# Milk Genomics

## Opportunities to improve the protein and fatty acid composition in raw milk

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**Jeroen M.L. Heck**

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## Abstract

The aim of the research described in this thesis is to identify the opportunities to improve the protein and fatty acid composition in milk. The main focus is on estimating the variation in milk protein and fatty acid composition between cows, between herds and between seasons, and on identifying the possibilities to change the protein and fatty acid composition by selecting for variants of specific genes.

It is shown that the variation in concentration, phosphorylation and genetic polymorphism of milk proteins can be estimated with capillary zone electrophoresis. Using this technique on approximately 2000 milk samples showed that large variation in protein composition between individual cows exists. Genetic variants of a number of milk proteins have a large effect on the protein composition in milk and explain a part of the genetic variation in milk protein composition.

Measurement of the fatty acid composition in milk samples of approximately 2000 cows showed that large variation in milk fatty acid composition between individual cows exist. The extent to which this variation could be attributed to genetic or herd effects varied between the individual fatty acids and was depending on how a fatty acid was synthesized. Genetic variants of SCD1 and DGAT1 have an effect on the fatty acid composition of milk and explain a part of the genetic variation in milk fatty acid composition. The protein variants and the SCD1 and DGAT1 variants can be used to select or breed for cows with a different milk composition. Selection for the β-LG B variant and the β-κ-CN haplotype $A^2B$ results in cows that produce milk that has a composition that is more suitable for cheese production and selection of the DGAT1 A and SCD1 V variants results in cows that produce milk that has a "better" fatty acid composition.

Studying the seasonal variation in milk composition in 2005 showed that milk composition varies throughout the year and has changed in time.

This thesis shows that there are good opportunities to exploit the variation in milk composition between cows to improve the protein and fatty acid composition in milk.

# Contents

# 1

# General introduction

## 1.1 Introduction

Milk and dairy products are important components of western diets. The composition of raw milk determines, to a large extent, the nutritional value and the technological properties of milk and dairy products, and the farmers milk price. Therefore, the composition of milk is of great importance for the farmers and dairy industry. Milk of many species is consumed by humans (e.g. goat, sheep, buffalo) but bovine milk (Bos Taurus) is economically the most important. This thesis deals with the composition of bovine milk.

Milk composition varies with health status, lactation stage and parity of the cow. Furthermore milk composition depends on feeding and genetics of the cow (Fox and McSweeney, 1998). In the last decades significant progress was made in improving the milk yield and composition of bovine milk in the Netherlands by both breeding and feeding practices as shown in Figure 1 (NRS, 2005).

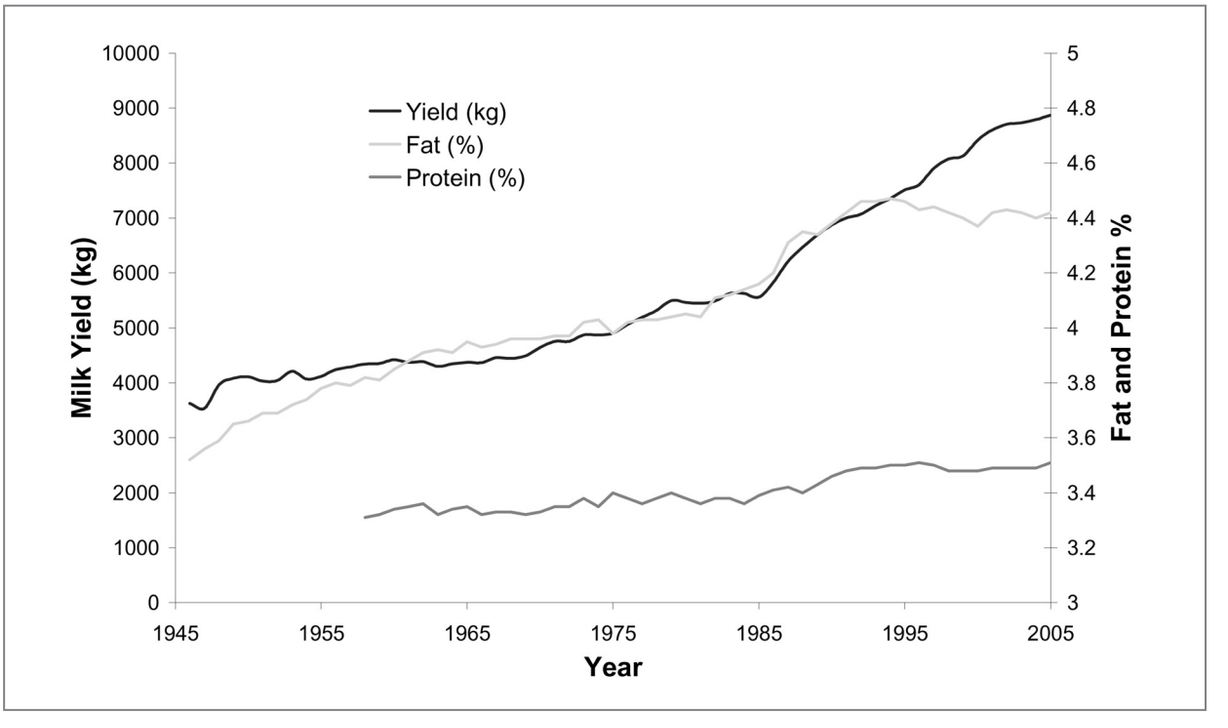


**Figure 1**: Changes in time in yield (kg), protein and fat (%) of milk from dairy cows in The Netherlands. (Adapted from NRS, 2005)

In less than 50 years the average milk yield of Dutch dairy cows more than doubled. During the same period there has been a large increase in the fat content and a smaller but significant increase in the protein content of the milk. This illustrates the opportunities to change the concentration of fat and protein in milk through genetic selection and optimal feeding and management practices. However, less is known about the possibilities to change the protein and fatty acid composition in milk by selection and feeding. The protein and fatty acid composition are of major importance for the technological properties and nutritional value of milk. Therefore, improving the protein and fatty acid composition in raw milk could further improve the quality of milk and increase the profitability of the dairy sector.

The protein and fatty acid composition in milk can be changed by both breeding and feeding. Many studies have shown that the fatty acid composition in milk can be changed by feeding (e.g. Moate et al., 2007). The protein composition, however, is not really influenced by the cow's diet (DePeters and Cant, 1992). Data about the possibilities to change the protein and fatty acid composition in milk by breeding is scarce and inconclusive. To be able to change the protein and fatty acid composition through breeding, genetic variation in the protein and fatty acid composition is required. It is of additional practical value to identify polymorphisms in genes that are responsible for the genetic variation in protein and fatty acid composition. These polymorphisms can then be used to select cows, based on the results of DNA tests, that produce milk with an improved protein or fatty acid composition.

## 1.2 Milk protein composition

Milk protein consists for a large part (± 90%) of the six main milk proteins α-lactalbumin (α-LA), β-lactoglobulin (β-LG), $\alpha_{S2}$-casein ($\alpha_{S2}$-CN), $\alpha_{S1}$-CN, κ-CN and β-CN. The other part of the protein fraction (± 10%) consists of minor proteins like bovine serum albumin, gamma caseins, proteose peptones, immunoglobulins, lactoferrine, lactoperoxidase, and a large number of other proteins that occur in very low concentrations (Farrell et al., 2004). The protein fraction shows even further heterogeneity because of post translational modifications like glycosylation, phosphorylation and disulfide binding, genetic polymorphism and the action of proteolytic enzymes on proteins (Fox and Mcsweeney, 1998). The six main milk proteins are all synthesized in the mammary gland and are products of the corresponding milk protein genes. The amino acid composition of these proteins is depending on the DNA sequence of these genes and their concentration in the milk depends on the level of expression of these genes and/or post-transcriptional control of their mRNA. The proteins in milk are traditionally classified in two groups based on their behavior at pH 4.6. The caseins are the proteins that precipitate and the whey proteins are the proteins that remain soluble at this pH. Most of the caseins in milk exist in colloidal particles called casein micelles. The main function of the micelle is thought to be the supply large amount of insoluble calcium phosphate to the mammalian young but its properties have also a major influence on the technological properties of the milk.



Milk protein composition is important for the nutritional value of milk which is determined by the amino acid composition of the different proteins. Furthermore, different proteins have different bioactive peptides encrypted in their amino acid sequences, which can be released upon digestion or by enzymatic processing (Meisel and Bockelmann, 1999). Milk protein composition is especially important for the technological properties of milk. Cheese yield for example, is directly related to the amount of casein in milk. With a larger fraction of the casein protein, i.e. a higher casein number, it is possible to produce more cheese from the same amount of protein.
The composition of the casein is also important for the renneting process of milk and the properties of cheese (Wedholm et al., 2006 ). These effects can be related to the effect of casein composition on micelle properties. It has been shown that micelles with a different size have a different protein composition (Lovisi et al., 2003).

The protein composition of milk can be changed when variation between cows exists. There is a lack of knowledge about the variation between cows in the concentration of all six main milk proteins. Previous research performed on variation in milk protein composition mainly focused on the variation in total protein and total casein (Ikonen et al., 2004). In this study we determined the relative concentration of all six main milk proteins in a large number of cows.
The casein number of milk (proportion of casein to total protein) is not influenced by feeding (Coulon et al., 1998, DePeters and Cant, 1992). However, the casein number is known to be influenced by polymorphisms in the milk protein genes of the cow. Multiple studies have reported the effect of genetic variants of the main milk proteins on the casein number of milk (Lunden et al., 1997, Mayer et al., 1997). Only in a few studies the effect of genetic variants on the protein composition was investigated in more detail than the casein number alone. These studies did show that genetic variants also influence the relative concentration of individual proteins (Bobe et al., 1999, Ng Kwai Hang and Sungwoo, 1996, Robitaille et al., 2002). However, in these studies only the genetic variants of β-LG and κ-CN were investigated and a small number of animals were used. Furthermore the analytical techniques applied could not quantify all six main milk proteins.

## 1.3 Milk fatty acid composition

Milk fat consist mainly (± 98%) out of triacylglycerols. Triacylglycerols are composed of 3 fatty acids esterified to a glycerol backbone. More than 400 different fatty acids have been found in milk (Jensen, 2002). Most of these fatty acids occur in trace amounts and only about twelve are present in concentrations higher than 1%. The fatty acids in milk can be classified on basis of their chain length (short chain, medium chain, long chain) or degree of unsaturation (saturated, monounsaturated, polyunsaturated). The fatty acids in milk are of dual origin. The fatty acids C4:0 – C14:0 and a part (±50%) of C16:0 are synthesised de novo in the mammary gland by the fatty acid synthetase enzyme complex (Fox and Mcsweeney, 1998). The rest of C16:0 and the longer chain fatty acids (mainly C18) are derived from the blood. The blood derived fatty acids mainly originate from the feed of the cow. The fat fraction of the cow's feed predominantly consists of unsaturated fatty acids. These unsaturated fatty acids are extensively bio hydrogenated by rumen bacteria to saturated fatty acids. In the mammary gland these saturated fatty acids and the de novo synthesised saturated fatty acids are partly unsaturated by the Stearoyl-CoA Desaturase (SCD1) enzyme (Pereira et al., 2003). Finally, the fatty acids are esterified to the glycerol backbone by multiple enzymes and subsequently secreted in the milk.



It is generally accepted that decreasing the amount of saturated fat is beneficial for human health (Mensink et al., 2003). Milk fat is different from other fats because it consists for a relatively large part of saturated fatty acids with a short chain length (≤10 carbon units), which are synthesised in the mammary gland. These shorter chain saturated fatty acid have never been negatively associated with human health (Steijns, 2008). Therefore, instead of simply decreasing the amount of saturated fat, decreasing the concentration of specific saturated fatty acids (e.g. C14:0 and C16:0), which are negatively associated with human health, might be a better objective.

Milk fat also contains fatty acids that are often associated with positive health effects (e.g. CLA). Increasing these fatty acids might therefore improve the nutritional value of milk. The fatty acid composition of milk also influences the processing and product properties of dairy products like cream and butter (Bobe et al., 2003). The spreadability of butter for example is better when produced from milk with a higher amount of unsaturated fatty acids.

Numerous studies have shown that the fatty acid composition of milk may be changed by altering the diet of the cow. Only a very few studies have investigated the opportunities to change fatty acid composition by breeding. The main focus in all studies has been on the fatty acids that occur in relative high concentrations in milk. Less attention has been paid to minor fatty acids like the odd and branched chain fatty acids and several rumen biohydrogenation intermediates.
These minor fatty acids can be of interest because they can be markers for defects like milk fat depression or indicators for specific rumen bacteria (Vlaeminck et al., 2006). Furthermore, studying the effects of genetics and feeding simultaneously can give more information on the biosynthesis of the different fatty acids. When milk fatty acid composition is genetically determined it is of interest to identify the genes responsible for this genetic variation. Most obvious candidates for genes that might have an effect on milk fatty acid composition are the genes directly involved in the synthesis of milk fat. A polymorphism in the SCD1 gene, has been associated with variability in carcass fatty acid composition (Taniguchi et al., 2004) and with milk fatty acid composition in Italian Holstein, Piedmontese, and Valdostana cattle (Mele et al., 2007, Moioli et al., 2007). Another candidate gene that may affect milk fatty acid composition, is acyl CoA:diacylglycerol acyltransferase 1 (DGAT1). A polymorphism in the DGAT1 gene explains 50% of the genetic variation in milk-fat percentage (Grisart et al., 2002).

## 1.4 Seasonal variation

In the Netherlands, the fat and protein content of farm milk are routinely recorded. Therefore, a good overview of the concentration of these components in Dutch milk exists. However, the exact composition of the fat and protein fractions in Dutch milk is unknown. An overview of the Dutch raw milk composition, including the fatty acid and protein composition, on a regular basis, is needed to monitor favorable or unfavorable changes in the future. An investigation of the Swedish dairy milk composition in the 1970s and 1996 has shown a substantial decrease in the casein number of Swedish raw milk during this period (Lindmark Mansson et al., 2003). Such a decrease in casein number would also be unfavorable for the Dutch dairy industry because in the Netherlands about half of the milk is used for cheese production (PZ, 2006). When determining the fatty acid and protein composition in raw milk it is important to realize that even within one country the composition is not constant; milk composition varies considerably throughout the season Therefore, to determine the average composition of Dutch raw milk, the seasonal variation in raw milk composition has to be taken into account.

## 1.5 Milk differentiation

Traditionally, raw milk of a uniform quality is used as starting material for all possible dairy products. This means that raw milk with the same composition is used for the production of consumption milk as for the production of cheese. This is suboptimal because optimal milk composition differs for different dairy applications. As mentioned before, milk composition differs considerably between individual cows. However, virtually all of this variation is evened out because the milk from multiple milkings and a large number of animals from one herd is pooled together and mixed with the milk from other herds in one storage tank at the dairy factory.
Differentiation is possible when cows that produce milk with a composition that is more suitable for a specific application can be grouped together. Their milk can be collected separately and used for that specific application. The concept of milk differentiation based on composition could result in a more efficient production of dairy products or in the production of products with an enhanced quality. Cows may receive specific feeding to produce milk with an improved composition for a specific application. In addition to feeding it is also possible to use cows that based on their genes produce milk with an additional value for that specific application. Finding genes responsible for the genetic variation would create new opportunities for milk differentiation based on composition. For example, one may select cows with a variant of a gene that is associated with the production of milk with a better fatty acid composition or milk that is more suitable for cheese production because of a higher casein content.

## 1.6 Aim and outline of this thesis

The aim of the research described in this thesis is to identify the opportunities to improve the protein and fatty acid composition in milk. The main focus is on estimating the variation in milk protein and fatty acid composition between cows, between herds and between seasons, and on identifying the possibilities to change the protein and fatty acid composition by selecting for variants of specific genes.

**Chapters 2** and **3** deal with milk protein composition. In **chapter 2** a capillary zone electrophoresis method is presented and used to determine the variation in the concentration, phosphorylation and genetic polymorphism of milk proteins in a large number of milk samples. In **chapter 3** the data from **chapter 2** are used to establish the effect of genetic variants and casein haplotypes on the protein composition of raw milk.
**Chapters 4** and **5** deal with milk fatty acid composition. In **chapter 4** the variation in milk fatty acid composition between cows is described. Furthermore, genetic and herd parameters were estimated and used to characterize the fatty acids in milk. In **chapter 5** the genetic parameters for milk fat unsaturation and the effects of variants of the SCD1 and the DGAT1 gene on milk fat unsaturation are reported.
**Chapter 6** deals with milk protein and milk fatty acid composition. In this chapter the seasonal variation in milk composition is given in milk samples that were representative for the complete Dutch milk supply during 2005. Furthermore, the difference in milk fatty acid composition of Dutch milk between 2005 and 1992 is shown.
In **chapter 7** it is discussed how the results obtained in this thesis can be used to change the protein and fatty acid composition in milk. Furthermore, it is speculated how the results might be beneficial for the dairy industry.

# 2

# Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis

J. M. L. Heck, C. Olieman, A. Schennink, H. J .F. van Valenberg, M. H. P. W. Visker, R. C. R Meuldijk, A. C. M. van Hooijdonk. (2008). *Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis.* International Dairy Journal 18:548-555.

## Abstract

Capillary zone electrophoresis (CZE) was tested for reproducibility and was shown to be a suitable method for estimating the relative concentration of α-lactalbumin, β-lactoglobulin, $\alpha_{S2}$-casein, $\alpha_{S1}$-casein, κ-casein, and β-casein in milk. This study showed how the method can also be used to determine the relative concentration of two different phosphorylation states of $\alpha_{S1}$-casein ($\alpha_{S1}$-CN-8P and $\alpha_{S1}$-CN-9P ) and three of $\alpha_{S2}$-casein ($\alpha_{S2}$-CN-10P, $\alpha_{S2}$-CN-11P and $\alpha_{S2}$-CN-12P). Furthermore, with CZE it was possible to determine most common genetic variants including the κ-casein E variant which has not been identified by CZE before. Analyses of milk samples of 1948 Dutch Holstein-Friesian cows showed large variation in both the relative protein concentration and the relative concentration of different phosphorylation states for all milk proteins studied. Correlations between different phosphorylation states of $\alpha_{S1}$- and $\alpha_{S2}$-casein indicated that the relative concentration of specific phosphorylation states of $\alpha_{S1}$- and $\alpha_{S2}$-casein were related to each other.

## 2.1 Introduction

Milk protein composition determines, to a large extent, the nutritional value and the technological properties of milk. Protein composition of bovine milk varies with season, stage of lactation, feeding and health status of the cow, but is predominantly determined by genetic factors (Ng Kwai Hang et al., 1987; Groen et al., 1994). Information on genetic variation in protein composition can be used to breed cows producing milk with added value such as a higher level of casein or altered concentrations of other proteins. To determine the possibilities of changing protein composition by selective breeding, the variation in protein composition among individual cows has to be known.

The bovine milk protein fraction consists, for a large part (±90% w/w), of six proteins known as α-lactalbumin (α-LA), β-lactoglobulin (β-LG), $\alpha_{S2}$-casein ($\alpha_{S2}$-CN), $\alpha_{S1}$-casein ($\alpha_{S1}$-CN), κ-casein (κ-CN) and β-casein (β-CN). These main milk proteins are heterogeneous because they are subject to genetic polymorphism and posttranslational modifications. Genetic polymorphism in the milk protein genes can result in protein variants that differ in one or more amino acids, which are especially of interest when they are associated with protein composition and milk production traits (Bobe et al., 1999). An important posttranslational modification of milk proteins is phosphorylation, occurring exclusively at caseins and not at whey proteins. Due to their strong calcium binding properties these phosphate groups are not only important for the nutritional value of milk but also for the physical chemical properties of the casein micelles.

Previous research performed on variation in milk protein composition mainly focused on the variation in total protein and total casein (Ikonen et al., 2004). Research on variation in concentration of the individual main milk proteins is limited and mostly performed only on a small number of animals (Groen et al., 1994; Bobe et al., 1998; Wedholm et al., 2006). Additionally, knowledge is lacking about variation in the degree of phosphorylation of milk proteins among individual cows. To get a detailed view on the variation in milk protein composition, a large number of milk samples has to be analysed using a technique capable of separating the main milk proteins and proteins that differ in their degree of phosphorylation.



Capillary zone electrophoresis (CZE) is a suitable technique that provides rapid separations showing high resolutions and good possibilities for protein quantification. De Jong et al. (1993) first used CZE to separate whey and casein proteins simultaneously. This method was used for separation of genetic variants (Recio et al., 2001) and it was shown that CZE enabled the separation of $\alpha_{S1}$-CN containing 8 phosphate groups from $\alpha_{S1}$-CN containing 9 phosphate groups (Recio et al., 1997). $\alpha_{S2}$-CN has multiple phosphorylation states, which would result into the appearance of multiple peaks. Several authors (Otte et al., 1997; Ortega et al., 2003) have proposed that some unidentified peaks are different phosphorylation states of $\alpha_{S2}$-CN. However, the actual assignment of peaks as different phosphorylation states of $\alpha_{S2}$-CN has not been performed yet.
The CZE method has also been used for quantitative determination of some individual proteins (Otte et al., 1997; Strickland et al., 2001; Gomez Ruiz et al., 2004), but has not been applied to determine the variation in concentration of all separated proteins in a large number of samples.

Therefore, to estimate variation in concentration, phosphorylation and genetic polymorphism of milk protein by applying CZE, two actions have to be undertaken. (1) The nature of some previously unidentified peaks, that might be genetic variants or proteins that represent different phosphorylation states from known proteins, has to be established. (2) The method of de Jong et al. (1993) has to be tested for its reproducibility in quantifying all main milk proteins in a large number of samples.
The objective of this study is to estimate the variation in detailed protein composition in milk of 1948 Dutch Holstein-Friesian cows. This is the first time variation in protein composition has been studied in such detail on such a large number of animals.

## 2.2 Materials and methods

### 2.2.1 Milk samples

From February to March 2005 milk samples from 1948 Dutch Holstein-Friesian cows in their first lactation, located in 398 herds, were collected as described in Schennink et al. (2007). The samples were preserved with 0.03 % w/w sodium azide, transported refrigerated and stored frozen at – 40 °C within one day.

### 2.2.2 Preparation of sample solution and run buffer

The sample solution and the run buffer were prepared as described by Recio and Olieman (1996). The sample solution was prepared by mixing 335 µL of homogenous milk sample with 500 µL sample buffer. After mixing, the sample solution was left at room temperature for 1 h and was subsequently centrifuged for 5 min at 5000 g to remove remaining fat. The sample buffer (pH 8.6 ± 0.1) consisted of 167 mM hydroxymethyl-aminomethane (TRIS) (Sigma, St. Louis, MO, USA), 42 mM 3-morpholino-propanesulfonic acid (MOPS) (Sigma), 67 mM ethylene-diamine–tetra-acetic acid disodium salt dihydrate (EDTA) (Merck, Darmstadt, Germany), 17 mM DL-dithiothreitol (DTT) (Sigma), 6 M urea (Merck) and 0.05 % w/w methylhydroxyethyl cellulose 30000 (MHEC) (Serva, Heidelberg, Germany). The run buffer (pH 3.0 ± 0.1) consisted of 0.19 M citric acid (Merck), 20 mM sodium citrate (Merck), 6 M urea and 0.05 % w/w MHEC.



### 2.2.3 CZE analyses

CZE analyses were carried out with a Beckman P/ACE MDQ Capillary Electrophoresis system controlled by 32 KaratTM Software, version 7.0 (Beckman Instruments, Fullerton, CA, USA). Separations were performed using a fused-silica OV-1701-OH deactivated capillary (TSP-050375-P-10), (BGB Analytik, Boeckten, Switzerland) with dimensions 57 cm X 50 µm I.D. and a slit opening of 100 x 800 µm.
Separations were carried out at 45 °C and a linear voltage gradient from 0 to 25 kV in 3 min was used, followed by constant voltage at 25 kV. Before each separation, the capillary was flushed in reverse direction for 3 min with run buffer. Sample solutions were injected at the anode by pressure injection at 0.5 psi for 20 s. One series consisted of approximately 20 milk samples, six standard samples prepared with known amounts of whey or casein, and two reference milk samples. A total of 103 series was performed in which 1948 milk samples were analyzed.

### 2.2.4. Identification

Identification was done by spiking with milk protein standards: α-LA, β-LG, $\alpha_S$-CN, κ-CN and β-CN (all from Sigma). The $\alpha_S$-CN standard was run also after incubation with sample buffer containing 0.6 M 2-mercaptoethanol instead of DTT and after incubation with sample buffer without DTT. Genetic variants of the main milk proteins were determined by comparison of the electropherograms with literature electropherograms and analyses with milk samples containing known genetic variants. For κ-CN, two peaks could be distinguished. To identify the genetic variants representing these peaks, exon 4 of κ-CN was sequenced in a subset of 95 cows. Primers for PCR and sequencing were designed based on the κ-CN genomic sequence (Genbank accession no. X14908): forward, 5'-CTCTGCTTCTGCTGCTGCTA-3' and reverse, 5'- TTGCCTTATTTACCTGCGTTG -3'.

### 2.2.5 Quantification

UV detection was performed at 214 nm (data collection rate 5 Hz). The absorbance of a protein at 214 nm is mainly proportional to the number of peptide bonds (Hutterer & Dolnik, 2003). It cannot be excluded, however, that aromatic amino acid residues have a minor influence on the absorbance at 214 nm. The number of peptides bonds per gram of protein is nearly equal for all milk proteins. When using CZE, peak area depends not only on the response of the protein at 214 nm but also on its migration time (Beckers & Bocek, 2004). Peak area doubles when migration velocity is halved. Therefore, we used this equation to estimate the relative concentration of an individual protein:

$$C_x = \frac{\frac{A_x}{t_x}}{\sum_{i=1}^{n} \frac{A_i}{t_i}} \times 100\%$$

where $C_x$ is the relative concentration, $A_x$ the area in the electropherogram and $t_x$ the migration time of protein x and n the total number of peaks which together comprise 100% of the area. Method reproducibility was determined by calculating the coefficient of variation (CV) of relative peak area, for all individual proteins, from a reference milk sample that was added in all 103 series.

## 2.3 Results and discussion

### 2.3.1 Protein identification

*2.3.1.1 Separation of milk proteins*

Milk proteins separated in the order: α-LA, β-LG, $\alpha_{S2}$-CN, $\alpha_{S1}$-CN, $\alpha_{S0}$-CN, κ-CN and β-CN (Figure 1). κ-CN separates into multiple minor and one major peak as shown in previous studies (Otte et al., 1997; Miralles et al., 2000; Ortega et al., 2003). The major peak comprises ±50% of the total κ-CN peak area. The major part (>50%) of κ-CN is in the mono-phosphorylated non-glycosylated form (Farell et al., 2004). Therefore, the major κ-CN peak was assigned as κ-CN-1P. The minor peaks most likely represent different glycosylated or phosphorylated forms of κ-CN and migrate at the same migration time as β-CN $A^1$ or β-CN $A^2$ (Otte et al., 1997). Therefore, these minor forms of κ-CN can only be identified and quantified in the absence of β-CN $A^1$ or β-CN $A^2$, i.e. in milk samples of cows homozygous for β-CN.

Most of the peaks migrating between 23 and 29 minutes in Figure 1 were also present in the electropherogram of the $\alpha_S$-CN standard (Figure 2a). The electropherogram of the standard contained the peaks assigned in milk samples as $\alpha_{S2}$-CN, $\alpha_{S1}$-CN and $\alpha_{S0}$-CN. The electropherogram also contained 9 other peaks which also occurred in milk samples but had not been assigned yet. We decided to use the $\alpha_S$-CN standard to further investigate the nature of these 9 unidentified peaks migrating between 23 and 29 minutes.

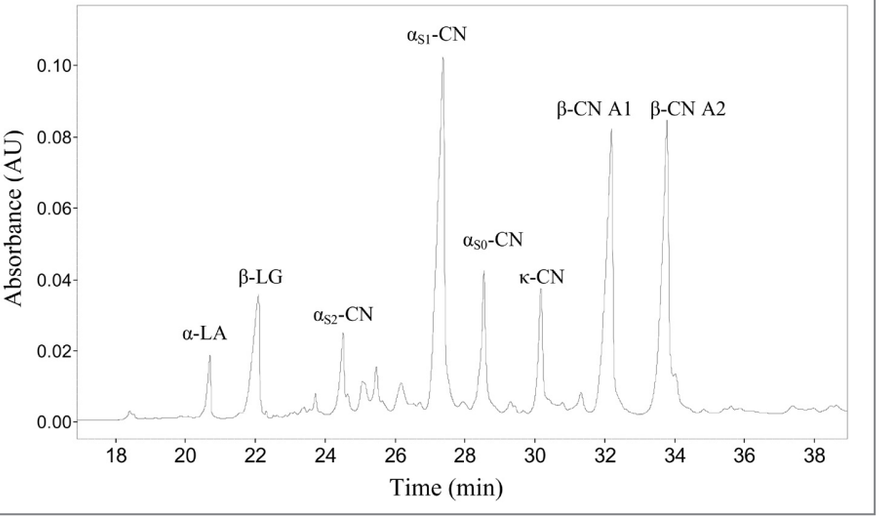


**Figure 1:** Electropherogram of raw bovine milk from an individual cow.

*2.3.1.2. Identification of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN by means of the $\alpha_S$-CN standard*

In raw milk, $\alpha_{S2}$-CN is partly present as disulphide linked dimers (Farrell et al., 2004). $\alpha_{S1}$-CN differs from $\alpha_{S2}$-CN because it does not contain any cysteine residues and is therefore unable to form dimers. This difference can be used to determine which of the unidentified peaks (1 to 9) in the $\alpha_S$-CN standard are $\alpha_{S2}$-CN's and which are $\alpha_{S1}$-CN's. During normal sample preparation, DTT (17 mM) is added as a reducing agent to convert dimers to their monomer form. When we do not use a reducing agent during sample preparation, a part of the $\alpha_{S2}$ -CN will still be present as dimers. Dimers have a different migration time during CZE analysis than monomers (Strickland et al., 2001). Before we performed this experiment, we first determined whether the reducing conditions used during normal analyses (17 mM DTT) were sufficient to reduce all dimers of $\alpha_{S2}$-CN to their monomer form.

We incubated the $\alpha_S$-CN standard with 0.6 M 2-mercaptoethanol in the sample buffer, a condition known to reduce all disulfide linked dimers of $\alpha_{S2}$-CN to their monomer form (Rasmussen & Petersen, 1991). No difference was seen between electropherograms from samples incubated with sample buffer containing 17 mM DTT and samples incubated with 0.6 M 2-mercaptoethanol. Thus, all the disulfide bridges were fully reduced in the presence of 17 mM DTT and none of the unidentified peaks 1 to 9 were disulfide linked dimers of $\alpha_{S2}$-CN. Subsequently, the $\alpha_S$-CN standard was incubated without a reducing agent in the sample buffer to differentiate between $\alpha_{S2}$ and $\alpha_{S1}$-CN's. Figure 2b shows that by omission of DTT the size of peaks 1 to 6 was reduced while peaks 7 to 9 are unaffected. This demonstrated that peaks 1 till 6 come from the reduction of DTT and are $\alpha_{S2}$-CN's. These six peaks have previously been assigned correctly as $\alpha_{S2}$-CN's by Otte et al. (1997) and Ortega et al. (2003). Both authors proposed these peaks to be disulphide linked dimers or different phosphorylation states of $\alpha_{S2}$-CN. Our results showed that these peaks are no dimers of $\alpha_{S2}$-CN. Therefore, the next step was to determine whether these peaks could be different phosphorylation states of $\alpha_{S2}$-CN.

*2.3.1.3 Identification of different phosphorylation states of $\alpha_{S2}$-CN*

To determine which peaks corresponded to the different phosphorylation states of $\alpha_{S2}$-CN, the separation pattern of the $\alpha_S$-CN standard and the difference in migration time between $\alpha_{S1}$-CN and $\alpha_{S0}$-CN was used. $\alpha_{S0}$-CN, also known as $\alpha_{S1}$-CN-9P, differs from $\alpha_{S1}$-CN-8P in one extra

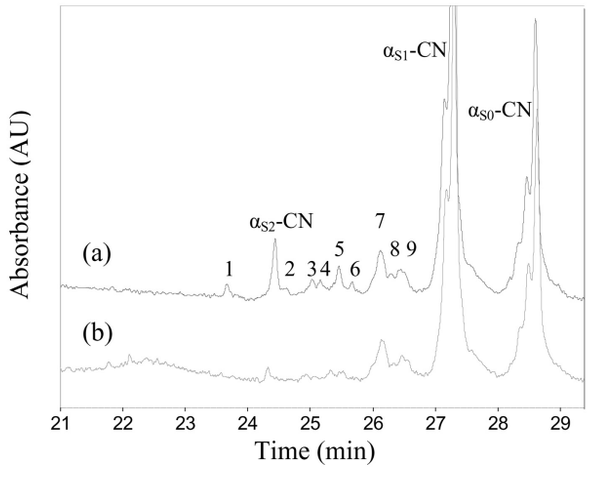


**Figure 2:** Electropherogram of 1 mg $mL^{-1}$ $\alpha_S$-CN standard
(a): with 0.17 mM DTT
(b): without DTT

Observed migration times:
1: 23.7; $\alpha_{S2}$-CN: 24.5; 2: 24.6; 3: 25.0; 4: 25.2; 5: 25.4, 6: 25.7; 7: 26.2, 8: 26.4; 9: 26.5; $\alpha_{S1}$-CN: 27.4; $\alpha_{S0}$-CN: 28.7

Predicted migration times:
$\alpha_{S2}$-CN-10P: 23.7 (peak 1); $\alpha_{S2}$-CN-12P: 25.4 (peak 5); $\alpha_{S1}$-CN-7P: 26.2 (peak 7); $\alpha_{S2}$-CN-13P: 26.3

phosphate group located at a serine residue at position 41 (Farrell et al., 2004). At the conditions used, an additional phosphate group results in additional negative charge and, therefore, in a longer migration time (Recio et al., 1997). $\alpha_{S2}$-CN also has multiple phosphorylation states containing 10, 11, 12 or 13 phosphate groups (Farrell et al., 2004) and is separated into multiple peaks as shown in Figure 2. To determine which of the separated $\alpha_{S2}$-CN peaks are different phosphorylation states of $\alpha_{S2}$-CN, the difference in migration time between $\alpha_{S1}$-CN-8P and $\alpha_{S1}$-CN-9P was used.

The migration time of a protein during CZE depends linearly on its charge but is also influenced by mass and shape of the protein (Adamson et al., 1993). We assumed no significant changes in mass or shape when different phosphorylation states of the same protein were compared.

