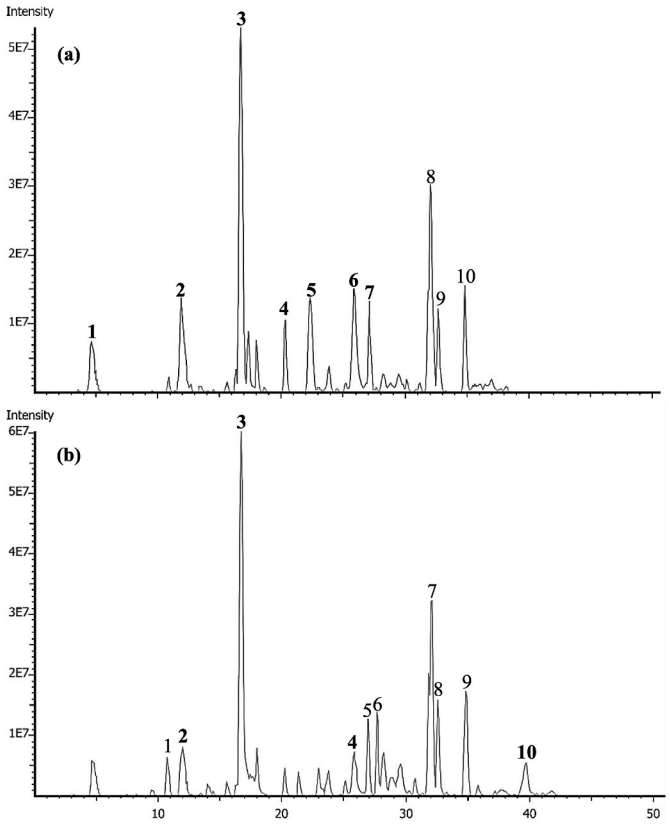


Fig. 6. Peptide mapping of β-CN-derived peptides from (a) β-CN B in A1B milk and (b) β-CN I A1I milk after 24-h incubation with plasmin. The x-axis is retention time, and the y-axis is peak intensity. Main peaks are labelled 1–10, and bold numbers indicate peptides relating to plasmin cleavage sites.

Considering the five principal cleavage sites of plasmin on β-CN, Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, Lys_{107}-Glu_{108}, Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184} (Fox et al., 1994), most of the peptides in β-CN B were related to plasmin-derived proteolysis (6 peptides out of 10), while only four peptides in β-CN I were related to plasmin-derived proteolysis.

The peptides in unincubated or incubated samples with different β-CN genetic variants were also compared in Tables 1 and 2, and three peptides (β-CN 106–113, 114–122, 177–190) were only determined in incubated milk. Two peptides seemed to be associated with the B variant only (β-CN 106–122, 108–122), and one peptide, β-CN 114–122, was only determined in incubated β-CN B, but not in the I variant. No peptides containing the 122nd AA as their C-terminal in β-CN I variant before or after 24 h incubation with plasmin were determined in this study (data not shown). This may be due to the difference on AA position 122 between the B and I variant, where the B has an Arg and I has a Ser (Martin et al., 2012). Thus, the Arg at position 122 might be more sensitive to the plasmin-driven proteolysis, since plasmin preferentially cleaves the C-terminal side of Arg residues (Visser, 1981). Petrat-Melin et al. (2015) investigated the effect of different primary structures on milk digestion patterns and reported that Arg_{122} of B β-CN introduced a novel trypsin cleavage site, leading to the absence of peptide β-CN (106–113) after digestion by pepsin and pancreatic enzymes. However, this peptide was detected in milk containing β-CN B after 7-d natural incubation and 24-h incubation with plasmin in this study.

Since the B variant of β-CN has a different AA profile at position 122 to all the other β-CN genotypes, comparisons between β-CN B variant and other genetic variants including A1, A2, and I were carried out using milk with main genotypes and minor genotypes (data not shown). Overall, more peptides were obtained by cleaving Arg_{122}-related sites, i.e., Glu_{121}-Arg_{122} and Arg_{122}-Gln_{123} from β-CN B, compared to Ser_{122} in other genotypes. This finding emphasized that the B variant behaves differently to A1 variant, as its urea-PAGE pattern showed double bands, and their peptide profiles were significantly different. In this case, A1B milk cannot be simply grouped as 'A1-type' milk in terms of proteolysis, which may have significance for, for example, proteolysis during cheese ripening.

