Natural variation in casein composition of milk

Etske Bijl

Thesis committee

Promotor
Prof. Dr A.C.M. van Hooijdonk
Professor of Dairy Science and Technology
Wageningen University

Co-promotors
Dr T. Huppertz
Principal Scientist Dairy and Ingredient Technology
NIZO food research, Ede

H.J.F. van Valenberg
Assistant professor, Food Quality and Design Group
Wageningen University

Other members
Prof. Dr H. Gruppen, Wageningen University
Prof. A.L. Kelly, University College Cork, Ireland
Prof. Dr C.G. de Kruif, Utrecht University
Dr A.J. Buitenhuis, Aarhus University, Denmark

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Thesis

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Abstract

Considerable natural variation in casein content and composition exists between milk samples from individual cows. The objective of the work described in this thesis was to increase our understanding of the natural variation in casein composition of bovine milk and its implications for casein structure as well as for some relevant technological properties of milk. This study shows that the expression of caseins and their post-translational modification as well as inclusion of calcium in casein micelles are well-balanced processes. It is concluded that variation in α_{s1}-casein phosphorylation results in changes in the core of casein micelles and glycosylation of κ-casein results in changes in the surface of casein micelles. Both factors are therefore relevant to consider for optimization of dairy products and the design of future breeding strategies.

Contents

1

General introduction

1.1 Introduction

1.1. Dutch Milk Genomics Initiative

This PhD project was part of the multidisciplinary Dutch Milk Genomics Initiative (MGI). The MGI project partners are the Animal Breeding & Genetics and Dairy Science & Technology groups from Wageningen University as well as the cooperative cattle improvement cooperation CRV, NIZO food research and the Dutch Dairy Organization. The Dutch MGI was started in 2004 and is this year celebrating its 10-year anniversary.

The aim of the Dutch MGI is to identify genes that contribute to natural variation in milk quality characteristics, with a focus on milk fat and protein composition. Furthermore, the project aims to provide tools to optimize breeding strategies and to contribute to scientific knowledge that can help in the creation of innovative dairy products. In the past 10 years, 7 PhD theses have been published on these topics (Bouwman, 2014; Demeter, 2011; Heck, 2009; Lu, 2013; Schennink, 2009; Schopen, 2010; Stoop, 2009). For these projects, the extensive milk genomics sample set was used, which consisted of milk and blood samples of a population of 2000 individual Dutch Holstein-Friesian cows distributed over 400 commercial herds in the Netherlands.

Within the framework of the Dutch MGI, this PhD thesis contributes to the determination of detailed milk protein composition, in particular casein composition and its influence on technological properties of milk.

1.2 Introduction

Bovine milk contains 3–4% protein and almost 80% of the milk protein fraction consists of four caseins; α_{s1}-casein (α_{s1}-CN), β-casein (β-CN), α_{s2}-casein (α_{s2}-CN) and κ-casein (κ-CN), which occur at a ratio of ~ 4:1:3.5:1.5, respectively (Davies & Law, 1977). Most of the caseins in milk are assembled in casein micelles, which are highly hydrated association colloids consisting of several thousands of individual casein molecules and salts (Dalgleish & Corredig, 2012). The unique structure of casein micelles allows the delivery of large amounts of calcium and phosphate to the neonate, without risk of pathological calcification of the maternal mammary gland (Holt & Carver, 2012; Neville, 2005). Casein micelles are also the key constituents determining milk functionality in traditional dairy processes, such as rennet coagulation of cheese milk and acid coagulation of yoghurt milk. Nowadays, caseins are also used to prepare innovative products, such as functional foods with bioactive casein-derived peptides or delivery vesicles for nutraceuticals (Abd El-Salam & El-Shibiny, 2012; Phelan, Aherne, FitzGerald, & O'Brien, 2009).

In the past, breeding and feeding strategies have been successfully applied to increase milk yield and the protein and fat content of milk. This increase is economically favorable for dairy product manufacture, of which cheese is the most important in the Netherlands. For the production of cheese and cheese yield, not the total protein content of milk, but the casein content is especially important. A parameter directly related to cheese yield is the casein index, which is the total casein content divided by the total protein content of milk. The higher the casein index, the higher the cheese yield. Recently, promising scenarios have been developed to increase the casein index of milk using genetic selection (Schopen, 2010). Besides variation in casein content of milk, considerable natural variation exists in casein composition between milk samples from individual cows (Heck et al., 2008). The caseins have a high heritability and can therefore be of use in the design of breeding strategies (Schopen et al., 2009). However, the question that remains is, if this natural variation in casein composition can result in a difference of the casein micelle properties, which can affect the technological properties of dairy products, in specific its suitability for cheese production.
The natural variation in casein composition may result from genetic variation and post-translational modifications (PTM) (Holland, 2009; Huppertz, 2013). A detailed overview of natural variation in caseins will be given in the following sections.

1.3 Genetic variation

The whole bovine genome contains 29 chromosomes and two sex chromosomes and is estimated to contain 3 billion base pairs of DNA and an estimated 22000 of protein-coding genes (Elsik et al., 2009). Within this genome four casein genes are closely linked within a 250 kb region on chromosome 6 (119 Mb in size) in the order of α_{s1}-CN, β-CN, α_{s2}-CN and κ-CN (Ferretti, Leone, & Sgaramella, 1990; NCBI, 2014; Threadgill & Womack, 1990). During gene expression, DNA is transcripted into RNA and is subsequently translated into a protein amino acid sequence, which forms the primary structure of a protein. The primary structure of caseins is shown in Figure 1.1 and 1.2. For all of the caseins, genetic variants have been detected (Farrell et al., 2004). The genetic variants can results from single nucleotide polymorphism (SNP) in the DNA, as well as from nucleotide insertions or deletions (Caroli, Chessa, & Erhardt, 2009). Protein variants differ between species, genus and breeds. An overview of genetic variants detected in Dutch Holstein-Friesian cows and their frequencies in 2005 are shown in Table 1.1. Furthermore, Table 1.1 contains information on the substituted amino acids in comparison to the reference variant of each casein. The substituted amino acids have different side

Table 1.1 Genetic variants of caseins, frequencies and substituted amino acids in Holstein-Friesian cows.

Protein	Genetic variant	Frequency in 2005[1]	Substituted amino acid[2]
α_{s1}-casein	B	0.997	reference variant
	C	0.003	207 Glu → Gly
α_{s2}-casein	A	1	
β-casein	A1	0.283	82 Pro → His
	A2	0.504	reference variant
	A3	0.001	121 His → Gln
	B	0.020	82 Pro → His; 137 Ser → Arg
	I	0.192	108 Met → Leu
κ-casein	A	0.599	reference variant
	B	0.309	157 Thr → Ile; 169 Asp → Ala
	E	0.092	176 Ser → Gly

[1] Frequencies of genetic variants of Dutch Holstein Friesian cows in 2005 (Heck et al., 2009; Visker et al., 2011).
[2] Substituted amino acids compared to the reference genetic variants of the caseins (Caroli et al., 2009; Farrell et al., 2004).

groups that can vary in polarity, charge, size, shape, hydrophobicity and reactivity. Therefore, amino acid substitution can result in changes in the secondary and tertiary structure of caseins and quartenary structure of casein micelles.

1.4 Post-translational modifications

1.4.1 Phosphorylation

Phosphorylation of caseins is catalyzed by kinase enzymes that attach phosphate groups to specific amino acids residues Ser and Thr, that are present in a Ser/Thr-Xxx-Glu/pSer/Asp motif (Mercier, 1981) within the primary protein structure. This PTM occurs in the Golgi apparatus of the mammary epithelial cells after formation of the polypeptide chain (Bingham & Farell, 1977) and is influenced by factors such as protein sequence, gene expression of kinase enzymes, substrate availability, and phosphorylation site accessibility (Holland, 2009). Phosphorylation is one of the key factors responsible for the stabilization of calcium phosphate nanoclusters in casein micelles (De Kruif & Holt, 2003; Huppertz, 2013). Phosphorylation sites of the caseins are highlighted in Figure 1.1 and 1.2. The reference protein of α_{s1}-CN has eight phosphorylated residues (α_{s1}-CN-8P) and the minor form has nine phosphorylated residues (α_{s1}-CN-9P) (Manson, Carolan, & Annan, 1977). This extra phosphate group is present on Ser56 and follows the Ser-Xxx-Asp motif. The

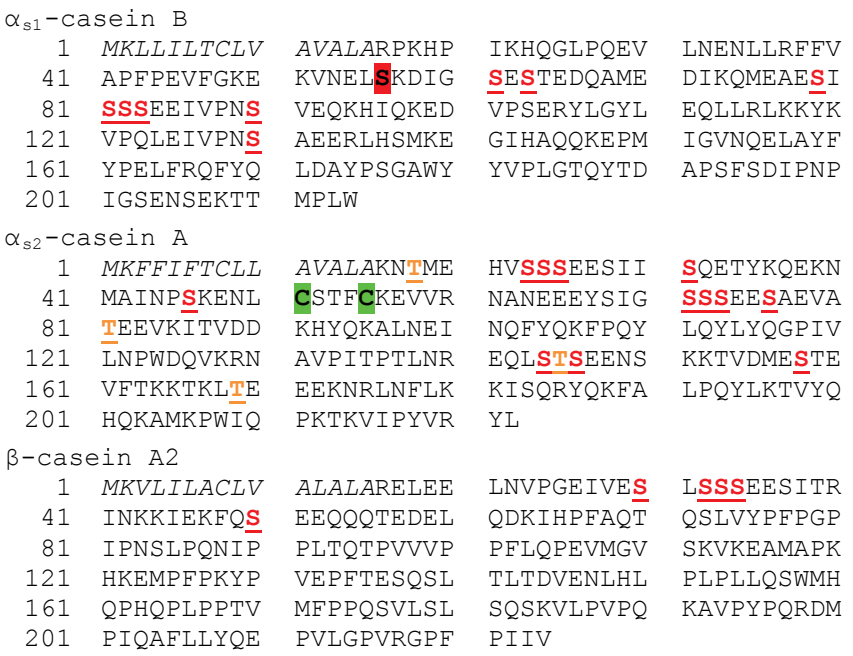

Figure 1.1 Primary structure of the reference proteins of α_{s1}-CN, α_{s2}-CN, β-CN. The signal peptides are printed in italics. Confirmed phosphorylated residues of α_{s1}-CN-8P and β-CN-5P are marked bold, red and underlined. The exact location of the phosphorylated residues in α_{s2}-CN-11P have not been confirmed but are presumable 11 of the 12 confirmed phosphorylated serine residues (marked bold, red and underlined), the theoretical Thr phosphorylation sites in α_{s2}-CN are marked bold and orange (Holland, 2009; Mercier, 1981). The additional phosphorylated Ser residue in α_{s1}-CN-9P is marked bold and highlighted red (Manson et al., 1977). Cysteine residues that can engage in disulphide bond formation are printed bold and highlighted green (Rasmussen et al., 1992a, 1992b).

reference protein of α_{s2}-CN has 11 phosphate groups (α_{s2}-CN-11P). In theory 12 Ser and 4 Thr residues can be phosphorylated but only minor forms with 10, 12 and 13 phosphorylated residues have been detected (Holland, 2009). β-CN is usually present with five phosphorylated residues. The reference protein of κ-CN has one phosphorylated residue, but κ-CN with either two or three phosphorylated groups have been detected as well (Mollé & Leonil, 1995; Vreeman, Visser, Slangen, & Van Riel, 1986). The two Ser residues in κ-CN are phosphorylated in the strict order of Ser170, Ser148. In κ-CN A the position of the third phosphorylated residue has not been confirmed yet and can be either Thr166 or Thr156 (Mercier, 1981; Mollé & Leonil, 1995). In κ-CN-B the third residue to be phosphorylated is Thr166 (Holland,

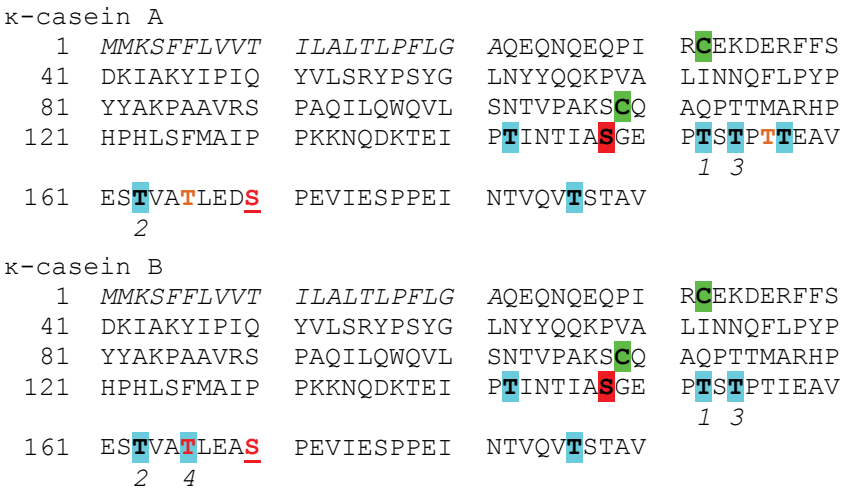

Figure 1.2 Primary structure of the genetic variants A and B of κ-CN. The signal peptides are printed in italics. The confirmed phosphorylated residue of κ-CN-1P is marked bold, red and underlined. The additional phosphorylated Ser residue in κ-CN-2P is marked bold and highlighted red (Holland, 2009). The confirmed phosphorylated Thr residue in κ-CN-B-3P is marked bold and red (Holland et al., 2006), the theoretical phoshorylated residues in κ-CN-A-3P are marked bold and orange (Mercier, 1981; Mollé & Leonil, 1995; Vreeman et al., 1986). The Thr residues that can be glycosylated in κ-CN are marked bold, and highlighted blue (Holland et al., 2006; Pisano et al., 1994), the confirmed order of glycosylation is indicated below the Thr residues in italic numbers. Cysteine residues that can engage in disulphide bond formation are printed bold and highlighted green (Rasmussen et al., 1992a, 1992b).

Deeth, & Alewood, 2006). This last residue is the only confirmed phosphorylated Thr residue in the caseins. In addition, Thr166 can also be glycosylated in κ-CN B and is therefore also the only confirmed residue in the caseins that can be subject to multiple PTMs.

1.4.2 Glycosylation

Another important PTM is glycosylation. Of the four caseins, κ-CN is the only casein to be glycosylated. On average, 60% of the κ-CN is glycosylated (Vreeman et al., 1986). Glycans can be linked at Thr residues of κ-CN molecules by glycosyltransferase enzymes via O-glycosidic bonds. The κ-CN A and B variant both have six confirmed Thr residues that can be glycosylated (Figure 1.2) (Holland et al., 2006; Pisano, Packer, Redmond, Williams, & Gooley, 1994). However, not all of these six positions are the same in the A and B variant: Thr157 cannot be glycosylated in the κ-CN B variant, since this amino acid residue is substituted by

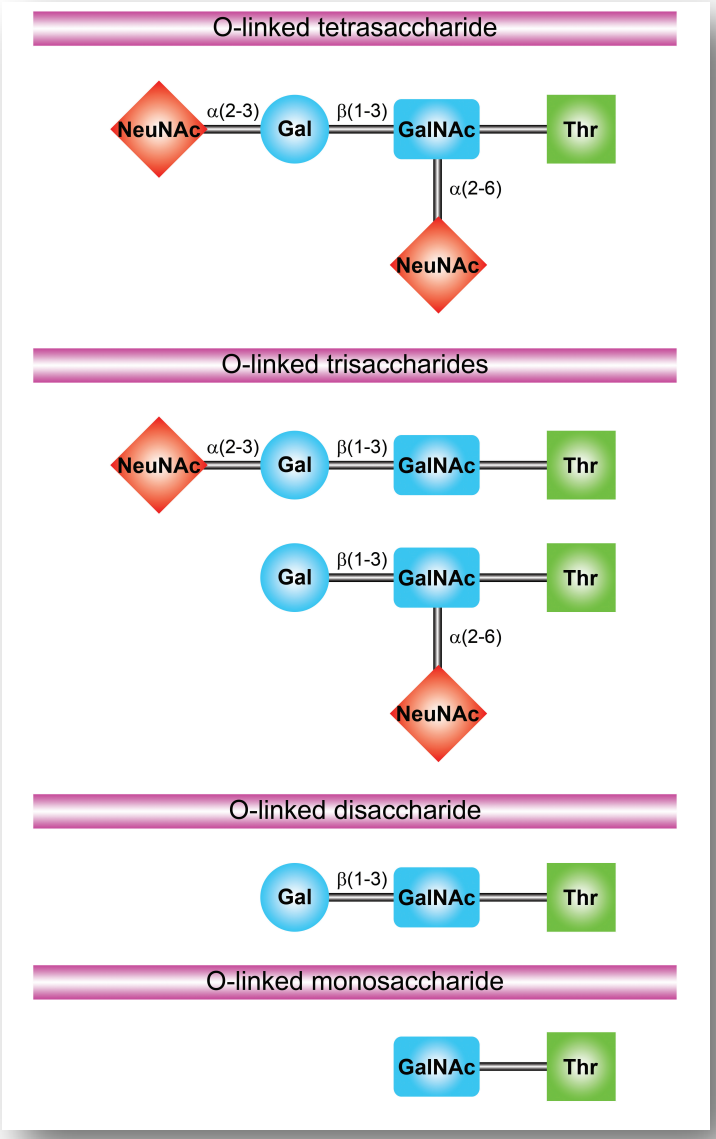

Figure 1.3 Variation in O-glycosidically linked sugar chains of κ-CN (Saito & Itoh, 1992). Carbohydrates in the sugar chains are N-acetylneuraminic acid (NeuNAc), N-acetylgalactosamine (GalNAc) and Galactose (Gal).

the hydrophobic amino acid Ile (Table 1.1) and in the A variant glycosylation of Thr166 has not been confirmed. As with phosphorylation, glycosylation of κ-CN also seems to follow a distinct order. In both genetic variants κ-CN is glycosylated in the order of Thr152, Thr163, Thr154, respectively (Holland, Deeth, & Alewood, 2005). In κ-CB-B, the fourth glycosylated group is located at Thr166. In general, κ-CN B was found to contain more glycosylated groups than κ-CN A (Coolbear, Elgar, & Ayers, 1996). Most glycans on κ-CN are present in the form of a tetrasaccharide (56%), but also a monosaccharide (0.8%), disaccharide (6.3%) and two trisaccharides with either a straight or a branched chain (18.4% and 18.5% respectively) have been found by HPLC analysis (Saito & Itoh, 1992). The structures of these glycans are presented in Figure 1.3. The number and size of the glycans, as well as the negative charge of the neuraminic acid residues caused by the deprotonation of the carboxylate group (pKa 2.6) (Vimr, Kalivoda, Deszo, & Steenbergen, 2004) are all factors that can influence the properties of κ-CN.

1.4.3 Disulphide bonds

Another post-translational modification is the formation of disulphide bonds between Cys residues in caseins. α_{s2}-CN and κ-CN contain Cys residues (Figure 1.1 and 1.2). α_{s2}-CN is present in milk as a monomer or as a dimer (Annan & Manson, 1969). The alignment of the Cys residues in the dimers can be either parallel or antiparallel (Rasmussen, Hojrup, & Petersen, 1992a). κ-CN has been found in milk as monomers and oligomers. Only 10% of κ-CN seems to be present as monomers and oligomers consisting of two or more monomers (Huppertz, 2013). The Cys residues in the oligomers appear to be randomly linked (Rasmussen, Hojrup, & Petersen, 1992b).

1.5 Casein micelles

Casein micelles are heat-stable, highly hydrated colloidal structures that contain approximately 3.5 g of water per g of protein (Jeurnink & Dekruif, 1993). The dry matter of casein micelles consists of approximately 94% proteins and 6% of minerals (Fox, 2003). Casein micelles have a typical diameter of 200 nm in bulk milk (De Kruif & Holt, 2003), while in milk of individual cows a variation in hydrodynamic diameter between 154 nm to 230 nm was found (De Kruif & Huppertz, 2012). The exact mechanism of formation and structure of casein micelles has been reviewed and debated extensively (Dalgleish, 2011; De Kruif & Holt, 2003; De Kruif, Huppertz, Urban, & Petukhov, 2012; Farrell Jr., Malin, Brown, & Qi, 2006; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2006; McMahon & Oommen, 2013).

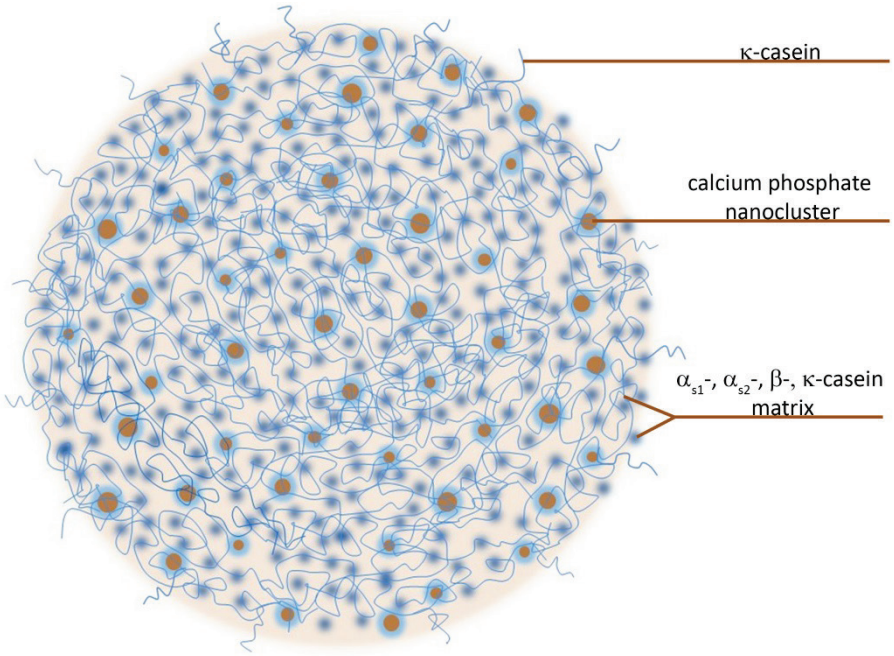

Figure 1.4 Representation of a casein micelle, adapted from De Kruif et al. (2012). The internal structure of the casein micelle consists of a matrix of αs1-CN, αs2-CN, β-CN and κ-CN and calcium phosphate nanoclusters. The external region consists of a hairy layer of κ-CN.

1.5.1 Casein micelle structure

In Figure 1.4, a representation of the casein micelle is shown, which is based on the internal structure that was proposed by De Kruif et al. (2012). The internal structure of casein micelles is composed of a protein matrix in which calcium phosphate nanoclusters are dispersed. These calcium phosphate nanoclusters are about 2 nm in radius and an average sized casein micelle, with a DLS radius (R65) of 80 nm, contains approximately 285 of these clusters De Kruif et al. (2012). The phosphorylated residues of the three calcium sensitive caseins, α_{s1}-CN, β-CN and α_{s2}-CN, can bind to calcium phosphate nanoclusters (Farrell Jr, Kumosinski, Malin, & Brown, 2002). It has been shown by use of phosphopeptides that a phosphorylation centre with at least three phosphorylated amino acids within a short range of each other, is needed to form a stable calcium phosphate nanocluster (Aoki, Umeda, & Kako, 1992). The maximum number of phosphorylation centres in the caseins are 0, 1, 2 and 3 for κ-CN, β-CN, α_{s1}-CN and α_{s2}-CN, respectively. Parts of the caseins that are not involved in the stabilization of calcium phosphate nanoclusters, can engage in weak interactions, such as

hydrophobic interactions, hydrogen bonding, ion bonding and weak electrostatic interactions, thereby forming the protein matrix. Some of the hydrophobic parts of the caseins form denser clusters of about 2 nm in size, and 5-fold more of the clusters are present in an average sized casein micelle compared to the number of nanoclusters (De Kruif et al., 2012). Casein micelles are stabilized for a large part by the calcium-insensitive κ-CN (Dalgleish, Horne, & Law, 1989), which can stabilize the calcium sensitive caseins by about ten times its mass (Fox & Brodkorb, 2008). The hydrophilic C-terminal of κ-CN forms a hairy layer also known as a polyelectrolyte brush and sterically stabilizes the casein micelles (De Kruif, 1999; Tuinier & de Kruif, 2002). The κ-CN layer has a hydrodynamic thickness of approximately 7 nm (Holt & Horne, 1996).

1.5.2 Interaction with the milk salts

The salts in casein micelles are in thermodynamic equilibrium with the salts in the milk serum (Gaucheron, 2005; Holt, 1985; Lucey & Horne, 2009). On average, 70% of calcium, 50% of inorganic phosphate, 30% of magnesium, and 10% of citrate in milk are located in the casein micelle (Holt, 2004). The remainder of these salt fractions are present in the milk serum, together with the majority of sodium, potassium and chloride ions. Ions in the milk serum phase are present as free ions, such as Ca^{2+}, or ion complexes such as CaCitrate^{-}. In the casein micelle, the main forms are amorphous micellar calcium phosphates and cation-protein complexes. The phosphorus fraction in the casein micelle consists of the organic phosphate attached to the casein polypeptides and inorganic phosphate that is present in the calcium phosphate nanoclusters. The calcium phosphate nanoclusters are the most complex salt fractions in milk. Calcium and phosphate in the nanoclusters can be present in different ratios and can exist in primarily amorphous and possibly some different crystallized forms. Because of the complexity the thermodynamic equilibrium between the salts in the milk serum and the salt in the casein micelles is slowly attained. It has been predicted that 49 phosphorylated casein centres can stabilize one calcium phosphate nanocluster and that there are 13.2 calcium ions, 6.5 inorganic phosphate ions, 1 magnesium ion and 1.3 citrate ions present per phosphorylated casein centre (Holt, Timmins, Errington, & Leaver, 1998; Holt, 2011). Modification of the salt equilibria by different conditions such as heating, acidification or addition of NaCl or calcium chelators, has a large influence on the structure and stability of casein micelles and the stability of milk and dairy products during processing (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; De La Fuente, 1998; Huppertz & Fox, 2006; Lucey & Horne, 2009). Due to the complexity of the interactions between the caseins, calcium phosphate

nanoclusters and the salt in the milk serum, it is difficult to predict the exact behaviour of ions in milk upon the application of the different physico-chemical conditions (Gao, 2010; Mekmene, Le Graet, & Gaucheron, 2009).

