Options for selecting dairy cattle
for milk coagulation ability

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Academic dissertation

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Abstract

The main objective of this thesis was to evaluate means of selecting dairy cattle for milk coagulation ability (MCA) to improve milk quality for cheese production. The possibilities for direct selection were examined by obtaining sufficiently reliable heritability and repeatability estimates for milk coagulation traits; estimates obtained in earlier studies have been based on fairly small data sets. In addition, possibilities for indirect selection and marker assisted selection for MCA were considered by obtaining sufficiently reliable estimates of genetic correlations between milk coagulation and production and quality traits, and by studying the non-coagulation of milk and associated factors. The effect of non-genetic factors on MCA and the breed differences between Finnish Ayrshire and Holstein-Friesian cows in MCA were assessed as well.

To estimate the genetic parameters for MCA and to study the effects of non-genetic and breed factors on MCA, three separate data sets were collected: a longitudinal Finnish Ayrshire data set, a data set of Finnish Ayrshire and Holstein-Friesian cows and a data set of 91 Finnish Ayrshire sires with large daughter groups. The last data set was also used as source data for gene mapping of the genomic regions associated with the non-coagulation of milk. The traits used to describe MCA were milk renneting time, curd firming time and curd firmness.

The results revealed significant variation in MCA among cows, sires, herds, breeds and lactation stage. Despite the large differences in herd bulk milks, herd explained only a minor part of the variation in MCA. There was some indication that good management and feeding decrease the proportion of poorly coagulating milks. However, breed differences and genetic differences within breeds are probably a greater cause for large variation in herd bulk milks than herd management and feeding. Holstein-Friesian cows were superior to Finnish Ayrshire cows in MCA. Poor coagulation or non-coagulation of milk was only a minor problem (10%) in Holstein-Friesian cows, whereas one-third of Finnish Ayrshire cows produced poorly or non-coagulating milk.

Almost 40% of the variation among animals was additive genetic. Selection is thus the most effective way to improve MCA. Direct selection is the most effective selection method. Based on the high repeatability estimates, only three measurements are needed to reliably estimate cows' average MCA. However, the current measuring devices are not suitable for the large-scale measurement required to include the trait in routine milk recording.

Options for selecting MCA indirectly or via marker-assisted selection were evaluated by studying the following means: selection based on production and udder health traits in the total merit index, selection based on protein or casein content or on the pH of milk and selection based on reduction of the prevalence of non-coagulating milk.

The findings indicate that the udder health index both improves MCA genetically and decreases the prevalence of non-coagulating milk through somatic cell count. Under the present weighting scheme, however, hardly any response to MCA is expected. No genetic correlation between test-day milk yield and milk coagulation traits or non-coagulation of milk was observed.

Neither the protein or casein content of milk nor the pH of milk was found to be a viable option for indirect selection. The results for casein content were identical to those for protein content, which is already included in routine milk recording. The genetic correlation between MCA and protein and casein content of milk was, however, almost zero. Further investigation of the relationship between predicted breeding values for curd firmness and protein and casein contents suggested that selection based on the latter would maintain NC-carriers in the Finnish Ayrshire population. The pH of milk was moderately genetically correlated with milk coagulation traits, but was not clearly genetically associated with the non-coagulation of milk. Therefore, its inclusion in the index would likely not decrease the frequency of non-coagulating milk.

About 10% of Finnish Ayrshire cows produced non-coagulating milk in all data sets analysed; some of the cows produced non-coagulating milk at almost every sampling. The non-coagulation of milk is thus a worryingly common problem in the Finnish Ayrshire population. None of the environmental factors studied could fully explain it. However, several indications of a genetic cause emerged and two associated loci were mapped to chromosome 2 (BMS1126) and chromosome 18 (BMS1355). No association between casein genes themselves and non-coagulation of milk was observed. Instead, two candidate genes, LOC538897 in chromosome 2 and SIAT4B in chromosome 18, were found, both of them involved in the post-translational modification of amino acids. LOC538897 functions as a non-specific serine/threonine kinase and SIAT4B, or sialyltransferase, catalyses the last step of glycosylation of κ-casein. The potential role of the candidate genes in the non-coagulation of milk warrants further investigations. Currently, the elimination of the carrier bulls of non-coagulation genes would be the most effective way of genetically improving MCA in Finland.

Contents

1. List of original articles

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I Tyrisevä, A.-M., Ikonen, T., and Ojala, M. 2003. Repeatability estimates for milk coagulation traits and non-coagulation of milk in Finnish Ayrshire cows. Journal of Dairy Research 70: 91-98.

II Tyrisevä, A.-M., Vahlsten, T., Ruottinen, O., and Ojala, M. 2004. Noncoagulation of milk in Finnish Ayrshire and Holstein-Friesian cows and effect of herds on milk coagulation ability. Journal of Dairy Science 87: 3958-3966.

III Ikonen, T., Morri, S., Tyrisevä, A.-M., Ruottinen, O., and Ojala, M. 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content, and pH of milk. Journal of Dairy Science 87: 458-467.

IV Tyrisevä, A.-M., Elo, K., Kuusipuro, A., Vilva, V., Jänönen, I., Karjalainen, H., Ikonen, T., and Ojala, M. 2008. Chromosomal regions underlying non-coagulation of milk in Finnish Ayrshire cows. Manuscript, submitted for publication in Genetics.

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Contribution of the author to papers I-IV:

I: The author prepared the data for statistical analyses, conducted the statistical analyses, interpreted the results and was the main author of the paper.

II: The author participated in planning the study and in performing laboratory and statistical analyses of the data, interpreted the results and was the main author of the paper.

III: The author participated in collection of milk samples, in performing laboratory analyses, in preparing the data for statistical analyses, in interpretation of the results and in reviewing drafts of the manuscript.

IV: The author participated in planning of the study, in collection of samples, and in laboratory analyses, conducted the statistical analyses, interpreted the results and was the main author of the paper.

2. Abbreviations

E	excellently coagulating
FA	Finnish Ayrshire
HF	Holstein-Friesian
MCA	milk coagulation ability
NC	non-coagulating, curd firmness value 0 mm after testing time of 30-31 min
PBV	predicted breeding value
PC	poorly coagulating, curd firmness value <20 mm after testing time of 30-31 min
QTL	quantitative trait locus/loci
SCS	somatic cell score, natural logarithm of somatic cell count

3. Introduction

3.1. Consumption of milk and milk products in Finland

Finns have traditionally been a milk-drinking population, but the consumer habits of milk and milk products have changed over the last few decades. A 20% decrease in the consumption of liquid milk has occurred within the last 20 years, while consumption of processed milk products has increased. Consumption of cheese shows an impressive increase of 50% during the last two decades, and about 40% of milk produced is used for cheese production (Finnish Food and Drink Industries' Federation 2007). At present, the Finnish annual cheese consumption has nearly attained the level of southern European countries like Italy and France, being 19 kg/capita in 2005 (Eurostat 2007).

Because cheese production has such a large impact on the dairy industry, it is important that the milk is of good quality for cheese-making. This raises many questions: How is good milk for cheese production determined? Which traits best describe it? Are those traits easy to measure? What environmental factors affect them? Do genetic differences in these traits exist between animals? What are the options for selection? This thesis attempts to find answers to these questions.

3.2. Milk coagulation – a critical step in cheese-making

Milk caseins, i.e., α_{s1}-, α_{s2}-, β- and κ-casein, are important compounds in cheese because they form the gel network that encompasses the other constituents of cheese. Caseins are in micelle form in milk. The exact structure is still under debate, mostly because the micelle structure cannot be crystallized (Kumosinski et al. 1991). To date, many competing models of the micelle structure have been proposed, as reviewed by Farrell et al. (2006) and Horne (2006); some consensus exists. It is generally agreed that α_{s1}-, α_{s2}- and β-caseins, as highly phosphorylated and hydrophobic caseins (Swiss-Prot database at http://au.expasy.org/sprot/sprot-top.html), are mainly located in the micelle core, whereas the only modestly phosphorylated, but heavily glycosylated κ-casein (Swiss-Prot) is located on the micelle surface. All of the phosphorylated and glycosylated sites of κ-casein are at its c-terminal end, making it very hydrophilic (Farrell et al. 2006). These c-terminal ends protrude from the micelle surface, preventing overly early aggregation of micelles (Shekar et al. 2006). Further, α_{s1}-, α_{s2}- and β-caseins bind a large amount of calcium through Ca-P crosslinks (Farrell et al. 2006).

Milk coagulation, the first step in cheese-making, begins when chymosin splits κ-casein into two parts: para-κ-casein and glycomacropeptide. After the cleavage of the hydrophilic glycomacropeptide to whey, micelles can aggregate with each other and form the cheese curd. The physicochemical mechanism of this step is not thoroughly known, but hydrophobic interactions, colloidal calcium phosphate crosslinks and +/- charge bridges most probably play a role in it (Horne 1998, Lucey et al. 2003).

