Influence of β-CN Genotype on Physicochemical Properties and Functionality of Bovine Milk

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ABSTRACT

Several studies have been focused on the effect of milk protein genetic variants on milk physicochemical properties and functionality in recent years. β-casein, an important protein related to milk processibility, has been reported to have 2 main genetic variants A1 and A2, for which cows may be homozygous or heterozygous. In this study, several physicochemical properties of milk with β-casein variants A1A1, A1A2 and A2A2 from 3 collection occasions were analyzed. Higher manganese content and lower pH were found to be associated with the A1A1 variant compared with the other 2 genotypes. Better rennet and acid coagulation were found in A1A1 milk compared with A1A2 and A2A2 milk (although \( P > 0.05 \)), while A2A2 milk was more stable to creaming compared with the other 2 genotypes, which may be linked to its smaller fat globule size. Thus, milk from cows with A1A1 genotype could be preferable for cheese making, while that with A2A2 variant can be used in formulations requiring good stability against creaming, and for, e.g., yogurt making, where the softer yogurt texture may be easier to digest.

Key words: Milk, β-casein, genotype, physicochemical properties, functionality, processing properties

Interpretative summary: Proteins in milk are expressed as different genetic variants, which may be linked to milk physico-chemical properties and functional properties. This study focused on the effect of β-CN genotypes on milk composition, coagulation properties, heat stability and creaming properties. The findings of how β-CN genotypes may affect these properties can be applied in specific milk selection for optimal dairy product processing in industry.

INTRODUCTION

Bovine milk contains 3.5% protein (Miller et al., 2006), which comprises about 80% whey protein and 20% casein (Hoffman and Falvo, 2004). These proteins have been found to be expressed in different genetic variants, and these variants influence milk yield, composition, and milk processability (Hallén et al., 2007; Bonfatti et al., 2010). To improve milk quality for processing, the selection of certain milk protein genotypes is regarded as an increasingly important consideration.

β-casein plays an important role in the structure of casein micelles, and its genetic variants are linked to fat percentage (Hanusová et al., 2009), fat and protein yields (Heck et al., 2009), as well as cheese yield and quality (Massella et al., 2017). Twelve genetic variants of β-CN were listed by Farrell et al. (2004), including A1, A2, A3, B, C, D, E, F, G, H1, H2 and I. Of these variants, A1, A2, A3, B and C are the most frequent alleles of β-CN (Jann et al., 2002), and the A2 variant has been reported to be the most ancient allele in the Bos genus (Caroli et al., 2016). Since the A2 milk market has been commercially successful in some parts of the world, e.g., Australia, (Fernández-Rico et al., 2022), studies on possible effect of β-CN genotypes on milk physicochemical properties and functionality are necessary to identify issues that might arise if farmers select their herds on the basis of genotype that is A2-dominant.

An influence of β-CN A1 and A2 variants on milk processability has, however, been noted by several researchers, i.e., milk of genotype A1 is more favorable than A2 for rennet coagulation (Ketto et al., 2017; Gai et al., 2021; Vigolo et al., 2021). Daniloski et al. (2022a) reported poor acid gelation properties associated with milk containing the A2A2 β-CN, while better emulsifying properties are found in sodium caseinate obtained from the A2A2 milk (Daniloski et al. 2022b).

In the current study, the effects of A1A1, A1A2 and A2A2 β-CN variants on physicochemical properties and functional properties of milk were investigated. If dif-
ferences are identified, dairy processors may in the future select milk with specific β-CN genotype for specific applications.

MATERIALS AND METHODS

Milk Samples

Whole bovine milk was obtained from Teagasc Food Research Centre, Moorepark (Cork, Ireland) on 3 separate occasions from the same cows. All the milk samples were from spring-calving 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 (HPLC-HRMS) (Fuerer et al., 2020). On each collection, milk samples were obtained from 15 cows, 5 milk samples had the A1A1 variant of β-CN, 5 samples were the A1A2 variant, and 5 were the A2A2 variant. Each individual fresh milk sample was divided in 2, and 0.05% sodium azide was added to one to inhibit the growth of microorganisms for longer preservation and stored at 4°C; this portion was used for gross composition, freezing point, pH, ionic concentration, casein micelle size and polydispersity index (PDI), zeta potential and fat globule size determination, as well as for rennet coagulation, acid coagulation and heat stability analyses; the other portion was frozen at −80°C and, after slow thawing at 4°C, was used for mineral profiling and determination of creaming properties. Preliminary work (data not shown) has indicated that such treatments do not affect the creaming properties of milk.