1.6 Aim and outline of this thesis

The first aim of the research described in this thesis was to increase our understanding of natural variation in casein composition of milk and its impact on casein micelle structure. Since casein micelles are in thermodynamic equilibrium with salts in milk serum, understanding of the natural variation in the salt fraction milk was necessary as well. The other aim of the work described in this thesis was to increase understanding how natural variation in casein composition can influence related technological properties of milk and how this understanding can be of use in the development of breeding strategies.

First, the study in **Chapter 2** describes natural variation in casein content and milk salt composition. The salt composition and milk protein content of milk of individual cows and bulk milk was determined during one year. Using these data, correlation analyses between salt and protein fractions and a detailed overview of bulk milk composition were made. Furthermore, the results were placed in a historical perspective to highlight evolution in milk composition over time.

Secondly, information on genetic background of the phosphorylation of α_{s1}-CN was studied in depth in **Chapter 3**. Therefore, genetic correlations and a genome wide association study were made of α_{s1}-CN with eight and nine phosphate groups, using information obtained from almost 2000 individual Dutch Holstein-Friesian cows.

Subsequently, the influence of natural variation in both casein and salt composition on casein micelle size was studied in **Chapter 4**. Casein micelle size was determined of 50 individual Holstein-Friesian cows. A sub-set of samples with small and large average casein micelle size was selected. For this set, a comparison of detailed milk composition was made, including salt composition, relative protein composition and glycosylation of κ-CN. The influence of genotypes of milk proteins on casein micelle size was determined as well.

The next step was to determine the influence of natural variation in casein composition on related technological properties of milk. For this purpose, proteolysis of caseins was studied, which is an important process for the preparation of cheese. The study in **Chapter 5** describes the influence of natural variation in α_{s1}-CN phosphorylation and genetic variation of β-CN in milk on chymosin-induced hydrolysis of caseins in milk gels and sodium caseinate solutions.

Chapter 6 contains a general discussion how the results obtained in this thesis contribute to our understanding of milk composition and formation in the mammary gland. And it is speculated how the results presented in this thesis can be applied for the optimization of dairy products and how the results can be of use in the development of breeding strategies.

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2

Protein, casein, and micellar salts in milk: Current content and historical perspectives

Etske Bijl[1], Hein van Valenberg[1], Thom Huppertz[2], Toon van Hooijdonk[1]

[1] Dairy Science and Technology Group, Wageningen University, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands; [2] NIZO food research, PO Box 20, 6710 BA, Ede, the Netherlands

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Abstract

The protein and fat content of Dutch bulk milk has been monitored since the 1950s and has increased considerably, by 11 and 20%, respectively, whereas milk yield has more than doubled. The change in protein and fat content of milk is advantageous for dairy industry, as these are the 2 most economically valuable constituents of milk. Increases in protein and fat content of milk have allowed increases in the yield of various products such as cheese and butter. However, for cheese and other applications where casein micelles play a crucial role in structure and stability, it is not only casein content, but also the properties of the casein micelles, which determine processability. Of particular importance herein is the salt partition in milk, but it is unknown whether increased protein content has affected the milk salts and their distribution between casein micelles and milk serum. It was, therefore, the objective of this research to determine salt composition and protein content for individual cow milk and bulk milk over a period of 1 yr and to compare these data to results obtained during the 1930s, 1950s, and 1960s in the last century. Calcium, magnesium, sodium, potassium and phosphorus content were determined by inductive coupled plasma atomic emission spectrometry and inorganic phosphate, citrate, chloride and sulphate content by anion-exchange chromatography in bulk milk and milk ultracentrifugate. In addition, ionic calcium and ionic magnesium concentration were determined by Donnan membrane technique. It was concluded that historical increase in milk yield and protein content in milk have resulted in correlated changes in casein content and the micellar salt fraction of milk. In addition, the essential nutrients, calcium, magnesium and phosphorus in milk have increased the past 75 yr; therefore, the nutritional value of milk has improved.

2.1 Introduction

Accurate knowledge on the distribution and total concentration of milk salts is relevant throughout the dairy chain. In nutrition science, great interest exists in the salt content and composition of milk and other dairy food products because they contribute substantial amounts of essential nutrients to the Western diet (i.e., 52-75% of calcium, 30-45% of phosphorus, 19% of potassium and 16-21% of magnesium; Cashman, 2011; Gueguen and Pointillart, 2000; Huth et al., 2006). Furthermore, milk salt composition is important for the technological properties of milk. The partition of salts, especially the distribution of calcium phosphate between the casein micelle and serum phase, has a large influence on the structure and stability of casein micelles (Dalgleish and Corredig, 2012; Holt, 1985; Lucey and Horne, 2009). The salt partition can be altered by changes in physicochemical conditions, such as heating, cooling and acidification (De La Fuente, 1998; Gaucheron, 2005) and can, therefore, have major consequences for several dairy processes in which casein micelles are involved, such as acid coagulation of yogurt products and stability of concentrated milk during heating and evaporation (Hardy et al., 1984; Nieuwenhuijse et al., 1988). In addition, colloidal calcium phoshate and free calcium play an essential role during rennet coagulation in the cheese-making process (Lucey and Fox, 1993).

The major fraction of salts in milk is composed of the cations calcium, magnesium, potassium, and sodium and the anions citrate, phosphate, and chloride (Holt, 1985; Pyne, 1962). They can be either dissolved as free ions or ion pairs in milk serum or dispersed in colloidal calcium phosphate nanoclusters bound to caseins (De Kruif et al., 2012). Calcium phosphate nanoclusters are in dynamic equilibrium with milk serum (Holt et al., 1989; Little and Holt, 2004). On average, 70% of calcium, 50% of inorganic phosphate, 30% of magnesium and 10% of citrate in milk are located in the casein micelle (Holt, 2004) and are of vital importance for casein micelle stability (Farrell et al., 2006; De Kruif and Holt, 2003; Walstra et al., 2005). Because of the close relation between micellar salts and casein, calcium phosphate in milk varies in proportion to the casein content of milk (Holt, 2011; Holt and Jenness, 1984; Lucey and Horne, 2009).

It is clearly relevant for many disciplines within the field of dairy science and for the dairy industry to have an accurate understanding of the casein content and salt composition of the milk used on a daily basis. However, the detailed salt composition and casein content is currently not monitored on a regular basis. Although it is recognized that the salt composition in milk of individual cows is influenced by breed, season, stage of lactation, diet, and mastitis within the time

frame of a lactation period (Gaucheron, 2005), until now insufficient data have been available to determine variation over a longer time frame of decades. During the past 50 yr, several trends between casein, salt fractions, and several other factors have been observed using individual milk samples, bulk milk, mineral depleted milk and even interspecies analysis. White and Davies (1958) examined the bulk milk of 1 herd of Ayrshire cows during 1 yr as well as individual milk samples of cows at different stages of lactation. The analysis of differences in milk composition between cows showed multiple direct and inverse correlations between different salt fractions, pH, lactose, and stage of lactation. Correlations between lactose, sodium and potassium were also observed in milk from 8 individual Friesian cows which were followed during the first 90 d of their lactation (Tsioulpas et al., 2007a). In addition, correlations between calcium in serum and citrate in serum were found after analysis of bulk milk from 5 creameries in Scotland during 1 yr (Holt and Muir, 1979). Mineral depletion of bulk milk and 3 individual cow milk samples showed a strong correlation between colloidal calcium and colloidal phosphate (Holt, 1982) and were later also found between species (Holt and Jenness, 1984).

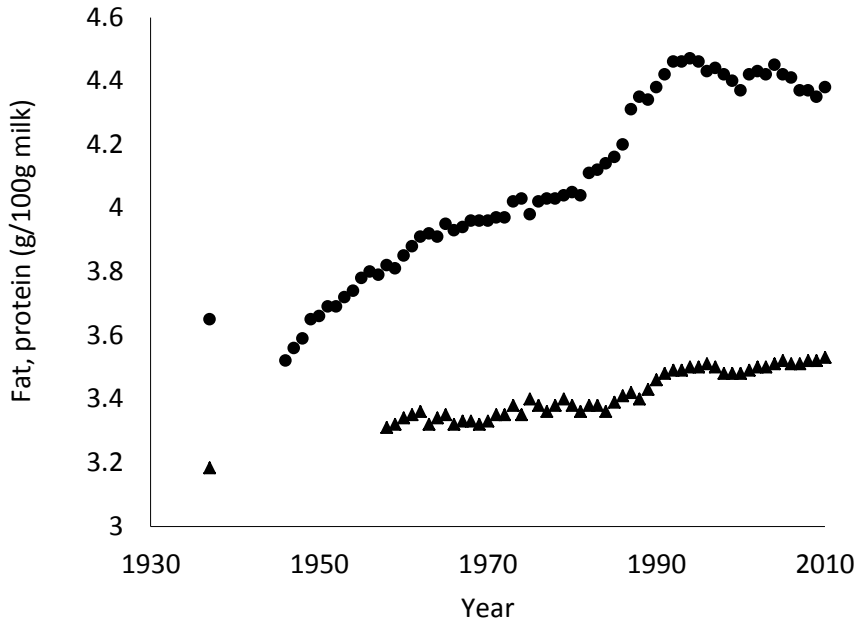

Figure 2.1 Historical trend for fat (●) and protein (▲) content (g/100g milk) of Dutch bovine raw milk from year 1945 to 2010 (CRV, 2012). Data for 1937 are from Ter Horst (1963).

The protein and fat content of Dutch bulk milk has been monitored yearly since the 1950s and it can be seen that both have increased considerably (Figure 2.1). Also milk yield per cow has approximately doubled and the average milk protein yield in the Netherlands has increased from 148 kg in 1960 to 331 kg in 2010 per lactation per cow (CRV, 2012). These changes are the result of changes in management, especially feed composition, and breeding strategies (Heck et al., 2009). Breeding goals in most countries consist of milk yield and milk composition. Selection might result in correlated changes in other traits. For example, selection for a higher fat content resulted in more saturated and less unsaturated fatty acids (Stoop et al., 2008). Similarly, selection for higher protein content can alter milk protein composition (Schopen et al., 2009). Breeding and payment schemes for the dairy industry have been aimed to increase protein content or protein yield of milk (Boland et al., 2001; Schopen et al., 2009). The change in protein and fat content of milk is advantageous for dairy industry, as these are the 2 most economically valuable constituents of milk. Increases in protein and fat content of milk have allowed increases in the yield of various products derived from milk, including cheese, butter as well as milk protein- and milk fat-based ingredients. However, for the applications listed above, particularly those where casein micelles play a crucial role in structure and stability, it is not only casein content, but also the properties of the casein micelles that determine processability. Of particular importance herein is the mineral balance in milk, but it is unknown whether increased protein content has affected the milk salts and their distribution between casein micelles and milk serum. These changes could influence structure and stability of casein micelles as well as processes in which casein micelles play a crucial role such as rennet coagulation, acid coagulation, and concentration of milk. Besides that, changes in salt composition might have an impact on the nutritional value of milk.

The objective of this research was, therefore, to determine salt composition and protein content for individual cow milk and bulk milk over a period of 1 yr, and to place these recent data in a historical perspective, using results from milk salt analysis performed during the 1930s, 1950s and 1960s of the last century. This was done by creating 2 data sets, containing information on detailed salt composition and the general parameters, protein, fat, lactose content, and pH. The first data set consisted of individual cow milk samples of normal somatic cell count that were taken during one yr from multiple farms, from animals in different stages of lactation, thus allowing high variation in milk composition. For the second dataset, bulk milk which was representative for average milk composition in The Netherlands was analyzed monthly during 1 yr. Variation in the latter data set was lower, but made it a good representative of current milk composition, to be placed

in historical perspective using published results on Dutch bulk milk composition, starting from the 1930s.

2.2 Materials and methods

2.2.1 Milk Samples

From January to December 2010, milk samples were collected each week from bulk milk tanks of 20 dairy plants. The dairy plants collected milk from farms in their region. These 20 regions cover almost all farms in The Netherlands and contained predominantly milk from Holstein-Friesian cattle. The 20 samples were mixed together to make 1 representative bulk milk sample for The Netherlands. Sodium azide (0.03 wt%) was added as a preservative and milk protein, fat, casein, lactose, dry matter, non-protein nitrogen (NPN) and urea content, as well as freezing point, were measured according to methods described by Heck et al. (2009). Additional measurements to determine salt content were performed on two 2-L samples of the bulk milk and 3 individual 2-L samples of cow milk the third week of each month. The individual cow milk samples originated from 4 different farms. In total, milk samples were taken from ten cows in early lactation (4-81 d; colostrum was excluded), ten cows in mid-lactation (95-262 d) and ten cows in late lactation (>272 d). To obtain skim milk, raw milk was centrifuged (1,500 × *g* for 15 min at 10°C; Avanti J-26 XP, Beckman Coulter GmbH, Krefeld, Germany). The pH of skim milk samples was determined by pH electrode (Orion 8127BNWP, Thermo Orion Inc., Beverly, MA, USA). Skim milk ultracentrifugate (100,000 × *g*, 20°C for 1 h) was obtained using Beckman Coulter L-60 ultracentrifuge with a 70 Ti rotor (Beckman Coulter GmbH).

2.2.2 Salt Measurements

To measure total content of calcium, magnesium, sodium, potassium and phosphorus, samples were prepared by microwave-assisted wet digestion, followed by inductive coupled plasma atomic emission spectrometry (**ICP-AES;** Varian Australia Pty Ltd., Mulgrave, Australia; ISO, 2010). Ion content was determined by ICP-AES as milligrams per kilogram of skim milk. Concentrations of chloride, sulphate, inorganic phosphate, and citrate were determined by anion exchange chromatography (IonPac AS19 column, 4 × 250 mm, Dionex; Thermo Scientific, Sunnyvale, CA, USA) after 500-fold dilution in ultrapure water (PURELAB Ultra System; Veolia Water Solutions, Ede, the Netherlands; Gaucheron et al., 1996). The concentrations of ionic calcium and magnesium in skim milk samples were determined by the Donnan membrane technique (Gao et al., 2009).

Distribution of calcium, magnesium, phosphorus and inorganic phosphate in the sedimentable phase, which will be further referred to as the micellar phase, was calculated by substracting nonsedimentable content in milk serum ultracentrifugate from total content in milk. Ion speciation of organic ester phosphate in micelles and organic ester phosphate in milk serum was calculated using the formulas used by White and Davies (1958). Sodium content of milk was corrected for the presence of added sodium azide, by substracting 4.6 mmol/kg from the total sodium content.

2.2.3 Statistical Analysis

Statistical analyses of correlations were performed in IBM SPSS statistics (version 19; IBM Corp., Armonk, NY, USA), using Pearson correlation coefficients on n = 30 individual milk samples [** and * indicate correlation is significant at the 0.01 level or 0.05 level (2-tailed), respectively].

2.3 Results and discussion

Results for analysis of protein content and salt composition for individual cow milk and bulk milk are displayed in Figure 2.2. The trends are clustered based on different families of correlations in milk composition, as discussed by Holt (1985). The first family of correlations (Figure 2.2) consisted of multivalent micellar salts, which were closely related to casein and protein content of the milk. For the individual milk samples, total calcium, phosphorus, and magnesium had highly significant correlations ($p<0.01$) with the protein content of milk. Also, the micellar calcium and phosphorus had highly significant correlations ($p<0.01$) with total protein, whereas micellar magnesium had a slightly lower correlation with protein content ($p<0.05$). Also, micellar calcium, phosphorus, and magnesium showed highly significant correlations ($p<0.01$) with their total contents in milk (Figure 2.2). Furthermore, strong correlations were observed between micellar phosphorus, calcium and magnesium ($p<0.01$). This is in agreement with literature, since they were present in the colloidal calcium phosphate (**CCP**) nanoclusters in casein micelles at a fixed ratio (Holt, 1982).

The second family of correlations (Figure 2.3) consisted of lactose, chloride, sodium and potassium; these constituents are involved in maintaining the osmotic pressure of milk, which is always closely related to the osmotic pressure of blood. The correlations of lactose content with chloride and sodium ($p<0.01$) have been observed before (Rook and Wood, 1958; White and Davies, 1958). Sodium and potassium and sodium and chloride were highly correlated ($p<0.01$). This strong

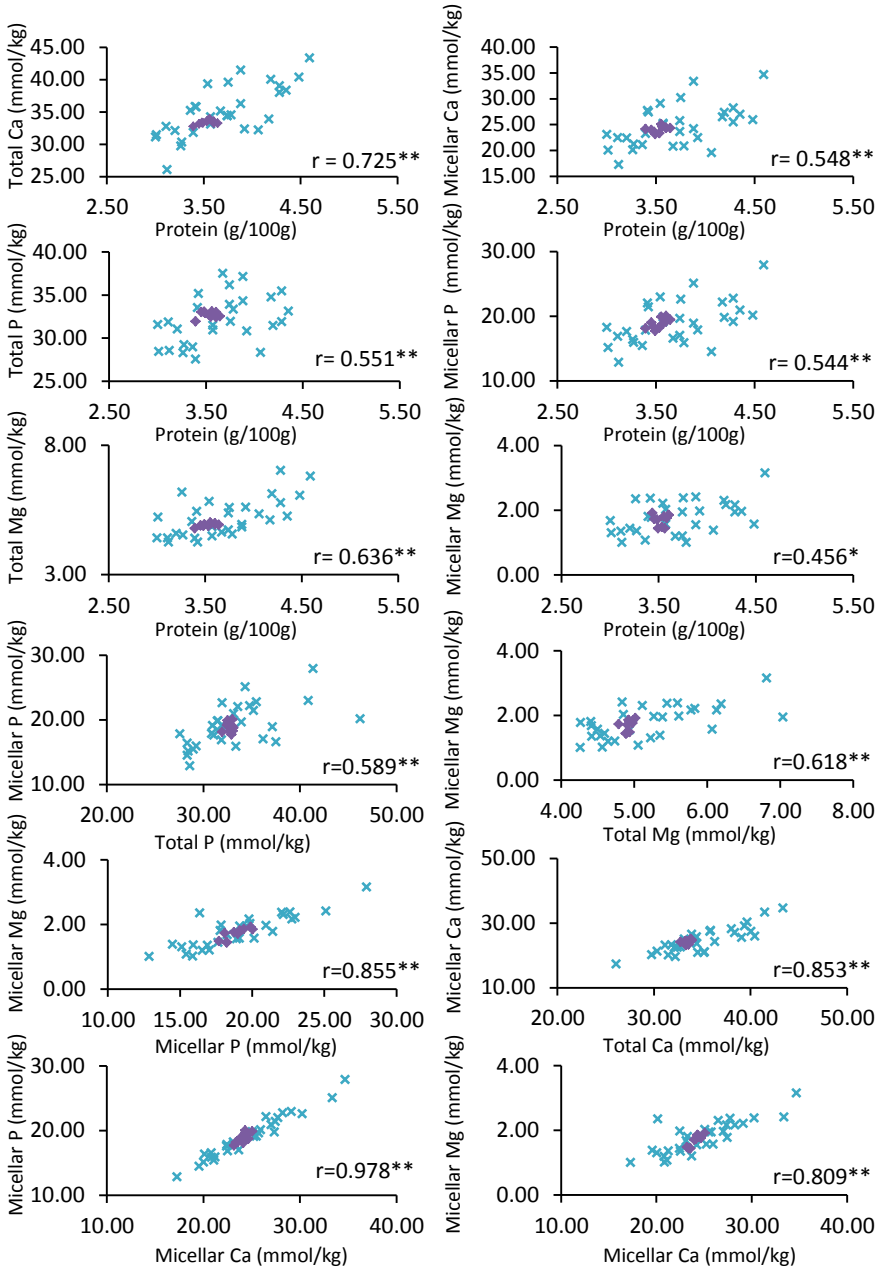

Figure 2.2 Trends for micellar multivalent salts and protein for individual cow milk (×) and bulk milk (◆). r indicates the Pearson correlation for individual cow milk data; ** and * indicate that the correlation is significant at the 0.01 level or 0.05 level (2-tailed), respectively.

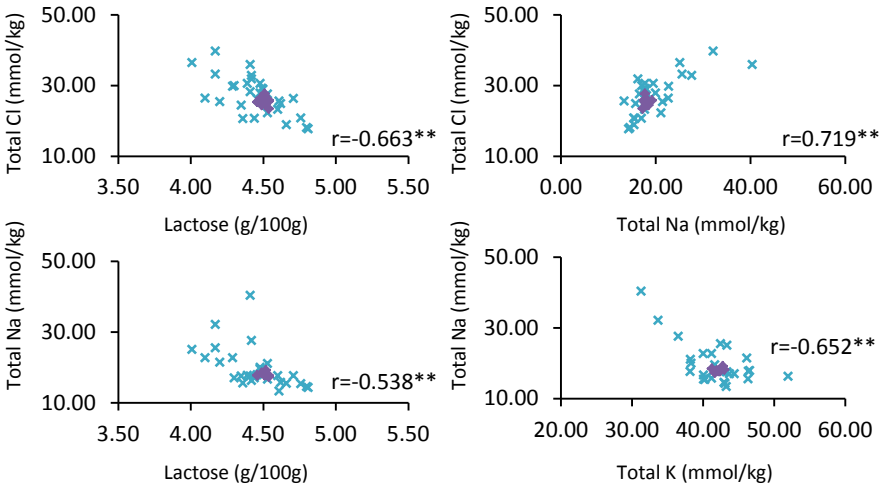

Figure 2.3 Trends for lactose, sodium, potassium and chloride for individual cow milk (×) and bulk milk (◆). r indicates the Pearson correlation for individual cow milk data; ** and * indicate that the correlation is significant at the 0.01 level or 0.05 level (2-tailed), respectively.

correlation was mainly due to the increase in sodium in comparison to potassium for 3 cows that were at the end of their lactation period. This phenomenon was also observed by White and Davies (1958) and can be explained by the opening of tight junctions at the end of lactation, when milk secretion rate decreases (Nguyen and Neville, 1998).

The third cluster of correlations (Figure 2.4) contained information on nonsedimentable multivalent ions, pH and free ions in solution. Nonsedimentable calcium and nonsedimentable magnesium were highly correlated with nonsedimentable citrate ($p<0.01$) and nonsedimentable calcium and nonsedimentable phosphorus were also highly correlated ($p<0.01$), but showed a wider distribution. This was also observed by Holt and Muir (1979), and can be explained by the higher affinity of calcium to form complexes with citrate in milk, as the logarithm of association constants (pK_{assoc}) of the calcium-citrate ($CaCit^-$) complex is 5.2, which is over a hundred times higher than the pK_{assoc} of 3.0 of the CaHPO4 complex formed between calcium and inorganic phosphate (Holt et al., 1981). For the second groups of correlations, free calcium and free magnesium were highly correlated with their nonsedimentable counterparts and with each other ($p<0.01$). Finally, pH and free calcium and nonsedimentable phosphorus were

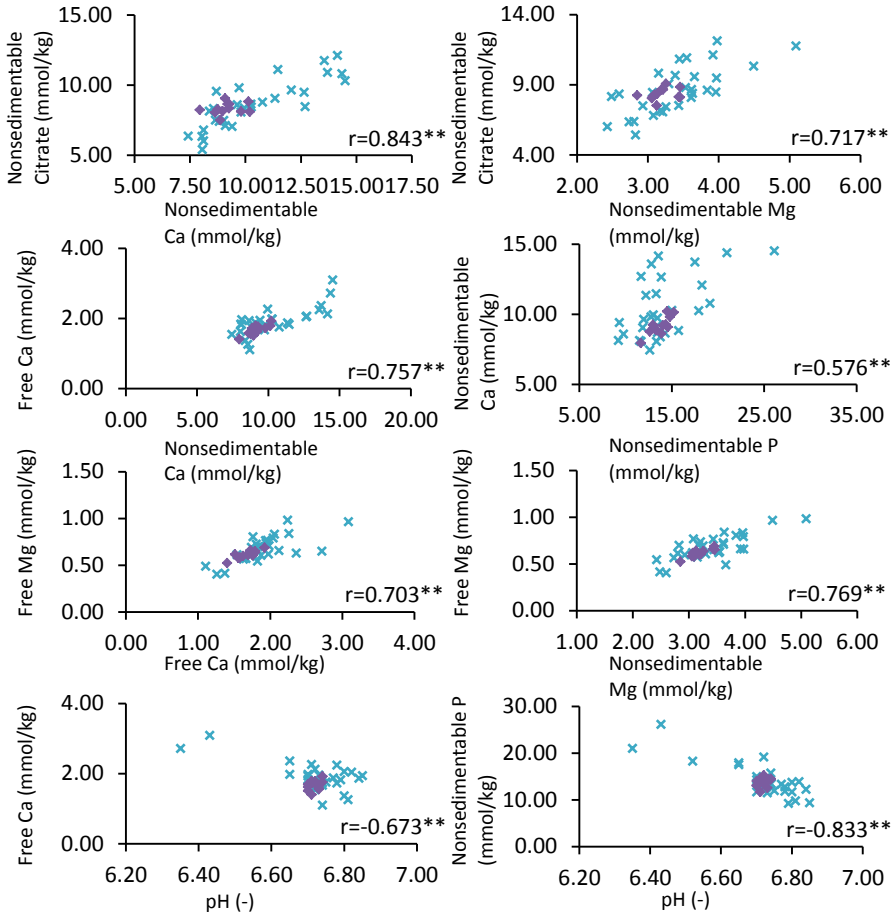

Figure 2.4 Trends for nonsedimentable multivalent ions, free Ca, free Mg, and pH in milk serum for individual cow milk (×) and bulk milk (◆). r indicates the Pearson correlation for individual cow milk data; ** and * indicate that the correlation is significant at the 0.01 level or 0.05 level (2-tailed), respectively.

highly correlated ($p<0.01$) because 3 cows at the beginning of their lactation (4, 5, and 6 d) had a milk pH below 6.6. This was also observed by Tsioulpas et al. (2007a) for cows up to 15 d in lactation, as well as by White and Davies (1958).