Based on many studies, milk coagulation affects cheese yield (Martin et al. 1997, Ikonen et al. 1999b, Johnson et al. 2001, Malacarne et al. 2006). Even though yield differences between well and poorly coagulating milks may not be large on an experimental scale, they can be substantial when extrapolated to a typical cheese plant scale (Ikonen et al. 1999b). Milk coagulation ability is therefore a good candidate for determining milk quality for cheese-making. Also important is that it is a measurable trait. Caseins are good candidates as well because they form the cheese curd and are major constituents of cheese. According to van Hooydonk et al. (1986) and Udabage et al. (2001), a certain minimum amount of casein and calcium is needed for milk coagulation to occur. However, the role of the casein is dependent on milk coagulation ability because the latter determines the proportion of caseins transformed into cheese. Further, most of the other factors impacting cheese-making, such as calcium content and pH of milk, have an explicit effect via the milk coagulation process. The emphasis of this thesis is on milk coagulation ability because of its central role in cheese-making. The milk coagulation traits used here to describe milk coagulation ability are milk renneting time (R), curd firming time (K_{20}) and curd firmness (E_{30}). The definitions of these traits are presented in the Materials and methods section.

3.3. Factors affecting milk coagulation ability

Differences exist between milk in milk coagulation ability (MCA). After decades of research, much is already known about the factors affecting MCA. However, the magnitude of many genetic parameters and the influence of many non-genetic factors associated with MCA need to be established or confirmed before selection for better milk quality for cheese production through MCA is possible.

3.3.1. Systematic environmental factors

Systematic environmental factors include parity, lactation stage and season. Results for the effect of parity on MCA are scarce and have been contradictory. In Lindström et al. (1984), MCA slightly deteriorated with parity, in Schaar (1984) MCA improved with parity and in the studies of Pagnacco and Caroli (1987), Davoli et al. (1990) and Ikonen et al. (1999a) parity had no effect on MCA.

Except for the reports of Lindström et al. (1984) and Ikonen et al. (1999a), the effect of lactation stage on MCA has only been studied in small sample sizes and/or data sets have not covered the entire lactation period. However, lactation stage seems to have a clear effect on MCA (Davoli et al. 1990, Kefford et al. 1995, Kreuzer et al. 1996a, b, Ostersen et al. 1997, Ikonen et al. 1999a). In some studies, this effect disappeared when MCA was corrected for such milk composition traits as casein content and pH of milk (Lindström et al. 1984, Schaar 1984, Pagnacco and Caroli 1987, Bastian et al. 1991). This is easily understood since environmental factors likely influence MCA through their effect on milk

composition. In studies where lactation stage affected MCA, the beginning and the end of lactation were associated with good MCA.

Grazing season has been found to have a favourable effect on MCA in some studies (Grandison et al. 1984, Macheboeuf et al. 1993), whereas some other studies reported that MCA was at its best in autumn (Schaar 1984, Bastian et al. 1991). Because climate conditions can drastically differ among countries, general conclusions are difficult to draw.

3.3.2. Herd management and feeding

No estimates of herd effects (proportion of herd variance of the total phenotypic variance) on MCA are available. Ikonen et al. (1999b) did, however, observe a considerable variation in bulk milks between herds. In addition, some results for factors embedded in herd effects exist. Studies have clearly emphasized the importance of dietary energy level on MCA (Macheboeuf et al. 1993, Kreuzer et al. 1996b, Malossini et al. 1996) and on cheese yield and quality (Kefford et al. 1995). Cows on a low-energy diet had worse MCA than cows on a well-balanced or high-energy diet. Further, the proportion of cows producing poorly coagulating milk (i.e., the curd is not firm enough for cutting in a standard 30-min time in cheese dairies) was higher for the cows on a low-energy diet (Malossini et al. 1996). Ostersen et al. (1997), among others, reported that body condition at calving affects MCA of cows throughout lactation, a good body condition being associated with a higher MCA.

Udder health has a clear phenotypic effect on MCA. Based on many studies reviewed by Bergère and Lenoir (2000), mastitis has a detrimental influence on the MCA of cows. Mastitis changes the mineral balance of the milk, decreasing for example the calcium content and increasing the pH of milk. It also decreases the casein content of milk (Le Roux et al. 2003, Coulon et al. 2004). All of these changes affect the micelle structure and the ability to form a firm curd (Bergère and Lenoir 2000, Lucey 2002). Researchers have in several studies confirmed mastitis milk's detrimental effect on both cheese yield and structure (Bergère and Lenoir 2000, O'Brien et al. 2005).

Farmers can affect the MCA of the herd bulk milk also through the selection of breed. There are indigenous breeds (e.g., Montbéliarde) and high-dry-matter-content breeds (e.g., Jersey) that have repeatedly turned out to be superior in MCA to such high-yielding dairy breeds as Holstein-Friesian (Macheboeuf et al. 1993, Kreuzer et al. 1996b, Malossini et al. 1996, Auldist et al. 2002, Kübarsepp et al. 2005b). Several explanations for this exist: for instance protein and casein content of milk is higher and the κ-casein B-allele is more common in indigenous and high-dry-matter-content breeds than in high-yielding dairy breeds (Macheboeuf et al. 1993, Kreuzer et al. 1996b, Malossini et al. 1996, Auldist et al. 2002, Kübarsepp et al. 2005b). On the other hand, compared with the high-yielding breeds, many indigenous and high-dry-matter-content breeds produce much less milk. Because the main profit comes from the amount of milk produced, the indigenous and high-dry-matter-content breeds are not as tempting a choice as the high-yielding dairy breeds.

3.3.3. Dairy technological factors

Some technological manipulations can be performed to improve MCA. Lowering pH directly or indirectly by adding starter to the milk, adding $CaCl_2$, increasing the amount of rennet, or raising the temperature of the milk coagulation process can improve MCA (van Hooydonk et al. 1986, van den Berg et al. 1992, Horne 1998, Brulé et al. 2000, Udabage et al. 2001, Lucey et al. 2003). However, these manipulations have limits and beyond these, MCA can start to deteriorate or some unfavourable side-effects can occur (Udabage et al. 2001).

3.3.4. Genetic factors

The most efficient and permanent way to improve milk quality for cheese production is to select breeding animals for fundamental traits. To be able to do this, genetic differences must exist between animals. Indeed, a considerable degree of variation has been observed in MCA both between breeds and between animals within breeds. About 20-40% of the phenotypic variation has been additive genetic in nature (Lindström et al. 1984, Tervala et al. 1985, Oloffs et al. 1992, Ikonen et al. 1999a), whereas only 16% of the phenotypic variation in the test day milk yield (Ikonen et al. 1999a) was additive genetic – the latter being the trait successfully selected for for decades. However, the estimates of heritability for renneting time and curd firmness have been based on relatively small data sets (300-1900 cows), and only the results of Ikonen et al. (1999a) were based on a mixed model methodology with an animal model, effectively utilizing the relationships between animals. In general, a mixed model methodology is not as sensitive to unbalanced data structure, i.e., unequal family sizes, as the ANOVA methodology (Lynch and Walsh 1998) used in the other studies.

3.4. Options for selection

3.4.1. Direct selection for MCA

Despite the limitations of earlier studies, MCA seems to be a characteristic with moderate heritability, and thus, possibilities exist for direct selection. With direct selection, genetic improvement of MCA is the most effective. Reliable estimates of the heritability and repeatability of milk renneting time and curd firmness are still needed. The estimates of repeatability are important since the measurement of milk coagulation traits is both laborious and time-consuming. Due to the lack of high capacity milk renneting devices, MCA is presently not a characteristic that can be included in routine monthly milk recording. Information on the minimum number of samples per cow for reliably estimating their average MCA is therefore required.

Thus far, only three studies have estimated repeatability for milk coagulation traits (Schaar 1984, Caroli et al. 1990, Ikonen et al. 1997). The magnitude of the estimates has ranged from 0.43 to 0.66 for milk renneting time and from 0.57 to 0.64 for curd firmness, indicating that MCA is a very repeatable characteristic. However, these results were based on small and sparse data sets. In Schaar (1984), 62 cows were sampled twice during either one lactation or two successive lactations. In Caroli et al. (1990), 32 cows were sampled eight times during a single lactation. Ikonen et al. (1997) sampled 59 Finnish Ayrshire and 55 Finnish Friesian cows three times during a single lactation and analysed them as separate data sets.

3.4.2. Indirect selection and marker-assisted selection for MCA

Because measurement of MCA is laborious, studying means for indirect selection and marker-assisted selection for MCA is warranted. Indirect selection can be done via traits that are sufficiently strongly correlated genetically with milk coagulation traits. Marker-assisted selection for MCA comes into question when some of the quantitative genes for MCA with sufficiently large effects are known and can be utilized in selection.

