Chapter 7

Milk Protein Genotype Associations with Milk Coagulation and Quality Traits

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1. Introduction

Cheese production is of substantial economic importance in most European countries, where an increasing amount of the produced milk is used for manufacturing cheese (Eurostat 2010a). In Estonia 60% (Statistics Estonia, 2010), in Italy more than 75% (De Marchi et al., 2008), and in Scandinavian countries 33% (Wedholm et al., 2006) of milk is used for cheese production. Recent trends indicate that per capita consumption of cheese is also increasing (Eurostat 2010b).

Milk quality is an essential factor to the dairy industry due to its economic impact. Milk coagulation ability is one of the most important factors affecting cheese yield and quality and has been reviewed (Jakob & Puhan, 1992; Johnson et al., 2001), and therefore is becoming more important as an increasing percentage of milk is used for cheese manufacturing. Milk coagulation properties (MCP) are commonly defined by milk coagulation time (RCT) and curd firmness (A_{30}). It is feasible to design raw milk according to its specific technological use.

Improving cheese yield and quality, through the direct selection of breeding animals on the basis of milk coagulation property traits, is an option due to genetic variation of MCP traits (Ojala et al., 2005). MCP are heritable, quantitative traits; up to 40% of the variation among animals is caused by genetic factors (Ikonen et al., 2004). Estimates of heritability for MCP traits are from 0.30 to 0.40 (Bittante et al., 2002; Ikonen et al., 1999a), and from 0.25 for RCT , and 0.15 for A_{30} (Cassandro et al., 2008) to 0.28 for RCT to 0.41 for A_{30} (Vallas et al., 2010). Predictions of MCP provided by mid-infrared spectroscopy (MIR) techniques have been proposed as indicator traits for the genetic enhancement of MCP (Cecchinato et al., 2009; De Marchi et al., 2009). The expected response of RCT and A_{30} ensured by the selection using MIR predictions as indicator traits was equal to, or slightly less than, the response achievable through a single measurement of these traits. Accordingly, breeding strategies

for the enhancement of MCP based on MIR predictions as indicator traits could be easily and immediately implemented for dairy cattle populations where the routine acquisition of spectra from individual milk samples is already measured (Cecchinato et al., 2009). Nevertheless, MCP traits analyzed with different methodologies have significantly different values, due to the diversity of the instruments used and the coagulant activity (Pretto et al., 2011). The type of coagulant could also have an effect, since different coagulants have been used. The method proposed for the prediction of non-coagulation probability of milk samples showed that non coagulating samples from one methodology were highly predictable based on the rennet coagulation time measured with another methodology (Pretto et al., 2011). A standard definition of MCP traits analysis is needed to enable reliable comparisons between MCP traits recorded in different laboratories, and in different animal populations and breeds.

More than 95% of the proteins contained in ruminant milk are coded by six structural genes (Martin et al., 2002). The four casein genes (*CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3)* are linked in a cluster, referred to as the CN locus, mapped on chromosome 6 and encode α_{s1}-CN, β-CN, α_{s2}-CN, and κ-CN, respectively, as previously reviewed (Caroli et al., 2009). κ-CN is a key element in renneting, but interactions with the other milk protein systems have to be taken into account, in particular, β-CN and β-LG. Monitoring milk protein variation in different breeds of cattle avoids an increase of alleles with unfavourable effects on cheesemaking (Caroli et al., 2000; Comin et al., 2008; Erhardt et al., 1997; Ikonen et al., 1994, 1999a; Lodes et al., 1996). Therefore, another option for enchancing cheese yield and quality is indirect selection against, or for, some milk protein alleles. Selection against κ-CN E-allele would be a good means to indirectly improve milk quality for cheese production because the E-allele is unfavourably associated with non-coagulating milk, which is common (10%) in Finnish Ayrshire cows (Elo et al., 2007). Likewise the κ-CN G-allele in the Pinzgauer breed (Erhardt et al., 1997) and the κ-CN E-allele in the Italian Friesian breed (Caroli et al., 2000) were both associated with unfavourable coagulation properties. As for a positive association, it is well known that the B variants of β- and k-casein and β -lactoglobulin (β-LG) are favourable for milk coagulation and cheese-making (Dovc & Buchberger, 2000; Losi et al., 1973; Patil et al., 2003; Schaar et al., 1985; Walsh et al., 1998). k-CN allelic variants have been associated with variation in total casein and k-CN concentrations in milk (Hallén et al., 2008; Van den Berg et al., 1992), variation in casein micelle size (Di Stasio & Mariani, 2000; Walsh et al., 1998) and differences in coagulating properties and the cheesemaking quality of milk (Di Stasio & Mariani, 2000). Genetic variants of β -LG have been shown to have an indirect effect on cheese yield through their effect on the ratio of casein to total protein (Coulon et al., 1998; Lundén et al., 1997; McLean, 1986).