The obtained correlations confirm that the results described earlier, obtained from individual milk samples, bulk milk, mineral-depleted milk, or interspecies analysis, are equally valid for our data set of individual cow milk. When comparing

Table 2.1 Overview of composition and characteristics of bulk milk (n = 12)

	Bulk milk 2010	
Parameter	Mean	SD
Whole milk		
Protein (%, wt/wt)	3.53	0.08
Fat (%, wt/wt)	4.39	0.16
Lactose (%, wt/wt)	4.51	0.03
DM (%, wt/wt)	13.38	0.21
NPN (%, wt/wt)	0.173	0.005
Urea (mg/100g)	23	1.13
Casein (%, wt/wt)	2.75	0.06
Freezing point (°C)	-0.519	0.00
Total Ca (mg/kg)	1,279	13
Total Mg (mg/kg)	114	1
Total K (mg/kg)	1,567	23
Total Na (mg/kg)	394	12
Skim milk		
pH	6.72	0.01
Protein (%, wt/wt)	3.69	0.09
Casein (%, wt/wt)	2.88	0.07
Lactose (%, wt/wt)	4.71	0.03
DM (%, wt/wt)	9.40	0.08
Total Ca (mmol/kg)	33.37	0.34
Nonsedimentable Ca (mmol/kg)	9.17	0.64
% Micellar Ca (%, wt/wt)	72.5	4.3
Micellar Ca (mmol/kg)	24.20	0.59
Micellar Ca/ g of casein (mmol/g)	0.84	0.02
Ionized Ca (mmol/kg)	1.67	0.14
Total Mg (mmol/kg)	4.92	0.06
Nonsedimentable Mg (mmol/kg)	3.19	0.18
% Micellar Mg (%, wt/wt)	35.1	1.9
Micellar Mg (mmol/kg)	1.73	0.18
Ionized Mg (mmol/kg)	0.61	0.04
Total P (mmol/kg)	32.70	0.37
Total inorganic PO_4 (mmol/kg)	21.47	1.21
Nonsedimentable P (mmol/kg)	13.74	1.03
Nonsedimentable inorganic PO_4 (mmol/kg)	10.10	0.62
% Micellar inorganic PO_4 (%, wt/wt)	53.0	2.0
Micellar inorganic PO_4 (mmol/kg)	11.38	0.80
Organic ester PO_4 (mmol/kg)	3.64	0.84
Micellar organic PO_4 (mmol/kg)	7.59	1.24
Micellar P/ g of casein (mmol/g)	0.66	0.02
Total citrate (mmol/kg)	9.15	0.42
Nonsedimentable citrate (mmol/kg)	8.33	0.41
% Micellar citrate (%, wt/wt)	9.0	0.3
Total Na (mmol/kg)	17.95	0.55
Total K (mmol/kg)	42.05	0.61
Total Cl (mmol/kg)	25.55	1.07
Total SO_4 (mmol/kg)	0.94	0.18

individual milk to bulk milk, it can be seen that data points for bulk milk are in the middle of individual milk distribution and show low variation, as was expected. The detailed composition of bulk milk is given in Table 2.1. An important aspect of this list is the content of ionic calcium and magnesium in milk, because these are 2 of the most important factors for stability of milk (Lewis, 2011). The value of ionic calcium in bulk milk was 1.67 ± 0.14 mmol/kg. This result is in agreement with the value of 1.90 ± 0.4 mmol of free calcium/kg of skim milk reported by Tsioulpas et al. (2007b), who averaged results from 234 individual milk samples. The content of ionic magnesium in milk was measured for the first time in bulk milk and was 0.61 ± 0.04 mmol/kg.

The detailed information of bulk milk was also placed in a historical perspective. From the historical trend displayed in Figure 2.5, it can be seen that the casein content of milk in the Netherlands has increased around 9% from 2.64 ± 0.13 g/100g skim milk to 2.88 ± 0.07 g/100g skim milk since 1937. The increase in casein is in the same order of magnitude as the 11% increase of protein content of bulk milk in Figure 2.1, which increased from 3.30 ± 0.10 to 3.69 ± 0.09 g/100g skim milk. Current and historical analysis all have used the Kjeldahl method to determine casein nitrogen and a conversion factor of 6.38 was used for all data. Therefore, the comparison to historic measurements should be reliable. Using the results of these measurements it can be calculated that serum proteins did not substantially increase (0.66 ± 0.16 g/100g skim milk in 1937 to 0.81 ± 0.11 g/100g skim milk in 2010). It is still feasible, however, that serum protein content will increase more than casein content of milk in the future. Heck et al. (2008) described allele frequencies for 6 major milk proteins in 1989 and 2005. Since 1989, the A allele frequency of β-lactoglobulin (β-LG) has increased from 0.425 to 0.583. The A allele is strongly associated with a higher relative concentration of β-LG and a lower relative concentration of the caseins (Heck et al., 2008).

Besides casein content, Figure 2.5 shows that total calcium, phosphorus and magnesium content in milk have increased as well (by 12.4, 9.6 and 7.2% respectively). The increase in calcium is larger than increase in magnesium; therefore this is most likely caused by an increase in micellar salt fraction, as 72.5% of calcium, 53% of inorganic phosphate and 35.1% of magnesium was present in the micellar phase (Table 2.1). In addition, nonsedimentable calcium did not increase. This is in agreement with the fact that milk serum is supersaturated with calcium phosphate. Thus, any increase in calcium, magnesium, and phosphorus was mainly caused by the increase in casein content. The strong positive correlation between casein content and micellar calcium has also been found for interspecies

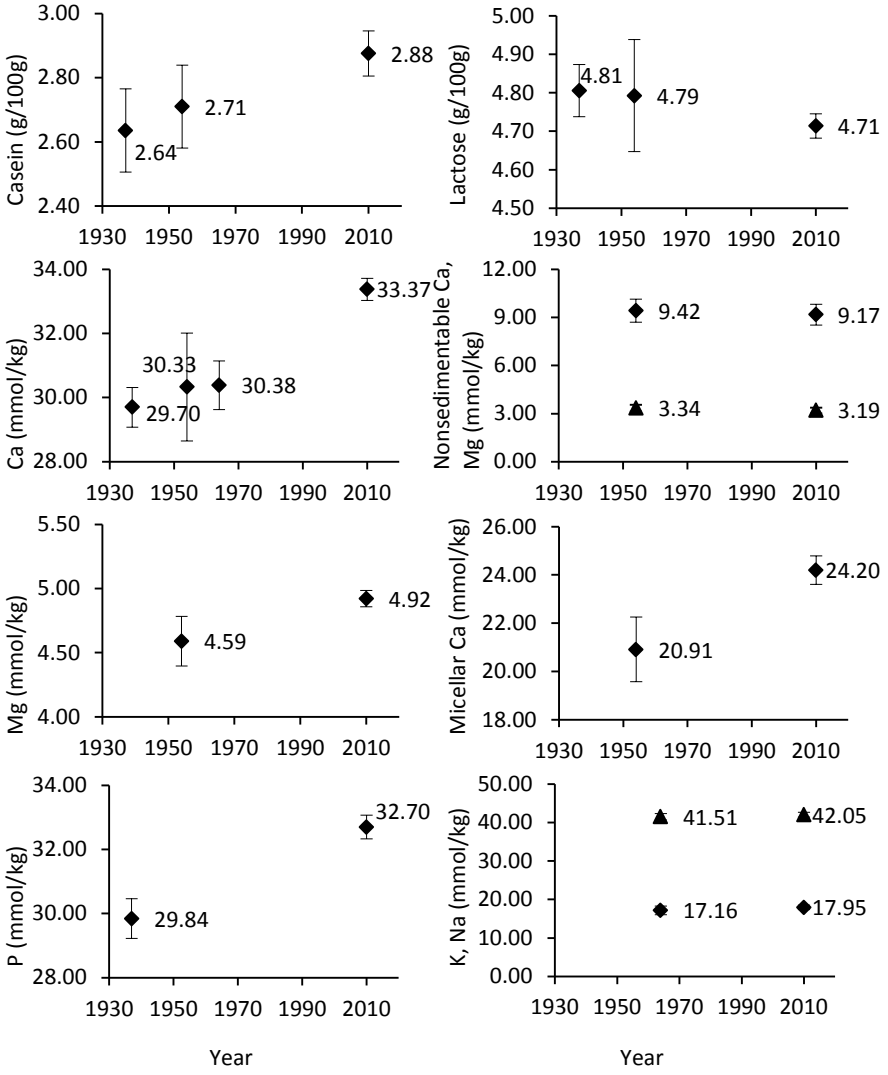

Figure 2.5 Historical trend for casein, lactose, Ca, Mg, nonsedimentable Ca (◆), nonsedimentable Mg (▲), micellar Ca, P, K (▲), and Na (◆) content in Dutch bulk skim milk. Values for casein, lactose, Ca and P were obtained from Ter Horst (1963), who used bulk milk from all cooperative dairies in the province of Friesland in the period 1934 to 1937. In addition, values for casein, lactose, and different fractions of Ca and Mg were obtained from Mulder et al. (1956), who determined composition of Dutch bulk milk in the period 1953 to 1956 based on milk composition of 10 individual cows during lactation. Values for Ca, K, Na were obtained from Eisses and de Ruig (1966); results were averaged for bulk milk from dairy plants of 4 different regions in the Netherlands in the period 1962 to 1965. The error bars represent the standard deviation of the mean.

analyses (Holt and Jenness, 1984). Unfortunately, insufficient historical data are available on different phosphate fractions in milk serum ultracentrifugate to make a reliable comparison. However it may be expected that the increase in total phosphorus is mainly caused by an increase in micellar inorganic phosphate and micellar organic phosphate. Using data on casein, total calcium, and nonsedimentable calcium, it can be calculated that the content of micellar calcium per gram of casein has not substantially increased in Dutch bulk milk (0.77 +/- 0.06 in 1954 to 0.84 +/- 0.02 in 2010).

On further examination of milk serum, it can be seen that lactose, potassium, and sodium content of Dutch bulk milk, which are almost completely present in the serum phase of milk, have not significantly increased over the years (Figure 2.5). The rather stable concentrations can be explained by the fact that they are the main determinants of osmolality in milk, together with chloride. Variation in osmolality of blood is low in healthy cows to maintain homeostasis. Since osmotic pressure in blood and in milk are closely related (Bjerg et al., 2005; Wheelock et al., 1965), as outlined previously, it is logical that variation in determinants of osmolality of milk is low as well over time.

The historical trends in Figure 2.5 were made using data of Dutch bulk milk, but even when we compare current values in Table 2.1 to the results of White and Davies (1958), which were obtained from a different breed (Ayrshire) at 1 farm in the United Kingdom more than 50 yr ago, no significant differences exist in nonsedimentable calcium, nonsedimentable magnesium, nonsedimentable phosphate fractions, and micellar calcium per gram of casein. This suggests that our findings are not only applicable for the Netherlands, but also for other countries.

2.4 Conclusions

Casein content and related micellar fraction of calcium, phosphorus and magnesium have increased significantly the past 75 yr, whereas salt content of serum and the salt composition of casein micelles have remained almost constant. Therefore, it can be concluded that historical increase in milk yield and protein content in milk have resulted in correlated changes in casein content and the micellar salt fraction of milk. Furthermore correlations observed for protein and micellar salt composition between individual cows over short time periods have also been consistent over more than 7 decades. Finally the essential nutrients, calcium, magnesium and phosphorus in milk have increased; therefore the nutritional value of milk has improved. All the observed changes are related to CCP nanoclusters in milk. As CCP nanoclusters have great structural importance, it will

be interesting to monitor the future trend to gain better insights into how the lactating mammary gland is coping with increased production of caseins, and if structure of casein micelles is changing as a result of this increased production.

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3

Phosphorylation of alpha-s1-casein is regulated by a different set of genes

E. Bijl[1], H. J. F. van Valenberg[1], T. Huppertz[2], A. C. M. van Hooijdonk[1], H. Bovenhuis[3]

[1] Dairy Science and Technology Group, Wageningen University, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands; [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands; [3] Animal Breeding and Genomics Centre, Wageningen University, PO Box 338, 6700 AH, Wageningen, the Netherlands

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Abstract

Casein phosphorylation is a post-translational modification catalyzed by kinase enzymes that attach phosphate groups to specific amino acids in the protein sequence. This modification is one of the key factors responsible for the stabilization of calcium phosphate nanoclusters in casein micelles and for the internal structure of the casein micelles. α_{s1}-Casein (α_{s1}-CN) is of special interest, since it constitutes up to 40% of the total casein fraction in milk and it has two common phosphorylation states, with eight (α_{s1}-CN-8P) and nine (α_{s1}-CN-9P) phosphorylated serine residues. Factors affecting this variation in the degree of phosphorylation are not known. The objective of this research was to determine the genetic background of α_{s1}-CN-8P and α_{s1}-CN-9P. The genetic and phenotypic correlation between α_{s1}-CN-8P and α_{s1}-CN-9P was low (0.18 and 0.19, respectively). This low genetic correlation suggests a different genetic background. These differences were further investigated by means of genome wide association study (GWAS). GWAS showed that both α_{s1}-CN-8P and α_{s1}-CN-9P were affected by a region on *Bos Taurus* autosome (BTA) 6, but only α_{s1}-CN-8P was affected by a region on BTA11, which contains the β-lactoglobulin (β-LG) gene, and only α_{s1}-CN-9P was affected by a region on BTA14, which contains the DGAT1 gene. Estimated effects of β-LG concentration and protein genotypes showed that only α_{s1}-CN-8P was associated with the β-LG gene polymorphism; the AA genotype of β-LG was associated with a lower concentration of α_{s1}-CN-8P (-0.32), and the BB genotype with a higher concentration of α_{s1}-CN-8P (0.41). Estimated effects of DGAT1 genotypes showed that only α_{s1}-CN-9P was associated with the DGAT1 gene polymorphism; DGAT1 AA genotype was associated with a higher α_{s1}-CN-9P concentration (0.53) and DGAT1 KK genotype with a lower concentration of α_{s1}-CN-9P (-0.44). The results give insight in phosphorylation of α_{s1}-CN-8P and α_{s1}-CN-9P, which seem to be regulated by a different set of genes.

3.1 Introduction

Phosphorylation of caseins is catalyzed by kinase enzymes that attach phosphate groups to specific amino acids residues Ser and Thr (Mercier, 1981). This post-translational modification (PTM) occurs in the Golgi apparatus of the mammary epithelial cells after formation of the polypeptide chain (Bingham and Farell, 1977) and is influenced by factors such as protein sequence, gene expression of kinase enzymes, substrate availability, and phosphorylation sites accessibility (Holland, 2009).

In general, phosphorylation and dephosphorylation of a protein can regulate almost every aspect of protein function, e.g., its biological activity, stabilization and initiation in protein-protein interactions (Cohen, 2002). Particularly in caseins, phosphorylation is one of the key factors responsible for the stabilization of calcium phosphate nanoclusters in casein micelles and the internal structure of the casein micelles (De Kruif and Holt, 2003, Huppertz, 2013). This unique micellar structure allows milk to deliver large amounts of calcium and phosphate to the neonate without enhanced risk of biocalcification in the mammary gland of the mother (Holt and Carver, 2012, Neville, 2005).

There is a large variation in the phosphorylation state between the caseins. Although α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN) and κ-casein (κ-CN) are all phosphoproteins, the amount of phosphorylated groups (n*P) they carry varies widely; ascending from 1P–3P on κ-CN, 4P–5P on β-CN, 8P–9P on α_{s1}-CN to 10P–13P on α_{s2}-CN (Farrell et al., 2004, Holland, 2009). The causes for variation in phosphorylated states of caseins and the effects on internal structure of casein micelles are not known and might differ per type of casein. Differences in phosphorylation of α_{s1}-CN are of special interest, since total α_{s1}-CN constitutes up to 40% of the total casein fraction in milk and it has two common phosphorylated states; i.e., α_{s1}-CN-8P and α_{s1}-CN-9P. The α_{s1}-CN-9P fraction contains an additional phosphorylated serine residue at position 56, including the signal peptide, and was previously known as α_{s0}-CN (Manson et al., 1977). Analysis of variation in casein phosphorylation on the milk samples of almost two thousand individual Holstein Friesian cows showed that the α_{s1}-CN-8P fraction occurred in a 3-fold excess over the α_{s1}-CN-9P fraction (Heck et al., 2008). The heritability of total α_{s1}-CN concentration that was determined for the same set of samples and was 0.47 (Schopen et al., 2009). However the genetic background for individual phosphorylated fractions, α_{s1}-CN-8P and α_{s1}-CN-9P is not known.

The objective of this research was to determine the genetic background of the α_{s1}-CN-8P and α_{s1}-CN-9P fractions in milk. The results for the 1912 cows taken during

winter period were used to estimate genetic parameters and correlations and to perform a genome wide association study (GWAS) in order to identify genomic regions associated with phosphorylation of α_{s1}-CN-8P and α_{s1}-CN-9P.

3.2 Materials and methods

3.2.1 Phenotypes

Milk samples of almost two thousand Holstein-Friesian cows in their first lactation from 398 commercial herds in the Netherlands were collected as part of the Dutch Milk Genomics Initiative (Bouwman et al., 2011, Schennink et al., 2007, Schopen et al., 2009). The pedigree of the cows was supplied by the NRS (Arnhem, the Netherlands). In the current study of 1857 milk samples taken in the winter period, milk protein composition was determined by capillary zone electrophoresis (CZE) (Heck et al., 2008). Using this method it was possible to estimate the relative protein concentration (%) on the basis of peak area of the protein fraction and total peak area in the electropherogram. The concentrations were determined of α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). Besides total α_{s1}-CN it was also possible to determine the concentration of the individual fractions of α_{s1}-CN-8P and α_{s1}-CN-9P.

3.2.2 Genotypes

DNA was isolated from blood samples of 1736 cows for genotyping. As described in detail by Schopen et al. (2011), a 50k SNP chip developed by CRV (Cooperative Cattle Improvement Organization, Arnhem, the Netherlands) was used to genotype cows as well as the sires of those cows using the Infinium assay (Illumina Inc, San Diego, CA, USA). The map positions of the single nucleotide polymorphisms (SNPs) were based on bovine genome assembly BTAU 4.0 (Liu et al., 2009). In total, 589 SNPs were located on the X chromosome, and 775 SNPs could not be mapped to a Bos taurus (BTA) chromosome and therefore were assigned to BTA 0. The SNPs on BTA 0 and the X chromosome were included in the study.

The dataset used in the association study consisted of 1667 animals with both α_{s1}-CN-8P and α_{s1}-CN-9P phenotypes and genotypes. Polymorphisms responsible for the known protein variants of β-CN (A1, A2, A3, B, and I) and κ-CN (A, B and E) were genotyped using a SNaPshot assay, as described by Visker et al. (2011). Based on the protein variants, β-κ-CN haplotypes were derived (Visker et al., 2011). In total, 1606 cows were available with β-κ-CN haplotypes and α_{s1}-CN-8P and α_{s1}-CN-9P phenotypes. Protein variants A and B for β-LG were genotyped as described by Ganai et al. (2009). To estimate the effect of β-LG protein variants on α_{s1}-CN-8P

and α_{s1}-CN-9P, 1671 cows were available. Genotypes for the DGAT1 K232A polymorphism were obtained as described by Schennink et al. (2007). The effect of the DGAT1 K232A polymorphism on α_{s1}-CN-8P and α_{s1}-CN-9P was estimated based on 1704 cows.

3.2.3 Statistical analyses

Genetic parameters

To estimate genetic parameters and variance components the following model was used:

$$y_{klmn} = \mu + \beta_1 dim_{klmn} + \beta_2 e^{-0.05 dim_{klmn}} + \beta_3 ca_{klmn} + \beta_4 ca^2{}_{klmn} + season_k + scode_l + animal_m + herd_n + e_{klmn} \quad [1]$$

where y_{klmn} is the observation for α_{s1}-CN-8P or α_{s1}-CN-9P. The overall mean of the trait is μ, dim_{klmn} is a covariate describing the effect of days in lactation, ca_{klmn} is a covariate describing the effect of age at first calving, $season_k$ is the fixed effect of the k^{th} class of calving season (three classes: summer [June-August 2004], autumn [September-November 2004], and winter [December 2004-February 2005]), $scode_l$ is the fixed effect accounting for possible differences in genetic level between proven bull daughters and young bull daughters, $animal_m$ is the random additive genetic effect of animal m, $herd_n$ is the random herd effect of the n^{th} herd, and e_{klmn} is the random residual. The animal effects were assumed to be distributed as $N(0, A\sigma_a^2)$, herd effects were assumed to be distributed as $N(0, I\sigma_{herd}^2)$ and the residuals were assumed to be distributed as $N(0, I\sigma_e^2)$, where A is the additive genetic relationships matrix and I the identity matrix.

The statistical package ASReml (Gilmour et al., 2009) was used to perform the analysis. The intraherd heritability was calculated as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

The proportion of variance due to differences among herds was calculated as:

$$h_{herd} = \frac{\sigma_{herd}^2}{\sigma_{herd}^2 + \sigma_a^2 + \sigma_e^2}$$

For estimating genetic and phenotypic correlations between α_{s1}-CN-8P and α_{s1}-CN-9P bivariate analyses were performed using model [1].

GWAS and Single Genes

The single SNP association study was performed using model [1], extended with a fixed SNP effect. In the association analysis the variance components were fixed to estimates obtained from a model without a SNP effect. To estimate effects of β-LG protein variants and the DGAT1 K232A polymorphism model [1] was extended with a genotype effect.

Haplotype analysis

To estimate the variance explained by the β-κ-CN haplotypes, the following model was used:

$$y_{klmnop} = \mu + \beta_1 dim_{klmnop} + \beta_2 e^{-0.05dim_{klmnop}} + \beta_3 ca_{klmnop} + \beta_4 ca^2{}_{klmnop} + season_k + scode_l + haplo1_o + haplo2_p + animal_m + herd_n + e_{klmnop} \quad [2]$$

where the variables were the same as in model [1], but extended with $haplo1_o$ and $haplo2_p$; $haplo1_o$ is the random effect of the first β-κ-CN haplotype and $haplo2_p$ is the random effect of the second β-κ-CN haplotype. Effects for haplo1 were assumed to be distributed as $N(0, I\sigma^2_{haplo-group})$, where I is the identity matrix and $\sigma^2{}_{haplo\text{-}group}$ is the haplo-group variation. The same distribution was assumed for haplo2 effects. The proportion of variation explained by haplo-groups was calculated as:

$$h_{haplo\text{-}group} = \frac{\sigma^2_{haplo\text{-}group}}{(\sigma^2_{haplo\text{-}group} + \sigma^2_a + \sigma^2_e)}$$

Significance Thresholds

The genome-wide false discovery rate (FDR) was based on the P-values from the animal model using the qvalue package (Storey and Tibshirani, 2003) in R. The FDR was calculated for each trait individually. Associations with a FDR < 0.1 were considered to be significant.

3.3 Results and discussion

3.3.1 Genetic parameters

The means, standard deviation (SD), coefficient of variation (CV) and genetic parameters for α_{s1}-CN fractions are given in Table 3.1. α_{s1}-CN-8P accounted for 21.3% of total protein in milk and α_{s1}-CN-9P 7.4%. The CV for α_{s1}-CN-9P concentration was larger (14%) compared to α_{s1}-CN-8P (5%). The proportion of

Table 3.1 Means (% w/w of total protein in milk), standard deviations (SD), coefficients of variation (CV), phenotypic variance (σ_p^2), intraherd heritability (h^2), proportion of variance explained by herd (h_{herd}) and correlations (genetic below diagonal and phenotypic above diagonal) among α_{s1}-caseins of 1857 Holstein Friesian cows (SE in brackets).

Trait	Mean (%)	SD (%)	CV (%)	σ_p^2	h^2	h_{herd}	Correlations		
							α_{s1}-CN	α_{s1}-CN-8P	α_{s1}-CN-9P
α_{s1}-CN	33.6	1.7	5	2.80	0.52 (0.11)	0.11 (0.02)	-	0.75 (0.02)	0.73 (0.02)
α_{s1}-CN-8P	21.3	1.1	5	1.32	0.48 (0.10)	0.12 (0.02)	0.73 (0.08)	-	0.19 (0.04)
α_{s1}-CN-9P	7.4	1.1	14	1.18	0.76 (0.12)	0.08 (0.02)	0.79 (0.06)	0.18 (0.15)	-

variation due to differences between herds was similar for the α_{s1}-CN-8P and α_{s1}-CN-9P concentrations and was relatively low (~0.10). In contrast, the intra-herd heritability for α_{s1}-CN-9P concentration was high (0.76). The heritability for α_{s1}-CN-8P concentration was lower (0.48). The correlations between total α_{s1}-CN concentration and both underlying traits α_{s1}-CN-8P and α_{s1}-CN-9P were high. However, the correlations among α_{s1}-CN-8P and α_{s1}-CN-9P concentration, which account for a large part of total α_{s1}-CN, were surprisingly low: the phenotypic correlation was 0.19 ± 0.04 and the genetic correlation was 0.18 ± 0.15. The low genetic correlations indicate that α_{s1}-CN-8P and α_{s1}-CN-9P concentration are genetically different traits.

3.3.2 Genome wide association study

The genetic differences between α_{s1}-CN-8P and α_{s1}-CN-9P concentration were further explored by means of GWAS, results of which are displayed in Figure 3.1. It can be seen that both components were affected by a region on Bos Taurus autosome (BTA) 6, but only α_{s1}-CN-8P concentration was affected by a region on BTA 11 and only α_{s1}-CN-9P concentration was affected by a region on BTA 14. This is the first time that a different genetic origin is shown for α_{s1}-CN-8P and α_{s1}-CN-9P fractions in milk. The effects on BTA 6, 11 and 14 will be further discussed in the following sections.

