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EFFECT OF MILK PROTEIN COMPOSITION AND GENETIC POLYMORPHISM ON MILK RENNET COAGULATION PROPERTIES

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To my mother Tiiu Jõudu.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following papers, which are referred to by Roman numerals in the text. The papers are reproduced by kind permission of the publishers.

I **Kübarsepp, I.,** Henno, M., Kärt, O., Tupasela, T. 2005. A comparison of the methods for determination of the rennet coagulation properties of milk. Acta Agriculturae Scandinavica, Section A - Animal Science, 55(4), 145–148.

II **Kübarsepp, I.,** Henno, M., Viinalass, H., Sabre, D. 2005. Effect of κ-casein and ß-lactoglobulin genotypes on the milk rennet coagulation properties. Agronomy Research, 3(1), 55–64.

III **Kübarsepp, I.,** Henno, M., Pärna, E., Viinalass, H., Sabre, D. 2006. Frequencies of κ-Cn and β-Lg genetic variants among Estonian cattle breeds and their effect on the milk renneting properties. Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, August 13-18, 2006, Belo Horizonte, Brazil, Communication No 01 - 65. http://www.wcgalp8.org.br/wcgalp8/articles/paper/1_66-1731.pdf

IV **Jõudu, I.,** Henno, M., Värv, S., Kaart, T., Kalamees, K., Kärt, O. 2007. Milk protein genotypes and milk coagulation properties of Estonian Native cattle. Agricultural and Food Science, 16, 222-231.

V **Jõudu, I.,** Henno, M., Kaart, T., Püssa, T., Kärt, O. 2008. The effect of milk proteins contents on the rennet coagulation properties of milk from individual dairy cows. International Dairy Journal, 18(9), 967-970 [in press].

ABBREVIATIONS

aa	amino acid
Cn	casein
DMY	daily milk yield
E_{30}	curd firmness
EHF	Estonian Holstein
EN	Estonian Native
ER	Estonian Red
K_{20}	curd-firming time
La	lactalbumin
Lg	lactoglobulin
MCP	milk coagulation properties
NCM	noncoagulated milk
NIR	near infrared
NK_{20}	milk having curd firmness less than 20 mm (poorly coagulated milk)
RCT	rennet coagulation time
RHF	Red-and-White Holstein
SCC	somatic cell count
SCS	somatic cell score (logSCC)

1. INTRODUCTION

The food industry yields a quarter of the total industrial output of Estonia; the dairy industry is the largest (~⅓ of the total food industry). Dairy farming is the main source of income for agricultural holdings in Estonia. Within the dairy industry, cheese is a product of major importance; about one third of the milk produced in Estonia is converted into cheese and cheese production is increasing. Based on Estonian dairies' data, for the production of 1 kg of a similar cheese, 1 kg more milk than the European average is required in Estonia.

The coagulation properties of milk are of great importance because they influence cheese yield and quality. Milk with favourable coagulation properties (short coagulation and curd firming times, and a firm curd) is expected to give more cheese with desirable composition than milk with unfavourable properties (Ng-Kwai-Hang et al., 1989; Johnson et al., 2001). Milk used for cheese production, has to have, in addition to good quality parameters, also good rennet coagulation properties to ensure conversion of milk solids to cheese and to prevent losses in profit to the dairy companies. In many countries it has been found that, as a result of the cattle breeding, there has been an increase in milk production, but the coagulation properties of milk have decreased, and the number of cows in the population producing non-coagulated milk has increased (Malossini et al., 1996; Tyriseva et al., 2003).

Since the discovery of genetic polymorphism in β-lactoglobulin by Aschaffenburg and Drewry (1955), genetic variants have been found in all major milk proteins and many researchers from different countries have demonstrated that milk composition, milk yield and technological properties are connected with milk protein genetic variants. As reviewed by Buchberger and Dovč (2000), several studies have demonstrated the influence of genetic variants of milk proteins on the contents of protein and casein in milk. These findings have aroused the interest of many research groups around the world because of the potential of using milk protein genes as markers to aid in selection for milk yield and technological quality.

Previous studies in Estonia (Mihhejev, 2002; Kübarsepp et al., 2003, 2003a) showed, that about 8–9% of milk did not coagulate and additionally

17–20% of milk had poor rennet coagulation properties. To improve the efficiency of cheese production it is necessary to identify strategies to improve raw milk rennet coagulation properties.

Since the most sustainable conservation strategy is to promote self-supporting productive populations, it would be beneficial to establish a well-functioning selection programme for the Estonian Native cattle breed. Genetic improvement could concentrate on maintaining or increasing the profitability of production in traits for which the breed still possesses a competitive edge (Toro and Mäki-Tanila, 1999). Suitability of milk for cheese production could be one such trait. A preliminary comparison of milk coagulation properties among Estonian dairy breeds in an earlier study showed certain advantages of milk from Estonian Native cows, despite the limited number of Estonian Native cows in the study (Mihhejev, 2002; Kübarsepp et al., 2003).

This study involves a more extensive analysis to ascertain specific markers that could be used to identify milk suitable for cheese-making and thereby provide an economic advantage to the dairy industry.

