Association between technological properties of bovine milk, cold induced depletion and genetic variants of β- and κ-casein

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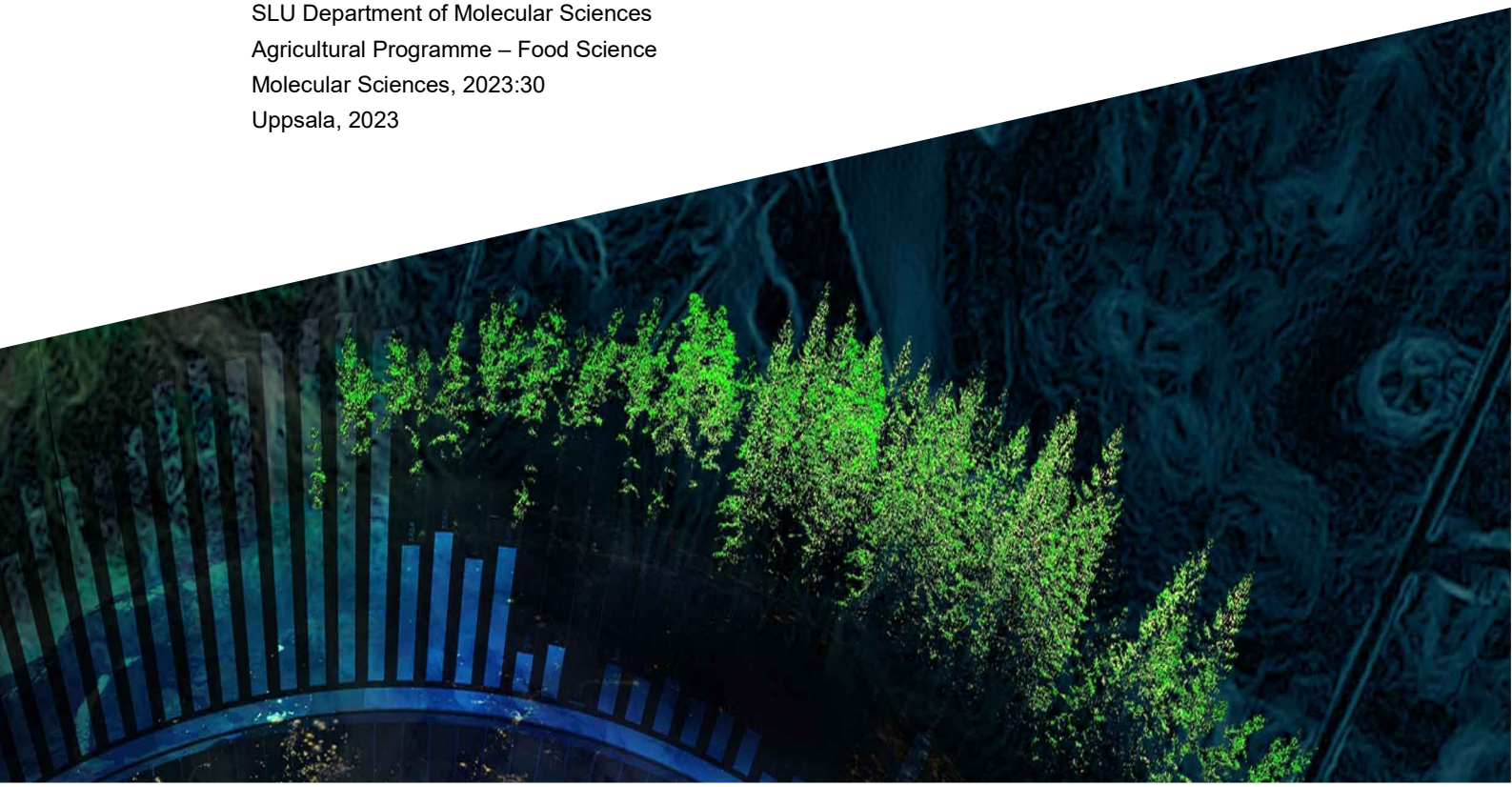

Association between technological properties of bovine milk, cold induced depletion and genetic variants of β-, and κ-casein
Samband mellan mjölkens teknologiska egenskaper, kylrelaterad dissociation och genetiska varianter av β- och κ-kasein

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Abstract

Caseins (CN) represent about 80% of the total protein in bovine milk and are of major importance in dairy production. The four casein types, i.e., αs1-, β-, αs2-, and κ-casein are in turn divided into different genetic variants. The genetic variants of interest in this study were β-CN variants A1, A2 and I, and κ-CN variants A and B. The genetic variants of caseins are believed to influence many milk properties including gross composition, micelle size, coagulation properties (rennet coagulation time and curd firmness) and protein profile. The aim of the study was to investigate the relation between these properties, the three genetic variants of β-CN and to some extent also genetic variants of κ-CN. The aim was also to investigate to what extent different genetic variants of β-CN were able dissociate from the micelle into milk serum due to cooling. The results indicated relation between milk gross composition and genetic variants of β-CN, where β-CN variant I showed highest protein content (3.80 %) compared to β-CN variants A1 (3.35 %, $p<0.001$) and A2 (3.45 %, $p<0.001$). The rennet coagulation time in cold milk (milk with depleted micelles) with β-CN genotype A1A1 was up to 90 % shorter than for cold milk with β-CN genotype II ($p<0.001$). Meanwhile, no significant difference between the two genotypes was found in tempered milk (native micelles). However, there was no clear association between cooling and curd firmness. The casein profile and the casein number were analysed both in tempered and cold milk serum phase. When comparing the relative concentration of β-CN in milk serum phases of cold and tempered milk, respectively, the increase associated to β-CN II milk was 235 % and 72 % higher, than the increase associated to β-CN A1A1 and A2A2, respectively. Furthermore, the casein number in cold milk serum phase associated to the β-CN genotype II was 18 % and 16 % lower, respectively, than in the serum phases corresponding to the A1A1 and A2A2 genotypes; though, also here the observed differences were only numerical. This might indicate that the level of cold induced depletion might vary between different genetic variants of β-CN.

Keywords: Casein, genetic variants of caseins, casein micelle, β-casein depletion, milk coagulation properties

Sammanfattning

Kaseiner (KN) utgör cirka 80 % av det totala proteininnehållet i komjölk och är av yttersta vikt för mejeriindustrin. De fyra typerna av kasein, dvs. αs1-, β-, αs2-och κ-kasein är i sin tur uppdelade i olika genetiska varianter. De genetiska varianterna som var av relevans för denna studie var β-KN A1, A2 och I, samt κ-KN A och B. Kaseiners genetiska varianter anses ha en inverkan på flertalet egenskaper hos mjölk, som mjölkens sammansättning, micellstorlek, koaguleringsegenskaper och proteinprofil. Målet med studien var att undersöka sambandet mellan dessa egenskaper och olika genetiska varianter av främst β-KN, men även till viss del genetiska varianter av κ-KN. Ett annat mål var att undersöka till vilken grad olika genetiska varianter av β-KN dissocierar från kaseinmicellen i mjölkserumet vid nedkylning. Resultaten indikerade ett samband mellan mjölkens sammansättning och β-KN I, varvid denna genetiska variant var associerad till ett högre proteininnehåll (3,80 %) jämfört med variant A1 (3,35 %) och A2 (3,45%) ($p<0.001$). Koaguleringstiden för mjölk med β-KN genotypen A1A1 var upp till 90 % kortare än för β-JN II ($p<0.001$), när mjölken kylts och β-KN dissocierat från micellerna. Ingen signifikant skillnad i koaguleringstiden kunde urskiljas mellan de två genetiska varianterna när mjölken var rumstempererad. Inga likvärdiga samband observerades gällande gelstyrkan. Kaseinprofil och kaseininnehåll i förhållande till det totala proteininnehållet mättes i mjölkserum både före och efter kylning. När man jämförde den relativa koncentrationen av β-KN i mjölkserumfaser av kall respektive tempererad mjölk, var ökningen associerad med β-KN II-mjölk 235 % och 72 % högre än ökningen associerad med β-KN A1A1 respektive β-CN A2A2. Dessutom hade mjölkserum från nedkyld β-KN variant II 18 % respektive 16 % högre kaseininnehåll i förhållande till det totala proteininnehållet, jämfört med motsvarande A1A1 respektive A2A2. Detta skulle kunna indikera att olika genetiska varianter av β-KN dissocierar i olika utsträckning vid kylning av mjölken.

Nyckelord. Kasein, genetiska varianter av kasein, kasein micell, läckage av β-kasein, mjölkens koaguleringsegenskaper

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Abbreviations

CN	Casein
CF	Curd Firmness
DLS	Dynamic Light Scattering
DH	Danish Holstein
DTE	1,4-Dithioerythritol
FTIR	Fourier Transform Infrared Spectroscopy
HOL	Holstein
HPLC	High Performance Liquid Chromatography
α-La	Alpha-Lactalbumin
LG	Lactoglobulin
LC/ESI-MS	Liquid Chromatography Electron Spray Ionization Mass Spectrometry
MSP	Milk Serum Phase
MS-DWS	Multi-Speckle Diffusing Wave Spectroscopy
PTM	Post Translational Modification
RCT	Rennet Coagulation Time
SLB	Solid Liquid Balance
SM	Skim Milk
SMUF	Simulated Milk Ultra Filtrate
WM	Whole milk

Background

1.1 Introduction to caseins

Casein (CN) is the most abundant milk protein, constituting around 80 % of the total protein in bovine milk (Dalgleish & Corredig 2012; Duerasch et al. 2020). In turn, based on amino acid sequence/primary structure, there are four different families of caseins, i.e., αs1-, αs2-, β-, and κ-CN (Farrell et al. 2004). The ratio between αs1-, αs2-, β-, and κ-CN in bovine milk is approximately 4:1:4:1 (Jensen et al. 2012). The number of amino acid residues in αs1-, αs2-, β-, and κ-CN is 199, 207, 209 and 169 respectively (Farrell et al. 2004). While β-CN is the most hydrophobic casein (Jensen et al. 2012; Duerasch et al. 2020), all caseins have both hydrophobic and hydrophilic regions, making them amphiphilic.

Caseins are essential for many dairy products, e.g. cheese. These products are of high importance for the modern dairy industry. Cheese is for example one of the few dairy products in Sweden of which consumption have increased in the last few decades (Jordbruksverket 2023). In several countries, e.g. Sweden and Denmark (Dalgleish & Corredig 2012; Cendron et al. 2021; Mejeriforeningen 2021; Jirskog 2022), cheese is also the most exported dairy product.

The other large group of milk proteins consists of whey proteins, dispersed in the milk serum (Farrell et al. 2004; Jensen et al. 2012), constituting about 20 % of total protein in milk. The two main whey proteins are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La).

1.2 Casein polymorphism

The four genes encoding the four different types of caseins are all located in the CN-locus, a gene cluster, located on chromosome six, consisting of 250 000 base pairs. The physical order of the genes and their names are *CSN1S1, CSN2, CSN1S2,* and *CSN3*. They respectively encode αs1-CN, β-CN, αs2-CN, and κ-CN protein. These genes and their nomenclature are shared between the species cow/bovine

(*Bos taurus),* yak (*Bos grunniens*), Zebu (*Bos indicus*) and Bali cattle (*Bos indicus*) (Martin et al. 2002; Caroli et al. 2009).

There are several genetic variants, genotypes, for each casein gene. The scientific and commercial focus on different genetic variants is usually on variants with different amino acid sequences. Silent mutations, where a change in a codon still codes for the same amino acid, are seldom of interest (Farrell et al. 2004; Hewa Nadugala et al. 2022). Casein genotypes occur both as homozygotes and heterozygotes, where homozygotes have two identical alleles of a gene e.g., κ-CN AA, and heterozygotes have two different alleles e.g., κ-CN AB (Hallén et al. 2007). Research has shown that different genotypes affect properties of milk differently, and subsequently also the dairy products. Some properties where significant correlations with casein genotypes have been found include casein micelle size and the content of protein and urea (Walsh et al. 1998; Bijl et al. 2014; Cendron et al. 2021; Hewa Nadugala et al. 2022). Furthermore, gelation and coagulation properties, e.g., curd firming rate, RCT and CF were also found to be associated to genotypes (Poulsen et al. 2013; Cendron et al. 2021).