In the Dutch Holstein-Friesian population selection for *CSN2-CSN3* haplotype A_2*-B,* together with *LGB* B, would result in cows that produce milk more suitable for cheesemaking (Heck et al., 2009). It has also been argued that haplotypes have similar effects in the different breeds and the CN genes themselves were responsible for the haplotype effects observed, rather than genes physically linked to the CN complex (Boettcher et al., 2004).

The objective of this study was to estimate the contribution of the aggregate β-κ-CN and β-LG genotypes on first lactation milk coagulation and quality traits of Estonian Holstein cows. A parallel objective was to identify the variation in genetic polymorphism of milk proteins with the aim to improve the protein composition in milk by selecting for variants of specific genes.

2. Material and methods

2.1. Performance of Estonian cattle populations

In Estonia there are three breeds of dairy cattle – Estonian Red (ER), Estonian Holstein (EHF) and Estonian Native (EN). Distribution of breeds has come to favour EHF (Fig.1). In Estonia, 93.0% of cows are enrolled in an official milk recording programme (Results of Animal Recording in Estonia 2010, 2011) (Fig. 2). Since 1995, average milk yield in Estonia has risen 3947 kg (48%, Fig. 3). The 2010 average actual production for Estonian Holstein herd that were enrolled in producing-testing programs and eligible for genetic evaluation was 7778 kg milk, 317 kg of fat and 260 kg of protein per year. Holstein dairy cattle dominate in Estonian milk production industry because of their excellent production and greater income.

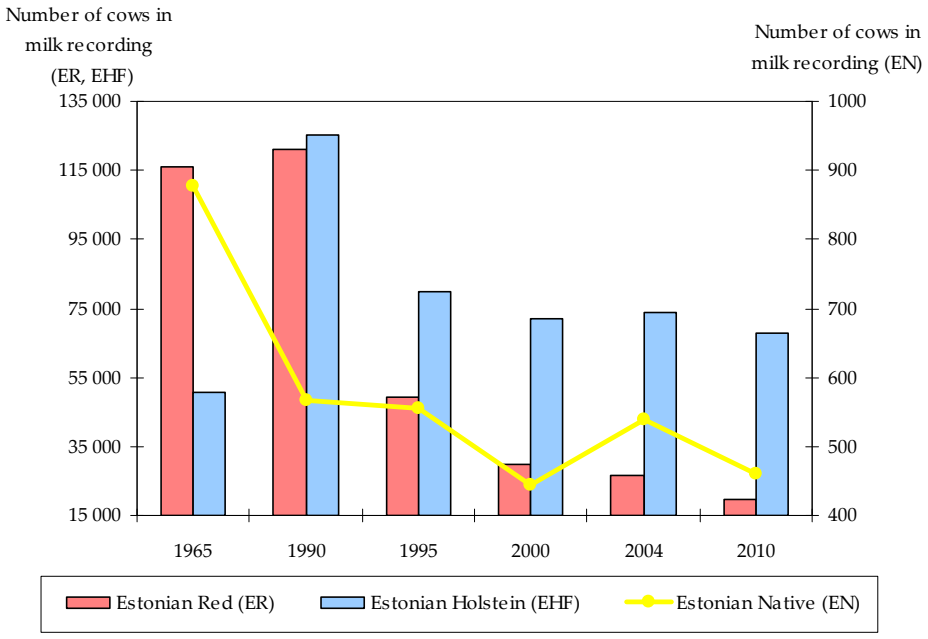

Figure 1. Changes over time in number of cows of each indicated breed on a milk recording programme

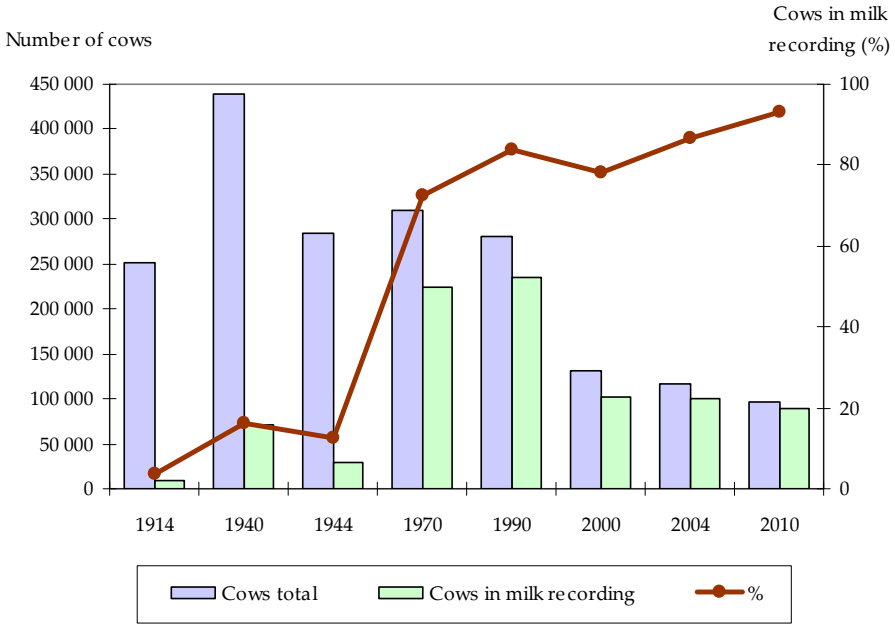

Figure 2. Changes over time in number of Estonian dairy cows and the proportion on a milk recording programme