Therefore we assumed that the migration time of a $\alpha_{S1}$-CN-9P (28.7 min) relative to $\alpha_{S1}$-CN-8P (27.4 min) is equal to the charge of $\alpha_{S1}$-CN-9P relative to the charge of $\alpha_{S1}$-CN-8P. At pH 3, $\alpha_{S1}$-CN casein without any phosphate groups has a charge of +24.3, calculated with pKa values for amino acids as in Damodaran, (1996). Using this charge and the observed migration times, an extra charge of -0.81 for every phosphate group at pH 3 could be calculated.
The most prevalent form of $\alpha_{S2}$-CN is the protein containing 11 phosphate groups (Farrell et al., 2004). Therefore we assigned the $\alpha_{S2}$-CN peak with the largest area as $\alpha_{S2}$-CN-11P. We estimated the expected migration time of $\alpha_{S2}$-CN-10P, -12P and -13P by calculating the charge of these phosphorylation states relative to $\alpha_{S2}$-CN-11P using the value -0.81 as the extra charge of one phosphate group. The predicted migration time of $\alpha_{S2}$-CN-10P (23.7 min) and $\alpha_{S2}$-CN-12P (25.4 min) resembled the observed migration time of peak 1 and 5 in Figure 2. It was not possible to assign a peak to $\alpha_{S2}$-CN-13P because it was predicted to migrate at the same time (26.3 min) as peaks which are not $\alpha_{S2}$-CN. Its concentration was probably also too small (Leonil et al., 1995). The relative areas of the peaks assigned agreed with the relative concentration of the different phosphorylation states (Leonil et al., 1995; Farrell et al., 2004).

In most studies $\alpha_{S1}$-CN is reported to have only two phosphorylation states with $\alpha_{S1}$-CN-8P being more abundant than $\alpha_{S1}$-CN-9P (Farrell et al., 2004). However, Leonil et al., (1995) found a small part of $\alpha_{S1}$-CN containing only 7 phosphate groups. The predicted migration time of $\alpha_{S1}$-CN-7P (26.2 min) resembled that of a partly separated $\alpha_{S1}$-CN peak (Figure 2, peak 7). This was a good indication that a small part of $\alpha_{S1}$-CN actually contains only 7 phosphate groups. Unfortunately, peak 7 was not a well resolved peak making quantification difficult. Therefore, we conclude that the presented CZE method is a suitable method to quantify all known phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN except $\alpha_{S1}$-CN-7P and $\alpha_{S2}$-CN-13P. It remains unclear what the exact nature of peaks 2, 3, 4, 6, 8 and 9 was. Possibly, these peaks represented proteins that have been partly hydrolysed by the action of proteolytic enzymes on $\alpha_{S1}$-CN or $\alpha_{S2}$-CN. It is known that both proteins are susceptible to proteolytic enzymes and breakdown products of them have been found in raw milk (Crudden et al., 2005; Kelly, et al., 2006).



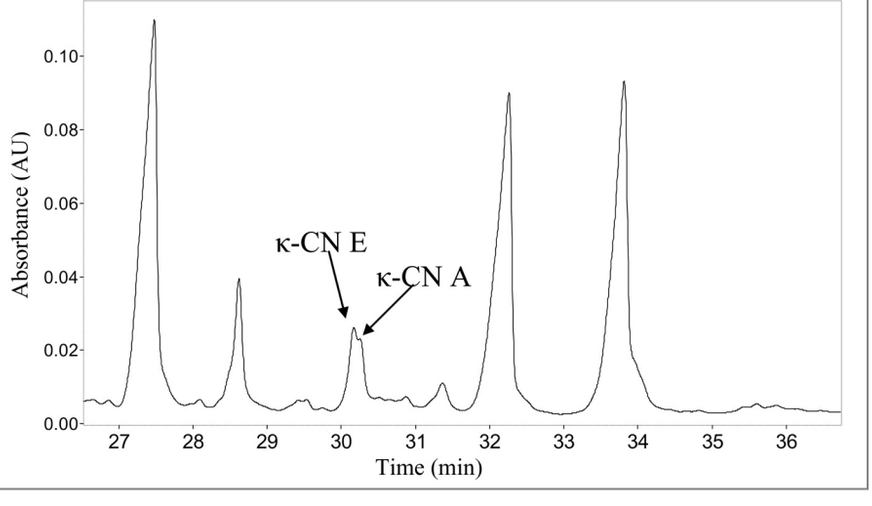


**Figure 3:** Electropherogram of a raw milk sample of an individual cow containing the genetic variants E and A of κ-CN.

*2.3.1.4. Identification of genetic variants*

In the electropherograms of 1948 milk samples we could distinguish the variants A and B of β-LG, B and C of $\alpha_{S1}$-CN and $A^1$, $A^2$, $A^3$ and B of β-CN. The separation patterns of samples containing these variants have been shown before (Recio et al., 1997). We also found samples showing a separation pattern which has not been shown before. Figure 3 shows an electropherogram of a milk sample showing an extra peak migrating just before the κ-CN peak. In samples where this extra peak was present, the area of the original κ-CN peak was approximately halved. The nature of this protein variant was determined by sequencing of exon 4 of the κ-CN genomic DNA in a subset of 95 cows. This subset included 28 cows that showed the additional peak in the electropherogram of their milk. The peak was identified as κ-CN E which differs from the A variant in a substitution of a serine by a glycine residue at position 155 of the protein. The original peak contained both the A and B variant which can not be separated at the pH of analysis. This is the first time κ-CN E has been separated by CZE. It is surprising that we were able to separate κ-CN E because the substitution involves two neutral amino acids which will normally not influence the charge of the protein. The E variant of κ-CN could be important because of its negative association with milk coagulation properties found in a growing number of studies (Hallen et al., 2007).

### 2.3.2 Protein quantification

To determine the variation in protein composition of a large number of samples by CZE, the peak area has to increase linearly as a function of injected protein, and the results should be reproducible after a large number of analytical series. Indeed, peak area increased linearly ($R^2$ >0.99 for all main milk proteins) when different amounts of protein standard were injected as shown in previous studies (Otte et al., 1997; Miralles et al., 2001; Gomez Ruiz et al., 2004). Method reproducibility was determined by calculating the coefficient of variation (CV) of relative peak area, for all individual proteins, using a reference milk sample that was analysed in all 103 series. CV values for reproducibility of the relative protein area were below 6% for all of the six main milk proteins (Table 1). These CV values were better than the reproducibility values obtained in previous studies (Otte et al., 1997; Bobe et al., 1998; Ortega et al., 2003). Moreover, the number of samples analysed (1948) and series performed (103) were larger than in any other study using CZE or RP-HPLC.



| Protein | Mean (% w/w) | CV (%) | Reproducibility CV(%) |
|---|---|---|---|
| α-LA | 2.4 | 13.0 | 4.3 |
| β-LG | 8.3 | 14.4 | 4.2 |
| $\alpha_{S2}$-CN | 10.1 | 13.7 | 5.7 |
| $\alpha_{S1}$-CN | 33.6 | 5.2 | 1.5 |
| κ-CN | 8.4[1] | 14.3[2] | 3.4[2] |
| β-CN | 27.2 | 5.8 | 2.1 |
| $\alpha_{S2}$-CN-10P | 1.0 | 35.4 | 15.7 |
| $\alpha_{S2}$-CN-11P | 3.5 | 16.4 | 5.7 |
| $\alpha_{S2}$-CN-12P | 2.2 | 10.0 | 5.8 |
| $\alpha_{S1}$-CN-8P | 21.2 | 6.5 | 2.2 |
| $\alpha_{S1}$-CN-9P | 7.4 | 14.9 | 3.8 |

**Table 1:** Mean and coefficients of variation (CV) of relative protein composition of 1948 Dutch Holstein Friesian cows and CV for CZE method reproducibility.

[1]Sum of κ-CN-1P with a mean 4.0% w/w and minor forms.
[2]κ-CN-1P only.

The mean relative concentration of the six main milk proteins determined with our CZE method is shown in Table 1. Total $\alpha_{S1}$-CN and total $\alpha_{S2}$-CN was determined by adding the concentrations of the different proteins of $\alpha_{S1}$-CN ($\alpha_{S1}$-CN-8P, $\alpha_{S1}$-CN-9P and peak 7 to 9) and $\alpha_{S2}$-CN ($\alpha_{S2}$-CN-10P, $\alpha_{S2}$-CN-11P, $\alpha_{S2}$-CN-12P, peak 2 to 4 and peak 6) respectively. Simple addition of the κ-CN peaks was not possible because one or more of the minor κ-CN peaks in all samples were invisible due to overlap with a β-CN peak. A mean value for these minor peak(s), based on samples where these peaks were visible, was added to the main κ-CN peak, and the same value was subtracted from the overlapping β-CN peak. Mean protein composition determined by our method was in line with values of Karman & van Boekel (1986) and well in the range of other studies (Bobe et al., 1998; Farrell et al., 2004; Wedholm et al., 2006).

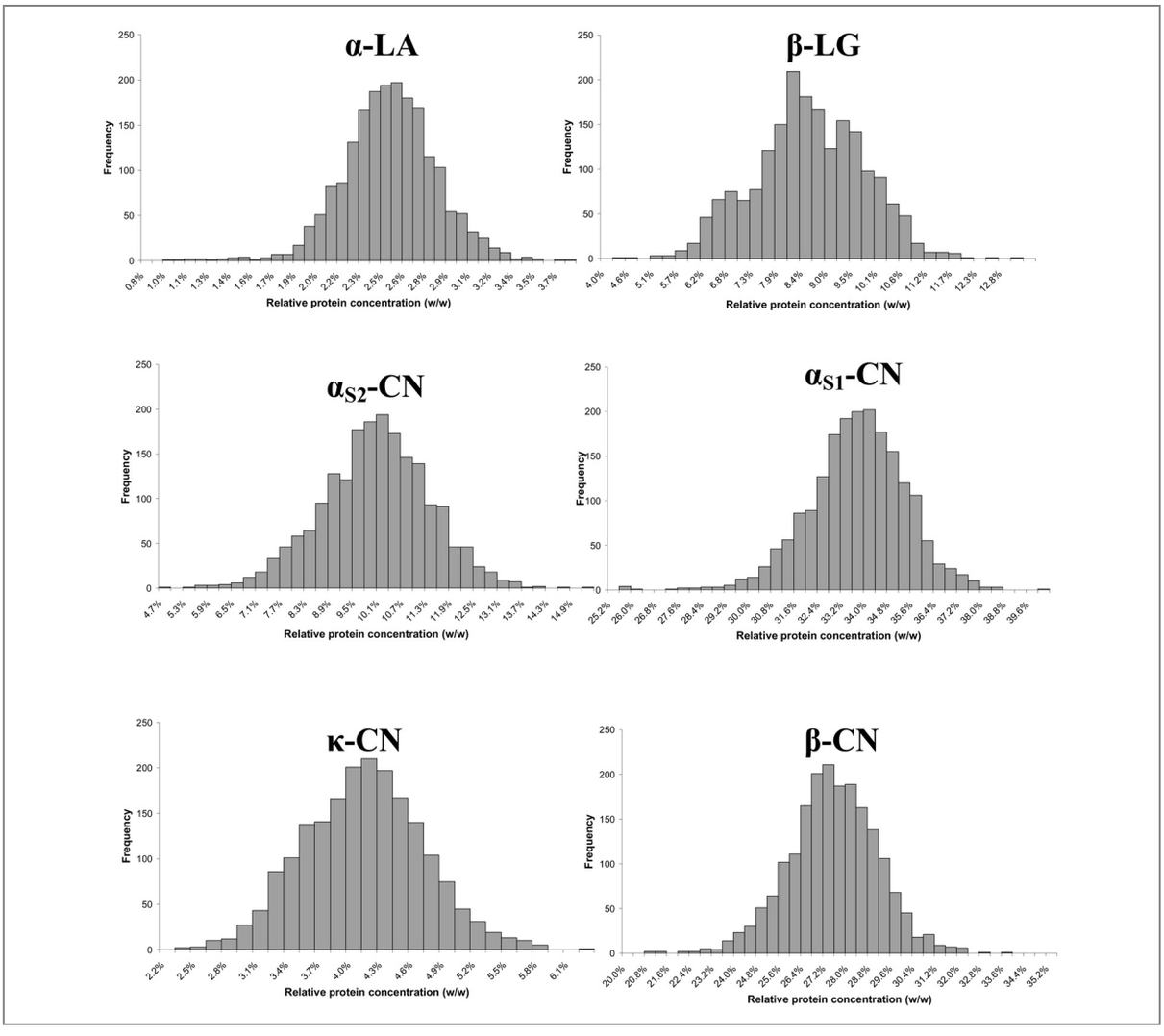


**Figure 4:** Histogram of relative protein concentration of: α-LA, β-LG, $\alpha_{S2}$-CN, $\alpha_{S1}$-CN, κ-CN and β-CN measured in milk samples of 1948 cows. The histogram of κ-CN represents the relative protein concentration of κ-CN-1P only.

### 2.3.3 Variation in protein composition

For all proteins studied, CV values for relative protein concentration were larger than CV values for reproducibility (Table 1). Consequently, the considerable variation in concentration of all studied milk proteins among individual animals was significant. Figue 4 shows large variation in relative protein concentration of the six main milk proteins in 1948 milk samples. When not considering 5% of the observations that represent the highest and lowest values, we found an 1.7 fold difference in the relative protein concentration of α-LA, β-LG, $\alpha_{S2}$-CN, and κ-CN and a 1.2 fold difference in $\alpha_{S1}$-CN and β-CN, calculated by dividing the highest and lowest values of the remaining observations. The shape of the histogram of β-LG can be explained by the occurrence of the A and B variants of β-LG in our population and the large effect of this variant on the concentration of β-LG.

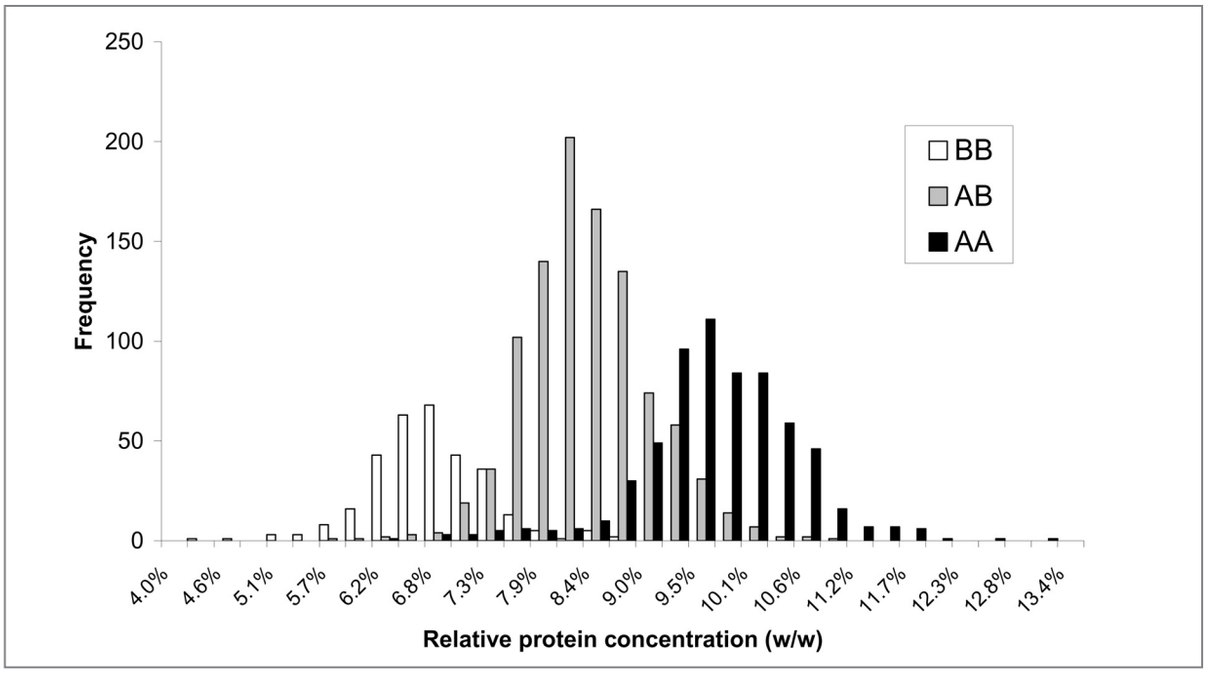


**Figure 5:** Histogram of relative β-LG concentration of 210 cows with BB, 1001 with AB, and 637 with AA genotype of β-LG.



| Protein | $\alpha_{S2}$-CN-10P | $\alpha_{S2}$-CN-11P | $\alpha_{S2}$-CN-12P | $\alpha_{S1}$-CN-8P |
|---|---|---|---|---|
| $\alpha_{S2}$-CN-11P | 0.73 | | | |
| $\alpha_{S2}$-CN-12P | 0.09 | 0.47 | | |
| $\alpha_{S1}$-CN-8P | -0.19 | -0.12 | 0.09 | |
| $\alpha_{S1}$-CN-9P | -0.73 | -0.74 | 0.01 | 0.14 |

**Table 2:** Correlations[1] between the different phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN measured in milk samples of 1948 HF ows. [1]Standard errors of correlations were between 0.01 and 0.03.

When comparing cows with the same genotype for β-LG we found three distributions with a mean value of 6.6% for BB, 8.2% for AB and 9.5% w/w for BB (Figure 5). This showed that in the Dutch Holstein-Friesian population both the A and B variant occur frequently and have an effect on the relative concentration of β-LG similar to what was found in other studies (Ng Kwai Hang & Sungwoo, 1996; Bobe et al., 1999).

Until now the variation in concentration of different phosphorylation states of milk proteins has not been studied. Large variation in the relative concentration of the different phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN exists (Table 1). We found an average of 74% of $\alpha_{S1}$-CN containing 8 phosphate groups and 26% containing 9 phosphate groups. For $\alpha_{S2}$-CN the values are, 15% having 10 , 52% having 11, and 33% having 12 phosphate groups. Table 2 shows the very remarkable correlations between the relative concentration of the different phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN. Most striking is that both $\alpha_{S2}$-CN-10P and $\alpha_{S2}$-CN-11P but not $\alpha_{S2}$-CN-12P, had a low correlation with $\alpha_{S1}$-CN-8P and a very high correlation with $\alpha_{S1}$-CN-9P. This indicated that the relative concentration of specific phosphorylation states of $\alpha_{S1}$- and $\alpha_{S2}$-CN were related to each other.

To change protein composition by breeding, variation in protein composition is essential and a part of this variation has to be genetic. This study shows large variation in protein composition and phosphorylation of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN among individual cows in the Dutch Holstein-Friesian population. The next step in our research will be to identify which part of this variation is genetic and which genes are responsible for the genetic differences in protein composition among individual cows.

## 2.4 Conclusions

The high reproducibility of the CZE method used in this study demonstrates its capability to determine protein composition and its variation in a large number of milk samples. This method enables the determination of the relative concentrations of α-LA, β-LG, $\alpha_{S2}$-CN, $\alpha_{S1}$-CN, κ-CN and β-CN. Moreover, it is possible to determine which part of $\alpha_{S2}$-CN contains 10, 11 or 12 phosphate groups and which part of $\alpha_{S1}$-CN contains 8 or 9 phosphate groups. Furthermore, it is possible to separate the κ-CN E variant and most of the common genetic variants. This study shows large variation in the relative concentration of the main milk proteins and the phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN among individual cows. This is to our knowledge the first time it is shown that the relative concentration of specific phosphorylation states of $\alpha_{S1}$- and $\alpha_{S2}$-CN are related to each other.

# 3

# Effects of milk protein variants on the protein composition of bovine milk

J. M. L. Heck, A. Schennink, H. J. F. van Valenberg, H. Bovenhuis, M. H. P. W. Visker, J. A. M. van Arendonk, A. C. M. van Hooijdonk (2008). *Effects of milk protein variants on the protein composition of bovine milk*. Journal of Dairy Science, accepted.

## Abstract

The effects of β-lactoglobulin (β-LG), β-casein (β-CN), and κ-CN variants and β-κ-CN haplotypes on the relative concentrations of the major milk proteins α-lactalbumin (α-LA), β-LG, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, and κ-CN and milk production traits were estimated in the milk of 1,912 Dutch Holstein Friesian cows. We show that in the Dutch Holstein Friesian population, the allele frequencies have changed in the past 16 years. In addition, genetic variants and casein haplotypes have a major impact on the protein composition of milk and explain a considerable part of the genetic variation in milk protein composition. The β-LG genotype was associated with the relative concentrations of β-LG (A >> B) and of α-LA, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, and κ-CN (B > A) but not with any milk production trait. The β-CN genotype was associated with the relative concentrations of β-CN and $\alpha_{S2}$-CN ($A^2 > A^1$) and of $\alpha_{S1}$-CN and κ-CN ($A^1 > A^2$) and with protein yield ($A^2 > A^1$). The κ-CN genotype was associated with the relative concentrations of κ-CN (B > E > A), $\alpha_{S2}$-CN (B > A), α-LA, and $\alpha_{S1}$-CN (A > B) and with protein percentage (B > A). Comparing the effects of casein haplotypes with the effects of single casein variants can provide better insight into what really underlies the effect of a variant on protein composition. We conclude that selection for both the β-LG genotype B and the β-κ-CN haplotype $A^2B$ will result in cows that produce milk that is more suitable for cheese production.

## 3.1 Introduction

Bovine milk protein largely (± 90% w/w) consists of 6 major milk proteins that are products of the milk protein genes α-lactalbumin (α-LA), β-lactoglobulin (β-LG), $\alpha_{S1}$-casein ($\alpha_{S1}$-CN), $\alpha_{S2}$-CN, β-CN, and κ-CN. The protein composition of milk determines, to a large extent, the nutritional value and the technological properties of milk. Cheese yield, for example, increases with casein concentration, and cheese properties like milk coagulation time and curd firmness depend on the casein composition (Wedholm et al., 2006). The protein composition of bovine milk varies with season, stage of lactation, feeding, and health status of the cow but is predominantly determined by genetic factors (Bobe et al., 1999b).

In all 6 major milk proteins, amino acid changes have been detected that are caused by polymorphisms in the corresponding genes (Farrell et al., 2004). When these genetic variants are associated with the protein composition of milk, this information can be used to breed for cows that produce milk with improved value, such as a higher level of casein or altered concentration of other proteins. Most research concerning milk protein variants has focused on its association with milk production traits rather than with milk protein composition. Previous research into the effects of milk protein variants on protein composition mainly reported the effects of the A and the B variants of β-LG and κ-CN on the concentration of β-LG, κ-CN, and total casein in milk (Ng-Kwai-Hang and Kim, 1996; Lunden et al., 1997; Mayer et al., 1997; Robitaille et al., 2002). These studies consistently showed that the A variant of β-LG is associated with a higher concentration of β-LG and a lower concentration of casein, and that the B variant of κ-CN is associated with a higher concentration of κ-CN in milk. The effects of these genotypes on the concentrations of all 6 major milk proteins, however, have only been investigated in a few studies with a relatively small number of animals (Bobe et al., 1999b). Moreover, in addition to the A and B variants of β-LG and κ-CN, the E variant of κ-CN and the $A^1$, $A^2$, and B variants of β-CN frequently occur in the Holstein Friesian and other cow populations (Bovenhuis and van Arendonk, 1991; Lunden et al., 1997; Ojala et al., 1997; Boettcher et al., 2004). The effects of these variants on the relative concentration of all 6 major milk proteins are unknown.



The 4 casein genes are closely linked and organized in a casein locus of around 250 kb, located on bovine chromosome 6 (Threadgill and Womack, 1990). Therefore one casein variant might be in linkage disequilibrium with another casein variant. This construct makes it difficult to determine whether the effect of a casein variant is specific to that variant or is an effect of another closely linked casein gene. Casein haplotypes are combinations of casein variants that are inherited together. Comparing the effects of casein haplotypes with the effects of single casein variants can provide better insight into what really underlies the effect of a variant on protein composition. The effect of casein haplotypes on protein composition has, to our knowledge, not been reported before.

The aim of our study was to estimate the effects of milk protein variants and casein haplotypes on the relative concentrations of all 6 major milk proteins in the milk of Dutch Holstein Friesian cows.

## 3.2 Materials and methods

### 3.2.1 Animals

This study is part of the Dutch Milk Genomics Initiative, which focuses on the genetic background of detailed milk composition. As part of this study, morning milk samples and blood samples were collected from 1,912 first-lactation cows from 398 commercial herds in the Netherlands. From February to March 2005, a 0.5-L milk sample was collected from each cow at one morning milking and preserved with 0.03% w/w sodium azide. Blood samples for DNA isolation were collected between April and June 2005. At least 3 cows per herd were sampled, and cows were milked twice a day. Cows descended from one of 50 young bulls (848 cows), from one of 5 proven bulls (873 cows), or from other proven bulls (191 cows). The NRS (Arnhem, the Netherlands) provided the pedigrees of the cows and the milk yield records. Each cow was over 87.5% Holstein Friesian and was in lactation between Day 66 and Day 247.

### 3.2.2 Sample analysis

Fat and true protein percentages were measured by infrared spectroscopy, using a MilkoScan FT6000 (Foss Electric, Hillerod, Denmark) at the Milk Control Station (Zutphen, the Netherlands). Fat and protein yields were calculated by multiplying each percentage by the morning milk yield. Morning milk yield data were missing for 155 cows. Aliquots taken for analyses of milk protein composition were frozen at -40°C until analysis.

The relative concentrations of the 6 major milk proteins α-LA, β-LG, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, κ-CN, and β-CN were estimated in milk samples from all 1,912 cows by capillary zone electrophoresis (CZE) (Heck et al., 2008). κ-CN is a heterogeneous protein containing multiple phosphorylation and glycosylation states (Farrell et al., 2004). With CZE, we could only quantify the non-glycosylated, mono-phosphorylated state (κ-CN-1P) (Heck et al., 2008). About 50% of the κ-CN is in this state; therefore, measuring only κ-CN-1P leads to an underestimation of the total amount of κ-CN. The other 50% of κ-CN (glycosylated, multi-phosphorylated forms) partly co-migrate with β-CN leading to a less accurate estimation of the total amount of β-CN (Heck et al., 2008).



### 3.2.3 Protein variants

Protein variants of α-LA, β-LG, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, and β-CN were determined by CZE (Heck et al., 2008). Protein variant identification for β-LG and β-CN by CZE was confirmed by genotyping the β-LG polymorphism A3984G (D64G; A/B variant) and the β-CN polymorphisms A8101C (H67P; $A^1/A^2$ variant) and C8267G (S122R; $A^2$/B variant) for 43% of the animals using the Illumina Golden Gate Assay (Illumina, San Diego, CA). Protein variant identification for $\alpha_{S1}$-CN by CZE was confirmed by sequencing of exon 17 of $\alpha_{S1}$-CN in a subset of 96 randomly selected cows and by sequencing exons 9 and 17 for the 6 cows genotyped as $\alpha_{S1}$ BC using CZE.

For analysis, we used the protein variants determined by CZE for all proteins except for κ-CN, which could not be determined by CZE. Therefore, genotypes for the κ-CN polymorphisms C5309T (A/B variant), A5345C (A/B variant), and A5365G (A/E variant) were assayed by the SNaPshot single-base primer extension method (Applied Biosystems, Foster City, CA, USA).

The primer designs were based on the GenBank® sequence (X14908): forward PCR primer, 5'-CTCTGCTTCTGCTGCTGCTA-3'; reverse PCR primer, 5'-TTGCCTTATTTACCTGCGTTG

-3'; genotyping primer for C5309T, 5'- GAGCCTACAAGTACACCTACCA - 3'; genotyping primer for A5345C, 5'- AGCACTGTAGCTACTCTAGAAG- 3'; and genotyping primer for A5365G, 5'- GTGTTGATCTCAGGTGGGC- 3'. Experimental details for this assay have been described by Schennink et al. (2008). In total, 1,732 animals were genotyped for κ-CN. Genotypes for κ-CN were missing for 180 animals because either no DNA sample was available (n = 177) or the sample could not be genotyped unambiguously (n = 3).

### 3.2.4 Haplotypes

From the 1,732 genotyped cows, only cows descending from sires with at least 10 genotyped daughters (1,629) were used to deduce $\alpha_{S1}$-β-κ-CN haplotypes. The CN haplotypes of the sires were inferred from offspring genotypes, assuming no recombination events between the CN genes. Subsequently, the haplotypes inherited by each daughter were inferred. The $\alpha_{S1}$-β-κ-CN haplotypes could be assigned unambiguously for 1,517 cows. Daughters that inherited the rare haplotypes $BA^2E$, BBA, $CA^2A$, $CA^2B$, and $CA^1E$ (n = 9, 3, 2, 1, and 1, respectively) and daughters for which haplotypes could not be reconstructed were included in the statistical analysis as a separate class. Expected haplotype frequencies were calculated by multiplying the corresponding allele frequencies of $\alpha_{S1}$-CN, β-CN, and κ-CN assuming linkage equilibrium. To quantify linkage disequilibrium between the β-CN and κ-CN variants, $r_{ij}^2$ was calculated as described by Hill and Robertson (1968). Linkage disequilibrium between different pairs of alleles were combined by pooling ($r^2$) as described by Zhao et al. (2005).

$$r^2 = \sum_{i=1}^{k} \sum_{j=1}^{m} p(A_i) * p(B_j) * r_{ij}^2$$

where k and m are the numbers of alternate alleles at locus A and B, respectively and

$$r_{ij}^2 = \frac{D_{ij}^2}{p(A_i) * (1 - p(A_i)) * p(B_j) * (1 - p(B_j))}$$

where

$$D_{ij} = p(A_i B_j) - p(A_i) * p(B_j)$$

where $p(A_i)$ is the frequency of allele $A_i$ at locus A, $p(B_j)$ the frequency of allele $B_j$ at locus B, and $p(A_iB_j)$ the frequency of haplotype $A_iB_j$.

### 3.2.5 Statistical analysis

Variance components were estimated using an Animal Model in ASReml (Gilmour et al., 2002):

$$y_{ijklmno} = \mu + b_1*dim_i + b_2*e^{-0.05*dim} + b_3*afc_j + b_4*afc_j^2 + season_k + scode_l + g_m + herd_n + U_o + e_{ijklmno}$$

where y was the dependent variable; μ was the general mean; dim was the covariate describing the effect of days in milk modeled with a Wilmink curve (Wilmink, 1987); afc was the covariate describing the effect of age at first calving; season was the fixed effect of the class of calving season (June to August 2004, September to November 2004, or December 2004 to February 2005); scode was the fixed effect of the differences in genetic level between groups of proven bull daughters and young bull daughters; g was the fixed effect of the genotype or number of copies (0, 1, or 2) of the casein haplotype; herd was the random effect of groups of animals sampled in the same herd; Uo was the random additive genetic effect of animal o; and e was the random residual effect.

Ungenotyped individuals were included as a separate group and appeared to be randomly distributed across other effects in the model. The variance-covariance structure of the additive genetic effects was $Var(U) = A\sigma_U^2$, where A was a matrix of additive genetic relationships between individuals and $\sigma_U^2$ was the additive genetic variance. The proportion of additive genetic variance explained by the genotype was defined as:

$$r^2_{genetic} = \frac{\sigma^2_{genotype}}{\sigma^2_U + \sigma^2_{genotype}}$$

where $\sigma^2_{genotype}$ was calculated by estimating the variance of the genotype based on the estimated genotype effects and the observed genotype frequencies.



**Table 1:** Allele frequencies of the six major milk proteins in Dutch Holstein Friesian cows in 1989 and in 2005.
[a] Allele frequencies of the imported HF in 1989 reported by Bovenhuis and van Arendonk (1991).
[b] Not recognized.

| Protein | Allele | Frequency in 1989[a] | Frequency in 2005 |
|---|---|---|---|
| α-LA | B | 1 | 1 |
| β-LG | A | 0.425 | 0.583 |
| | B | 0.575 | 0.417 |
| $\alpha_{S1}$-CN | B | 0.950 | 0.997 |
| | C | 0.050 | 0.003 |
| $\alpha_{S2}$-CN | A | 1 | 1 |
| β-CN | $A^1$ | 0.462 | 0.285 |
| | $A^2$ | 0.498 | 0.692 |
| | $A^3$ | 0.014 | 0.001 |
| | B | 0.026 | 0.022 |
| κ-CN | A | 0.847 | 0.599 |
| | B | 0.153 | 0.309 |
| | E | -[b] | 0.092 |

## 3.3 Results

### 3.3.1 Allele and haplotype frequencies

Table 1 shows the allele frequencies, obtained by gene counting, of α-LA, β-LG, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, and κ-CN in the Dutch Holstein Friesian population in 1989 (Bovenhuis and van Arendonk, 1991) and in 2005 (this study). The frequencies of the rare alleles $\alpha_{S1}$-CN C, β-CN $A^3$, and β-CN B have decreased since 1989. The frequency of β-LG A has increased at the expense of β-LG B, the frequency of β-CN $A^2$ has increased at the expense of β-CN $A^1$, and the frequency of κ-CN B has increased at the expense of κ-CN A. Furthermore, in 2005, at least one copy of the κ-CN E allele was found in 18% of the studied cow population, but this variant was not reported by Bovenhuis and van Arendonk (1991).

Table 2 shows the observed and expected $\alpha_{S1}$-β-κ-CN haplotype frequencies. The observed haplotype frequencies differed from the expected frequencies ($P < 0.001$, tested by chi-square test), especially for haplotypes containing κ-CN E and β-CN B. The frequencies of the haplotypes $BA^1E$ and BBB were much higher than expected for a population in linkage equilibrium. To quantify linkage disequilibrium we calculated the correlation coefficient for the casein variants ($r^2$) as described by Hill and Robertson (1968). We found an overall $r^2$ based on all haplotypes of 0.056 and $r^2$ below 0.06 for all individual haplotypes, except for $A^1E$ which had an $r^2$ of 0.278.

### 3.3.2 Effects of β-LG variants

The estimated effects of the β-LG genotypes on test day morning milk production traits and milk protein composition are shown in Tables 3a and 3b. The β-LG genotype was associated with differences in the relative concentration of all 6 milk proteins (all $P < 0.001$) but not with any milk production trait (all $P > 0.23$). In comparison to the B variant, the A variant was strongly associated with a higher relative concentration of β-LG and a lower relative concentration of the other 5 major milk proteins. Cows with the BB, BA, and AA genotypes had a mean relative β-LG concentration of 6.58%, 8.15%, and 9.52% (w/w), respectively (Heck et al., 2008). The β-LG genotype explained 90% ($r^2$genetic) of the genetic variation in β-LG and between 2% and 10% of the genetic variation in the other proteins.



| $\alpha_{s1}$-β-κ-CN haplotype | Observed (n) | Observed frequency | Expected[a] frequency |
|---|---|---|---|
| $BA^1A$ | 348 | 0.115 | 0.170 |
| $BA^1B$ | 164 | 0.054 | 0.088 |
| $BA^1E$ | 287 | 0.095 | 0.026 |
| $BA^2A$ | 1446 | 0.476 | 0.413 |
| $BA^2B$ | 715 | 0.236 | 0.213 |
| $BA^2E$ | 9 | 0.003 | 0.063 |
| BBA | 3 | 0.001 | 0.013 |
| BBB | 58 | 0.019 | 0.006 |
| $CA^2A$ | 2 | 0.001 | 0.001 |
| $CA^2B$ | 1 | 0.000 | <0.001 |
| $CA^1E$ | 1 | 0.000 | <0.001 |

**Table 2:** Observed and expected frequencies of the $\alpha_{S1}$-β-κ-CN haplotypes in 1518 Dutch HF cows.
[a]Expected $\alpha_{S1}$-β-κ-CN haplotype frequency when independent segregation of the casein alleles is assumed. All observed frequencies differed from the expected frequencies ($P < 0.05$, tested with a chi square test). Haplotypes with an expected frequency lower than 5 (BBA, $CA^2A$, $CA^2B$ and $CA^1E$) were not tested.