3.4.2.1. Traits in the total merit index

The easiest way to indirectly improve MCA is when the milk coagulation traits are genetically correlated with the traits included in the total merit index of breeding bulls. In Finland, the current traits (weights) in the total merit index of Finnish Ayrshire bulls (Faba breeding 2007) are production (0.9), udder health (0.3), udder conformation (0.4), fertility (0.3) and feet and legs (0.1). The production index is a combined index of production traits (4 x protein production index + 1 x fat production index - 1 x milk production index). The udder health index is estimated utilizing information on veterinary treatments for mastitis, somatic cell count and udder conformation. The udder conformation, fertility and feet and leg indices are multi-trait indices. Genetic correlations between milk coagulation traits and milk production traits and somatic cell count have thus far been estimated from limited data sets (Lindström et al. 1984, Oloffs et al. 1992, Ikonen et al. 1999a), and no reliable estimates are available. No genetic correlation estimates between milk coagulation traits and the other traits in the total merit index exist. Udder health traits might be good candidates to indirectly improve MCA because their phenotypic associations with MCA are strong (Le Roux et al. 2003, Coulon et al. 2004, O'Brien et al. 2005), suggesting the possibility of a genetic association.

3.4.2.2. Other candidate traits

If sufficiently strong correlations do not exist between milk coagulation traits and the traits included in the total merit index, it is still possible to look for other associated traits to

include in the index. The candidate traits must fulfil two important criteria: they must be highly genetically correlated with milk coagulation traits, and they must be easy to measure in order to be included in routine milk recording. Three possible candidate traits are evident: protein content, casein content and pH of milk; protein content is already included in routine milk recording. The justification for choosing these candidate traits has been discussed in earlier section. Reliable estimates of genetic correlations of the milk coagulation traits with the protein and casein contents and pH of milk are not yet available.

3.4.2.3. Selection for casein genotypes

In her thesis, Ikonen (2000) thoroughly evaluated the options for selection for casein genotypes. According to Ikonen (2000) and many other studies (Macheboeuf et al. 1993, Kübarsepp et al. 2005b, Comin et al. 2006), the effect of the κ-casein B-allele on MCA is favourable and large. This allele is rare in high-yielding dairy breeds as compared with indigenous breeds. Its prevalence in Finnish Ayrshire is only about 8% (Ikonen et al. 1996). The most obvious reason for its rareness is its tight linkage disequilibrium with the β-casein A_1-allele, which in turn is associated with low milk and protein yields (Ikonen 2000), the main traits of selection in dairy breeds. Thus, selecting FA cows for the κ-casein B-allele under the present selection scheme is difficult since the number of β-κ-casein A_2-B cows with favourable effects on both MCA and yield traits is small. Selection against the κ-casein E-allele, by contrast, is an attractive choice. It is common (31%) in Finnish Ayrshire, the main dairy breed in Finland (Ikonen et al. 1996), and its effect on MCA is unfavourable (Ikonen 2000). In addition, in Finnish Ayrshire, the κ-casein E-allele is mainly linked to the β-casein A_1-allele, which is associated with low milk and protein yields (Ikonen et al. 2001). Further, the E-allele has a negative effect on protein content and somatic cell score (Ojala et al. 2004b, 2005).

3.4.2.4. Selection against non-coagulation of milk

Selection against the κ-casein E-allele would be an option worth considering to improve MCA, but it would not solve the problem of non-coagulation of milk (Ikonen et al. 1999a). Based on Ikonen et al. (1999a), non-coagulation of milk was not associated with κ-casein polymorphism in Finnish Ayrshire cows.

Non-coagulation of milk is a rather common (about 8%) phenomenon in Finnish Ayrshire (Ikonen et al. 1999a). No curd forms in the standard 30-min testing time in non-coagulating (NC) milk, and such a milk is poor raw material for cheese production (Ikonen et al. 1999b), even though the addition of $CaCl_2$ can re-establish its ability to coagulate (van Hooydonk et al. 1986, Nyholm 2002). However, as much as 0.04% $CaCl_2$ addition was needed for some NC-milk to regain normal coagulation ability (Nyholm 2002). In many countries, the highest allowable $CaCl_2$ addition is 0.02% since a higher

concentration can produce a bitter flavour in cheese (Buch Kristensen 1999, Nyholm 2002).

To date, rather little is known about the non-coagulation of milk, and thus, more knowledge about the phenomenon and associated causes and factors is needed. However, according to several studies (Tervala and Antila 1985, van Hooydonk et al. 1986, Resmini et al. 1995), the cleavage of κ-casein, the first step in milk coagulation, occurs more or less normally in NC-milk. In addition, many studies (van Hooydonk et al. 1986, Resmini et al. 1995, Wedholm et al. 2006, Tsioulpas et al. 2007) have reported that the ratio of soluble casein to total casein is higher, the moisture content of micelles is higher and the content of total and colloidal calcium is lower in NC-milk than in normal milk. Some studies (Okigbo et al. 1985, van Hooydonk et al. 1986) have observed that the pH of NC-milk is higher than that of normal milk. This may, however, result from the lower level of calcium in NC-milk relative to normal milk (van Hooydonk et al. 1986). Based on the variation among sires in the proportion of daughters producing NC-milk, Ikonen et al. (1999a) suggested that the causes for non-coagulation are partly genetic.

4. Aims of the study

The main objective of this thesis was to evaluate means of selecting dairy cattle for MCA to improve milk quality for cheese production. Options for direct and indirect selection and selection against non-coagulation of milk were examined. The effects of non-genetic factors on MCA were also investigated. Studies I-IV contributed to these objectives by providing the following:

I. Estimates of repeatability for milk coagulation traits, persistence of non-coagulation of milk and effects of environmental factors on MCA.
II. Breed differences in MCA and herd effects on MCA.
III. Estimates of heritability for milk coagulation traits and genetic and phenotypic correlations between milk coagulation and production and quality traits.
IV. Mapping of genes affecting non-coagulation of milk.

5. Materials and methods

5.1. Materials

Three separate data sets were collected for Studies I-III, as shown in Figure 1. The data set in Study IV was a sub-sample of the data set in Study III. As the main objectives in Study I were to establish the repeatability estimates for milk coagulation traits and to evaluate the persistence of NC-milk, monthly samples were collected in one herd over a two-year period. This data set was also appropriate for evaluating the influence of systematic environmental factors, such as lactation stage, on MCA. All cows were of Finnish Ayrshire breed.

The data set in Study II was collected as part of the milk quality project of one dairy cooperative in Central Finland. The dairy had specialized in producing high-quality Emmental cheese and wanted to assess the MCA of individual cows and herd bulk milks of milk-producers, and to train milk-producers to improve the quality of milk. The data set proved to be good for evaluating the breed differences in MCA and the prevalence of non-coagulation of milk among Finnish Ayrshire (FA) and Holstein-Friesian (HF) cows. In addition, the data set proved to be good for evaluation of herd effects and factors embedded in there, determined by farmers filling out a questionnaire concerning the management and feeding practices in their herds.

The main objectives of Study III were to establish the estimates of heritability for milk coagulation traits and the genetic and phenotypic correlations between milk coagulation and production and quality traits. Further, the data collected had to also be suitable for the gene mapping of the non-coagulation of milk (Study IV). Hence, the aim of the sampling was to collect sufficiently large half sib-groups to estimate the genetic parameters reliably, and to ensure that the data would serve as a good source for gene mapping. Only half sib-groups of the sires assumed to be heterozygous for the hypothesized NC-genes and with large daughter groups were selected for the gene mapping study. The classification of sires as heterozygous was based on their daughters' distribution for MCA (Figure 3, Sire B). All cows in the sample were of Finnish Ayrshire breed.

Item	Study I	Study II	Study III
time of sampling	September 1993 - August 1995	April - May 1999 before grazing season	February - May 1999 before grazing season
main objectives	estimates of repeatability for MCA[a], persistence of NC-milk, effects of non-genetic factors on MCA	effect of herd on MCA and differences between breeds in MCA and non-coagulation of milk	estimates of heritability for MCA, genetic correlations between MCA and milk production and quality traits, genetic factors associated with NC-milk
design	monthly measurements of MCA in the experimental herd of Helsinki University, Viikki	125 milk-producers of one cooperative dairy, one MCA measurement / cow + herd bulk milk sample	5095 daughters of 91 bulls in 693 herds in Southern and Central Finland, one MCA measurement / cow
breed	Finnish Ayrshire (FA)	FA, Holstein-Friesian (HF), Crossbred (CB)	FA
number of cows in statistical analyses	83	1408	4664
number of samples in statistical analyses	979: 2 to 22 / cow, mean 12	1408 (959 FA, 399 HF, 50 CB) + 84 herd bulk milk samples	4664, number of daughters / bull range 17-271, mean 51
traits	MCA, test-day milk yield, fat and protein content, pH and SCS of milk	MCA, 305-d milk, fat, and protein yields, and protein and fat content, pH of milk	MCA, test-day milk yield, fat and protein content, casein content, pH and SCS of milk
other	records of veterinary treatments kept by cattlemen	information on feeding and management of herds	

[a]MCA= milk renneting time in min: time from addition of rennet to the start of coagulation; curd firming time in min: time of start of coagulation to the diagram width of 20 mm; curd firmness in mm: diagram width 30 min after addition of rennet. The wider the diagram drawn by the measuring device, the better the milk coagulation ability.