In the evaluation programme for young bulls of Estonian Holstein breed, ca 25 bulls are tested each year, parallel testing is carried out on 10-12 foreign bulls. The selection of bulls is made from imported American and Canadian embryos, the Estonian Holstein best bull dams and imported young bulls. Often the sons of imported cows are used whose sires are world-known top bulls. The bulls come mainly from the USA, Canada, Germany and the Netherlands.

Currently, estimated breeding values (EBV) for production, conformation and udder health traits for bulls and cows in Estonia are computed by the Animal Recording Centre four times per year (Pentjärv & Uba, 2004). Breeding value estimation is carried out separately for the EHF and the Estonian Red breed (ER), using the best linear unbiased predictor (BLUP) test day animal model for production and udder health traits and the BLUP animal model for conformation traits. The EBV for each production trait – milk (kg), fat (kg) and protein (kg) – is the mean breeding value of the first, second and third lactations, adjusted by the mean average breeding value of the cows born in a defined base year (currently, 1995).

The milk production index (SPAV) is expressed as relative breeding value (RBV) with a mean of 100 and a standard deviation of 12 points for base animals, combining breeding values for milk, fat and protein yield weighted by relative economic values of 0:1:4 for EHF and 0:1:6 for ER (Pentjärv & Uba, 2004).

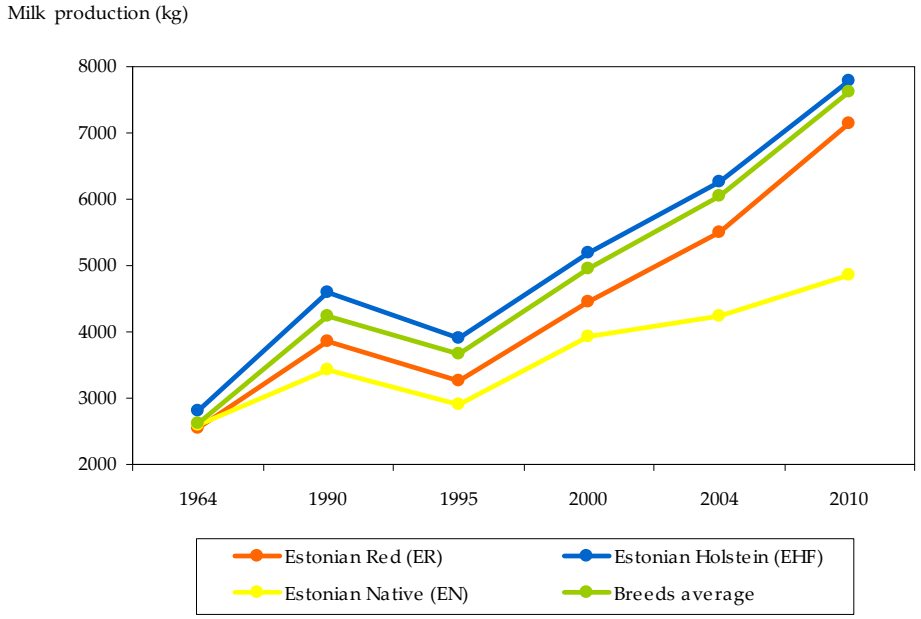

Figure 3. Changes over time in annual milk yield per cow of each indicated breed

The information source for breeding value estimation of udder health traits is somatic cell count (SCC) in one millilitre of milk, transformed into a somatic cell score (SCS) using the formula $SCS = \log_2 (SCC/100000) + 3$ (Pentjärv & Uba, 2004).

The udder health index SSAV is calculated as the sum of EBVs of the first, second and third lactations with index weights 0.26, 0.37 and 0.37, respectively, and is expressed as RBV for genetic evaluation of conformation traits. Data from first lactation cows are used to compute RBVs for 16 linear traits for EHF and 14 linear traits for ER, as well as for three general traits. The conformation index SVAV is expressed as RBV, combining relative breeding values for type, udder and feet by relative economic weights of 0.3:0.5:0.2 for ER and 0.3:0.4:0.3 for EHF.