### 3.3.3 Effects of β-CN variants

The estimated effects of the β-CN genotypes on milk production traits and milk protein composition are shown in Tables 4a and 4b. The β-CN genotypes were associated with protein yield ($P < 0.01$) and differences in the relative concentrations of all 6 main milk proteins (all $P < 0.01$). Cows with the $A^1$ allele had a lower protein yield than cows with the $A^2$ allele, resulting from decreased milk production. The $A^1$ variant was also associated with higher relative concentrations of $\alpha_{S1}$-CN and κ-CN and with lower relative concentrations of $\alpha_{S2}$-CN and β-CN compared to the $A^2$ variant. The B variant of β-CN was associated with higher relative concentrations of κ-CN compared to either the $A^1$ and $A^2$ variants. Cows heterozygous for β-CN ($A^1A^2$, $A^1B$, or $A^2B$) had a higher relative concentration of β-CN and a lower relative concentration of $\alpha_{S1}$-CN compared to homozygous ($A^1A^1$ or $A^2A^2$) individuals. The genotypes of β-CN explained 54% ($r^2$genetic) of the genetic variation in β-CN and between 1% and 13% of the genetic variation in the other proteins.

**Table 3a:** Effects of β-LG genotypes on milk production traits.

| | Milk (kg) | Protein (kg*100) | Fat (kg*100) | Protein (%) | Fat (%) |
|---|---|---|---|---|---|
| **Trait mean** | **13.44** | **47.03** | **57.73** | **3.51** | **4.36** |
| AA (n = 621) | 0 | 0 | 0 | 0 | 0 |
| AB (n = 987) | -0.17 (0.13) | -0.54 (0.42) | -0.18 (0.54) | 0.01 (0.01) | 0.03 (0.03) |
| BB (n = 304) | -0.28 (0.18) | -0.96 (0.59) | -0.62 (0.77) | 0.00 (0.02) | 0.03 (0.05) |
| P value[2] | 0.25 | 0.23 | 0.73 | 0.73 | 0.64 |

**Table 4a:** Effects of β-CN genotypes on milk production traits.

| | Milk (kg) | Protein (kg*100) | Fat (kg*100) | Protein (%) | Fat (%) |
|---|---|---|---|---|---|
| **Trait mean** | **13.44** | **47.03** | **57.73** | **3.51** | **4.36** |
| $A^1A^1$ (n = 159) | 0 | 0[a] | 0 | 0 | 0 |
| $A^1A^2$ (n = 752) | 0.34 (0.21) | 1.46 (0.70)[b] | -0.38 (0.91) | 0.02 (0.02) | -0.13 (0.06) |
| $A^2A^2$ (n = 916) | 0.62 (0.22) | 2.54 (0.72)[c] | 0.72 (0.94) | 0.03 (0.02) | -0.14 (0.06) |
| $A^1B$ (n = 21) | 0.04 (0.56) | 0.92 (1.83)[abc] | 0.24 (2.39) | 0.04 (0.06) | 0.00 (0.16) |
| $A^2B$ (n = 62) | 0.69 (0.38) | 3.03 (1.23)[bc] | 1.88 (1.61) | 0.02 (0.04) | -0.12 (0.11) |
| P value[2] | 0.03 | < 0.01 | 0.21 | 0.82 | 0.21 |



**Table 5a:** Effects of κ-CN genotypes on milk production traits.

| | Milk (kg) | Protein (kg*100) | Fat (kg*100) | Protein (%) | Fat (%) |
|---|---|---|---|---|---|
| **Trait mean** | **13.44** | **47.03** | **57.73** | **3.51** | **4.36** |
| AA (n = 601) | 0 | 0 | 0 | 0[a] | 0 |
| AB (n = 686) | -0.06 (0.14) | 0.78 (0.45) | 0.54 (0.59) | 0.07 (0.01)[b] | 0.02 (0.04) |
| BB (n = 141) | -0.23 (0.23) | 0.89 (0.75) | -0.02 (0.98) | 0.11 (0.02)[b] | 0.01 (0.06) |
| EE (n = 15) | 1.22 (0.63) | 2.92 (2.07) | 2.86 (2.69) | -0.08 (0.07)[a] | -0.18 (0.18) |
| AE (n = 187) | -0.11 (0.22) | -0.31 (0.71) | -0.15 (0.93) | 0.00 (0.02)[a] | -0.03 (0.06) |
| BE (n = 102) | -0.16 (0.27) | 0.50 (0.88) | -1.26 (1.14) | 0.08 (0.03)[b] | -0.08 (0.07) |
| P value[2] | 0.04 | 0.03 | 0.01 | < 0.001 | 0.63 |

[1]CN-index: $100*(\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN}) / (\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN} + \alpha\text{-LA} + \beta\text{-LG})$.
[2]When P value < 0.01, superscripts were used to indicate which genotypes differ ($P < 0.05$).

### 3.3.4 Effects of κ-CN variants

The estimated effects of the κ-CN genotypes on milk production traits and milk protein composition are shown in Tables 5a and 5b. The κ-CN genotypes were associated with protein percentage ($P<0.001$) and differences in the relative concentrations of all 6 main milk proteins (all $P<0.001$). The κ-CN B variant was associated with a higher protein percentage and the E variant with a lower protein percentage compared to the A variant. The B variant of κ-CN was associated with a lower relative concentration of α-LA and $\alpha_{S1}$-CN and a higher relative concentration of $\alpha_{S2}$-CN compared to the A variant. The B variant of κ-CN was associated with a higher relative concentration of κ-CN and the A variant with a lower relative κ-CN concentration compared to the E variant. Cows with the AA, AE, AB, EE, BE, or BB genotypes had mean relative κ-CN concentrations of 3.77, 3.98, 4.12, 4.15, 4.17, and 4.55% (w/w), respectively. The κ-CN genotype explained 29% ($r^2$genetic) of the genetic variation in κ-CN and between 2% and 11% of the genetic variation in the other proteins.

**Table 3b:** Effects of β-LG genotypes on milk protein composition.

| | α-LA | β-LG | $\alpha_{S1}$-CN | $\alpha_{S2}$-CN | β-CN | κ-CN | CN index[1] |
|---|---|---|---|---|---|---|---|
| **Trait mean** | **2.44** | **8.35** | **33.63** | **10.38** | **27.17** | **4.03** | **87.46** |
| AA (n = 621) | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] |
| AB (n = 987) | 0.03 (0.02)[b] | -1.32 (0.03)[b] | 0.31 (0.08)[b] | 0.23 (0.07)[b] | 0.46 (0.08)[b] | 0.10 (0.03)[b] | 1.46 (0.04)[b] |
| BB (n = 304) | 0.09 (0.02)[c] | -2.84 (0.05)[c] | 0.80 (0.12)[c] | 0.54 (0.10)[c] | 0.71 (0.11)[c] | 0.23 (0.04)[c] | 3.14 (0.06)[c] |
| P value[2] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

**Table 4b:** Effects of β-CN genotypes on milk protein composition.

| | α-LA | β-LG | $\alpha_{S1}$-CN | $\alpha_{S2}$-CN | β-CN | κ-CN | CN-index[1] |
|---|---|---|---|---|---|---|---|
| **Trait mean** | **2.44** | **8.35** | **33.63** | **10.38** | **27.17** | **4.03** | **87.46** |
| $A^1A^1$ (n = 159) | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] |
| $A^1A^2$ (n = 752) | -0.06 (0.03)[b] | -0.41 (0.10)[b] | -1.60 (0.14)[b] | 0.33 (0.11)[b] | 2.45 (0.11)[b] | -0.24 (0.05)[b] | 0.63 (0.11)[b] |
| $A^2A^2$ (n = 916) | -0.02 (0.03)[a] | -0.24 (0.10)[c] | -1.19 (0.14)[c] | 0.99 (0.11)[c] | 1.26 (0.12)[c] | -0.30 (0.05)[c] | 0.38 (0.12)[c] |
| $A^1B$ (n = 21) | -0.07 (0.07)[ab] | -0.52 (0.26)[bc] | -1.21 (0.36)[bc] | 0.47 (0.29)[abc] | 2.40 (0.30)[bd] | 0.45 (0.12)[d] | 0.91 (0.30)[bc] |
| $A^2B$ (n = 62) | -0.11 (0.05)[b] | -0.35 (0.17)[bc] | -1.74 (0.24)[b] | 0.61 (0.19)[b] | 2.79 (0.20)[d] | 0.28 (0.08)[d] | 0.77 (0.20)[b] |
| P value[2] | < 0.01 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

**Table 5b:** Effects of κ-CN genotypes on milk protein composition.

| | α-LA | β-LG | $\alpha_{S1}$-CN | $\alpha_{S2}$-CN | β-CN | κ-CN | CN-index[1] |
|---|---|---|---|---|---|---|---|
| **Trait mean** | **2.44** | **8.35** | **33.63** | **10.38** | **27.17** | **4.03** | **87.46** |
| AA (n = 601) | 0[a] | 0[a] | 0[ab] | 0[a] | 0[a] | 0[a] | 0[a] |
| AB (n = 686) | -0.11 (0.02)[b] | -0.30 (0.06)[bc] | -0.43 (0.09)[c] | 0.18 (0.07)[b] | 0.03 (0.09)[a] | 0.34 (0.03)[b] | 0.43 (0.07)[bd] |
| BB (n = 141) | -0.19 (0.03)[c] | -0.27 (0.10)[bc] | -1.20 (0.15)[d] | 0.45 (0.12)[c] | 0.09 (0.14)[a] | 0.75 (0.05)[c] | 0.47 (0.12)[bcd] |
| EE (n = 15) | 0.03 (0.07)[ab] | 0.01 (0.29)[abc] | 0.68 (0.41)[a] | -0.63 (0.33)[a] | -0.99 (0.40)[b] | 0.35 (0.13)[bd] | -0.11 (0.34)[abc] |
| AE (n = 187) | -0.02 (0.03)[a] | -0.19 (0.10)[abc] | -0.18 (0.14)[bc] | -0.22 (0.11)[a] | 0.19 (0.13)[a] | 0.21 (0.04)[d] | 0.20 (0.11)[ac] |
| BE (n = 102) | -0.12 (0.03)[bc] | -0.52 (0.12)[c] | -0.79 (0.17)[e] | 0.05 (0.14)[ab] | 0.68 (0.16)[c] | 0.45 (0.05)[b] | 0.68 (0.14)[d] |
| P value[2] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

[1]CN-index: $100*(\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN}) / (\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN} + \alpha\text{-LA} + \beta\text{-LG})$.
[2]When P value < 0.01, superscripts were used to indicate which genotypes differ ($P < 0.05$).

### 3.3.5 Effects of β-κ-CN haplotypes

To estimate the effects of the haplotypes, we used the β-κ-CN haplotype instead of the $\alpha_{S1}$-β-κ-CN haplotype because our population was almost completely monomorphic for $\alpha_{S1}$-CN (Table 2). The estimated effects of the β-κ-CN haplotypes on milk protein composition and milk production traits are shown in Table 6a and 6b. The $A^1A$ haplotype was associated with a higher fat percentage ($P = 0.01$) and a lower protein yield ($P < 0.001$) and milk yield ($P < 0.01$).

The $A^2A$ haplotype was associated with a higher milk yield ($P < 0.01$) and a lower protein percentage ($P < 0.01$). The $A^1B$ and $A^2B$ haplotypes were associated with higher protein percentages ($P < 0.01$), and the $A^2B$ haplotype was also associated with a higher protein yield ($P < 0.01$).

**Table 6a:** Effects of β-κ-CN haplotypes[1] on milk production traits.

| | Milk (kg) | Protein (kg*100) | Fat (kg*100) | Protein (%) | Fat (%) |
|---|---|---|---|---|---|
| **Trait mean** | **13.44** | **47.03** | **57.73** | **3.51** | **4.36** |
| ***$A^1A$*** | | | | | |
| 0 (n = 1172) | 0[a] | 0[a] | 0 | 0 | 0 |
| 1 (n = 312) | -0.27 (0.16)[ab] | -1.38 (0.51)[b] | -0.61 (0.67) | -0.03 (0.02) | 0.05 (0.04) |
| 2 (n = 17) | -1.45 (0.60)[b] | -5.57 (1.96)[c] | -1.52 (2.58) | -0.05 (0.07) | 0.55 (0.17) |
| P value[3] | < 0.01 | < 0.001 | 0.03 | 0.16 | 0.01 |
| ***$A^1B$*** | | | | | |
| 0 (n = 1344) | 0 | 0 | 0 | 0 | 0 |
| 1 (n = 152) | -0.28 (0.23) | 0.01 (0.76) | 0.75 (0.99) | 0.08 (0.03) | 0.15 (0.06) |
| 2 (n = 5) | -1.41 (1.03) | -3.48 (3.41) | -1.88 (4.40) | 0.12 (0.12) | 0.34 (0.30) |
| P value[3] | 0.02 | 0.04 | 0.03 | 0.01 | 0.09 |
| ***$A^1E$*** | | | | | |
| 0 (n = 1230) | 0[a] | 0 | 0 | 0 | 0 |
| 1 (n = 256) | -0.10 (0.18)[a] | -0.60 (0.60) | -0.74 (0.79) | -0.02 (0.02) | -0.06 (0.05) |
| 2 (n = 15) | 1.24 (0.63)[b] | 2.47 (2.06) | 2.61 (2.68) | -0.12 (0.07) | -0.18 (0.18) |
| P value[3] | < 0.01 | 0.02 | 0.02 | 0.23 | 0.57 |
| ***$A^2A$*** | | | | | |
| 0 (n = 384) | 0[a] | 0 | 0 | 0[a] | 0 |
| 1 (n = 796) | 0.37 (0.15)[b] | 0.67 (0.50) | 0.47 (0.66) | -0.05 (0.02)[b] | -0.07 (0.04) |
| 2 (n = 321) | 0.44 (0.19)[b] | 0.44 (0.62) | 0.41 (0.81) | -0.07 (0.02)[b] | -0.06 (0.05) |
| P value[3] | < 0.01 | 0.03 | 0.03 | < 0.01 | 0.44 |
| ***$A^2B$*** | | | | | |
| 0 (n = 877) | 0 | 0[a] | 0 | 0[a] | 0 |
| 1 (n = 536) | 0.03 (0.14) | 1.15 (0.46)[b] | 0.82 (0.60) | 0.07 (0.02)[b] | 0.00 (0.04) |
| 2 (n = 88) | -0.23 (0.29) | 0.86 (0.93)[ab] | -1.47 (1.22) | 0.11 (0.03)[b] | -0.07 (0.08) |
| P value[3] | 0.05 | < 0.01 | < 0.01[4] | < 0.001 | 0.84 |
| ***BB*** | | | | | |
| 0 (n = 1443) | 0 | 0 | 0 | 0 | 0 |
| 1 (n = 58) | 0.03 (0.33) | 0.00 (1.07) | 0.19 (1.39) | -0.02 (0.04) | -0.04 (0.09) |
| P value[3] | 0.01 | 0.01 | < 0.01[4] | 0.49 | 0.94 |

[1]The number below the β-κ-CN haplotype indicates the number of copies of the haplotype a cow carries (0,1 or 2).
[2]Casein index: $100*(\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN}) / (\alpha_{S1}\text{-CN} + \alpha_{S2}\text{-CN} + \beta\text{-CN} + \kappa\text{-CN} + \alpha\text{-LA} + \beta\text{-LG})$.
[3]When P value < 0.01, superscripts were used to indicate which number of copies of a haplotype differ ($P < 0.05$).
[4]Only the group with unknown haplotypes (not in table) differed ($P < 0.05$).

All haplotypes were associated with a difference in the relative concentrations of the 6 major milk proteins. In general, haplotypes containing the β-CN A[1] variant were associated with higher concentrations of $\alpha_{S1}$-CN and κ-CN and lower relative concentrations of β-CN and $\alpha_{S2}$-CN compared to haplotypes containing the A[2] variant. In general, haplotypes containing the A variant of κ-CN were associated with a higher relative concentration of α-LA and $\alpha_{S1}$-CN and a lower relative concentration of κ-CN and $\alpha_{S2}$-CN.

**Table 6b:** Effects of β-κ-CN haplotypes[1] on milk protein composition.

| | α-LA | β-LG | $\alpha_{S1}$-CN | $\alpha_{S2}$-CN | β-CN | κ-CN | CN-index[2] |
|---|---|---|---|---|---|---|---|
| **Trait mean** | **2.44** | **8.35** | **33.63** | **10.38** | **27.17** | **4.03** | **87.45** |
| ***A[1]A*** | | | | | | | |
| 0 (n = 1172) | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0 | 0[a] |
| 1 (n = 312) | 0.04 (0.02)[b] | 0.17 (0.07)[b] | 0.10 (0.10)[a] | -0.59 (0.08)[b] | 0.36 (0.10)[b] | -0.09 (0.03) | -0.25 (0.08)[b] |
| 2 (n = 17) | 0.12 (0.07)[ab] | 0.55 (0.28)[b] | 1.99 (0.40)[b] | -1.28 (0.32)[c] | -1.52 (0.38)[c] | -0.09 (0.13) | -0.78 (0.33)[b] |
| P value[3] | < 0.01 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.02 | < 0.001 |
| ***A[1]B*** | | | | | | | |
| 0 (n = 1344) | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] |
| 1 (n = 152) | -0.12 (0.03)[b] | -0.32 (0.11)[b] | 0.33 (0.15)[b] | -0.39 (0.12)[b] | -0.09 (0.14)[a] | 0.39 (0.05)[b] | 0.49 (0.12)[b] |
| 2 (n = 5) | -0.20 (0.13)[ab] | 0.94 (0.48)[a] | 0.73 (0.70)[ab] | -0.92 (0.56)[ab] | -1.77 (0.67)[b] | 0.82 (0.23)[b] | -0.91 (0.57)[a] |
| P value[3] | < 0.001 | < 0.001 | < 0.01 | < 0.01 | < 0.01 | < 0.001 | < 0.001 |
| ***A[1]E*** | | | | | | | |
| 0 (n = 1230) | 0 | 0[a] | 0[a] | 0[a] | 0[a] | 0 | 0 |
| 1 (n = 256) | 0.02 (0.02) | -0.17 (0.08)[b] | -0.14 (0.12)[a] | -0.30 (0.10)[b] | 0.51 (0.11)[b] | 0.08 (0.04) | 0.16 (0.10) |
| 2 (n = 15) | 0.08 (0.08) | 0.09 (0.29)[ab] | 0.86 (0.41)[b] | -0.76 (0.33)[b] | -0.88 (0.39)[c] | 0.19 (0.14) | -0.24 (0.34) |
| P value[3] | 0.04 | < 0.01 | < 0.01 | < 0.01 | < 0.001 | 0.05 | < 0.001[4] |
| ***A[2]A*** | | | | | | | |
| 0 (n = 384) | 0[a] | 0[a] | 0[a] | 0 | 0[a] | 0[a] | 0[a] |
| 1 (n = 796) | 0.05 (0.02)[b] | 0.07 (0.07)[a] | 0.09 (0.10)[a] | -0.06 (0.08) | 0.45 (0.09)[b] | -0.33 (0.03)[b] | -0.10 (0.08)[a] |
| 2 (n = 321) | 0.13 (0.02)[c] | 0.27 (0.09)[b] | 0.75 (0.12)[b] | 0.18 (0.10) | -0.29 (0.12)[c] | -0.59 (0.04)[c] | -0.40 (0.10)[b] |
| P value[3] | < 0.001 | < 0.001 | < 0.001 | 0.04 | < 0.001 | < 0.001 | < 0.001 |
| ***A[2]B*** | | | | | | | |
| 0 (n = 877) | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] | 0[a] |
| 1 (n = 536) | -0.08 (0.02)[b] | -0.26 (0.06)[b] | -0.66 (0.09)[b] | 0.53 (0.07)[b] | -0.05 (0.09)[ab] | 0.23 (0.03)[b] | 0.35 (0.08)[b] |
| 2 (n = 88) | -0.17 (0.03)[c] | -0.19 (0.13)[ab] | -1.47 (0.18)[c] | 0.90 (0.15)[c] | -0.35 (0.18)[b] | 0.55 (0.06)[c] | 0.30 (0.15)[b] |
| P value[3] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.01 | < 0.001 | < 0.001 |
| ***BB*** | | | | | | | |
| 0 (n = 1443) | 0 | 0 | 0 | 0 | 0[a] | 0[a] | 0[a] |
| 1 (n = 58) | -0.07 (0.04) | -0.12 (0.15) | -0.35 (0.22) | -0.03 (0.18) | 0.92 (0.21)[b] | 0.56 (0.07)[b] | 0.36 (0.18)[b] |
| P value[3] | < 0.01[4] | < 0.001[4] | < 0.01[4] | 0.95 | < 0.001 | < 0.001 | < 0.001 |

[1]The number below the β-κ-CN haplotype indicates the number of copies of the haplotype a cow carries (0,1 or 2).
[2]Casein index: 100*($\alpha_{S1}$-CN + $\alpha_{S2}$-CN + β-CN + κ-CN) / ($\alpha_{S1}$-CN + $\alpha_{S2}$-CN + β-CN + κ-CN + α-LA+ β-LG).
[3]When P value < 0.01, superscripts were used to indicate which number of copies of a haplotype differ (P < 0.05).
[4]Only the group with unknown haplotypes (not in table) differed (P < 0.05).

## 3.4 Discussion

### 3.4.1 Frequencies

Allele frequencies were within the range of allele frequencies of other studies in Holstein Friesian cattle (Lunden et al., 1997; Bobe et al., 1999b). The allele frequencies of β-LG, β-CN, and κ-CN in the Dutch Holstein Friesian population have changed over the last 16 years. The most remarkable change we found is the frequency of the κ-CN E variant in 2005. This variant was not detected in the Dutch Holstein Friesian population in 1989. Possible reasons for this difference could be that the E variant was actually present but not recognized with the technique used in 1989 (Bovenhuis and Verstege, 1989) or that the E variant was introduced into the Dutch Holstein Friesian population in the past 16 years. The κ-CN E variant has been found to be associated with poor coagulation properties (Ikonen et al., 1999a; Hallen et al., 2007).
In the Netherlands, there has been no selection for specific protein variants in breeding programs; therefore, changes in allele frequencies could be caused by the association of specific variants with the applied selection criteria or by random drift. We show a large increase in the frequencies of β-CN $A^2$ and κ-CN B. These variants are associated with a higher protein yield and a higher protein percentage, respectively. Because protein yield is an important selection criterion, selection for these variants may have occurred.

The difference between the observed and expected haplotype frequencies clearly shows that in our population, the β-CN and κ-CN variants are in linkage disequilibrium. The close linkage between casein variants has been demonstrated before in the Dutch Holstein Friesian (Bovenhuis and van Arendonk, 1991) and in other cow populations (Ikonen et al., 2001; Boettcher et al., 2004).
The overall $r^2$ of 0.056 indicates that, in general, the effect of a specific casein locus can be attributed to a linked casein locus only to a small extent. However, the κ-CN E variant predominantly occurs with the β-CN $A^1$ variant, and the β-CN B variant predominantly occurs with the κ-CN B variant. Thus, for κ-CN E and β-CN B, it is hard to determine whether the observed effects are a result of the variant itself or are caused by the linked variant.



### 3.4.2 β-LG

In our study, the β-LG genotype was not associated with any milk production trait, and the literature concerning the effect of this genotype on milk production traits is not consistent. Some studies also have found no effect (Lunden et al., 1997; Ojala, et al., 1997), but other studies have (Bovenhuis et al., 1992; Ikonen et al., 1999b). We show that the A variant of β-LG is associated with a higher relative concentration of β-LG compared to the B variant. The favorable effect of the A variant on the relative concentration of β-LG that we identified is in agreement with other studies (Ng-Kwai-Hang and Kim, 1996; Lum et al., 1997).
The large effect of the β-LG variant on the β-LG concentration in milk might be explained by differences in expression of β-LG arising from polymorphisms in the promoter region that are in linkage disequilibrium with the protein variants (Wagner et al., 1994; Lum et al., 1997; Folch et al., 1999). Another explanation might be a post-transcriptional control, such as differences in stability of the mRNA between the A and B variant.

We showed that the genotype of β-LG explains 90% of the genetic variation in the relative concentration of β-LG. This result agrees with that from the study of Bobe et al. (1999b), who concluded that the β-LG genotype almost completely controls the proportion of β-LG in total milk protein.

The effect of the β-LG variants on relative concentrations of α-LA and the individual caseins is less well known. Our results show that the higher relative concentration of β-LG associated with the A variant is compensated by a lower relative concentration of all other milk proteins. This finding is also concordant with those of Bobe et al. (1999b), who concluded that the A allele of β-LG increased the proportion of β-LG at the expense of $\alpha_{S1}$-CN and β-CN and with those of Ng-Kwai-Hang et al. (1987), who found that the A variant was associated with lower concentrations of α-LA, $\alpha_S$-CN ($\alpha_{S1}$-CN + $\alpha_{S2}$-CN), κ-CN, and β-CN. The actual mechanism causing the effect of the β-LG variant on the concentrations of the other milk proteins is unknown. However, the observed strong effect on the relative concentrations of all proteins combined with the absence of an effect on the protein percentage suggests that the 6 major milk protein genes are co-regulated (Bobe et al., 1999a).

### 3.4.3 β-CN

We show that the β-CN genotype is associated with milk yield and protein yield. Similar effects of the β-CN genotype on milk production traits have been reported in other studies (Bovenhuis et al., 1992; Ikonen et al., 1999b; Ikonen et al., 2001; Boettcher et al., 2004).

The β-CN $A^1$ variant was associated with higher relative concentrations of $\alpha_{S1}$-CN and κ-CN and lower relative concentrations of β-CN and $\alpha_{S2}$-CN compared to the $A^2$ variant. The association of the β-CN B variant with a higher relative κ-CN concentration can be explained by the observation that this variant predominantly occurs with the B variant of κ-CN, which has a favorable effect on the relative κ-CN concentration. The effects of the β-CN alleles on α-LA, β-LG, $\alpha_{S1}$-CN, and β-CN concentrations did not seem to be additive. The heterozygous $A^1A^2$ animals had lower α-LA, β-LG, and $\alpha_{S1}$-CN and higher β-CN concentrations in their milk than homozygous $A^1A^1$ or $A^2A^2$ animals. This phenomenon, called dominance, is known in livestock and plant breeding and has been reported before in association with β-CN variants (Graml and Pirchner, 2003).



### 3.4.4 κ-CN

We show that the κ-CN genotype is associated with protein percentage. Similar effects of the κ-CN genotype on milk production traits have been reported in other studies (Bovenhuis et al., 1992; Ikonen et al., 1999b; Ikonen et al., 2001; Boettcher et al., 2004). We show that the κ-CN B variant has a favorable effect on the κ-CN concentration, which is consistent among studies (Ng-Kwai-Hang et al., 1987; Van Eenennaam and Medrano, 1991; Bobe et al., 1999b). The effect of the A and B variants of κ-CN on the other milk proteins is less well known. Ng-Kwai-Hang et al. (1987) found just as we did that the B variant was associated with a lower α-LA concentration, but Bobe et al. (1999b) reported no effect on α-LA. The higher $\alpha_{S2}$-CN and lower $\alpha_{S1}$-CN associated with the B variant of κ-CN partly agrees with the results of Bobe et al. (1999b), who found no effect on $\alpha_{S2}$-CN but a similar effect on $\alpha_{S1}$-CN. The results concerning the effects of the κ-CN variants on the relative concentration of κ-CN and β-CN should be taken with consideration in mind.

With the CZE technique used, we were only able to estimate the relative concentration of the non-glycosylated mono-phosphorylated κ-CN. When phosphorylation or glycosylation patterns differ for the κ-CN variants, conclusions might not be valid for the total amount of κ-CN. Coolbear et al. (1996) showed that the B variant is equally phosphorylated but more highly glycosylated compared to the A variant. Therefore, the positive effect of the B variant on the total amount κ-CN might even be larger than the effect on κ-CN-1P reported in Table 5b. The glycosylated and multi-phosphorylated κ-CN partly co-migrated with β-CN. Therefore, variation in the relative concentrations of these κ-CN forms influence the relative concentration of β-CN.

The effect of κ-CN E on technological properties of milk has been reported before (Ikonen et al., 1999a; Lodes et al., 1996; Hallen et al., 2007). This study, however, is the first to report the effect of the κ-CN E variant on milk protein composition. The E variant had an effect on the relative κ-CN concentration that averaged between the effects of the A and B variants. Therefore, the association found by Ikonen et al. (1999a) and Hallen et al. (2007) with poor coagulation properties of the κ-CN E variant would most likely not have been caused by a difference in protein composition. We show that cows with the κ-CN BB genotype have approximately 20% more κ-CN than cows with the AA genotype. The genotype of κ-CN explains only 29% of the total genetic variation in κ-CN. This finding suggests that there are polymorphisms other than those in the κ-CN gene that influence the relative κ-CN concentration. Locating these genes offers great opportunities to substantially change the relative κ-CN concentration through selective breeding.

### 3.4.5 Casein haplotypes

We show that casein haplotypes are associated with milk production traits and milk protein composition. Similar effects of casein haplotypes on milk production traits have been reported in other studies (Ikonen et al., 2001; Boettcher et al., 2004). The effects of casein haplotypes on specific milk protein composition has not been reported before. Comparing the effects of casein haplotypes with the effects of single casein variants can provide better insight into what really underlies the effect of a variant on protein composition. For example, in our population, the E variant of κ-CN is associated with a lower relative $\alpha_{S2}$-CN concentration. The haplotype frequencies show that the κ-CN E variant predominantly occurs with the β-CN $A^1$ variant. The other haplotypes containing the $A^1$ variant ($A^1A$ and $A^1B$) are also associated with a lower relative concentration of $\alpha_{S2}$-CN. These results suggest that the effect of κ-CN E on the relative $\alpha_{S2}$-CN concentration is not caused by the E variant itself but by the β-CN $A^1$ variant. Differences in linkage disequilibrium, i.e. haplotypes, between cow populations or breeds might therefore cause different associations of single variants with milk composition.

### 3.4.6 Impact

We have shown that genetic variants and casein haplotypes have a major impact on the protein composition of milk and explain a considerable part of the genetic variation in milk protein composition. Our results emphasize the need to monitor the distribution of milk protein variants in a cow population. The frequencies of the different genetic variants in the Dutch Holstein Friesian population have changed over the past 16 years. This change in frequencies might have affected the protein composition and technological aspects of the milk. The large effect of genetic variants on protein composition also indicates that selection for specific variants or haplotypes can be a useful tool in selecting for cows that produce milk with a desired protein composition.

Cheese production is of great importance for the Dutch dairy industry, using almost 50% of the milk produced in the Netherlands. Previous studies have shown that the B variant of β-LG increases casein concentration (Lunden et al., 1997) and cheese yield (Schaar et al., 1985; Van Den Berg et al., 1992; Wedholm et al., 2006). Our results show that the B variant reduces the relative concentration of β-LG and increases the relative concentrations of α-LA, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, and κ-CN. Selection for the β-LG B variant will result in milk with a very different whey composition and a higher casein concentration but with a similar casein composition. This similar casein composition implies that cheese quality is most likely not influenced by selection for the B variant. Therefore, selection for cows with the B variant of β-LG will improve cheese production without negatively affecting cheese properties.

Several studies have shown that the B variant of κ-CN is associated with favorable cheese properties (Schaar et al., 1985; Van den Berg et al., 1992; Walsh, et al., 1998; Marziali and Ng-Kwai-Hang, 1986). We have shown that the B variant is associated with increased κ-CN concentration but also with increased $\alpha_{S2}$-CN concentration and a decreased $\alpha_{S1}$-CN concentration. It is not clear whether the favorable cheese properties of the κ-CN B variant are a result of the increased κ-CN concentration only, the changed concentration of the other caseins, or a change in micelle properties. We also show that the β-CN variant has an effect on casein composition. At the moment, knowledge is scarce about the effect of casein composition on the technological properties of milk. However, the favorable effect of the $A^2$ allele of β-CN on protein yield makes it a good candidate in selecting for cows to produce milk for cheese production. We conclude that selection for both the β-LG genotype B and the β-κ-CN haplotype $A^2B$ will result in cows that produce milk that is more suitable for cheese production.

# 4

# Characterization of milk fatty acids based on genetic and herd parameters

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## Abstract

The objective of this study was to characterize the fatty acids (FA) in milk based on genetic and herd parameters to investigate the origin of the different FA in milk. Milk samples of 1912 Dutch Holstein-Friesian cows were analyzed on 39 different FA including odd and branched-chain fatty acids. The proportion of variation caused by genetic and herd effects was calculated. In addition, genetic and herd correlations among the fatty acids were estimated and a clustering technique was used to visualise these correlations. The results indicated that in Dutch milk C12:0 is not completely synthesised de novo but also partly blood derived. It was hypothesised that C20:0 in milk is formed from the action of elongase enzymes on C18:0 and that the odd-chain FA C5:0-C13:0 and a part of C15:0 and C17:0 are synthesised de novo while the other part of C15:0 and C17:0 is blood derived. Furthermore, this work gives an overview of the opportunities to change the concentration of individual FA both by breeding and feeding. It is clearly shown that the extent to which the individual FA can be changed varies greatly and is depending on the origin of the different FA in milk.

## 4.1 Introduction

Bovine milk contains around 3-5% fat which is mainly (± 98%) composed of triglycerides. The triglycerides are composed of three fatty acids (FA) esterified to a glycerol backbone. More than 400 different FA have been found in milk (Jensen, 2002). Most of these FA occur in trace amounts and only about twelve of them are present in concentrations higher than 1% of milk fat. The FA composition of milk is important because it influences the nutritional value (Mensink et al., 2003) and product properties (Walstra, 2006) of fat based dairy products. Therefore, changing the FA composition could result in milk fat with additional value for the dairy industry. Furthermore, the FA composition in milk is also of interest because it might be used as an indicator of the metabolic status of a cow (Stoop et al., accepted) or as an indicator of rumen bacterial metabolism (Vlaeminck et al., 2006a). Thus, in view of the improvement of value for the dairy industry as well as the potential use as indicator of metabolism, it is important to have knowledge about the origin of the individual FA in milk.