Item	Study IV
main objective	mapping DNA regions associated with non-coagulation of milk
design and number of cows in the analyses	a sub-sample of data in Study III: whole genome scan with 15 cM map density and selective DNA pooling of 33 NC[b]- and 49 E[c]-daughters over the 17 heterozygous sires for non-coagulation of milk → 11 chromosomes for individual genotyping with 10 cM map density using daughter design and selective genotyping: 18 heterozygous sires for non-coagulation of milk and their 188 NC-daughters (mean 10 / bull) and 289 E-daughters (mean 16 / bull) with phenotypes and genotypes, and 1561 moderate daughters for MCA (mean 87 / bull) with phenotypes only, each extreme representing about 12% of the sample
breed	FA
traits	square root of curd firmness pre-corrected for lactation stage, parity, age of milk sample, measuring unit of renneting device and herd, SCS of milk pre-corrected for lactation stage, parity and herd, and pH of milk pre-corrected for lactation stage, parity, age of milk sample and herd

[b]NC-daughter = a daughter with a non-coagulating milk sample
[c]E-daughter = a daughter with an excellently coagulating milk sample, i.e., a curd firmness value of 35 mm or more

Figure 1. *Overview of data sets in Studies I-IV.*

5.1.1. Traits evaluated and laboratory analyses

5.1.1.1. Milk quality traits

MCA was measured with a Formagraph (Foss Electric A/S, Hillerød, Denmark) in Study I and with a Computerized Renneting Meter, CRM (Polo Trade, Monselice, Italy) in Studies II and III. The traits describing MCA are for both devices: milk renneting time (R, min), curd firming time (K_{20}, min) and curd firmness (E_{30}, mm). Milk renneting time is the time from the addition of rennet to the start of coagulation. Curd firming time is the time from the start of coagulation to the diagram width of 20 mm, drawn by the renneting device. When the diagram width has reached 20 mm, the curd is normally firm enough for cutting at cheese dairies. If the width of the diagram does not reach 20 mm in a 30-min testing time, the milk is considered to be poorly coagulating (PC). In this case, curd firming time is assigned no value. Curd firmness is the width of the diagram after a testing time of 30-31 min. Up to a limit, the larger the width, the better the milk is for cheese production. Non-coagulating (NC) milk is assigned a curd firmness value of 0 mm. Curd firmness is therefore the best trait to describe MCA. Genetic correlations between the above milk coagulation traits are almost one (Ikonen et al. 1999a, Study III), so using all three traits is not even necessary.

Curd firmness was also examined as a binary trait in Study III, to investigate the heritability of non-coagulation of milk. Further, estimates of the genetic parameters for curd firmness were evaluated from two different data sets – the samples that coagulated and all samples – in order to evaluate the effects of deviation from normality and the effects of NC-samples on the estimates. In Study IV, curd firmness was pre-corrected for lactation stage, parity, age of the milk sample, measuring unit of the renneting device and herd since modelling of the environmental factors was impossible with the software that was used for linkage analyses. The trait was still bimodal after pre-corrections; to avoid spurious LOD score peaks with the Maximum Likelihood method, a square root transformation was used to normalize the distribution. This was done by balancing between normalization and minimal information loss. Prior to transformation, the distribution was inverted to enhance transformation of the more deviating tail.

In addition to the MCA measurements, pH of milk was measured with a PHM 83 Autocal pH Meter (Radiometer A/S, Copenhagen, Denmark) in Study I and with a 744 pH Meter (Metrohm, Herisau, Switzerland) in Studies II and III. The somatic cell count of milk was measured with a Fossomatic 360 (N. Foss & Co. A/S, Hillerød, Denmark) in Studies I and III. In statistical analyses, it was transformed to a natural logarithmic form (somatic cell score, SCS) to obtain an approximately normal distribution. In addition to curd firmness, pH of milk and SCS were used as traits in Study IV. They were also pre-corrected prior to linkage analyses: pH of milk for lactation stage, parity, age of the milk sample and herd effects, and SCS for lactation stage, parity and herd effects.

5.1.1.2. Milk production traits

Test-day milk yields and fat and protein contents – the latter measured with a MilcoScan 605 (N. Foss & Co. A/S, Hillerød, Denmark) – were used in Studies I and III. Because sampling in Study II was to be finished before the grazing season, measurements of the MCA did not match the milk recording days. Hence, 305-d milk, fat and protein yields and fat and protein contents were used in Study II. Casein content was measured with a MilcoScan FT 120 (Foss Electric A/S, Hillerød, Denmark) in Study III.

5.1.1.3. Molecular genetic analyses

Bull DNA was extracted from semen samples (Study IV) following a protocol by Zadworny and Kuhnlein (1990). The DNA of cows came from several sources: milk, hair and blood. The extraction of DNA from milk was accomplished using chloroform-phenol (Lipkin et al. 1993) and Chelex protocols (Amills et al. 1997), DNA from blood using a slightly modified protocol by Miller et al. (1988) and DNA from hair following a Chelex protocol by Walsh et al. (1991). Microsatellite markers were chosen from the Marc database (http://www.marc.usda.gov/genome/cattle/cattle.html) and from the NCBI database http://www.ncbi.nlm.nih.gov/genome/guide/cow/index.html). Samples were amplified with PTC100 and PTC200 PCR machines (MJ Research, Waltham, MA, USA) and were run with a Li-Cor Gene Readir 4200 DNA analyser (LI-COR, Lincoln, NE, USA) and with an ABI Prism 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). A basic PCR protocol for Li-Cor included 20 ng template DNA, 200 μM of each dNTP, 0.16 U Dynazyme II polymerase (Finnzymes, Espoo, Finland), the buffer provided with the enzyme and 0.25 μM of each primer in a reaction volume of 10 μl. A basic PCR protocol for ABI included 10 ng template DNA, 200 μM of each dNTP, 0.16 U Dynazyme II polymerase (Finnzymes, Espoo, Finland), the buffer provided with the enzyme and 0.05-0.2 μM of each primer in a reaction volume of 5 μl. A basic PCR program started with 4 min of denaturation in 94°C, followed by a cycle of 1 min in 94°C, 1 min in a marker-specific annealing temperature, 1 min in 72°C repeated 30 times, 10 min in 72°C and cooling to 4°C. The annealing temperature ranged from 50°C to 64°C. Two persons verified the genotypes using either Gene ImagIR software (Version 3.5.6, Scananalytics, Fairfax, VA, USA) or GeneMapper Software (Version 4.0, Applied Biosystems, Foster City, CA, USA).

5.1.2. Other information

In addition to the milk production traits, information on date of birth and calving, and on the pedigree was obtained for all studies from the Agricultural Data Processing Centre (Vantaa, Finland). In Study I, information on treatment of diseases was obtained from the records kept by cattlemen, and in Study II information on feeding practices and management of the herds was obtained from the questionnaires filled in by the farmers.

5.1.3. Data used in statistical analyses

5.1.3.1. Quantitative genetic data

Before statistical analyses, the data sets were pre-processed. All cows with missing or incorrect information, e.g. like errors in parentage information, parity or lactation stage, were excluded, as were samples collected within the first five days after parturition. As several measurements per cow were needed in Study I, the cows with only one measurement were also excluded. All herds outside the milk recording system were omitted from Study II due to missing information. In addition, Finncattle cows had to be excluded from the analyses in Study II because the number of cows was too small to be utilized. The sizes of the data sets are presented in Figure 1.

5.1.3.2. Molecular genetic data

A genome scan (Study IV) was carried out using selective DNA pooling (Darvasi and Soller 1994) and association analysis methods. Based on the hypothesis of the same ancestral mutation in all sire families, pools were made across sires. The hypothesis relied on three observations from the data: 1) an obvious spike of NC-samples in the distribution of MCA in the whole data set (Study III), 2) the sires in Study III could be divided into three different groups based on their daughters' distribution for MCA (Figure 3), indicating that they could be homozygous, heterozygous or non-carriers for the hypothesized NC genes and 3) sires were related due to several common ancestors. A pool of 33 cows producing NC-milk, and a pool of 49 cows producing excellently coagulating (E) milk were constructed. The cows were daughters of 17 sires, with an overall mean of 4.8 daughters per sire, and with a mean of 1.9 NC- and 2.9 E-daughters. In order to exclude the carriers of the NC-genes from the E-pool, only cows in mid-lactation with positive breeding values for curd firmness were accepted. Further, only cows with negative breeding values for curd firmness were accepted in the NC-pool without limitation on lactation stage. The number of microsatellites used for the genome scan was 194, with a density of 15 cM.