2.2. Data collection and laboratory milk analysis

First lactation milk samples were collected during routine milk recording as part of a development project for the Bio-Competence Centre of Healthy Dairy Products in Estonia during the period April 2005 – June 2008. The herds had twice-a-day or thrice-a-day milkings. The individual milk samples collected from the cows were either bulked test-day milkings or separate samples from each of the milkings on the test-day. Milk samples were immediately preserved after collection with Bronopol (2-bromo-2-nitropropane-1,3-diol, Knoll Pharmaceuticals, Nottingham, UK) and stored at 4°C during transportation and analyzing periods. Milk samples with a pH lower than 6.5, indicative of colostrum, and non-

coagulated milk samples (n=33) were excluded from the analysis. Furthermore, farms with less than 10 cows, and cows with fewer than three test-day records, were removed. The final dataset used for analyses consist of 11,437 test-day records from 2,769 Estonian Holstein cows which were located in 66 herds across the country and were daughters of 169 sires. The number of daughters per sire ranged from 1 to 267. Each cow had from 3 – 6 measurements collected during the different stages (7 – 305 DIM) of the first lactation. Information about the cows, herds and pedigree was obtained from the Estonian Animal Recording Centre (EARC), the Animal Breeders' Association of Estonia and a database, COAGEN®, was produced. The test-day milk yield was recorded and individual milk samples were analyzed for fat percentage, protein percentage and urea content using the MilkoScan 4000 and MilcoScan FT6000, and for SCC using the Fossomatic 4000 and Fossomatic 5000 cell counter at the Milk Analysis Laboratory of the EARC, using methods suggested by the International Committee for Animal Recording (2009). Values of SCC were log-transformed to SCS.

The pH and milk coagulation properties were determined at the Laboratory of Milk Quality of the Estonian University of Life Sciences, usually three days after sampling. The proportion of milk samples with a maximum storage age of seven days was very small, less than 1%. The pH level of the milk was determined using a pH meter (Seven Multi; Mettler Toledo GmbH, Greifensee, Switzerland) at a temperature of 20°C before analyzing the milk coagulation properties. The latter were milk coagulation time in minutes and firmness of curd in volts. Prior to the assessment of the milk coagulation properties, milk samples were heated to the renneting temperature (35°C). The rennet (Milase MRS 750 IMCU/ml; CSK Food Enrichment B.V., The Netherlands) used in the analyses was diluted 1:100 (v/v) with distilled water and 0.2 ml of the solution was added to 10 ml milk. The milk coagulation properties were determined using the Optigraph (Ysebaert, Frepillon, France), which was developed by YDD (Ysebaert Dairy Division) in partnership with the INRA (LGMPA, lab. G. CORRIEU) to define coagulation characteristics in the laboratory, specifically to answer the needs of cheese makers (Ysebaert Dairy Division, 2009).

Measurements made with the Optigraph are not based on a rheological method but on an optical signal in the near-infrared spectrum. During a coagulation test, the light transmitted through the milk gradually weakens because of changes in the micellar structure of casein. The Optigraph then calculates the coagulation parameters (coagulation time, curd firmness, speed of aggregation) by means of particular feature points extracted from the optical information acquired in real time (Optigraph User's Manual).

2.3. Data collection and laboratory blood analysis

Blood samples were collected as part of a development project for the Bio-Competence Centre of Healthy Dairy Products in Estonia during the period of June 2005 to December 2007. Blood samples (n=2,959) were stored in tubes containing K_3EDTA. DNA was extracted from whole blood according to the method described by (Miller et al., 1998) or by using a commercial Puregene Gentra Blood kit (Minneapolis, USA). The quantity of template DNA

was approximately 40 to 100 ng for Allele-specific oligonucleotide (ASO) PCR and PCR-RFLP, respectively. Polymorphisms of five milk protein genes were analyzed, four from the casein cluster *(CSN1S1, csn1s2, CSN2, CSN3)* and *LGB.* The list of single nucleotide polymorphisms (SNP) previously reported by (Chessa et al., 2007) was considered to distinguish genetic variants of milk proteins of the sampled cows. ASO primers were designed for the detection of polymorphisms in the *CSN2* (primer sequences in (Värv et al., 2009)) and *CSN1S1* (present study). *CSN1S1* genotyping included amplification of a 155-bp sequence of the gene at exon 17. In accordance with the SNP a26181g in *CSN1S1*, two specific forward oligos were designed to distinguish B and C alleles paired with one reverse primer. An extra mismatch was added to both forward primers at position 2 at the 3′ end. Restriction analysis was carried out to genotype *CSN1S2* (Ibeagha-Awemu et al., 2007), *CSN3* (Velmala et al., 1993) and *LGB* (Medrano & Aquilar-Cordova, 1990). In this study, amplified regions of *CSN1S2* with a digestion site to discriminate the protein variants from A-allele, were 330 bp (114 and 216 bp after restriction with *Mbo*II to detect B-allele), 354 bp *(Nla*IV restriction fragments 211 and 143bp to detect C-allele) and 356 bp (*Mnl*I restriction fragments 160 and 196 bp for non-D allele). Products of allele-specific PCR and digestion fragments were separated on agarose gel. Sequencing to verify the studied DNA regions was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA).