Gross composition, Freezing point, and pH

Gross composition of freshly collected milk samples was determined using a MilkoScan™ Mars (Foss, Hillerød, Denmark), carried out on each individual milk sample. Parameters measured include fat content (%), protein content (%), lactose content (%), solids-nonfat (SNF) (%), total solids (TS) (%) and freezing point (°C) (Tarazona Manrique eMVZ et al., 2019). A pH meter (Mettler Toledo, Switzerland) was used to determine milk pH. Milk samples were carefully invered a few times before measurement to disperse fat.

Mineral Content and Ionic Calcium Concentration determination

Twenty-five mls of each milk sample from 3 collections were used for mineral profiling using inductively coupled plasma optical emission spectroscopy (ICP-OES) using a modification of the method of Cunniff and Washington (1997). Ionic calcium concentration in milk samples was determined using a Titrando 907 (Metrohm Ireland Ltd., Carlow, Ireland) autotitrator with a calcium ion probe, operated using Tiamo v2.2 software. The ionic calcium concentration was calculated using a modification of the method of Crowley et al. (2014), using a standard curve with a set of 1, 2, 3, 4, 5 mmol/L calcium solutions prepared with 100 mM CaCl₂ and 67.5 mM KCl buffer, for determining ionic calcium concentration in milk samples, and all measurements were carried out at 25°C.

Casein Micelle Size, PDI and Zeta Potential determination

Raw milk samples were skimmed individually by centrifugation at 3500 rpm for 30 min at 4°C, followed by filtration through glass wool to remove fat, and diluted 1:100 using ultrapure water (18.2 MΩ.cm).

The mean diameter of casein micelles, PDI, and zeta potential of each skim milk (as described above) were determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The measurement was carried out at 25°C, with a scattering angle of 173° (Visentin et al., 2015) and each measurement had 3 automatic readings. Refractive index for protein was set to 1.45, with absorption coefficient of 0.001; for water, as dispersant, the refractive index was set to 1.33, viscosity was 0.8871 cP, and dielectric constant was 78.5. Sample cells used for casein micelle size and PDI determination were Malvern ZEN0040 disposable cuvettes. A Malvern DTS1070 disposable folded capillary tube was used for zeta potential measurement. These analyses were carried out in triplicate.

Fat globule size distribution

Fat globule size distribution of individual raw milk samples was determined using a Mastersizer 3000 (Malvern Instruments, Malvern, UK), equipped with a He-Ne Laser (λ = 633nm). The refractive index at 25°C was taken as 1.45, the absorption index was 0.001 and density was 1.33 (Di Marzo et al., 2016). Each measurement has 3 automatic readings. Surface volume mean diameter D [3,2], volume-weighed mean diameter D [4,3] and particle size distribution of diameters below which lie 10% (Dv10), 50% (Dv50) and 90% (Dv90) of the globule volume were recorded (Ménard et al., 2010). These analyses were carried out in triplicate.
**Rennet Coagulation properties**

The pH of raw milk samples was adjusted to 6.6 using 1 M HCl, and the milk samples were preheated to 32°C before mixing 100:1 with chymosin (Maxiren 180, 180 international milk-clotting units (IMCU, International Dairy Federation, 2007) per ml, DSM Food Specialties, Delft, the Netherlands; diluted 1:10 with de-ionized water). The Berridge method (Berridge, 1952) was used to determine rennet coagulation time (RCT) with modifications, where 2 mLs of milk was observed in thin-walled glass tubes. After adding diluted chymosin, tubes were gently rocked on a rack in a waterbath at 32°C and the time from addition of enzyme to the appearance of visual small flocs was recorded as milk RCT (Berridge, 1952; Klandar et al., 2007).