Verification of the genome scan results that indicated an association with non-coagulation of milk was carried out under daughter design and selective genotyping (Darvasi and Soller 1992). The cows were daughters of 18 sires. About 12% of each phenotypic extreme for MCA were genotyped. This yielded 188 NC-daughters (mean 10.4 daughters/sire) and 289 E-daughters (mean 16.1 daughters/sire). Further, 1561 daughters (mean 86.7 daughters/sire) with moderate MCA and only phenotypic information were included in the statistical analyses. The map density ranged from 2 to 18 cM, with a mean of 9.8 cM.

5.2. Statistical analyses

5.2.1. Analyses of quantitative genetic data

The effects of fixed (I-III) and random (III) effects and variance components of the random effects (I-III) were analysed with a univariate mixed model, and phenotypic (I, III) and genetic (III) correlations with multivariate (I) and bivariate (III) mixed models. Variance components were estimated using a Restricted Maximum Likelihood method under a repeatability animal model (I) and an animal model (II, III).

Parity and lactation stage were included as fixed effects in all studies and for all traits, except for 305-d milk production traits in Study II. Because of the long-term sampling in Study I, year-season was also included in the models as a fixed effect. As more than one breed was analysed in Study II, breed was included as a fixed effect. Due to the rather large variation among sensors of the CRM renneting device, the sensors were included as a fixed effect in the models for milk coagulation traits in Studies II and III, where the device was used. The age of the milk samples varied at the time of laboratory analyses in Study III. It was, thus, included as a fixed effect in the models for milk coagulation traits and pH of milk.

The additive genetic effect of the animals and the residual effect were included in all models as random effects. Due to several measurements per cow, permanent environmental effect was added as a random effect to the models in Study I. In Studies II and III, samples were obtained from many herds, and herd was therefore included as a random effect. As the effects of concentrate feeding frequency and type of concentrate were evaluated in Study II, herd effect was replaced with these fixed effects.

Pedigree information for the cows with observations included parents and grandparents (I-III), and for some cows also great grandparents (I) and great-great grandparents (I).

Solutions for the fixed and random effects were estimated using PEST (Groeneveld 1990). The statistical significance of the fixed effects was tested using F-test with PEST. The covariance components for the random effects were computed using REML (VCE 4.0, Groeneveld 1997).

Risk factors for the production of NC-milk were studied using logistic regression analyses in the sub-sample of 24 FA cows producing NC-milk at least once (I). The size of the sub-sample was 267 observations. The analyses were based on GENMOD procedure, logit link function and generalized estimating equations (GEE) methodology with SAS 8.2 (SAS Institute Inc. 1999). The risk factors studied were classified parity, lactation stage, year-season, continuous test-day milk yield, fat and protein content, pH and SCS of milk. The statistical significance of within-class estimates was based on standardized normal distribution. The statistical significance of overall estimates was based on Type 3 GEE analyses and χ^2 –distribution. The Type 3 analysis is analogous to Type III sums of squares in the GLM procedure, and it does not depend on the modelling order of the factors (SAS Institute Inc. 1999). Contrast estimates were also calculated for the differences between fixed effect classes. Their statistical significance was based on generalized score statistics and χ^2 –distribution.

5.2.2. Analyses of molecular genetic data

5.2.2.1. Analysis of selective DNA-pooling data

Before testing the intensities of marker alleles in NC- and E-pools for homogeneity, the intensities were corrected for PCR artefacts, i.e., shadow bands and differential amplification (IV). The corrections for shadow bands were made according to Lipkin et al. (1998), and the corrections for differential amplification according to Kirov et al. (2000). Homogeneity of the corrected allele intensities between the two pools was tested with χ^2-test using CLUMP (Sham and Curtis 1995), which simulates the test distribution using a Monte Carlo method because the test statistics no longer match the χ^2-distribution in typical large and sparse 2xN allele intensity tables.

5.2.2.2. Pre-corrections of traits and linkage analyses

Solutions for the fixed and random effects of curd firmness, SCS and pH of milk were obtained from the data set of 4664 observations (III) with PEST (Groeneveld 1990) using a univariate mixed model. The variance components for the herd effects were estimated with VCE4 using a Restricted Maximum Likelihood method (Groeneveld 1997). The marker map was constructed with a slightly modified version of ANIMAP software (Georges et al. 1995). The traits were analysed on a within-family basis using both non-parametric (Coppieters et al. 1998) and Maximum Likelihood (Georges et al. 1995) interval mapping methods developed for granddaughter/daughter designs.

6. Results

## 6.1. Variation in milk coagulation ability

Compared with milk production traits, the coefficient of variation in MCA was much larger, approximately 45% (vs. 20%, I-III). Part of the large variation was due to a spike in the distribution of FA cows, which in turn was caused by non-coagulation of milk. About 10% of FA cows produced NC-milk (I-III), whereas the prevalence was only 1% in HF cows (II). The distributions of curd firmness differed clearly between the two breeds (Figure 2), the distribution of FA cows being more skewed towards poor values. Besides the difference in the prevalence of NC-milk, breeds also differed in the proportion of PC-milk. About 30% of FA cows produced PC-milk (I-III), while the proportion in HF cows was only 12% (II).

The MCA of daughters differed clearly among sires (I-III). Differences in daughter distributions were most marked in data set III, in which the sires had relatively large daughter groups (Figure 3). Some of the sires had hardly any good daughters, with up to 50% of the daughters producing NC-milk (bull A in Figure 3). Conversely, some sires had only a few poor daughters, and their daughters' distribution was clearly skewed towards good values (bull C in Figure 3). Differences in the MCA of individual cows were also reflected in the MCA of herd bulk milks (II) and some of the herd bulk milks were non-coagulating (results not shown).
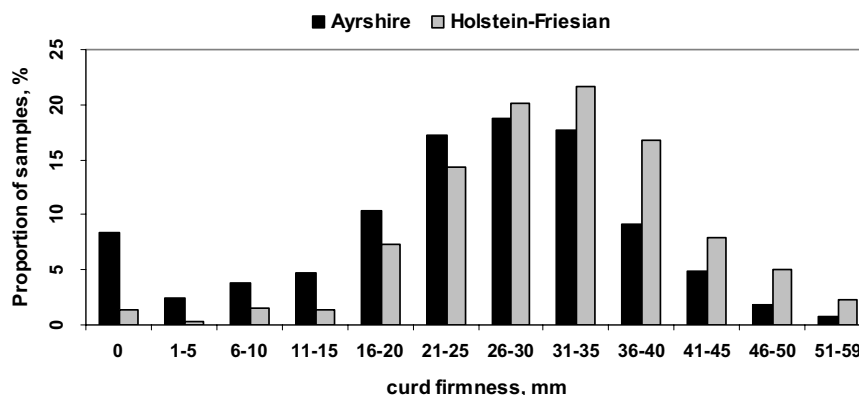
Figure 2. *Distributions of MCA of Finnish Ayrshire and Holstein-Friesian cows. To a set point, the higher the value for curd firmness, the better the milk is for cheese production. 0 mm refers to non-coagulating milk.*
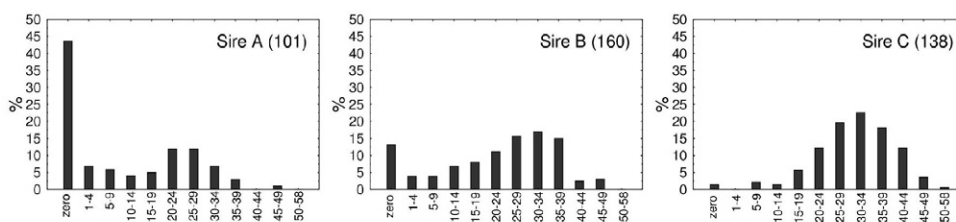
Figure 3. *Three different bulls' daughter distributions for curd firmness. To a set point, the higher the value for curd firmness, the better the milk is for cheese production. Zero refers to non-coagulating milk. The figure in parentheses refers to the number of daughters.*

6.2. Effects of parity, lactation stage and year-season

6.2.1. Parity

Test-day milk yield (I, III) and 305-d milk, fat and protein yields (II) increased and pH and SCS of milk deteriorated consistently with increasing parity in all data sets. The results of protein and fat content and MCA were more contradictory. Parity had either no effect on fat content (II) or fat content declined with increasing parity (I, III). Similarly, parity had no effect on protein content (III), or protein content declined with increasing parity (I, II). Casein content declined with increasing parity in Study III, the only data set in which it was analysed. The results for MCA were even more discrepant. MCA deteriorated with increasing parity in Study I, while parity had no effect on MCA in Study II. In Study III, parity had no effect on MCA when coagulated samples were analysed. However, MCA improved with increasing parity when NC-samples were included in the data. A probable reason for this was the larger proportion of primiparous cows than older cows producing NC-milk.

6.2.2. Lactation stage

The effect of lactation stage was strong ($P < 0.001$) on all studied traits and consistent among the data sets. Milk yield reached its maximum level about one month after parturition and then steadily declined towards the end of lactation (I, III). Fat (I, III), protein (I, III) and casein content of milk (III) as well as MCA (I-III) were at their best at the beginning and the end of lactation, in contrast to SCS, which was poorest at those stages (I, III). Milk pH was lowest at the very beginning of lactation, increasing steeply during the first two months and remaining high for the rest of lactation (I-III).