The FA in milk are of dual origin being de novo synthesised or blood derived. In general, the FA C4:0 – C14:0 and a part (±50%) of C16:0 are synthesised de novo in the mammary gland, and the other part of C16:0 and the longer chained FA are derived from the blood (Barber et al., 1997, Craninx et al., 2008). Within these two groups a further sub division can be made. In addition to the even-chain saturated FA, milk also contains odd-chain saturated FA. Odd-chain FA can be synthesised the novo in the same way as the even-chain saturated FA but with propionate (C3) instead of acetate and β-hydroxybutyrate as a precursor (Craninx et al., 2008, Vlaeminck et al., 2006a). The contribution of the de novo synthesised odd-chain FA to the total amount of odd-chain FA in milk is not known. The branched-chain FA and a part of the odd-chain longer chained FA C15:0 and C17:0 found in milk are believed to be largely products of rumen bacteria (Vlaeminck et al., 2006a). A large part of the cis 9 unsaturated FA in milk are produced by the action of the stearoyl-CoA desaturase (SCD) enzyme on both de novo synthesised as well as blood derived FA (Craninx et al., 2008, Schennink et al., 2008, Soyeurt et al., 2008). This shows that there are different metabolic pathways through which the FA can enter the milk. Although much is known about the pathways involved in the synthesis of the different FA in milk, the origin of some FA is still under debate. This is especially true for some minor FA that have not been studied in much detail before. To get insight in the origin of the FA in milk it is possible to analyze milk FA composition data with statistical techniques. It has been shown in previous studies that metabolic pathways of fatty acid synthesis can be elucidated with clustering techniques (Massart-Leen and Massart, 1981) and principal component analysis (Fievez et al., 2003). In these studies, correlations among fatty acids were used to investigate the origin of branched- chain fatty acids in goat milk (Massart-Leen and Massart, 1981) and the origin of heptadecenoic and conjugated linoleic acids in bovine milk (Fievez et al., 2003). These studies have focused on a limited number of different FA and used a low number of animals. Furthermore, the design of these studies did not enable the determination of genetic correlations among FA. Studying genetic correlations and correlations due to environmental factors like herd helps to get insight how fatty acid synthesis is affected by genetic and nutritional factors. The aim of this study is to look for patterns in genetic and herd parameters of 39 different FA including odd and branched-chain FA in milk samples of 1912 Dutch Holstein Friesian heifers, to investigate the origin of the different FA milk.

## 4.2 Materials and methods

### 4.2.1 Animals

Morning milk samples and blood samples were collected from 1912 first-lactation Holstein-Friesian cows from 398 commercial herds in the Netherlands. From February to March 2005, a 0.5-L milk sample was collected from each cow at one morning milking and preserved with 0.03% w/w sodium azide. At least three cows per herd were sampled, and cows were milked twice a day. Cows descended from one of fifty young bulls (848 cows), from one of five proven bulls (873 cows), or from other proven bulls (191 cows). The NRS (Arnhem, the Netherlands) provided the pedigrees of the cows and the milk yield records. Each cow was over 87.5% Holstein Friesian and was in lactation between Day 66 and Day 247.

### 4.2.2 Sample analysis

Milk FA composition was measured by gas chromatography at the COKZ laboratory (Netherlands Controlling Authority for Milk and Milk Products, Leusden, the Netherlands) as described by Schennink et al. (2007). The FA were expressed as weight-proportion of total fat weight. FA unsaturation indices, viz C10 index, C12 index, C14 index, C16 index, C17 index, C18 index and CLA index were used as proxies for the Δ9 desaturase activity in the mammary gland and were calculated from the ratio between the product and the sum of product and precursor FA as described by Schennink et al. (2008). The branched-chain FA C15:0 iso could not be quantified due to overlap with another FA peak in the chromatogram and was therefore not reported. The cis double bond of C10:1 and C12:1 could not be ascertained at the carbon 9 position and therefore these FA were reported as C10:1 and C12:1. For C5:0, C7:0 and C9:0 the concentration could not be determined in a number of samples (n = 262, 310 and 110 respectively). If a concentration was not determined it was treated as a missing value in the statistical analyses. The FA were grouped into 5 groups based on general knowledge about their synthesis; (1) even-chain saturated FA, (2) odd-chain saturated FA, (3) branched- chain FA, (4) C18FA and (5) cis 9 unsaturated FA. Results on 16 of the major FA have been published previously (Schennink et al., 2008; Stoop et al., 2008). Here we will additionally report parameters for 23 FA that occur in lower concentrations.



### 4.2.3 Statistical analysis

Variance components were estimated using an Animal Model in ASReml (Gilmour et al., 2002):

$$y_{ijklmno} = \mu + b_1*dim_i + b_2*e^{-0.05*dim} + b_3*afc_j + b_4*afc_j^2 + season_k + scode_l + herd_m + A_n + e_{ijklmn}$$

where y was the dependent variable; μ was the general mean; dim was the covariate describing the effect of days in milk modeled with a Wilmink curve (Wilmink, 1987); afc was the covariate describing the effect of age at first calving, a linear and a quadratic term were fitted; season was the fixed effect of the class of calving season (June to August 2004, September to November 2004, or December 2004 to February 2005); scode was the fixed effect of the difference in genetic level between groups of proven bull daughters and young bull daughters; herd was the random effect of groups of animals sampled in the same herd; An was the random additive genetic effect of animal n; and e was the random residual effect.

Proportion of variance due to herd reflects the relative importance of herd effects such as feed, hygiene, and management, and was estimated as:

$$\%herd = \frac{\sigma^2_{herd}}{\sigma^2_{herd} + \sigma^2_A + \sigma^2_e} * 100\%$$

Where $\sigma^2_{herd}$ = herd variation, $\sigma^2_A$ = additive genetic variation and $\sigma^2_e$ = the residual variation.

Proportion of the variance due to genetic effects was estimated as:

$$\%genetic \frac{\sigma^2_A}{\sigma^2_{herd} + \sigma^2_A + \sigma^2_e} * 100\%$$

Note that %genetic differs from the intraherd heritability as defined by Stoop et al. (2008) and Schennink et al. (2007; 2008) .
Herd correlations were estimated using bivariate analyses and model 1 as:

$$r_{herd} = \frac{\sigma_{herd1,herd2}}{\sqrt{\left(\sigma^2_{herd1} * \sigma^2_{herd2}\right)}}$$

Where $\sigma_{herd1,herd2}$ = herd covariance between trait 1 and trait 2, and $\sigma^2_{herd1}$ and $\sigma^2_{herd2}$ = herd variance of trait 1 and 2.

Genetic correlations were estimated using bivariate analyses and model 1 as:

$$r_g = \frac{\sigma_{A1,A2}}{\sqrt{\left(\sigma^2_{A1} * \sigma^2_{A2}\right)}}$$

Where $\sigma_{A1,A2}$ = additive genetic covariance between trait 1 and trait 2, and $\sigma^2_{A1}$ and $\sigma^2_{A2}$ = additive genetic variance of trait 1 and 2.

A principal component analysis PROC VARCLUS, in combination with PROC TREE in SAS (SAS 9.1, SAS Institute, 1999) was used to graphically visualize genetic and herd correlations among the FA.

## 4.3 Results

### 4.3.1 Means and coefficients of variation

Mean and coefficients of variation of the different FA, expressed as weight percentage of the total FA, and the unsaturation indices are presented in Table 1. The 39 different FA together comprise 97% of the total FA. The other 3% consists of a large number (> 100) of FA that occur in very low concentrations and that were not identified. Only 13 FA occur in concentrations higher than 1% of the fat. The concentrations of the different FA are in line with previously reported concentrations of FA in bovine milk on non-pasture diets (Jensen, 2002, Moate et al., 2007, Vlaeminck et al., 2006a). The FA C4:0, C6:0 and C14:0 and C16:0 have the lowest CV (7-9%) and C18:1 trans 10 has the highest CV (86%). The high CV for C18:1 trans 10 is caused by a small number of milk samples (n = 12) with extreme high concentrations of this FA (between 1 - 5%). If these 12 samples are excluded, the CV for C18:1 trans 10 is 42%. Such high concentrations of this specific FA have been reported frequently in conditions causing milk fat depression (Baumgard et al., 2001, Loor et al., 2005).

**Table 1:** Mean (g/100g fatty acids) and coefficients of variation for 39 FA in milk samples of 1912 HF cows.

| Fatty acid | Mean | CV(%) | Fatty acid | Mean | CV(%) |
|---|---|---|---|---|---|
| ***Even-chain saturated (1)*** | | | ***Even-chain saturated (1)*** | | |
| C4:0 | 3.50 | 8 | C12:0 | 4.11 | 17 |
| C6:0 | 2.23 | 7 | C14:0 | 11.62 | 8 |
| C8:0 | 1.37 | 10 | C16:0 | 32.61 | 9 |
| C10:0 | 3.03 | 14 | | | |
| ***Odd-chain saturated (2)*** | | | ***Odd-chain saturated (2)*** | | |
| C5:0 | 0.03 | 36 | C13:0 | 0.11 | 42 |
| C7:0 | 0.04 | 41 | C15:0 | 1.18 | 18 |
| C9:0 | 0.05 | 45 | C17:0 | 0.46 | 13 |
| ***Branched-chain (3)*** | | | ***Branched-chain (3)*** | | |
| C14:0 iso | 0.09 | 23 | C17:0 iso | 0.30 | 12 |
| C15:0 ante iso | 0.48 | 13 | C17:0 ante iso | 0.59 | 12 |
| C16:0 iso | 0.21 | 19 | | | |
| ***C18FA (4)*** | | | ***C18FA (4)*** | | |
| C16:1 trans 9 | 0.05 | 29 | C18:1 trans 10 | 0.23 | 86 |
| C18:0 | 8.73 | 16 | C18:1 trans 11 | 0.77 | 26 |
| C18:1 cis 11 | 0.41 | 27 | C18:1 trans 15 | 0.22 | 26 |
| C18:1 cis 12 | 0.20 | 30 | C18:2 cis 9,12 | 1.20 | 24 |
| C18:1 cis 13 | 0.09 | 30 | C18:3 cis 9,12,15 | 0.41 | 27 |
| C18:1 trans 4-8 | 0.21 | 24 | C20:0 | 0.13 | 18 |
| C18:1 trans 9 | 0.15 | 20 | | | |
| ***Cis 9 unsaturated (5)*** | | | ***Cis 9 unsaturated (5)*** | | |
| C10:1 | 0.37 | 18 | C17:1 cis 9 | 0.18 | 20 |
| C12:1 | 0.12 | 24 | C18:1 cis 9 | 18.02 | 12 |
| C14:1 cis 9 | 1.36 | 19 | C18:2 cis 9, trans 11 | 0.39 | 28 |
| C16:1 cis 9 | 1.44 | 22 | | | |
| ***Unsaturation indices*** | | | ***Unsaturation indices*** | | |
| C10 index | 10.9 | 17 | C17 index | 28.3 | 11 |
| C12 index | 2.7 | 20 | C18 index | 67.6 | 6 |
| C14 index | 10.5 | 17 | CLA index | 33.7 | 12 |
| C16 index | 4.2 | 19 | | | |

### 4.3.2 Variation due to genetic and herd effects

Figure 1 shows the proportion of the total variation explained by genetic and herd effects for the different FA and unsaturation indices in milk. Differences among herds most likely represent differences in feeding regimes among herds, but the effects of other management factors can not be excluded (Stoop et al., 2008). In general, the FA in the same groups (group 1-5) are to a similar extent affected by genetic and herd effects as illustrated by a comparable position in Figure 1 (dotted circles). The even-chain saturated FA have a high genetic effect (>30%) and a moderate herd effect (>15%). The odd-chain saturated and the branched-chain FA have a moderate to low genetic (<20%) and a moderate herd effect(>20%). The C18FA have a high herd effect (>30%) and a moderate to low genetic effect (<20%). Finally, some of the FA in the group of cis 9 unsaturated FA and all the unsaturation indices have a low herd effect (<10%) and a moderate to high genetic effect (>20%). Some FA have a clearly different position in Figure 1 than other FA from the same group. For example, C12:0 has a much higher herd effect than all the other even-chain saturated FA, C17:0 ante iso has a higher genetic effect than all the other branched-chain FA and C18:0 has a lower herd effect than all the other C18FA.

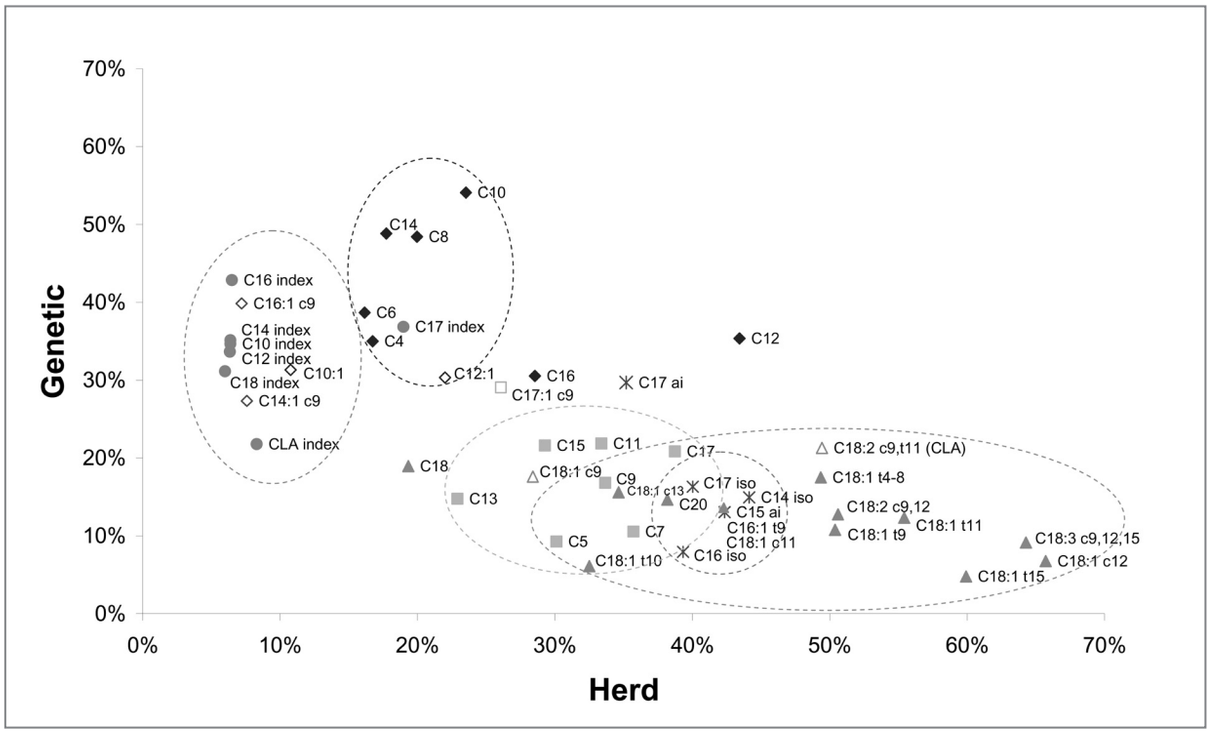


**Figure 1:** genetic and herd effects for FA composition (w/w%) in milk samples of 1912 Holstein Friesian cows. ◆, even-chain saturated FA (1); ■, odd-chain saturated FA (2); *, branched-chain (3); ▲, C18 FA (4); ◇, □, △, cis 9 unsaturated FA (5); ●, FA unsaturation indices;
Genetic = $(\sigma^2_A/(\sigma^2_A+\sigma^2_{herd}+\sigma^2_e))*100\%$, SE between 0.05 and 0.12.
Herd = $(\sigma^2_{herd}/(\sigma^2_A+\sigma^2_{herd}+\sigma^2_e))*100\%$, SE approximately 0.03.

### 4.3.3 Genetic and herd correlations

Genetic and herd correlations among the even and odd-chain saturated FA, among the branched-chain FA, among the C18FA, and among the unsaturation indices were calculated (in supplementary information). A cluster analysis was performed to visualise these genetic and herd correlations.

*Even and odd-chain FA*. Clustering based on correlations among herd effects of the even and odd-chain saturated FA shows that C6:0, C8:0, C10:0, C12:0 and C14:0 cluster to one group and C5:0, C7:0, C9:0, C11:0 and C13:0 cluster to another group (Figure 2). Furthermore, C15:0, C16:0 and C17:0 cluster together and C4:0 is more or less a separate group. Clustering based on genetic correlations shows C6:0, C8:0, C10:0, C12:0, C14:0 and C16:0 cluster together and C5:0, C7:0, C9:0, C11:0, C13:0, C15:0 and C17:0 cluster together. Again C4:0 is more or less a separate group.

*Branched-chain FA*. Herd clustering of the branched-chain FA shows that C14:0 iso and C16 iso cluster together, C15:0 ante iso and C17:0 iso cluster together and C17:0 ante iso is a separate

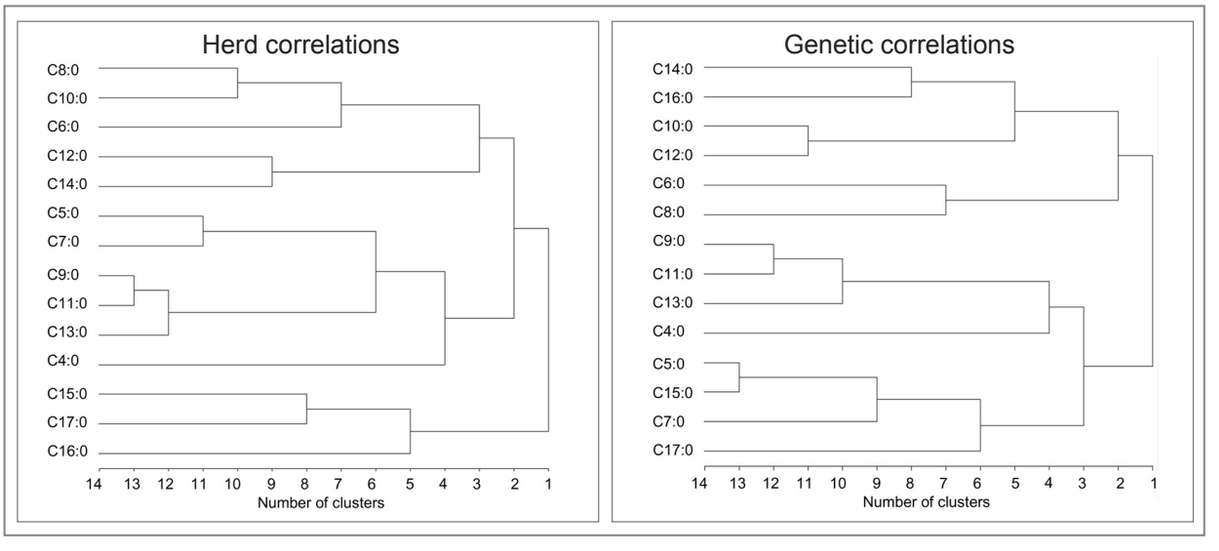


**Figure 2**: Cluster tree based on principal component analysis of herd and genetic correlations among the odd and even-chain saturated de novo synthesized FA.



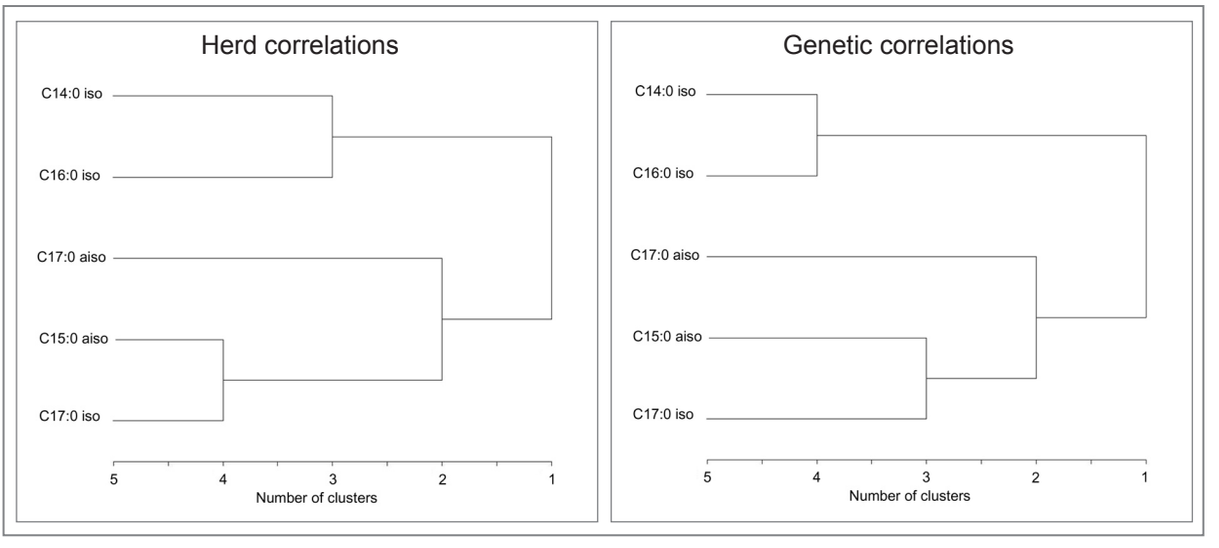


**Figure 3**: Cluster tree based on principal component analysis of herd and genetic correlations among the branched-chain FA.

group (Figure 3). Genetic clustering shows the same clusters as herd clustering.

*C18 FA.* Herd clustering of the C18FA shows that C18:1 trans 4-8, C18:1 trans 9, C18:1 trans 10, C18:1 trans 15 , C18:1 cis 11, C18:1 cis 12, C18:1 cis 13 and C18:2 cis 9,12 cluster together (Figure 4). Furthermore, C16:1 trans 9, C18:1 trans 11 and CLA cluster together and C18:0, C18:1 cis 9 and C20:0 cluster together. Genetic clustering shows that C18:0 and C20:0 clearly cluster together and C18:1 cis 9 is more or less a separate group.

Furthermore, C18:1 trans 4-8, C18:1 trans 10, C18:1 trans 15 , C18:1 cis 11 and C18:1 cis 13 cluster together and C16:1 trans 9, C18:1 trans 11, C18:1 cis 12, C18:2 cis 9,12, C18:3 cis 9,12, 15 and CLA cluster together.

*Unsaturation indices.* Herd Clustering of the unsaturation indices shows that the C18 index and CLA index cluster together and the C10 index, C12 index, C14 index and C16 index cluster together (Figure 5). The C17 index is a separate group. Genetic clustering shows the C18 index and CLA index cluster together and the C10 index, C12 index, C14 index cluster together. Furthermore the C16 index and C17 index cluster together.

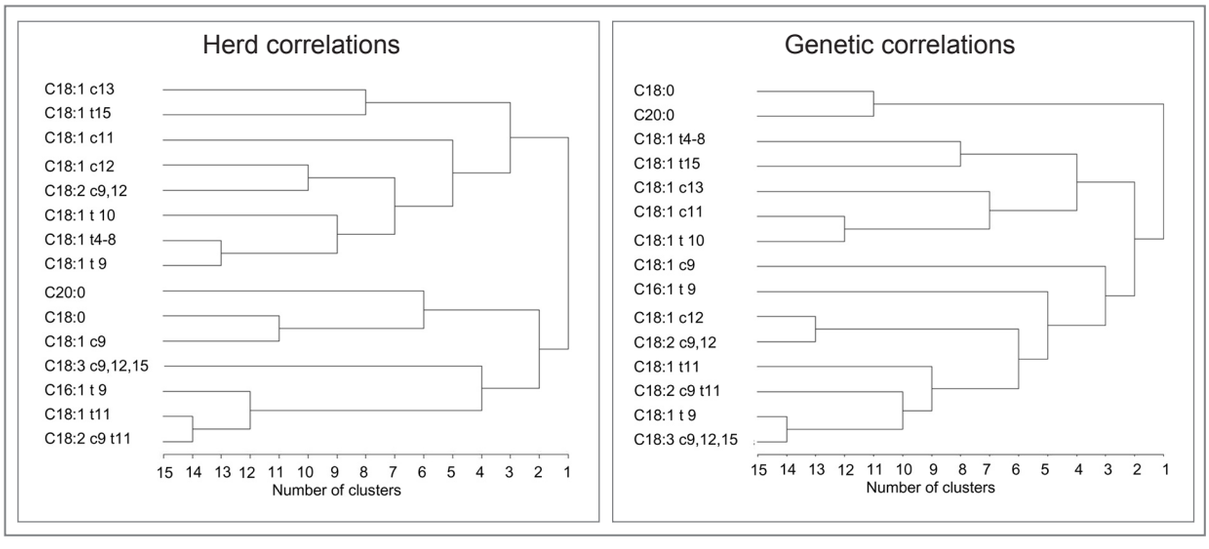


**Figure 4**: Cluster tree based on principal component analysis of herd and genetic correlations among the C18FA.



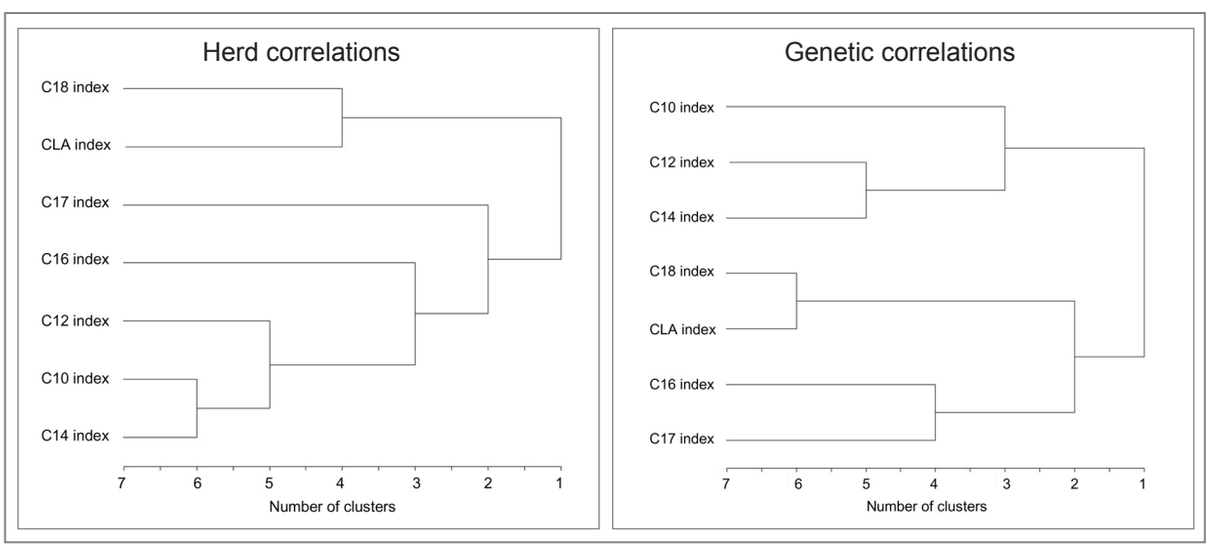


**Figure 5**: Cluster tree based on principal component analysis of herd and genetic correlations among the unsaturation indices.

## 4.4 Discussion

### 4.4.1 Even-chain saturated FA

The even saturated FA C4:0- C16:0 are synthesized de novo in the mammary gland by the multi enzyme acetyl-CoA carboxylase and fatty acid synthase complex (Barber et al., 1997). During this process acetate or butyrate produced by the rumen bacteria are elongated with 2 carbon units per cycle. This elongation does not proceed beyond a total of 16 carbon units but can be stopped earlier at any stage by the thioesterase enzyme, yielding FA with chain lengths between 4 and 16 carbon units.

The genetic and herd correlations indicate that C6:0, C8:0, C10:0, C12:0 and C14:0 cluster. This is in line with the described process about de novo synthesis. Figure 1 shows that genetic effects for the even-chain de novo synthesized FA are relatively high and herd effects are relatively low compared to all other FA. It was expected that genetic effects are higher for the novo synthesized FA than for the blood derived FA because their synthesis is highly dependent on the activity of different enzymes within the mammary gland and less depending on the composition of the fat in the diet. The de novo synthesized FA are synthesized mainly from acetate and butyrate which are produced in the rumen and hindgut by microorganisms. It is, however, unlikely that acetate and butyrate will be in short supply and limit de novo fatty acid synthesis and reduce milk fat content (Bauman and Griinari, 2000). However, the diet might contain specific FA or promote the formation of specific FA (e.g. C18:2 trans 10 cis 12) that inhibit the de novo FA synthesis (Baumgard et al. 2005). This could explain why the concentration of the even-chain de novo synthesized FA is also partly affected by herd. The herd and genetic correlations showed tight clustering between the FA that are only two carbon units apart. This might be related to the elongation with two carbon units at a time during the synthesis of the de novo synthesized FA. These high and positive genetic and herd correlations between de novo synthesized FA that are two carbon units apart are an important observation because they indicate that it might be difficult to increase the relative portion of for example C8:0 without increasing the relative proportion of C6:0 and C10:0 in milk fat both by selective breeding or feeding.



Our results show that C4:0, C12:0 and C16:0 differ from the general pattern of the other FA in the group of the de novo synthesized even-chain FA.

*C4:0.* Based on herd and genetic clustering butyric acid (C4:0) differs from the other FA. C4:0 is an unique FA because it does not, like all other the novo synthesized FA, require acetate for its production as it can be produced directly from β-hydroxybutyrate derived from the blood (Craninx et al., 2008). Changes in C4:0 concentration in milk FA upon a change in the diet have also been observed to differ from other FA shorter than 16 carbon atoms. For example, an increased concentrate:roughage ratio or the addition of DHA to the diet significantly decreased the concentration of saturated FA up to 14 carbon atoms, but not that of C4:0 (Bargo et al., 2006, Boeckaert et al., 2008). Furthermore, C4:0 is also unique because it is, unlike all other FA, esterified almost exclusively at the sn-3 position of the triacyl glycerol (Jensen, 2002). Although the exact mechanism is not known, these unique features in its synthesis might be related to the observed herd and genetic correlations with C4:0 and the other FA.

*C12:0.* Lauric acid (C12:0) deviates from the general pattern of the even-chain de novo synthesized FA because it has a much higher herd effect (Fig 1). This may be explained by the presence of considerable quantities of C12:0 in concentrates fed to Dutch dairy cows. In the Netherlands, concentrates are an important ingredient with on average 31% of DM in the diet in the winter (Van Bruggen, 2007). Ingredients like palm kernel expeller and extracted coconut are included in various Dutch concentrates and these ingredients contain a large amount of C12:0 (47% and 48% of the total FA, respectively) (CVB, 2007). The quantity and composition of the concentrates used by different farmers vary considerably explaining the relatively large herd effect for C12:0. It has been shown in previous studies that addition of C12:0 or C14:0 to the diet of cows will result in an increase of these FA in the secreted milk (Dohme et al., 2004, Odongo et al., 2007). The assumption that short chain FA like C12:0 are completely synthesized de novo (Craninx et al., 2008, Garnsworthy et al., 2006, Moate et al., 2008) is therefore unlikely to be valid for all the animals in the farms selected in the present study. This implies that feeding other FA that are considered to be fully synthesized de novo (e.g. C10:0, C8:0) could also result in an increase of these FA in milk.

*C16:0.* Palmitic acid (C16:0) has a lower genetic effect and a higher herd effect compared to the other even-chain de novo synthesized FA. Furthermore, it shows a negative genetic correlation to most other even-chain de novo synthesized FA (supplementary information). For example, the genetic correlation between C16:0 and C14:0 was - 0.87 while the herd correlation was + 0.12. This might be related to the origin of C16:0 in milk. C16:0 is considered to be partly synthesized de novo and partly derived from the blood (Craninx et al., 2008, Garnsworthy et al., 2006, Moate et al., 2008). This clear difference in herd and genetic correlations between C16:0 and C14:0 is important when one is interested in changing these FA in milk. The very high negative genetic correlation indicates that it will be difficult to decrease the concentration of both FA simultaneously by means of selective breeding. However, the low positive herd correlation suggest that C14:0 and C16:0 are almost independent and that by choosing the right feeding strategy it is possible to decrease both C14:0 and C16:0 in milk. Decreasing the relative proportion of C14:0 and C16:0 in milk fat simultaneously could be beneficial because both FA together comprise a main part (44%) of milk fat and increase the LDL cholesterol in humans (Mensink et al., 2003).



### 4.4.2 Odd-chain FA

Odd-chain saturated FA can be produced de novo in the mammary gland in the same manner as the even-chain FA but with propionate instead of acetate or butyrate as a precursor (Craninx et al., 2008, Vlaeminck et al., 2006a). The odd-chain FA C15:0 and C17:0 can also be produced by rumen bacteria (Craninx et al., 2008, Vlaeminck et al., 2006a). The contribution of the de novo synthesised odd-chain FA to the total amount of odd-chain FA in milk is not known.

The very high positive genetic correlations among odd-chain FA suggests that all odd-chain FA have a common origin. Furthermore, the positive genetic correlations among odd-chain and even-chain FA with a chain length form 7 to 14 (supplementary information) might indicate that these odd-chain and even-chain FA have a common origin. The genetic clustering clearly shows that the even and odd-chain saturated FA are separate groups. This agrees with the view that odd-chain

FA are synthesised de novo but with propionate instead of acetate as a precursor. Herd clustering showed that C15:0, C17:0 and C16:0 cluster together. C15:0, C17:0 and C16:0 are known to be both synthesised de novo and blood derived (Barber et al., 1997, Craninx et al., 2008, Vlaeminck et al., 2006a). These results suggest that it is likely that the odd-chain FA C5:0-C13:0 and a part of C15:0 and C17:0 are synthesised de novo and the other part of C15:0 and C17:0 is blood derived. This information is important when one is interested to use odd-chain FA as indicators of rumen bacteria. Although C5:0-C13:0 are not synthesised by the rumen bacteria they may be used as an indicator of the proportion of propionate available in the mammary gland.

### 4.4.3 Branched-chain FA

Branched-chain FA in milk are considered to originate for a large part from de novo production by rumen bacteria (Vlaeminck et al., 2006a). Three groups of branched-chain FA can be distinguished even-chain iso (C14:0 iso, C16:0 iso) odd-chain iso (C15:0 iso and C17:0 iso) and odd-chain ante iso (C15:0 ante iso C17:0 ante iso). These 3 groups are produced out of 3 different precursors; valine, leucine or isoleucine respectively (Vlaeminck et al., 2006a). Because different rumen bacteria have a different odd and branched-chain FA profile, it might be possible to use the odd and branched-chain FA profile in the milk as an indication of the relative abundance of specific bacterial populations in the rumen (Vlaeminck et al., 2006b).