Milk rennet coagulation time (RCT) was also determined using a Discovery Series Hybrid Rheometer (TA Instruments, Waters Corp., New Castle, Delaware, US), as well as storage modulus (G'), which indicates gel strength, as described by O’Connell et al. (2006) with modifications. The change of G' over a specific period, which was determined as described by Steffl et al. (1999), was used to evaluate gel firming rate, and data are presented as slope, ΔG'. Raw milk samples were prepared as above and 250 μL diluted chymosin, which was prepared as above, was added to 25 mL of preheated milk, the mix was stirred rapidly for 10 s using a spatula before the oscillation analysis. Oscillation was carried out at 32°C for 90 min, with a strain of 0.1% and angular frequency 6.28319 rad/s. The equilibration duration was 60 s, and G' was recorded continuously over 90 min. These analyses were carried out once on individual milk samples from 3 collections.

**Acid Coagulation properties**

Acid coagulation time (ACT) and acid gel strength were determined using an AR-G2 rheometer (TA Instruments, Waters Corp., New Castle, Delaware, US), as described by O’Connell et al. (2006) with modifications. The pH of 25 mLs raw milk was adjusted to 6.6 using 1 M HCl and pre-heated 32°C before mixing with 0.75 g glucono delta lactone (GDL), which was used to decrease milk pH gradually (Eshpari et al., 2014). The storage modulus (G') was determined to indicate the gel strength. The change of G' over time was calculated by the slope of acid coagulation profile, presented as ΔG', which represents the gel firming rate. Milk with added GDL was stirred rapidly for 10 s before oscillatory analysis. Analysis was undertaken at 32°C for 2.5 h, at 0.1% strain and an angular frequency of 6.28319 rad/s.

Changes in milk pH during acidification was recorded in parallel in a waterbath at 32°C, where the pH was recorded every 5 min after the addition of GDL until pH 4.6 was reached, and the relationship between pH and G’ as a function of time was plotted. The analyses were carried out once on an individual milk sample from 2 of the 3 collections (i.e., 10 measurements in total per genotype).

**Heat Stability**

Raw milk samples were adjusted to pH 6.4, 6.6, 6.8, 7.0, 7.2, 7.4 using 1 M HCl or 1 M NaOH, and were kept at 4°C overnight. The pH was checked again before analysis to ensure accuracy. Thermostable glass tubes with silicone seals were used for heat stability determination. Tubes were clipped to a rack and placed in a 140-degree oil bath (Singh, 2004); each glass tube contained 2.5 mLs of milk samples. The rack was rocked continuously, and the heat coagulation time (HCT) was recorded, which is regarded as time spent until visible flocs are observed in the glass tubes (Singh, 2004). A HCT-pH profile was then plotted, showing milk heat stability as a function of pH. The analyses were carried out once on an individual milk sample from 2 of the 3 collections.

**Creaming Properties**

Raw milk creaming properties were analyzed as described by Meng et al. (2022) using a TurbiScan LAB (Formulation, Ramonville St. Agne, France) with modifications. Each frozen raw milk sample was thawed overnight at 4°C and mixed invertedly before analysis. The migration of milk fat was captured by the measurement of backscattering (BS) signal change, which was carried out at 4°C for 24 h. Turbiscan Stability index (TSI) of the whole samples (TSI global) and the cream layer of milk (TSI cream) were recorded as a function of time as an indication of milk stability, as it reflects the extent of milk destabilization (Chen and Sagis, 2019), and higher TSI indicates lower milk stability. The mean value [(backscattering)%, ΔBS] and the peak thickness (ΔH) were also used to describe the creaming, which represent the changes in concentration and particle size and the depth of the cream layer (Meng et al., 2020). The analyses were carried out once on each milk sample from 2 of the 3 collections.

**Statistical analysis**

To determine if there were any differences between 3 β-CN genotypes, statistical analyses were carried out on all the parameters measured, as mentioned above.
Before statistical analysis, all data sets were tested for normality using the Shapiro-Wilk test at a significance level, \( \alpha \), of 0.05. A 2-way ANOVA was conducted that examined the effect of genotype and collection occasion on all the analyzed parameters. There was no interaction between the effects of genotype and collection occasion \(( P > 0.05)\) and, in this case, the effect of genotype on all the parameters can be analyzed independently.

Data was processed by one-way repeated measurement, considering the number of collections as replicate samplings. Tukey’s range test was used to compare mean values between samples at a significance level, \( \alpha \), of 0.05. Experimental results are expressed as mean ± standard deviation.

All statistical analyses were performed using SPSS (IBM, Version 26).