BTA 6

The SNP with the most significant association on BTA 6 for α_{s1}-CN-8P concentration was ULGR_BTC-043582 (-log10(p)= 34.5) at position 88.1 Mbp. This SNP was also

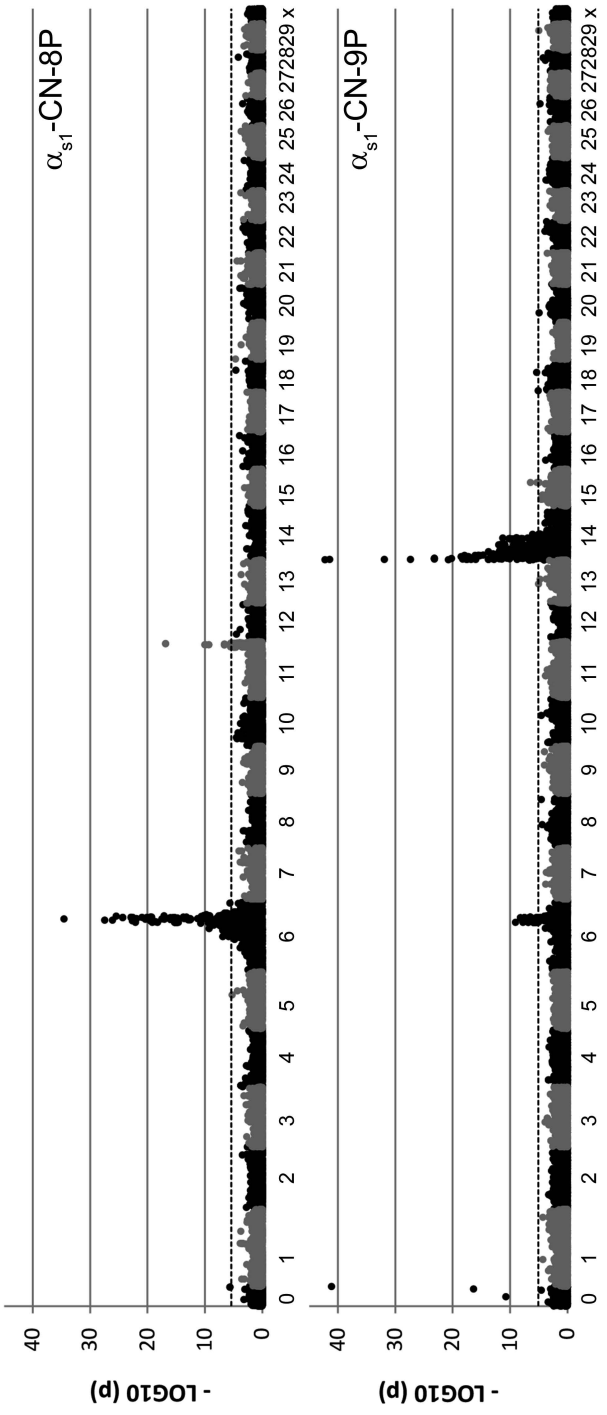

Figure 3.1 The –log10 (P-values) for all 48.874 SNPs for all 29 *Bos Taurus* autosomes (BTA) as well as BTA 0 and the X chromosome for α_{s1}-CN-8P and α_{s1}-CN-9P. The false discovery rate is indicated by the dotted line.

significant for α_{s1}-CN-9P concentration (-log10(p)=7.8) and was earlier demonstrated to be the lead SNP on BTA 6 for total α_{s1}-CN concentration by Schopen et al. (2011). The SNP with the most significant association on BTA 6 for α_{s1}-CN-9P concentration was ULGR_BTC-053514 (-log10(p)=9.0) at position 83566807 bp. This SNP was also significant for α_{s1}-CN-8P concentration (-log10(p)=22.1). Interestingly, this SNP was earlier demonstrated to be the lead SNP for total α_{s2}-CN concentration by Schopen et al. (2011).

The estimated effects of β-LG and DGAT1 genotypes and β-κ-CN haplotypes on α_{s1}-CN-8P and α_{s1}-CN-9P concentration are shown in Table 3.2. The IB β-κ-CN haplotype was associated with a lower concentration of α_{s1}-CN-8P and the A1B haplotype with a higher concentration of α_{s1}-CN-8P. The fraction of the total genetic variance that could be explained by the casein haplotypes on α_{s1}-CN-8P was 0.32 ± 0.15. In addition, a small effect was found of the IB haplotype on α_{s1}-CN-9P concentration. The fraction of the total genetic variance that could be explained by the casein haplotypes on α_{s1}-CN-9P was 0.02 ±0.02.

The fact that α_{s1}-CN-8P and α_{s1}-CN-9P were associated with BTA 6 can be well understood, because the α_{s1}-CN gene as well as the other casein genes are located in this region (Threadgill and Womack, 1990).

BTA 11

There was a highly significant effect (-log10(p)> 5.41) of chromosomal region on BTA 11 on α_{s1}-CN-8P concentration. This region contains the β-LG gene. The SNP that showed the strongest association with α_{s1}-CN-8P concentration was rs41255679. This SNP is located at 107.2 Mbp, in the promoter region of the β-LG gene, and is known to be in linkage disequilibrium with β-LG protein variants A and B (Ganai et al., 2009). Based on a GWAS and using mainly the same data as in the current study, Schopen et al. (2011) reported that the SNP rs41255679 showed the strongest association with β-LG concentration. It is known that β-LG protein variants A and B are associated with β-LG concentration (Heck et al. (2009) and therefore the observed effect on α_{s1}-CN-8P concentration might be either due to physical differences between protein variants A and B, or due to differences in β-LG concentration. Additionally, interactions between β-LG protein variants and β-LG concentration might exist. Analyses showed that there was no significant interaction (p=0.89) between with β-LG protein variants and β-LG concentration. However, there was a highly significant effect of both the β-LG protein variants (-log10(p)=12) and β-LG concentration (-log10(p)=6) on α_{s1}-CN-8P concentration. After adjusting for differences between β-LG protein variants, higher β-LG concentration was associated with a higher α_{s1}-CN-8P concentration. However,

when β-LG protein variants were not included in the model, the relationship between β-LG and α_{s1}-CN-8P concentration was negative. This can be explained by the higher β-LG concentration of β-LG AA milk as compared to β-LG BB milk (Heck et al. (2009). β-LG A was associated with a lower and β-LG B with a higher α_{s1}-CN-8P concentration, irrespective of accounting for β-LG concentration. The analysis convincingly showed that there was a distinct effect of β-LG protein variants and β-LG concentration, and that part of the negative effect of β-LG A on α_{s1}-CN-8P concentration was compensated by the higher β-LG concentration. No other evident genes that could explain the association with α_{s1}-CN-8P were located in this region.

The estimated effects of β-LG genotypes on α_{s1}-CN-8P and α_{s1}-CN-9P concentration (Table 3.2) showed that the AA genotype of β-LG was associated with a lower concentration of α_{s1}-CN-8P, and the BB genotype with a higher concentration (-log(p)= 11.3). For α_{s1}-CN-9P there was no significant effect of β-LG genotypes.

The function of β-LG in milk has not been elucidated yet. Possible functions that have been suggested do not include protein phosphorylation processes and first need to be further studied. Therefore the mechanism behind the established association between β-LG protein variants as well as β-LG concentration and α_{s1}-CN-8P concentration remains unclear.

BTA 14

There was a highly significant effect (-log10(p)>5.10) of chromosomal region on BTA14 on α_{s1}-CN-9P concentration. This region contains the DGAT1 gene. The SNP that showed the strongest association with α_{s1}-CN-9P concentration was ULGR_SNP_AJ318490_1c (-log10(p)=42.3) at position 0.4 Mbp followed by ULGR_SNP_AJ318490_1b (-log10(p)=41.4) as well at position 0.4 Mbp. These two SNPs are in full linkage disequilibrium with the DGAT1 K232A polymorphism, in which Lys (K variant) at AA position 232 is replaced by Ala (A variant) (Grisart et al., 2002, Winter et al., 2002). Besides the DGAT1 gene no other evident genes were located in this region.

The estimated effects of DGAT1 genotypes on α_{s1}-CN-8P and α_{s1}-CN-9P concentration (Table 3.2) showed that the DGAT1 AA genotype was associated with a higher α_{s1}-CN-9P concentration and the KK genotype with a lower concentration (-log(p)=12.1). There was no significant effect of DGAT1 genotypes on α_{s1}-CN-8P.

DGAT1 K232A influences milk fat percentage and composition: The K variant is associated with a higher fat content and more saturated fat, compared to the A variant (Grisart et al., 2002, Schennink et al., 2007, Winter et al., 2002). Although it

Table 3.2 Effect of β-LG (n= 1671 animals) and DGAT1 (n= 1704 animals) genotypes and β-κ-CN haplotypes (n= 1606 animals (Visker et al., 2011)) on α_{s1}-CN phosphorylation.

Genotype/ haplotype	n	α_{s1}-CN-8P	α_{s1}-CN-9P
β-LG			
AA	539	-0.32	0.06
AB	870	0.00	0.00
BB	262	0.41	-0.12
DGAT1			
AA	797	-0.06	0.53
AK	628	0.00	0.00
KK	279	0.01	-0.44
β-κ-CN[#]			
A2A	1503	0.22	0.05
IB	623	-0.49	-0.24
A1A	428	0.06	0.02
A1E	297	0.12	0.04
A1B	178	0.51	0.06
A2B	118	-0.01	-0.02
BB	65	-0.40	0.09

The estimated effect is the effect of having one copy of that haplotype

could be expected that DGAT1 polymorphism would mainly influence milk fat characteristics, ULGR_SNP_AJ318490_1b was earlier demonstrated to be the lead SNP on BTA14 for total α_{s1}-CN and α_{s2}-CN concentration and protein percentage in milk by Schopen et al. (2011). Furthermore Lu (2013) concluded that the membrane composition of the epithelial cells of the mammary gland could be different between cows with DGAT1 AA and KK genotypes. It therefore seems that the effect of DGAT1 polymorphism is not limited to milk fat characteristics.

3.3.3 Substrate specificity of casein kinases

The GWAS and estimated effect of β-LG and DGAT1 genotypes show that phosphorylation of α_{s1}-CN-8P and α_{s1}-CN-9P are not regulated by the same set of genes. Substrate specificity of casein kinases on α_{s1}-CN phosphorylation sites can provide understanding for the established differences. From the amino acid sequence of α_{s1}-CN-8P, we know that the eight Ser residues that can be phosphorylated follow the S-x-E/pS motif and that the Ser residue at position 56, which is additionally phosphorylated in α_{s1}-CN-9P, follows the S-x-D motif. The S-x-D motif is a unique recognition site that only occurs at this position in α_{s1}-CN and not in any of the other caseins. Previously, it was concluded that mammary gland

protein kinase was less active for this recognition site than for the common S-x-E/pS motif (Manson et al., 1977). However, in the meantime, more insight has been gained from the consensus phosphorylation sites of kinase enzymes (Ubersax and Ferrell Jr, 2007). With regard to casein kinases, Golgi-enriched fraction casein kinase (GEF-CK) from lactating mammary gland is thought to be responsible for phosphorylation of caseins (Moore et al., 1985). This fraction has the consensus sequence of S-x-E/pS and fails to recognize Asp for phosphorylation (Lasa-Benito et al., 1996). One of the candidates for the GEF-CK fraction has been identified as FAM20C. FAM20C is located on BTA 25 at position 42.7 Mbp. No polymorphism was found in this gene, and no effect was found of this gene on α_{s1}-CN-8P and α_{s1}-CN-9P concentration. However, it is known that FAM20C specifically phosphorylates S-x-E motifs (Tagliabracci et al., 2012). It therefore seems that another casein kinase in the Golgi apparatus of the lactating mammary gland could be responsible for the phosphorylation of the S-x-D motif in α_{s1}-CN-9P. This enzyme has not been identified as such yet. If two different enzymes are responsible for phosphorylation of α_{s1}-CN, this could explain why the genetic correlation between both fractions is low and why they are regulated by a different set of genes; the β-LG gene might be associated with the GEF-CK fraction that phosphorylates α_{s1}-CN-8P while DGAT1 gene might be associated with the phosphorylation of S-x-D motif in α_{s1}-CN-9P.

3.4 Conclusions

Considering the genetic correlations, GWAS and effect of β-LG and DGAT1 genotypes, it can be concluded that α_{s1}-CN-8P and α_{s1}-CN-9P are genetically different traits and are not regulated by the same set of genes. Both traits were associated with SNPs on BTA6, but only α_{s1}-CN-8P was associated with β-LG protein variants and β-LG concentration while only α_{s1}-CN-9P was associated with DGAT1 K232 polymorphism. Substrate specificity of casein kinases on α_{s1}-CN phosphorylation sites might be an important influencing factor; the unique S-x-D motif of α_{s1}-CN-9P fraction might be phosphorylated by a different enzyme than GEF-CK that has been recognized to phosphorylate the eight S-x-E/pS motifs of α_{s1}-CN-8P. The results suggest that there is a specific purpose towards phosphorylation of both α_{s1}-CN-8P and α_{s1}-CN-9P fractions.

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4

Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of κ-casein

Etske Bijl[1], Ruben de Vries[1], Hein van Valenberg[1], Thom Huppertz[2], Toon van Hooijdonk[1]

[1] Dairy Science and Technology Group, Wageningen University, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands; [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands

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Abstract

The average casein micelle size varies widely between milk samples of individual cows. The factors that cause this variation in size are not known but could provide more insight into casein micelle structure and into the physiology of casein micelle formation. The objective of this research was therefore to determine factors that influence average casein micelle size in milk from individual cows. Average casein micelle size of milk samples was associated with the A and B genetic variants of κ-casein, and differences in concentration of glycosylated κ-casein as a fraction of total milk protein. Milk samples with a low average casein micelle size were associated with the B variant of κ-casein and a higher relative concentration of glycosylated κ-casein, compared with milk samples with a high average casein micelle size. Differences observed may be attributed to the effect of glycosylated κ-casein groups on casein micelle formation in the mammary gland.

4.1 Introduction

The average hydrodynamic diameter of casein micelles in bulk milk is rather constant, at around 200 nm (De Kruif & Holt, 2003); however, casein micelle size of individual milk samples, although constant during lactation, can vary markedly between the milk from cows, between 154 and 230 nm (De Kruif & Huppertz, 2012). This variation in casein micelle size can influence several technological aspects of milk, such as the rennet-induced gel formation (Ekstrand, Larsson-Raznikiewicz, & Perlmann, 1980; Glantz et al., 2010). In addition, understanding the nature of differences in casein micelle size can provide more insight into casein micelle structure and the physiology of micelle formation in the mammary gland. To exploit the possibilities of differences in casein micelle size between cows, it is important to understand the factors that influence micelle size.

Besides the variation in average casein micelle size between cows, a large variation also exists in protein composition of the milk of individual cows (Heck et al., 2008). The sources of these variations in the major milk proteins include genetic polymorphism (Bobe, Beitz, Freeman, & Lindberg, 1999; Hallen, Wedholm, Andren, & Lunden, 2008; Heck et al., 2009) and post-translational modification (PTM) (Holland, 2009). Genetic polymorphism is mainly caused by the substitution or deletion of amino acids within the polypeptide chains, which result in genetic variants of milk caseins (Caroli, Chessa, & Erhardt, 2009; Farrell et al., 2004). The complexity of caseins is further increased by PTM. PTMs of caseins include the glycosylation of κ-casein (κ-CN), the phosphorylation of all caseins and the disulphide bridging of α_{s2}-casein (α_{s2}-CN) and κ-CN (Holland, 2009; Huppertz, 2013). The microheterogeneity of κ-CN is particularly high, since it can be present as the A, B and E genetic variant in Holstein-Friesian cows, with 0-6 glycans and 1-3 phosphate groups attached to specific threonine and serine residues, respectively, in its C-terminus (Holland, Deeth, & Alewood, 2006). In addition, the N-terminus of κ-CN contains two cysteine residues, which engage intermolecular disulphide-bridging (Bouguyon, Beauvallet, Huet, & Chanat, 2006; Holland, Deeth, & Alewood, 2008; Rasmussen, Hojrup, & Petersen, 1992). Glycosylation of κ-CN starts in the Golgi apparatus of milk epithelial cells (Vilotte, Whitelaw, Ollivier-Bousquet, & Shennan, 2003; Witsell, Casey, & Neville, 1990). These glycans can be present as mono-, di-, tri- and tetrasaccharides. The most common form is the tetrasacharide (56%), which is composed of galactose, N-acetylgalactosamine and two neuraminic acid groups (Saito & Itoh, 1992).

To determine correlations between size and milk composition, most studies have used bulk milk (Dalgleish, Horne, & Law, 1989; Davies & Law, 1983; Donnelly,

McNeill, Buchheim, & McGann, 1984; McGann, Donnelly, Kearney, & Buchheim, 1980; Yoshikawa, Takeuchi, Sasaki, & Chiba, 1982). In these studies on bulk milk, the casein micelles were fractionated into different size classes, and it was generally agreed that κ-CN content of these fractions was negatively correlated with size of the casein micelles therein. In contrast to κ-CN, there was no general agreement as to whether proportions of other caseins correlated with casein micelle size in these fractions. Also, there was no general agreement on the effect of glycosylation of κ-CN on casein micelle size; results showed that glycosylation of κ-casein was either positively correlated (Yoshikawa et al., 1982; O'Connell & Fox, 2000), inversely correlated (Slattery, 1978) or did not correlate with the size of fractionated casein micelles (Dalgleish, 1985, 1986) from bulk milk. Differences in methods of fractionation and analyses of different fractions of glycosylated κ-casein, as well as variation in bulk milk composition, might have caused these contradicting findings. Since the substructure of casein micelles is partly stabilized by calcium phosphate nanoclusters, correlations between micellar calcium and phosphate content and the size of casein micelle classes fractionated from bulk milk have also been investigated; such studies revealed that micellar inorganic phosphate decreases slightly, whereas micellar calcium remains constant, as casein micelle size decreases (Dalgleish et al., 1989).

For individual milk samples, average casein micelle size of the unfractionated skimmed milk is commonly compared between milk samples of individual cows. Also, for individual milk information on genetic variation can be included while for bulk milk this is often not available. It is therefore not surprising that, in general, different results have been obtained for individual milk samples compared to fractionated bulk milk. In the former, no correlation was found between total κ-CN level and casein micelle size (De Kruif & Huppertz, 2012). Studies on individual milk samples have also shown that average casein micelle size of individual cows' milk samples was correlated with genetic variants of κ-CN; milk samples with the κ-CN A variant had larger casein micelles than milk with the κ-CN B variant (Lodes, Buchberger, Aumann, & Klostermeyer, 1996; Walsh et al., 1998). These κ-CN variants differ at amino acid residues 136 (A: Thr; B: Ile) and 148 (A: Asp; B: Ala) (Farrell et al., 2004) and it has been argued that these differences in amino acid composition influence PTMs on κ-CN (Plowman, Creamer, Liddell, & Cross, 1997). For glycosylation, there appears to be a general agreement that κ-CN B is more extensively glycosylated than κ-CN A (Coolbear, Elgar, & Ayers, 1996; Robitaille, Ng Kwai Hang, & Monardes, 1991). However, a correlation between glycosylation of κ-CN and average casein micelle size of individual milk samples has not been reported before; furthermore, correlations with other PTMs have not been studied

before. With regard to salts composition of milk, analysis of total calcium, potassium and magnesium of individual milk samples obtained from Swedish-Holstein and Swedish-Red breeds from several herds showed a negative correlation between magnesium and potassium and casein micelle size ($p < 0.05$) (Glantz et al., 2010). No correlations between total calcium and casein micelle size were found (Devold, Brovold, Langsrud, & Vegarud, 2000; Glantz et al., 2010). However, correlations between micellar salts and micelle size have not been investigated before in individual bovine milk samples.

The objective of this research was therefore to determine factors that influence average casein micelle size of milk, by analysing differences in the salt composition and distribution and the casein composition of milk samples from individual cows with low or high average casein micelle size. For this purpose, two sets of 9 milk samples with high and low average sizes where selected from a larger set of milk samples of Holstein-Friesian cows. Significant results were confirmed using a second larger dataset, which consisted of milk samples of Montbéliarde cows. The Montbéliarde breed was selected in order to include the κ-casein BB genotype, which has been reported to be more common among Montbéliarde breed than in Holstein-Friesian cattle (Grosclaude, 1988).

4.2 Materials and methods

4.2.1 Sample selection and preparation

Raw milk was obtained from 50 Holstein-Friesian cows distributed over 5 herds, and 54 Montbéliarde cows of 1 herd, in the Netherlands. For each individual cow, 0.5 L of milk was taken during the afternoon milking. The selected Holstein-Friesian cows were in early or middle lactation (between 30 and 200 days) at the time of first sampling. Resampling was scheduled within 2 months after the first sampling. Samples from Montbéliarde cows were distributed over all three stages of lactation, except for the colostrum period (1-3 days after calving). Herds did not participate in any feeding trials and diets were not regulated. The samples were preserved with 0.02% (w/w) sodium azide (Sigma-Aldrich, Steinheim, Germany) immediately after cooled transport. To obtain skim milk, raw milk was centrifuged (1500 × *g*, 15 min at 10 °C) (Avanti J-26 XP, Beckman Coulter, Krefeld, Germany). Solubilized low-heat skim milk powder (Nilac; NIZO food research, Ede, The Netherlands) was used as a reference sample. The pH of defatted milk was measured, using an Orion 8102BN pH electrode (Thermo, Beverly, CA, USA). Skim

milk ultracentrifugate (100,000 × *g*, 20 °C for 1 h) was obtained using a Beckman Coulter L-60 ultracentrifuge with a 70 Ti rotor (Beckman Coulter).

4.2.2 Macronutrient composition

Fat, protein, dry matter, lactose content and somatic cell count (SCC) of milk was measured by CombiFossFT (Foss Electric, Hillerød, Denmark) combining Fourier transform infrared and flow cytometry technology at the Dutch milk control station (Qlip, Zutphen, The Netherlands).

4.2.3 Salt content and composition

Calcium, potassium, magnesium, sodium and phosphorus concentrations were measured by inductive coupled plasma atomic emission spectrometry (ICP-AES) (Varian, Mulgrave, Australia). Chloride, sulphate, inorganic phosphate and citrate concentrations were measured by Ion Chromatography (IC). Sample preparation for these analyses in milk and milk ultracentrifugate was performed as described previously (Gao et al., 2009).

4.2.4 Protein content and casein composition

For measurement of protein (6.38 x total N), casein and serum protein content of milk, samples were prepared according to the ISO protocol for determination of casein nitrogen in milk (ISO, 2011). Dumas analysis was performed in a Flash EA 1112 series protein analyser (Thermo). Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to determine casein composition (Visser, Slangen, & Rollema, 1991). Separation was performed on a reversed-phase analytical column C18 (Aeris 3.6μm XB-C18 Widepore, Phenomenex, Torrance, CA, USA). Temperature of the column was set to 40 °C, temperature in the sampler was 10 °C. The injection volume of the sample was 25 μL. A flow rate of 0.40 mL min^{-1} was applied during runs of 33 min. Peak profiles were analysed using Chromeleon software (Chromeleon 7.00, Dionex, Thermo). The fractions of the caseins and major whey proteins were estimated by dividing the integrated peak area of an individual protein by the total integrated peak area of whey proteins and caseins in the chromatogram of an individual milk sample. An example of peak assignment of an individual milk sample is given in Fig. 4.1. Phenotyping of glycosylated κ-CN (κ-CN-G) and non-glycosylated κ-CN with 1 phosphate group of genetic variants A/E/B (κ-CN-NG), genetic variants A and B of β-lactoglobulin (β-LG) and genetic variants A1, A2 and B of β-casein (β-CN) was based on the RP-HPLC method of Visser et al. (1991). The fraction of κ-CN-NG consisted of the κ-CN-A and κ-CN-B peaks and total

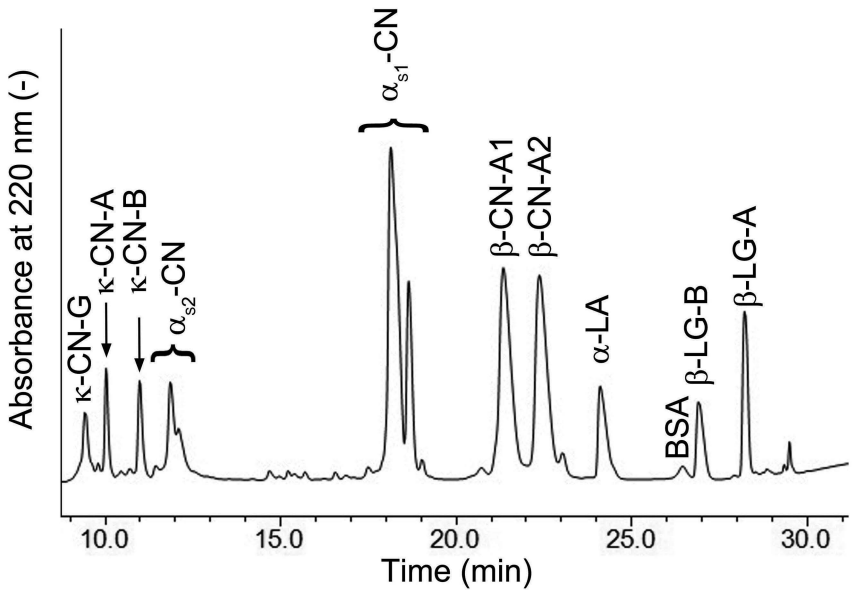

Figure 4.1 Peak assignment of proteins from high performance liquid chromatography of an individual milk sample.

κ-CN fraction consisted of κ-CN-G and κ-CN-NG peaks in the chromatogram (Fig. 4.1). The presence of the κ-CN E and β-CN A3 and I variants was verified using capillary zone electrophoresis (CZE) analysis (Heck et al., 2008).

4.2.5 Particle size analysis

The Z-average hydrodynamic diameter of casein micelles was determined by diluting defatted milk samples 50 times in milk ultrafiltrate (NIZO food research, Ede, the Netherlands). The diluted milk samples were filtered using a 1.2 µm MF Millipore syringe filter (Millipore, Billerica, MA, USA). Measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were measured at 22 °C using a scattering angle of 173°. For each sample, triplicate measurements were performed, each consisting of 13 sub-measurements.

4.2.6 Statistical analysis

IBM SPSS statistics 19 (IBM Corporation, Armonk, NY, USA) was used for data analysis. An independent sample t-test was performed to compare the groups with a low and a high average casein micelle size. One-way analysis of variance (one-way

ANOVA) was used to compare average casein micelle size between three groups with different genotypes of κ-CN. The least significant difference (LSD) post-hoc test was used for pairwise comparison of these groups. Pearson correlation coefficients were determined in the correlation analyses. Results were assigned to be significant at a 1% significance level.

4.3 Results and discussion

4.3.1 Variation in casein micelle size between milk of individual cows

To select cows that produced milk with small and large average casein micelle sizes, Z-average diameter was determined for skimmed milk of 50 Holstein-Friesian cows; the frequency distribution of the average casein micelle size of these samples is shown in Fig. 4.2. From the 50 milk samples analysed, 2 sets of 9 cows which produced milk with high and low average casein micelle size (below 171 nm or above 207 nm) were selected for resampling, which was conducted within two months of the first sampling. It was expected that micelle size of resampled cows would remain constant (De Kruif & Huppertz, 2012). A comparison of average casein micelle size of samples of the individual cows during the first and second round of sampling is shown in Fig. 4.3. There was no significant difference in average size between the first and second sampling round for the groups containing small and the large casein micelles. In addition, for most individual

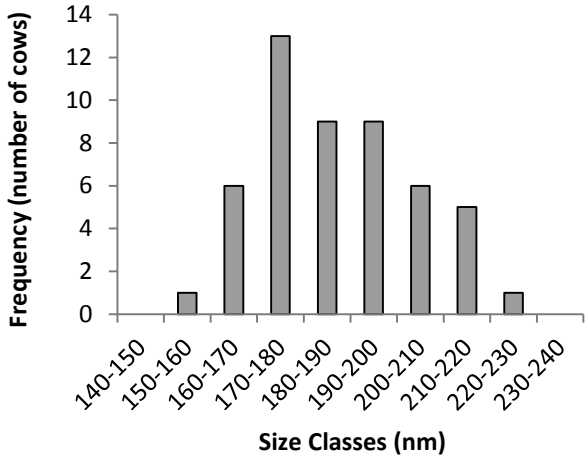

Figure 4.2 Frequency distribution of average casein micelle sizes of 50 individual Holstein-Friesian milk samples.