The two variants of κ-casein that has been deemed most common are κ-CN A and B, respectively. One other variant is κ-CN E, which has been considered quite common in some bovine populations (Farrell et al. 2004; Cendron et al. 2021). These are the three variants that have met the most interest among researchers. Other less common κ-CN variants include κ-CN B2, C, F, G, H, I and J (Farrell et al. 2004; Hallén et al. 2007; Caroli et al. 2009; Cendron et al. 2021; Sheng 2021). While κ-CN B differs from the A variant by an isoleucine residue instead of a tyrosine at position 136, and an alanine instead of aspartic acid residue at position 148, the κ-CN variant E differs from the A variant by a glycine residue instead of a serine residue at position 155 (Farrell et al. 2004).

The two β-CN variants that have gathered the most attention and research are β-CN A1 and A2 (Caroli et al. 2009; Poulsen et al. 2013). These variants are often considered the most abundant variants together with the β-CN B variant. (Nuomin et al. 2022). B-CN variant I is usually a less common variant, and its frequency and properties have been assessed only in a few studies (Caroli et al. 2009; Poulsen et al. 2013; Gustavsson et al. 2014). Other rare genetic β-CN variants include β-CN A3, D, E, F, G, H1 and H2 (Caroli et al. 2009; Nuomin et al. 2022). From an evolutionary perspective, B-CN A2 is believed to be the original variant of β-CN, B-CN A1 differing from A2 with one histidine residue instead of proline in position 67. β-CN variant B has a histidine instead of proline at position 67 and an arginine instead of a serine in position 122, compared to A2. β-CN variant I differs from variant A2 by having a one leucine instead of a methionine in position 93. Further β-CN variant I differs from variant A1 by having a proline instead of histidine at

position 67 and a leucine instead of methionine at position 93 (Farrell et al. 2004; Caroli et al. 2009; Shen 2021).

The most common αs1-CN variant is the B variant. Other variants include αs1-CN A, C, D, E, F, G, H and I (Farrell et al. 2004; Caroli et al. 2009; Hewa Nadugala et al. 2022). There are five known genetic variants of αs2-CN; αs2-CN A, B, C, D and E (Hewa Nadugala et al. 2022). Genetic variation also occurs in whey proteins. The two main variants of β-Lg are A and B and there are three known variants of α-La; A, B and C (Farrell et al. 2004; Caroli et al. 2009). Different genetic variants and genotypes of β-Lg has been shown to influence milk gross composition and coagulation properties (Cendron et al. 2021).

1.2.1 Post-translational modifications

Post-translational modification (PTM) is another factor which causes variation within the casein type/families. The two most important PTMs are phosphorylation and glycosylation.

Phosphorylation is a PTM in caseins which occurs when phosphate groups are attached to serine residues in the Golgi apparatus (Day et al. 2015; Hewa Nadugala et al. 2022). Phosphorylation occurs in all caseins, but the degree of phosphorylation varies between and within casein types. αs2-CN has the highest degree of phosphorylation with 10 to 13 phosphate groups. The most common αs1-CN genetic variant, variant B, normally has eight phosphate groups (αs1-CN 8P). However, αs1-CN 9P is also frequently found in milk. β-CN normally has five phosphates and κ-CN has one to two phosphates (Frederiksen et al. 2011; Boland et al. 2014; Day et al. 2015; Hewa Nadugala et al. 2022).

Glycosylation, i.e. PTM where glycan groups bind to threonine or serine residues, only occurs in κ-CN. κ-CN can have up to nine glycans and approximately 40 % of κ-CN in milk are glycosylated (Bonfatti et al. 2008; Boland et al. 2014; Shen 2021; Hewa Nadugala et al. 2022). In several earlier studies, κ-CN genotype BB has been linked with higher degree of glycosylation than genotype AA. This is even though κ-CN variant B has one less threonine group compared to κ-CN variant A (Coolbear et al 1996; Day et al 2015; Hewa Nadugala et al. 2022). The glycosylation level has also been shown to vary between κ-CNs with the same genetic variant. Levels of glysylation has been related to other factors such as lactation stage, milk yield and dry period (Coolbear et al. 1996; De Vries et al 2015).

A less known and poorly researched PTM is formation of κ-CN multimers/ oligomers. These occurs when cystine residues from two different κ-CN form a disulphide bridge. κ-CN has two cystine residues which yields three possible

disulphide bonding patterns between two κ-CN molecules. κ-CN with an intramolecular disulphide bond between its two cystine residues is called a monomer. The monomers of κ-CN can be separated from other proteins and visualized by the SDS-page method (Rasmussen et al. 1992; Boland et al. 2014; Shen 2021). In a study by Rasmussen et al. (1992), bands of κ-CN multimers in 1D-gels used for the SDS-page method, were found to have molecular mass from 130 kDa to 325 kDa. The average size difference between two consecutive bands was approximately 26 kDA, close to the molecular mass of one κ-casein molecule, i.e., 22 kDA. κ-CN can also form disulphide bonds with whey proteins such as β-Lg and α-lactalbumin. This usually happens during high temperature heat treatment of milk which causes the whey protein to denature and bind to κ-CN (Lowe et al. 2004; Boland et al. 2014).

1.2.2 Determination of different genetic variants and posttranslational modifications of milk proteins

There are several methods that can be used to determine genetic variants of the milk proteins and study which of the genotypes that are present in the milk (Bonfatti et al. 2008; Caroli et al. 2009).

Liquid chromatography (LC) can be used for separation of different types of caseins along with other milk proteins, e.g., β-Lg, followed by identification of the proteins based on their retention time. Furthermore, by this method different genetic variants of the same protein can be separated; for example κ-CN A and B. Different PTMs of the same protein can also be separated, which makes it possible to distinguish e.g., glycosylated κ-CN from unglycosylated, and αs1-CN with eight and nine phosphate groups, respectively. Relative quantities, but also numerical concentrations of different proteins with the help of references/standards, can be determined (Farrell et al. 2004; Bonfatti et al. 2008; Frederiksen et al. 2011; Duerasch et al. 2020; Ivanković 2021). One important LC method for identifying different types of milk proteins is a reversed-phase high-performance liquid chromatography (RP-HPLC) method developed by Bonfatti et al. (2008). However, in this method different genetic variants can have the same or very similar retention times, for example κ-CN A and E. Since these genetic variants have different molecular weights, they can be distinguish using methods which combine LC with electron spray ionization and mass spectrometry, i.e., liquid chromatography/ electron spray ionization- mass spectrometry (LC/ESI-MS). This technique has been modified and applied also for characterization of milk proteins (Fredriksen et al., 2011). LC methods show which proteins are present in the milk but not the DNA sequence of the milk protein genes. Thus LC is a phenotyping and not genotyping method (Bonfatti et al. 2008; Caroli et al. 2009).

Another way for identification of the genetic variants of proteins is genome sequencing of the DNA, which is a genotyping method. DNA can be extracted from e.g., blood or hair. Polymerase chain reaction (PCR) is then used to amplify the specific genes with the primers directed against the region of interest. To differentiate between genotypes, certain single nucleotide polymorphism (SNP) are identified (Bonfatti et al. 2008; Poulsen et al. 2013; Sebastiani et al. 2020; Nuomin et al. 2022).

1.3 Casein micelle

Caseins are mostly present in the form of casein micelles, as large colloidal aggregates. The diameter of a casein micelle is on average approximately 200 nm but can vary between 50-500 nm (Dalgleish & Corredig 2012; Jensen et al. 2012; Bijl et al. 2014; Duerasch et al. 2020). Except for casein, the micelles consist of calcium and phosphate, with trace amount of magnesium, citrate, and other minerals. There is approximately 3.5 g water per g of protein in micelles, resulting in the micelle taking up 10 % of the milk volume. In contrast, casein proteins only take up 2.5 % of the weight of milk (Dalgleish & Corredig 2012; de Kruif & Huppertz 2012).

The detailed structure of the casein micelle is not fully understood and different models have been presented over the decades. For example, in the 1980s and 1990s micelles were generally believed to consists of submicelles, the submicelle model. In the late 1990s and 2000s, studies with techniques like electron microscopy paved way for a new casein micelle model, called the nano cluster model. In this model, the micelle has no submicelles, instead the caseins are organised in a network structure, linked together via clusters of colloidal calcium phosphate (Walstra 1999; Dalgleish & Corredig 2012; Lucey et al. 2017; Sheng 2021). A third and recent model is the dual binding model which emphasises that CN can interact both via colloidal calcium phosphate and hydrophobic bonds (Horne 2016; Hewa Nadugala et al. 2022). In all the latter models, κ-CN is located on the outside/surface of the micelle, while the other more hydrophobic caseins, αs1-, αs2-, β-CN are located in the core of the micelle (Walstra 1999; Dalgleish & Corredig 2012; de Kruif & Huppertz 2012; Sheng 2021). The location of κ-CN on the surface of the micelle has been proven partly by the fact that the proportion of κ-CN increases when the micelle size decreases. κ-CN is thus related to the micelle's volume/surface ratio (Dalgeish et al. 1989). Further, the proportion of β-CN has been proven to decrease with smaller micelle size. The αs1- and αs2-CN seems to be unaffected by volume/surface ratio (Dalgleish et al. 1989; Dalgleish & Corredig 2012; Lucey et al. 2017).

1.3.1 Cold induced depletion of casein micelles

Cooling have been observed to induce depletion/dissociation/leakage of caseins from the micelle. This occurs with all caseins to some extent, but particularly in the case of β-CN, which is more sensitive than other caseins. The depletion process is reversible (Dalgleish & Corredig 2012; Duerasch et al. 2020; Guggisberg et al. 2022). The main mechanism behind the depletion is believed to be that lower temperatures weaken the hydrophobic bonds of caseins. Since β-CN is the most hydrophobic casein, and hydrophobically bond to other caseins inside the micelle, it quite easily disassociates into the milk serum (Raynal & Remeuf 2000; Dalgleish & Corredig 2012; Duerasch et al. 2020). In a study by Ono et al. (1990), the leakage of β-CN was greater from large micelles than from small micelles. In the same study, the leakage of κ-CN was larger in small micelles than in large micelles.
Due to increased solubility, calcium phosphate also dissociates and leaves the micelles during cooling. Since a significant share of β-CN inside the micelle is bond to the micelle structure via colloidal calcium-phosphate, "co-dissociation" with calcium could be another possible mechanisms behind the depletion (Dalgleish & Corredig 2012; Duerasch et al. 2020; Guggisberg et al. 2022). Earlier studies have shown that up to 40 to 50 % of the β-CN has been observed to leak out during cooling and subsequent cold storage (Duerasch et al. 2020). It is yet unknown whether κ-CN monomers depletes from the micelle in higher rate than multimers or vice versa (Rasmussen et al. 1992; Boland et al. 2014).

1.3.2 Factors influencing casein micelle size

Variation in casein micelle size has been associated to several factors, e.g., season and calcium content (Glantz et al. 2010; Hewa Nadugala et al. 2022). Several studies have pointed out genetic variants and genotypes of caseins, especially in the case of κ-CN, as important factors for the size of the micelle. Higher proportion of κ-CN in the casein profile has been linked with smaller average micelle size (Day et al. 2015). κ-CN B variant has been associated with smaller micelles compared to κ-CN A (Walsh et al. 1998; Bijl et al. 2014), and Jensen et al. (2015) linked κ-CN variant B with higher proportional κ-CN content than κ-CN A. Only few studies related to β-CN in relation to micelle size have been conducted. Day et al (2015) compared micelle size and genotypes of both β-CN and κ-CN. Large micelles (177-207 nm) were always associated to κ-CN genotype AA, in contrast to κ-CN genotypes AB or BB, which were typically associated to milk with small micelles (144-155 nm). β-CN I variant was only found in small micelles (144-155 nm). Lodes et al. (1996) showed that milk with β-CN genotype A1A1 had significantly larger average micelle size than genotypes A1A2 and A2A2.