The branched-chain FA have low genetic effect and a moderate herd effect. When these FA are for the major part synthesized by the rumen bacteria it is expected that genetic background of the cow is less important than the feed of the cow. Herd clustering showed that the even branched-chain FA C14:0 iso and C16:0 iso cluster together. Some of the predominant cellulolytic rumen bacteria contain relative high levels of C14:0 iso and C16:0 iso (Vlaeminck et al., 2006a). Therefore, feeding conditions that promote these kind of bacteria could result in an increase of both FA in milk. The FA C17:0 ante iso differs from the general pattern of the branched-chain FA. Because this FA shows a higher genetic effect (Fig 1) and forms a separate group both in the genetic and herd clustering. This might indicate that this FA has a different origin than the other branched-chain FA. It has been reported by Vlaeminck et al. (2006a) that the secretion of this FA into the milk was higher compared with its duodenal absorption. Therefore, we hypothesize that C17:0 ante iso can also be synthesized de novo. This agrees with the high genetic affect observed in our study. If C17:0 ante iso can be synthesized de novo this should be taken into account when it is used as an indicator of rumen bacteria.



### 4.4.4 C18 FA

The fatty acids with a chain length of 18 carbon units or longer are considered to be blood derived as they can not be synthesized de novo by the cow. These C18 FA mainly originate from the feed of the cow. The FA in dairy cattle diets are predominantly polyunsaturated FA (mainly C18:2 cis 9,12 and C18:3 cis 9, 12, 15). These unsaturated FA are for a large part biohydrogenated by the rumen bacteria to saturated C18:0. During this process several intermediates, mainly trans FA, are formed. A small part of the polyunsaturated FA from the feed and a small part of the intermediates are not completely hydrogenated to C18:0 and end up in the milk.

In general our results agree with this view of the synthesis of the C18 FA. Genetic effects are low and herd effects are high which is expected for FA that mainly originate from the feed. Grass and maize silage are the main forages for Dutch dairy cattle in the winter period (Van Bruggen, 2007). The relative contribution of these silages to the diet of Dutch cows varies greatly for different farms. The major FA in grass silage is C18:3 cis 9,12,15 and in maize silage C18:2 cis 9,12 is the most abundant FA (Kliem et al , 2008; Shingfield et al., 2005). Herd clustering showed that C18:3 cis 9, 12, 15 clustered together with a different group of FA than C18:2 cis 9, 12. Possibly, C18:3 cis 9, 12, 15 and the FA that cluster in this group (C16:1 trans 9, C18:1 trans 11 and CLA) are the FA that are associated with the feeding of grass silage and C18:2 cis 9, 12 and the FA in this cluster are associated with the feeding of maize silage. Herd clustering shows that C16:1 trans 9 clustered together with C18:1 trans 11 and CLA. This suggests that C16:1 trans 9 is formed during rumen conditions which also promotes the concentration of C18:1 trans 11 and CLA in milk.

Some results differ from the general pattern in the group of C18FA. Genetic correlations among the C18FA were all positive except for C18:0 and C20:0, which were negatively correlated to most other FA and strongly positive (+ 0.91) with each other. This high genetic correlation might be related to a possible origin of C20:0 in milk. C20:0 is believed to be formed by the action of elongase enzymes on C16:0 and C18:0 (Leonard et al., 2004, Moate et al., 2008). However, until now there has been no evidence for this hypothesis about the origin of C20:0 in milk. The very high genetic correlation between C18:0 and C20:0 reported in this study suggest that C20:0 can indeed be formed by the action of elongase enzymes on C18:0. It has not been reported that chain elongation occurs in the mammary gland of the cow. However, if C20:0 is formed out of C18:0 at a different place in the cow it can still enter the milk after mobilization of body fat.

### 4.4.5 Cis 9 unsaturated FA

Animals are capable of desaturating saturated FA to cis 9 unsaturated FA by the SCD enzyme, which catalyzes the insertion of a double bond between carbon atoms 9 and 10 of a FA (Pereira et al., 2003). In the bovine mammary gland the enzyme desaturates a large spectrum of different FA and a large part of the unsaturated FA are the products of the action of this enzyme (Mele et al., 2007). Multiple studies have used the ratio of FA in milk that are substrate and product of the SCD enzyme as a measure for the desaturase activity (Kelsey et al., 2003, Lock and Garnsworthy, 2003, Soyeurt et al., 2008).



In general our results confirm this view about the origin of cis 9 unsaturated FA in milk. Genetic and herd correlations between the different unsaturation indices were all positive (supplementary information). If all the cis 9 FA in milk are, for a large part, the product of the action of the same SCD enzyme it is expected that the unsaturation indices are positively correlated. It has been shown before that it is likely that the SCD enzyme is capable of deasturating C10:0, C12:0, C14:0, C16:0, C18:0 , C18:1 trans 11 (Schennink et al., 2008) and C17:0 (Fievez et al., 2003,). Next to these FA other FA in milk exist on which SCD might potentially work (e.g., C15:0, C18:1 trans 15, C20:0). Therefore, it is well possible that desaturase products of these potential substrates exist in milk.

The low herd and medium genetic effect for the unsatruration indices suggests that, for the activity of the SCD enzyme, genetic factors are more important than the diet of the cow. However, for the concentration of a product of the enzyme (e.g. CLA), the diet of the cow can be more important than genetic factors because the diet can have a major influence on the amount of substrate (e.g. C18:1 trans 11) available to the SCD enzyme. The low herd effect for the CLA index indicates that an increase in CLA as a result of changed feeding conditions will most likely be accompanied by an equal relative increase of the amount of C18:1 trans 11. This is also clearly reflected by a very high herd correlation between CLA and C18:1 trans 11 (+ 0.97).

Although all unsaturation indices show a positive genetic correlation, the size of the genetic correlations differs and genetic clustering clearly shows two groups of unsaturation indices. The C10, C12 and C14 index cluster together and the C16, C17 and C18 and CLA index cluster together. Is has been shown by Schennink et al. (2008) that the existence of these two groups on basis of genetic correlations can be explained by the SCD A293V polymorphism. It was hypothesized that this polymorphism affected the substrate specificity of SCD. In the study of Schennink et al. (2008) the C17 index was not taken into account. We show that the C17 index clusters together with the C16, C18 and CLA index. This agrees with the hypothesis that that the SCD A293V polymorphism alters specificity of SCD from shorter to longer chain FA.

## 4.5 Conclusions

Studying the genetic and herd parameters gave new insights in the possible origin of FA in milk. We show that it is likely that in Dutch milk C12:0 is not completely synthesised de novo but also partly blood derived. We hypothesise that C20:0 in milk is formed from the action of elongase enzymes on C18:0. We also show that it is likely that the odd-chain FA C5:0-C13:0 and a part of C15:0 and C17:0 are synthesised de novo and the other part of C15:0 and C17:0 is blood derived. Statistical analyses of milk FA composition data can not provide definite answers about the origin of FA in milk. However, it helps to form a hypothesis that can be tested in further, more controlled, studies. Furthermore, this work gives an overview of the opportunities to change the concentration of individual FA both by breeding and feeding. It is clearly shown that the extent to which the individual FA can be changed varies greatly and is depending on the origin of the different FA in milk.

**Table 2:** herd (above diagonal) and genetic (below diagonal) correlations among odd and even-chain saturated FA in milk samples of 1912 Holstein Frisian cows.

| Trait | C4:0 | C5:0 | C6:0 | C7:0 | C8:0 | C9:0 | C10:0 | C11:0 | C12:0 | C13:0 | C14:0 | C15:0 | C16:0 | C17:0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C4:0 | | -0.22[1] | 0.52[1] | -0.41[1] | -0.06[1] | -0.50[1] | -0.32[1] | -0.55[1] | -0.29[1] | -0.53[1] | -0.37[1] | -0.39[1] | 0.04[1] | -0.10[1] |
| C5:0 | -0.16[2] | | 0.25[1] | 0.97[1] | 0.41[1] | 0.75[1] | 0.53[1] | 0.69[1] | 0.18[1] | 0.73[1] | 0.22[1] | 0.48[1] | 0.12[1] | 0.23[1] |
| C6:0 | 0.48[2] | -0.15[3] | | 0.19[1] | 0.78[1] | 0.26[1] | 0.58[1] | 0.22[1] | 0.07[1] | 0.21[1] | 0.20[1] | -0.05[1] | 0.13[1] | -0.01[1] |
| C7:0 | -0.24[2] | 0.92[1] | -0.18[3] | | 0.56[1] | 0.90[1] | 0.71[1] | 0.87[1] | 0.30[1] | 0.90[1] | 0.37[1] | 0.56[1] | 0.05[1] | 0.24[1] |
| C8:0 | -0.05[2] | -0.02[3] | 0.82[1] | 0.17[2] | | 0.70[1] | 0.93[1] | 0.67[1] | 0.31[1] | 0.64[1] | 0.51[1] | 0.21[1] | 0.00[1] | 0.04[1] |
| C9:0 | -0.55[2] | 0.90[1] | -0.08[2] | 1.00[1] | 0.33[2] | | 0.84[1] | 0.99[1] | 0.41[1] | 0.98[1] | 0.47[1] | 0.57[1] | 0.01[1] | 0.19[1] |
| C10:0 | -0.40[2] | 0.12[3] | 0.55[2] | 0.37[2] | 0.92[1] | 0.52[2] | | 0.83[1] | 0.47[1] | 0.79[1] | 0.67[1] | 0.34[1] | 0.04[1] | 0.06[1] |
| C11:0 | -0.59[2] | 0.80[1] | -0.04[2] | 0.98[1] | 0.40[2] | 0.99[1] | 0.60[2] | | 0.45[1] | 0.99[1] | 0.49[1] | 0.60[1] | 0.03[1] | 0.20[1] |
| C12:0 | -0.56[2] | 0.11[2] | 0.34[2] | 0.49[2] | 0.81[1] | 0.65[2] | 0.96[1] | 0.72[2] | | 0.39[1] | 0.79[1] | -0.04[1] | -0.06[1] | -0.53[1] |
| C13:0 | -0.52[2] | 0.73[2] | 0.19[3] | 0.91[1] | 0.60[2] | 0.96[1] | 0.73[2] | 0.97[1] | 0.82[1] | | 0.50[1] | 0.60[1] | 0.03[1] | 0.30[1] |
| C14:0 | -0.49[2] | 0.08[3] | 0.08[2] | 0.20[2] | 0.50[2] | 0.33[2] | 0.72[1] | 0.43[2] | 0.76[1] | 0.62[2] | | 0.12[1] | 0.12[1] | -0.22[1] |
| C15:0 | -0.40[2] | 0.99[1] | -0.36[2] | 0.98[1] | -0.16[2] | 0.86[1] | 0.04[2] | 0.81[1] | 0.20[2] | 0.75[2] | 0.12[2] | | 0.33[1] | 0.70[1] |
| C16:0 | 0.52[2] | -0.16[3] | 0.15[2] | -0.31[3] | -0.33[2] | -0.30[2] | -0.64[2] | -0.36[2] | -0.67[2] | -0.42[3] | -0.84[1] | -0.10[3] | | 0.32[1] |
| C17:0 | -0.33[2] | 0.82[2] | -0.58[2] | 0.64[2] | -0.52[2] | 0.45[1] | -0.34[2] | 0.41[2] | -0.18[2] | 0.19[3] | -0.19[2] | 0.70[2] | -0.06[3] | |

[1]SE <0.1
[2]SE between 0.1 and 0.2
[3]SE between 0.2 and 0.3

**Table 3:** Herd (above diagonal) and genetic (below diagonal) correlations among the C18FA in milk samples of 1912 Holstein Frisian cows.

| Trait | C16:1 trans 9 | C18:0 | C18:1 cis 9 | C18:1 cis 11 | C18:1 cis 12 | C18:1 cis 13 | C18:1 trans 4-8 | C18:1 trans 9 | C18:1 trans 10 | C18:1 trans 11 | C18:1 trans 15 | C18:2 cis 9,12 | C18:2 cis 9, trans 11 | C18:3 cis 9,12,15 | C20:0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C16:1 trans 9 | | 0.27[1] | 0.45[1] | -0.30[1] | -0.17[1] | 0.00[1] | 0.08[1] | 0.09[1] | -0.03[1] | 0.88[1] | 0.26[1] | -0.06[1] | 0.89[1] | 0.38[1] | 0.19[1] |
| C18:0 | -0.48[3] | | 0.82[1] | -0.11[1] | 0.21[1] | 0.02[1] | 0.36[1] | 0.34[1] | 0.04[1] | 0.42[1] | 0.25[1] | 0.08[1] | 0.32[1] | 0.00[1] | 0.48[1] |
| C18:1 cis 9 | 0.30[3] | -0.41[3] | | 0.13[1] | 0.13[1] | 0.21[1] | 0.41[1] | 0.44[1] | 0.18[1] | 0.51[1] | 0.17[1] | 0.12[1] | 0.52[1] | 0.09[1] | 0.43[1] |
| C18:1 cis 11 | 0.67[2] | 0.02[3] | 0.35[3] | | 0.39[1] | 0.26[1] | 0.50[1] | 0.47[1] | 0.42[1] | -0.26[1] | -0.17[1] | 0.43[1] | -0.19[1] | -0.35[1] | -0.03[1] |
| C18:1 cis 12 | 0.58[2] | -0.27[3] | 0.47[3] | 0.33[3] | | 0.42[1] | 0.77[1] | 0.74[1] | 0.60[1] | 0.12[1] | 0.57[1] | 0.77[1] | 0.09[1] | -0.18[1] | 0.06[1] |
| C18:1 cis 13 | 0.33[3] | -0.07[3] | 0.37[3] | 0.76[2] | 0.57[2] | | 0.49[1] | 0.60[1] | 0.54[1] | 0.16[1] | 0.57[1] | 0.28[1] | 0.18[1] | 0.05[1] | -0.30[1] |
| C18:1 trans 4-8 | 0.53[2] | 0.05[3] | 0.25[3] | 0.47[2] | 0.68[2] | 0.77[2] | | 0.95[1] | 0.82[1] | 0.33[1] | 0.43[1] | 0.68[1] | 0.33[1] | -0.18[1] | 0.14[1] |
| C18:1 trans 9 | 0.86[2] | -0.44[3] | 0.63[2] | 0.59[2] | 0.80[2] | 0.45[3] | 0.66[2] | | 0.79[1] | 0.38[1] | 0.46[1] | 0.64[1] | 0.38[1] | -0.15[1] | 0.10[1] |
| C18:1 trans 10 | 0.59[3] | -0.32[4] | 0.46[3] | 0.94[2] | 0.53[3] | 0.68[2] | 0.78[2] | 0.82[2] | | 0.16[1] | 0.38[1] | 0.58[1] | 0.16[1] | -0.07[1] | -0.10[1] |
| C18:1 trans 11 | 0.45[2] | 0.05[3] | 0.12[3] | 0.40[3] | 0.61[2] | 0.32[3] | 0.69[2] | 0.78[2] | 0.54[3] | | 0.31[1] | 0.17[1] | 0.97[1] | 0.31[1] | 0.20[1] |
| C18:1 trans 15 | 0.11[3] | 0.28[3] | -0.00[4] | 0.57[3] | 0.79[2] | 0.56[3] | 0.73[2] | 0.51[3] | 0.55[3] | 0.58[2] | | 0.34[1] | 0.29[1] | 0.33[1] | -0.11[1] |
| C18:2 cis 9,12 | 0.57[2] | -0.27[3] | 0.41[3] | 0.33[3] | 0.97[1] | 0.45[2] | 0.52[2] | 0.86[1] | 0.44[3] | 0.63[2] | 0.66[2] | | 0.20[1] | -0.20[1] | 0.02[1] |
| C18:2 cis 9, trans 11 | 0.67[2] | -0.58[2] | 0.54[2] | 0.35[3] | 0.69[2] | 0.34[2] | 0.55[2] | 0.87[1] | 0.55[3] | 0.76[1] | 0.29[3] | 0.68[2] | | 0.34[1] | 0.21[1] |
| C18:3 cis-9,12,15 | 0.73[2] | -0.36[3] | 0.62[2] | 0.55[3] | 0.89[2] | 0.65[2] | 0.73[2] | 0.99[1] | 0.73[3] | 0.94[1] | 0.50[3] | 0.90[1] | 0.95[1] | | -0.05[1] |
| C20:0 | -0.26[3] | 0.91[1] | -0.32[3] | -0.04[3] | -0.23[3] | -0.20[3] | 0.07[3] | -0.17[3] | -0.29[4] | 0.42[2] | 0.30[3] | -0.21[3] | -0.14[3] | -0.06[3] | |

[1]SE <0.1
[2]SE between 0.1 and 0.2
[3]SE between 0.2 and 0.3

**Table 4:** Herd (above diagonal) and genetic (below diagonal) correlations among branched chain FA in milk samples of 1912 Holstein-Friesian cows.

| Trait | C14:0 iso | C15:0 ante iso | C16:0 iso | C17:0 iso | C17:0 ante iso |
|---|---|---|---|---|---|
| C14:0 iso | | 0.43[1] | 0.78[1] | 0.50[1] | 0.05[1] |
| C15:0 ante iso | -0.33[3] | | 0.18[1] | 0.80[1] | 0.74[1] |
| C16:0 iso | 0.96[1] | -0.15[3] | | 0.36[1] | 0.13[1] |
| C17:0 iso | -0.29[3] | 0.85[1] | -0.30[3] | | 0.63[1] |
| C17:0 ante iso | -0.59[2] | 0.58[2] | -0.51[3] | 0.55[2] | |

[1]SE <0.1
[2]SE between 0.1 and 0.2
[3]SE between 0.2 and 0.3

**Table 5:** Herd (above diagonal) and genetic (below diagonal) correlations among FA unsaturation indices in milk samples of 1912 Holstein-Friesian cows.

| Trait | C10 index | C12 index | C14 index | C16 index | C17 index | C18 index | CLA index |
|---|---|---|---|---|---|---|---|
| C10 index | | 0.86[1] | 0.98[1] | 0.56[2] | 0.70[2] | 0.28[2] | 0.33[2] |
| C12 index | 0.87[1] | | 0.98[1] | 0.73[2] | 0.51[2] | 0.58[2] | 0.61[2] |
| C14 index | 0.92[1] | 0.95[1] | | 0.91[2] | 0.72[2] | 0.64[2] | 0.68[2] |
| C16 index | 0.15[2] | 0.37[2] | 0.24[2] | | 0.62[1] | 0.75[2] | 0.50[2] |
| C17 index | 0.12[2] | 0.35[2] | 0.27[2] | 0.92[1] | | 0.43[2] | 0.27[2] |
| C18 index | 0.14[2] | 0.24[2] | 0.08[2] | 0.45[2] | 0.56[2] | | 0.80[1] |
| CLA index | 0.07[2] | 0.26[2] | 0.05[2] | 0.60[2] | 0.69[2] | 0.98[1] | |

[1]SE <0.1
[2]SE between 0.1 and 0.2

# 5

# Milk fatty acid unsaturation: genetic parameters and effects of Stearoyl-CoA Desaturase (SCD1) and Acyl CoA: Diacylglycerol Acyltransferase 1 (DGAT1)

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## Abstract

With regard to human health aspects of milk fat, increasing the amount of unsaturated fatty acids in milk is an important selection objective. The cow's diet has an influence on the degree of unsaturation, but literature suggests that also genetics plays a role. To estimate genetic variation in milk fatty acid unsaturation indices, milk fatty acid composition of 1,933 Dutch Holstein Friesian heifers was measured and unsaturation indices were calculated. An unsaturation index represents the concentration of the unsaturated product proportional to the sum of the unsaturated product and the saturated substrate. Intraherd heritabilities were moderate, ranging from 0.23 ± 0.07 for CLA index to 0.46 ± 0.09 for C16 index. We genotyped the cows for the SCD1 A293V and DGAT1 K232A polymorphisms, which are known to alter milk fatty acid composition. Both genes explain part of the genetic variation in unsaturation indices. The SCD1 V allele is associated with lower C10, C12 and C14 indices, and with higher C16, C18 and CLA indices in comparison to the SCD1 A allele, with no differences in total unsaturation index. In comparison to the DGAT1 K allele, the DGAT1 A allele is associated with lower C10, C12, C14 and C16 indices, and with higher C18, CLA and total indices. We conclude that selective breeding can contribute to higher unsaturation indices, and that selective breeding can capitalize on genotypic information of both the SCD1 A293V and the DGAT1 K232A polymorphism.

## 5.1 Introduction

Recent studies show that bovine milk fatty acid composition is determined to a large extent by genetics, indicating that selective breeding can be an effective means to alter the composition of milk fat (Schennink et al., 2007; Soyeurt et al., 2007). Milk fat is characterized by a high amount of saturated fatty acids, especially myristic acid (C14:0) and palmitic acid (C16:0), and by a low amount of (poly)unsaturated fatty acids. With regard to human health aspects, increasing the amount of unsaturated fatty acids in milk is an important selection objective.

The cow's diet plays a role in determining the degree of unsaturation of milk fat (Baumgard et al., 2000; Perfield et al., 2006; Perfield et al., 2007). Dietary fatty acids are hydrogenated in the rumen by bacteria and transported via the blood. In the mammary gland fatty acids originating from the blood or from de novo fatty acid synthesis can be desaturated. Eventually, the fatty acids that are secreted into the milk determine the degree of unsaturation of milk fat. This degree of unsaturation is often addressed by a so-called index: the concentration of the unsaturated product proportional to the sum of the unsaturated product and the saturated substrate.

Studies demonstrating a significant variation in unsaturation among breeds and cows on the same diet suggest that also genetics plays a role (Beaulieu and Palmquist, 1995; DePeters et al., 1995; Drackley et al., 2001; Kelsey et al., 2003; Lawless et al., 1999; Lock and Garnsworthy, 2002; Lock and Garnsworthy, 2003; Sol Morales et al., 2000). For example, Kelsey et al (2003) found that the milk-fat content of C18:2 cis 9, trans 11 (CLA) and the CLA index varied largely among individual cows on the same diet, namely over threefold. Lock and Garnsworthy (2002 and 2003) reported that the C14:1/C14:0 index differed significantly between cows. However, genetic parameters for milk fatty acid unsaturation indices are scarce in literature (Royal and Garnsworthy, 2005).



Animals are capable of desaturating saturated fatty acids to Δ9 unsaturated fatty acids by the stearoyl-CoA desaturase (SCD) enzyme, which catalyzes the insertion of a double bond between carbon atoms 9 and 10 of a fatty acid (Pereira et al., 2003). Two SCD isoforms have been identified in cattle, SCD1 and SCD5. SCD1 is located on chromosome 26 and expressed in a variety of tissues among which are adipose and mammary tissue, and SCD5 is located on chromosome 6 and expressed primarily in the brain (Chung et al., 2000; Lengi and Corl, 2007). A non-synonymous SNP in exon 5 of SCD1, causing the substitution of valine with alanine (A293V), has been associated with carcass fatty acid composition in Japanese Black cattle (Taniguchi et al., 2004) and with milk fatty acid composition in Italian Holstein, Piedmontese and Valdostana cattle (Mele et al., 2007; Moioli et al., 2007). The SCD1 A allele was associated with a higher mono-unsaturated fatty acids (MUFA) content.

Another candidate gene which may affect unsaturation, is acyl CoA:diacylglycerol acyltransferase 1 (DGAT1), which is located on chromosome 14 (Grisart et al., 2002). The DGAT1 enzyme plays a key role in triacylglycerol synthesis; it catalyzes the esterification of a fatty acyl-CoA to the

sn-3 position of a diacylglycerol. A lysine to alanine polymorphism in DGAT1 (K232A) explains 50% of the genetic variation in milk-fat percentage and also has a strong effect on milk fatty acid composition. The DGAT1 K allele was associated with a larger fraction of C16:0; and smaller fractions of C14:0, unsaturated C18 and CLA (Schennink et al., 2007).
Our study aims to estimate genetic variation for milk fatty acid unsaturation indices for specific fatty acids and their phenotypic and genetic correlations. Furthermore, we study the effects of polymorphisms in 2 candidate genes, namely SCD1 A293V and DGAT1 K232A, on milk fatty acid unsaturation indices.

## 5.2 Materials and methods

### 5.2.1 Animals

This study is part of the Dutch Milk Genomics Initiative, which focuses on the genetic background of detailed milk composition. As part of this study, morning milk samples and blood samples were collected from 1,933 first-lactation cows on 398 commercial herds in the Netherlands. A 0.5 liter milk sample was collected from each cow at 1 morning milking between February and March 2005. At least 3 cows per herd were sampled and cows were milked twice a day. Cows descended from 1 of 50 young bulls (845 cows), from 1 of 5 proven bulls (897 cows), or from other proven bulls (191 cows). The NRS (Arnhem, the Netherlands) provided pedigrees of the cows and the milk yield records. Total pedigree size was 26,300 records. Each cow was over 87.5 percent Holstein-Friesian and was in lactation between Day 63 and Day 282.

### 5.2.2 Phenotypes

Fat and protein percentages were measured by infra red spectroscopy, using a MilkoScan FT6000 (Foss Electric, Hillerod, Denmark) at the Milk Control Station (Zutphen, the Netherlands). Fat and protein yields were calculated by multiplying fat or protein percentage by the morning milk yield. Milk yield (kg) data were missing for 145 cows. Milk fatty acid composition was measured by gas chromatography at the COKZ laboratory (Netherlands Controlling Authority for Milk and Milk Products, Leusden, the Netherlands) as described by Schennink et al. (2007). With this method, the C18:1 cis9 peak was probably slightly overestimated due to coelution (Jensen, 2002). The cis double bond of C10:1 and C12:1 could not be ascertained at the carbon 9 position. The fatty acids were expressed as weight-proportion of total fat weight. Fatty acid unsaturation indices were calculated by expressing each product as a proportion of the product plus substrate, multiplied by 100 (Kelsey et al., 2003):

$$\frac{unsaturated}{unsaturated + saturated} * 100, \text{ e.g. } \frac{C14:1cis9}{C14:1cis9 + C14:0} * 100.$$

We calculated indices for the following product and substrate pairs: C10:1 and C10:0 (C10 index), C12:1 and C12:0 (C12 index), C14:1 cis 9 and C14:0 (C14 index), C16:1 cis 9 and C16:0 (C16 index), C18:1 cis 9 and C18:0 (C18 index), C18:2 cis 9, trans 11 (CLA) and C18:1 trans 11 (CLA index).

### 5.2.3 Genotypes

Blood samples for DNA isolation were collected between April and June 2005. Genotypes for the SCD1 A293V polymorphism were assayed by the SNaPshot single base primer extension method (Applied Biosystems, Foster City, CA, USA). The primer designs were based on the Genbank sequence (AY241932): forward PCR primer, 5'- TCATTTAACCCCTCATTACCTCA -3'; reverse PCR primer, 5'- TGTAAAATACTAGGCTTTCTGG -3'; genotyping primer, 5'- TGGTTTCCCTGGGAGCTG - 3'. To amplify the SCD1 fragment, 12 µl PCR reactions were set up containing 20 ng of genomic DNA, 0.2 µM of each primer and 2X AccuPrime Supermix II (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were 94°C for 5 min, 36 cycles of 94°C for 30 s, 55°C for 45 s, 68°C for 90 s, followed by an extension cycle of 68°C for 10 min. PCR products were purified by incubation with shrimp alkaline phosphatase (SAP) (USB, Cleveland, OH, USA) and Exo I (USB) at 37°C for 1 h and 72°C for 15 min. Extension reactions, using 3 µl of purified PCR product and 5 pmol of genotyping primer and SNaPshot multiplex Ready reaction mix (Applied Biosystems), were performed using 40 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. The extension products were incubated with SAP at 37°C for 1 h and 72°C for 15 min. Two microliters of extension product were added to 8 µl of Hi-Di formamide and electrophoresed on an ABI 3730 DNA analyzer. Results were analyzed using the GeneMapper Software v4.0 (Applied Biosystems). In total, 1,725 animals were genotyped for the SCD1 A293V polymorphism. Genotypes were missing for 208 animals because either no DNA sample was available (n = 141) or the sample could not be genotyped unambiguously (n = 67).
Genotyping of the DGAT1 K232A dinucleotide polymorphism was performed as described by Schennink et al. (2007). In total, 1,779 animals were genotyped for the DGAT1 polymorphism. Genotypes were missing for 154 animals because either no DNA sample was available (n = 141) or the sample could not be genotyped unambiguously (n = 13).

### 5.2.4 Statistical analysis

Analyses were performed first using SAS 9.1 (2002) procedures to determine significance of fixed effects. The model included days in milk (days between calving and date of sample), age at first calving, season of calving, and an effect of the differences in genetic level between proven bull daughters and young bull daughters. Variance components and genetic parameters were estimated using an Animal Model in ASReml (Gilmour et al., 2002):



$$y_{ijklmn} = \mu + b_1 * dim_i + b_2 * e^{-0.05 * dim} + b^3 * afc_j + b_4 * afc_j^2 + season_k + scode_l + herd_m + a_n + e_{ijklmn},$$

where y was the dependent variable, µ was the general mean, dim was the covariate describing the effect of days in milk modelled with a Wilmink curve (Wilmink, 1987), afc was the covariate describing the effect of age at first calving, season was the fixed effect of the class of calving season (June-August 2004, September-November 2004, or December 2004-February 2005), scode was the fixed effect of the differences in genetic level between proven bull daughters and young bull daughters, herd was the random effect of groups of animals sampled in the same herd, an was the random additive genetic effect of animal n, and e was the random residual effect. Effects of the polymorphisms were estimated using the same model, but extended with effect g: the fixed effect of the SCD1 genotype (AA, AV or VV) or the DGAT1 genotype (KK, KA or

AA). Ungenotyped individuals were included as a separate group, and appeared to be randomly distributed across other effects in the model.

The variance-covariance structure of the additive genetic effects was $Var(a)=A\sigma_a^2$, where A was a matrix of additive genetic relationships between individuals and $\sigma_a^2$ was the additive genetic variance. Heritabilities were estimated using univariate analyses. Heritability was defined as:

$$h_{IH}^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2} ,$$

where $\sigma^2_a$ was the additive genetic variation, and $\sigma^2_e$ was the residual variation. Heringstad et al. (2006) referred to this heritability as the intraherd heritability. The intraherd heritability is the parameter that is required to predict selection responses of alternative breeding strategies. The proportion of variance due to herd ($h_{herd}$) was defined as:

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

where $\sigma^2_{herd}$ was the herd variance. The proportion of additive genetic variance explained by the polymorphism was defined as:

$$r_{genetic}^2 = \frac{\sigma_{polymorphism}^2}{\sigma_a^2} ,$$

where $\sigma^2_{polymorphism}$ was calculated by estimating the variance of the polymorphism based on the estimated genotype effects and the genotype frequencies. Corrected means for the reference group in tables 3-5 were estimated by the PREDICT function in ASReml. Genetic correlations were estimated using bivariate analyses. Estimates of skewness and kurtosis for the fatty acid indices are in the same range as the estimates for milk production traits.

## 5.3 Results and discussion

### 5.3.1 Means and coefficients of variation

Fatty acid unsaturation indices were calculated from milk fatty acid profiles of 1,933 Dutch Holstein Friesian heifers (Table 1). Unsaturation indices of the short and medium-chain fatty acids C10, C12, C14 and C16 were between 2.7 and 10.9. Indices of the long-chain fatty acids C18 and CLA were higher, 67.6 and 33.7 respectively.

| Trait | Mean | CV (%) | 5% quantile | 95% quantile |
|---|---|---|---|---|
| C10 index[1] | 10.9 | 17 | 6.6 | 14.9 |
| C12 index[1] | 2.7 | 20 | 1.6 | 3.9 |
| C14 index[1] | 10.5 | 17 | 7.0 | 14.6 |
| C16 index[1] | 4.2 | 19 | 2.9 | 6.3 |
| C18 index[1] | 67.6 | 6 | 59.5 | 75.3 |
| CLA index[2] | 33.7 | 12 | 25.4 | 42.8 |
| Total index[3] | 26.4 | 10 | 21.5 | 33.1 |

**Table 1:** Mean, coefficient of variation (CV), 5% and 95% quantiles for fatty acid unsaturation indices, measured on 1 morning milk sample of 1,933 first-lactation Dutch Holstein Friesian cows.

[1]Indices are calculated according to the following example: C14 index = C14:1 cis9/(C14:1 cis9 + C14:0)*100.
[2]CLA index = CLA cis9, trans11/(CLA cis9, trans11 + C18:1trans11)*100.
[3]Total index = (C10:1 + C12:1 + C14:1 cis9 + C16:1 cis9 + C18:1 cis9 + CLA cis9, trans11)/( C10:1 + C12:1 + C14:1 cis9 + C16:1 cis9 + C18:1 cis9 + CLA cis9, trans11 + C10:0 + C12:0 + C14:0 + C16:0 + C18:0 + C18:1trans11)*100.

These values are in line with those reported for dairy cattle in other studies (Mele et al., 2007; Perfield et al., 2006; Perfield et al., 2007; Royal and Garnsworthy, 2005). Almost all C10, C12, C14 and approximately 50% of C16 fatty acids are synthesized in the mammary gland, whereas the longer-chain fatty acids as well as a proportion of the unsaturated long-chain fatty acids, are derived from the blood. The dual origin of the long-chain fatty acid unsaturation may play a role in the contrast between short/medium and long-chain fatty acid unsaturation indices. An alternative explanation for this contrast may be that long-chain fatty acids are unsaturated to a larger extent. The coefficient of variation was lowest for C18 index (6 %) and highest for C12 and C16 indices (about 20%).



| Trait | $h_{IH}^2$ (SE) | $h_{herd}$ (SE) | $\sigma^2_a$ | $\sigma^2_a / \sigma^2_{herd}$ |
|---|---|---|---|---|
| C10 index | 0.37 (0.09) | 0.06 (0.02) | 1.23 | 5.5 |
| C12 index | 0.37 (0.09) | 0.06 (0.02) | 0.09 | 5.6 |
| C14 index | 0.45 (0.09) | 0.06 (0.02) | 1.35 | 6.6 |
| C16 index | 0.46 (0.09) | 0.07 (0.02) | 0.30 | 6.2 |
| C18 index | 0.33 (0.08) | 0.06 (0.02) | 4.36 | 5.1 |
| CLA index | 0.23 (0.07) | 0.09 (0.02) | 3.49 | 2.5 |
| Total index | 0.30 (0.09) | 0.26 (0.02) | 1.58 | 0.8 |

**Table 2:** Intraherd heritability ($h_{IH}^2$), additive genetic variance estimate ($\sigma^2_a$), herd effect ($h_{herd}$), and ratio between additive genetic variance and herd variance ($\sigma^2_a/\sigma^2_{herd}$) for fatty acid unsaturation indices, measured on 1 morning milk sample of 1,933 first-lactation Dutch HF cows.

### 5.3.2 Heritabilities and herd Effects

Intraherd heritabilities range from 0.23 for CLA index to 0.46 for C16 index, and demonstrate a significant genetic effect on the variation in fatty acid unsaturation indices (Table 2). The heritability for total index was 0.30. Repeatabilities for C14, C16 and C18 indices, which are considered to be the upper limit of heritabilities, were estimated between 40 and 45% by Soyeurt et al. (2006), suggesting moderate heritabilities as well. Only Royal and Garnsworthy (2005) reported heritabilities for fatty acid unsaturation indices, based on 1,520 Holstein-Friesian cows, and reported similar values for C14 (0.30), C18 (0.19) and CLA (0.29) indices, but much lower values for C16 (0.01) and total (0.02) indices.