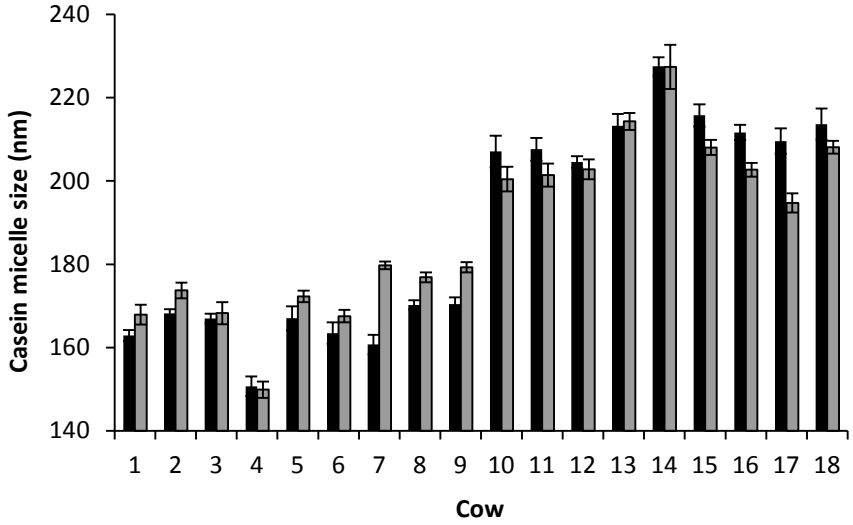

Figure 4.3 Casein micelle size of 18 individual cows at two different time points. Cows 1–9 were classified as producing milk with a low average casein micelle size, whereas cows 10–18 were characterized as producing milk with a high average casein micelle size.

samples, average micelle size remained rather constant. The average sizes of the micelles in the groups with small and large casein micelles used for further analysis were 170.6 +/- 9.1 nm and 206.7 +/- 5.0 nm, respectively (Table 4.1).

4.3.2 Influence of macronutrient composition and milk serum composition on casein micelle size

None of the macronutrients analysed differed significantly between groups of milk samples with low and high average casein micelle size (Table 4.1), similar to the observations for correlations between fat and protein with casein micelle size reported by De Kruif and Huppertz (2012). Furthermore, no significant difference in pH was found between milk samples with small and large casein micelles. This was also observed in earlier research on samples obtained in the early lactation, wherein casein micelle size remained constant from cows which were 2–30 days in lactation, while pH of milk gradually increased from 6.28 ± 0.11 towards 6.64 ± 0.10 (Tsioulpas, Grandison, & Lewis, 2007). The difference in SCC between the groups was not significant and well below the threshold value that indicates mastitis. In contrast, the concentration of soluble organic phosphate was significantly higher ($p < 0.01$) in the group of milk samples with large average casein

Table 4.1 Comparison of composition and properties of classes of milk samples with small and large average casein micelle size.[a]

Component or property	Casein micelle size class		
	Small micelles	Large micelles	*p*-value
Average casein micelle size diameter (nm)	170.6 ± 9.1	206.7 ± 5.0	<0.001
pH (-)	6.69 ± 0.04	6.69 ± 0.07	n.s.
Log somatic cell count (cells mL^{-1} milk)	4.43 ± 0.51	4.86 ± 0.40	n.s.
Macronutrient composition (g 100 g^{-1} milk)			
Protein	3.57 ± 0.43	3.54 ± 0.30	n.s.
Casein	2.62 ± 0.30	2.80 ± 0.52	n.s.
Serum protein	0.94 ± 0.47	0.74 ± 0.38	n.s.
Dry matter	12.79 ± 1.40	12.81 ± 1.36	n.s.
Lactose	4.53 ± 0.16	4.56 ± 0.17	n.s.
Fat	3.83 ± 1.14	3.87 ± 1.31	n.s.
Salt composition (mmol kg^{-1} defatted milk)			
Total Ca	31.99 ± 3.01	31.26 ± 1.56	n.s.
Soluble Ca	11.05 ± 1.35	10.91 ± 1.44	n.s.
Micellar Ca	20.94 ± 3.68	20.35 ± 1.50	n.s.
Total Mg	4.53 ± 0.41	4.60 ± 0.57	n.s.
Soluble Mg	3.15 ± 0.31	3.23 ± 0.45	n.s.
Micellar Mg	1.38 ± 0.41	1.37 ± 0.28	n.s.
Total P	32.99 ± 3.99	32.48 ± 3.58	n.s.
Total inorganic PO_4	19.53 ± 2.23	18.38 ± 2.56	n.s.
Soluble P	15.48 ± 1.88	15.64 ± 2.54	n.s.
Soluble inorganic PO_4	11.28 ± 1.53	9.78 ± 2.08	n.s.
Micellar inorganic PO_4	8.25 ± 1.64	8.59 ± 0.88	n.s.
Micellar organic PO_4	9.26 ± 2.04	8.24 ± 1.00	n.s.
Soluble organic PO_4	4.20 ± 1.14	5.86 ± 1.26	<0.01
Total Citrate	8.61 ± 1.75	8.47 ± 1.50	n.s.
Total K	43.12 ± 2.88	43.35 ± 3.28	n.s.
Total Na	14.86 ± 2.06	16.35 ± 3.01	n.s.
Total Cl	22.06 ± 2.86	25.63 ± 3.58	n.s.
Total SO_4	1.04 ± 0.17	1.26 ± 0.20	n.s.
Relative concentration of proteins (%)			
α_{s1}-casein	32.07 ± 1.30	33.12 ± 1.25	n.s.
α_{s2}-casein	8.33 ± 1.15	9.12 ± 1.18	n.s.
β-casein	33.32 ± 1.93	32.38 ± 1.71	n.s.
κ-casein (total)	10.45 ± 1.00	9.42 ± 1.23	n.s.
Glycosylated κ-casein	3.85 ± 0.76	2.84 ± 0.28	<0.002
Non-glycosylated κ-casein	6.60 ± 0.73	6.57 ± 1.08	n.s.
Blood serum albumin	0.72 ± 0.20	0.71 ± 0.26	n.s.
α-lactalbumin	4.15 ± 0.86	4.46 ± 0.54	n.s.
β-lactoglobulin	10.96 ± 1.02	10.78 ± 1.32	n.s.

[a] Values are means ± standard deviation (n = 9). Significance of difference between the groups was evaluated using a t-test. *p*-values are reported where significant differences were observed, whereas n.s. indicates no significant differences were observed between groups.

micelle size. The phosphate in this fraction is part of various organic constituents, such as phospholipids, ADP, non-sedimentable caseins and proteose peptones, in which minor fluctuations can occur. However, this fractions does not seem directly relevant for casein micelle size. Other soluble salts and the total amount of potassium, sodium, chloride, sulphate and citrate, which are mainly present in milk serum, as well as the whey proteins bovine serum albumin (BSA), α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), showed no significant difference between the groups.

4.3.3 Influence of casein composition on casein micelle size

Comparison of components related to the stabilization of the substructure of casein micelles, i.e., the concentrations of α_{s1}-CN, α_{s2}-CN and β-CN, as well as those of micellar calcium, magnesium and phosphate, showed no significant differences between the groups with low and high average casein micelle size (Table 4.1). However, Table 4.1 shows a significant difference ($P < 0.002$) in relative amount of κ-CN-G between the two groups; i.e., κ-CN-G was more abundant in milk samples with low average micelle size than in milk samples with high average micelle size. Differences in total κ-CN and κ-CN-NG were not significant between milk samples with low and high average casein micelle size. The results were confirmed using a second larger dataset (section 4.3.4).

4.3.4 Influence of genotypes of milk proteins on casein micelle size

Individual milk samples varied in genotype for κ-CN, β-CN and β-LG and the effect of these genotypes were related to casein micelle size. The comparison of κ-CN

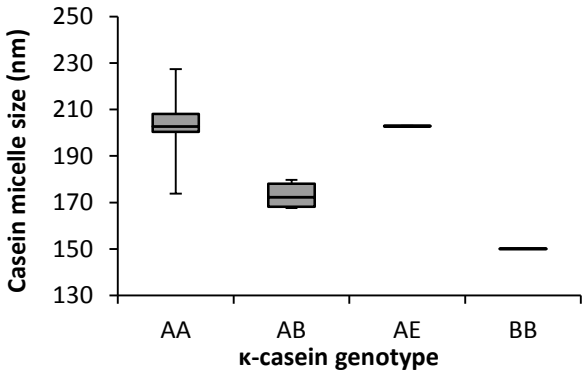

Figure 4.4 Boxplot of average casein micelle size as a function of κ-casein genotypes AA (n=9), AB (n=7), AE (n=1), BB (n=1) of individual Holstein-Friesian cows.

genotypes is shown in Fig. 4.4. Four different genotypes were found; AA (n=9), AB (n=7), AE (n=1), BB (n=1). There was a significant difference ($p < 0.001$) in casein micelle size between milks with the AA genotype (203.5 ± 14.6 nm) and the AB genotype (173.1 ± 5.4 nm). Interestingly, a significant difference ($p < 0.001$) was also found in κ-CN-G between groups of AA and AB genotypes of κ-CN; milk with the κ-CN AA genotype had less κ-CN-G compared with the κ-CN AB genotype. This is in agreement with the results of Robitaille et al. (1991), who reported that the milk from 33 cows with the κ-CN AA genotype contained, on average, a lower amount of neuraminic acid than the milk of 20 cows genotyped κ-CN AB. It therefore seems that there is an interaction between genetic variant of κ-CN, κ-CN-G and casein micelle size. In both cases, however, not enough results were available for the κ-CN BB genotype.

To determine to what extent genetic variants and κ-CN-G contribute to casein micelle size, these values were determined in another dataset, which will be presented and discussed below. Furthermore, 9 different genotypes of β-CN were found in the milks of Holstein-Friesian cows, containing combinations of the common A1, A2, A3, B and I variants. Subsets of groups were too small for statistical comparison. However, the large variation suggests that there was no influence of β-CN variants on average casein micelle size. With regard to β-LG, 8 samples had the AA genotype, 9 samples AB and 1 sample BB genotype. The AA to AB genotype showed no significant differences in size.

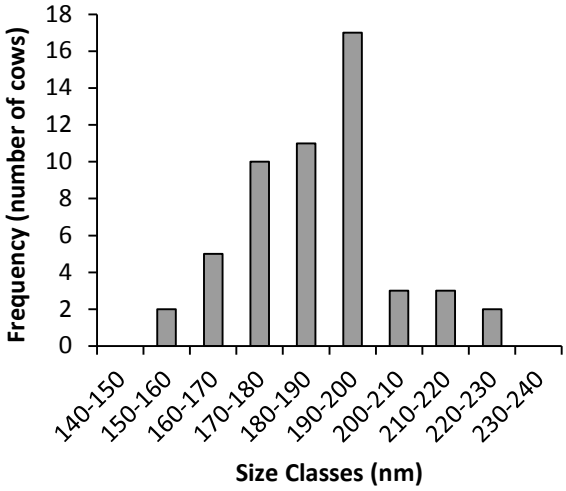

Figure 4.5 Frequency distribution of average casein micelle sizes of 53 individual Montbéliarde milk samples.

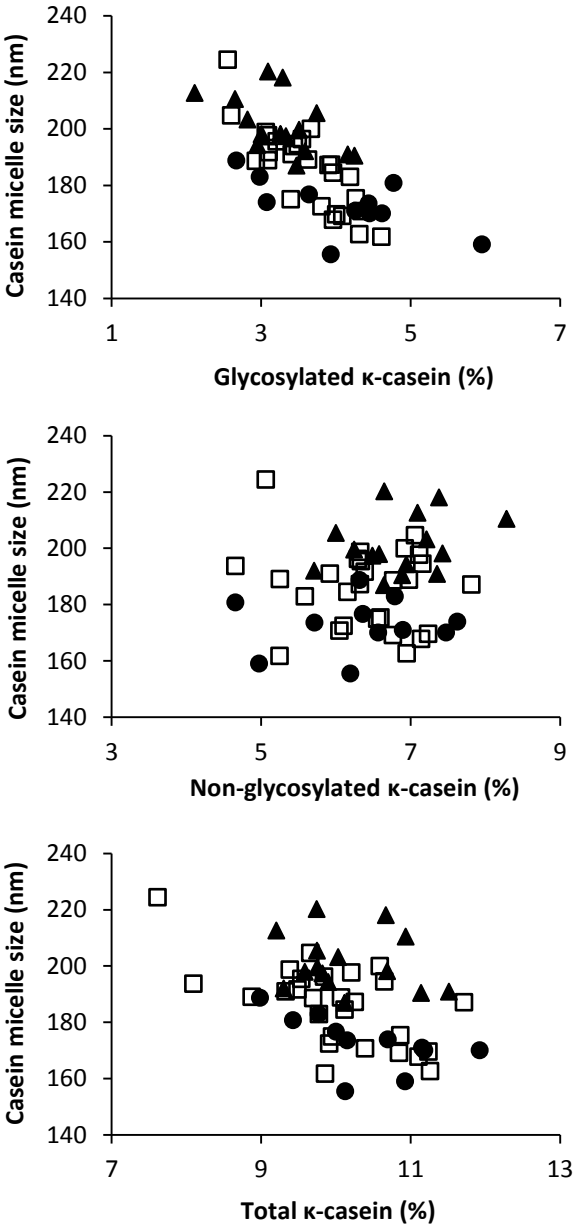

Figure 4.6 Average casein micelle diameter (nm) for different κ-casein genotypes (κ-CN; ●, BB; ▲, AA; □, AB) as a function of the percentage of glycosylated κ-CN, non-glycosylated κ-CN and total κ-CN, expressed as a fraction of total protein of 53 individual Montbéliarde milk samples.

From the aforementioned analysis between groups of milk samples containing small and large casein micelles, two variables were found to significantly affect average casein micelle size; i.e., κ-CN-G and genetic variants of κ-CN. To confirm and further evaluate these findings, a second, larger, dataset, containing milk samples of 53 Montbéliarde cows was created. The casein micelle size frequency distribution of the 53 Montbéliarde milk samples displayed in Fig. 4.5. Compared to the milk of Holstein-Friesians, average casein micelle size, total κ-CN and κ-CN-G for the milk of Montbéliardes did not differ significantly. Correlation analysis of casein micelle size of the 53 individual cows with the factors α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, κ-CN-G and κ-CN-NG, showed a significant correlation ($p < 0.01$) with total levels of κ-CN and κ-CN-G. The Pearson correlation coefficient with κ-CN-G (-0.718) was stronger than with total κ-CN (-0.448) and no significant correlation was found with κ-CN-NG (0.151). A large part, on average 36%, of total κ-CN was glycosylated, thus indicating that the correlation found with total κ-CN seems to originate from the κ-CN-G fraction. Genotypes of κ-CN were distributed as follows: 15 AA, 27 AB and 11 BB. There was a significant difference ($p < 0.001$) in casein micelle size between the three groups. Moreover the LSD post-hoc test showed that the average casein micelle size of the AA genotype (201.2 ± 10.3 nm) and AB genotype (186.0 ± 14.4 nm) and BB genotype (173.0 ± 9.7 nm) were significantly different ($p<0.01$) in all pairwise comparisons.

The factors κ-CN-G and genetic variants of κ-CN were used for further analysis. In Fig. 4.6, average casein micelle diameter for different genotypes of κ-CN as a function of levels of κ-CN-G, κ-CN-NG and total κ-CN is shown. It can clearly be observed that there is a strong correlation between average casein micelle size and κ-CN-G, whereas there is no apparent correlation with total κ-CN. Also, a significant

Table 4.2 Linear model with interaction of κ-casein (κ-CN) genetic variants and glycosylated κ-CN (κ-CN-G) on average casein micelle size (n=53) in milk samples from Montbéliarde cows.

Parameter	df	*F*	Sig.
Corrected model[a]	5	26.870	.000
Intercept	1	1088.650	.000
κ-CN variant * κ-CN-G	2	7.201	.002
κ-CN variant	2	8.324	.001
κ-CN-G	1	43.262	.000
Error	47		
Total	53		
Corrected Total	52		

[a] $R^2 = 0.741$.

difference ($p < 0.003$) was found in glycosylated κ-CN between groups homozygous for the A and B variant. The linear model with interaction for the effect of κ-CN genetic variants and κ-CN-G on average casein micelle size was significant ($p < 0.001$, $R^2 = 0.741$) (Table 4.2). This demonstrates that κ-CN-G and genetic variants of κ-CN, as independent factors and with interaction, have an effect on average casein micelle size. The F-value for κ-CN-G was higher than for κ-CN genetic variants and the interaction; therefore, it can be concluded that the effect of κ-CN-G is most important. Causes of a higher concentration of κ-CN-G in milk are not known.

4.4 Conclusions

Genetic variants A and B of κ-CN and glycosylation of κ-CN correlated significantly with average casein micelle size of milk of individual cows. Individual milk samples with a low average casein micelle size were associated with the B variant of κ-CN and a higher percentage of κ-CN-G as a fraction of total protein compared to individual milk samples with a high average casein micelle size. Macronutrient composition, whey proteins and constituents related to the stabilization of the substructure of casein micelles were not correlated with average casein micelle size.

The fact that average casein micelle size in milk of individual cows is specifically correlated with κ-CN-G has not been reported before, and can help increase our understanding of the formation and stabilization of casein micelles. Change in structure of κ-CN clusters caused by glycosylation can influence micellar stabilization during or after casein micelle formation in the mammary gland, and thereby influence casein micelle size. Because of the complex structure of casein micelles, presently, there are no mechanistic models available to accurately describe the underlying mechanism of this formation. However models that are available to describe self-assembly of surfactants and amphiphilic ionic block co-polymers could help to increase the understanding of casein micelle formation and stabilization (Borisov, Zhulina, Leermakers, & Müller, 2011; Israelachvili, Mitchell, & Ninham, 1976). Other PTMs that could play a role in this, such as disulphide bridging of κ-CN, need to be further examined as well.

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5

Chymosin-induced hydrolysis of caseins: Influence of degree of phosphorylation of alpha-s1-casein and genetic variants of beta-casein

Etske Bijl[1], Hein van Valenberg[1], Sybren Sikkes[1], Stijn Jumelet[1], Guido Sala[2,3], Kees Olieman[4], Toon van Hooijdonk[1], Thom Huppertz[4]

[1] Dairy Science and Technology Group, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands; [2] Physics and Physical Chemistry of Foods Group, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands; [3] Food Technology Centre, Food & Biobased Research, Wageningen University & Research centre, P.O. Box 17, 6700 AA, Wageningen, The Netherlands; [4] NIZO food research, P.O. Box 20, 6710 BA, Ede, The Netherlands

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Abstract

The objective of this study was to investigate the influence of natural variation in α_{s1}-casein and β-casein composition of milk on chymosin-induced hydrolysis of milk gels and sodium caseinate solutions. The results showed that 15% more of α_{s1}-casein with eight phosphate groups was broken down compared to α_{s1}-casein with nine phosphate groups at 50% total casein degradation in chymosin-induced milk gels. Furthermore, in sodium caseinate solutions, over 10% more of β-casein A2 was degraded compared to β-casein A1 and B variant at 50% total casein degradation. In general, comparison of sodium caseinate solutions to milk gels showed that small differences in either phosphorylation of α_{s1}-casein or amino acid substitution in genetic variants of β-casein can cause significant differences in degradation by chymosin, possibly due to changes in physical conformation of casein monomers, aggregates or casein micelles.

5.1 Introduction

Chymosin is the main proteinase in calf rennet, which initiates milk coagulation during cheese production. After removal of the whey, up to 30% of the chymosin is retained in the curd and plays a major role in the initial proteolysis of α_{s1}-casein (α_{s1}-CN) and β-casein (β-CN) during ripening of cheese (Fox & McSweeney, 1996; Grappin, Rank, & Olson, 1985; Upadhyay, McSweeney, Magboul, & Fox, 2004). The primary proteolysis products of caseins are the precursors of small peptides, amino acids and volatile flavour compounds (Sousa, Ardo, & McSweeney, 2001). Furthermore the hydrolysis of caseins leads to changes in textural properties (Lucey, Johnson, & Horne, 2003). Considering the impact on texture and flavour formation, proteolysis is often stated to be the most important biochemical event during the ripening of most cheese varieties (Fox, 1989; McSweeney, 2004).

The primary cleavage site of chymosin during cheese ripening is in α_{s1}-CN Phe23-Phe24 (Mulvihill & Fox, 1979). In cheese model solutions and cheese, proteolysis of β-CN by chymosin was low compared to degradation of α_{s1}-CN (Exterkate, Alting, & Slangen, 1995; Exterkate, Lagerwerf, Haverkamp, & Van Schalkwijk, 1997; Ledford, O'Sullivan, & Nath, 1966; McSweeney, Pochet, Fox, & Healy, 1994). In addition, the other two casein fractions in cheese curd, α_{s2}-casein and para-κ-CN, seem to be relatively resistant to proteolysis (Green & Foster, 1974; McSweeney, Olson, Fox, & Healy, 1994). Since chymosin mainly hydrolyzes α_{s1}-CN during cheese ripening, natural variation in α_{s1}-CN and β-CN in milk of individual cows can influence proteolysis and texture formation in cheese. Also variation in the individual α_{s1}-CN and β-CN fractions might influence proteolysis. Within the α_{s1}-CN fraction there is variation in phosphorylation of the proteins; α_{s1}-CN with eight phosphate groups (α_{s1}-CN-8P) and α_{s1}-CN with nine phosphate groups (α_{s1}-CN-9P) (Holland, 2009). Within β-CN fractions there is variation in genetic variants; in 2005, 29% of Dutch Holstein-Friesian cows had β-CN A1, 69% β-CN A2 and 2% β-CN B (Heck et al., 2009).

Information on the influence of natural variation in casein composition on proteolysis during ripening of cheese is scarce. The effect of genetic variants of caseins or casein content on milk coagulation properties have mainly been studied the first hours after rennet or chymosin addition (Bonfatti, Di Martino, Cecchinato, Degano, & Carnier, 2010; Delacroix-Buchet, Lefier, & Nuyts-Petit, 1993; Hallen, Allmere, Naslund, Andren, & Lunden, 2007; Ikonen, Morri, Tyriseva, Ruottinen, & Ojala, 2004; Jõudu, Henno, Kaart, Püssa, & Kärt, 2008; Ostersen, Foldager, & Hermansen, 1997; Poulsen et al., 2013). Some other studies have determined the influence of variation in casein composition of milk on proteolysis and texture

formation during ripening of cheese (O'Mahony, McSweeney, & Lucey, 2008; St-Gelais & Hache, 2005). However, in these studies casein fractions were added to or removed from the milk in order to vary casein composition. Although the use of a model system is required to study effects in detail, the approach of addition or removal of caseins has several drawbacks. First of all, removal of β-CN from milk by cold membrane filtration has been shown to influence renneting time and stiffness of cheese gels by changes in ionic composition of milk (Holland, Corredig, & Alexander, 2011). Secondly, addition of β-CN to milk will alter total casein content and it is not known whether the additional β-CN is associated with the casein micelle in the same way as in the original micelles. Altered casein micelle structure might result in different coagulation behaviour and proteolysis.
The objective of this research was therefore to study the influence of natural variation in α_{s1}-CN and β-CN composition of milk on chymosin-induced hydrolysis of milk gels. In order to take the influence of casein micelle structure into account, a comparison was made between proteolysis in milk gels and sodium caseinate (NaCas) solutions.

5.2 Materials and Methods

5.2.1 Sample selection and preparation

Raw milk was obtained during the afternoon milking from 49 Holstein-Friesian cows (between 36 and 271 d in lactation) distributed over 2 herds in the Netherlands, with resampling done 1 month after first sampling. The samples were preserved with 0.1 g kg^{-1} thiomersal and 0.02 mg kg^{-1} aprotinin (Sigma-Aldrich, Steinheim, Germany) immediately after cooled transport. Raw milk was centrifuged twice (1100 × *g*, 15 min at 7 °C) (Avanti J-26 XP, Beckman Coulter, Krefeld, Germany) to obtain defatted milk. Samples were selected and mixed based on their α_{s1}-CN/β-CN ratio, determined as outlined in section 5.2.3; seven samples were mixed in equal amounts to obtain a representative mixed sample with a low α_{s1}/β-CN ratio and eight samples were mixed in equal amounts to obtain a representative mixed sample with a high α_{s1}/β-CN ratio.

5.2.2 Sample analysis

Fat, dry matter, lactose content and somatic cell count (SCC) of the individual milk samples was measured by CombiFossFT (Foss Electric, Hillerød, Denmark) combining Fourier transform infrared and flow cytometry technology. Protein (6.38 × total nitrogen), casein and serum protein were measured according to ISO (2011). All these analyses were executed at the Dutch milk control station (Qlip, Zutphen,

The Netherlands). pH was measured, using an Orion 8102BN pH electrode (Thermo, Beverly, CA, USA). Casein micelle size was measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) as described previously (Bijl, de Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014). Dry matter content of milk gels was determined by difference in weight of gels before and after heating in a stove (106 °C, 16 h).

IBM SPSS statistics 19 (IBM Corporation, Armonk, NY, USA) was used for data analysis. An independent sample t-test was performed to compare the groups with a low and a high α_{s1}/β-CN ratio. Results were assigned to be significant at a 1% significance level.