Whether cold induced depletion of caseins influences casein micelle size or not is unclear and results vary between studies (Duearsch et al. 2020). In studies by Maciel et al. (2014) and Duerasch et al. (2020), micelle size was measured with Dynamic Light Scattering (DLS) using skim milk diluted in simulated milk ultra-filtrate. Both studies reported that there was no significant difference in micelle size between cold and tempered milk. A slight increase of micelle size after cooling was reported by Yahimi et al. (2014), measuring micelle size with dynamic light scattering using casein micelle suspension diluted in milk permeate.

1.4 Casein genetic variants and protein content

Several studies examining protein content of milk in relation to the genetic variants of caseins have been conducted. κ-CN variant B has been associated with higher protein content than κ-CN A and E (Heck et al. 2009; Cendron et al. 2021). Cendron et al (2021) reported the following order of protein content based on κ-CN genotypes in Italian Holstein cattle: BB > AB > AA/AE/BE/EE. Most studies did not find a difference in protein content between β-CN A1 and A2 (Heck et al. 2009; Cendron et al. 2021).

1.5 Coagulation

The κ-casein layer on the micelle surface contributes to the stability of the micelle. Glycosylated κ-casein causes steric hindrance and electrostatic repulsion which causes the micelles to repel from each other. The part of the κ-casein responsible for this repulsion is called the macropeptide, spanning from amino acid residue 106 to 169 (Farrell et al. 2004; Dalgleish & Corredig 2012; Lucey et al. 2017; Sheng 2021) When the macropeptide is cut off by enzymes, e.g., chymosin and pepsin from cow rennet, the micelles will start to aggregate and eventually coagulate. (Dalgleish & Corredig 2012; Jensen et al. 2012). The corresponding coagulum, also known as curd, is important for the production of cheese. Thus, milk with good coagulation properties is sought after. Higher protein content is associated with better coagulation and higher cheese yield. Therefore, milk payment systems usually have premium for higher protein content (Jensen et al. 2012; Mejeriforeningen 2021; Poulsen 2021).

Coagulation properties of milk can be evaluated using parameters obtained from oscillatory rheology. For example RCT, curd firming rate and curd firmness (CF) can be measured by this method (Poulsen 2021; Cendron et al. 2021; Guggisberg et al. 2022). CF is a measure of the strength of the curd, determined by its elastic modulus or elasticity index at a certain time point after the rennet addition. RCT is

a way to measure the rate of the coagulation and is often measured as the time when the solid-liquid balance is 0.5 Solid liquid balance is the proportion between forces that causes solidity and liquidity, respectively, and thus solid liquid balance is unitless (Wedholm et al. 2006; Guggisberg et al. 2022). Good coagulation properties are usually described as short RCT, high curd firming rate and high curd firmness. These qualities are in turn related to rapid coagulation process and a strong curd, which are sought after in e.g., cheese production (Poulsen et al. 2013; Bijl et al. 2014; Cendron et al. 2021; Guggisberg et al. 2022).

1.5.1 Casein genetic variants and coagulation properties

One of the main interests associated to the different genetic variants and genotypes of caseins has been their effect on milk coagulation properties and yield in e.g., cheese production.

β-CN A2 and κ-CN A, often in combination, has been associated with milk which has poor or no coagulation (Jensen et al. 2012; Poulsen et al. 2013). β-CN A1 has been linked to longer RCT, lower curd firming rate and lower CF than β-CN A2 in e.g., Holstein cattle (Poulsen et al. 2013; Cendron et al. 2021). β-CN variant I has been related to lower curd firming rate than β-CN A1 and A2 (Poulsen et al. 2013).

Studies have established, that κ-CN variant B is associated to more desirable coagulation properties than variant A. κ-CN B has been shown to have shorter RCT, higher curd firming rate and higher CF than κ-CN A (Poulsen et al. 2013; Bijl et al. 2014; Cendron et al. 2021). Cendron et al. (2021) found that the genotype κ-CN AB had CF and curd firming rate inbetween κ-CN genotype AA and BB. It has been suggested that the smaller micelle size is correlated with the desirable coagulation properties. The idea is that smaller micelles allow for tighter packing of micelles in the curd, which yields more intermicellar bonds and thus a stronger curd. This could explain why κ-casein B, which has also been related to smaller micelle size, seems to yield better coagulation properties (Walsh et al. 1998; Poulsen et al. 2013; Bijl et al. 2014; Cendron et al. 2021). Further, smaller micelle size has been related to better coagulation properties also in studies which did not focus on genotypes, e.g. the study by Maciel et al. (2014).

1.5.2 Effect of cooling on coagulation and production properties

Cooling has been shown to affect the milk coagulation and properties of the resulting gel negatively, and thus dairy production qualities. Maciel et al. (2014), investigated how different levels of cooling affected the coagulation properties of milk from individual cows. Each cow was categorised as either having good or poor coagulating milk, and the study showed prolonged RCT and decreased gel firmness after cooling of milk. Likewise, Guggisberg et al. (2022) found prolonged RCT,

decreased CF and decreased cheese yield in cooled cheese milk. On the other hand, cooling had no significant effect on cheese milk rheological parameters like RCT in the study by Maciel et al. (2014). Raynal and Remeuf (2020) investigated how cooling at 4 °C for up to 48 hours affected rheological properties, casein and calcium depletion in cow's milk. The RCT was 30 % longer in milk that had been stored at 4 °C for 48 hours compared to milk that had been stored for zero hours (fresh milk). Also, the depletion/solubilization of casein and calcium was higher in cow milk after 48 hours compared to zero hours.

1.6 Casein polymorphism and cow breeds

Studies related to frequencies of different genetic variants and genotypes of caseins have been performed in many countries and for different breeds. When it comes to frequencies of genetic variants and genotypes, β-CN is the most studied one (Jann et al. 2002; Poulsen et al. 2013; Gustavsson et al. 2014; Massella et al. 2017; Chessa et al. 2020; Sebastiani et al. 2020; Cendron et al. 2021; Ivanković 2021; Hewa Nadugala et al. 2022; Roin et al. 2022), β-CN A2, followed by A1, being the most common variants in perhaps the most common and examined dairy breed, i.e., Holstein.

Some of the most comprehensive studies regarding frequencies of genetic variants and genotypes have been performed in Italy. Cendron et al. (2021) mapped out β-CN and κ-CN genetic variants and genotypes in over 5 300 cows from 122 herds in the Veneto region, Italy, over a period of 9 years. Chessa et al. (2020), analysing over 200 000 samples that had been collected worldwide, mainly from Holstein cows in Italy, USA, and Canada, reported that the frequency of β-CN A2 in Holstein had increased from 1990 to 2017. β-CN I, B and A3 are three other genetic variants that are often found in Holstein, however, these variants are less common (Poulsen et al. 2013; Gustavsson et al. 2014; Nuomin et al. 2022). Gustavsson et al. (2014) and Poulsen et al. (2013) found that around 7 % of Danish Holstein cows from 20 herds had a β-CN I allele. In several studies on the Jersey breed, the β-CN B variant has been the second most frequent variant instead of A1. Noumin et al (2022), reported the same trend in a study on Japanese Jersey in Western Japan and the same trend was also observed in Danish Jersey by Gustavson et al (2014). When it comes to β-CN genotypes, A1 is usually more common in heterozygotes, e.g., A1A2 in Holstein or A1B in Jersey, than in the homozygotic A1A1 form. The most common β-CN genotype for A2 varies between different studies, although heterozygotes tend to constitute the most common form in cows with the A2 allele in both Jersey and Holstein. However, there are also studies on Holstein cows reporting that homozygotic β-CN A2A2 was the most common β-CN genotype with the A2 allele, and most common β-CN genotype overall (Poulsen et al. 2013;

Gustavsson et al 2014). In contrast, there are other studies reporting that A1A2 was the most common β-CN genotype in Holstein cows (Massella et al. 2017; Cendron et al. 2021).

For κ-CN, the A variant tends to be the most frequent one in Holstein, while B has been found to be more frequent in some Jersey populations (Gustavsson et al. 2014). In the study on Danish Holstein by Poulsen et al (2013) the frequency for the varieties of κ-CN A, B and E was 70 %, 24 % and 6 %, respectively. Cendron et al. (2021), investigating κ-CN variants in Italian Holstein, reported frequencies of 57 %, 33 % and 10 % for κ-CN A, B and E, respectively. Chessa et al. (2020) found that κ-CN variants B and E had increased while A had decreased, both in Italian and worldwide Holstein populations between 1990 to 2017 (Chessa et al 2020). The most common κ-CN genotype varies between studies and breeds. For Holstein, Poulsen et al. (2013) and Gustavsson et al. (2013) reported κ-CN genotype AA being the most common one, while Cendron et al. (2021) reported κ-CN genotype AB. Some studies have also investigated the frequency of composite genotype, the combination of several different milk protein genotypes. Poulsen et al. (2013) showed that the most common composite genotypes of αs1-CN-β-CN-κ-CN in Holstein were BB-A2A2-AA and BB-A1A2-AA.

Frequencies of genetic variants and genotypes can vary greatly between farms. Noumen et al. (2022) showed that the frequency of the β-CN I in Jersey cows varied from 2 % to 15 % in a study on eight different farms in western Japan. Some variants, e.g., κ-CN A and B, and β-CN A2, can be found in all cow breeds and β-CN A1 in most cow breeds. Some genetic variants can only be found in a few or even a single cow breed. β-CN E has only been found in Italian Piemontese (Caroli et al. 2009; Massella et al. 2017). Mapping of genotypes present in different breeds is an active ongoing research area. As an example, a recent study by Roin et al. (2022), was the first to report the existence of the rare variants αS1-CN A and D in the traditional Norwegian dairy breed Telemark.

Genetic variants of β-CN and κ-CN is accounted for in the breeding of dairy cattle in some countries, e.g., Sweden and Italy (Chessa et al. 2020; Växa 2023). An indication of how farmers might perceive different genetic variants and gentoypes of β-CN and κ-CN can be seen in the communication from the Swedish Livestock orgnaisaiton Växa (2023). On its homepage, Växa (2023), provides members with the information that scientific studies has found κ-CN genotype BB to have "supreme cheese making properties" in contrast to κ-CN genotype EE, which they say is associated with "non-coagulating milk" and thus "unsuitable for cheese production". Research in the early 90s is the reason for the β-CN variant A2 receiving substantial interest and for different "health claims"; e.g., β-CN A2 variant having better digestion properties than β-CN A1 variant (A2 milk 2023).

This has resulted in a product called A2-milk, originally being sold in Australia and New Zealand, but recently also in several other regions, e.g., US, Denmark, and the Netherlands. In their evaluation, the European Food Safety Authority (EFSA) concluded that there is no scientific consensus supporting these "health claims" (European Food Safety Authority 2009; Massella et al. 2017; Chessa et al. 2020). Chessa et al. (2020) reported that the frequency of β-CN A2 and κ-CN B had increased in the Holstein population in e.g., Italy and US since the 1990s. They hypothesised that it might be due to deliberate breeding targeting these two genetic variants, however, also that it could be a side effect in breeding for other qualities.

1.7 Objective and aim

The aim of this study was to investigate the effect of different genetic variants and genotypes of β-CN, and to some extent κ-CN, on selected milk properties, namely gross composition, casein content, level of glycosylated κ-CN, micelle size and coagulation properties. Furthermore, the study aimed to investigate whether cold induced depletion varied between different genetic variants of β-CN.