2.4. Statistical analyses

Preliminary analyses for testing the significance of fixed effects and single genotype effects were carried out on the SAS System® (SAS, Cary, NC, USA) using the MIXED procedure. Aggregate β-κ-CN genotypes were formed for further analysis. The genotypes with relative frequencies of less than 1% were grouped together into rare β-κ-CN genotype (A_1A_1-BB, A_1A_1-BE, A_1A_1-EE, A_1B-BB, A_1B-BE, A_2A_2-AE, A_2A_2-BE, A_2A_3-AA, A_2B-AA, A_2B-BB, BB-AB, BB-BB). Further statistical analysis was carried out using ASReml (VSN International Ltd., Hemel Hempstead, UK), using the following univariate repeatability animal model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Cg} + \mathbf{Za} + \mathbf{e},$$

where **y** – vector of observations of the dependent variable (log-transformed RCT, A_{30}, milk yield, milk protein percentage, fat percentage, SCS); **b** – vector of fixed effects (quadratic polynomial of day in milk, calving age, sample age, sampling year-season, calving year-season); **g** – fixed effects of the β-κ-CN genotypes or β-LG genotypes; **a** – vector of random effects (herd $N(0,\mathbf{I}\sigma_h^2)$, additive genetic $N(0,\mathbf{A}\sigma_a^2)$ and permanent environmental effect $N(0,\mathbf{I}\sigma_{pe}^2)$); **e** – vector of residual random error effects $N(0,\mathbf{I}\sigma_e^2)$; **X**, **C**, **Z** – known incidence matrices for fixed, genotype and random effects, respectively.

Sample age was included as a covariate in the model only for milk coagulation traits. Sampling year-season and calving year-season were grouped into 3-month classes, 14 classes from April 2005 to June 2008 and 11 classes from December 2004 to August 2007, respectively. Three generations of ancestors with a total number of 17,185 animals in the relationship matrix were included in the analysis.

The class with the largest number of observations, genotype A_2A_2-AA was used as the class of comparison. It is also homozygous for both loci. Accordingly, the standard errors of the genotype effects are standard errors of the differences between each genotype and the most frequent A_2A_2-AA genotype (Comin et al., 2008).

3. Results and discussion

3.1. Genetic structure of milk proteins

Allelic variants of casein and *LGB* loci and genotypes are presented in Tables 1 and 2. Four allelic variants of *CSN2*, three allelic variants of *CSN3* and two variants of the *LGB* gene in Estonian Holstein population were detected. *CSN2* occured at a significantly higher frequency for the A_2 allele. The B-allele and A_3 allele at *CSN2* were rare. The further scan of β-CN, to discriminate protein variant I from variant A_2 performed in the Dutch Holstein Friesian population revealed frequency of I allele of 0.14 for proven bulls, 0.27 in young bulls and 0.192 in cows and demonstrated that it is actually one of the common variants for the Holstein population (Visker et al., 2011). This frequency of the β-CN protein variant in Dutch Holstein Friesian population is relatively high compared with the frequencies of other cattle breeds. The Italian Holstein's frequency of the I allele was 0.12 (Jann et al., 2002), while a survey of 30 cattle breeds yielded frequencies up to 0.14 (Jann et al., 2004). As pointed out (Visker et al., 2011), the associations of β-CN protein variant A_2 should still interpreted with care, because they may have been combined associations of protein variants A_2 and I, which are very different in some traits. Therefore further investigation is needed for Estonian Holstein β-CN I allele. The mean frequency of the β-CN A_2 allele in Estonian Holstein was 0.647 (Table 1), ranging in farms in Estonia from 0.35 to 0.73. The mean frequency of the β-CN A_1 allele was 0.320, ranging in farms in of Estonia from 0.19 to 0.50. At the *CSN2* locus the A_2 allele was highly predominant over other alleles. The heterozygous genotype A_1A_2 and the homozygous genotype A_2A_2 at β-CN were represented at almost the same frequencies (0.434 and 0.411, respectively). The most common β-CN genotypes A_1A_2 and A_2A_2 frequencies comprised jointly 84%. The occurrence of milk protein genetic variants revealed somewhat higher frequencies of the β-CN A_2 allele and the homozygotic genotype A_2A_2 (Table 1 and 2) in the Estonian Holstein population in comparison with the common European cattle breeds (European Food Safety Authority, 2004). In some large Estonian farms the β-CN A_2 allele mean frequency was about 0.70 and homozygotic genotype mean frequency about 0.50.