6.2.3. Year-season

Except for test-day milk yield, no clear year-season trend was observed in milk production traits (I). Year-season had no effect on SCS. MCA and pH of milk were, however, at their best during the grazing season. Because the calvings in the experimental herd occurred mainly in autumn, the favourable effect of grazing season on MCA could partially be explained by the late lactation stage of the cows.

6.3. Effect of herd

Herd explained only a minor part of the variation in MCA (5-9% in Studies II and III), pH of milk (11-15% in Studies II and III) and SCS of milk (8% in Study III), compared with 305-d milk production traits (>40% in Study II) or test day milk production traits (20% in Study III).

Cows fed concentrate only twice a day produced about 1000 kg less milk, 25 kg less fat and 30 kg less protein per year than cows fed four times a day (II). Concentrate feeding frequency had no clear or strong effect on 305-d protein content, but 305-d fat content decreased with increasing frequency. Twice a day feeding with concentrate had a weak unfavourable influence on MCA and pH of milk compared with more frequent feeding with concentrate. It increased, however, the production of PC-milk by 10 percentage units, but no association with NC-milk was detected.

The type of concentrate in Finland is normally either a farm mixture of barley and oats or a compound feed, or both. The type of concentrate had to some extent opposite effects on milk production traits and MCA (II). The higher the proportion of oats in the farm mixture, the lower the 305-day milk, fat and protein yields. However, a moderate proportion of oats in the diet slightly improved MCA. Differences between the type of concentrate groups for milk renneting time and curd firmness were, however, small and of the same magnitude as their standard errors. The combination of compound feed and farm mixture was associated with the highest 305-d yield traits.

Dividing the herds into two groups consisting of the 10 highest and the 10 lowest production herds based on 305-d protein yields disclosed more than a one or two phenotypic SD unit difference in 305-milk production traits, but no difference in MCA. The proportion of PC-milks was, however, 18 percentage units higher in the lowest production herds compared with the highest ones, but no association with NC-milk was detected.

6.4. Effect of breed

HF cows produced milk that started to coagulate 2.4 min earlier (0.5 phenotypic SD in overall data) and was 4.1 mm firmer (0.3 phenotypic SD in overall data) than that of FA

cows (II). They were also superior to FA cows in pH of milk and 305-d milk and protein yields, whereas FA cows produced milk with a higher protein and fat content.

6.5. Genetic parameters of milk coagulation traits

6.5.1. Estimates of heritability and repeatability

MCA is a very heritable and repeatable characteristic. The heritability estimate for milk renneting time was almost 0.30, and for curd firmness 0.40 (III). The magnitude of the heritability estimates for milk coagulation traits was about the same as those for the protein and casein content of milk. By contrast, the heritability estimate for test-day milk yield was only 0.13. Repeatability estimates for milk renneting time and curd firmness were almost 0.70 (I). Again, the magnitude of the repeatability estimates for milk coagulation traits was about the same as that for protein content. The repeatability estimate for test-day milk yield was about 0.40.

6.5.2. Genetic and phenotypic correlations

The genetic correlations of milk coagulation traits with test-day milk yield and fat, protein and casein content were very low or zero (III). The genetic correlations of milk coagulation traits with pH and SCS of milk were moderate and favourable (III). The milk coagulation traits and SCS were, however, not phenotypically correlated (III). The phenotypic correlations between milk coagulation traits and pH of milk were lower than the genetic correlations. Protein and casein content of milk were highly genetically correlated (III, 0.92).

6.6. Non-coagulation of milk

6.6.1. Persistence

The FA cows could be classified into three groups according to their MCA (I). Two-thirds of the cows produced milk that always coagulated. One-third produced NC-milk at least once. Those cows could be classified into two groups of equal size: cows producing NC-milk only a few times during lactation and cows producing NC-milk at almost every sampling. The production of NC-milk was most common at peak or mid-lactation in Study I.

6.6.2. Heritability

The heritability estimate for curd firmness as a binary trait was 0.26 (III). This result as well as the clear differences in daughters' MCA among bulls (Figure 3) lend strong support for some of the causes of non-coagulation of milk being genetic.

6.6.3. Phenotypic and genetic associations

Non-coagulation of milk was not consistently found to be associated with parity and lactation stage. Non-coagulation of milk was not associated with parity in Studies I and II; however, in Study III, the prevalence of NC-milk was higher for primiparous cows than for older cows. Further, NC-milk of FA cows was clearly associated with peak and mid-lactation in Studies I and III, but no association was detected in FA in Study II. All HF cows that produced NC-milk were in late lactation (II), but no general conclusions can be drawn because the number of NC-cows was low (5). The probability of producing NC-milk differed between year-season classes (I).

A high pH of milk increased the probability of producing NC-milk in Study I. Based on the magnitude of phenotypic correlations between curd firmness and pH of milk in all samples (-0.18) and in coagulated samples (-0.26) in Study III, pH of milk seemed to be more strongly associated with coagulated samples. No phenotypic association was detected between non-coagulation of milk and SCS of milk (I, III) or mastitis treatment (I).

The predicted breeding values (PBVs) for milk production and quality traits were compared between cows producing NC-milk and cows producing coagulating milk (III). The latter cows were classified into four groups based on their phenotypic records: cows with curd firmness values of 1-19 mm, 20-29 mm, 30-39 mm and 40-58 mm. The class 1-19 mm represents poorly coagulating (PC) milk and the class 40-58 mm excellently coagulating (E) milk.

According to the results, a non-linear relationship existed between MCA and protein and casein contents since the mean PBVs were best in both NC- and E-groups. However, in studying the scatter plots of PBVs for curd firmness and protein and casein contents (Figure 4), the non-linear relationship was less evident.

Even though no phenotypic association was present between non-coagulation of milk and SCS, they were genetically associated. The association could be observed in two ways (III). First, the genetic correlation was higher between SCS and curd firmness of all samples (-0.45) than of coagulated samples (-0.33). Second, the mean PBV for SCS was highest (i.e., poorest) in the NC-group, and it decreased linearly with increasing curd firmness. Consistent with the phenotypic association, pH of milk was more clearly associated with coagulated samples. Again, this could be observed in two ways (III). First, the genetic correlation was higher between pH and curd firmness of coagulated samples (-0.51) compared with that of all samples (-0.32). Second, the poorest mean PBV was in the PC-group, not in the NC-group, and the best mean PBV was in the E-group.
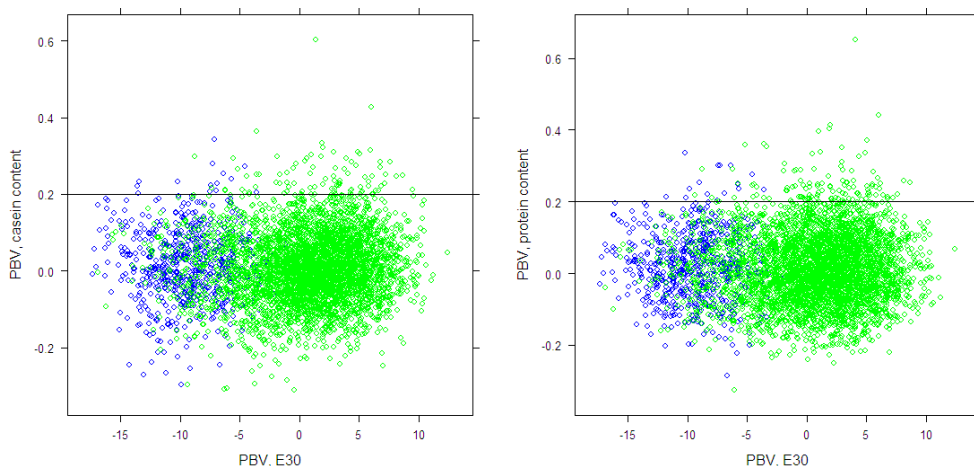
Figure 4. *Scatter plots of predicted breeding values (PBVs) for curd firmness and casein and protein contents from the data set of 4664 cows. Blue points refer to cows with non-coagulating milk samples and green points to cows with coagulating milk samples.*

6.6.4. Gene mapping

Several genomic regions associated with the non-coagulation of milk or MCA were discovered. This was to be expected due to the nature of the data. The strongest results were detected in chromosomes 2, 18 and 24. In chromosome 2, near marker BMS1126, and in chromosome 18, near marker BMS1355, the results were statistically significant over all families at the experiment-wise 0.1% significance level. The result in chromosome 24, near marker BM7151, was statistically significant at the experiment-wise 0.1% significance level, but only in one family. The results revealed chromosomes 2 and 18 to be the most likely candidate regions for the non-coagulation of milk. Further, based on the distribution of the sire alleles in the NC- and E-daughters, the region near marker BMS1355 was the most encouraging.