Methods and Materials

2.1 Collection of milk samples

Bovine raw milk was collected from 216 Danish Holsteins (DH), six Danish Red dairy cattle (RDM) and seven DH/RDM hybrids (KRY). The age of the cows varied from two to seven years. Number of lactations were between one and six, and stage of lactation between two and 40 weeks. The cows were milked in the morning.
Milk from all cows were used in the initial/first screening of β-CN, κ-CN, and β-Lg genotypes. The β-CN genotypes A1A1, A2A2 and II were of major interest in the study. Thus, milk from three cows with β-CN genotype A1A1, four with A2A2 and three with II were chosen for further analysis. For the analysis, equal amount of milk from all chosen cows with the same β-CN was mixed to make so called "pooled milk".

2.2 Preparation of milk samples

Different fractions of milk were used for different analyses. Whole milk (WM) was used to determine the gross composition (total protein, casein, and lactose), with fourier-transform infrared spectroscopy (FTIR). Skim milk (SM) was used for dynamic light scattering (DLS) with *Zetasizer*, liquid chromatography electron spray ionization mass spectrometry (LC/ESI-MS) and multi speckle-diffusing wave spectroscopy (MS-DWS). Milk serum phase (MSP), i.e., after removing casein micelles by ultracentrifugation, was also used for LC/ESI-MS analysis,

FTIR, DLS and MS-DWS were conducted on fresh milk. The milk samples used in LC/ESI-MS were frozen at -80 °C overnight and then stored at -20 °C for up to 16 days.
For FTIR, six technical replicates were done for each pooled milk. Number of measurements for the other analyses are mentioned in their respective method.

2.2.1 Cooling treatment

DLS, MS-DWS, LC/ESI-MS were all performed on SM and MSP, made from WM with either native or depleted micelles. Milk with native micelles, is also referred to as tempered milk. Tempered SM and MSP were prepared from fresh milk that had been stored at room temperature for up to 12 hours after milking. Milk with depleted micelles was also referred to as cold milk. To prepare cold milk, WM was stored in a fridge at 4 ℃ overnight. Cold SM and MSP was then prepared from cold WM.

2.2.2 Preparation of skim milk

To separate fat from skim milk, WM was centrifuged in Falcon tubes of 15 or 50 ml (*Megafuge ST Plus Series – Centrifuge*, Thermo Scientific, Stockholm Sweden; or SL40R -Centrifuge, Themro Scientific, Stockholm, Sweden) with a *TX750* rotor of 195 mm radius (Thermo Scientific*),* at 3500 rpm for 30 minutes at 4 ℃. After the centrifugation, the fat phase was discarded.

2.2.3 Preparation of milk serum phase

The aim with preparing MSP was to have a phase where casein micelles had been separated and removed from the milk serum. Thus it was possible to measure the proportions of casein that had disassociated from the casein micelles. MSP was prepared using ultracentrifuge (*Optima^{TM} L-80 XP Ultracentrifuge)* with a *Type 70 Ti Fixed-Angle Titanium* Rotor (Beckman Coulter Inc, Brea, USA) of 65.7 mm radius. Ultracentrifugation was conducted 31 00 rpm for 1 hours and at 21 °C for tempered milk and 4 °C for cold milk. The aims were to separate the casein micelles from the rest of the milk serum. The difference in temperature was used to avoid initiation respective reversion of casein micelle depletion.

2.3 Dynamic light scattering

Dynamic light scattering was used to measure Z-average hydrodynamic diameter of casein micelles (nm), i.e., the average micelle size (nm), in the SM samples. DLS was done with a *Zetasizer LAB* (Malvern Instruments, Malvern, UK). To remove any remaining fat, samples were filtered with 1.2 µm, GF membrane syringe filter (Phenomex, Californien, USA). The filtered samples were mixed with simulated milk ultra filtrate (SMUF) buffer in a ratio of 1:100. Sample and SMUF were mixed in a *DTS0012 disposable 10x10 plastic cell cuvette* (67.754, Sarstedt AG & Co., Nümbrecht, Germany). SMUF buffer was prepared according to Jenness and Koops (1962). The SMUF buffer consisted of 11.61 mM potassium dihydrogen phosphate, 3.70 mM tripotassiumcitrate dihydrate, 6.09 mM sodium citrate dihydrate, 1.03

mM potassium sulphate, 2.17 mM potassium carbonate, 8.05 mM potassium chloride, 8.98 mM calcium chloride dihyrate and 3.21 mM magnesium dichloride hexahydrate. All chemicals used in the SMUF buffer were from Sigma Aldrich (Darmstadt, Germany). The SMUF buffer was stored in a freezer when not used. SMUF buffer was placed in a fridge to thaw overnight the day before DLS. Before mixing, the SMUF buffer had been filtered two times with a 0.45 μm syringe filter (Phenomenex, CA, USA). The samples were measured with a method using a side scattering angle of 90°, at 20 °C with an equilibrium time of two minutes. Measurements were done in technical triplicates. The results were evaluated with the *ZS XPLORER* (Malvern Instruments, Malvern, UK) software.

2.4 Multi speckle diffusing wave spectroscopy

Multi speckle diffusing wave spectroscopy (MS-DWS) was conducted with a *Rheolaser Master*® (Formulaction, Toulouse, France). MS-DWS light scattering was used to measure changes in microstructure throughout aggregation and coagulation of casein micelles. Thus, the coagulation properties, i.e., RCT and curd firmness (CF) could be measured.

Both tempered and cold skim milk samples were analysed with MS-DWS, both at native pH of 6.72±0.04 and at pH adjusted to pH 6.5±0.02. pH of SM samples was measured with a pH-meter (826 pH Mobile, Metrohm, Herisau, Switzerland). The pH was adjusted with 10 % lactic acid, added dropwise. The tempered and the cold SM, respectively, combined with native and adjusted pH, respectively, for the three different β-genotypes, resulted in 12 different sample types, which were analysed in technical duplicates.

The tempered SM samples were heated in 33 °C water bath for 30 minutes. Cold samples were not heated to avoid the casein depletion to reverse.

The rennet solution was prepared by mixing 203 IMCU/ml chymosin (*Chy-Max*® *Plus*, Christian Hansen, Hørsholm, Denmark) with MilliQ in a 1:10 ratio. Before MS-DWS, 10 ml SM sample at either native or adjusted pH, was transferred to a 15 ml Falcon tube. Diluted chymosin (20 μl) was added to the SM and mixed, and 4 ml of the SM sample was analysed with MS-DWS. Six SM samples could be analysed at the same time, and the MS-DWS analysis ran for 1 hour after the samples had been placed in the instrument.
The results were evaluated with the *Rheosoft Master_1.4.0.10* software (Formulaction, Toulouse, France). RCT was defined as the time (s) when the solid-liquid balance (SLB) reached a value of 0.5, or the maximum SLB in cases when

0.5 was never reached. Curd firmness was defined as the value of the Elasticity Index, 20 min into the run.

2.5 Determination of genotypes with liquid chromatography – electrospray ionisation/mass spectrometry

Liquid chromatography/electrospray inonisation-mass spectrometry (LC-ESI/MS) was used to determine the genotypes of β-CN, κ-CN, and β-Lg. By determining genotypes, milk from cows which were homozygotes (β-CN: A1A1, A2A2 and II), could be chosen for further analysis. Given that κ-CN and β-Lg have also been reported to have an effect on milk properties, they where also genotyped (Cendron et al 2021). LC/ESI-MS was also used to determine the proportional content of αs1-, αs2-, β-, and κ-CN, the whey proteins β-Lg and α-La. Further, LC/ESI-MS was used to determine the genetic variants of these proteins. LC/ESI-MS was also used to determine the level of glycolysation of κ-CN. The method used was based on Fredriksen et al. (2011) method.

Two separate screenings were conducted with LC/ESI-MS. The first screening was as mentioned above conducted on skim milk from 229 individual cows, of which 216 where HOL. The second screening was performed on MSP from the pooled milk samples representing the three different β-CN genetic variants in DH cows. MSP derived from tempered and cold milk, respectively, was screened, resulting in six samples for the second screening.

In preparation for LC-ESI/MS, frozen skim milk or MSP samples were thawed at room temperature. If any fat residues were visually observed, the skim milk samples were centrifuged with an *Eppendorf 5417R Refrigerated Centrifuge* (Eppendorf, Germany), at 14 000 rpm for 5 min at 7 ℃ to separate the fat. The skim milk (150 µl) was then mixed with 450 µl working solution and 12 µl of 1M 1,4-Dithioerythritol (DTE; Sigma Aldrich, Darmstadt, Germany). The DTE had previously been dissolved in 100 mM Bis-Tris. The working solution contained 6 M guanidine hydrochloride (Sigma Aldrich, Darmstadt, Germany) and 5.37 mM sodium citrate dihydrate (Sigma Aldrich, Darmstadt, Germany) in 100 mM Bis-Tris (Sigma Aldrich, Darmstadt, Germany). The pH of Bis-Tris had previously been adjusted to pH 6.8. The mixed samples were incubated in room temperature for 1 hour and then centrifuged at 14 000 rpm for 10 min at 7 ℃ (*Eppendorf 5417R Refrigerated Centrifuge*, Eppendorf, Germany). Each sample (400 µl) was then filtered through a *Mini Uni-Prep syringeless filter* 0.2 µm.

The LC equipment was an *Agilent 1260 Infinity II Series* (Agilent Technologies, Santa Clara, Ca, USA) equipped with a G7112B binary pump including degasser, a G7129A vial sampler, a G7116A thermostat and a G7117C diode array detector (DAD) 214/280 nm. The mass spectrometer was an *Agilent LC/MSD XD* G6135B (Agilent Technologies). The column used for separation was a Jupiter C4 column, 300 Å, 250 x 2.00 mm, (Phenomenex, USA) with column temperature set at 40 °C. Solvent A and solvent B were used in the system. Solvent A consisted of 0.05 triflouroacetic acid (TFA; (Sigma Aldrich, Darmstadt, Germany) in Milli Q water and solvent B consisted of 0.05 % TFA in acetonitrile (Merck). As mobile phase, used to separate the proteins, a binary solvent system with linear gradient (33-50% solvent B) was used. The LC had a flow of 0,3 ml/min and the injection volume for samples was 6 µl. *Agilent ChemStation for LC Systems* software was used to control the program.

The MS had the following settings: The drying gas flow was 9 L/min, with a drying gas temperature at 350 °C, nebulize pressure at 40 pound per square gauge, capillary voltage at 3 500 volt and quadruple temperature of 99 °C.

Each run with SM from individual cows contained 40 milk samples except one with 29. Each run included one reference milk sample and one blank sample. The reference milk was prepared in the same way as the other milk samples. The blank consisted of 600 µl working solution + 12 µl 1M DTE, and it was incubated, centrifuged, and filtered with the same method as the milk samples. A cleaning program where the column was cleaned with 95 % solvent B was used between the runs.

The software *Agilent ChemStation* (Agilent, Santa Clara, CA, USA) was used to evaluate the data, chromatograms and molecular masses, obtained from LC/ESI-MS. Integration was used to measure relative contents of different proteins in SM and MSP samples. Deconvolution was used to determine the genotype of β-CN, κ-CN and β-Lg.

2.5.1 Determination of casein profile and glycosylation rate in relation to genotype

Milk samples from 34 DH were analysed by LC/ESI-MS to determine the casein profile in milk with different genotypes of β-CN respective κ-CN. The cows had one of 15 different homozygotic genotypes in regards to β-CN, κ-CN and β-Lg (*Table 10, Appendix 1*). All comspoite genotypes consisted of one of the following β-CN homozygote genotypes, A1A1, BB or II. κ-CN genotype could either be AA, AB, BB or BE. β-Lg genotype could either be AA, AB or BB. There where

between one to four cows with each of the composite genotypes, (*Table 10, Appendix 1).* Milk form the same cows where used when determining casein profile and glycolysation in relation to both β-CN and κ-CN genotype.

The Casein profile was determined as the share (%) of different casein types of the total casein content. The glycosylation rate of κ-CN in milk with the different κ-CN genotypes was also measured.