Protein	Allele	n	Frequency	Protein	Allele	n	Frequency
κ-CN	A	4,356	0.737	α_{s1}-CN	B	5,446	0.983
	B	1,163	0.197		C	92	0.017
	E	391	0.066	α_{s2}-CN	A	5,329	0.962
β-CN	A_1	1,888	0.320		B	164	0.030
	A_2	3,818	0.647		D	45	0.008
	A_3	5	0.001	β-LG	A	2,947	0.498
	B	191	0.032		B	2,967	0.502

Table 1. Allele frequencies of κ-CN, β-CN, α_{s1}-CN, α_{s2}-CN and β-LG in cows of the Estonian Holstein breed

This finding, of a high frequency of the A_2 allele, confirms the advantage of the Estonian Holstein breed that their milk naturally might lower health risks associated with the occurence of the β-CN A_1 allele. The other advantage of the β-CN A_2 allele is its positive association with protein yield (Olenski et al., 2010). The positive effect of the rare A_2A_2-BB genotype, that is the *A_2-B* haplotype, on milk and protein yields has been reported in previous studies on Californian Holstein (Ojala et al., 1997), Finnish Ayrshire (Ikonen et al., 2001) and Dutch Holstein-Friesian cows (Heck et al., 2009).

Protein	Genotype	n	Frequency	Protein	Genotype	n	Frequency
κ-CN	AA	1,606	0.544	α_{s1}-CN	BB	2,877	0.967
	AB	850	0.288		BC	92	0.033
	AE	294	0.100	α_{s2}-CN	AA	2,764	0.926
	BB	116	0.039		AB	159	0.057
	BE	81	0.027		AD	42	0.015
	EE	8	0.003		BB	1	0.001
β-CN	A_1A_1	270	0.092		BD	3	0.001
	A_1A_2	1,282	0.434	β-LG	AA	631	0.214
	A^1B	66	0.022		AB	1,685	0.570
	A_2A_2	1,212	0.411		BB	641	0.216
	A_2A_3	5	0.002				
	A_2B	107	0.036				
	BB	9	0.003				

Table 2. Genotype frequencies of κ-CN, β-CN, α_{s1}-CN, α_{s2}-CN and β-LG in cows of the Estonian Holstein breed

CSN3 had A, B and E allelic variants (Table 1 and 2). κ-CN shows a prevalence of the A allele at a mean frequency of 0.737, followed by the B allele at frequency of 0.197 and the E allele at frequency of 0.066. The most frequent κ-CN genotype of all genotyped Estonian Holstein cows was AA, which was found in slightly more than half of the cows (54.4%), followed by AB (28.8%) and AE (10%). A favourable genetic marker for protein yield, MCP and cheese production, κ-CN B, was rare (3.9% overall) in the homozygous state. The unfavourable κ-CN E allele was also very rare, only eight of the 2,954 sampled cows had the EE genotype.

CSN1S1 had B and C allelic variants (Table 1 and 2), showing the prevalence of the B allele (0.983) and *CSN1S2* revealed three allelic variants: A, B, and D, showing the prevalence of the A allele (0.962). Because α_{s1}-CN and α_{s2}-CN were almost monomorphic (Table 1), they were excluded from the aggregate casein genotypes.

β-LG was represented with two allelic variants A (0.498) and B (0.502). Comparing the results of the genetic structure of milk proteins of the Estonian Holstein breed of Jõudu, 2008 and Värv et al., 2009 to those of this study, the quantity of detected alleles, and their frequencies, are somewhat different. The reason for this could be attributed to the sampling capacity (n=42) in (Värv et al., 2009) and the sampling procedure. Sampling was carried out

in this study on a large population (n=2,954, 42 farms) across the whole breed. With a small sampling size the rare allele was not exposed and in one previous paper (Jõudu, 2008) the sampling was not performed across the whole breed, causing some different conclusions about allele frequencies of Estonian cattle breeds in their investigations compared to those of this study.

Expected frequencies of the β-κ-CN genotypes were calculated by multiplying the expected frequencies of the β-CN and κ-CN genotypes. Some alleles at one locus were associated only with certain alleles at the other locus, causing distinct differences between observed and expected frequencies of certain β-κ-CN genotypes (Table 3).

κ-CN	β-CN genotype						
genotype	A_1A_1	A_1A_2	A_1B	A_2A_2	A_2A_3	A_2B	BB
AA	4.0 (117)	23.1 (683)	0.0	27.1 (801)	0.2 (5)	0.1 (2)	0.0
	5.0	23.6	1.2	22.4	0.1	2.0	0.2
AB	1.6 (48)	10.2 (301)	1.5 (45)	12.5 (369)		2.8 (82)	0.1 (3)
	2.6	12.5	0.6	11.8		1.0	0.1
AE	2.5 (73)	7.2 (214)	0.0	0.2 (7)		0.0	0.0
	0.9	4.3	0.2	4.1		0.4	0.0
BB	0.1 (3)	1.2 (34)	0.4 (12)	1.2 (36)		0.8 (24)	0.2 (6)
	0.4	1.7	0.1	1.6		0.1	0.0
BE	0.7 (20)	1.7 (51)	0.3 (9)	0.0 (1)		0.0	
	0.2	1.2	0.1	1.1		0.1	
EE	0.3 (8)	0.0		0.0			
	0.0	0.1		0.1			