The proportion of total variance explained by herd was small, ranging from 0.06 to 0.09 of total variance in individual unsaturation indices. For total unsaturation index the herd effect was larger (0.26). The ratio of genetic variance to herd variance showed that for all indices the genetic variance was much larger than the herd variance, except for total index, for which the herd variance was slightly larger than the genetic variance. The total unsaturation index, in fact, mainly represents the ratio of C18:1 cis9 to C16:0, because these fatty acids are the largest saturated and unsaturated fatty acid fractions in milk. The proportion of variance explained by herd was 0.28 for C18:1 cis9 fraction and 0.29 for C16:0 fraction in milk, which explains the relative large herd variance of the total unsaturation index (Stoop et al., 2008). The moderate to high heritabilities for unsaturation indices in combination with the moderate to high coefficients of variation indicate that the unsaturation indexes can be changed by means of selection.

**Table 3:** Effect of the SCD1 A293V polymorphism on milk fatty acid composition (g/100g of total fatty acids).

| Trait | Predicted mean of AA group | AA (n=919) | VA[1] (n=689) | VV[2] (n=117) | P value[3] |
|---|---|---|---|---|---|
| C10:0 | 2.95 | 0 | 0.10 (0.02) | 0.16 (0.04) | <0.001 |
| C10:1 | 0.38 | 0 | -0.03 (0.00) | -0.06 (0.01) | <0.001 |
| C12:0 | 4.09 | 0 | 0.09 (0.03) | 0.15 (0.06) | 0.003 |
| C12:1 | 0.12 | 0 | -0.01 (0.00) | -0.02 (0.00) | <0.001 |
| C14:0 | 11.38 | 0 | 0.22 (0.04) | 0.42 (0.09) | <0.001 |
| C14:1 cis9 | 1.46 | 0 | -0.17 (0.01) | -0.33 (0.02) | <0.001 |
| C16:0 | 32.67 | 0 | -0.12 (0.13) | -0.25 (0.25) | 0.58 |
| C16:1 cis9 | 1.38 | 0 | 0.17 (0.02) | 0.34 (0.03) | <0.001 |
| C18:0 | 8.83 | 0 | -0.30 (0.07) | -0.43 (0.13) | <0.001 |
| C18:1 cis9 | 18.28 | 0 | 0.08 (0.09) | 0.17 (0.18) | 0.55 |
| C18:1 trans11 | 0.77 | 0 | -0.02 (0.01) | -0.03 (0.02) | 0.01 |
| CLA cis9, trans11 | 0.38 | 0 | 0.01 (0.00) | 0.02 (0.01) | 0.003 |

[1]Contrast of VA-AA genotypes; [2]Contrast of VV-AA genotypes; [3]Statistical significance of the SCD1 A293V polymorphism.

### 5.3.3 Effects of the SCD1 A293V polymorphism

To study the effect of the SCD1 A293V polymorphism, 1,725 cows were genotyped. The frequency of 293A was 0.73 and the genotypes were in Hardy-Weinberg equilibrium. A higher frequency of the A allele is also reported for Italian Holsteins (0.57), Valdostana (0.65), Jerseys (0.94) and Japanese Black cattle (0.59) (Mele et al., 2007; Moioli et al., 2007; Taniguchi et al., 2004). The SCD1 genotype did not significantly affect fat or protein percentage, nor fat, protein or milk yield (results not shown). Effects of the SCD1 genotype on milk fatty acid composition are in Table 3. In comparison to the A allele, the V allele was associated with a higher proportion of C10:0, C12:0, C14:0, C16:1 and CLA, and with a lower proportion of C10:1, C12:1, C14:1, C18:0 and C18:1 trans 11. Moioli et al. (2007), who studied 27 Piedmontese and 27 Valdostana cows, found similar results, but no significant effects on C10:0 and C12:0. Mele et al. (2007), who studied 297 Italian Holstein Friesian cows, only found significant effects on C14:1 and on total mono-unsaturated fatty acids (MUFA), similar to ours, and on C18:1 cis9, on which we found no significant effect.

The SCD1 A293V polymorphism had significant effects on the unsaturation indices for all individual fatty acids, but not on the total unsaturation index (Table 4). The V allele was associated with lower indices of C10, C12 and C14, and higher indices of C16, C18 and CLA. The negative effect of the V allele on C10 and C14 index is in agreement with Moioli et al. (2007); however, they reported no significant effects on the other indices, which could be due to their limited sample size.

**Table 4:** Effect of the SCD1 A293V polymorphism on milk fatty acid unsaturation indices.

| Trait | Predicted mean of AA group | AA (n=919) | VA[1] (n=689) | VV[2] (n=117) | P value[3] | $r^2_{genetic}$(%)[4] |
|---|---|---|---|---|---|---|
| C10 index | 11.68 | 0 | -1.18 (0.09) | -2.16 (0.17) | <0.001 | 41 |
| C12 index | 2.92 | 0 | -0.29 (0.02) | -0.56 (0.05) | <0.001 | 34 |
| C14 index | 11.42 | 0 | -1.34 (0.08) | -2.61 (0.16) | <0.001 | 52 |
| C16 index | 4.05 | 0 | 0.48 (0.04) | 0.98 (0.08) | <0.001 | 31 |
| C18 index | 67.57 | 0 | 0.87 (0.19) | 1.43 (0.37) | <0.001 | 6 |
| CLA index | 33.56 | 0 | 1.32 (0.20) | 2.39 (0.40) | <0.001 | 18 |
| Total index | 26.63 | 0 | 0.05 (0.13) | 0.10 (0.25) | 0.85 | <1 |

[1]Contrast of VA-AA genotypes; [2]Contrast of VV-AA genotypes; [3]Statistical significance of the SCD1 A293V polymorphism; [4]Percentage of genetic variance explained by the SCD1 A293V polymorphism.

**Table 5:** Effect of the DGAT1 K232A polymorphism on milk fatty acid unsaturation indices.

| Trait | Predicted mean of KK group | KK (n=293) | KA[1] (n=837) | AA[2] (n=649) | P value[3] | $r^2_{genetic}$(%)[4] |
|---|---|---|---|---|---|---|
| C10 index | 11.54 | 0 | -0.29 (0.12) | -0.54 (0.13) | <0.001 | 3 |
| C12 index | 2.92 | 0 | -0.09 (0.03) | -0.20 (0.04) | <0.001 | 5 |
| C14 index | 11.47 | 0 | -0.47 (0.11) | -0.97 (0.12) | <0.001 | 9 |
| C16 index | 4.57 | 0 | -0.25 (0.05) | -0.56 (0.06) | <0.001 | 14 |
| C18 index | 66.46 | 0 | 1.23 (0.24) | 2.31 (0.26) | <0.001 | 15 |
| CLA index | 32.87 | 0 | 1.15 (0.27) | 1.90 (0.29) | <0.001 | 12 |
| Total index | 25.67 | 0 | 0.62 (0.16) | 1.80 (0.17) | <0.001 | 29 |

[1]Contrast of KA-KK genotypes; [2]Contrast of AA-KK genotypes; [3]Statistical significance of the DGAT1 K232A polymorphism; [4]Percentage of genetic variance explained by the DGAT1 K232A polymorphism.

The negative effect on C14 index was also found by Mele et al. (2007), but they didn't find a significant effect on other indices. The finding that the SCD1 polymorphism has highly significant effects on individual indices, but not on the total index suggests that the activity of the enzyme is not affected. An altered substrate specificity, however, ís supported by the differential effects on the individual fatty acid indices. Substrate specificity of SCD enzymes has been demonstrated for SCD isoforms in knockout studies in mice (Miyazaki and Ntambi, 2003).

In vitro studies in rat liver microsomal preparations have shown that acyl-CoA derivatives with 12 to 19 carbon atoms were required for activity of the SCD1 enzyme and that the enzyme has substrate specificity with preference for longer-chain fatty acids (Enoch et al., 1976). Based on Enoch et al. (1976) is would be unlikely that C10:1 is desaturated from C10:0, which is, however, suggested by our finding that the C10 index is affected by the SCD1 A293V polymorphism.

The enzyme function of SCD1 may be affected by the polymorphism, since it causes a valine to alanine substitution on position 293, which is located in the third histidine-rich region of the enzyme. These histidine-rich regions are important for catalytic activity (Shanklin et al., 1994).

The SCD1 A293V polymorphism contributed considerably to the genetic variance (Table 4): it explained 31% to 52% of the genetic variance for C10, C12, C14 and C16 indices, but only 18% for CLA and 6% for C18 index (Mosley and McGuire, 2007).

### 5.3.4 Effects of the DGAT1 K232A polymorphism

A total of 1,779 cows was genotyped for the DGAT1 K232A polymorphism. The frequency of 232K was 0.40 and the genotypes were in Hardy-Weinberg equilibrium. The DGAT1 A allele was associated with lower indices of C10, C12, C14 and C16, and with higher indices of C18 and CLA (Table 5). The A allele was also associated with a higher total unsaturation index. The vast majority of fatty acids in milk is present in the form of triacylglycerols.

DGAT1 catalyzes the last step in triacylglycerol synthesis: the esterification of a fatty acyl-CoA to the sn-3 position of a diacylglycerol. The DGAT1 K232A polymorphism was reported to have an effect on, among others, C14:0, C16:0 and unsaturated C18 fatty acids in milk. Given these effects of the polymorphism on milk fatty acid composition, an effect on milk fatty acid unsaturation indices was conceivable. The effects on composition and unsaturation may be explained by different causes: a higher activity of DGAT1 and an alteration of specificity of DGAT1 (Schennink et al., 2007).



| | DGAT1 K232A genotype | | | |
|---|---|---|---|---|
| **SCD1 A293V genotype** | KK | KA | AA | **Total** |
| AA | 159 | 439 | 316 | 914 |
| AV | 106 | 313 | 264 | 683 |
| VV | 16 | 59 | 41 | 116 |
| Total | 281 | 811 | 621 | 1713[1] |

**Table 6:** Number of animals per combined genotype of the SCD1 A293V and DGAT1 K232A polymorphisms.

[1] 220 animals could not be genotyped for one or both polymorphisms.

### 5.3.5 Joint effects of the SCD1 and DGAT1 polymorphisms

The contribution of the DGAT1 K232A polymorphism to the genetic variance of unsaturation indices was lower than the contribution of the SCD1 A293V polymorphism for all indices, except for C18 and total index (Table 4, 5). When the statistical model was extended to include both the SCD1 genotype and the DGAT1 genotype as fixed effects, the effects of DGAT1 on the unsaturation indices did not change considerably, meaning that the two polymorphisms do not explain the same part of the genetic variation (results not shown). In other words, the genetic variation explained by DGAT1 and the genetic variation explained by SCD1 is additive. Cows with the SCD1 VV genotype and the DGAT1 AA genotype (n = 41) have 9%, 6% and 14% higher indices of C16, C18 and CLA, respectively, compared with cows with the SCD1 AA and DGAT1 KK genotype (results not shown, frequencies of combined genotypes in table 6). This shows that selective breeding can improve when both the polymorphisms in SCD1 and DGAT1 are used in genetic selection.

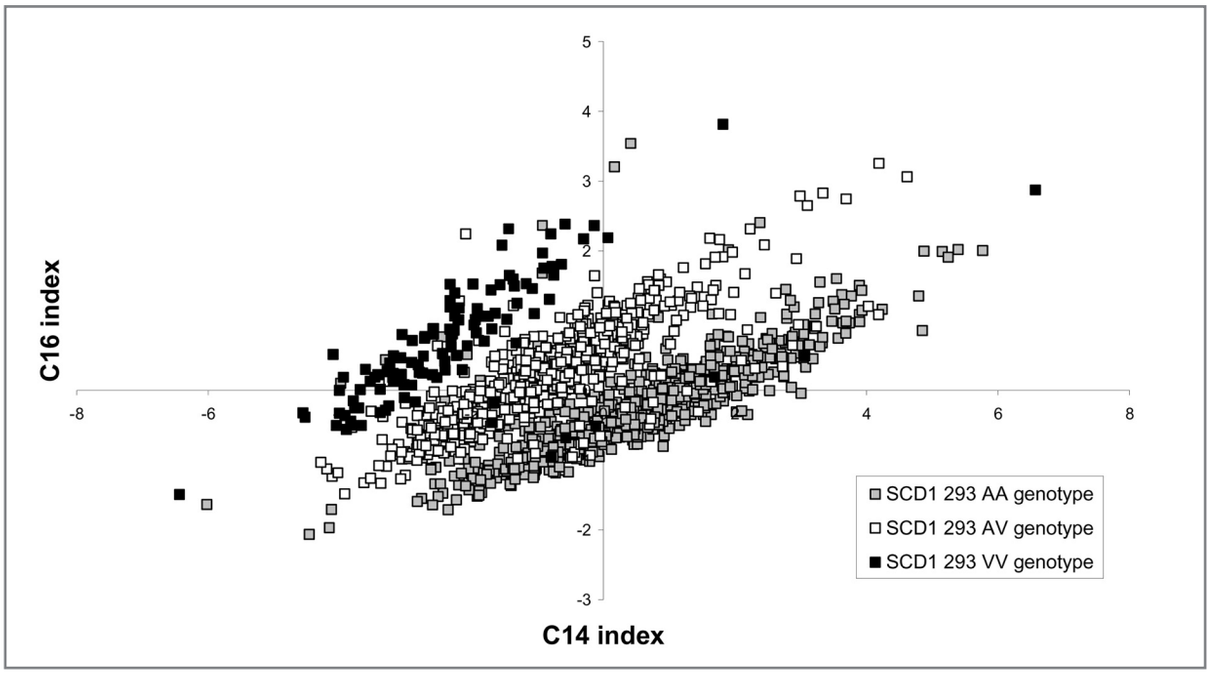


**Figure 1:** Relation between C14 and C16 unsaturation indices of individual cows. Phenotypes are corrected for DIM modeled with a Wilmink curve, age at first calving, calving season, differences in genetic level between groups of proven bull daughters and young bull daughters, and herd.

### 5.3.6 Correlations

Phenotypic and genetic correlations were high and positive among the medium-chain fatty acid unsaturation indices C10, C12 and C14 and among the long-chain fatty acid indices C18 and CLA, ranging from 0.86 through 0.98 (Table 7). Genetic correlations were low between the medium-chain and the long-chain fatty acid indices (0.05-0.26). C16 index showed low to moderate genetic correlations with C10 (0.15), C12 (0.37) and C14 (0.24) indices. Genetic correlations of C16 index with C18 and CLA indices were also moderate, although slightly higher (0.45 and 0.60). Our observations are similar to the phenotypic correlations reported in previous studies (Kelsey et al., 2003; Peterson et al., 2002). Genetic correlations of fat percentage with C10 (0.25), C12 (0.26), C14 (0.31) and C16 (0.17) indices were low, but positive, whereas genetic correlations of fat percentage with C18 (-0.35) and CLA (-0.48) indices were negative.

Accordingly, an increase in fat percentage will lead to slightly higher C10, C12, C14 and C16 indices, but to lower C18 and CLA indices. Fat percentage also showed a negative genetic correlation with total index (-0.52). This might partly reflect the effects of the DGAT1 K232A polymorphism, for which the K allele is known to have a very large effect on fat percentage. In this study, it has been shown that the K allele is also associated with higher C10, C12, C14 and C16 indices, and lower C18 and CLA indices. It is likely that breeding in the Dutch dairy in the past years has resulted not only in increased fat percentage and fat yield, but also in higher C10 to C16 indices, and lower C18 and CLA indices.

The moderate genetic correlation between C14 and C16 index can be partly explained by the SCD1 polymorphism (Figure 1). Three groups can be identified in this figure, which almost completely coincide with the 3 genotypes of SCD1 A293V (AA, AV and VV). Correction for the SCD1 polymorphism, by adding the SCD1 genotype to the model as a fixed effect, results in a higher genetic correlation between C14 and C16 index (0.93 with vs. 0.24 without correction, Table 7), but also in higher correlations between C10 index and C16 index (0.69 vs. 0.15) and between C12 index and C16 index (0.91 vs. 0.37), and between the medium chain C10, C12 and C14 indices and the long chain C18 and CLA indices (0.2 to 0.4 higher after correction; data not shown). Thus, the correlation due to the SCD1 A293V polymorphism is negative for these indices, whereas the correlation due to other genetic effects is positive.

### 5.3.7 Impact

We have demonstrated that milk fatty acid unsaturation indices have a substantial genetic component, indicating that it is possible to change unsaturation indices by selective breeding. Our results also show that both the SCD1 A293V and the DGAT1 K232A polymorphism explain part of the genetic variation in the unsaturation indices of milk fat. Even though the SCD1 A293V polymorphism does not affect the overall degree of unsaturation, its effects on the individual fatty acid indices offer opportunities for improving milk fatty acid composition. Human trial studies show that not all saturated fatty acids affect cholesterol concentrations to the same extent and that some are more unfavorable than others (Mensink et al., 2003). C16:0 is the most unfavourable, hypercholesterolemic fatty acid. Therefore, an increase of certain fatty acid indices (i.e. a shift from saturated to unsaturated) at the expense of others would be beneficial.



Our study gives more insight into the process of unsaturation. Not only SCD1 plays a significant role by desaturating saturated fatty acids into unsaturated fatty acids, but also DGAT1 is important by influencing the composition of the triacylglycerols. The entire pathway of lipogenesis, which next to SCD1 and DGAT1 involves other enzymes like fatty acid synthase and acetyl-coenzyme A carboxylase, is regulated by the transcription factor sterol regulatory element-binding protein (SREBP)-1c and activated by dietary fatty acids. We made an important start to unravel the genetics of unsaturation and this could be the start of other studies looking into the interaction between genetics and feeding.

**Table 7:** Phenotypic (below diagonal) and genetic (above diagonal, SE in parentheses) correlations[1] between fatty acid unsaturation indices and milk production traits.

| Trait | C10 index | C12 index | C14 index | C16 index | C18 index | CLA index | Total index | Fat % | Fat yield | Protein yield | Milk Yield |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C10 index | | 0.87 (0.05) | 0.92 (0.03) | 0.15 (0.17) | 0.14 (0.18) | 0.07 (0.20) | 0.33 (0.18) | 0.25 (0.17) | -0.05 (0.19) | -0.14 (0.23) | -0.22 (0.19) |
| C12 index | 0.87 | | 0.95 (0.02) | 0.37 (0.15) | 0.24 (0.17) | 0.26 (0.18) | 0.39 (0.18) | 0.26 (0.17) | -0.21 (0.19) | -0.25 (0.22) | -0.39 (0.18) |
| C14 index | 0.86 | 0.94 | | 0.24 (0.15) | 0.08 (0.17) | 0.05 (0.19) | 0.30 (0.17) | 0.31 (0.16) | -0.13 (0.18) | -0.29 (0.20) | -0.39 (0.17) |
| C16 index | 0.34 | 0.48 | 0.44 | | 0.45 (0.13) | 0.60 (0.12) | 0.44 (0.16) | 0.17 (0.16) | -0.21 (0.18) | -0.32 (0.21) | -0.37 (0.17) |
| C18 index | 0.50 | 0.58 | 0.49 | 0.63 | | 0.98 (0.02) | 0.83 (0.08) | -0.35 (0.16) | -0.36 (0.18) | 0.17 (0.22) | 0.01 (0.20) |
| CLA index | 0.47 | 0.58 | 0.46 | 0.63 | 0.90 | | 0.83 (0.09) | -0.48 (0.16) | -0.44 (0.19) | 0.10 (0.25) | 0.05 (0.22) |
| Total index | 0.38 | 0.35 | 0.39 | 0.47 | 0.66 | 0.53 | | -0.52 (0.15) | -0.43 (0.18) | 0.09 (0.25) | 0.14 (0.21) |
| Fat% | -0.07 | -0.03 | 0.02 | 0.07 | -0.29 | -0.28 | -0.37 | | 0.51 (0.14) | -0.29 (0.20) | -0.58 (0.13) |
| Fat yield | -0.03 | -0.04 | -0.06 | -0.04 | -0.13 | -0.12 | -0.29 | 0.45 | | 0.59 (0.15) | 0.38 (0.16) |
| Protein yield | 0.09 | 0.11 | 0.03 | 0.01 | 0.20 | 0.20 | 0.01 | -0.26 | 0.67 | | 0.88 (0.05) |
| Milk yield | 0.04 | 0.00 | -0.07 | -0.09 | 0.14 | 0.13 | 0.08 | -0.47 | 0.57 | 0.89 | |

[1] Standard errors of phenotypic correlations were between 0.004 and 0.03.

# 6

# Seasonal variation in the Dutch bovine raw milk composition

J. M. L. Heck, H. J. F. van Valenberg, J. Dijkstra, A. C. M. van Hooijdonk. *Seasonal variation in the Dutch bovine raw milk composition.* Submitted.

## Abstract

In this study we determined the detailed composition and seasonal variation in Dutch dairy milk. Raw milk samples representative for the complete Dutch milk supply were collected weekly from February 2005 until February 2006. Large seasonal variation exists in the concentration of the main components and milk fatty acid composition. Milk lactose concentration was rather constant throughout the season. Milk true protein content was somewhat more responsive to season with lowest values in June (3.21 g/100g) and highest values in December (3.38 g/100g). Milk fat concentration increased from a minimum of 4.10 g/100g in June to a maximum of 4.57 g/100g in January. Largest, up to 2 fold, seasonal changes in the fatty acid composition were found for trans fatty acids, including CLA. Milk protein composition was rather constant throughout the season. Milk unsaturation indices which were used as an indication of desaturase activity were lowest in spring and highest in autumn. Compared with a previous investigation of Dutch dairy milk in 1992, the fatty acid composition of Dutch raw milk has changed considerably, with in particular a higher content of saturated fatty acids in 2005 milk.

## 6.1. Introduction

Milk and dairy products are important components of western diets. The composition of raw milk determines, to a large extent, the nutritional value and the technological properties of milk and dairy products. Therefore, the composition of milk is of great importance for the dairy industry and there is large interest in changing the composition of milk. The composition of milk varies with stage of lactation, feeding, health status of the cow and genetic factors (Fox & McSweeney 1998). Because different countries use different breeds and feeding regimes and have different calving patterns and breeding practices, milk composition will also differ among countries. To get insight in the milk composition in the Netherlands, it is therefore essential to examine the composition of the milk produced by Dutch cows.

The Dutch raw milk composition has changed during the past decades due to changes in the feeding regime and breeding practices or other changes in dairy husbandry. For example, fat percentage has increased from 3.8 % in 1960 to 4.4 % in 2005 (Eurostat, 2008). It is unclear, however, whether the composition of milk fat has also changed during this period. A detailed overview of the milk composition is also needed to set a standard to detect favorable or unfavorable changes in the future. An investigation of the Swedish dairy milk composition in the 1970s and 1996 has shown a substantial decrease in the casein content of Swedish raw milk during this period (Lindmark-Månsson et al., 2003). Such a decrease in casein content would also be unfavorable for the Dutch dairy industry, because in the Netherlands about 50% of the milk is used for cheese production.

When determining the composition of raw milk it is important to realize that within one country the composition is not constant. Milk composition varies considerably throughout the seasons as shown in multiple studies (Auldist et al., 1998; Jahreis et al., 1996; Lindmark-Månsson et al., 2003; Lock & Garnsworthy, 2003). These seasonal changes offer problems and opportunities for dairy manufactures. Spreadability of butter, for example, is better when it is produced from summer fat compared to winter fat. Studies concerning seasonal variation tended to be small-scale and studied milk samples from a limited number of cows from 1 herd (Auldist et al., 1998; Jahreis et al., 1996; Lock & Garnsworthy, 2003). However, milk composition between herds differs markedly even within herds from the same breed (Stoop et al., 2008). Therefore, these studies may not give a reliable overview of the seasonal variation of the milk supplied to the dairy factories. To our knowledge, only a very limited number of studies analyzed raw milk composition that represents the total milk supply of a whole country (Lindmark-Månsson et al., 2003). Furthermore, most studies concerning seasonal variation only analyzed samples once a month or once a season (Jahreis et al., 1996; Muuse et al., 1986; Palmquist et al., 1993; Wolff et al., 1995). Kelly et al. (1998) and Elgersma et al. (2004) showed that milk composition changes when cows switch from a silage based diet to a fresh grass based diet and back. Because such changes in the diet of cows occur very rapidly, e.g. with changing weather conditions, milk composition can change markedly even on a week to week basis. Therefore, to get better insight the factors that cause the seasonal changes in milk composition in the Netherlands a higher sampling interval is preferred.

The aim of this study is to provide an up to date standard for the Dutch raw bovine milk composition and to investigate the seasonal variation in the main components, and the composition of the protein and fat fractions. To achieve this we analyzed during 1 year, every week, 1 milk sample that was representative for the total Dutch milk supply on detailed milk composition.

## 6.2 Materials and methods

### 6.2.1 Samples and methods of analysis

The samples used were the same dairy milk samples that are collected routinely by the Dutch milk control station (MCS) as a representative Dutch milk sample. From February 2005 until February 2006, every week, bulk milk samples from 17 dairy plants situated in the Netherlands were collected, pooled together and conserved with 0.03% sodium azide.

In total 52 milk samples, which were representative for the Dutch dairy milk composition during every week, in one year, were analyzed. All 52 samples were analyzed on all the components using analytical methods as summarized in Table 1. Total saturated fat was calculated as the sum of C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0 iso, C14:0, C15:0, C15:0 iso, C15:0 ante iso, C16:0, C16:0 iso, C17:0, C17:0 iso, C17:0 ante iso, C18:0, C19:0, and C20:0. Fatty acid unsaturation indices, viz. C10 index, C12 index, C14 index, C16 index, C18 index, and CLA index, as proxies for the Δ9 desaturase activity in the mammary gland were calculated from the ratio between the product and the sum of product and precursor fatty acid as described by (Schennink et al., 2008).

**Table 1:** Analysis methods used to determine the raw milk composition.

| Component | Method | Reference |
|---|---|---|
| Protein, NPN | ISO 8968-1 | (ISO, 2001) |
| True protein | Calculated; Protein-NPN | |
| Casein | ISO 17997-1 | (ISO, 2004a) |
| Casein number | Calculated; Casein/True protein | |
| Fat | ISO 1211 | (ISO, 1999) |
| Lactose | ISO 22662 | (ISO, 2007) |
| Dry matter | NEN 6844 | (NEN, 1991) |
| Cell count | ISO 13366-1 | (ISO, 2008) |
| Urea | ISO 14637 | (ISO, 2004b) |
| Freezing point | ISO 5764 | (ISO, 2002) |
| Protein composition | Capillary zone electrophoresis | (Heck et al., 2008) |
| Fatty acid composition | Gas chromatography | (Schennink et al., 2007) |

### 6.2.2. Statistical analysis

The mean value for a component was calculated as the weighted mean of all 52 samples. For components that showed seasonal variation the coefficient of variation (CV), and the minimum and maximum value were calculated. The minimum and maximum values were the monthly averages based on the 4 or 5 samples that represented these months. To determine the presence of seasonal variation in a milk component, the month of sampling was tested as a fixed effect in a general linear model using Genstat, tenth edition.

## 6.3 Results and discussion

### 6.3.1 Main components

Holstein-Friesian is the predominant dairy breed in the Netherlands (NRS, 2008) and therefore the analyzed milk samples largely reflect the milk composition of Holstein-Friesian cows.

Table 2 shows the mean values of the main components of Dutch raw milk in 2005. In 2005, fat and protein percentage were higher in raw milk from the Netherlands than in milk from all other European countries in the same year (Eurostat, 2008).

When comparing milk composition with literature values of other countries it is important to take into account the year in which the samples were taken. Large changes in milk composition in the past decades have occurred in many countries (Eurostat, 2008) due to changes in feeding, breeding and cattle management practices.

**Table 2:** Mean, coefficient of variation (CV) and seasonal variation of the main components of Dutch bovine raw milk in 2005.

| Component | Mean | CV | Minimum | | Maximum | | P value |
|---|---|---|---|---|---|---|---|
| Protein (g/100g) | 3.48 | 1.7 | 3.39 | (July) | 3.56 | (December) | < 0.001 |
| True protein (g/100g) | 3.30 | 1.9 | 3.21 | (July) | 3.38 | (December) | < 0.001 |
| Casein (g/100g) | 2.72 | 1.9 | 2.64 | (July) | 2.78 | (December) | < 0.001 |
| Fat (g/100g) | 4.38 | 3.9 | 4.10 | (July) | 4.57 | (January) | < 0.001 |
| Lactose (g/100g) | 4.51 | 0.6 | 4.46 | (October) | 4.55 | (May) | < 0.001 |
| Dry matter(g/100g) | 13.3 | 1.6 | 12.9 | (July) | 13.5 | (December) | < 0.001 |
| Cell count ($10^3$ cells/mL) | 186 | 11.1 | 167 | (November) | 217 | (August) | < 0.001 |
| NPN (g/100g) | 0.182 | 3.6 | 0.172 | (January) | 0.190 | (August) | < 0.001 |
| Urea (mg/100g) | 24 | 9.0 | 22 | (December) | 26 | (August) | < 0.001 |
| Freezing point (°C) | -0.519 | 0.4 | -0.517 | (July) | -0.521 | (February) | 0.012 |

The most recent study on composition of raw dairy milk composition, on a more detailed level than fat and protein percentage only, has been performed in 1996 in Sweden (Lindmark-Månsson et al., 2003).

Compared to Swedish milk in 1996, Dutch milk in 2005 contains more fat, protein, casein and dry matter and less lactose, non protein nitrogen (NPN) and urea. Furthermore, Dutch milk has a higher freezing point and a lower cell count. Table 2 shows seasonal variation in all components.

The weekly variation in fat, protein and lactose percentage is shown in Figure 1. In general fat, protein, true protein, casein and dry matter have a minimum in the summer and a maximum in the winter. Cell count, NPN and urea, which is the main constituent of the NPN fraction, have a minimum in the winter and a maximum in the summer. Lactose has a minimum in autumn and a maximum in spring.

Although the level of the main components can differ, similar seasonal patterns have been found in other studies (Fox & McSweeney 1998; Lindmark-Månsson et al., 2003). In the Netherlands, calving patterns are non seasonal (NRS, 2008) and therefore seasonal changes of the main components are most likely of dietary origin. Of the main milk components lactose, fat and protein, lactose had the smallest and fat the highest variation with protein in between. This is in line with the general observation that fat is the most sensitive component of milk to dietary changes and lactose the least sensitive, with protein in between again (Jenkins and McGuire, 2006).

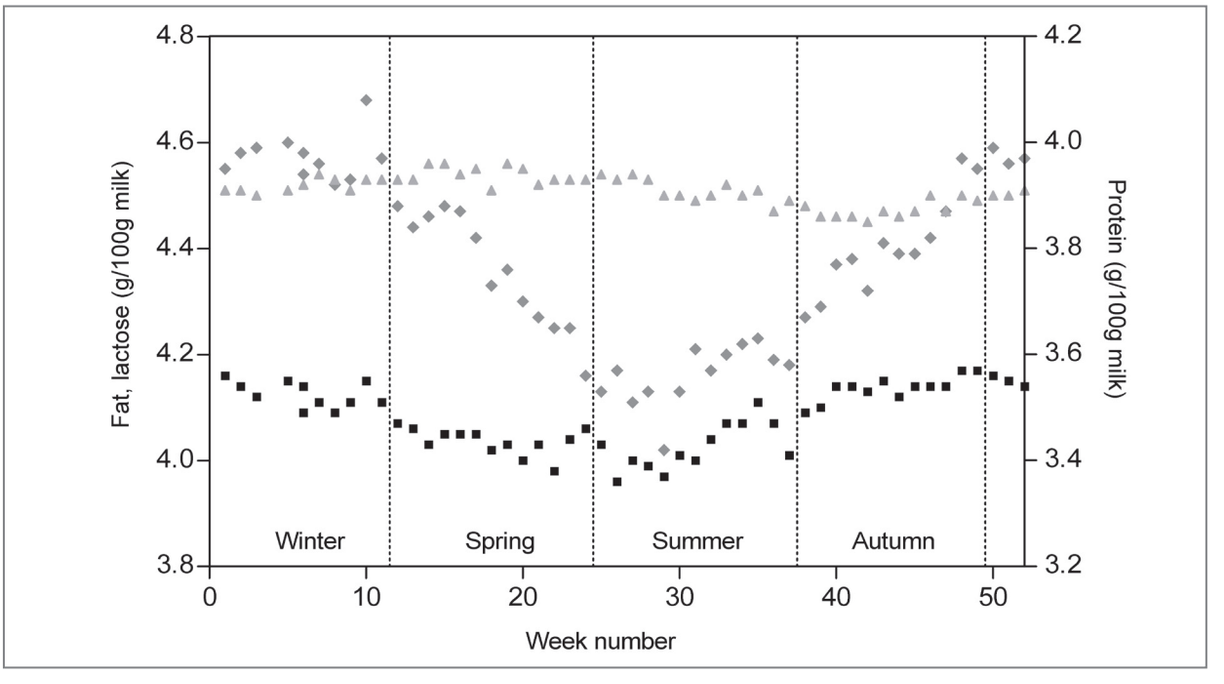


**Figure 4**: Weekly variation in the concentration of protein (■), fat (◆) and lactose (▲) (g/100g milk) in Dutch bovine raw milk in 2005.

General changes in the average Dutch dairy diet composition in 2005 can help to explain the seasonal variation in the protein and fat percentage in the milk. The major ingredients in Dutch dairy cattle rations include fresh grass, grass silage, maize silage, and concentrates (Van Bruggen, 2007). During the grazing season (roughly April until September), fresh grass is consumed by most cows (on average 25% of diet DM), but the dietary proportion of grass silage is lower than in the winter season (28 and 44%, respectively). Also, the concentrate proportion is lower in the grazing season (22%) than in the winter season (31%), whilst the proportion of maize silage (25%) does not differ. The higher concentrate to forage ratio in the winter season is generally associated with lower levels of fibre and higher levels of starch in the diet, which gives rise to an increased production of propionic acid in the rumen (Bannink et al., 2006) and increased microbial protein supply (Dijkstra et al., 1998).

Propionic acid is the major precursor of glucose followed by amino acids, and glucogenic nutrient supply leads to various hormonal signals in the cow that result in an increased milk protein concentration (Jenkins & McGuire, 2006), in line with the observed higher milk protein content in the winter period.
A lower milk fat content with fresh pasture (summer) compared with silage (winter) is commonly observed (e.g., Elgersma et al., 2004; Couvreur et al., 2006). The linolenic acid content in fresh grass is much higher than in grass silage, and high levels of linolenic acid are associated with the production of specific long chain unsaturated fatty acids that inhibit de novo fatty acid synthesis in the mammary gland and reduce the milk fat content (Baumgard et al., 2000).