5.2.3 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) analysis was performed on a Beckman P/ACE MDQ Capillary Electrophoresis system (Beckman Instruments, Fullerton, CA, USA) to determine protein composition and the degree of phosphorylation. For this purpose, the CZE method of (Heck et al. (2008) and Recio and Olieman (1996) was optimized for separation of α_s-caseins by increasing the length of the capillary to 85 cm and increasing the pH of the run buffer to 3.15, while reducing the concentration of sodium citrate to 5 mM. Separations were carried out at 45 °C and a linear voltage gradient from 0 to 30 kV in 10 min was used. In addition to the sample buffer described by Heck et al. (2008), the sample buffer solutions contained 0.8 mg/mL L-leucine-p-nitroanilide (Sigma-Aldrich) as internal standard (IS). Defatted milk samples were mixed with sample buffer at a ratio of 2:3. Samples were injected at the anode by pressure injection at 1.05 bar for 40 s. UV detection was performed at 214 nm and peak area was corrected for migration time in order to determine relative protein concentration (Heck et al., 2008). Chromeleon 7 software version 7.1 (Dionex, Thermo Scientific, Sunnyvale, CA, USA) was used for the analysis of results.

The method was used to determine the relative concentration of α_{s1}-CN-8P and α_{s1}-CN-9P, total α_{s1}-CN (sum of α_{s1}-CN-8P and α_{s1}-CN-9P), α_{s2}-CN, β-CN, non-glycosylated κ-CN-1P, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). Also, the proteolysis products after chymosin-induced hydrolysis of the Phe23- Phe24 bond of α_{s1}-CN, α_{s1}-CN fragments f1-23 and f24-199, were determined (Recio, Amigo, Ramos, & Lopez-Fandino, 1997). The results of the proteolysis experiments for decrease in casein fractions were expressed as a function of total casein degradation, which was defined as the sum of α_{s1}-CN-8P, α_{s1}-CN-9P and β-CN since these were the main chymosin degraded fractions in the samples after κ-CN was hydrolysed during the renneting stage.

5.2.4 Sodium caseinate solutions

Acid curd was prepared from defatted milk samples preheated to 40 °C by decreasing pH to 4.6 using 1M HCl and washing the curds 3 times over a cheese cloth with ultrapure water at 40 °C with 15 min intervals. Next, pH was adjusted to 7.0 with 1M NaOH and 0.1 g kg^{-1} thiomersal and 0.02 mg kg^{-1} aprotinin were added again since most preservatives were lost in the whey. Samples were freeze dried (-53 °C, p<1.5 mbar) (ALPHA 1-4 LDplus, Martin Christ, Osterode am Harz, Germany). NaCas solutions with a casein content of 3% were prepared by dissolving freeze-dried powder in ultrapure water at 32 °C. The pH of the solution was set to 5.6 using 0.23 % (w/w) D-gluconic acid δ-lactone (GDL, Sigma-Aldrich), followed by the addition of 0.01 IMCU mL^{-1} (international milk clotting units) chymosin (Maxiren 600, DSM, Delft, The Netherlands). The samples were subjected to the same temperature-time treatments as for the milk gels as described in the next section and were stored in a climate chamber at 15 ± 1 °C until analysis. Measurements were performed 2.5 h and 2, 4, 6 and 8 d after the addition of chymosin.

5.2.5 Milk gels

Defatted milk was concentrated using a stirred ultrafiltration cell (Stirred Cell Model 8400, Amicon, Millipore, Billerica, MA, USA) equipped with a regenerated cellulose membrane with a cut-off value of 10 kDa (Ultracel PLC membrane 10, Millipore). The ultrafiltration cell was pressurized to 2.5- 3.0 bar. A concentration factor of 5 was obtained based on the mass reduction of the sample. In order to prepare a milk gel 1.5 % (w/w) GDL was added to simulate the pH drop caused by starter bacteria in cheese, immediately followed by the addition of chymosin at a final concentration of 0.13 IMCU mL^{-1}. The concentrated milk was stirred gently, poured into pre-weighed 15 mL tubes, incubated in a water bath at 32 °C for 45 min, followed by incubation in a water bath at 40 °C for 30 min. Subsequently the tubes were centrifuged (2000 × *g*, 60 min, 40 °C). Whey was discarded and the tubes were stored in a climate chamber at 15 ± 1 °C until analysis. Measurements were performed 5 h and 5, 15, 30 and 44 d after addition of chymosin. Using this procedure stable gels with a dry matter content of 50% could be obtained that showed no syneresis during storage.

5.2.6 Texture analysis

Large deformational properties of milk gels were analysed by Texture Analyser (TA.XT.Plus, Stable Micro Systems, Godalming, UK) equipped with a cylindrical probe with a diameter of 50 mm. The gels were cut with a stainless steel gel slicer into pieces with a height of 10 mm each. Gels were compressed to 90% strain at 0.1

mm s^{-1}. The stress acting on the sample during compression (true stress) was calculated as follows:

$$\sigma_t = \frac{F}{A} \qquad \text{(eq. 5.1)}$$

where F is the force measured during compression and A is the cross-sectional area of the sample. The toughness (integral of the stress-strain curve, using a strain of 90%) and gel strength (maximum stress) were determined. Measurements were performed in duplicate.

5.3 Results and discussion

5.3.1 Variation in milk composition of individual cows with high or low α_{s1}/β-CN ratio

To select cows that produced milk with a wide variation in α_{s1}-CN and β-CN composition, α_{s1}-CN over β-CN ratio (α_{s1}/β-CN ratio) was determined of milk of 49 Holstein-Friesian cows. The frequency distribution of α_{s1}/β-CN ratio of these samples is shown in Fig. 5.1. From the 49 milk samples analysed, two sets of ten cows that produced milk with either low or high α_{s1}/β-CN ratio (below 0.72 or

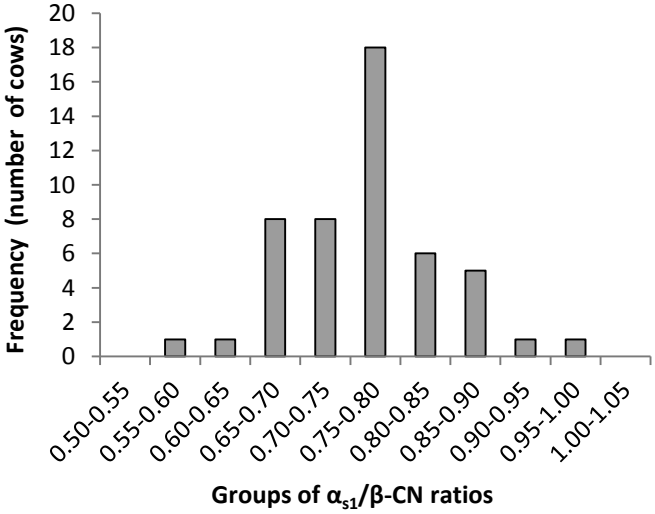

Figure 5.1 Frequency distribution of α_{s1}/β-CN ratio of 49 Holstein-Friesian milk samples.

above 0.79) were selected for resampling. It was expected that α_{s1}/β-CN ratio of resampled cows would remain rather constant since the heritability of milk protein is high (Schopen et al., 2009) and protein composition of milk for individual cows has previously been shown not to vary significantly as a function of lactation stage (De Kruif & Huppertz, 2012). However three cows from the group with low α_{s1}/β-CN ratio had a ratio above 0.73 and two cows from the high α_{s1}/β-CN ratio group had a ratio below 0.80 and were therefore not included for further analysis. Also, there was a slight increase in mean α_{s1}/β-CN ratio for both groups; the mean of the low ratio group increased from 0.68 ± 0.06 to 0.72 ± 0.02 and the mean of the high ratio group increased from 0.86 ± 0.08 to 0.94 ± 0.13. Despite the variations for individual cows in α_{s1}/β-CN ratio the difference between the groups with low and high α_{s1}/β-CN ratio was highly significant ($p<0.002$) (Table 5.1).

In Table 5.1, a comparison of composition and properties of milk samples with low and high α_{s1}/β-CN ratio is presented. None of the macronutrient properties as well as somatic cell count and days in lactation differed significantly between the two groups. Furthermore, in Table 5.1 a comparison between relative concentrations of the major proteins between the group with low and high α_{s1}/β-CN ratio is shown. Only total α_{s1}-CN and β-CN were significantly different; on average the low α_{s1}/β-CN ratio group had 9.5 % less α_{s1}-CN and 15.8 % more β-CN. The individual α_{s1}-CN-

Table 5.1 Comparison of composition and properties of classes of milk samples with high (n=8) and low (n=7) α_{s1}/β-CN ratio. Values represent means ± standard deviation.

Component or property	α_{s1}/β-CN ratio class		
	High ratio	Low ratio	*p*-value
α_{s1}/β-CN ratio (-)	0.94 ± 0.13	0.72 ± 0.02	<0.002
Log Somatic cell count (cells mL^{-1} milk)	4.63 ± 0.34	4.64 ± 0.54	n.s.
Days in lactation	172 ± 71	189 ± 50	n.s.
Macronutrient composition (g 100 g^{-1} defatted milk)			
Protein	4.00 ± 0.55	3.89 ± 0.43	n.s.
Casein	3.02 ± 0.49	3.06 ± 0.33	n.s.
Serum protein	0.98 ± 0.14	0.84 ± 0.11	n.s.
Dry matter	9.47 ± 0.59	9.46 ± 0.42	n.s.
Lactose	4.64 ± 0.25	4.88 ± 0.15	n.s.
Fat	0.05 ± 0.03	0.03 ± 0.03	n.s.
Relative concentration of proteins (%)			
α_{s1}-casein	27.76 ± 1.60	25.13 ± 1.24	<0.004
α_{s1}-casein-8P	20.70 ± 2.11	19.21 ± 0.78	n.s
α_{s1}-casein-9P	7.06 ± 1.52	5.93 ± 0.73	n.s
β-casein	30.05 ± 3.49	34.81 ± 1.78	<0.006
α_{s2}-casein	9.78 ± 1.40	10.46 ± 1.29	n.s.
Non-glycosylated κ-casein-1P	5.61 ± 1.44	5.37 ± 0.69	n.s.
α-lactalbumin	2.04 ± 0.42	2.06 ± 0.40	n.s.
β-lactoglobulin	10.50 ± 1.21	8.26 ± 1.78	n.s.

8P and α_{s1}-CN-9P were not significantly different, due to the large standard deviation in the high ratio group.

In Fig. 5.2, a ternary plot is given with the casein composition of the individual milk samples with high and low α_{s1}/β-CN ratio. On the axis the α_{s1}-CN, β-CN and κ-CN concentrations are given, since these fractions are most important for proteolysis by chymosin. This figure shows that the variation within the low α_{s1}/β-CN ratio group was less compared to the high α_{s1}/β-CN ratio group; the high ratio group had the highest variation in β-CN percentage between 40.8% and 51.8 % and a variation in α_{s1}-CN between 39.6% and 47.5%, while the low ratio group had only a variation between 52.3% and 54.6% in β-CN and 37.6% and 39.5% in α_{s1}-CN. Between the two groups, there was less difference in κ-CN concentration; all samples of the low ratio group and five samples of the high ratio group had values below 9.6%.

Besides the variation in casein composition, there was also variation in genetic

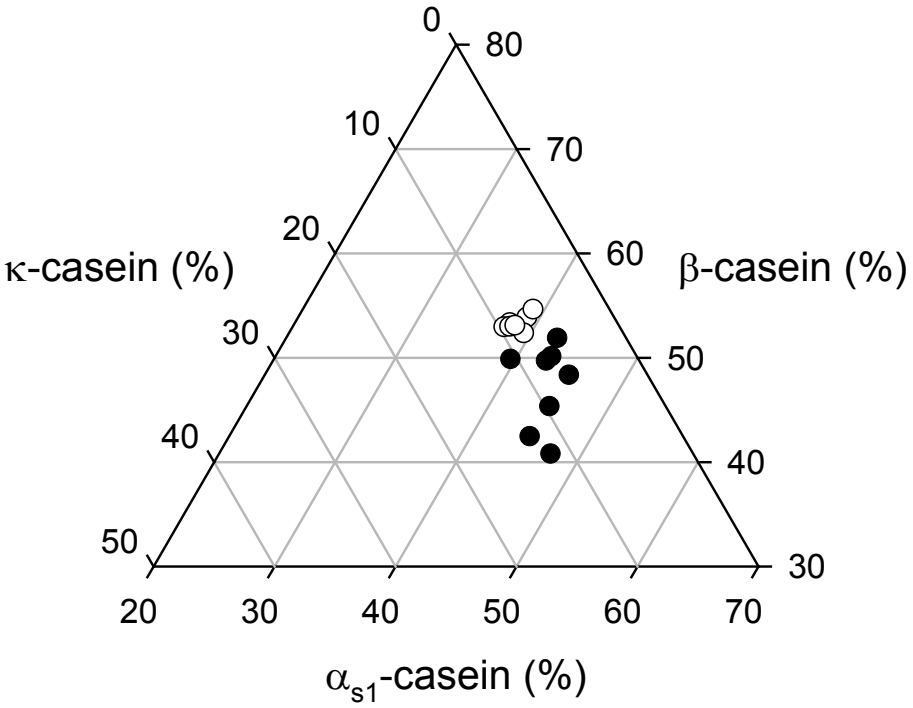

Figure 5.2 Casein composition of individual milk samples selected to prepare mixed milk with high α_{s1}/β-CN ratio (●, n=8) and low αs1/β-CN ratio (○, n=7).The concentrations of κ-CN, β-CN and α_{s1}-casein were normalized so that the sum of the components was 100%.

variants of β-CN between the two groups; within the low ratio group, four cows had the B variant of β-CN and of those cows 3 had the A2B genotype and 1 the BB genotype, while the B variant was not present in the high α_{s1}/β-CN ratio group. The frequency of the B variant within the Dutch Holstein-Friesian cow population is low (2 % in 2005), but the A2B genotype has the highest β-CN and lowest α_{s1}-CN concentration compared to other genotypes (Heck et al., 2009). It can therefore be expected that selection for a low α_{s1}/β-CN ratio will result in a relatively large amount of cows in the sample set with the B variant of β-CN.

5.3.2 Parameters of mixed milk, sodium caseinate solutions and milk gels

From individual milk samples with known casein composition, 2 mixed milk samples were prepared with either high or low α_{s1}/β-CN ratio. The casein composition of mixed milk with high α_{s1}/β-CN ratio (H) and low α_{s1}/β-CN ratio (L) determined by CZE (Fig. 5.3). The figure shows clearly that the peak for the β-CN B was present in the low ratio sample, and that it was absent in the high ratio sample. Further analysis of the mixed milk samples showed that there was a significant difference in casein micelle size; the low ratio sample had a mean size of

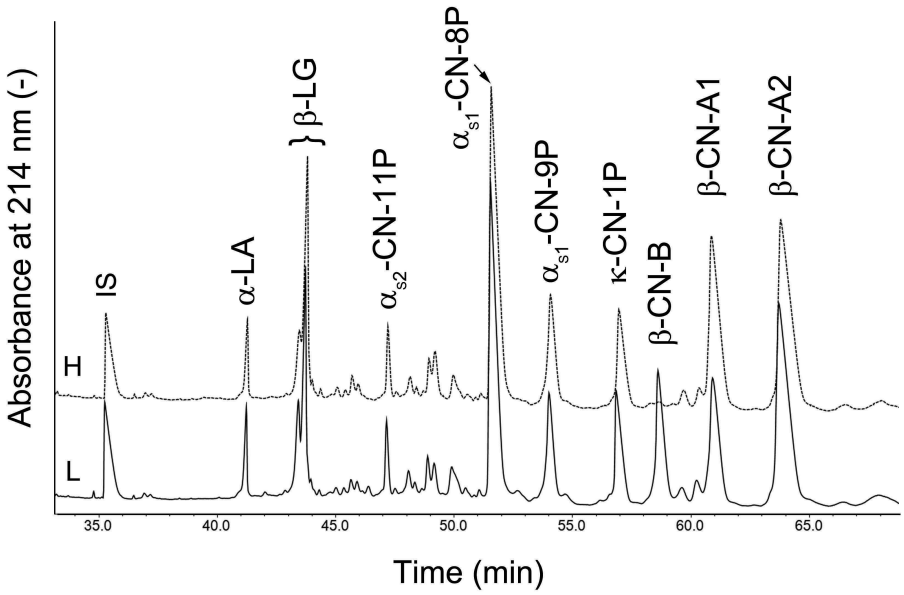

Figure 5.3 Casein composition of milk with high ratio (H) and low (L) α_{s1}/β-CN ratio determined by capillary zone electrophoresis.

181 ± 1.4 nm and the high ratio sample had a mean of 197 ± 3.2 nm. There was no significant difference in pH of both samples.; The dry matter content was similar for the high and low α_{s1}/β-CN ratio milk gels; 48.9 ± 0.7 % (H) and 49.9 ± 0.6 % (L). The milk gels did not fracture during compression to 90 % strain.

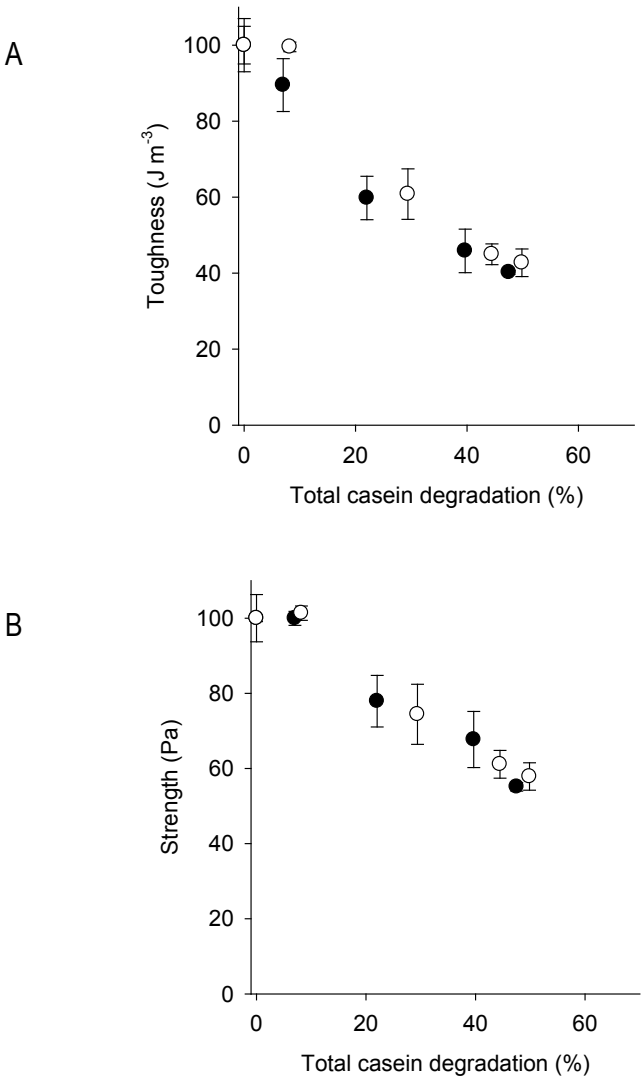

Figure 5.4 Toughness (A) and gel strength (B) of milk gels with high (●) and low (○) ratio of α_{s1}/β-CN as function of total degradation (%). Values represent means with standard deviations indicated as error bars.

In Fig. 5.4, toughness and gel strength of milk gels with high and low ratio of α_{s1}/β-CN are shown as function of total casein degradation. It can be observed that the toughness of all milk gels decreased during storage and that they all became softer. These results were expected, since soluble peptides in the cheese models increase upon degradation of caseins by chymosin. These peptides are released from the gel matrix and result in softening of gels during the initial stage of ripening (Lucey et al., 2003). Furthermore, it can be seen that there was no difference between the milk gels with high and low ratio of α_{s1}/β-CN in gel strength during degradation of caseins by chymosin. However, there was a small difference in toughness during initial degradation (above 80% residual intact casein) of the milk gels; the toughness of the high ratio milk decreased faster than that of the low ratio milk. This difference was most likely caused by the initial difference in casein micelle size of the milk samples. Glantz et al. (2010) showed that milk with smaller casein micelles formed stronger gels at 30 min after chymosin addition. In this case the strength between samples with large and small average micelle size did not differ, but the toughness of the high ratio sample with large average micelle size decreased faster.

5.3.3 Proteolysis in sodium caseinate solutions and milk gels

In Fig. 5.5, the decrease in α_{s1}-CN and β-CN fractions in NaCas solution and that in milk gels as a function of total casein degradation are compared. There was no substantial difference in decreases of caseins between sample with high or low α_{s1}/β-CN ratio in both NaCas solutions and milk gels. Nevertheless, there was a clear difference in decrease in α_{s1}-CN and β-CN fractions between NaCas solutions and milk gels; there was almost 45% decrease in α_{s1}-CN and 55% decrease in β-CN fractions in the NaCas solutions compared to an almost 80% decrease in α_{s1}-CN and less than 30% decrease in β-CN in milk gels at 50% total casein degradation. This indicates that there was a large impact of the structure of the casein aggregates on the degradation by chymosin. Similar observations have been made previously. The efficiency of chymosin towards β-CN has been reported to be over 10-fold higher than for α_{s1}-CN in pure substrate solutions; in citrate solutions at pH 6.2, chymosin hydrolysed β-CN monomers most efficiently, followed by β-CN aggregates and α_{s1}-CN aggregates (Carles & Ribadeau-Dumas, 1984, 1985). However, in cheese it was shown that the specificity of chymosin towards β-CN was limited (McSweeney et al., 1994; Visser & de Groot-Mostert, 1977) and probably only β-CN was hydrolysed during the first weeks of ripening (Exterkate et al., 1997).

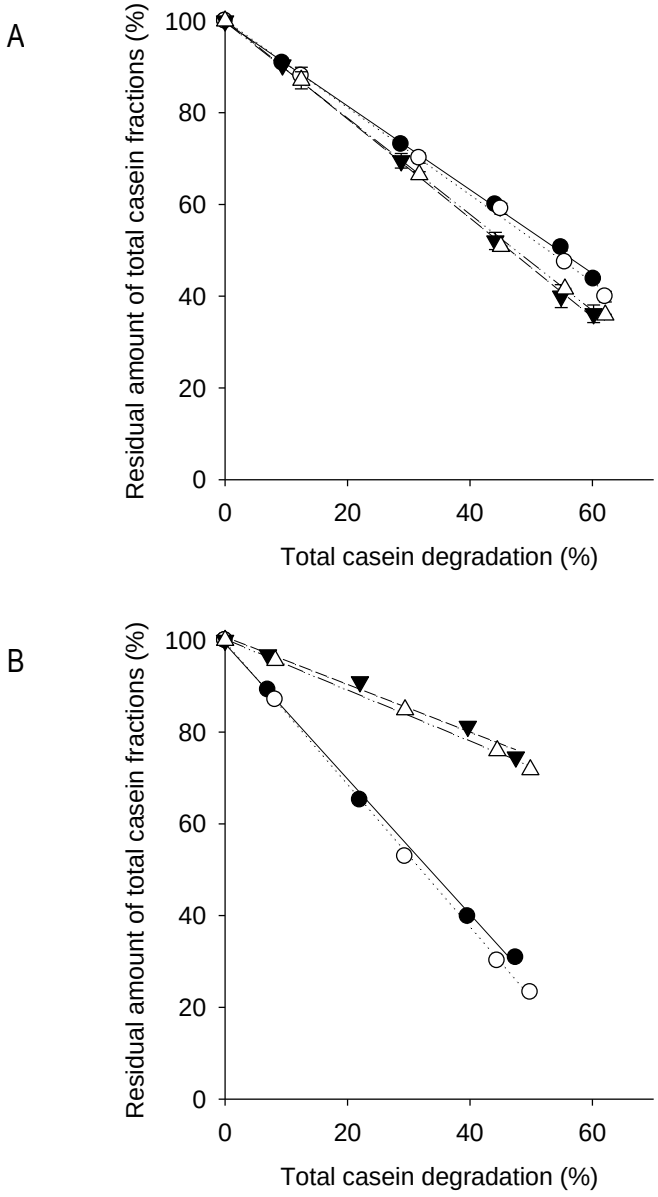

Figure 5.5 α_{s1}-CN and β-CN fractions (%) in sodium caseinate (A) and milk gels (B) as a function of total casein degradation (%) in samples with high (H) or low (L) α_{s1}/β-CN ratio; ●, α_{s1}-CN (H); ○, α_{s1}-CN (L); ▼, β-CN (H); △ β-CN (L). Casein fractions (%) were calculated based on initial protein composition of sodium caseinate (A) and milk gels (B). Values represent means with standard deviations indicated as error bars.

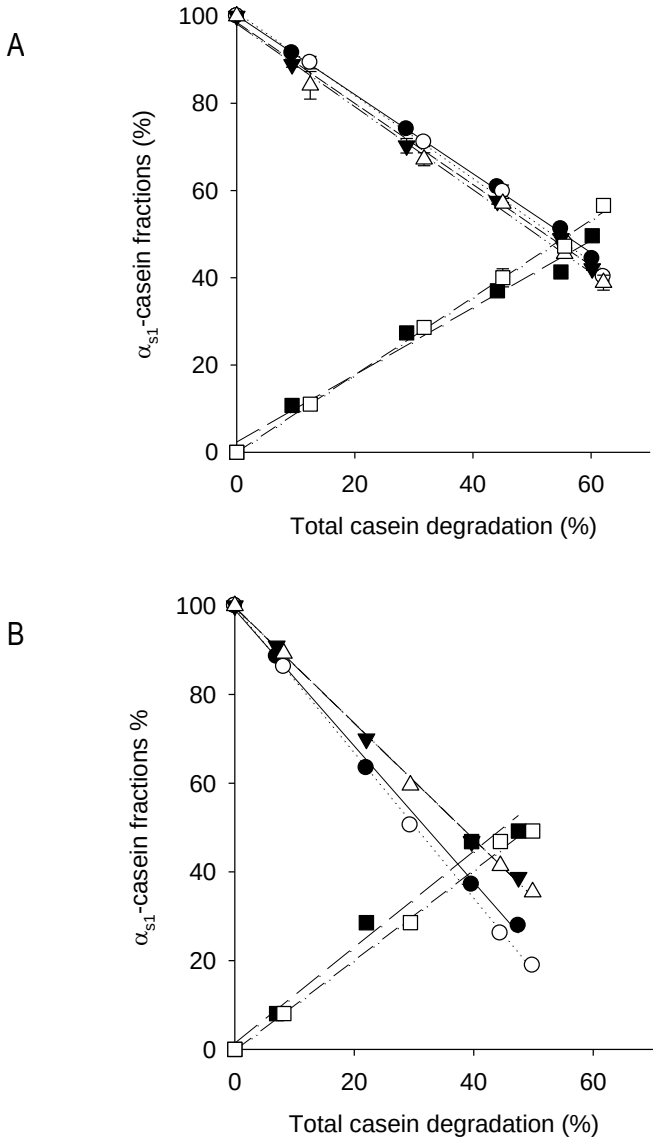

Figure 5.6 α_{s1}-CN-8P, α_{s1}-CN-9P fractions and α_{s1}-CN peptide fragments f1-23 and f24-199 (%) in sodium caseinate (A) and milk gels (B) as a function of total casein degradation (%) in samples with high (H) or low (L) α_{s1}/β-CN ratio; ●, α_{s1}-CN-8P (H); ○, α_{s1}-CN-8P (L); ▼, α_{s1}-CN-9P (H); △, α_{s1}-CN-9P (L); ■ sum of peptide fragments f1-23 and f24-199 (H), □ sum of peptide fragments f1-23 and f24-199 (L). Casein fractions (%) were calculated based on initial protein composition of sodium caseinate (A) and milk gels (B). Values represent means with standard deviations indicated as error bars.