2.5.2 Determination of casein profile and casein number in milk serum phase

LC/ESI-MS was used to determine the casein profile in MSP with different genotypes of β-CN. Casein number, which is the "total amount of casein" divided by "total amount of protein" was also measured with LC/ESI-MS using MSP derived from tempered and cold milk, respectively.

2.6 1D-SDS-Page

1D-SDS-Page was performed in order to investigate and compare the leakage of κ-CN monomers and multimers in tempered and cold MSP. The relevance of κ-CN mono- and multimers for the rest of the study was deemed to be low, thus method, results and discussion for the 1D-SDS-Page experiment are found in *Appendix 2*.

2.7 Statistical analysis

Statistical analyses were performed when the samples were analysed in duplicates or more. Statistical analysis was also used when a large number of individual samples where used, i.e. determination of casein profile and glycosylation in relation to genotype. ANOVA at 95 % confidence interval was performed to test for statistical significance. $p<0.05$ was established as the significance level. Tukey pairwise comparison test was used for investigation of the differences between and within groups of samples. Both ANOVA and Tukey test were performed using Minitab® 19.2020.1 (Minitab Inc., State College, PA, USA)

Results

3.1 Genotype screening by LC/MS-ESI

Due to the low number of cows from other breeds, only the genetic variants and genotypes found in Holstein cows have been included in the results.

The two most common β-CN genotypes, A2A2 and A2I, were present in a majority of DH cows (62 %). The most common κ-CN genotype, AB, was present in 50 % of the cows. The most common β-Lg genotype, AA, was present in 43 % of the cows (*Table 1*).

The most common β-CN allele was A2, which was three times more frequent than the β-CN A1 allele and 2.5 times more frequent than the I allele. The most common κ-CN allele was B, which was 62 % more frequent than the κ-CN A allele. The β-Lg allele A was 73 % more frequent than the β-Lg B allele (*Table 1*).

In total, 42 different composite genotypes with respect to κ-CN, β-CN, β-Lg, were found among the 216 DH cows, as seen in *Table 2.* Most of the cows (58 %) had one of the 10 most common composite genotypes (see the first 10 composite genotypes in *Table 2*). The β-CN A2 allele was found in 29 of the 42 composite genotypes, of which nine were β-CN A2A2 homozygotes and 20 β-CN heterozygotes with one A2 allele. The β-CN A1 allele was present in 23 of the 42 composite genotypes, of which five were A1A1 homozygotes. The β-CN I allele was present in 16 composite genotypes of which two were II homozygotes. The three most common κ-CN genotypes, AB, BB and AA were present in 14, 17 and 7 of the composite genotypes, respectively. Of the 16 composite genotypes with β-CN I allele, nine were homozygotes for the κ-CN genotype BB and only two did not have a κ-CN B allele (*Table 2*).

Table 1 Frequencies of genotypes and alleles of β-CN, κ-CN and β-Lg in Danish Holstein cows

Protein type	*Genotype frequency[1] (%)*								*Allele frequency[2] (%)*				
β-CN	*A1A1*	*A1A2*	*A1I*	*A2A2*	*A2I*	*A2B*	*II*	*A3A3*	*A1*	*A2*	*A3*	*B*	*I*
	3.24	21.30	7.41	32.87	29.17	0.46	5.09	0.46	17.59	58.33	0.46	0.23	23.37
κ-CN	*AA*		*AB*		*BB*		*AE*	*BE*	*A*		*B*		*E*
	12.96		49.54		35.19		0.46	1.85	37.96		60.88		1.16
β-Lg	*AA*			*AB*			*BB*		*A*			*B*	
	42.59			41.67			15.74		63.43			36.57	

[1]*Genotype frequency: the percentage of cows with a certain genotype (N=216)*

[2]*Allele frequencies: the percentage of alleles of a certain genetic variant. (N=432, since there are two alleles per genotype). CN = casein. Lg = lactoglobulin.*

Table 2 List of the composite genotypes (C. genotype) with respect to κ-CN, β-CN and β-Lg in Danish Holstein cows. The number of cows with each composite genotype is indicated (N=216)

C. genotype	**Nr. of cows**	**C. genotype**	**Nr. of cows**	**C. genotype**	**Nr. of cows**	**C. genotype**	**Nr. of cows**	**C. genotype**	**Nr. of cows**
AB A2A2 AB[1]	19	BB II AA	7	AB A2I BB	4	AA A2I BB	1	BB II BB	1
AB A1A2 AA	17	AA A2A2 AA	6	BB A1I AA	4	AB A1A1 AB	1	BE A1A1 AA	1
AB A2I AB	16	BB A1A2 AA	6	AB A1I AB	3	AB A1A1 BB	1	BE A1A2 AA	1
BB A2I AA	15	BB A2A2 AA	6	BB A1A2 AB	3	AB A1I AA	1	BE A1A2 AB	1
AB A2I AA	12	AA A2A2 BB	5	BB II AB	3	AB A2B AA	1		
BB A2I AB	10	BB A1I AB	5	AA A1A1 AB	2	AB A3A3 AA	1		
AA A2A2 AB	10	BB A2A1 BB	5	AA A1A2 AA	2	AE A1A2 AA	1		
AB A1A2 AB	9	BB A2A2 AB	5	AA A1A2 AB	2	BB A1A1 AB	1		
AB A2A2 AA	9	BB A2I BB	5	BB A2A2 BB	2	BB A1I BB	1		
AB A2A2 BB	9	AB A1A2 BB	4	BE A1I AA	2	BB A1A2 BB	1		

[1]*The composite genotypes are written in the order of κ-CN, β-CN, β-Lg. CN= casein. Lg = lactoglobulin*

3.2 Milk composition

The gross composition, i.e. content of protein, casein and lactose, in the WM were compared between the ß-CN genotypes (*Table 3)*. The protein content was on average 10 % and 13 % higher in β-CN II genotype compared to A2A2 genotype and A1A1 genotype, respectively ($p<0.001$). Further, the protein content was on average 3 % higher in β-CN A2A2 compared to A1A1 ($p<0.001$). The casein content in milk associated with β-CN II genotype was on average 16 % and 20 % higher compared to the A2A2 and A1A1 genotype, respectively. Further, the casein content was on average 3 % higher in β-CN A2A2 compared to A1A1 ($p<0.001$). The lactose content in milk associated with β-CN II was on average 4 % and 2 % higher compared to β-CN A2A2 and A1A1, respectively. Further, the lactose content was on average 2 % higher in β-CN A1A1 compared to A2A2 ($p<0.001$).

Table 3 Gross composition of protein, casein and lactose content in whole, pooled milk samples from Danish Holstein cows with different β-casein genotypes. Differences between the groups (row wise) were evaluated by Tukey pairwise comparison test and were considered significant at p<0.05.

	Gross composition in β-CN genotype (%)			*p-value*
	A1A1[1]	**A2A2**	**II**	
Protein	3.35±0.01[a]	3.45±0.00[b]	3.80±0.00[c]	p<0.001
Casein	2.47±0.02[a]	2.55±0.01[b]	2.97±0.01[c]	p<0.001
Lactose	4.73±0.01[a]	4.65±0.00[b]	4.82±0.00[c]	p<0.001

Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. A1A1, A2A2 and II represent the β-CN genotypes. CN = Casein. WM = Whole Milk; Each mean value is based on six tehcncial replciates of one milk sample, N=6.

3.3 Micelle size

The size of native- and depleted micelles were compared within and between genotypes (*Table 4)*. Between the groups, the native micelles associated with the β-CN genotype A1A1 were on average 11 % and 7% larger compared to A2A2 and II, respectively ($p=0.002$). The depleted micelles were on average 14 % and 8 % larger in A1A1 genotype compared to A2A2 and II, respectively ($p<0.001$). The depleted micelles in milk associated to the II genotype, were on average 6 % larger compared to A2A2. ($p<0.001$). Within the group A1A1 the native micelles were on average 4 % smaller compared to depleted micelles ($p=0.027$). No significant difference in micelle size were observed between native and depleted micelles within the A2A2 and II genotype groups.

Table 4 Average micelle size in skim milk samples, based on the β-casein genotype and temperature treatment. Differences between (row wise) and within (column wise) genotypes were evaluated by Tukey pairwise comparison test and were considered significant at p<0.05.

	Average micelle size (nm) of β-CN genotypes			*p-Value*
	A1A1	**A2A2**	**II**	
Native micelles	164.80±3.00[1,a]	148.00±4.21[b]	153.40±1.93[b]	p=0.002
Depleted micelles	171.10±1.04[2,a]	150.00±1.80[b]	158.83±3.10[c]	p<0.001
p-Value	p=0.027	p=0.492	p=0.062	

Native micelles = tempered milk; depleted micelles = cold milk. Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. Means within a column that do not share a number are significantly different. A1A1, A2A2 and II represent the β-CN genotypes. CN = casein. Each mean value is based on three tehcncial replicates of one milk sample, N=3.

3.4 Coagulation properties

The average RCT was compared both between and within the groups *(Table 5).* Each group in *Table 5* was a combination of a temperature treatment (tempered or cold) and a β-CN genotype. combination of the the β-CN genotypes and different temperature treatments (*Table 5).* The average RCT was also coampared within and between group consisting of all the temperature treatment, in *Table 11, Appendix 3.* For the tempered milk, there was no significant difference in RCT, neither between nor within β-CN genotypes, when coagulation was compared at native and adjusted pH, respectively.

For the cold milk at pH 6.72, milk associated to genotypes A1A1 and A2A2 had on average 30 % and 39 %, respectively, shorter RCT than genotype II (p=0.017). At pH 6.5, the RCT was on average 90 % longer in II genotype compared to A1A1 (p<0.001). For cold milk associated to the A2A2 genotype at pH 6.5, the RCT was extremely short compared to the other two genotypes. This is likely due to an artifact and considering that all the measurements consisted of a few technical replicates of the same milk sample, it might not be considered as a true value (p<0.001).

Within the group cold milk A1A1, milk at pH 6.72 had on average 29 % longer RCT than at pH 6.5 (p=0.002).

Table 5 Rennet coagulation time (s) in skim milk based on the β-casein genotypes, temperature treatment and pH. Differences between (row wise) and within (column wise) genotypes were evaluated by Tukey pairwise comparison test and were considered significant at p<0.05.

Cooling treatment	*pH-treatment*	*Average Rennet coagulation time (s)*			*p-value*
		A1A1	**A2A2**	**II**	
Tempered milk	pH 6.72	404±282	532±82	852±30	p=0.157
	pH 6.5	717±96	556±180	833±26	p=0.209
	p-value	p=0.276	p=0.879	p=0.577	
Cold milk	pH 6.72	739±9[1,a]	645±45[1,a]	1050±101[b]	p=0.017
	pH 6.5	574±8[2,a]	49±2.[2,b]	1087±89[c]	p<0.001
	p-value	p=0.002	p=0.003	p=0.736	

Tempered milk = milk with native micelles. Cold milk = milk with depleted micelles. Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. Means within a column that do not share a number are significantly different. A1A1, A2A2 and II represent the β-casein genotypes. Each mean value is based on two technical replicates of one milk sample.

The curd firmness (CF) was determined by the elasticity index after twenty minutes from the rennet addition. The curd firmness was compared both between and within groups, β-CN genotypes (*Table 6)*. Groups in *Table 6* had the same definition as in *Table 5.* The average CF was also compared within all the temperature treatments in the different β-CN groups in *Table 12, Appendix 3.* In the tempered milk at pH 6.72, the A1A1 genotype had on average 103 % higher CF than milk from the II genotype (p=0.031). In the cold milk at pH 6.5, the A1A1 genotype had on average 76 % and 461 % higher CF than milk from the A2A2 and II genotypes, respectively. Moreover, the A2A2 genotype had on average 219 % higher CF than genotype II (p<0.001). In tempered milk, the genotype A2A2 had on average 27 % higher CF at pH 6.72 than at pH 6.5 (p=0.008). In cold milk, genotype A1A1 had on average 57 % lower CF at pH 6.72 than at pH 6.5 (p=0.028). Also in cold milk, A2A2 had

on average 31 % lower CF at pH 6.72 than in milk at pH 6.5 (p=0.035). Genotype II, had on average 46 % lower CF at pH 6.72 than at pH 6.5 in cold milk (p=0.003).