### 6.3.2 Protein composition

Table 3 shows the mean relative concentrations of the 6 main milk proteins and the casein number. The 6 main proteins together comprise 90.8 % w/w of the true protein. The other 9.2 % w/w consists of minor proteins including bovine serum albumin, gamma caseins, proteose peptones, immunoglobulins, lactoferrine, lactoperoxidase, and multiple other proteins that occur only in low concentrations. Relative concentrations of the 6 main milk proteins are in line with literature values (Karman & van Boekel, 1986).

**Table 3:** Mean (g/100g true protein), coefficient of variation and seasonal variation in the protein composition of Dutch bovine raw milk in 2005

| Component | Mean | CV | Minimum | Maximum | P value |
|---|---|---|---|---|---|
| Casein number[1] | 82.3 | 0.3 | 81.8 (October) | 82.6 (January) | 0.023 |
| α-lactalbumin | 2.3 | 5.7 | 2.2 (November) | 2.4 (June) | < 0.001 |
| β-lactoglobulin | 8.4 | 3.6 | 8.1 (May) | 8.8 (February) | 0.024 |
| $\alpha_{S1}$-casein | 33.5 | * | * | * | n.s. |
| $\alpha_{S2}$-casein | 10.8 | * | * | * | n.s. |
| β-casein | 27.5 | * | * | * | n.s. |
| κ-casein | 8.2 | * | * | * | n.s. |

[1]Casein number: (casein/true protein)*100



Comparing the casein number with literature values has to be done with care because some studies use casein divided by total protein rather than casein divided by true protein to calculate the casein number. Dividing casein by total protein would yield a casein number of 78.0 instead of 82.3. The casein number is in line with other studies reporting on the casein number of milk (Auldist et al., 1998; Coulon et al., 1998; Lindmark-Månsson et al., 2003).
Table 3 shows that protein composition is rather constant throughout the season. This agrees with other studies that have shown that the casein number and protein composition can be influenced by feeding practices only to a small extent (Coulon et al., 1998; Schopen et al., submitted). The only protein that showed highly significant ($P < 0.001$) seasonal variation was α-lactalbumin. This protein shows a seasonal pattern similar to the amount of lactose in milk. This relation between lactose and α-lactalbumin might be related to the fact that α-lactalbumin is a coenzyme required for lactose production.

### 6.3.3 Fatty acid composition

*6.3.3.1 Mean fatty acid composition*

Table 4 shows the mean fatty acid composition of the Dutch bovine milk in 2005. The fatty acids C5:0, C7:0, C9:0 and C11:0 occurred in concentrations below 0.1 % and were not reported in Table 4. The cis double bond of C10:1 and C12:1 could not be ascertained at the carbon 9 position and therefore these fatty acids were reported as C10:1 and C12:1 in Table 4. The 34 fatty acids listed in the table together comprise about 96 % of the total fat. The other 4 % is composed of a very large (> 100) number of different fatty acids which were not identified and occurred in very low concentrations. These fatty acids comprise almost exclusively unsaturated fatty acids, because the saturated fatty acids known to exist in milk were all identified. Therefore, the total of the saturated fatty acids in Table 4 yields an accurate value for the total amount of saturated fat in milk. On average 70.6 % of the total fat was saturated. Mean fatty acid composition was in line with values reported by Jensen (2002).

*6.3.3.2. Seasonal variation in fatty acid composition*

Table 4 shows the seasonal variation in all fatty acids. The largest seasonal variation was found in the trans fatty acids. The concentration of C16:1 trans 9, C18:1 trans 11, and C18:2 cis 9 trans 11 (CLA) was twice as high in summer milk compared to winter milk. Based on the seasonal pattern, two groups of fatty acids could be distinguished, viz. fatty acids that have a minimum in the summer and a maximum and the winter, and fatty acids that have a minimum in the winter and a maximum in the summer. In general, the fatty acids that have a minimum in the summer are the fatty acids that are synthesized de novo, while the fatty acids that have a minimum in the winter are blood derived fatty acids. Milk C16:0 originates for some 50% from arterial blood and 50% from de novo synthesis (Barber et al., 1997), and this fatty acid was also lowest in the summer period. Similar seasonal patterns, i.e. lower values of de novo synthesized and higher values for the blood derived fatty acids in the summer, have been reported in French (Wolff et al., 1995), German (Precht & Molkentin, 1999), American (Palmquist et al., 1993), Swiss (Collomb et al., 2008) and Dutch (Muuse et al., 1986) milk. In New Zealand, rather different seasonal patterns in fatty acids have been observed (Auldist et al., 1998). The New Zealand dairy farming is mainly based on the use of pasture and seasonal calving is adopted to maximize pasture utilization. France and Germany use similar feeding strategies throughout the season as the Netherlands and also do not have a strong seasonal calving pattern.



The weekly changes in the total amount of saturated fatty acids are shown in Figure 2. The amount of saturated fatty acid is highest during the winter period and lowest during the summer. Figure 3 shows the weekly variation of C18:1 cis 9, C18:1 trans 11, C18:2 cis 9 trans 11 (CLA), and C18:3 cis 9, 12, 15. These fatty acids show a low value throughout the winter and maximum in the summer. The position of the maximum for C18:3 cis 9, 12, 15 (June) differs from the position of the maximum of the other 3 fatty acids (August).

**Table 9:** Mean (g/100 fatty acids), coefficient of variation and seasonal variation in the fatty acid composition of Dutch bovine raw milk in 2005.

| Fatty acid | Mean | CV | Minimum | | Maximum | | P value |
|---|---|---|---|---|---|---|---|
| C4:0 | 3.58 | 3.6 | 3.47 | (April) | 3.72 | (October) | 0.002 |
| C6:0 | 2.18 | 4.3 | 2.07 | (July) | 2.3 | (February) | < 0.001 |
| C8:0 | 1.33 | 4.9 | 1.25 | (July) | 1.41 | (December) | < 0.001 |
| C10:0 | 2.88 | 6.1 | 2.65 | (August) | 3.11 | (February) | < 0.001 |
| C10:1 | 0.37 | 7.7 | 0.34 | (July) | 0.41 | (December) | < 0.001 |
| C12:0 | 3.85 | 5.4 | 3.56 | (August) | 4.15 | (February) | < 0.001 |
| C12:1 | 0.11 | 7.9 | 0.10 | (July) | 0.12 | (December) | < 0.001 |
| C13:0 | 0.10 | 7.6 | 0.09 | (July) | 0.11 | (December) | < 0.001 |
| C14:0 | 11.35 | 3.4 | 10.84 | (August) | 11.86 | (February) | < 0.001 |
| C14:0 iso | 0.10 | 6.5 | 0.09 | (December) | 0.1 | (July) | < 0.001 |
| C14:1 cis 9 | 1.23 | 6.0 | 1.13 | (June) | 1.34 | (December) | < 0.001 |
| C15:0 | 1.13 | 3.1 | 1.09 | (August) | 1.18 | (December) | < 0.001 |
| C15 iso | 0.19 | 10.8 | 0.16 | (January) | 0.23 | (June) | < 0.001 |
| C15 ante iso | 0.51 | 5.6 | 0.47 | (January) | 0.56 | (June) | < 0.001 |
| C16:0 | 31.17 | 5.4 | 29.03 | (June) | 33.2 | (January) | < 0.001 |
| C16:0 iso | 0.22 | 4.0 | 0.21 | (January) | 0.24 | (July) | < 0.001 |
| C16:1 trans 9 | 0.07 | 26.8 | 0.05 | (February) | 0.1 | (August) | < 0.001 |
| C16:1 cis 9 | 1.64 | 3.7 | 1.55 | (June) | 1.72 | (October) | < 0.001 |
| C17:0 | 0.49 | 3.3 | 0.47 | (October) | 0.52 | (July) | < 0.001 |
| C17:0 iso | 0.35 | 8.9 | 0.32 | (February) | 0.41 | (July) | < 0.001 |
| C17:0 ante iso | 0.62 | 3.5 | 0.6 | (January) | 0.65 | (July) | < 0.001 |
| C17:1 cis 9 | 0.22 | 5.3 | 0.21 | (January) | 0.24 | (September) | < 0.001 |
| C18:0 | 10.25 | 6.3 | 9.42 | (December) | 11.16 | (July) | < 0.001 |
| C18:1 trans 4-8 | 0.23 | 11.6 | 0.2 | (February) | 0.27 | (July) | < 0.001 |
| C18:1 trans 9 | 0.16 | 8.6 | 0.14 | (December) | 0.17 | (July) | < 0.001 |
| C18:1 trans 10 | 0.23 | 13.0 | 0.2 | (January) | 0.27 | (September) | < 0.001 |
| C18:1 trans 11 | 1.12 | 29.9 | 0.76 | (February) | 1.6 | (August) | < 0.001 |
| C18:1 trans 15 | 0.22 | 12.3 | 0.19 | (December) | 0.25 | (July) | < 0.001 |
| C18:1 cis 9 | 17.60 | 6.8 | 16.06 | (February) | 19.18 | (August) | < 0.001 |
| C18:1 cis 11 | 0.49 | 6.2 | 0.45 | (February) | 0.52 | (June) | < 0.001 |
| C18:2 cis 9 trans 11 | 0.54 | 28.1 | 0.38 | (February) | 0.76 | (August) | < 0.001 |
| C18:2 cis 9, 12 | 1.28 | 3.1 | 1.25 | (September) | 1.34 | (May) | < 0.001 |
| C18:3 cis 9,12, 15 | 0.50 | 9.3 | 0.43 | (December) | 0.57 | (June) | < 0.001 |
| C20:0 | 0.13 | 8.5 | 0.12 | (September) | 0.14 | (December) | < 0.001 |
| Saturated fatty acids | 70.63 | 2.7 | 68.1 | (August) | 73.11 | (February) | < 0.001 |
| | | | | | | | |
| C10 index | 11.4 | 3.7 | 10.9 | (June) | 12.0 | (October) | < 0.001 |
| C12 index | 2.9 | 4.6 | 2.7 | (June) | 3.1 | (October) | < 0.001 |
| C14 index | 9.8 | 4.2 | 9.3 | (June) | 10.14 | (October) | < 0.001 |
| C16 index | 5.0 | 4.5 | 4.7 | (March) | 5.4 | (October) | < 0.001 |
| C18 index | 63.2 | 1.2 | 62.5 | (March) | 64.7 | (October) | < 0.001 |
| CLA index | 32.7 | 3.5 | 31.1 | (June) | 33.8 | (October) | < 0.001 |

*6.3.3.3 Possible causes of seasonal variation in fatty acid composition*

The seasonal patterns in the concentration of the individual fatty acids are most likely the result of many interdependent factors. However, 3 main factors, that are known to affect the fatty acid composition, might play an essential role; (1) the supply of fatty acids through the diet, (2) rumen microbial metabolism, and (3) the activity of the stearoyl-CoA desaturase (SCD) enzyme.

(1) Diet of the cow has a large impact on the fatty acid composition of the milk (Palmquist et al., 1993). In the Netherlands, an average dairy diet in the summer period has 25% (DM basis) fresh grass (van Bruggen, 2007). The winter diet does not contain fresh grass but comprises more concentrate (31%) than the summer diet (22%) and also more grass silage (44 and 28%, respectively). During wilting and ensiling of grass, extensive lipolysis and oxidative losses of fatty acids in grass occur (Dewhurst et al., 2006). Also, grass used for grazing is generally less mature than grass cut for silage making, which further decreases the levels of PUFA in grass silage compared with fresh grass, especially that of C18:3 (Ferlay et al., 2006).

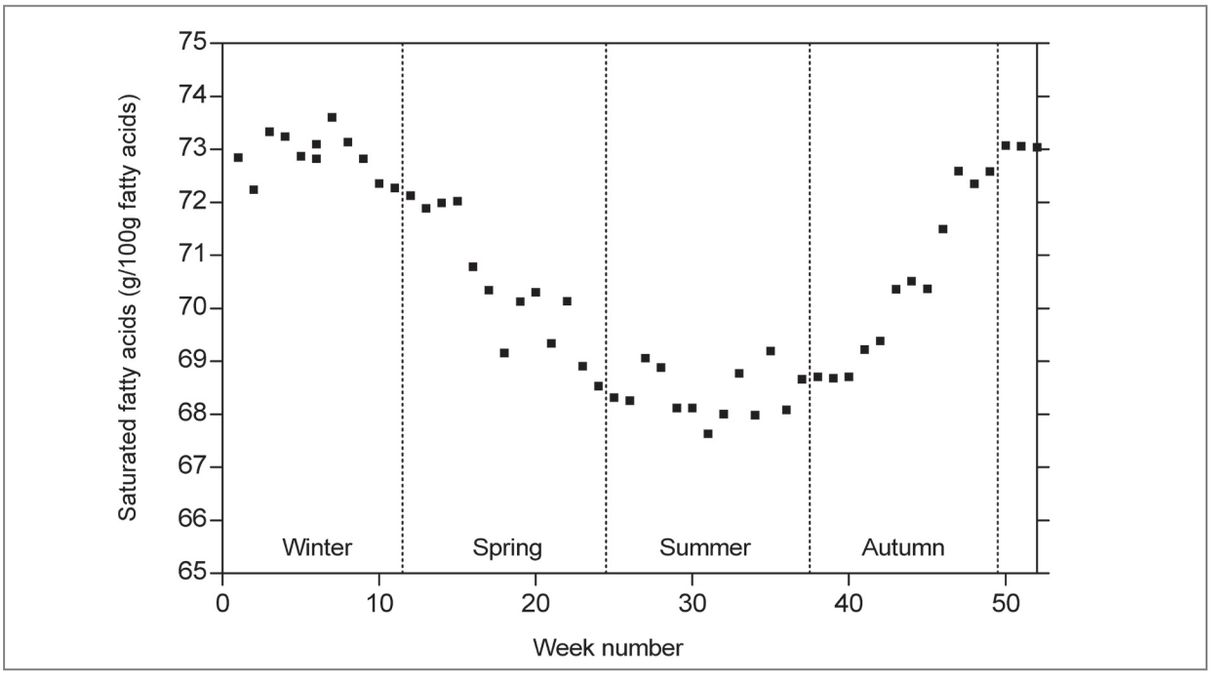


**Figure 2:** Weekly variation in the concentration of saturated fatty acids (g/100 fatty acids) in Dutch bovine raw milk in 2005.

Therefore, increasing the dietary fresh grass content generally increases C18:3 concentration of the diet and consequently raises milk fat concentrations of C18:0, C18:1 cis 9, C18:1 trans 11, CLA, and C18:3 cis 9, 12, 15, and decreases concentrations of de novo synthesized shorter chain fatty acids (review Chilliard et al., 2001).

Our findings of milk fatty acid composition in summer compared with winter are in line with these observations. The switch from the winter to the summer diet and back occurs in the Netherlands around April (week 16) and October (week 41). The exact week numbers in which this transition occurs will vary depending on weather conditions and choice of the farmers, but in general this transition will occur for most herds in the Netherlands within a relatively short time period of a couple of weeks. These transitions will cause an abrupt change in the supply of different fatty acids through the diet (Elgersma, 2004).

Figures 2 and 3 show a marked change in fatty acid composition around these transition periods. However, except for some weeks during the winter, milk fatty acid composition changes continuously throughout the season also well before and after this transition periods.

The amount of C18:3 cis 9, 12, 15, for example, decreases from the beginning of the stable period and increases again towards the end of the stable period. Furthermore, concentrations of C18:2 cis 9 trans 11 (CLA) keep increasing well after the spring transition period to a maximum in late summer. These continuous changes in the fatty acid composition might be caused by the fact that even within the summer and winter diets the composition of the diet varies. Within the grazing period, grass quality and grass supply varies and the intake of fatty acids during this period may not be constant.

The amount of C18:3 cis 9, 12, 15 in perennial ryegrass in the Netherlands in 2005 decreased slightly from April to May, in July reached the same level as April, and continued to increase afterwards (Witkowska et al., 2008).

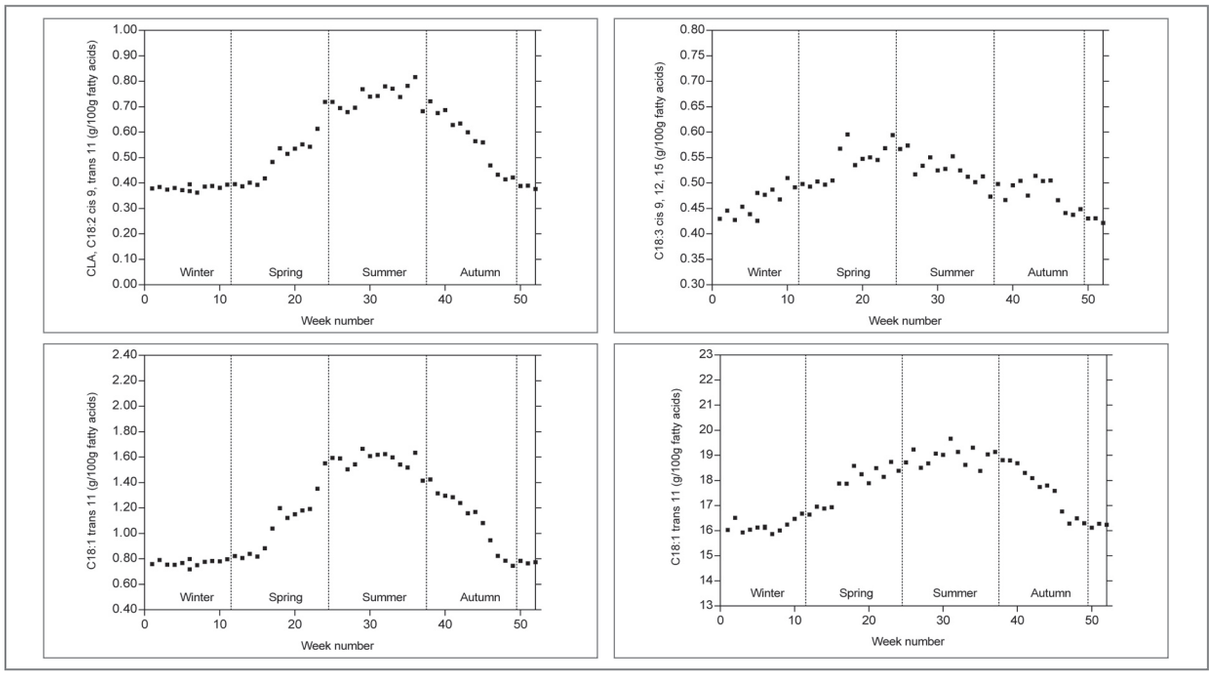


**Figure 3:** Weekly variation in the concentration of C18:2 cis 9 trans 11 (CLA), C18:3 cis 9,12,15, C18:1 trans 11 and C18:1 cis 9 (g/100 g fatty acids) in Dutch bovine raw milk in 2005.

From late summer onwards, grass growth is reduced and the proportion of grass in the total diet decreases or cows have less opportunity to select the fatty acid rich top layers of the grass, which will counterbalance the increased fatty acid content of the grass. Furthermore, depending on the quality of the grass, the farmer will supplement feeds other than fresh grass. Grass yield and voluntary intake of grass starts to decline towards the end of the summer into autumn, and farmers tend to supplement the diet to a larger extent than in spring and early summer. This might explain the increase in saturated fatty acids starting from the middle of August.

(2) The unsaturated fatty acids from the feed are extensively biohydrogenated by the rumen micro-organisms. During this process, saturated fatty acids and several intermediates, mainly trans fatty acids, are formed. Based on their metabolic pathways, different groups of bacteria can be classified that are involved in different steps of the biohydrogenation process (Lock & Bauman, 2004).

Diets that induce a marked reduction in rumen pH, such as high concentrate diets, cause a shift in bacterial populations with increased growth of Megasphera elsdenii and reduced growth of Butyrivibrio fibrisolvens (Klieve et al., 2003). Such a shift is related with incomplete biohydrogenation of poly-unsaturated fatty acids and increased formation of trans-10 fatty acids rather than trans-11 fatty acids, which reduces de novo fatty acid synthesis in the mammary gland.

Therefore, next to the direct supply of fatty acids to the milk, the diet also indirectly influences the fatty acids composition of milk by altering the rumen bacteria population or their metabolism.

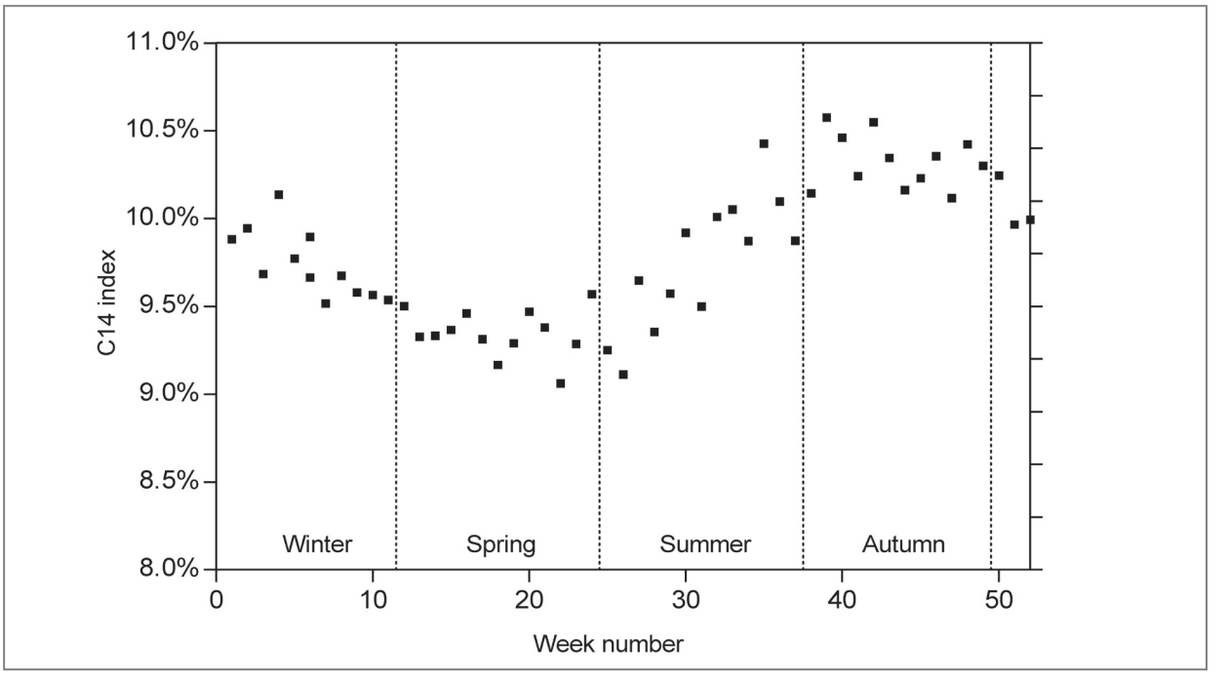




**Figure 4**: Weekly variation in the C14 index (C14:1 cis 9/(C14:1 cis 9 + C14:0)) calculated from the fatty acid composition of Dutch bovine raw milk in 2005.

(3) In various animals including ruminants, Δ-9 desaturase (stearoyl-CoA desaturase; SCD) adds a cis 9 double bond to different fatty acids (Pereira et al., 2003). Multiple studies have used the ratio of fatty acids in milk that are substrate and product of the SCD enzyme as a measure for the desaturase activity (Kelsey et al., 2003; Lock & Garnsworthy, 2003; Soyeurt et al., 2008). Although this unsaturation index can give an indication of the activity of the enzyme, it is important to realize that the index is not a direct measurement of the enzyme activity and it is also influenced by other factors (Schennink et al., 2008).

Table 4 shows the seasonal variations in the unsaturation indices. Comparing the CV values of the indices with the CV values of the substrates suggests that the contribution of the seasonal variation in the desaturase activity to the seasonal variation of the fatty acids that are products of this enzyme varies for the different fatty acids. For some desaturase products in particular

C14:1 cis 9 this contribution was considerable but for C18:1 cis 9 and CLA this contribution was very small. This indicates that the seasonal variation of in particular CLA is mainly caused by the seasonal variation in the fatty acid supply to the mammary gland (C18:1 trans 11).
The weekly pattern of the C14 index (Figure 5) shows that the C14 index was lowest at the end of the spring and highest at the beginning of the autumn. Similar seasonal patterns were found for the other indices (Table 4), although the lowest values for the C16 index and C18 index were found earlier in spring (March instead of June). These results disagree with the study of Lock & Garnsworthy (2003) who showed that SCD activity was highest in May and June (C14 index) or July (C16 and CLA index), which could partly explain the higher value of unsaturated fatty acids in summer milk. However, the present findings for C18:0 are also not in line with those of Lock and Garnsworthy (2003), which was lowest in June in their study but almost highest in ours. In addition C18:1 cis 9 and C18:3 cis 9, 12, 15 did not show a clear seasonal pattern in Lock & Garnsworthy (2003) but had a distinct seasonal pattern in our study.

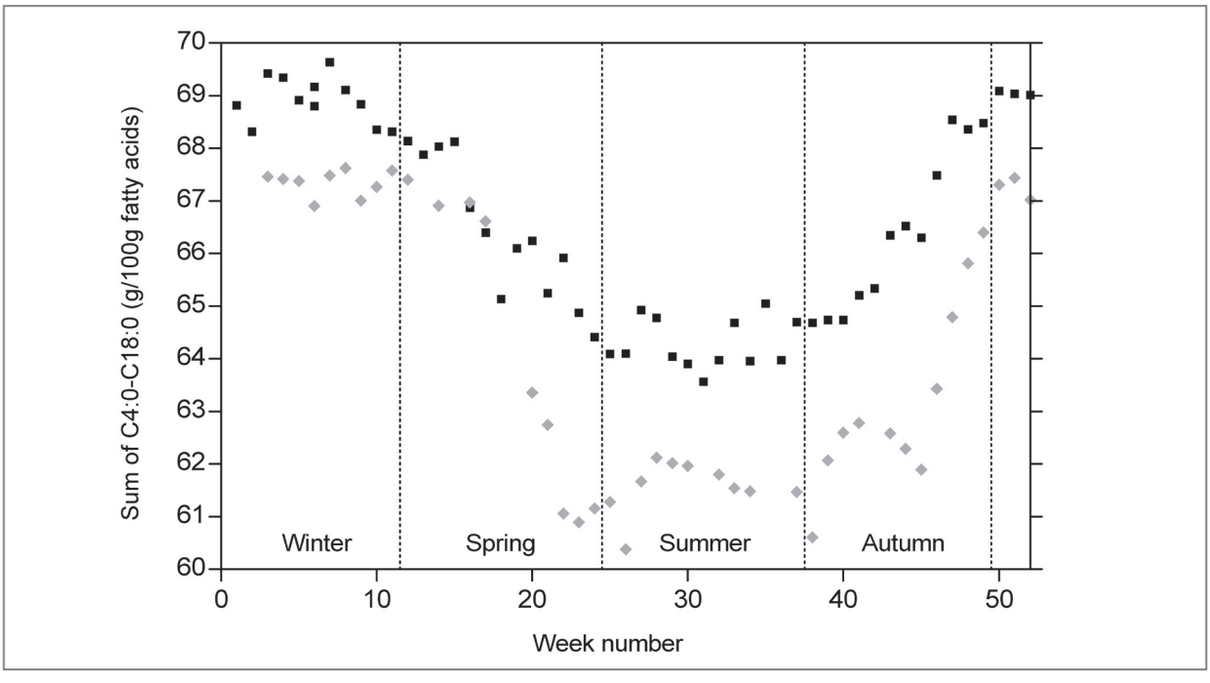


**Figure 5:** Weekly variation in the concentration of C4:0-C18:0[1] (g 100/g fatty acids) in Dutch bovine raw milk in 2005 (■) and in 1992 (♦). [1]Sum of C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0 and C18:0.

*6.3.3.4 Comparison of the fatty acid composition between 1992 and 2005*

Figure 5 shows the sum of the even saturated fatty acids C4:0-C18:0 in Dutch raw milk in 1992 and in 2005. The samples of 1992 were collected in the same manner as in 2005 and analyzed with the same technique on the same location as in 2005. The figure shows that the seasonal pattern in both years is similar. However, in 1992, the amount of C4:0-C18:0, and most likely the total amount of saturated fatty acids, was lower compared to 2005 (64.2 % and 66.6 %, respectively). This change might be caused by changes in feeding practices or by the breeding strategies used. It is also shown in Figure 5 that in summer the difference in the amount of C4:0-C18:0 between 2005 and 1992 was bigger than in winter. This might be caused by the fact that during the grazing season in 2005 a higher percentage of the cows did not graze outside at all and a smaller percentage of cows had unrestricted (day and night) grazing compared to 1992, while

in the winter period all cows were confined both in 2005 as well as in 1992. Data on fresh grass intake in 1992, obtained in the same way as described by Van Bruggen (2007), also showed a higher dietary grass proportion in 1992 than in 2005 (34 and 12%, respectively).
As discussed previously, a higher proportion of fresh grass in the diet reduces the content of saturated fatty acids in milk. Moreover, the proportion of maize silage in the diet increased from 15% (1992) to 25% (2005) which may result in an increased saturated fatty acid content in milk compared with grass silage. Finally, due to environmental pollution issues, N fertilization levels of grassland were lower in 2005 than in 1992, resulting in grass and grass silages with a lower crude protein content. Since the levels of fatty acid in grass, and in particular that of C18:3, are positively related to the crude protein content of grass (Boufaïed et al., 2003), this might further explain the reduced milk contents of saturated fatty acids in 1992 compared with 2005, both in the grazing and non-grazing season. The difference between 2005 and 1992 might also be partly the result of the breeding strategies applied. Stoop et al. (2008) showed that selection for higher fat % might result in higher proportions of C16:0. In 1980-1984, Dutch butterfat had a mean proportion of C16:0 of 27.5 % (Muuse et al., 1986). In 1992, the mean proportion of C16:0 in raw milk was 28.3 % and in 2005 it was 31.2 %. The differences in fatty acid composition between 1992 and 2005 clearly shows that although milk production and fat and protein content of the milk of dairy cows in the Netherlands have improved continuously, the quality of the milk fat might have changed in an undesirable manner from a human health perspective. This emphasises the need to monitor the detailed milk composition including the concentration of individual fatty acids, to identify how changes due to breeding and feeding affect the nutritional value and technological properties of milk.

## 6.4 Conclusions

This work provides a standard for the composition of Dutch bovine raw milk in 2005 and provides insight in the seasonal changes in milk composition. Large seasonal variation exists in the concentration of the main milk components and milk fatty acid composition. The most pronounced changes due to season occurred for fatty acids, with a clear decrease in proportion of saturated fatty acid and a large increase in trans fatty acids like CLA, during the grazing season. Compared with a similar, representative sample of bovine milk in 1992, the level of saturated fatty acids in milk markedly increased. To get insight in future changes and to speculate whether these changes will negatively influence the nutritional value or technological properties of milk, this research should be repeated in the next decade.

# 7

# General discussion

## 7.1 Milk protein composition

### 7.1.1 Variation in milk protein composition

In chapter 2 the variation in detailed milk protein composition between 1948 individual cows was reported. A major difference with previous studies is the applied analytical method. We used capillary zone electrophoresis (CZE) while previous studies on variation in milk protein composition mainly used High Performance Liquid Chromatography (HPLC) (Bobe et al., 1998, Wedholm et al., 2006) or gel electrophoresis (Ng Kwai Hang et al., 1987). With the CZE method it was possible to determine the relative concentrations of the major milk proteins α-LA, β-LG, $\alpha_{S2}$-CN, $\alpha_{S1}$-CN, κ-CN and β-CN with a good reproducibility. With HPLC and gel electrophoresis reproducibility is less and quantification of $\alpha_{S2}$-CN is difficult. The CZE technique makes it possible to determine the relative concentration of $\alpha_{S2}$-CN that contains 10, 11 and 12 phosphate groups and the relative concentration of $\alpha_{S1}$-CN that contains 8 and 9 phosphate groups. The results presented in Chapter 2 are unique and give insight in the variation in the degree of phosphorylation of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN. Analysis revealed that the relative concentration of specific phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN were related to each other. Both $\alpha_{S2}$-CN-10P and $\alpha_{S2}$-CN-11P but not $\alpha_{S2}$-CN-12P, had a low correlation with $\alpha_{S1}$-CN-8P and a very high correlation with $\alpha_{S1}$-CN-9P. The cause and implication of this remarkable observation is not known and is worth further investigating.

With CZE we could identify most of the common genetic variants of the main milk proteins. However, the CZE technique was unable to separate the κ-CN A and B. Therefore, a DNA technique was used to identify the genetic variants of κ-CN. We sequenced the exon 4 of the κ-CN gene of 95 animals and identified the genetic variants A, B and E. Subsequently, all animals were genotyped for these three κ-CN variants by a SNaPshot assay. The κ-CN E variant was not detected before in Dutch cows. Most likely the κ-CN E variant was not detected before in Dutch cows because the identifying techniques used in previous studies could not identify this variant. Therefore it is likely that the κ-CN E variant also occurs in other cow populations but has not been detected. The detection of this E variant might be important because of its possible association with poor milk coagulation properties (Hallen et al., 2007, Ikonen et al., 1999). For identification of all genetic variants of milk proteins, sequencing of the DNA is a better technique than separation of the proteins in milk. We did not sequence the complete coding regions of all six main milk protein genes in all animals. Therefore, it is possible that in our population genetic variants of the main milk proteins exist other than those reported in Chapter 2.

The milk protein composition between individual cows shows large variation (Chapter 2). Schopen et al. (2008) used these data to estimate how much of the variation is caused by genetic and by herd effects. The main results of that study are summarised in Table 1. In general, herd effects are very low, indicating that feeding can influence the milk protein composition only to a very small extent. This agrees with other studies (Coulon et al., 1998, DePeters and Cant, 1992) and with the rather constant protein composition throughout the season as reported in Chapter 6. The moderate to high heritabilities indicate that a large part of the variation in milk protein composition is due to genetic variation between cows, implicating that milk protein composition can be changed through selective breeding.

### 7.1.2. Effects of genetic variants of the milk proteins

In chapter 3 it is shown that genetic variants of the main milk protein explain a part of the genetic variation in milk protein composition. Therefore, protein variants can be a useful tool to breed or select animals that produce milk with an improved milk protein composition. In the Netherlands more than 50% of the raw milk is used for cheese production and therefore an increase in the amount of casein in the milk would be very beneficial for the Dutch dairy industry.