Table 3. Observed and expected frequencies (upper and lower line respectively, each given as percentage) of the aggregate β-κ-CN genotypes (numbers of cows in the brackets) in 2,954 Estonian Holstein cows

Some genotypes were observed two to fourfold more frequently than expected (A_1A_1-BE, A_1B-BE, A_1B-BB etc) and A_2B-BB at eightfold more frequently than expected. All cows carrying the κ-CN EE genotype had association only with the β-CN A_1A_1 genotype as has also been reported for Finnish Ayrshire cows (Ikonen et al., 1999a). Some genotypes were less frequent than expected (A_1A_1-BB a quarter and A_2A_2-AE one-twentieth of the expected frequency). Linkage disequilibrium in the casein loci has been observed in dairy cattle population differing in breed and geographical location, leading to unbalanced data (Bovenhuis et al., 1992; Ikonen et al., 1999b; Van Eenennaam & Medrano, 1991). The probable reason for unbalanced data in the Estonian Holstein population could be also the frequent use of few sires carrying, and transmitting, specific casein haplotypes to their offspring. Disequilibrium in the *CSN2* and *CSN3* loci can also be produced and maintained by selection favouring one combination of alleles over another (Falconer & Mackay, 1996).

3.2. Associations between milk protein genotypes and milk coagulation and quality traits

The associations of β-CN and κ-CN genotypes with milk coagulation (RCT, A_{30}), quality traits (SCS, fat and protein contents), and milk yield was investigated (Table 4).

3.2.1. Milk coagulation traits

Milk coagulation traits (RCT and A_{30}) were affected by aggregate β-κ-CN genotypes ($p<0.001$, Table 4). The most favourable β-κ-CN genotypes for RCT included the B allele at both loci, as has also been reported elsewhere (Comin et al., 2008) for Italian Holstein cows. Favourable aggregate genotypes for RCT were A_1B-AB and A_2B-AB. The best aggregate genotypes for A_{30} had two B alleles κ-CN, A_1A_2-BB, and the second best had the genotype A_2A_2-BB. κ-CN B was the most favourable for MCP, in every combination with β-CN, as has also been reported by (Comin et al., 2008).

		RCT* (min)		A_{30} (V)		MILK (kg)		PROTEIN (%)		FAT (%)		SCS***	
Genotype	N	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE
β-κ-CN		p<0.001		p<0.001		p=0.015		p<0.001		p=0.007		p=0.127	
A_1A_1-AA	110	-0.037	0.014	-0.506	0.255	-1.237	0.424	0.008	0.019	0.041	0.047	0.141	0.139
A_1A_1-AB	42	-0.039	0.021	2.164	0.388	-0.703	0.645	0.073	0.029	0.008	0.071	-0.095	0.213
A_1A_1-AE	70	-0.033	0.016	-0.894	0.305	-1.132	0.508	0.053	0.023	0.035	0.056	0.311	0.168
A_1A_2-AA	633	-0.019	0.007	-0.237	0.130	-0.639	0.217	0.019	0.010	0.046	0.024	-0.049	0.072
A_1A_2-AB	271	-0.056	0.009	2.401	0.173	-0.708	0.288	0.054	0.013	0.064	0.032	0.072	0.095
A_1A_2-AE	207	-0.009	0.010	-0.683	0.190	0.028	0.317	-0.004	0.014	-0.035	0.035	0.051	0.105
A_1A_2- BB	34	-0.073	0.023	4.357	0.422	-2.013	0.705	0.130	0.031	0.246	0.077	-0.560	0.235
A_1A_2- BE	49	-0.052	0.019	2.214	0.355	-0.963	0.595	0.039	0.026	-0.008	0.065	0.084	0.197
A_1B -AB	42	-0.137	0.021	2.390	0.390	0.057	0.648	0.030	0.029	0.009	0.071	-0.081	0.214
A_2A_2-AA	768	0		0		0		0		0		0	
A_2A_2-AB	337	-0.017	0.009	2.157	0.161	-0.383	0.268	0.057	0.012	0.036	0.029	-0.144	0.088
A_2A_2- BB	34	-0.072	0.023	3.865	0.418	-0.460	0.699	0.076	0.031	-0.042	0.077	-0.237	0.233
A_2B -AB	75	-0.092	0.016	1.966	0.295	-0.443	0.493	0.006	0.022	-0.121	0.054	-0.056	0.163
Rare**	93	-0.084	0.014	2.018	0.267	-0.453	0.445	0.029	0.020	-0.051	0.049	-0.168	0.148
β-LG		p<0.001		p<0.001		p=0.462		p=0.648		p=0.356		p=0.571	
AA	589	-0.015	0.006	-0.426	0.127	0.181	0.194	0.000	0.009	-0.016	0.021	-0.047	0.065
AB	1,569	0		0		0		0		0		0	
BB	609	0.033	0.006	0.367	0.131	0.200	0.198	-0.008	0.009	0.023	0.022	0.037	0.065