Table 6 Curd firmness in skim milk, based of the β-casein genotypes, cooling/tempering treatment and pH. Differences between (row wise) and within (column wise) genotypes were evaluated by Tukey pairwise comparison test and were considered significant at p<0.05.

Cooling treatment	*pH-treatment*	*Average curd firmness (elasticity index, 20 min after addition of rennet)*			*p-value*
		A1A1	A2A2	II	
Tempered milk	pH 6.72	$0.00701 \pm 0.00117^{1,a}$	$0.00542 \pm 0,00009^{1,ab}$	$0.00346 \pm 0.00013^{1,b}$	p=0.031
	pH 6.5	0.00433 ± 0.00009^{12}	0.00426 ± 0.00012^{2}	0.0041 ± 0.00028^{2}	p=0.499
	p-value	p=0.084	p=0.008	p=0.098	
Cold milk	pH 6.72	0.00236 ± 0.00074^{2}	0.00216 ± 0.00024^{3}	0.00053 ± 0.00004^{3}	p=0.048
	pH 6.5	$0.00550 \pm 0.00011^{1,a}$	$0.00313 \pm 0.00011^{4,b}$	$0.00098 \pm 0.0^{3,c}$	p<0.001
	p-value	p=0.028	p=0.035	p=0.003	

Tempered milk = milk with native micelles. Cold milk = milk with depleted micelles. Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. Means within a column that do not share a number are significantly different. A1A1, A2A2 and II represent the β-casein genotypes. Each mean value is based on two technical replicates of one milk sample, N = 2

3.5 Casein and protein profile in milk with different genotypes

The share of the caseins αs1-CN, αs2-CN, β-CN, κ-CN for the different β-CN genotypes were compared (*Table 7)*. No significant differences were observed between different genotypes of β-CN, and their share of the caseins αs1-CN, αs2-CN, β-CN, κ-CN.

Table 7 The share of the caseins αs1-CN, αs2-CN, β-CN, κ-CN (%) of total casein content in milk from Danish Holstein cows with different β-CN genotypes. Differences between the genotypes (row wise) were evaluated by Tukey pairwise comparison and were considered significant at p<0.05.

Casein share	*β-CN genotype*			*p-value*
	A1A1	**A2A2**	**II**	
αs1-CN	9.76±0.76	10.20±1.15	10.33±0.36	p=0.638
αs2-CN	34.78±1.64	34.47±1.04	34.32±0.64	p=0.777
β-CN	42.26±1.59	41.25±1.09	41.90±0.54	p=0.131
κ-CN	12.62±0.87	12.30±1.49	12.48±0.50	p=0.881

Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. A1A1, A2A2, and II represent the β-CN genotypes. CN = casein. Number of cows with the different genotypes; A1A1 N=4, A2A2 N=20, II N=8.

The share of the caseins αs1-CN, αs2-CN, β-CN, κ-CN, and the glycosylation levels for different κ-CN genotypes were also compared (*Table 8)*. κ-CN AA genotype had on average 10 % higher share of αs1-CN in its casein profile than in BB genotype (p=0.028). κ-CN genotype AA had on average 14 % lower share of κ-CN in its casein profile than κ-CN AB- and BB genotype. The level of glycosylated κ-CN was 16.04 % higher in the κ-CN AA genotype compared to BB (p=0.009).

Table 8 The share of the caseins αs1-CN, αs2-CN, β-CN, κ-CN (%),of total casein content, and the level of glycosylated κ-CN in milk from Danish Holstein cows with different κ-CN genotypes. Differences between the genotypes (row wise) were evaluated by Tukey pairwise comparison and were considered significant at p<0.05.

Caseins share	*κ-CN genotype*			*p-value*
	AA	**AB**	**BB**	
αs1-CN	10.96 ± 0.85^{a}	10.02 ± 0.95^{ab}	9.98 ± 0.8^{b}	p=0.028
αs2-CN	34.02±0.84	34.68±0.99	34.63±1.01	p=0.301
β-CN	41.73±1.55	41.52±1.01	41.45±0.78	p=0.825
κ-CN	10.90 ± 1.14^{a}	12.57 ± 0.44^{b}	12.83 ± 0.87^{b}	p<0.001
Gκ-CN (%)	46.31 ± 6.10^{a}	42.17 ± 5.10^{ab}	38.88 ± 4.96^{b}	p=0.009

Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. AA, AB, and BB represents the κ-CN *genotypes. CN = casein. Gκ-CN = glycosylated k-casein. Number of cows within different* κ-CN *genotypes;* κ-CN *AA N=8,* κ-CN *AB N=9,* κ-CN *BB N=17.*

3.5.1 Casein and protein profile in milk serum phase

The share (%) of the the caseins αs1-CN, αs2-CN, β-CN, κ-CN out of total casein content, and the casein number, determined in MSP using LC/ESI-MS analysis, is shown in *Table 9*. Only one measurement of one sample per genotype and cold/tempered treatment was analysed. Thus, statistical analysis was not possible

to provide, and the results and differences between genotypes and temperature treatments are only numerical.

Tempered milk A2A2 genotype had 27 % and 46 % higher share of β-CN than corresponding A1A1 and II, respectively. Tempered milk A1A1 genotype had on average 15 % higher share of β-CN than corresponding II. Tempered milk A2A2 genotype had 78 % and 22 % higher share of casein number than corresponding A1A1 and II, respectively.

Cold milk A2A2 genotype had 15 % and 16 % higher share of β-CN than corresponding A1A1 and II, respectively. Cold milk from II genotype had 134 % and 56 % higher casein number than corresponding A1A1 and A2A2, respectively. β-CN share was always higher in cold MSP samples compared to corresponding tempered samples. It was 24 % higher in cold milk from A1A1 genotype compared to tempered, 12 % higher in A2A2 and 41 % higher in II. The percentual difference of β-CN in II genotype was 235 % and 72 % higher than the percentual difference for A1A1 and A2A2, respectively. The casein number in tempered MSP associated with A1A1 genotype was 18 % lower than in corresponding cold MSP. The casein number in tempered MSP with A2A2 genotype was 16 % lower compared with corresponding cold MSP. The casein number was 60 % higher in cold MSP from II genotype compared to tempered *(Table 13, Appendix 4).*

Table 9 Share (%) of the the caseins αs1-CN, αs2-CN, β-CN, κ-CN out of total casein content and casein number in the milk serum phase after different temperature treatments and for different β-casein genotypes.

Genotype end treatments	*αs1-CN*	*αs2-CN*	*β-CN*	*κ-CN*	*CN number*
A1A1 Tempered	11.67	28.08	44.60	12.88	19.65
A1A1 Cold	13.10	7.31	55.35	17.16	16.07
A2A2 Tempered	13.30	8.84	56.59	18.66	28.58
A2A2 Cold	13.51	12.19	63.57	8.87	24.06
II Tempered	19.05	24.49	38.77	17.17	23.36
II Cold	14.85	12.63	54.80	10.59	37.47

Tempered = milk with native micelles. Cold = milk with depleted micelles. Casein number = CN/total protein. CN = casein. MSP = milk serum phase.

Discussion

4.1 Frequencies of genotypes and alleles

All the cows in this study were from one single research herd. This is opposed to most other studies investigating frequencies of genetic variants and genotypes within several herds. The frequencies of alleles and genotypes should thus not be considered as representative for Danish Holstein as a whole. However, some interesting comparisons can be done with other frequency studies. The frequency of the β-CN I allele and κ-casein allele B were clearly higher than in most studies on Holstein. Moreover, in this study, κ-casein B was the most common κ-casein allele, while in other studies on Holstein, e.g., Poulsen et al (2013), and Cendron et al (2021), κ-casein A was the most represented allele. In this study, the β-CN I allele frequency was exceptionally high, 23.37 % (*Table 1*), compared to other studies sush as Poulsen et al. (2013) and Massella et al. (2021) where the frequency was found to vary between 2-8 %. Further, the β-CN A2 allele frequency in this study was within the same range as in most other studies on Holstein. In contrast, the frequency of the β-CN A1 allele was lower compared to other studies on Holstein. (Poulsen et al. 2013; Gustavson et al. 2014; Cendron et al. 2021; Massella et al. 2021).

Interestingly, the genetic variant β-CN I mostly occurred in cows with at least one κ-casein B allele in this study (*Table 2*), indicating an association between β-CN I and κ-casein B. This finding is in accordance with the study by Poulsen et al. (2013), where it was hypothesised to be a result of genetic association. κ-casein B is commonly associated with better coagulation properties. Breeding for κ-casein B is thus of interest and could lead to an increase in κ-casein B frequency in various bovine populations (Chessa et a.l 2020; Växa 2023). If this would correspond to an increased frequency of the β-CN I allele, further knowledge of the effect of β-CN I on different technological properties of milk and dairy products would be of clear interest. However, Chessa et al. (2020), studying frequencies of genetic variants in large Holstein populations over time, did not find an increase of β-CN I despite observering an increase in κ-casein B frequency. Therefore, relation between β-CN

I and κ-casein B should be furthered studied to be able to conclude if there is an association or not.

4.2 Milk gross composition

In this study, the content of protein and casein in milk with different β-CN genotypes were in the following order: II > A2A2 > A1A1 (*Table)3*. Earlier studies, e.g., Cendron et al. (2021), have also observed higher protein and casein content associated to the β-CN A2 variant than to the β-CN A1. It is important to note that both κ-CN genetic variants A and B were present in the pooled milk with β-CN genotype A1A1, whereas the the pooled milk with β-CN genotype A2A2 and II only had κ-CN genetic variant B. This is relevant, given that κ-CN genetic variant B has also been associated with higher levels of protein and casein by e.g. Cendron et al. 2021. Therefore, κ-CN B variant might have contributed to higher protein and casein content in the milk from β-CN A2A2- and β-CN II genotype in this study.
The indication that milk associated to the β-CN II genotype could have higher content of protein, casein and lactose is of clear interest. Especially considering that milk payment systems include protein content as one of the major parameters contributing to the milk price (Arla 2023). To get a better understanding about the relation between β-CN genotype II and milk gross composition, studies with a larger sample set from several herds should be conducted. Further, the milk composition from cows with β-CN genotype II should be compared with milk associated to other β-CN genotypes, including heterozygotes with only one I allele.

4.3 Micelle size

The results given by dynamic light scattering indicated that pooled milk with β-CN genotype A1A1 had larger average micelle size than the genotypes A2A2 and II, both when micelles were native and depleted (*Table 4)*. To our knowledge, there are only a few previous studies of the relation between β-CN genotype and micelle size that have been conducted. Day et al (2015) found β-CN A1 and A2 alleles associated to both large and small micelles. The size of the large micelles in their study was comparable with the size of β-CN A1A1 micelles in this study. Also, the size of small micelles in their study was in agreement with the size of β-CN A2A2 and II micelles in this study. In several studies, e.g., Bijl et al (2014), no relation between β-CN genotype and average micelle size was found. It is also important to note that in this study, the milk from cows with A1A1 genotype partly consisted of milk from cows with the κ-casein genotypes AA, AB and BB as opposed to milk with β-CN A2A2 and II, which only had κ-casein BB genotype. Indeed, other studies e.t., Bijl et al. (2014) and Walsh et al. (1998), have shown that milk

associated to the κ-CN AA genotype, and sometimes the AB genotype, had larger average micelle size than milk with the κ-CN BB genotype. In Day et al. (2015), κ-CN AA, was mostly found in large micelles and κ-CN AB and BB in small micelles. The results of this study in combination with the results in earlier studies, could thus indicate that κ-CN had larger impact on micelle size than β-CN.