**RESULTS**

### Milk Composition, Mineral Profile and pH

Statistically significant differences in the majority of milk composition analysis were not found between the 3 genotypes \(( P > 0.05)\), as shown in Table 1. The pH value of A1A2 milk was the highest, while that of A2A2 was the lowest \(( P < 0.05)\) among the milk samples containing 3 different \( \beta \)-CN genotypes. The manganese content was found to be significantly different between genotypes \(( P < 0.05, \text{Table 2})\); none of the other mineral concentrations were significantly different.

### Casein Micelle Size, PDI, Zeta Potential and Fat Globule Size

No statistically significant differences in casein micelle size, PDI or zeta potential were found between genotypes \(( P > 0.05, \text{Table 3})\). Statistically significant differences in fat globule size were found between the 3 \( \beta \)-CN genotypes for D \([3,2]\) \(( P < 0.05, \text{Table 3})\), where A1A2 > A1A1 > A2A2. The fat globule size distribution, Dv90, was also found to be different in milk with different genotypes, where A1A1 > A1A2 > A2A2 \(( P < 0.05, \text{Table 3})\).

### Rennet Coagulation properties

Figure 1 shows the rennet coagulation profiles of raw milk samples with 3 \( \beta \)-CN genotypes from 3 collections. Milk of variant A2A2 had the poorest performance compared with the other 2 variants, as it coagulated more slowly (longer RCT), and gel firmness was lower (lower \( G' \)) throughout renneting, but these differences were not statistically significant \(( P > 0.05, \text{Table 4})\). The slopes of the plot of \( \Delta G' \) versus time, which indicates the gel-firming rate, were in the order A2A2 < A1A1 < A1A2 (Table 4).

### Acid Coagulation properties

Statistically significant differences were found between the 3 genotypes on \( G' \) values at 1 h \(( P < 0.05, \text{Table 5})\), A1A1 > A1A2 > A2A2; and the acid coagulation profile of A1A1 milk was always above that of the other 2 genotypes, i.e., the acid gel formed by A1A1 was the lowest \(( P < 0.05)\) among the milk samples containing 3 different \( \beta \)-CN genotypes. The manganese content was found to be significantly different between genotypes \(( P < 0.05, \text{Table 2})\); none of the other mineral concentrations were significantly different.

### Table 1. Composition and pH of milk with three \( \beta \)-CN genotypes sampled on three different occasions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A1A1 (n = 5)</th>
<th>A1A2 (n = 5)</th>
<th>A2A2 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.70 ± 0.07</td>
<td>6.76 ± 0.05</td>
<td>6.72 ± 0.06</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>5.31 ± 1.87</td>
<td>5.26 ± 1.54</td>
<td>5.24 ± 1.50</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>3.58 ± 0.40</td>
<td>3.56 ± 0.57</td>
<td>3.56 ± 0.29</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>4.50 ± 0.22</td>
<td>4.40 ± 0.19</td>
<td>4.39 ± 0.35</td>
</tr>
<tr>
<td>SNF (%)</td>
<td>8.91 ± 0.49</td>
<td>8.66 ± 0.49</td>
<td>8.64 ± 0.45</td>
</tr>
<tr>
<td>TS (%)</td>
<td>14.25 ± 2.21</td>
<td>13.97 ± 1.84</td>
<td>13.93 ± 1.56</td>
</tr>
<tr>
<td>Freezing point (°C)</td>
<td>−0.49 ± 0.03</td>
<td>−0.49 ± 0.02</td>
<td>−0.50 ± 0.03</td>
</tr>
</tbody>
</table>

\( ^a-b \)Means within a row with different superscripts differ \(( P < 0.05)\).

1Values were compared between three \( \beta \)-CN genotypes.

\( \text{Table 2. Mineral contents (mg/100 g) of milk with three \( \beta \)-CN genotypes}^{1,2,3} \)