In Fig. 5.6, α_{s1}-CN-8P and α_{s1}-CN-9P fractions and peptide fragments α_{s1}-CN f1-23 and α_{s1}-CN f24-199 are compared in sodium caseinate solutions and milk gels as a function of total casein degradation. Proteolysis by chymosin of these individual fractions has not been studied before. Similar to proteolysis of total α_{s1}-CN, there was no substantial difference in decrease of α_{s1}-CN-8P and α_{s1}-CN-9P between fractions with high or low α_{s1}/β-CN ratio in both sodium caseinate solutions and milk gels. Also, there was no substantial difference in degradation between α_{s1}-CN-8P and α_{s1}-CN-9P in NaCas solutions. This indicates that the position of the 9th phosphate group in α_{s1}-CN-9P, which is located at Ser41 (Manson, Carolan, & Annan, 1977), does not influence chymosin-induced hydrolysis of its primary cleavage site, Phe23-Phe24. In contrast to NaCas solutions, 15% more of α_{s1}-CN-8P was broken down than α_{s1}-CN-9P at 50% total degradation in milk gels. It seems therefore that the accessibility of α_{s1}-CN-9P within large casein micelle aggregates differs from α_{s1}-CN-8P, in such a way that chymosin-induced hydrolysis of Phe23-Phe24 bond is reduced. Since the protein arrangement within the inner structure of the casein micelles has not been elucidated to date (De Kruif, Huppertz, Urban, & Petukhov, 2012), no direct evidence is available on the difference in position of α_{s1}-CN-8P and α_{s1}-CN-9P within casein micelles. According to predictions of the secondary structure of α_{s1}-CN-8P, the 9th phosphate group on Ser41 would just be positioned in front of an α-helix region (Creamer, Richardson, & Parry, 1981; Kumosinski, Brown, & Farrell, 1991), which is likely to influence the secondary structure of α_{s1}-CN-9P. Furthermore, the additional phosphate group might influence calcium phosphate nanocluster formation within casein micelles. According to Holt (2004), at least three phosphorylated residues are needed to form a stable nanocluster. The position of Ser41 close to two other phosphorylated residues, Ser46 and Ser48, in α_{s1}-CN-9P might result in different, and possibly more stable, nanocluster formation than with α_{s1}-CN-8P.

Besides the influence of variation in phosphorylation of α_{s1}-CN, the influence of genetic variants of β-CN on proteolysis by chymosin was also studied. In Fig. 5.7, residual β-CN genetic variants B, A1 and A2 are displayed as a function of total casein degradation (%) in sodium caseinate and milk gels. Also in this case, there was no substantial difference in degradation between fractions with high or low α_{s1}/β-CN ratio in both NaCas solutions and milk gels. In NaCas solutions over 10% more of the β-CN A2 variant was degraded than of the A1 and B variants at 50% total degradation. In the milk gels, there was a tendency for β-CN B variant to be more efficiently degraded until 40% of total degradation, although it stabilized at the same level as the genetic variants A1 and A2 at 50% total degradation.

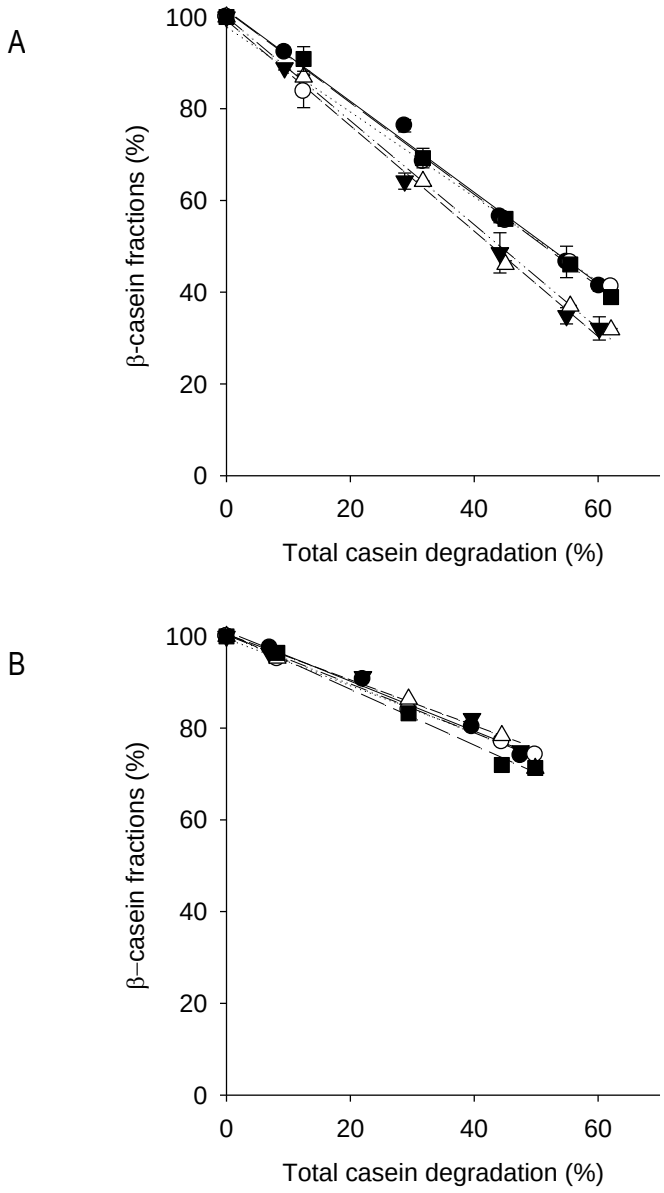

Figure 5.7 β-CN genetic variants B, A1 and A2 (%) in sodium caseinate (A) and milk gels (B) as a function of total casein degradation (%) in samples with high (H) or low (L) α_{s1}/β-CN ratio; ●, β-CN A1 (H); ○, β-CN A1 (L); ▼, β-CN A2 (H); △, β-CN A2 (L); ■, β-CN B (L). Casein fractions (%) were calculated based on initial protein composition of sodium caseinate (A) and milk gels (B). Values represent means with standard deviations indicated as error bars.

Compared to the A1 variant, in the A2 variant His67 is replaced by Pro67 and in the β-CN B variant Ser122 is replaced by Arg122 (Caroli, Chessa, & Erhardt, 2009). The amino acid substitutions of the genetic variants are not in close range of the cleavage sites Leu192-Tyr193 and Ala189-Phe190 of chymosin and will therefore not directly influence chymosin activity, but they might influence physical structure of β-CN and thereby chymosin accessibility. In the β-CN A2 variant, Pro67 is part of two successive Pro-X-Pro sequences. In theory these sequences can be part of a polyproline II structure (Adzhubei, Sternberg, & Makarov, 2013; Benedetti et al., 1983; Cubellis, Caillez, Blundell, & Lovell, 2005) and evidence for the presence of this structure in β-CN has been found by Raman optical activity measurements (Syme et al., 2002). Kumosinski, Brown, and Farrell (1993) modelled the two successive Pro-X-Pro sequences as an open spring structure within the secondary structure of β-CN. The other two genetic variants of β-CN A1 and B do not possess this spring structure. Therefore, in NaCas solution, the A2 variant might form a more accessible type of casein monomer or aggregate to chymosin than the A1 and B variant. In milk gels this effect was not found, most likely due to the large influence of calcium phosphate nanoclusters on the structure of casein micelles.

5.4 Conclusions

Proteolysis by chymosin was not influenced by natural variation in α_{s1}/β-CN ratio of sodium caseinate solutions and milk gels. Also natural variation in α_{s1}/β-CN ratio did not influence firmness and gel strength in milk gels. However, study of individual casein fractions of α_{s1}-CN and β-CN showed that proteolysis by chymosin of α_{s1}-CN-8P was more efficient than α_{s1}-CN-9P in milk gels and proteolysis by chymosin of the A2 variant of β-CN was more efficient in NaCas solutions. These findings have several implications. First of all α_{s1}-CN phosphorylation is an important factor to consider in further research on influence of natural variation in milk composition on proteolysis during ripening of cheese. Possibly, cheese with a high α_{s1}-CN-8P concentration and low α_{s1}-CN-9P concentration will be subject to more efficient degradation by chymosin and generate a higher amount of peptides and flavour precursors during ripening. Furthermore, this finding makes it important to characterize α_{s1}-CN and β-CN fractions of milk, either bulk milk or milk from individual cows, into more detail because these casein fractions influence the degradation efficiency of chymosin. In general, comparison of NaCas solutions to milk gels showed that small chemical differences in either phosphorylation or amino acid substitution can cause significant differences in degradation by

chymosin possibly due to changes in physical conformation of casein monomers, aggregates or casein micelles.

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6

General discussion

The objective of the work described in this thesis was to increase our understanding of the natural variation in casein and salt composition in bovine milk and its implications for casein micelle structure and for some relevant technological properties of milk. The studies in **Chapters 2, 4 and 5** showed that natural variation observed in casein micelles can be divided in factors that are associated with the casein micelle core (section 6.1 and 6.2) and factors associated with the casein micelle surface (section 6.3). First, in section 6.1, current protein content, casein content and salt composition of milk are discussed, with a focus on the micellar salt fraction of milk, and placed in a historical perspective (**Chapter 2**). Next, in section 6.2 the genetic background (**Chapter 3**) of phosphorylation of α_{s1}-caseins (α_{s1}-CN) is described. Subsequently, in section 6.3, the impact of natural variation in κ-casein glycosylation (κ-CN-G) on casein micelle size is discussed (**Chapter 4**). New insights gained on the natural variation in casein micelle surface are also discussed in section 6.3. Next, in section 6.4 the implications of variation in casein composition for the technological properties of milk and design of future breeding strategies are presented (**Chapters 2 - 5**). Finally, in section 6.5 an overview is made of the main conclusions in this thesis.

6.1 The micellar salt fraction and the casein micelle core

The protein content, casein content and salt composition in individual cow milk and bulk milk were studied in **Chapter 2**. Correlation analyses were performed and the results were divided into clusters. The first cluster showed highly significant correlations ($P < 0.01$) between the protein content and the content of total and micellar calcium, phosphorus and magnesium. This was explained by the fixed ratio of these salts in the calcium phosphate nanoclusters of the casein micelles (Holt, 1982). The second cluster showed several significant correlations between the contents of sodium, potassium, chloride and lactose in milk, all of which are jointly involved in maintaining the osmotic pressure of milk (Rook & Wood, 1958; White & Davies, 1958).

In addition to the analysis of individual milk samples, a detailed overview was made of current bulk milk composition. The objective of this aspect of the study was to place the results in a historical perspective in order to see which changes had occurred over the past decades, especially in milk casein content and salt composition of milk. Many salt fractions had not changed; non-sedimentable magnesium and calcium as well as potassium and sodium had all remained virtually stable since 1934. The results with Dutch bulk milk are in agreement with the results of White and Davies (1958), obtained more than 50 years ago with a

different breed (Ayrshire) from one farm in the United Kingdom. The only significant change observed, was that casein content and the content of micellar calcium, phosphorus and magnesium had increased significantly over the past 75 years. These changes were associated with the calcium phosphate nanoclusters in milk, which are the driving force in the formation of casein micelles. Although, they are important in the formation of the casein micelles, the associated micellar calcium fraction did not show substantial variation in milk the past decades; micellar calcium per gram of casein did not substantially increase per kg of Dutch bulk milk (0.77 ± 0.06 mmol/g in 1954 to 0.84 ± 0.02 mmol/g in 2010). It was therefore concluded that the successful application of breeding management in the past that resulted in an increase in yield and protein content of milk, has also resulted in correlated changes in casein content and the micellar salt content of milk. However, there are no indications that the composition of calcium phosphate nanoclusters has changed. Therefore, the natural variation in casein composition became the focus of further study.

6.2 The casein micelle core: Phosphorylation of α_{s1}-CN

The objective of the study described in **Chapter 3** was to determine the genetic background in concentration of α_{s1}-CN with eight and nine phosphate groups (α_{s1}-CN-8P and α_{s1}-CN-9P, respectively) in milk. The genetic and phenotypic correlation between α_{s1}-CN-8P and α_{s1}-CN-9P was low (0.18 and 0.19, respectively) and suggests a different genetic background. This was an unexpected result, because only one gene was always believed to be responsible for expression of α_{s1}-CN and no genetic correlations are known for the two different fractions (Schopen et al., 2009). The differences between α_{s1}-CN-8P and α_{s1}-CN-9P traits were further investigated by means of genome wide association study (GWAS), which showed that both α_{s1}-CN-8P and α_{s1}-CN-9P were affected by a region on Bos Taurus autosome (BTA) 6, which contains the casein genes. However, only α_{s1}-CN-8P was affected by a region on BTA 11, which contains the β-lactoglobulin (β-LG) gene, and only α_{s1}-CN-9P was affected by a region on BTA 14, which contains the diacylglycerol acyltransferase 1 (DGAT1) gene. A different genetic origin of the α_{s1}-CN-8P and α_{s1}-CN-9P fractions in milk is an important new finding and will be discussed in more detail in the next sections.

6.2.1 Substrate specificity of casein kinases

In **Chapter 3**, it was discussed that another casein kinase in the Golgi apparatus of the lactating mammary gland might be responsible for the different genetic origin of the α_{s1}-CN-8P and α_{s1}-CN-9P fractions in milk. To date, the Golgi-enriched

fraction casein kinase (GEF-CK) is the only casein kinase, thought to be responsible for phosphorylation of caseins (Moore, Boulton, Heid, Jarasch, & Craig, 1985). GEF-CK has the consensus sequence of Ser-Xxx-Glu/pSer (Lasa-Benito, Marin, Meggio, & Pinna, 1996). In addition, one of the candidates for the GEF-CK fraction has been identified as FAM20C. This enzyme was shown to specifically phosphorylate Ser-Xxx-Glu motifs (Tagliabracci et al., 2012). The FAM20C gene is located in on BTA 25 at position 42.7 Mbp. No polymorphism was found in this gene, implicating that this gene has no effect on the α_{s1}-CN-8P and α_{s1}-CN-9P concentration in milk. Since the phosphorylated group at Ser56 in α_{s1}-CN-9P has the unique Ser-Xxx-Asp motif, which is unlikely to be phosphorylated by GEF-CK or FAM20C, another casein kinase might be involved in the phosphorylation of α_{s1}-CN-9P. However, this enzyme has not been identified yet. If genes for other kinase enzymes can be identified, it would be interesting to determine if they are located in the vicinity of the DGAT1 gene. It is therefore recommended that further studies focus on the role of the DGAT1 and β-LG genes. Up to now the biological significance of the difference in phosphorylation of the one phosphate group at Ser56 is unknown. It might be that this residue is involved in the formation of a more stable calcium phosphate nanocluster (**Chapter 5**). Previously it was suggested that the casein kinase in milk was less active for the Ser-Xxx-Asp recognition site than for the common Ser-Xxx-Glu/pSer motif (Manson, Carolan, & Annan, 1977), which suggests that the final concentration of α_{s1}-CN-8P and α_{s1}-CN-9P in milk is not specifically regulated. However, in the light of the findings in this **Chapter 3**, it seems more likely that there is a specific purpose towards phosphorylation of both α_{s1}-CN-8P and α_{s1}-CN-9P fractions. Whether this is of importance in the formation and stability of casein micelles or the degradation in the stomach of the neonate needs to be further studied.

6.2.2 Effect β-LG concentration and genotypes

The GWAS showed that only α_{s1}-CN-8P concentration was affected by a region on BTA 11, which contained the β-LG gene. Further analyses showed that the β-LG A variant was associated with a lower and the β-LG B variant with a higher α_{s1}-CN-8P concentration. Also, there was a highly significant effect of total β-LG concentration on α_{s1}-CN-8P concentration but not on α_{s1}-CN-9P concentration. No significant interaction ($p=0.89$) between the β-LG protein variants and β-LG concentration was found and no other evident genes were located in the region. Although the function of β-LG in milk has not been elucidated yet (Sawyer, 2013) and possible functions that have been suggested do not include protein phosphorylation processes, some further insights can be gained from these results. The observed

effect on α_{s1}-CN-8P concentration might be caused by physical differences in genetic variants of β-LG A and B. In general, β-LG has the ability to bind a wide range of polar and apolar ligands (Dominguez-Ramirez, Del Moral-Ramirez, Cortes-Hernandez, Garcia-Garibay, & Jimenez-Guzman, 2013). In addition, the amino acid substitutions in the β-LG A variant compared to the β-LG B variant, lead to structural changes (Qin, Bewley, Creamer, Baker, & Jameson, 1999) and differences in processing properties such as heat stability (Jakob & Puhan, 1992; Manderson, Hardman, & Creamer, 1998; Nielsen, Singh, & Latham, 1996). Also, enzyme binding was influenced by differences in the β-LG genetic variants; Farrell and Thompson (1990) showed that variant A inhibited alkaline phosphatase more than variant B. It is feasible that the β-LG A variant is associated with less phosphorylation by GEF-CK in the lactating mammary gland than β-LG B. This is possibly induced by more binding of the enzyme or its substrate by the A variant, thereby inhibiting phosphorylation. The higher concentration of β-LG in milk with the β-LG A variant might be associated with a higher expression of GEF-CK and thereby compensate for this effect.

A theory of competition in expression of secretory proteins has been proposed and was tested in milk from transgenic mice (McClenaghan, Springbett, Wallace, Wilde, & Clark, 1995). Higher expressed levels of β-LG resulted in suppression of levels of α_s-CNs and β-CN. This theory was supported by Hallen, Wedholm, Andren, and Lunden (2008) who found that an increase in the fraction of κ-CN as a proportion of the major proteins was accompanied by a decrease in the α_{s1}-CN fraction and vice versa. In this study, however, only associations between β-LG concentration and α_{s1}-CN-8P concentration were found and not between β-LG concentration and α_{s1}-CN-9P concentration. It seems therefore that the expression of α_{s1}-CN-9P does not follow the theory of competition and a well-balanced cooperation in gene expression seems to be more likely.

6.2.3 Effect of DGAT1 genotypes

The GWAS showed a highly significant effect of chromosomal region on BTA14 on α_{s1}-CN-9P concentration. The strongest associations with α_{s1}-CN-9P concentration were found between two single nucleotide polymorphisms (SNPs) that are in full linkage disequilibrium with the DGAT1 K232A polymorphism. In the K232A polymorphism, the Lys (K variant) at amino acid position 232 is replaced by Ala (A variant) (Grisart et al., 2002; Winter et al., 2002). In addition, the estimated effects of DGAT1 genotypes showed that the DGAT1 AA genotype was associated with a higher α_{s1}-CN-9P concentration (0.53) and DGAT1 KK genotype with a lower concentration of α_{s1}-CN-9P (-0.44). DGAT1 K232A influences milk fat content and

composition: The K variant is associated with a higher fat content and more saturated fat, compared to the A variant (Grisart et al., 2002; Schennink et al., 2007; Winter et al., 2002). It was therefore surprising to find an association specifically with α_{s1}-CN-9P concentration, which has no known or obvious relation with milk fat synthesis. A few studies mention an association between DGAT1 and milk proteins (Lu, 2013; Schopen et al., 2011). Suggesting that the role of DGAT1 in milk is not limited to fat synthesis only. It might well be that the DGAT1 gene is associated with the expression of the kinase enzyme that specifically phosphorylates the ninth phosphate group of α_{s1}-CN.

6.2.4 Ser56 and calcium phosphate nanocluster stability

The casein micelle inner structure has not been fully elucidated to date, particularly with respect to the location and interactions of specific proteins (De Kruif, Huppertz, Urban, & Petukhov, 2012). Therefore, no direct evidence is available where α_{s1}-CN-8P and α_{s1}-CN-9P are positioned within the casein micelle. In **Chapter 5**, an association was shown between α_{s1}-CN-8P and α_{s1}-CN-9P concentration and chymosin induced hydrolysis of these caseins in chymosin-induced milk gels. The fact that 15% more of α_{s1}-CN-8P was broken down than α_{s1}-CN-9P at 50 % total casein degradation in milk gels, suggested that the accessibility of α_{s1}-CN-9P within para-casein micelle aggregates differed from α_{s1}-CN-8P. Models of the secondary structure of α_{s1}-CN-8P showed that Ser56, which is phosphorylated in α_{s1}-CN-9P, is positioned in front of an α-helix region (Creamer, Richardson, & Parry, 1981; Kumosinski, Brown, & Farrell, 1991). Therefore phosphorylation of Ser56 is likely to influence the secondary structure of α_{s1}-CN-9P. Another important effect of phosphorylation of Ser56 might be its influence on calcium phosphate nanoclusters formation. The region in α_{s1}-CN with amino acids 56 to 65 can contain two or three phosphorylated amino acids (Holt, 2004). It was shown previously that, after tryptic digestion, peptides with three or more phosphorylated amino acids remained attached to micellar calcium phosphate, while peptides with less phosphorylated groups were not retained or poorly retained (Ono, Ohotawa, & Takagi, 1994). Together with Ser56 in α_{s1}-CN-9P, Ser61 and Ser63, might form more stable nanoclusters than within α_{s1}-CN-8P.

6.3 Glycosylation of κ-casein and the casein micelle surface

The objective of the study described in **Chapter 4** was to determine the influence of natural variation in casein and salt composition of milk on casein micelle size.

Samples of 50 individual Holstein-Friesian cows were selected for determination of casein micelle size. The casein micelle diameter of individual cows varied between 150 and 230 nm. Nine cows with a large average casein micelle size were and nine cows with a low average casein micelle size were selected for resampling. De Kruif and Huppertz (2012) showed that casein micelle size does not change significantly for individual cows during lactation. Also in this study, casein micelle size of most cows remained rather constant. This suggests that casein micelle size is a trait with a high heritability.

6.3.1 Difference in small and large casein micelles

The main differences found in composition between groups with small or large average casein micelle size, were associated with κ-CN-G concentration and with the AA and AB genotype of κ-casein (κ-CN). Our study showed for the first time an association between κ-CN-G in milk of individual cows and casein micelle size. The results fitted well with previous findings; an association was found between genetic variants of κ-CN and casein micelle size (Lodes, Buchberger, Aumann, & Klostermeyer, 1996; Walsh et al., 1998) and an association was found between the AA and AB genotype of κ-CN and glycosylation of κ-CN (Coolbear, Elgar, & Ayers, 1996; Robitaille, Ng Kwai Hang, & Monardes, 1991). The association with κ-CN-G and genotypes of κ-CN were confirmed using 54 milk samples of Montbéliarde cows, in which the genetic variant B of κ-CN is more common (Grosclaude, 1988). In bulk milk generally a negative correlation between casein micelle size and total κ-CN is found (Dalgleish, Horne, & Law, 1989; Davies & Law, 1983; Donnelly, McNeill, Buchheim, & McGann, 1984; McGann, Donnelly, Kearney, & Buchheim, 1980; Yoshikawa, Takeuchi, Sasaki, & Chiba, 1982). Such a correlation was also found in our study, when using the individual milk samples. However, the correlation between casein micelle size and κ-CN-G was stronger (-0.718) than with total κ-CN (-0.448). Since the total κ-CN fraction consisted for a considerable part (37%) of κ-CN-G, the strong correlation seemed to originate from the κ-CN-G fraction. Moreover, the non-glycosylated fraction of κ-CN did not significantly correlate with casein micelle size.

6.3.2 Insights gained on variation in casein micelle surface

In **chapter 4** it was discussed that glycosylation of κ-CN can help increase our understanding of the formation and stabilisation of casein micelles in the mammary gland. Glycosylation of κ-CN occurs in the Golgi apparatus (Keller, Keenan, & Eigel, 1979) where casein micelles are also formed (Farrell & Thompson,

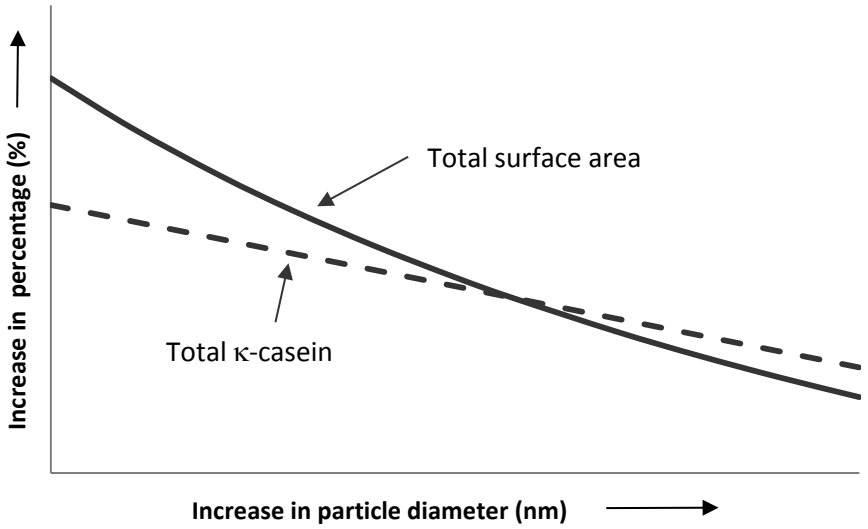

Figure 6.1: Increase in total surface area of particles (%) and total κ-casein (%) as a function of particle diameter.

1971; Farrell Jr., Malin, Brown, & Qi, 2006). It is unknown, however, whether glycosylation occurs before, during or after incorporation of κ-CN in the micelle. After the formation of casein micelles, the brush density of κ-CN influences casein micelle stability (De Kruif, 1999; Tuinier & de Kruif, 2002). It might be that the brush density of κ-CN also influences the formation of casein micelles itselves. The increased density of κ-CN caused by glycosylation could mean that κ-CN-G molecules can stabilize a larger surface area compared to their non-glycosylated counterparts. A decrease in particle diameter means a larger total surface area (Figure 6.1). Furthermore, Figure 6.1 shows qualitatively that the additional amount of total κ-CN in milk with small particles is not sufficient to cover the additional surface area. Therefore κ-CN-G molecules may stabilize a larger surface area compared to its non-glycosylated counterpart during casein micelle formation, causing an increase in the total surface area.