The NCBI gene database (http://www.ncbi.nlm.nih.gov/genome/guide/cow/ index.html) revealed two possible candidate genes within selected regions in chromosomes 2, 18 and 24: LOC53889, located 1.4 Mbp downstream from BMS1126 in chromosome 2, and SIAT4B, located 1.2 Mbp downstream from BMS1355 in chromosome 18. Both of these catalyse the post-translational modification of amino acids.

Some indication was found that the same regions that were associated with MCA in chromosomes 4 and 27 were also associated with pH of milk. In chromosome 24, the maxima of the MCA and pH of milk were 20 cM apart. Due to the long confidence interval, it is possible, however, that the exact positions are the same. Only one quantitative trait locus (QTL) for SCS of milk, located in linkage group 13a, was detected. No QTL for MCA was found in that linkage group.

7. Discussion

7.1. Milk coagulation ability as a study trait

MCA is a quantitative trait. Thus, many genes affect it, some with larger effects than others such as casein genes. Based on our understanding, some alleles of these genes can even have dramatic effects, causing the non-coagulation of milk. Especially in a gene mapping, this inevitably affects the structure of the collected data set in a way that should be borne in mind. NC-cows may be a very heterogeneous group for the genes affecting MCA in general, whereas E-cows are likely to be carrying predominantly good genes for MCA. Thus, even if the main aim in Study IV was to look for NC-genes, some of the genomic regions found are likely those affecting milk coagulation ability, not non-coagulability.

E-cows are also a group worth considering in more detail. Since cows carrying NC-genes can occasionally produce excellently coagulating milk, especially at the beginning and end of lactation, the probability of selecting only non-carriers to the E-group would be higher if the selection was based on several MCA measurements. This would, however, increase the cost of gene mapping considerably.

7.2. Design of Study IV

Conducting a genome scan with a 15 cM marker map within families would have been less risky than the association analysis across families that were conducted. The data set was also small at both stages of gene mapping. It was, thus, possible to find only major genes. However, this suited our objectives since the preliminary analyses supported that only a few genes cause the non-coagulation of milk.

Another option of genomic study of non-coagulation of milk would be a gene expression study of the extreme cows for MCA using a whole genome array. Because the number of animals in a gene expression study can be noticeably lower than in gene mapping, MCA could be measured in the smaller group of animals over a long period to ensure that the cows selected for gene expression analysis are extreme examples of MCA.

7.3. Effect of lactation stage

According to the results of Study I, where lactation stage, parity and season were investigated simultaneously, the effect of lactation stage on MCA was the largest. MCA was at its poorest in mid-lactation. The results were consistent in all data sets and are in agreement with most other studies (e.g., Kreuzer et al. 1996a, Ostersen et al. 1997, Ikonen et al. 1999a, Kübarsepp et al. 2003, 2005b).

7.4. Effect of herd

Herd accounted for only a minor proportion of the total variance in MCA compared with the milk production traits (II, III). This indicates that the selection of animals for MCA is much more efficient than herd management and feeding – unless a new management factor or feed additive with a strong impact on MCA emerges. Fairly significant differences nevertheless existed between herd bulk milks in MCA (II). This can be partly due to the differences in the proportion of breeds and genetic differences within breeds.

Even though herd had only a small impact on MCA compared with genetic effects, the results in Study II suggest that good management and feeding of cows can have a substantial effect on yield traits at the year-level, and thus, on farm profits, with a positive effect on MCA as well.

Frequent feeding of concentrate had a strong positive effect on 305-d milk yield traits. The positive effect on MCA was more modest, with no association with non-coagulation of milk. However, the proportion of PC-milk was clearly lower for cows fed frequently with concentrate than for cows fed with concentrate only twice a day. The same kind of effect has been observed with a well-balanced or high-energy diet (Macheboeuf et al. 1993, Malossini et al. 1996). Frequent feeding of concentrate can thus be associated with a better energy balance of cows, even though the results in the literature have been quite contradictory (Yang and Varga 1989, Macleod et al. 1994, Robinson and McNiven 1994, Shabi et al. 1999). Another explanation for the strong effect can be the association of frequent concentrate feeding with more professional herdsmanship. The division of the herds to the 10 highest and the 10 lowest production herds based on 305-d protein yield gave some support for this hypothesis. The best herds were larger and more often pure FA and/or HF herds (supporting selection), concentrate was fed more frequently, and it was more often a combination of compound feed and farm mixture with a lower proportion of oats (the most positive effect on yield traits) compared with the poorest herds.

7.5. Effect of breed

HF cows were superior to FA cows with regard to MCA (II). The size of the Holstein-Friesian data set was not very large, but it was the largest thus far collected for this purpose in Finland. The results were in accord with findings obtained earlier with smaller Finnish data sets (Ikonen et al. 1997, 1999a). Only 12% of the HF cows produced PC-milk, and non-coagulation of milk was only a minor problem (1%) in this breed. By contrast, 30% of FA cows produced PC-milk, and about one-third of this was non-coagulating. In accordance with foreign studies (e.g., Kübarsepp et al. 2005b, De Marchi et al. 2007), MCA of Finncattle has been better than that of highly selected dairy breeds (Tervala et al. 1983, 1985 and Study II, results not shown), although sizes of data sets have been small. The present milk pricing system does not, however, favour Landrace cattle. However, the two major Finnish dairy breeds, FA and HF, also clearly differed in MCA. At first glance, this seems to open a possibility to favour HF cows over FA cows. However, cautious interpretation is warranted. The Finnish Holstein breeding strategy is

strongly based on utilization of foreign bulls, similar to strategies in Estonia and Italy. According to Estonian and Italian studies, about 6% of Estonian Holstein and Red-and-White Holstein cows (Kübarsepp et al. 2005b) and 10% of Italian HF cows (Cassandro et al. 2008) produced NC-milk. Further, based on an other Italian study (Cassandro and Marusi 2001), a large variation existed in the proportion of daughters producing PC- or NC-milk among HF bulls, indicating a genetic cause for the non-coagulation of milk also in HF.

7.6. Estimates of heritability and repeatability

As earlier studies have indicated (h^2: Lindström et al. 1984, Tervala et al. 1985, Oloffs et al. 1992, Ikonen et al. 1999a, and r: Schaar 1984, Caroli et al. 1990, Ikonen et al. 1997), MCA is a very heritable (III) and repeatable characteristic (I). Both estimates were also in good agreement with the more recent study by Bittante et al. (2002). By contrast, the heritability estimate for the curd firmness of the coagulated samples reported by Cassandro et al. (2008) was somewhat lower than that in Study III. Differences in breeds and modelling might explain some of the discrepancy. The sensors of the CRM device were not included in the model in Cassandro et al. (2008). Based on our knowledge, variation in the sensors of the CRM renneting device is statistically significant – despite accurate calibration of the device – and modelling for this source of variation should result in an increase in the estimate of heritability.

The estimates of heritability and repeatability for milk coagulation traits are much higher than those for milk yield. In principle, good possibilities for direct selection exist and only three MCA measurements per cow should be sufficient for a reliable estimation of average MCA (e.g., Van Vleck et al. 1987).

7.7. Genetic and phenotypic correlations

Somewhat surprisingly, no or only low genetic correlation existed between milk coagulation traits and milk protein and casein content (III). However, SCS of milk and pH of milk were both moderately genetically correlated with milk coagulation traits. This indicates that good udder health and good MCA are genetically associated with each other even if there was no phenotypic correlation between milk coagulation traits and SCS of milk. Under the present weighting in the total merit index, hardly any progress is gained in MCA via SCS.

In general, the estimates of the genetic correlations of curd firmness with milk yield and quality traits were in good accordance with those in HF cows in the studies by Bittante et al. (2002) and Cassandro et al. (2008). However, Cassandro et al. (2008) reported genetic correlations of the curd firmness of coagulated samples with the protein and casein content of milk to be higher than those in Study III.

7.8. Non-coagulation of milk

Non-coagulation of milk is a common problem in the FA cows (I-III), and some FA cows can produce NC-milk at almost every sampling (I). Even though some environmental factors, such as peak and mid-lactation (I, III), were associated with this phenomenon, none could explain it fully. Interestingly, several indications of a genetic predisposition accumulated: a) a substantial variation was present in the proportion of daughters producing NC-milk among sires (III), b) the heritability estimate for the curd firmness as a binary trait was 0.26 (III) and c) highly significant (0.1% experiment-wise statistical significance) gene mapping results emerged over families in chromosome 2 and 18, near loci BMS1126 and BMS1355 (IV). No earlier studies of the heredity of the non-coagulation of milk exist, except for the study by Ikonen et al. (1999a), where a possible genetic cause of the non-coagulation of milk in FA cows was for the first time suggested. This was based on the observation that many cows producing NC-milk were daughters of two closely related sires.

Three potential causes of non-coagulation of milk exist: the occurrence of an unfavourable mutation in a) the casein gene/genes or in b) the gene/genes responsible for their post-translational modification (phosphorylation and glycosylation) or in c) the genes controlling the activity of the above genes. The post-translational modification of the caseins to a large extent influences their ability to bond calcium and their degree of hydrophilicity, both of which further affect the micelle structure (e.g., Horne 1998, Farrell et al. 2006).