The B variant of β-LG is associated with a lower relative concentration of β-LG and a higher relative concentration of the other 5 main milk proteins. The β-LG BB genotype is associated with 3% more casein than the AA genotype and is not associated with a difference in any of the other milk production traits. These findings are consistent with other studies (Bobe et al., 1999, Lunden et al., 1997), indicating that the effect of β-LG variants on the casein concentration in milk is a major effect that exists in multiple cow populations. A new finding is that the composition of the casein is not influenced by the A or B variant of β-LG. This is of practical importance because this implies that selection for the β-LG B variant will most likely have no influence on the coagulation process of the milk and the properties of the cheese. This means selection for the B variant will increase the profitability of the cheese production without any negative effects on cheese properties  It is important to realize that producing cheese automatically implies producing whey. The economical value of whey is significant and mainly based on the protein content. Selection for the B variant of β-LG will have a major impact on the value of the whey as milk from cows homozygous for the B variant has a 23% lower concentration of β-LG compared to average Dutch milk. However, because prices for casein are much higher than prices for whey, the decrease of the value of the whey will not outweigh the benefits of a more profitable cheese production.  The change in composition of the whey associated with selection for the B variant will also have an effect on the properties of the whey products such as whey protein concentrates because the ratio between α-LA and β-LG is different (relatively more α-LA). This changed composition of the whey is beneficial when using the whey as ingredient for instant formula since human milk contains no β-LG and a relatively high concentration of α-LA. Furthermore, a lower concentration of β-LG in milk might result in a decrease of the fouling rate of heating equipment because this fouling rate depends on the β-LG concentration (Elofsson et al., 1996). Apart from the effects of the β-LG variant on the concentration of the protein, the B variant is also a different protein than the A variant with a slightly different amino acid composition. This also has an effect on the properties of the protein such as the thermal stability (Boye, 2004). Based on all these effects it can be concluded that selection for the B variant of β-LG will increase the profitability of the dairy industry.



| Protein | Heritability | Herd effect |
|---|---|---|
| α-LA | 0.55 | 0.16 |
| β-LG | 0.80 | 0.05 |
| $\alpha_{S1}$-CN | 0.47 | 0.12 |
| $\alpha_{S2}$-CN | 0.73 | 0.13 |
| β-CN | 0.25 | 0.16 |
| κ-CN | 0.64 | 0.12 |

**Table 1:** Heritabilities and herd effects of the six main milk proteins. (adapted from Schopen et al. (2008))

The B variant of κ-CN is associated with increased κ-CN concentration but also with increased $\alpha_{S2}$-CN concentration and a decreased $\alpha_{S1}$-CN concentration. The effect of the B variant of κ-CN on the concentration of κ-CN is consistent with other studies in other cow populations. Several studies have shown that the B variant of κ-CN is associated with favorable cheese properties (Van Den Berg et al., 1992; Walsh et al., 1998; Marziali and Ng-Kwai-Hang, 1986; Schaar et al., 1985). It is not clear whether the favorable cheese properties of the κ-CN B variant are a result of the increased κ-CN concentration only, the change in concentration of the other caseins, or a change in micelle properties. The B variant of κ-CN is also associated with the protein content and composition of rennet whey. Most cheese is produced by the addition of rennet that contains chymosin (Fox and McSweeney, 1998). Chymosin cleaves κ-CN into para-κ-CN and the casein macro peptide (CMP). The CMP represents about 30% of the κ-CN molecule and is lost in the whey. A higher κ-CN concentration in the milk will therefore result in a higher concentration of CMP in the whey.

The β-CN $A^2$ variant was associated with a higher concentration of β-CN and $\alpha_{S2}$-CN and with a lower concentration of $\alpha_{S1}$-CN and κ-CN compared to the $A^1$ variant. The effect of β-CN variants on the concentration of individual proteins as reported in literature is not consistent (Graml and Pirchner, 2003, Ng Kwai Hang et al., 1987). In this study the effects of the β-CN variants do not seem to be additive for all proteins. For example, cows that possess both the $A^1$ and the $A^2$ variant (heterozygous individuals) produced milk with more β-CN than cows that are homozygous for $A^1$ or $A^2$ ($A^1A^1$ or $A^2A^2$). This phenomenon, called dominance, is known in livestock and plant breeding and has been reported before in association with β-CN variants (Graml and Pirchner, 2003). Based on our results selection for β-CN variants can be used to change the casein composition in milk. However, little is known about the effect of casein composition on the technological properties of milk (Hallen et al., 2007) and more research is needed.

In chapter 3 we show that, in our population, the overall effect of a specific casein locus could only to a small extent be attributed to a linked casein locus. However, the κ-CN E variant predominantly occurred with the β-CN $A^1$ variant, and the β-CN B variant predominantly occurred with the κ-CN B variant. Thus, for κ-CN E and β-CN B, it is hard to determine whether the observed effects on protein composition are a result of the variant itself or are caused by the closely linked variant on the other locus. Differences in haplotypes, i.e. linkage variants, between cow populations or breeds might therefore cause different associations of single variants with milk composition. For example, if in another cow population the κ-CN E variant does not predominantly occur with the β-CN $A^1$ variant it is well possible that the E variant in that population is associated with a different protein composition. Comparing the effects of casein haplotypes with the effects of single casein variants can provide better insight into what really underlies the effect of a variant on protein composition. The close linkage of the casein genes is important if one is interested in selecting cows with a specific casein variant because selection of a favorable casein variant could result in the indirect selection of an unfavorable linked casein variant.

Selecting specific genetic variants of multiple proteins simultaneously could result in even larger changes in milk protein composition than selecting a specific variant of only one protein. Based on the effects reported in this study the β-LG B variant and the β-κ-CN haplotype $A^2B$ are associated with a milk protein composition that is more suitable for cheese production (see Table 2).

**Table 2:** Relative difference between mean protein composition of milk of cows homozygous for β-LG B and β-κ-CN $A^2B$ (Cheese cows) and mean protein composition of milk of the 2000 Holstein-Friesian cows used in this study.

| | α-LA | β-LG | $\alpha_{s1}$-CN | $\alpha_{s2}$-CN | β-CN | κ-CN | CN-index |
|---|---|---|---|---|---|---|---|
| Cheese cows | -4% | -23% | = | +3% | = | +13% | +2.5% |

### 7.1.3. Future possibilities

We have shown that large variation in milk protein composition between cows exists and genetic variants of the main milk proteins explain a part of the genetic variation in milk protein composition. These genetic variants can be used as a DNA marker to select and breed for cows that produce milk with an improved milk protein composition. However the protein variants studied in this thesis explain only a part of the genetic variation in milk protein composition. This means that more genes with an effect on milk protein composition exist. Identifying these genes or markers closely linked to these genes can help to further improve the protein composition in milk. It is also possible to change the protein composition in milk without the use of DNA markers. Changes in protein composition can also be achieved with traditional breeding techniques that involve phenotyping of offspring of breeding bulls. However such a strategy involves analyzing of a larger number of samples. At the moment determination of the protein composition of milk is costly and time consuming. The development of quick, cheap and robust methods to determine the protein composition in milk can help in breeding for protein composition.

There are limits to the extent in which the milk protein composition can be changed. Especially for proteins that have a clear biological function it is unlikely that very large changes in its concentration are achievable. For example κ-CN plays a major role in stabilizing the micelle. It has been shown that mice in which the κ-CN gene was knocked out, fail to lactate (Shekar et al., 2006). Therefore, it is not likely that a cow can produce milk without κ-CN. However, the variation in relative κ-CN concentration in milk samples of our population showed that a cow can produce milk with only half of the concentration of κ-CN found in normal milk. For other proteins, like β-LG, the biological function is not as clear. Furthermore, β-LG is also not expressed in milk of some of other species (e.g. human). Therefore, it can be hypothesized that it might be possible also for a cow to produce milk with only very low concentrations of this protein.

## 7.2 Milk fatty acid composition

### 7.2.1 Variation in milk fatty acid composition

In Chapter 4 the variation in milk fatty acid composition was reported and it was shown which part of this variation was caused by genetic and by herd effects. The results show that milk fatty acid composition varies considerably between individual cows and can be changed both by feeding and by breeding. Previous studies focussed on the possibilities to change fatty acid composition by feeding. Very few studies investigated the possibilities to change the fatty acid composition of milk by breeding. We studied a large number of different fatty acids which gave us also insight in the genetic and herd variation in for example the odd and branched chain fatty acids. These fatty acids are of interest because they can possibly be used as indicators of rumen bacteria (Vlaeminck et al., 2006).

The extent to which the individual FA can be changed by breeding and feeding varies and depends on the way the FA is synthesised. In general the fatty acids that can be changed best by feeding are the blood derived fatty acids and the fatty acids that can be best changed by breeding are the de novo synthesised fatty acids. This observation agrees with most other studies where it is shown that feeding has a major impact on especially the blood derived fatty acids (Moate et al., 2007). This also agrees with the results given in Chapter 6 where it is shown that the concentration of especially the blood derived fatty acids varies considerably throughout the season while the variation in the de novo synthesised fatty acids is smaller. Seasonal changes in fatty acids are considered to be of dietary origin.

The genetic and herd parameters were used to investigate the origin of FA in milk. In general the results confirm the well known origin of some FA in milk but they also provide some new insight in the possible origin of other FA in milk. For example, analyzing genetic and herd parameters showed that C12:0 had a much higher herd effect than all other de novo synthesised FA. Research to the diet of Dutch cows revealed that C12:0 occurs in high concentrations in palm kernel flakes which are a major component of Dutch concentrates. This explained the high herd effect for C12:0 but also indicated that it might be possible to increase the concentration of FA, that are considered to be fully synthesised de novo, by incorporating these FA in the feed of the cow. This shows that although, statistical analyses of milk FA composition data can not provide definite answers about the origin of FA in milk it helps to form a hypothesis that can be tested in further, more controlled, studies.



Knowledge about the origin of FA is important when one is interested in changing the fatty acid composition of milk. For example, if the objective is to increase the concentration of CLA in milk, it is important to realize that this fatty acid mainly originates from the action of desaturase on C18:1 trans 11 and therefore, when desaturase activity is constant, an increase in CLA will most likely be accompanied by an increase in C18:1 trans 11. However, if desaturase activity can be altered, it might be possible to increase the concentration of CLA at the expense of C18:1 trans 11.

### 7.2.2. Effects for variants of DGAT1 and SCD1

In chapter 5 it is shown that unsaturation of milk fatty acids has a clear genetic component and that variants of DGAT1 and SCD1 gene explain a part of this genetic variation. Therefore, the SCD1 and DGAT1 polymorphism could be a useful tool to select cows that produce milk with a different fatty acid composition. The degree of unsaturation of fatty acids might be relevant from a human health perspective (Mensink et al., 2003) but it might also influence the technological properties of fat based dairy products (Walstra, 2006).

We show that the SCD1 V variant is associated with a lower C10, C12 and C14 index and with a higher C16, C18 and CLA index and no effect on the total unsaturation index. This suggests that this polymorphism does not affect the overall activity of the enzyme, but alters the substrate specificity. Substrate specificity of SCD enzymes has been demonstrated for SCD isoforms in knockout studies in mice (Miyazaki and Ntambi, 2003). The DGAT1 polymorphism also affects the unsaturation indices. This is an important observation because these unsaturation indices are frequently used as a proxy for the desaturase activity (Lock and Garnsworthy, 2003, Soyeurt et al., 2008). Our results show that the unsaturation index is influenced by other factors than the activity of the desaturase enzyme alone.

Based on the current general opinion about the effects of specific FA on human health, selection of cows with the DGAT1 A and SCD1 V polymorphism would result in milk with a better FA composition. Table 3 shows the relative difference in fatty acid composition of milk of a better fat cow compared to average Dutch milk.

**Table 3:** Relative difference between mean fatty acid composition of milk of cows homozygous for DGAT1 A and SCD1 V (better fat cow) and mean fatty acid composition of milk of the 2000 Holstein Friesian cows used in this study.

| | Unsaturated | C16:0 | CLA | Linoleic acid | α-linoleic acid |
|---|---|---|---|---|---|
| Better fat cow | +5% | -5% | +21% | +7% | +2% |

### 7.2.3. Future possibilities



We have shown that large variation between cows exists in milk fatty acid composition and that a part of this variation is due to genetic differences. Furthermore we have shown that variants of SCD1 and DGAT1 explain a part of the genetic variation in the concentration of different fatty acids. For most fatty acid only a relatively small part of the genetic variation is explained by the variants studied (Chapter 5, (Schennink et al., 2007). This means more genes exist that affect the fatty acid composition in milk. Identifying these genes or markers closely linked to these genes can help to further improve the fatty acid composition in milk. Changes in fatty acid composition can also be achieved with traditional breeding techniques that involve phenotyping of offspring of breeding bulls. Fatty acid composition might be determined fast and cheap by mid-infrared spectrometry (Soyeurt et al., 2006). This technique is already applied during routine milk recording of individual cows. If this technique can be used to accurately determine the fatty acid composition in milk it would be possible to cost effectively breed for a specific fatty acid composition without the use of DNA markers. However, compared to gas chromatography the

accuracy and the number of different fatty acids that can be measured by mid-infrared spectrometry is much lower. Therefore, it depends on which fatty acids are of interest whether this infrared technique will provide sufficient information.

There will be biological limitations in the concentration of individual fatty acids in milk fat. Fluidity of the milk fat has to be between certain limits in order for the cow to be able to lactate. The fluidity is determined by the chain length, and the number, position and orientation (cis or trans) of the double bonds of the individual fatty acids but also by the stereospeifi c arrangement of the different fatty acids in the triglyceride molecule (Beaulieu and Palmquist, 1995). The cow has a wide range of possibilities (desaturating of fatty acids, producing fatty acids with different chain lengths, or esterification on specific position in the triglyceride) to counter balance changes in individual fatty acids while remaining fluidity of milk fat as a whole. Knowledge about these processes is important when developing strategies to further change the fatty acid composition of milk.

## 7.3 Seasonal variation in the composition of Dutch bovine milk

In Chapter 5 the composition of Dutch milk and the seasonal variation in this composition was determined by analyzing raw milk samples that were representative for the complete Dutch milk supply. The results reported in chapter 6 show that large seasonal variation exists in all main components in milk. For example, fat and protein percentage of milk showed a maximum monthly average of 4.57 % and 3.56 % during winter and a minimum monthly average of 4.10 % and 3.39 % respectively, during summer. Similar seasonal trends in the concentration of fat and protein in dairy milk has been observed in a study by (Lindmark Mansson et al., 2003). It is shown that milk protein composition was rather constant throughout the season. This agrees with other studies that have shown that the casein number and protein composition can only to a small extent be influenced by feeding practices (Coulon et al., 1998); Schopen et al., accepted). Milk fatty acid composition varied considerably during the season. In general, the fatty acids that have a minimum in the summer are the fatty acids that are synthesized de novo, while the fatty acids that have a minimum in the winter are blood derived fatty acids. Similar seasonal patterns, i.e. lower values of de novo synthesized and higher values for the blood derived fatty acids in the summer, have been reported in French (Wolff et al., 1995), German (Precht and Molkentin, 1999), American (Palmquist et al., 1993), Swiss (Collomb et al., 2008) and Dutch (Muuse et al., 1986) milk. The seasonal patterns in the concentration of the individual fatty acids are most likely the result of many interdependent factors. In chapter 6 the possible causes of the seasonal variation in fatty acid composition are discussed. Seasonal changes in the diet of Dutch dairy cows might partly explain the seasonal changes in the fatty acid composition of milk because changes in the diet that occur in the Netherlands during the season (van Bruggen, 2007) alter the supply of fatty acids (Elgersma, 2004) and the rumen microbial metabolism (Klieve et al., 2003). Furthermore it is shown that the activity of SCD, as measured by unsaturation indices, also varies throughout the season. The contribution of the seasonal variation in the desaturase activity to the seasonal variation of the fatty acids that are products of this enzyme varies for the different fatty

acids. For some desaturase products in particular C14:1 cis 9 this contribution was considerable but for C18:1 cis 9 and CLA this contribution was very small. This indicates that the seasonal variation of in particular CLA is mainly caused by the seasonal variation in the fatty acid supply to the mammary gland (C18:1 trans 11).

The results in chapter 6 provide a standard for the detailed composition of Dutch milk in 2005. A standard is important to detect favorable and unfavorable changes in milk composition in the future. An investigation of the Swedish dairy milk composition in the 1970s and 1996 has shown a substantial decrease in the casein number of Swedish raw milk during this period (Lindmark Mansson et al., 2003). Such a decrease in casein number would also be unfavorable for the Dutch dairy industry, because in the Netherlands about 50% of the milk is used for cheese production. It is not known whether the protein composition in milk in the Netherlands has changed in the past decades. However, the results in chapter 3 show that the frequency of specific genetic variants of the main milk proteins has changed compared to 1989. For example, the frequency of the β-LG B variant had decreased from 0.58 to 0.42 and the frequency of the κ-CN B variants had increased from 0.15 to 0.31. It is also shown in this chapter that these variants have an effect on the protein composition in milk. Based on these effects it can be hypothesized that, compared to 1989, in 2005 the concentration of β-LG and κ-CN in Dutch milk has increased. An increase in the concentration of β-LG would be unfavorable and an increase in κ-CN would be favorable for cheese production. Therefore, it is difficult to state whether compared to 1989 the protein composition in 2005 is better or worse for the profitability of the dairy industry. In Chapter 6 it is shown that the fatty acid composition of milk in 2005 has changed compared to 1992. The concentration of C4:0-C18:0 (sum of the even saturated fatty acids C4:0 to C18:0) was higher in 2005 compared to 1992 (66.6 % and 64.2 %, respectively). These changes in milk composition in time are the results of changes in the feed of the cows, changes in herd management and breeding practices.

## 7.4 Opportunities for milk differentiation

The results described in this thesis provide a tool to select and breed for cows, that produce milk with a protein or fatty acid composition that is more suitable for a specific application. It is possible to use protein or fatty acid composition as a breeding objective for the whole Dutch cow population. The main question remains where to select for. An optimal milk composition for one application might differ from an optimal milk composition for another application. Applying milk differentiation could help to overcome this problem.

Milk differentiation on composition can be applied in different ways but two possible scenario's for milk differentiation are likely. (1) Milk differentiation on composition in which complete herds are used to produce one type of milk for one specific application and (2) Milk differentiation on composition in which in one herd two types of milk are produced. Which scenario is preferred depends on multiple factors like logistical costs, farm size, farm management, costs of an extra milk storage tank, milk prices and future developments within the dairy sector but the opportunities described in this thesis can be helpful for both. In the Netherlands dairy factories are specialized, producing only a few and not the whole range of dairy products. Therefore, it would be beneficial to use herds that are located near a cheese factory to produce milk that is more suitable for cheese production. As discussed previously different types of milk can be produced by using the genetic variation in milk composition or the effect of the diet of the cow on milk composition. By combining these effects even larger changes can be achieved. For example if one is interested in producing milk with a higher CLA concentration it is possible to use cows with the DGAT1 and SCD1 variants that are associated with higher CLA levels and feed these cows a diet that further promotes the CLA concentration in their milk.

Next to the dairy industry the scenario's of milk differentiation should also involve the dairy farmers and the breeding companies. An economical incentive for the farmers is a prerequisite because an effort from the farmers is needed. An incentive is to pay farmers based on the casein concentration or on the amount of unsaturated fat in their milk. Furthermore, farmers can be paid on the relative occurrence of specific genetic variants in their milk. The breeding companies can provide the information on the breeding values for composition of the breeding bulls available for most farmers. Therefore, to optimally benefit from the opportunities described in this thesis cooperation between all operators within the dairy chain (i.e. the breeding companies, farmers and the dairy industry) is needed and the use of milk differentiation is preferred.

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# S

# Summary

Milk and dairy products are important components of western diets. The composition of raw milk determines, to a large extent, the nutritional value and the technological properties of milk and dairy products, and the farmers milk price. Therefore, the composition of milk is of great importance for the farmers and the dairy industry. This thesis deals with the opportunities to improve the protein and fatty acid composition in milk.

**Chapter 1** is a general introduction to this thesis. In **chapter 2** a capillary zone electrophoresis (CZE) technique was tested and it was shown that CZE was a good technique to determine the variation in protein composition in milk. The method was used to measure the relative concentration of the six main milk proteins in milk of 1948 cows. The results showed that there was large variation in the protein composition in milk of individual cows. The method was also used to measure the relative concentration of two different phosphorylation states of $\alpha_{S1}$-casein ($\alpha_{S1}$-CN-8P and $\alpha_{S1}$-CN-9P) and three of $\alpha_{S2}$-casein ($\alpha_{S2}$-CN-10P, $\alpha_{S2}$-CN-11P and $\alpha_{S2}$-CN-12P) in all 1948 milk samples. These analyses revealed that the relative concentration of specific phosphorylation states of $\alpha_{S1}$-CN and $\alpha_{S2}$-CN were related to each other. Both $\alpha_{S2}$-CN-10P and $\alpha_{S2}$-CN-11P but not $\alpha_{S2}$-CN-12P, had a low correlation with $\alpha_{S1}$-CN-8P and a very high correlation with $\alpha_{S1}$-CN-9P. Furthermore, it was shown that CZE could be used to identify most common genetic variants including the κ-casein E variant which had not been identified by CZE before.

In **chapter 3** the relative protein concentrations and genetic variants measured in milk of 1912 Dutch Holstein Friesian cows were used to determine the effects of genetic variants on the relative concentrations of the six major milk proteins and milk production traits. It is shown that these genetic variants have a major impact on the protein composition of milk and explain a considerable part of the genetic variation in milk protein composition. This indicates that protein genetic variants can be a useful tool to select and breed for cows that produce milk with an improved protein composition. The frequencies of the genetic variants in the Dutch Holstein-Friesian population in 2005 were compared with the frequencies in 1989. This comparison showed that the frequencies of specific genetic variants have changed in time. Based on the effects of genetic variants on protein composition, this suggests that the protein composition of Dutch milk has changed in time.

In **chapter 4**, a total of 39 different FA were characterized based on genetic and herd parameters to investigate the origin of these FA in milk. Fatty acid composition in milk of 1912 Dutch Holstein Friesian cows was measured by gas chromatography. The proportion of the variation in milk fatty acid composition caused by genetic and herd effects was calculated. In addition,

genetic and herd correlations among the fatty acids were estimated and a clustering technique was used to visualise these correlations. The results of these analyses provided new insights in the possible origin of some FA in milk. It was suggested that in Dutch milk C12:0 is not completely synthesised de novo but also partly blood derived. The results also indicated that C20:0 in milk is formed from the action of elongase enzymes on C18:0 and that the odd-chain FA C5:0-C13:0 and a part of C15:0 and C17:0 are synthesised de novo while the other part of C15:0 and C17:0 is blood derived. Furthermore, the genetic and herd parameters reported provided an overview of the opportunities to change the concentration of individual FA both by breeding and feeding. It is shown that the extent to which the individual FA can be changed varies greatly and depends on the origin of the different FA in milk.

In **chapter 5** the fatty acid composition of milk of 1933 Dutch Holstein-Friesian cows was measured and used to estimate the genetic variation in milk fatty acid unsaturation indices and the effect of the SCD1 A293V and DGAT1 K232A polymorphisms on these indices. The results showed that unsaturation of milk fatty acids has a clear genetic component and the variants of DGAT1 and SCD1 gene explained a part of this genetic variation. This indicates that the SCD1 and DGAT1 polymorphism could be a useful tool to breed or select cows that produce milk with an improved fatty acid composition. In this chapter it is also shown that the SCD1 V variant was associated with a lower C10, C12 and C14 index and with a higher C16, C18 and CLA index and no effect on the total unsaturation index. This suggests that this polymorphism does not affect the overall activity of the SCD1 enzyme, but alters the substrate specificity.

In **chapter 6** the detailed composition and seasonal variation in Dutch dairy milk was determined. For this purpose raw milk samples that were representative for the complete Dutch milk supply were collected weekly from February 2005 until February 2006 and analyzed on detailed composition. The results provided a standard for the concentration of the main components and the protein and fatty acid composition in Dutch milk in 2005. The weekly samples showed large seasonal variation in the concentration of the main components and in milk fatty acid composition while milk protein composition was rather constant throughout the season. The seasonal variation in milk fatty acid composition in 2005 was compared with the seasonal variation in milk fatty acid composition in 1992. Compared to 1992 the fatty acid composition of Dutch raw milk in 2005 had changed considerably and had a higher proportion of saturated fat.



In **chapter 7** it is discussed how the result obtained in this thesis can be used to improve the protein and fatty acid composition in milk. It is concluded that selection of cows with β-LG B variant and β-κ-CN haplotype $A^2B$ results in milk that is more suitable for cheese production. Selection of cows with the DGAT1 A and SCD1 V polymorphism results in milk that has a better fatty acid composition. It is discussed why milk differentiation should be used to optimally benefit from the opportunities described in this thesis.
This thesis shows that there are good opportunities to exploit the variation in milk composition between cows to improve the protein and fatty acid composition in milk.

# S

# Samenvatting

Melk en andere zuivelproducten zijn een belangrijk onderdeel van onze voeding. De nutritionele waarde van deze melkproducten wordt in belangrijke mate bepaald door de samenstelling van de rauwe melk. De samenstelling van de rauwe melk heeft ook invloed op de verwerkingseigenschappen van de melk en de eigenschappen van de producten die ervan gemaakt worden. Ook is de prijs die de boeren betaald krijgen voor de melk die zij leveren afhankelijk van de samenstelling van deze melk. de samenstelling van rauwe melk is daarom van groot belang voor zowel de zuivelindustrie als de boeren. In dit proefschrift is er gekeken naar de mogelijkheden om de eiwitsamenstelling en de vetzuursamenstelling in rauwe melk te verbeteren.

**Hoofdstuk 1** is een introductie tot dit proefschrift. In **hoofdstuk 2** wordt aangetoond dat een bepaalde CZE methode gebruikt kan worden voor het meten van de variatie in eiwitsamenstelling in rauwe melk. Deze CZE methode is vervolgens gebruikt voor het meten van de variatie in eiwitsamenstelling in melk van 1948 verschillende koeien. In elk melkmonster is de relatieve concentraties van de zes eiwitten die met de hoogste concentraties voorkomen in melk (α-LA, β-LG, $\alpha_{S1}$-CN, $\alpha_{S2}$-CN, β-CN, en κ-CN) bepaald. De resultaten van deze analyses lieten zien dat er grote variatie was in de concentratie van deze zes eiwitten in de melk van verschillende koeien. Verder werd ook aangetoond dat de CZE methode gebruikt kan worden voor het bepalen van de fosforyleringsgraad van $\alpha_{S1}$-CN ($\alpha_{S1}$-CN-8P en $\alpha_{S1}$-CN-9P) en $\alpha_{S2}$-CN ($\alpha_{S2}$-CN-10P, $\alpha_{S2}$-CN-11P en $\alpha_{S2}$-CN-12P). Bepaling van de concentratie van $\alpha_{S1}$-CN en $\alpha_{S2}$-CN met een verschillende fosforyleringsgraad in de melk van de verschillende koeien liet zien dat de mate waarin $\alpha_{S1}$-CN gefosforyleerd was afhankelijk was van de mate waarin $\alpha_{S2}$-CN gefosforyleerd was. Tenslotte werd aangetoond dat de CZE methode ook goed gebruikt kan worden voor het identificeren van de meeste genetische varianten van melkeiwitten, inclusief de κ-CN E variant. Deze κ-CN E variant was nog niet eerder geïdentificeerd met behulp van CZE.



In **hoofdstuk 3** wordt het effect beschreven van een aantal genetische varianten van melkeiwitten op melkproductiekenmerken en de eiwitsamenstelling in melk. Voor deze analyses zijn de gegevens van 1912 verschillende koeien gebruikt. De resultaten lieten zien dat een aantal van de geteste genetische varianten een groot effect heeft op de samenstelling van het eiwit en een aanzienlijk deel van de genetische variatie in de eiwitsamenstelling bepaalt. Dit betekent dat deze genetische varianten gebuikt kunnen worden om koeien te selecteren of te fokken voor koeien met een betere melkeiwitsamenstelling. Vergelijking van de frequentie van de verschillende genetische varianten in de onderzochte populatie met de frequentie van deze varianten in de Nederlandse Holstein-Friesian populatie in 1989 liet zien dat in 2005 de frequenties van een aantal genetische varianten sterk veranderd was ten opzichte van 1989.

Omdat deze genetische varianten gerelateerd waren aan de eiwitsamenstelling in melk is het goed mogelijk dat de eiwitsamenstelling in de Nederlandse melk ook in de tijd veranderd is.

**Hoofdstuk 4** beschrijft de karakterisatie van 39 verschillende vetzuren uit melk op basis van genetische en bedrijfs parameters met als doel om meer inzicht te verkrijgen in de synthese van deze vetzuren. Voor de bepaling van deze parameters werd de vetzuursamenstelling gemeten met behulp van gas chromatografie in de melk van 1912 verschillende koeien. Het gedeelte van de variatie in vetzuursamenstelling als gevolg van genetische en bedrijfs effecten en de genetische en bedrijfscorrelaties tussen de verschillende vetzuren werden bepaald. Een cluster techniek werd gebruikt om patronen in deze genetische en bedrijfscorrelaties overzichtelijk weer te geven. Voor een aantal vetzuren leverde deze analyses informatie op over de mogelijke herkomst van dit vetzuur dat afweek of extra informatie gaf ten opzichte van wat al bekend was over de synthese van dit vetzuur. Zo kon geconcludeerd worden dat het zeer waarschijnlijk is dat C12:0 in Nederlandse melk niet volledig de novo gesynthetiseerd wordt maar ook gedeeltelijk afkomstig is uit het voer en via het bloed in de melk kan komen. Met behulp van de resultaten kon ook aannemelijk gemaakt worden dat C20:0 in melk gevormd wordt door de werking van elongase enzymen op C18:0. Verder is het waarschijnlijk dat de vetzuren met een oneven ketenlengte C5:0-C13:0 en een gedeelte van C15:0 en C17:0 de novo gesynthetiseerd worden terwijl de rest van het C15:0 en C17:0 uit het bloed afkomstig is. De genetische en bedrijfsparameters bepaald in dit hoofdstuk geven ook een goed beeld van wat de mogelijkheden zijn om de vetzuursamenstelling in melk te veranderen door gericht te fokken of door veranderingen in het voer van de koeien. Deze parameters laten zien dat de mate waarin de concentratie van een bepaald vetzuur veranderd kan worden erg varieert tussen de verschillende vetzuren en afhankelijk is van hoe dit vetzuur gesynthetiseerd wordt.

In **hoofstuk 5** zijn de vetzuurpatronen in de melk van 1933 verschillende koeien gebruikt om inzicht te krijgen in de genetische factoren die van invloed zijn op de onverzadiging van melkvet. Hiervoor is er gekeken naar de genetische variatie in een aantal onverzadigings indexen en naar het effect van de SCD1 A293V en DGAT1 K232A polymorphismen op deze onverzagigingsindexen. Deze analyses lieten zien dat de onverzadiging van melkvet een duidelijke genetische component heeft en dat de varianten van DGAT1 en SCD1 een deel van deze genetische variatie verklaren. Dit betekent dat deze varianten gebuikt kunnen worden om koeien te selecteren of te fokken voor koeien met een betere vetzuursamenstelling. De resultaten lieten ook zien dat de SCD1 V variant geen effect had op de totale onverzadiging van melkvet maar wel geassocieerd was met een lagere C10, C12 en C14 index en met een hogere C16, C18 en CLA index. Dit suggereert dat de SCD1 V variant geen effect heeft op de activiteit, maar wel op de specificiteit van het SCD1 enzyme.



**Hoofdstuk 6** beschrijft de variatie in de samenstelling van Nederlandse rauwe melk gedurende het seizoen. Voor het bepalen van deze seizoensvariatie is gebruik gemaakt van monsters die vanaf februari 2005 tot februari 2006 elke week verzameld zijn en die representatief waren voor de samenstelling van de totale Nederlandse melkplas in die week. Deze 52 monsters zijn vervolgens geanalyseerd op een groot aantal componenten. De resultaten van deze analyses

lieten zien dat de concentratie van de belangrijkste componenten en de vetzuursamenstelling in de melk sterk varieerde gedurende het jaar. De eiwitsamenstelling in de melk was echter redelijk constant gedurende het seizoen. Vergelijking van de vetzuursamenstelling in 2005 met de vetzuursamenstelling in Nederlandse melk in 1992 liet zien dat, vergeleken met 1992, melkvet in 2005 een groter aandeel aan verzadigde vetten bevat.

In **hoofdstuk 7** wordt bediscussieerd hoe de resultaten beschreven in dit proefschrift gebruikt kunnen worden voor het verbeteren van de eiwit en vetzuursamenstelling in melk. Er wordt geconcludeerd dat selectie van koeien of fokken voor koeien met de β-LG B variant en het β-κ haplotype $A^2B$ resulteert in een samenstelling van de melk die gunstiger is voor het maken van kaas. Selectie voor de DGAT1 A en SCD1 V varianten resulteert in melk met een “betere” vetzuursamenstelling. Verder wordt in dit hoofdstuk besproken waarom melk differentiatie toegepast moet worden om optimaal gebruik te maken van de mogelijkheden beschreven in dit proefschrift.

Dit proefschrift laat zien dat er goede mogelijkheden zijn om de variatie in melksamenstelling tussen koeien te gebruiken om de eiwit en vetzuursamenstelling in melk te verbeteren.

# D

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Jeroen

# C

# Curriculum Vitae

Jeroen Heck werd geboren op 2 oktober 1978 in Eindhoven. In 1997 behaalde hij zijn gymnasium diploma aan het Augustinianum te Eindhoven. In datzelfde jaar begon hij aan de opleiding levesmiddelentechnologie aan de Wageningen Universiteit. Hij specialiseerde zich in de richtingen levensmiddelen natuurkunde en levensmiddelen proceskunde en liep stage bij Massey University en het Dairy Research Institute in Nieuw Zeeland en bij Friesche Vlag in Nijkerk. Na zijn afstuderen in 2003 werkte hij als procestechnoloog bij Friesche Vlag in Nijkerk tot hij in augustus 2004 begon aan zijn promotieonderzoek bij de Dairy Science & Technology groep aan de Wageningen Universiteit. De resultaten van dit onderzoek zijn beschreven in dit proefschrift genaamd; “Milk genomics, opportunities to improve the protein and fatty acid composition in raw milk”. Vanaf oktober 2008 is hij werkzaam als researcher bij het corporate researchcentrum van FrieslandCampina in Deventer.

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# Education

## Discipline specific activities

### Courses

Dietary influences on blood pressure, Wageningen University, 2006
Industrial proteins, Wageningen University, 2006

### Meetings

The milk genome and human health, Napa, USA, 2004
The milk genome and human health, Brussel, Belgium, 2006
World Dairy summit, Dublin, Ireland, 2007
ADSA-ASAS joint annual meeting, Indianapolis, USA, 2008
NZO research days (3X), Wageningen, 2004-2008
Genootschap ter bevordering van de zuivelkunde (3X), The Netherlands, 2004-2008

## General courses

VLAG PhD week, Bilthoven, 2004
Toegepaste statistiek, Wageningen Business School, 2005
Mutivariate analysis, PE&RC, 2006
Organising and supervising MSc thesis projects, Wageningen University, 2006
Scientific writing, Wageningen University, 2006
Bioinformatics, EPS, 2007
PhD competence assessment, Wageningen University, 2007
Career perspectives, VLAG, 2008

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