Only β-CN genotype A1A1 showed a significant difference in micelle size between native and depleted micelles. No potential explanation for this could be found in the literature. One hypothesis could be that the difference is associated to the presence of κ-CN genotype AA and AB in β-CN genotype A1A1 milk. It could also be due to small sample size. Overall, there is no scientific consensus whether cold depletion has any effect on micelle size. Whether there is an effect or not seem to vary with method of sample preparation and measurement of micelle size (Duerash et al. 2020). In studies by Maciel et al. (2014) and Duerach et al. (2020), who used methods for sample preparation and measurement of casein micelle size similar to the ones used in this study, no significant difference between micelle sizes in tempered and cold milk, respectively, were observed.

4.4 Coagulation

The RCT for milk with the β-CN II genotype was significantly longer compared to that of β-CN A1A1 and A2A2 genotypes in cold milk, while there was no significant difference between the genotypes when the milk was tempered (*Table 5*). This was the case for milk both at pH 6.7 and pH 6.5. This could indicate that the cooling and subsequent depletion of caseins had a greater effect on milk with the β-CN II genotype than for A1A1/A2A2 genotypes. However, the same trend could not be observed for curd firmness (*Table 6*).

There were observed inconsistencies in association with the water bath treatment. Water bath treatment of tempered milk at pH 6.72 and 6.5, respectively, were conducted at the same time point. However, tempered milk at pH 6.72 was subject to MS-DWS analysis approximately one hour before the tempered milk at pH 6.5. Lower pH usually yield shorter RCT, which was not the case for the tempered milk in this study. The uneven water treatment could explain the lack of a significant difference, but its important to note that there where only one sample for every combination of pH and temperature treatment, and thus the data is overall unreliable. Further, the A2A2 cold milk and adjusted pH showed a remarkably low RCT which is difficult to explain. One factor to consider is colloidal calcium, where higher levels are associated with better coagulation properties. Further, it is known that calcium is partly dissociated from the micelle during cooling (Farrell et al.

2004; Caroli et al. 2009). However, calcium concentration was not measured in this study and thus it could be considered as an possible factor.
The β-Lg genotype is another factor that is belived to effect coagulation properties (Cendron et al. 2021). However, all the pooled milk samples (β-CN genotypes A1A1, A2A2 and II) had the same β-Lg genotype, i.e., β-Lg AB.

4.5 Casein profile in relation to the κ-CN and β-CN genotypes

In this study, an association between κ-CN genotype and casein profile was observed (*Table 7*), while genetic variants of β-CN did not appear to affect the casein profile (*Table 8*). Milk with κ-CN genetic variant B (κ-CN genotypes BB and AB) was associated with a higher relative concentration of κ-CN in this study. This has also been shown in earlier studies, e.g. Jensen et al (2015). Higher level of κ-CN has in turn been associated with smaller micelles in earlier studies (Dalgleish & Corredig 2012). In this study, pooled milk with only the κ-CN genetic variant B (i.e. homozygotes) had smaller micelles than pooled milk with both κ-CN genetic variant A and B. Thus, this study might indicate a relation between κ-CN genetic variant B, smaller micelle size and higher κ-CN content. The higher κ-CN proportion in the milk with κ-CN AB and BB genotypes in this study could hypothetically be connected to the lower relative content of αs1-CN in milk with the κ-CN genotypes AB and BB. The higher content of κ-CN might compensate the lower levels of αs1-CN. However, the results only indicate differences in the relative concentrations (proportion) of the caseins, and not the actual concentrations. Conducting RP-HPLC analysis using casein standards, as in Duerach et al. (2020) could give the true concentrations.

The glycosylation level of κ-CN was higher in milk with κ-CN genotype AA than in milk with the BB genotype. Earlier studies have shown the opposite, with higher glycosylation levels in κ-CN genotype BB than AA (Hewa Nadugala et al. 2022). No earlier studies showing higher levels of glycosylation in κ-CN genotype AA compared to κ-CN BB could be found. Coolbear et al. (1996) showed that glycosylation level can vary between samples with the same genotype. The higher glycosylation levels in κ-CN genotype AA could thus be explained by variation within the small sample set of κ-CN genotypes in this study. The variation could have been affected by other factors sush as stage of lactation and lactation numbers of the cows (DeVries et al. 2015). These factors where not taken into account for in the selection of cows for the pol milk and could thus have affected the different results of this study.

4.6 Cold induced leakage of caseins

To the authors knowledge, this is the first study to investigate the relationship between different genetic variants and/or genotypes of β-CN, and depletion of caseins induced by cooling. The relative concentration of β-CN was higher in MSP from cold milk compared to tempered for all genotypes, *Table 9*. This was expected, given that earlier studies have shown that β-CN deplete to a higher extent than other caseins as a result of cooling (Dalgleish & Corredig 2012). In this study, both tempered and cold milk with β-CN A2A2 genotype had the highest share of β-CN. However, the ß-CN share in tempered and cold MSP, respectively, differed most in MSP associated to the ß-CN II genotype, which, thus, possibly had the highest depletion rate of β-CN (Table 10, Appendix 2). β-CN genotype A2A2 had highest casein number in tempered milk while β-CN genotype II had the highest casein number in the same milk type (*Table 9*). In fact, β-CN II was the only genotype where casein number was higher in cold than tempered milk. This indicates that β-CN variant I can be more prone to the cold depletion. Further supporting this indication, milk with β-CN II genotype had significantly longer RCT than A1A1 and A2A2 with tempered milk, but not with cold milk (*Table 5*). However, the same trend was not seen with curd firmness (*Table 6*). Furthermore, it is difficult to draw any conclusions without knowing the actual concentrations of the different caseins, and thus, as proposed earlier, a study using RP-HPLC with standards should be done. Further, the differences are only numerical, because of the low number of biological replicates, which makes the results uncertain.

No tests looking into potential mechanisms that could investigate the extent of leakage of different β-CN genetic variants were conducted. However, the amino acids that differ between the A1 and I alleles (i.e., histidine and leucine, respectively) compared to the A2 allele (proline and methionine, respectively) have higher hydrophobicity index (Monera et al. 1995). β-CN has been proposed to be more prone to cold leakage due to its relatively high hydrophobicity (Dalgleish & Corredig 2012). Hence, if cold induced leakage would be higher for the β-CN genotypes A1 and I, than for A2, it might be due to A1 and I having higher hydrophobicity. However, when it comes to the amino acid difference between II and A1A1, proline and arginine instead of histidine and methionine, proline is more, arginine less hydrophobic in the A1A1 genotype (Monera et al. 1995). As stated before, the sample size was minimal with only one sample for each combination of genotype and temperature treatment. More data is needed to see if there is any relation between β-CN genetic variants and the levels of cold induced leakage. The level of hydrophobicity of the genetic variants of β-CN could be a potential factor/mechanism worth looking more into.

Conclusion

While several associations between the genetic variants and genotypes of β-CN/κ-CN, and different properties of the milk could be observed, the sample size was usually very small. This makes it difficult to draw any conclusions, although the results still gave some interesting indications. Thus, this project should be considered a pilot study.

This study indicated that the β-CN variant I was more prone to cold induced depletion than β-CN variants A and B. The β-CN variant I was also associated to a higher relative concentration of β-CN in the milk, and a higher casein number in the milk serum phase compared to the β-CN A and B variants, although the results were only numerical and thus associated with a high uncertainty. However, this is to the authors knowledge the first study indicating a relation between the genetic variant of a casein and cold induced depletion. Further, the differences in RCT between the β-CN I and β-CN A or B variants were significant with cold but not with tempered milk. This might indicate that the effect of cooling on coagulation properties could differ between different genetic variants/genotypes of β-CN.
β-CN variant I is relatively rare, which is why comparisons with β-CN A1 and A2, have been overlooked in the research. This study showed that the β-CN variant I could be linked with higher protein and casein content. Further, results suggested that β-CN I has an association with the κ-CN variant B, which is of interest given that κ-CN B is positively associated with better coagulation properties. All these indications show that the impact of the β-CN I on different milk properties certainly should be of higher interest in future studies.
Another interesting indication in this study was that genetic variants/genotypes of κ-CN seemed to have greater impact on micelle size than genetic variants/genotypes of β-CN.

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Popular science summary

Cheese is a popular milk product of high importance for milk producers and dairy industry. Cheese making is possible thanks to a group of milk proteins named caseins. Caseins are normally arranged in spheric structures, known as micelles. The addition of certain enzymes to milk, usually chymosin and/or pepsin, causes the micelles to aggregate, forming a gel or curd which is the basis for the resulting cheese.

There are four main types of caseins, i.e., αs1-, β-, αs2-, and κ-casein. κ-casein is located on the outside of the micelles and is the casein that hinders the micelles from aggregating when the "curd forming" enzymes are not present. β-casein is the most abundant casein, and located inside the micelle. Cooling can cause caseins, especially β-casein, to leak out of the micelle, and this can in turn lead to less effective cheese production due to lowering of the cheese yield.

There are several genetic variants of the four casein types. Genetic variants have been shown to affect different properties of milk, e.g., the amount of protein in milk and cheese production qualities. This study aimed to investigate the relation between different genetic variants of β-casein and milk properties. There was a special interest in investigating if some genetic variants leak out more than others from the micelle in connection to cooling of the milk. The genetic variants of interest were β-casein A1, A2 and I. β-casein A1 can be called number **#1**, being linked to better production qualities in earlier studies. β-casein I has barely been studied before, which makes it extra interesting.

In the study, β-casein variant I had the highest protein and relative casein content compared to β-casein variants A1 and A2. This makes β-casein variant I even more interesting given that milk payment systems are partly based on protein content. The results regarding the effect of genetic variants on technological properties were overall quite inconclusive. However, when the milk had been stored cold, there was a significant difference between milk with β-casein variants A1 or A2, and β-casein variant I for "rennet coagulation time", i.e. the time it takes after addition of the enzyme until the milk begins to form a gel. Meanwhile, there was no significant difference in tempered milk. This result become extra interesting as it indicates that β-casein variant I compared to β-casein variants A1 and A2, may leak more due to

cooling. The study thus indicates that the level of cold induced depletion of caseins from the micelles might vary between different genetic variants of β-casein, affecting the technological quality of the milk.

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Appendix

Appendix 1. Composite genotype of cows used when determing casein profile and glycosylation rate in relation to genotype

Table 10. List of the composite genotypes used in determination of casein profile and glycosylation rate in relation to genotype with LC/ESI-MS, with respect to κ-CN, β-CN and β-Lg in Danish Holstein cows. The number of cows with each composite genotype is indicated (N=34)

Composite genotype	Number of cows
AA A1A1 AB[1]	2
AB A1A1 AB	1
BB A1A1 AB	1
BE A1A1 AA	1
AA A2A2 AA	2
AA A2A2 AB	2
AA A2A2 BB	1
AB A2A2 AA	4
AB A2A2 BB	4
BB A2A2 AA	2
BB A2A2 AB	3
BB A2A2 BB	3
BB II AA	4
BB II AB	3
BB II BB	1

[1]*The composite genotypes are written in the order of κ-CN, β-CN, β-Lg. CN = casein. Lg = lactoglobulin*

Appendix 2. 1D-SDS-Page

Method

1D-SDS-page sample preparation

To prepare samples for 1D-SDS-Page, 44 µl SM and 44 µl Laemmli samplebuffer (1:1 ratio) was added to a 1.5 ml Eppendorf tubes (Eppendorf, Hamburg, Germany). The Laemmli samplebuffer consisted of 20 mM Tris-buffer, 2 % sodium dodecyl sulphate/SDS (Serva, Heidelbergt, Germany), 85 % glycerol (Merck), MilliQ water (56 ml/L) and Bromphenol blue (Merck). The tube was then vortexed for a few seconds with an *IKA® VORTEX 3* (IKA, Staufen, Germany). The milk samples used in 1D-SDS-Page were frozen at -80 ℃ overnight and then stored at -20 ℃ for up to 18 days.