<table>
<thead>
<tr>
<th>Mineral</th>
<th>A1A1 (n = 5)</th>
<th>A1A2 (n = 5)</th>
<th>A2A2 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>128.09 ± 18.21</td>
<td>121.86 ± 14.03</td>
<td>126.71 ± 14.22</td>
</tr>
<tr>
<td>Magnesium</td>
<td>11.07 ± 1.12</td>
<td>10.66 ± 1.61</td>
<td>11.39 ± 1.44</td>
</tr>
<tr>
<td>Manganese (μg/100g)</td>
<td>5.26( ^a-b ) ± 3.05</td>
<td>3.45( ^a-b ) ± 1.22</td>
<td>3.31( ^a ) ± 0.93</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>106.72 ± 21.94</td>
<td>103.95 ± 14.69</td>
<td>106.77 ± 17.87</td>
</tr>
<tr>
<td>Potassium</td>
<td>146.40 ± 15.09</td>
<td>144.64 ± 17.51</td>
<td>153.64 ± 15.95</td>
</tr>
<tr>
<td>Sodium</td>
<td>38.55 ± 14.03</td>
<td>46.96 ± 12.88</td>
<td>48.03 ± 14.28</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.40 ± 0.08</td>
<td>0.29 ± 0.07</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>Ionic calcium (mmol•L(^{-1}))</td>
<td>2.45 ± 0.49</td>
<td>2.51 ± 0.44</td>
<td>2.49 ± 0.51</td>
</tr>
</tbody>
</table>

\( ^a-b \)Means within a row with different superscripts differ \(( P < 0.05)\).

1Values were compared between three \( \beta \)-CN genotypes.

2Mean ± SD.

3Unit for mineral content is (mg/100 g), unless specified.
milk was stronger than for the other 2 genotypes. The gel firming rate (slope \( \Delta G' \)) was in the order \( A_{1A1} > A_{1A2} > A_{2A2} \).

The pH of milk with \( A_{1A1} \) variant was the slowest to drop to 4.6, while \( A_{1A2} \) was the fastest, and \( A_{2A2} \) was in between, suggesting a better resistance to pH change, which can be concluded to be a higher buffering capacity, of milk with the \( A_{1A1} \) variant, compared with \( A_{1A2} \) and \( A_{2A2} \) (Figure 3).

**Heat Stability**

Heat coagulation profiles of milk with 3 \( \beta-CN \) genotypes are shown in Figure 5; the heat stability of \( A_{2A2} \) milk was always the lowest, except at pH 6.8, while

<table>
<thead>
<tr>
<th></th>
<th>( A_{1A1} (n = 5) )</th>
<th>( A_{1A2} (n = 5) )</th>
<th>( A_{2A2} (n = 5) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCT_B3 (min)</td>
<td>3.61 ± 0.69</td>
<td>3.67 ± 1.42</td>
<td>4.04 ± 1.14</td>
</tr>
<tr>
<td>RCT_R4 (min)</td>
<td>2.42 ± 1.05</td>
<td>2.42 ± 1.84</td>
<td>3.52 ± 2.24</td>
</tr>
<tr>
<td>( G'_{2hr} ) (Pa)</td>
<td>161.08 ± 72.60</td>
<td>157.89 ± 77.96</td>
<td>132.53 ± 81.40</td>
</tr>
<tr>
<td>( G'_{7hr} ) (Pa)</td>
<td>204.50 ± 75.78</td>
<td>189.22 ± 94.56</td>
<td>177.52 ± 82.48</td>
</tr>
<tr>
<td>( G'_{9hr} ) (Pa)</td>
<td>225.36 ± 80.26</td>
<td>205.60 ± 101.28</td>
<td>199.92 ± 75.91</td>
</tr>
<tr>
<td>Slope ( \Delta G' )</td>
<td>0.124</td>
<td>0.157</td>
<td>0.115</td>
</tr>
</tbody>
</table>

1Values were compared between three \( \beta-CN \) genotypes.
2Mean ± SD.
3RCT measured using the Berridge method.
4RCT measured using a rheometer.

**Table 4.** Rennet coagulation properties of milk with three \( \beta-CN \) genotypes from three collections

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**Figure 2.** Storage modulus (G') change as a function of incubation time during acid coagulation. Milk with 3 \( \beta-CN \) genotypes is presented as, \( A_{1A1} (●) \), \( A_{1A2} (▲) \), \( A_{2A2} (♦) \). Data shown are average values of data from 2 collections. Error bars represent standard deviation.
A1A2 milk was the most stable at alkaline pH. No significant differences were found on heat coagulation time at different pH values between genotypes (\( P > 0.05 \)).