No association between the non-coagulation of milk and the casein genes themselves was detected in Study IV. Further, based on several studies, the cleavage of the κ-casein occurs more or less normally in NC-milks (Tervala and Antila 1985, van Hooydonk et al. 1986, Resmini et al. 1995). Two possible candidate genes catalysing the post-translational modification were, however, found in Study IV. LOC538897 is a predicted gene that functions as a non-specific serine/threonine kinase (Ensembl database at http://apr2007.archive.ensembl.org/Bos_taurus/index.html). Golgi casein kinase, responsible for the phosphorylation of caseins, has not yet been molecularly characterized, nor located in the bovine genome. According to Tibaldi et al. (2006), the Golgi casein kinase recognizes the Ser-x-Glu/pSer sequence and accounts for all casein kinase activity of the Golgi apparatus with non-specific kinase activity. It is thus possible that the novel gene is the Golgi casein kinase. The second candidate gene, SIAT4B, is a sialyltransferase that catalyses the addition of N-acetylneuraminic acid (NeuAc) to galactose (Gal) (Ensembl database and KEGG database at http://www.genome.jp/kegg/kegg2.html). κ-casein is the only glycosylated casein and its major glycoform is a branched tetrasaccharide: NeuAcα(2-3)Galβ(1-3)[NeuAcα(2-6)]GalNAc, attached to the threonine (Holland et al. 2006). Thus, sialyltransferase is the enzyme catalysing the last step in the glycosylation of κ-caseins. Both of these genes are strong candidates, but their role in the non-coagulation of milk must still be verified.

The results in Study III indicated that SCS and the non-coagulation of milk are genetically associated, whereas the pH of milk is more clearly associated with good MCA. In Study IV, no evidence of a QTL associated with SCS of milk in chromosomes 2 or 18

was detected, but some loci (chromosomes 4, 27 and possibly 24) were associated with both MCA and pH of milk, in accordance with findings in Study III. The power of the analyses was, however, low since the data were selected based on MCA and the number of observations was small. Based on the QTL databases of Iowa State University (2007), University of Sydney (2007) and Texas A&M University (2007), one QTL for SCS has been discovered in chromosome 2 and several QTLs for SCS in chromosome 18. However, all QTLs for SCS in both chromosomes were located within a few dozen cM of the loci found in Study IV.

7.9. Options for selection

7.9.1. Direct selection for MCA

Direct selection would be an effective and tempting option for selecting breeding animals for MCA if a high-throughput renneting device were developed. The advantage of the CRM renneting device over Formagraph, which is no longer on the market, is automated data collection. However, the device is not as accurate (SE +/- 2-3mm) or as technically developed and reliable as needed. An Estonian group has started to use a new NIR-based Optigraph (Alliance Instruments, Frépillon, France, Kübarsepp et al. 2005a). It seems to be more accurate than the CRM, but unfortunately is not more efficient (Ivi Kübarsepp, personal communication). Based on the review by Lucey (2002), new renneting devices have been steadily developed through the decades so the possibility of a larger scale measuring device coming on the market in the future is likely. Currently, based on the three measurements per cow to estimate average MCA (I), selecting bull dams and young AI-bulls directly for MCA is possible (Ikonen 2000). However, direct selection remains a laborious option.

7.9.2. Indirect selection and marker assisted selection for MCA

Selection of sires for udder health also improves MCA through the moderate genetic correlation between SCS and milk coagulation traits, and decreases the prevalence of non-coagulation of milk. However, the response is probably weak because of the low weight placed on udder health. The other candidate traits – protein content and casein content of milk as well as pH of milk – also offered no solution. The genetic correlation between casein and protein content was very high (0.92), indicating that protein content, which is routinely measured in milk recordings, reflects casein content well. The genetic correlation between MCA and protein and casein content of milk was, however, almost zero. Further investigation of the relationship between PBVs for curd firmness and protein and casein contents (Figure 4) suggested that selection based on the latter would maintain NC-carriers in the FA population. Further, even though pH of milk was moderately

genetically correlated with MCA, it was not clearly associated with the non-coagulation of milk, i.e., hardly any change in its prevalence occurred.

Selection against the κ-casein E-allele at first glance appears to be a very tempting option because of E-allele's strong unfavourable genetic associations with milk yield and milk quality and coagulation traits (Ikonen 2000, Ojala et al. 2004b, 2005). Further, selection against the κ-casein E-allele would have no negative side-effects on fertility (Ruottinen et al. 2004) or body weight (Ojala et al. 2004a). Unfortunately, it however, would not have an impact on the non-coagulation of milk (Ikonen et al. 1999a, IV).

Because non-coagulation of milk is such a common problem (10%) in FA cows and its cause is genetic, the most effective way to improve MCA in Finland would be to eliminate carrier bulls from the selection program.

8. Conclusions

Significant variation was evident in MCA among cows, sires, herds, breeds and lactation stage. Despite the large differences in herd bulk milk, herd explained only a minor part of the variation in MCA. There was some indication that good management and feeding decrease the proportion of PC-milk. However, breed differences and genetic differences within breeds are probably a more significant cause of large variation in herd bulk milk than herd management and feeding. HF cows were superior to FA cows in MCA. Poor coagulation and non-coagulation of milk were only a minor problem (12%) in HF cows, whereas one-third of FA cows produced PC- or NC-milk. Because the breeding strategy of HF cows is based on extensive use of foreign bulls, favouring HF cows may not be the solution to improving MCA in Finland. According to foreign studies, non-coagulation of milk can be as serious a problem in HF cows in some countries as in FA cows in Finland.

Almost 40% of the variation among animals was additive genetic. Selection is thus the most effective way to improve MCA. Direct selection would be the most effective selection method. Based on the high repeatability estimates, only three measurements are needed to reliably estimate cows' average MCA. However, the current measuring devices are not suitable for the large-scale measurement required to include the trait in routine milk recording. While evaluation of breeding values for bull dams and young AI-bulls is possible (Ikonen 2000), direct selection would be laborious given the current renneting devices.

Other possibilities for improving MCA genetically are indirect selection and marker-assisted selection for MCA. The following three options were evaluated here: selection based on production and udder health traits in the total merit index, selection based on protein or casein content or on the pH of milk and selection based on reduction of the prevalence of NC-milk.

The findings indicate that the udder health index both improves MCA genetically and decreases the prevalence of NC-milk through somatic cell count. Under the present weighting scheme, however, hardly any response to MCA is expected. No genetic correlation between test-day milk yield and milk coagulation traits or non-coagulation of milk was observed.

Neither the protein or casein content of milk nor the pH of milk was found to be viable options for indirect selection. The results for casein content were identical to those for protein content, which is already included in routine milk recording. The genetic correlation between them was almost one, indicating that the protein content reflects the casein content well. The genetic correlation between MCA and protein and casein content of milk was, however, almost zero. Further investigation of the relationship between PBVs for curd firmness and protein and casein content suggested that selection based on the latter would maintain NC-carriers in the FA population. The pH of milk was moderately genetically correlated with milk coagulation traits, but was not clearly genetically associated with the non-coagulation of milk. Therefore, its inclusion in the index would likely not decrease the frequency of NC-milk.

If the non-coagulation of milk were not such a common problem in the Finnish dairy cattle population, selection against the κ-casein E-allele would be a very tempting means

to improve MCA. The E-allele is common in FA cows, and it is unfavourably genetically associated with both milk coagulation traits and many yield and quality traits (Ikonen 2000, Ojala et al. 2004b, 2005). It would be a readily available tool for selection. Unfortunately, selection against the E-allele would not have an impact on the non-coagulation of milk.

About 10% of FA cows produced NC-milk in all data sets analysed; some of the cows produced NC-milk at almost every sampling. The non-coagulation of milk is thus a worryingly common problem in the FA population. None of the environmental factors studied could fully explain it. However, several indications of a genetic cause emerged, and two associated loci were mapped to chromosome 2 (BMS1126) and chromosome 18 (BMS1355). In conclusion, currently, the elimination of carrier bulls of NC-genes would be the most effective way of genetically improving MCA.

Taken together, the evident implication of these findings is the need to develop a tool for selection against NC-genes. Further goals are to identify the genes affecting the non-coagulation of milk and to understand the mechanisms behind the phenomenon. Two candidate genes, LOC538897 in chromosome 2 and SIAT4B in chromosome 18, were found in Study IV. However, their role in the non-coagulation of milk awaits verification. Another future study is to thoroughly evaluate the financial impact of NC-milk on cheese plants. Based on a moderate-scale cheese-making trial, poor coagulation appears to cause significant losses to cheese plants (Ikonen et al. 1999b). But, as the authors also suggested, a thorough evaluation is needed. This would help breeding organizations, in collaboration with dairy industry, to make decisions on how to perform selection in the Finnish dairy cattle population in the future.

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