4. Conclusions

There were no significant differences in milk composition between the B and I variants of β-CN; however, the B variant was associated with larger casein micelles, lower rennet coagulation time and firmer acid gels compared to the I variant. The remaining parameters, including protein content, rennet curd firmness, acid coagulation time, were not significantly different among the four milk types, A1B, A1I, A2B and A2I or, between the B and I genetic variants. Rennet coagulation profiles across all seven genotypes (including A1A1, A1A2, A2A2) were broadly similar, with the minor B and I variants showing coagulation properties comparable to the main A1 and A2 genotypes. Plasmin-derived proteolysis was similar across the four milk types, but the bond around Arg_{122} in the B variant appeared more susceptible to hydrolysis than that with Ser_{122} in the I variant, resulting in more peptides obtained by cleaving Arg_{122}-related sites. The double β-CN band unique to the B variant further highlighted its distinctiveness. For applications requiring specific coagulation properties, such as cheese or yogurt production, B and I variants can be grouped into 'A1-type' and 'A2-type,' respectively. Further investigation should explore the origin of the double β-CN bands in the B variant, and perhaps investigate bioactive peptides released from different genetic variants.

CRediT authorship contribution statement

Nan Gai: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Therese Uniacke-Lowe:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition,

Table 1
Principal β-casein-derived peptides in A1B milk before and after incubation at 37 °C for 24 h with plasmin[a].

No. peak	AA sequence[a]	Peptide	Before incubation				24 h incubation + p[3]			
			A1B	A1I	A2B	A2I	A1B	A1I	A2B	A2I
1	VKEAMAPK	f98-105					*	*	*	*
2	HKEMPFPK	f106-113					*	*	*	*
3	KIEKF … ELQDK	f29-48	*	*	*	*	*	*	*	*
4	YPVEPFTER	f114-122					*		*	
5	HKEMP … PFTER	f106-122	*		*		*		*	
6	NVPGE … TRINK	f7-28	*	*	*	*	*	*	*	*
7	EMPFP … PFTER	f108-122			*		*		*	
8	YQEPV … GPFPI	f193-207	*	*	*	*	*	*	*	*
9	AVPYP … IQAFL	f177-191	*	*	*		*	*	*	*
10	YQEPV … PFPII	f193-208	*	*	*	*	*	*	*	*

[2]+p represents the incubation with added plasmin.
[a] Presence of the listed principal peptides in other milk samples (*).

Table 2
Principal β-casein-derived peptides in A1I milk before and after incubation at 37 °C for 24 h with or without plasmin[a].

No. peak	AA sequence	peptide	Before incubation				24 h incubation + p[2]			
			A1B	A1I	A2B	A2I	A1B	A1I	A2B	A2I
1	VKEAMAP	f98-104	*				*	*	*	*
2	HKEMPFPK	f106-113					*	*	*	*
3	KIEKF … ELQDK	f29-48	*	*	*	*	*	*	*	*
4	NVPGE … TRINK	f7-28	*	*	*	*	*	*	*	*
5	AVPYP … PIQAF	f177-190					*	*	*	*
6	VLPVP … PIQAF	f170-190		*	*	*	*	*	*	*
7	YQEPV … GPFPI	f193-207	*	*	*	*	*	*	*	*
8	AVPYP … IQAFL	f177-191	*	*	*		*	*	*	*
9	YQEPV … PFPII	f193-208	*	*	*	*	*	*	*	*
10	VYPFP … AMAPK	f59-105	*	*	*	*	*	*	*	*

[2]+p represents the incubation with additional plasmin.
[a] Presence of the listed principal peptides in other milk samples (*).

Conceptualization. **David A. Goulding:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jonathan O'Regan:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Michael Affolter:** Writing – review & editing, Methodology, Data curation. **Alan L. Kelly:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2025.106352.

Data availability

Data will be made available on request.

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