**Creaming Properties**

Creaming properties of milk samples were evaluated using the TSI (global) value (Table 6), TSI\(_{\text{cream}}\), \( \Delta \text{BS} \) (%) and \( \Delta \text{H} \) (mm) profiles (Figure 5). The profiles of TSI\(_{\text{cream}}\) and \( \Delta \text{H} \) (mm) of A2A2 milk were always below the other 2 milk samples, the difference between \( \Delta \text{BS} \) (%) was not significant. The TSI (global) of A2A2 milk was always the lowest among the 3 genotypes after 24 h, and statistically significant differences were found at 4, 8, 12 and 20 h (\( P < 0.05 \), Table 6), which indicated that milk containing the A2 \( \beta \)-CN variant was the most stable to creaming.

**DISCUSSION**

Differences in physicochemical properties and functional properties, including pH, some mineral contents, zeta potential, fat globule size, rennet coagulation properties, acid coagulation properties and creaming properties were found between milk with 3 \( \beta \)-CN genotypes A1A1, A1A2 and A2A2. In general, A1A1 milk was associated with the lowest natural pH values (\( P < 0.05 \)), the highest manganese content (\( P < 0.05 \)), as well as the lowest stability to creaming (\( P < 0.05 \)). Milk with the A1A2 variant was found to have the highest initial pH (\( P < 0.05 \)). A2A2 milk was found to have the smallest fat globules (\( P < 0.05 \)) and a better creaming stability compared with the other 2 genotypes (\( P < 0.05 \)). A1A1 milk was associated with better rennet coagulation properties and acid coagulation properties, while the gel firmness was much stronger than the other 2 genotypes, although the differences were not statistically significant (\( P > 0.05 \)).

Composition and casein micelle size of milk samples with 3 different genotypes were not statistically significantly different (\( P > 0.05 \)), which indicated that the differences on functional properties, including rennet coagulation, acid coagulation and creaming properties, were most likely not due to these parameters in this study, but are possibly affected by \( \beta \)-CN structural differences between the variant A1 and A2, or milk fat globule size, as discussed below.

Our findings for milk composition were consistent with what was reported by Devold et al. (2000) and Hanusová et al. (2011), where no effects of \( \beta \)-CN genotypes were found for fat and protein levels in milk. Cardak (2005) reported no effect of \( \beta \)-CN genotypes on fat content in Holstein cows, whereas the A1A1 milk was associated with higher fat and protein content in comparison to the A1A2 and A2A2 milk in Simmental cows.

### Table 6. Turbiscan Stability Index (global) of milk samples with three \( \beta \)-CN genotypes\(^{1,2}\)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>A1A1 (n = 5)</th>
<th>A1A2 (n = 5)</th>
<th>A2A2 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>3.46 ± 2.52</td>
<td>2.05 ± 2.02</td>
<td>2.64 ± 3.32</td>
</tr>
<tr>
<td>2</td>
<td>6.72 ± 2.64</td>
<td>5.19 ± 1.47</td>
<td>4.69 ± 3.08</td>
</tr>
<tr>
<td>4</td>
<td>9.69 ± 2.96</td>
<td>8.75 ± 2.43</td>
<td>6.80 ± 3.42</td>
</tr>
<tr>
<td>8</td>
<td>12.82 ± 3.01</td>
<td>12.87 ± 3.47</td>
<td>9.23 ± 3.54</td>
</tr>
<tr>
<td>12</td>
<td>14.81 ± 3.07</td>
<td>15.67 ± 3.97</td>
<td>11.02 ± 3.55</td>
</tr>
<tr>
<td>20</td>
<td>18.86 ± 3.87</td>
<td>19.73 ± 4.43</td>
<td>16.04 ± 3.37</td>
</tr>
<tr>
<td>24</td>
<td>20.75 ± 4.87</td>
<td>20.97 ± 4.71</td>
<td>17.29 ± 3.54</td>
</tr>
</tbody>
</table>

\(^{1}\)Means within a row with different superscripts differ (\( P < 0.05 \)).

\(^{2}\)Values were compared between three \( \beta \)-CN genotypes.