* Log-transformed

** Genotypes with an occurrence of less than 1%

*** SCS = log2(SCC/100,000) + 3

Table 4. Statistical significance of milk protein genotypes (p), the number of Estonian Holstein cows (n) per β-κ-CN aggregate genotype and β-LG genotype, estimated genotype effects (Est.) with their standard errors (SE) on milk coagulation time (RCT), curd firmness (A_{30}) and milk production and composition

As for the impact of *CSN2* locus, the β-CN A_2 allele was more favourable for A_{30} than A_1. The A_1 allele did not show, in this investigation, the most favourable effect (superior to β-CN A_2) which was described by (Comin et al., 2008). The composite β-κ-CN genotypes, including the κ-CN B allele, were also associated with the best MCP in Finnish Ayrshire cattle (Ikonen et al., 1999a; Ojala et al., 2005), but the *CSN2* locus in that sampled population did not include the β-CN B allele. Comparing both casein loci, it seems that *CSN3* affected milk coagulation traits more than *CSN2*. Since the discovery of the micelle-stabilizing protein κ-casein, in 1956, it became evident that κ-CN had an important effect on the stabilization of casein micelles (Waugh & Von Hippel, 1956). In our study, the most frequent β-κ-CN genotype, A_2A_2-AA, and genotype A_1A_2-AE were associated with poor milk coagulation time, which is consistent with (Comin et al., 2008). Also, the rare E allele of the κ-CN in the β-κ-CN aggregate genotype had an unfavourable effect on milk coagulation properties. The association of this rare allele of κ-CN with poor milk coagulation has been previously reported (Ikonen et al., 1999a) and (Comin et al., 2008).

3.2.2. Milk yield and protein and fat percentage, SCS

Milk yield and protein and fat contents were affected by aggregate β-κ-CN genotype ($p<0.05$, Table 4). β-κ-CN genotype A_1B-AB is favourable for milk yield. Similarly to A_{30}, the most favourable β-κ-CN genotypes for milk protein content were homozygous for the B allele for κ-CN, A_1A_2-BB and A_2A_2-BB. The most favourable aggregate genotype for fat content was also A_1A_2-BB and unfavourable genotype for fat percentage, containing E allele in κ-CN locus, A_1A_2-AE, but also genotype A_2B-AB.

The most favourable for protein content was BB for κ-CN and A_1A_2 for β-CN (the second best was A_2A_2, where the A_2A_2 genotype of β-CN had a slight advantage over the A_1A_1 genotype). These results were in agreement with those previously reported (Heck, 2009), that the κ-CN genotype was associated with protein content (B>A). Milk with the aggregate genotype A_1A_2-BB had the best firmness of curd and also the best protein and fat contents. This is in agreement with another investigation (Vallas et al., 2010), where curd firmness had the highest genetic correlation with milk protein percentage (0.48), suggesting that a high protein percentage results in a favourable curd firmness. It has been reported (Cassandro et al., 2008) that there is a correlation coefficient of 0.44 between curd firmness and protein percentage, which is in agreement with the results found in this experiment. The genetic correlations of −0.24 and −0.07 reported (Ikonen et al., 1999a, 2004) for the same traits, however, are different. These inconsistencies indicate that different methodologies used for the investigations may influence the results (Pretto et al., 2011). Curd firmness showed a weak positive genetic correlation (Vallas et al., 2010) with milk fat percentage (0.25) and a weak negative genetic correlation with milk yield (−0.29). Therefore, selection for improved curd firmness may be associated with a somewhat higher protein and fat percentage and slightly reduced milk yield. Genetic correlations for curd firmness with milk yield and fat percentage were negligible in previous studies (Cassandro et al., 2008; Ikonen et al., 1999a). As for the impact of the *CSN2* A_1 and A_2 alleles on milk production, the A_2 allele seems to have slight advantage over A_1 in the aggregate β-κ-CN genotype. Genotypes of β-LG were

associated with both milk coagulation traits ($p<0.001$), but had no a significant effect on either milk yield ($p=0.462$), protein percentage ($p=0.648$), nor fat percentage ($p=0.356$) and SCS ($p=0.571$).

4. Conclusion

The β-κ-CN locus had a strong effect on protein and fat content and milk coagulation properties. Milk with the β-κ-CN aggregate genotype A_1A_2-BB had the best firmness of curd and also the best protein and fat contents. The aggregate genotype A_2A_2-BB, haplotype *A_2-B*, was also favourable for milk coagulation property traits and protein content. The β-LG locus had no impact on SCS, milk production nor protein and fat contents. The β-LG BB genotype had better curd firmness and AA better milk coagulation time. Linkage disequilibrium in the *CSN2* and *CSN3* loci, which most probably led to unbalanced data, provided justification for the use of aggregate β-κ-CN and β-LG in selection for better milk technological and quality traits.

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