A schematic illustration of the surface of casein micelles is given in Figure 6.2. Casein micelles in milk with a low average micelle size contain more glycosylated κ-CN on the outside covering a larger surface area. Since no differences were found in components related to the inner structure and casein micelle size, the calcium phosphate nanoclusters are equally distributed within milk with small and large

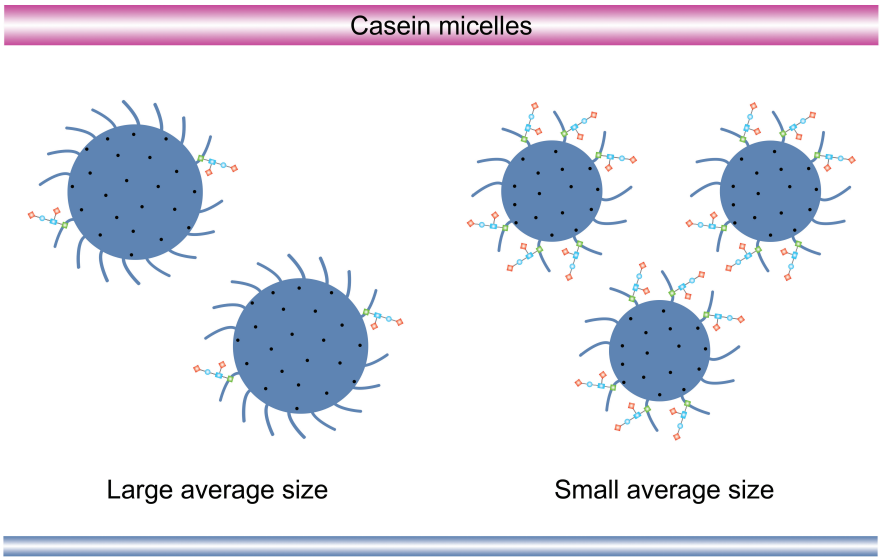

Figure 6.2: Impression of the difference in the surface of casein micelles of milk with small and large average casein micelle size.

average casein micelle size. In this impression, the influence of disulphide bonding has not been taken into account. Oligomerization of κ-CN will influence stabilization of the casein micelles. Whether the model also applies to differences in casein micelle size within the milk sample of individual cows, still needs to be confirmed. Based on these results, it seems likely that the smaller casein micelles in a distribution of casein micelles of one individual cow contain more glycosylated κ-casein.

6.4 Natural variation in casein composition: implications for technological properties of milk and opportunities for breeding strategies

In the previous sections, it was discussed that natural variation in phosphorylation of α_{s1}-CN results in changes in the core of casein micelles whereas variation in κ-CN-G results in changes in the surface of the casein micelles. In this section the implications of these findings for some relevant technological properties of milk and the design of future breeding strategies will be discussed.

6.4.1 Influence of natural variation in casein composition cheese- and yoghurt-making properties of milk

Rennet coagulation is an important process for cheese making. There are only a limited amount of studies on the effects of natural variation in casein composition on rennet coagulation, and all of these studies focus on the initial gelling stage (Bonfatti, Di Martino, Cecchinato, Degano, & Carnier, 2010; Delacroix-Buchet, Lefier, & Nuyts-Petit, 1993; Hallen, Allmere, Naslund, Andren, & Lunden, 2007; Ikonen, Morri, Tyriseva, Ruottinen, & Ojala, 2004; Jõudu, Henno, Kaart, Püssa, & Kärt, 2008; Ostersen, Foldager, & Hermansen, 1997; Poulsen et al., 2013). After the initial gelling, chymosin is partly retained in the curd and plays a major role in the proteolysis of α_{s1}-CN and β-casein (β-CN) during ripening of cheese and thus in the formation of flavour compounds. It was therefore the objective of the study described in **Chapter 5** to take the research on natural variation one step further than the initial gelling stage and study the influence of in α_{s1}-CN and β-CN composition of milk on chymosin-induced hydrolysis of these caseins in milk gels. An important finding was that proteolysis by chymosin of α_{s1}-CN-8P was more efficient than of α_{s1}-CN-9P in casein gels. Therefore, it was concluded that natural variation in α_{s1}-CN phosphorylation is an important factor to consider during ripening of cheese. As discussed in section 6.2, the three phosphorylated residues in α_{s1}-CN-9P in the region of amino acid 56 to 65 might result in more stable nanoclusters compared to α_{s1}-CN-8P. This can result in a lower accessibility of α_{s1}-CN-9P to chymosin during ripening of cheese. When the initial milk casein composition has a high α_{s1}-CN-8P concentration and low α_{s1}-CN-9P concentration, this could result in cheese with a higher amount of peptides and flavour precursors during ripening. It is therefore recommended to further study the role of phosphorylation of α_{s1}-CN in proteolysis during cheese ripening.

In **Chapter 5**, a study of the texture of milk gels during ripening is described. Initial differences in casein micelle size between the milk samples were associated with an initial difference in toughness (J m^{-3}) of the milk gels. These differences disappeared after several days of ripening. In an earlier study by Glantz et al. (2010), it was shown that milk with smaller casein micelles formed stronger gels 30 minutes after chymosin addition. These results indicate that casein micelle size is important for the initial texture of cheese. In addition to cheese, the natural variation in casein micelle size between cows might be an important determinant for the texture of yogurt products after acid coagulation. Also glycosylation of κ-CN, which is associated with casein micelle size, might influence texture formation. In section 6.3 it was discussed that natural variation in κ-CN-G is associated with differences in the surface of the casein micelles. Differences in surface properties

could be especially important in the acid coagulation of yogurt milk, since the glycosylated groups will remain attached to κ-CN, in contrast to cheese where the glycomacropeptides are mostly lost in the whey. Furthermore, the neuraminic acid groups of glycosylated κ-CN will stay negatively charged at the pH of yoghurt. Therefore, the glycosylated groups of κ-CN can interact with whey proteins and free cations in the milk serum. Due to the complexity of the reactions it is not possible to predict the influence on the final texture of the product. It is therefore recommended to further study whether natural variation in κ-CN-G influences acid coagulation of yogurt milk.

6.4.3 Design of future breeding strategies

In the previous sections, it was shown that natural variation in glycosylation and phosphorylation of caseins are both factors that influence casein micelle properties and can be of use for the development and optimization of dairy products. However, the casein composition of milk is not used in the design of breeding strategies at the moment. This partly arises from the fact that in dairy processing large quantities of bulk milk are used, which has a relatively constant casein composition. This constant composition is necessary to guarantee a constant processing quality. The downside of this practice is that it creates a blind spot to the natural variation in protein composition of milk. After confirmation that variation in casein composition can be used for the creation of dairy products, inclusion of technologically relevant parameters would pose great opportunities for the optimization of breeding strategies. In the past, breeding strategies have successfully resulted in the increase of milk yield and protein and fat content of milk. Currently, promising scenarios to increase the casein index of milk are under consideration. The next step for the future would be to take casein composition of milk into account as well. A good start towards determining such scenarios would be the setting-up of a logistic network for the collection and study of milk samples from individual cows in corporation with animal breeders and quality control institutes. This would result in a large database with information of individual cows on genetic variants and casein composition, from which cows could be randomly selected for further experiments. All parties involved could benefit from the exchange of information. When individual milk samples become more readily available, there are potentially many more technological relevant parameters, such as the phosphorylation of α_{s2}-CN and disulphide bonding of κ-CN that can be studied and incorporated in the design of breeding strategies.

6.5 Conclusions

- The casein and micellar salt content in milk have increased significantly the past 75 years, but the amount of micellar calcium per gram of casein did not change. Therefore, there are no indications that the composition of calcium phosphate nanoclusters in the core of the casein micelle have changed.
- Phosphorylation of α_{s1}-CN-8P and α_{s1}-CN-9P is regulated by a different set of genes; α_{s1}-CN-8P concentration was associated with β-LG content and genetic variants and α_{s1}-CN-9P concentration with DGAT1 genotypes. The differences might result from a second casein kinase that is active on the Ser-Xxx-Asp motif of α_{s1}-CN-9P.
- α_{s1}-CN-8P is more efficiently degraded by chymosin than α_{s1}-CN-9P in milk gels. This could be explained by the formation of a more stable nanoclusters by α_{s1}-CN-9P.
- Casein micelles with a small average diameter are associated with a higher concentration of glycosylated κ-CN and the B variant of κ-CN, while casein micelles with a large average diameter are associated with a lower concentration of glycosylated κ-CN and the A variant of κ-CN. Stabilization of a larger surface area by glycosylated κ-CN compared to unglycosylated κ-CN may play a role in this.
- Natural variation in α_{s1}-CN phosphorylation results in changes in the core of casein micelles and natural variation in glycosylation of κ-CN results in changes in the surface of casein micelles. Both factors are therefore relevant to consider for optimization of dairy products and the design of future breeding strategies.

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Summary

Summary

Considerable natural variation in casein content and composition exists between milk samples from individual cows. The heritability of casein composition is high and can therefore be of use in the design of breeding strategies. The objective of the work described in this thesis was to increase our understanding of the natural variation in casein composition of bovine milk and its implications for casein structure as well as for some technological properties of milk. The natural variation in casein composition is extensive and comprises genetic variation and the post-translational modifications, including phosphorylation and glycosylation.

The study in **Chapter 2** describes the salt composition and protein content for individual cow milk and bulk milk and places the results in a historical perspective. Correlation analyses on these results were performed and a detailed overview of the composition of bulk milk was made. Next, the detailed information of bulk milk was placed in a historical perspective. It was found that casein content and related micellar fraction of calcium, phosphorus and magnesium had increased significantly the past 75 years, whereas salt content and composition of serum and the salt composition of casein micelles had remained almost constant.

In the study in **Chapter 3** the genetic background of α_{s1}-casein (α_{s1}-CN) phosphorylation with eight (α_{s1}-CN-8P) and nine (α_{s1}-CN-9P) phosphate groups was analyzed using information obtained from almost 2000 individual Dutch Holstein-Friesian cows. A genome wide association study (GWAS) showed that both α_{s1}-CN-8P and α_{s1}-CN-9P were affected by a region on chromosome 6, but only α_{s1}-CN-8P was affected by a region on chromosome 11, which contains the β-lactoglobulin (β-LG) gene, and only α_{s1}-CN-9P was affected by a region on chromosome 14, which contains the diacylglycerol acyltransferase 1 (DGAT1) gene. It was concluded that α_{s1}-CN-8P and α_{s1}-CN-9P are genetically different traits and are not regulated by the same set of genes. Furthermore, it was discussed that substrate specificity of casein kinases on α_{s1}-CN phosphorylation sites might be an important influencing factor; the unique Ser-Xxx-Asp motif of α_{s1}-CN-9P fraction might be phosphorylated by a different enzyme than the other eight Ser-Xxx-Glu/pSer motifs.

The study in **Chapter 4** describes the influence of natural variation in both casein and salt composition in milk of individual cows on casein micelle size. Casein micelle size was determined in the milk of 50 individual Holstein-Friesian cows. A sub-set of samples with small and large average casein micelle size was selected and salt composition, relative protein composition, genetic variants of κ-casein (κ-CN) and glycosylation of κ-casein (κ-CN-G) were determined. Results were confirmed using a second dataset with milk samples from 54 individual

Montbéliarde cows. Milk samples with a small average casein micelle size were associated with the B variant of κ-CN and a higher relative concentration of κ-CN-G, compared to milk samples with a large average casein micelle size which were associated with the A variant of κ-CN and a lower relative concentration of κ-CN-G.
The objective of the study in **Chapter 5** was to investigate the influence of natural variation in α_{s1}-CN and β-casein (β-CN) composition of milk on chymosin-induced hydrolysis of milk gels and sodium caseinate solutions. The results showed that 15% more of α_{s1}-CN-8P was broken down compared to α_{s1}-CN-9P at 50% total casein degradation in chymosin-induced milk gels. Furthermore, in sodium caseinate solutions, over 10% more of β-CN variant A2 was degraded compared to β-CN A1 and B variant at 50% total casein degradation. In general, comparison of sodium caseinate solutions to milk gels showed that small differences in either phosphorylation of α_{s1}-CN or amino acid substitution in genetic variants of β-CN can cause significant differences in degradation by chymosin.
In **Chapter 6** it was discussed how the results in this thesis increase our understanding of natural variation in casein composition of milk and its impact on casein micelle structure. This study shows that the expression of caseins and their post-translational modification as well as inclusion of calcium in casein micelles are well-balanced processes. Furthermore, implications of natural variation in casein composition of milk for some technological properties of milk and breeding strategies are discussed. It is concluded that variation in α_{s1}-CN phosphorylation results in changes in the core of casein micelles and glycosylation of κ-CN results in changes in the surface of casein micelles. Both factors are therefore relevant to consider for optimization of dairy products and the design of future breeding strategies.

Samenvatting (Dutch summary)

Samenvatting

Er bestaat een aanzienlijke natuurlijke variatie in caseïne gehalte en samenstelling tussen melk van individuele koeien. De erfelijkheid van caseïne samenstelling is hoog en kan daarom van nut zijn voor het ontwerp van fok strategieën. Het doel van het werk beschreven in dit proefschrift was het vergroten van de kennis van de natuurlijke variatie in caseïne samenstelling van koemelk en de implicaties daarvan voor caseïne structuur en sommige technologische eigenschappen van melk. De natuurlijke variatie in caseïne samenstelling is omvangrijk en bevat genetische variatie en post-translationele modificaties, met inbegrip van fosforylering en glycosylering.

De studie in **Hoofdstuk 2** beschrijft de zoutsamenstelling en het eiwitgehalte voor individuele koemelk en bulk melk en plaatst de resultaten in historisch perspectief. Er werden correlatie analyses op de resultaten uitgevoerd en er werd een gedetailleerd overzicht van de samenstelling van bulk melk gemaakt. Vervolgens werd de gedetailleerde informatie van bulk melk in historisch perspectief geplaatst. De resultaten wezen uit dat het caseïne gehalte en gerelateerde micellaire fractie van calcium, fosfor en magnesium significant waren gestegen de afgelopen 75 jaar, terwijl het zoutgehalte en samenstelling van het serum en de zoutsamenstelling van de caseïne micellen vrijwel constant waren gebleven.

In de studie in **Hoofdstuk 3** werd de genetische achtergrond van fosforylering van α_{s1}-caseïne (α_{s1}-CN) met acht (α_{s1}-CN-8P) of negen (α_{s1}-CN-9P) fosfaatgroepen geanalyseerd met behulp van informatie die was verkregen van bijna 2000 individuele Nederlandse Holstein-Friesian koeien. Een genoom brede associatie studie wees uit dat α_{s1}-CN-8P en α_{s1}-CN-9P beiden werden aangedaan door een regio op chromosoom 6, maar alleen α_{s1}-CN-8P werd beïnvloed door een regio op chromosoom 11, dat het β-lactoglobuline (β-LG) gen bevat en alleen α_{s1}-CN-9P werd beïnvloed door een regio op chromosoom 14, dat het diacylglycerol acyltransferase 1 (DGAT1) gen bevat. Er werd geconcludeerd dat α_{s1}-CN-8P en α_{s1}-CN-9P genetisch verschillende eigenschappen zijn en niet worden gereguleerd door dezelfde set genen. Verder werd er bediscussieerd dat substraat specificiteit van caseïne kinases op fosforyleerbare posities van α_{s1}-CN hierin een belangrijke rol kan spelen; het unieke Ser-Xxx-Asp motief van de α_{s1}-CN-9P fractie wordt misschien gefosforyleerd door een ander enzym dan de andere acht Ser-Xxx-Glu/pSer motieven.

De studie in **Hoofdstuk 4** beschrijft de invloed van natuurlijke variatie in caseïne en zout samenstelling in melk van individuele koeien op caseïne micel grootte. Caseïne micel grootte was bepaald in de melk van 50 individuele Holstein-Friesian koeien.

Een sub-set van monsters met gemiddeld kleine of grote micellen werd geselecteerd en zout samenstelling, relatieve eiwitsamenstelling, genetische varianten van κ-caseïne (κ-CN) en glycosylering van κ-CN (κ-CN-G) werden bepaald. De resultaten werden bevestigd met behulp van een tweede dataset met melk van 54 individuele Montbéliarde koeien. Melk met gemiddeld kleine caseïne micellen was geassocieerd met de B variant van κ-CN en een hogere relatieve concentratie van κ-CN-G, in vergelijking met melk met gemiddeld grote caseïne micellen die was geassocieerd met de A variant van κ-CN en een lagere relatieve concentratie van κ-CN-G.

Het doel van de studie in **Hoofdstuk 5** was het onderzoeken van de invloed van natuurlijke variatie in α_{s1}-CN en β-caseïne (β-CN) samenstelling van melk op chymosine geïnduceerde hydrolyse van melk gels en natrium caseïnaat oplossingen. De resultaten toonden aan dat 15% meer α_{s1}-CN-8P werd afgebroken dan α_{s1}-CN-9P bij 50% totale caseïne afbraak in chymosine geïnduceerde melk gels. Verder werd 10% meer β-CN variant A2 afgebroken in natrium caseïnaat oplossingen in vergelijking tot de β-CN varianten A1 en B bij 50% totale caseïne afbraak. In het algemeen liet de vergelijking van natrium caseïnaat oplossingen met melk gels zien dat kleine verschillen in fosforylering van α_{s1}-CN of de vervanging van een aminozuur in de genetische varianten van β-CN significante verschillen in afbraak door chymosine kan veroorzaken.

In **Hoofdstuk 6** werd bediscussieerd hoe de resultaten in dit onderzoek ons begrip van natuurlijke variatie in caseïne samenstelling van melk en de invloed op de structuur van caseïne micellen vergroten. Deze studie toont aan dat de expressie van caseïnes en hun post-translationele modificaties met daarbij de opname van calcium in de caseïne micellen uitgebalanceerde processen zijn. Verder werden de implicaties van natuurlijke variatie in caseïne samenstelling van melk op sommige technologische eigenschappen van melk en fok strategieën bediscussieerd. Er werd geconcludeerd dat de variatie in α_{s1}-CN fosforylering resulteert in veranderingen in de kern van de caseïne micellen en dat glycosylering van κ-CN resulteert in veranderingen in het oppervlak van caseïne micellen. Beide factoren zijn daarom relevant om rekening mee te houden bij het optimaliseren van zuivel producten en het ontwerp van toekomstige fok strategieën.

List of publications

Peer-reviewed publications

Bijl, E., R. de Vries, H. J. F. van Valenberg, T. Huppertz and A. C. M van Hooijdonk. 2014. Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of kappa-casein. International Dairy Journal. 34:135-141.

Bijl, E., H. J. F. van Valenberg, T. Huppertz and A. C. M van Hooijdonk. 2013. Protein, casein and micellar salts in milk: Current content and historical perspectives. Journal of Dairy Science. 96:5455-5464

Sagis, L. M. C., E. Bijl, L. Antono, N. C. A. de Ruijter and H. J. F. van Valenberg. 2013. Protein transfer to membranes upon shape deformation. European Physics Journal Special Topics. 222:61-71

Bijl, E., H. J. F. van Valenberg, S. Sikkes, S. Jumelet, G. Sala, C. Olieman, A. C. M. van Hooijdonk and T. Huppertz. Chymosin-induced hydrolysis of caseins: Influence of degree of phosphorylation of alpha-s1-casein and genetic variants of beta-casein. Submitted for publication.

Bijl, E., H. J. F. van Valenberg, T. Huppertz, A. C. M van Hooijdonk and H. Bovenhuis. Phosphorylation of alpha-s1-casein is regulated by a different set of genes. Submitted for publication.

Conference abstracts

Bijl, E., R. de Vries, H. J. F. van Valenberg, T. Huppertz and A. C. M. van Hooijdonk. Factors influencing casein micelle size in milk of individual cows: kappa-casein genetic variants and glycosylation. 10th International Symposium Milk Genomics & Human Health, Davis, CA, USA, October 1-3, 2013.

Bijl, E., S. Jumelet, H. Bovenhuis, G. Sala and H. van Valenberg. Influence of α_{s1}-/β-casein ratio on proteolysis and texture formation in rennet induced milk gels. 9th International Symposium on Milk Genomics & Human Health, Wageningen, The Netherlands, October 8-10, 2012.

Bijl, E., H. J. F. van Valenberg, T. Huppertz and H. J. F. van Hooijdonk. Natural variation in casein composition of milk. IMGC Workshop Techniques of Measuring Milk Phenotypes. Wageningen, The Netherlands, October 10-12, 2012.

Bijl, E., J. M. L. Heck, H. Bovenhuis, H. J. F. van Valenberg, A. C. M. van Hooijdonk. Milk genomics and its opportunities for sustainable production of cheese and new product development. IDF Cheese Ripening & Technology Symposium, Madison, WI, USA, May 21-24, 2012.

About the author

Curriculum vitae

Etske Bijl was born on the 22nd of December 1982 in Delft, the Netherlands. In 1999 she obtained her high school diploma at the Theresia Lyceum in Tilburg. In the same year she started military officer training and a study military business administration at the Royal Military Academy in Breda. In 2004 she switched careers and started the study Food Technology at Wageningen University. In 2007 she obtained her BSc diploma in Food Technology and started a Master in Food Technology with a specialization in Dairy Science & Technology. She performed her post-graduate research at the groups of Dairy Science and Technology and Physics and Physical Chemistry of Foods, where she focused on the effect of milk fat globule deformation on lipase interfacial binding. Next, she completed a traineeship at FrieslandCampina Corporate Research Deventer in the Food Structuring department. After her MSc graduation in 2009, she started her PhD research in the Milk Genomics project at the Dairy Science & Technology group in cooperation with the Animal Breeding and Genomics Centre of Wageningen University. The results of her PhD research are described in this thesis, entitled: "Natural variation in milk casein composition". Currently, she is working as a researcher at the FrieslandCampina Innovation Centre in Wageningen.

Curriculum vitae

Etske Bijl werd geboren op 22 december 1982 te Delft. In 1999 behaalde ze haar gymnasiumdiploma aan het Theresia Lyceum in Tilburg. In datzelfde jaar begon ze de officiersopleiding en studie militaire bedrijfskunde aan de Koninklijke Militaire Academie in Breda. In 2004 veranderde ze van carrière en begon de studie Levensmiddelentechnologie aan Wageningen Universiteit. In 2007 behaalde ze haar BSc diploma Levensmiddelentechnologie en startte de master Levensmiddelentechnologie met als specialisatie *Dairy Science & Technology*. Ze deed haar afstudeeronderzoek bij de vakgroepen Zuivelkunde en Levensmiddelennatuurkunde, waarbij ze keek naar het aanhechten van een enzym aan het oppervlak van een melkvetbolletje tijdens vervorming. Vervolgens liep ze stage bij de onderzoeksafdeling *Food Structuring* van FrieslandCampina Corporate Research in Deventer. Na het behalen van haar MSc diploma in 2009, startte ze met haar promotieonderzoek aan Wageningen Universiteit binnen het *Milk Genomics* project bij de Zuivelkunde groep in samenwerking met Fokkerij en Genetica. De resultaten van haar promotieonderzoek zijn beschreven in dit proefschrift, genaamd “Natuurlijke variatie in caseïne-samenstelling van melk”. Sinds februari 2014 is ze werkzaam als onderzoeker bij het FrieslandCampina Innovation Centre in Wageningen.

Training and education

Overview of completed training activities

Discipline specific activities

International conferences	
10th International Symposium on Milk genomics & Human Health, Davis, CA, USA	2013
9th International Symposium on Milk Genomics & Human Health, Wageningen, NL	2012
6th IDF symposium on Cheese Ripening & Technology, Madison, WI, USA	2012
Symposia, seminars & workshops	
Yogurt Innovation, Wageningen, NL	2013
Techniques for measuring milk phenotypes, Wageningen, NL	2012
WIAS science day, Wageningen, NL	2010
Genetics of milk quality, Wageningen, NL	2010
Insight in development and functional differentiation of the mammary gland, Wageningen, NL	2010
Impact of insoluble calcium phosphate interactions on dairy products, Wageningen, NL	2010
Biomolecular sciences, Wageningen, NL	2010
Milk protein functionality , Apeldoorn, NL	2010
Courses	
Speciation and Bioavailability, SENSE, Wageningen, NL	2011
Reaction Kinetics in Food Science, VLAG, Wageningen, NL	2009

General courses

"Our Future Leaders", Recess college London, Oegstgeest, NL	2013
High-impact writing in Science, WIAS	2012
Voice and presentation skills training, WGS	2012
NWO Talent day, Utrecht, NL	2011
Scientific writing, WGS	2011
Teaching and supervising thesis students, WGS	2010
PhD Competence Assessment, WGS	2010
PhD introduction week, VLAG	2010

Optionals	
PhD excursion, Food Quality and Design, UK	2012
Advanced statistics, Wageningen, NL	2010
PhD excursion, Dairy Science and Technology, Ghent, Belgium	2009
Preparation of PhD proposal	2009

Teaching & didactic skills training	
Supervision of 12 MSc theses and 2 BSc theses	’09-‘13
Teaching assistant, Dairy Science & Technology, MSc practical course	’10-‘12
Teaching assistant, Dairy Chemistry & Physics, MSc practical course	’10-‘12

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Before I started my PhD thesis, I worked on a student research project in the basement of a science laboratory. In this basement worked several highly committed young people every day until late at night. These people, I could only assume, were PhD students. At that time, I thought of PhD students as devoted scientists, who worked mainly in isolation. However, during my own PhD journey I came to learn that, although at some moments you find yourself indeed for many hours in a basement (not because of the dramatic scenery, but for practical reasons), research is not an individual exercise and starts to come alive upon discussion of plans and results with others. I have had many valuable discussions with my supervisors, colleagues and many other interested people. Without their support and feedback this thesis wouldn't have its current form. Therefore, I would like to take a few pages to thank everyone who had an impact on this work.

First of all, I would like to thank my promotor Prof. Toon van Hooijdonk for giving me the opportunity to start a PhD in the milk genomics project. Toon thanks for guarding the headlines of the research and help me look with an helicopter view to my results so that all pieces of the puzzle came together. Your approach towards finding a balance between relevance of the research and scientific depth will be something that I will take with me during my career.

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Colophon

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