\(^{3}\)Mean ± SD.
cows. However, Puhan (1997) reported that the A1A1 variant is associated with higher protein content compared with the other 2 variants, while an association between β-CN A2A2 and higher protein and fat content was reported by Caroli et al. (2009). The pH of milk can be affected by several factors, such as lactic acid formation in milk, temperature (Walstra, 1999), season, protein and fat content (Chen et al., 2014), feeding, stage of lactation (Millogo et al., 2010). Its variation can also be due to milk buffering capacity, which is affected by mineral distribution between aqueous and solid phases (Salaün et al., 2005). As temperature and cow feeding and collection conditions were controlled, and no differences were found on milk composition, the pH variation may be linked to the buffering capacity in this study. However, the pH values lay in normal fresh milk pH range (6.6–6.8), and for all the analyses related to pH, i.e., coagulation, the pH of each individual milk was adjusted to the same value.

The salt contents of milk, including calcium, magnesium and inorganic phosphorus, are known to influence rennet coagulation properties (Frederiksen et al., 2011; Vigolo et al., 2022). It was also reported by Frederiksen et al. (2011) that the potassium content has a negative effect on rennet coagulation time, while ionic calcium content is positively correlated with rennet coagulation properties (Glantz et al., 2011), with higher ionic calcium content being linked to shorter RCT and firmer gels. In our study, these parameters did not significantly differ (P > 0.05) between the 3 β-CN genotypes, thus, the differences in their rennet coagulation properties are considered to be due to other factors, i.e., concentration and genotype of other proteins, especially κ-CN, which highly impacts milk coagulation properties (Bisutti et al., 2022). In addition, although the manganese content was found to be significantly different between the 3 genotypes (P < 0.05), this may be due to other factors, i.e., other sources of variation between cows, as only 10% of total Mn binds to casein (Pechová, et al., 2008).

Casein micelle size in bovine milk from individual cows varies between 154 and 230 nm (de Kruif and Huppertz, 2012), and the results of the present study lie within this range. In line with the results reported by Ketto et al. (2017) and Freitas et al. (2017), β-CN genotypes were found to have no effect on casein micelle size. However, several other studies reported a correlation between casein micelle size and β-CN genotypes. Day et al. (2015) and Daniloski et al. (2022a) found that milk with β-CN A2A2 variant showed larger casein micelles, while Devold et al. (2000) found that casein micelle sizes in milk were in the order of A2A2 < A1A2 < A1A1, although the differences were not statistically significant (P > 0.05).

Zeta potential is an indicator of the stability of a colloidal dispersion, and the lower the absolute value is, the less stable the colloidal dispersion (Hanaor et al., 2012). A different trend to this study (A1A2 > A2A2 > A1A1, Table 3) was reported by Daniloski et al. (2022a), who studied reconstituted milk powder, for which the zeta potential of A1A1 milk was the highest, while that of A2A2 was the lowest.

The average fat globule size in the A2A2 milk was slightly smaller than that of the other 2 genotypes, and its creaming rate was the slowest. According to Stoke’s law, as larger fat globules have a larger diameter, and large fat globules have a smaller fat membrane to fat

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**Figure 5.** Turbiscan stability index (TSI<sub>crem</sub>), mean value (ΔBS) and peak thickness profile of milk with 3 β-CN genotypes. Milk with 3 β-CN genotypes is presented as, A1A1 (●), A1A2 (▲), A2A2 (♦). Data shown are typical individual milk samples representing the 3 β-CN genotypes. Error bars represent standard deviation.
volume ratio, resulting in lower density, their rising rate is higher than that of smaller fat globules (Mulder and Walstra, 1974; Walstra, 1995; Ma and Barbano, 2000), which may be linked to the faster creaming rate of A1A1 and A1A2 milk.

In previous studies, the A1 allele of β-CN was found to be favorable for cheese-making, while the A2 allele was associated with poor rennet coagulation (Hallén et al., 2007; Comin et al., 2008; Jensen et al., 2012; Bisutti et al., 2022), which is consistent with the results in the present study, but the difference was not significant. Better rennet coagulation properties of A1A1 milk were also reported by Glantz et al. (2010) and Logan et al. (2014). The non-significant differences on rennet coagulation properties between 3 β-CN genotypes in this study may be due to the large standard deviation, which may be affected by the rheometry method used.

In our study, milk with β-CN variant A2A2 had significantly smaller fat globule size compared with the other 2 genotypes (P < 0.05). Interactive effects of milk fat globule size and casein micelle size on rennet gel strength were reported by Logan et al. (2014), where rennet gels formed from milk with larger fat globules tend to be firmer than those with smaller globules, possibly due to more efficient fitting of large fat globules within pores of the casein micelle network (Logan et al., 2014). In contrast, Michalski et al. (2002) reported that G’ in rennet gels is higher when milk fat globule size is smaller.