1D-SDS-Page

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D-SDS-Page) was used to visualize the caseins that had leaked out into the MSP, and other proteins present in MSP sush as whey proteins. The MSP was derived from both tempered and cold milk. AnykD™ Criterion™ TGX™ Precast Midi Protein Gels (Biorad Laboratories Inc, Hercules, USA) were used. MSP samples diluted 1+1 with Laemmli sample buffer were thawed at room temperature. For every type of pooled MSP, four different samples were prepared: tempered MSP without and with reducing conditions, respectively, and cold MSP without and with reducing conditions, respectively. Reducing conditions were achived with DTE as a reducing agent. Reducing agents break disuplhide bonds, thus breaking down quartenary structure, separating the subunits of the quaertenary structure (Medschoolcoach 2023). For samples with reducing conditions, 0.8 µl 1 M DTE was added after thawing. After thawing, all samples were vortexed for a few seconds each with an *IKA® VORTEX 3* (IKA, Staufen, Germany). After that, the samples were placed on a heating block set to 90 ℃, for 2 minutes. Afterwards they were centrifuged in a Eppendorf 5417R Refrigerated Centrifuge (Eppendorf, Germany) at 10 000 RCF, for 1 minute at 20 ℃.

The gel was placed in a criterion cell (Bio-Rad, Hercules, USA) filled with Laemmli working soloution. The Laemmli running buffer consisted of 25 mM Tris-base, 192 mM glycine (WWR Chemicals), 0.1 % SDS (Serva, Heidelbergt, Germany), and MilliQ water. 15 µl of each MSP sample was pipeted in each well of the gel. As reference 5 µl of *Spectra Multicolor Broad Range Protein Ladder* (Thermo Scientific, Rockford, USA) was used. The voltage was set to 200 V (constantly) and the run took approximately 30 minutes.

After the run, the gel was covered in fix soloution consisting of 50 % ethanol, 8 % phopsporic acid and 42 % MiliQ water. A container with gel and fix soloution was

placed on shaking table for 2 hours. Afterwards the gel was stained. The staining consisted of 0.1461 M aliminium sulphate, 87.6 % MiliQ water, 1.99 % phopsoric acid (Supenco, Bellefonte, PA, USA), 0.004 % coomasie brilliant blue (Sigma Aldrich, Darmstadt, Germany) and 9.57 % ethanol (VWR Chemicals, Radnor, PA, USA).

Results

For each β-CN genotype there were a non-reduced tempered, a reduced tempered, a non-reduced cold and reduced tempered sample. With the protein ladder the gel had 13 lanes in total, *Figure 1*. For the non-reduced samples, for all β-genotypes, and both tempered and cold, several thick bands in the upper half of their lanes were observed.For the reduced samples, both tempered and cold samples of all β-genotypes, these thick bands in the upper half of the lane were either not observed, or they were thinner, located at the same position as the thick bands observed for the non-reduced samples.
No visible differences could be found between the bands in the lower half of the gel, between the different lanes of MSP sample. No visible differences could be seen between the bands from tempered and cold MSP, respectively.
Protein bands from the tempered and non-reduced β-CN A1A1 MSP were observed to partly leak into the protein ladder.

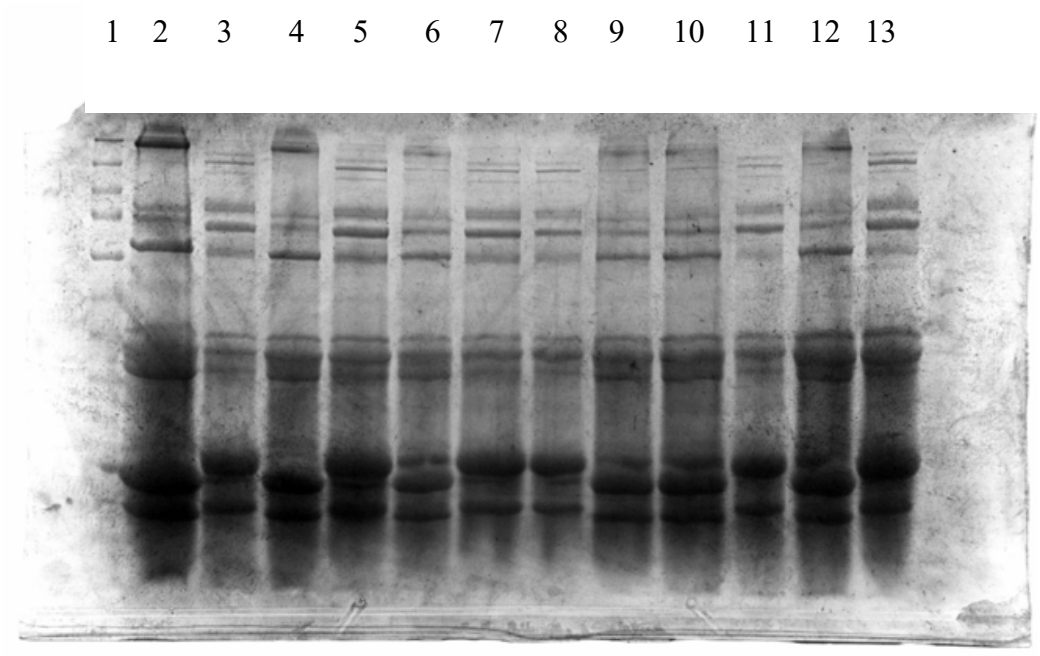

Figure 1. Proteins from milk serum phase (MSP) in 1D-SDS-Page. Sample lanes from left to right; Lane 1: Protein Ladder. Lane 2: Non-reduced tempered β-CN A1A1. Lane 3: Reduced tempered β-CN A1A1. Lane 4: Non-reduced cold β-CN A1A1. Lane 5: Reduced cold β-CN A1A1. Lane 6: Non-reduced tempered β-CN A2A2. Lane 7: Reduced tempered β-CN A2A2. Lane 8: Reduced cold β-CN

A2A2. Lane 9: Non-reduced cold β-CN A2A2. Lane 10: Non-reduced tempered β-CN II. Lane 11: Reduced tempered β-CN II. Lane 12: Non-reduced cold β-CN II. Lane 13: Reduced cold β-CN II.

Discussion

With the contamination of the protein ladder, it was difficult to predict the molecular mass of different bands (*Figure 1*). Comparisions of results from former studies, e.g., Resmussen et al. (1992) helped in indicating the identities of proteins present in different bands. The thick bands that in this study were exclusively observed in non-reduced samples in the upper half of the gel, seems to match the κ-CN multimer bands seen in the study by Rasmussen et al (1992). No visible difference in thickness of these bands could be observed between MSP samples from tempered respective cold milk.

Appendix 3. Coagulation properties; additional tables

The average RCT was compared within the groups; column wise *Table 11.* Cold milk from β-CN genotype A2A2 at pH 6.5 had very short RCT, which might be due to an artifact and could does be considered to not be a true value ($p<0.001$).

Table 11 Rennet coagulation time (s) in skim milk (SM), based on the β-CN genotypes, cooling/tempering treatment and pH treatment. Differences between (row wise) and within (column wise) the groups were evaluated by Tukey pairwise comparison and were considered significant at $p<0.05$.

Cooling treatment	*pH-treatment*	*Average rennet coagulation time (s)*			*p-value*
		A1A1	**A2A2**	**II**	
Tempered milk	pH 6.72	404±282	532±82[1]	852±30	p=0.157
	pH 6.5	717±96	556±180[1]	833±26	p=0.209
Cold milk	pH 6.72	739±9[a]	645±45[1,a]	1050±102[b]	p=0.017
	pH 6.5	574±8[a]	49±2[2,b]	1087±89[c]	p<0.001
	p-value	p=0.236	p=0.014	p=0.046	

Tempered milk = milk with native micelles. Cold milk = milk with depleted micelles. Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. Means within a column that do not share a number are significantly different. A1A1, A2A2 and II represents different β-CN genotypes. CN = casein RCT = rennet coagulation time. SM = skim milk; N = 2

The average curd firmness (CF) was compared within the groups; column wise *Table 12.* Within the group skim milk (SM) with β-CN genotype A1A1, tempered milk with pH 6.72 had on average 197 % higher CF than A1A1 cold milk pH 6.72 Further the A1A1 with combination of cold milk and pH 6.5 had on average 122 % higher CF than A1A1 cold milk pH 6.72 ($p=0.011$). Within the group SM with β-CN genotype A2A2, tempered milk with pH 6.72 had on average 27 %, 151 % and 73 % higher CF than corresponding tempered milk pH 6.5 and cold milk pH 6.72 and cold milk pH 6.5, respectively. A2A2 tempered milk with pH 6.5 had on average 97 % and 36 % higher CF than corresponding cold milk pH 6.72 and cold milk pH 6.5, respectively. A2A2 cold milk pH 6.72 had on average 31 % lower CF than corresponding cold milk pH 6.5 ($p<0.001$). Within the group SM with β-CN genotype II, tempered milk with pH 6.72 had on average 16 % lower CF than corresponding tempered milk pH 6.5. Further, II tempered milk pH 6.72 had on average 553 % and 353 % higher CF than corresponding cold milk pH 6.72 and

cold milk pH 6.5, respectively. II tempered milk with ph 6.5 had on average 774 % and 418 % higher CF than corresponding cold milk pH 6.72 and cold milk pH 6.5, respectively ($p<0.001$).

Table 12 Curd firmness in skim milk (SM), based on the β-CN genotype, cooling/tempering treatment and pH treatment. Differences between (row wise) and within (column wise) the groups were evaluated by Tukey pairwise comparison and were considered significant at $p<0.05$.

Cooling treatment	*pH-treatment*	*Average curd firmness (elasticity index, 20 min after addition of rennet)*			*p-value*
		A1A1	A2A2	II	
Tempered milk	pH 6.72	$0.00701 \pm 0.00117^{1,a}$	$0.00542 \pm 0,00009^{1,ab}$	$0.00346 \pm 0.00013^{1,b}$	p=0.031
	pH 6.5	0.00433 ± 0.00009^{12}	0.00426 ± 0.00012^{2}	0.00410 ± 0.00028^{2}	p=0.499
Cold milk	pH 6.72	0.00236 ± 0.00074^{2}	0.00216 ± 0.00024^{3}	0.00053 ± 0.00004^{3}	p=0.048
	pH 6.5	$0.00550 \pm 0.00011^{1,a}$	$0.00313 \pm 0.00011^{4,b}$	$0.00098 \pm 0.00000^{3,c}$	p<0.001
	p-value	p=0.011	p<0.001	p<0.001	

Tempered milk = milk with native micelles. Cold milk =milk with depleted micelles. Mean values ± standard deviation. Means within a row that do not share a letter are significantly different. Means within a column that do not share a number are significantly different. A1A1, A2A2 and II represents different β-CN genotypes. CF = curd firmness; N = 2

Appendix 4 Percentual change of casein profile and casein number between tempered and cold Milk Serum Phase (MSP)

The percentual difference between the cold and tempered MSP share (%) of the the caseins αs1-CN, αs2-CN, β-CN, κ-CN of total casein content, and casein number in MSP, is shown in *Table 10*. The II genotype had the largest percentual difference for β-CN and αs1-CN share, A1A1 had the largest difference for αs2-CN and A2A2 had the largest difference for κ-CN. The percentual difference of β-CN in II was 71,58 % and 235,36 % higher than the percentual difference for A2A2 and A1A1, respectively.

Table 13 Differences (%) in the share of the four different types of caseins, and casein number, between cold milk serum phase (MSP) compared to tempered MSP

Genotype	*αs1-CN*	*αs2-CN*	*β-CN*	*κ-CN*	*Casein number*
A1A1	+12.25	-73.97	+24.10	+33.23	-18.22
A2A2	+1.58	+37.8	+12.33	-52.47	-15.81
II	-22.05	-48.43	+41.35	-38.32	+60.40

CN = Casein. Casein number = CN/Total protein. MSP = Milk serum phase

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