Effects of genetic variants on rennet coagulation properties may also relate to chaperone activity, which has an inhibitory effect on milk coagulation (Holt et al., 2013) and is also affected by protein secondary structure (Raynes et al., 2015). β-CN A2 contains an additional proline residue compared with A1 and so more polyproline-II helix is included in its structure (Raynes et al., 2015), which contributes to a more flexible polypeptide chain (Adzhubei et al., 2013) and ultimately gives milk with β-CN A2 a greater self-assembly behavior (Raynes et al., 2015). Such structural differences may enhance the ability of β-CN A2 to interact with other proteins as a molecular chaperone, and hinder protein aggregation (Raynes et al., 2015).

In this study, milk with β-CN A2A2 variant had the least favorable acid coagulation properties, which is consistent with what was reported by Daniloski et al. (2022b), but in contrast to the finding of Juan and Trujillo (2022), who found that milk with the β-CN A2A2 variant gave firmer gels than the A1A2 variant. Michalski et al. (2002) reported that the G’ values of acid gels are lower when milk fat globule size is smaller, which can also be associated with the softer acid gel in milk containing A2 β-CN. However, larger fat globule size was found to impair acid coagulation by Ketto et al. (2017), whereas β-CN has been reported to have no effect on milk acid coagulation properties (Hallén et al., 2009; Ketto et al., 2019); Hallén et al. (2009) concluded that acid coagulation properties are only associated with β-lg genotypes while, after standardizing protein content, acid coagulation was only found to be affected by κ-CN genotypes (Ketto et al., 2017).

Effects of protein genotypes on milk heat stability have been reported by several authors on κ-CN and β-lg only (Friedhelm 2018), as heat-induced dissociation of κ-CN is the main reason for milk heat coagulation (Huppertz, 2016), and β-lg, as the main whey protein, is the prime contributor to heat-induced whey protein coagulation (Huppertz, 2016). In brief, milk with the AA and AB variants of κ-CN are more heat resistant than the BB variant, while the BB variant of β-lg is the most heat stable (Friedhelm 2018). This may explain the similar heat stability between different β-CN genotypes in this study, as the κ-CN and β-lg genotypes are variable in cows with the same β-CN genotype. Although A1A1 and A2A2 milk had a type A profile, while the profile of A1A2 milk tended to be closer to a type B profile in this study, by plotting the average values from milk samples having the same β-CN genotype, the cow-to-cow variation cannot be ignored.

Although some differences for milk physicochemical properties and functional properties between 3 β-CN genotypes were found in this study, some were found to not be statistically significant. This might be different if the sample size is larger, which was not possible in this study but should be taken into consideration in future studies. The A2 variant of β-CN is known to be the original variant in bovine milk, and the mutation from proline to histidine on position 67 on amino acid sequence occurred in European dairy cattle, leading to the A1 variant (Dantas et al., 2023). The A2 variant is present in human milk whereas A1 is not (Gatica & Alomar, 2017). Up to now, the A2 variant is the most frequent in cow milk in Europe (except France), USA, Australia, and New Zealand, whereas the A2 variant is predominant in Asian and African breeds (Hoque and Mondal, 2019). Although the A2 milk market has become important in many countries, its negative effect on milk rennet coagulation properties and acid coagulation properties must be taken into consideration in cheese and yogurt processing, as its longer coagulation time may lead to a higher cost and lower production efficiency. This is possibly the reason for the higher frequency of A1 variant in the countries or areas listed above, which have a higher demand for milk to manufacture cheese. However, the porous coagulum produced from A2 milk might be used for specific products, e.g., more easily digestible yogurt (Nguyen et al., 2018). Also, as the separation of cream layer from skim milk
in the A2A2 milk is slower than in A1A2 and A1A1 milk, this can be a benefit in certain dairy product processing applications.

This study was designed to determine if there were any differences between the genotypes on milk physicochemical properties and functionalities. It has been shown that differences exist, such as better rennet and acid coagulation of A1A1 milk and the greater stability to creaming in A2A2 milk, which may assist processors in selecting milk for specific products and processes. In further studies, larger sample sizes could further eliminate inter-individual variations.

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### Table A1. MILK PROTEIN GENOTYPING

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