2. REVIEW OF LITERATURE

2.1. Proteins in milk

Milk proteins are a very heterogeneous group of molecules and can be divided into five main classes: caseins, whey proteins, milk fat globule proteins, enzymes and other minor proteins. In addition to the high heterogeneity between the five different milk protein classes, there is also heterogeneity among proteins within each class. Milk proteins range from 10 to >1,000 kDa in molecular mass and have different amino acid compositions and sequences. The degree of posttranslational modification (proteolysis, phosphorylation, glycosylation, formation of disulphide bridges) and the existence of genetic variants further contribute to the observed heterogeneity (Table 1) (Dalgleish, 1992; Ng-Kwai-Hang, 2002; Farrell et al., 2004). Physiochemical properties of individual proteins vary on a very large scale. The fractionation and isolation techniques of proteins based on these differences (Tremblay et al., 2003) are described as follows. Differences in net electrical charge, sensitivity to ions (e.g. calcium) and solubility in the presence of denaturing agents (urea) is used for the isolation of some proteins by precipitation with different concentrations of salts ($CaCl_2$, $(NH_4)_2SO_4$) and alcohol under different pH and temperature conditions. Ultracentrifugation, size-exclusion chromatography, ultrafiltration and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), based on differences in molecular mass are used, as are chromatographic and electrophoretic methods based on the charge and ionic strengths of the proteins (Ng-Kwai-Hang, 2002).

Caseins in milk are defined as phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20 °C (Dalgleish, 1992). Bovine milk contains four caseins: α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein. In cattle four casein genes are clustered on chromosome 6 occupying a genomic region of about 250 kb, tightly linked together and behave as one Genetic Unit (Martin et al., 2002; Rijnkels, 2002). Caseins contain ester bond phosphate and due to their relatively high content of proline, they tend to have very little secondary structure.

Table 1. Major proteins of bovine milk and some of their properties (Dalgleish, 1992; Ng-Kwai-Hang, 2002; Farrell et al., 2004)

Protein	Mw	No. of aa residues			No. of PO_4	Glyco-sylation	Conc. (gL^{-1})	Genetic variants
	(kDa)	Total	Pro	Cys				
α_{S1}-Cn	23	199	17	0	8-9	-	10	A, **B**, **C**, D, E, F, G, H
α_{S2}-Cn	25	207	10	2	10-13	-	2.6	**A**, B, C, D
β-Cn	24	209	35	0	4-5	+	9.3	**A^1**, **A^2**, A^3, **B**, C, D, E, F, G, H^1, H^2, I
κ-Cn	19	169	20	2	1	0/+	3.3	**A**, **B**, C, E, F^1, F^2, G^1, G^2, H, I, J
β-Lg	18	162	8	5	0	+	3.2	A, **B**, C, D, E, F, G, H, I, J, W
α-La	14	123	2	8	0	(+)	1.2	A, **B**, C

Mw – molecular weight; Pro – proline; Cys – cysteine; Conc. - concentration

α_{S1}-, α_{S2}- and β-caseins, richer in phosphate groups, are distinguished from κ-casein for their more or less marked tendency to "precipitate" in the presence of calcium ions (Dalgleish, 1992). κ-casein is constituted of two different moieties with regard to their solubility: one of these (1-105 aa) is characterised by the presence of hydrophobic residues, the other (106-169 aa), to which are attached also the carbohydrate groups, manifests a marked hydrophilic nature (Ng-Kwai-Hang, 2002). Because of these ampholitic properties, κ-Cn plays the role of colloid-protector towards the other caseins, and constitutes with them dispersion in the milk by the formation of micelles. Each of these micelles is composed of several thousand molecules of all the four caseins. The micelles maintain the hydrophobic portions protected inside, and expose outside the hydrophilic moieties of the κ-casein (Dalgleish, 1992).

The whey protein fraction is the group of milk proteins that remain soluble in milk serum after precipitation of the casein fraction at pH 4.6 (20 °C) and accounts for ~20% of total protein in bovine milk. Whey proteins are a more heterogeneous group of compounds than caseins. Unlike caseins they have organized secondary and tertiary structures and most are globular proteins. β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins account for >95% of the noncasein proteins. Of these four major whey proteins β-lactoglobulin and α-lactalbumin are synthesized in the mammary gland whereas other two are transported to the mammary gland via the blood (Dalgleish, 1992; Ng-Kwai-Hang,

2002; Fox, 2003; Farrell et al., 2004). The genes coding for β-Lg and α-La, are located, in the bovine, on the 11th and 5th chromosomes respectively, justifying their independent segregation with respect to the casein loci (Martin et al., 2002).

Milk protein composition and genetic polymorphism have great significance in applied fields, such as animal sciences or the dairy industry: these studies are focused on clarifying the associations between genetic variants and production traits, reproduction efficiency, the adaptation capacity of the cattle, and detection of influences on milk nutritional and technological properties (Puhan and Jacob, 1994; Ng-Kwai-Hang, 1998; Ng-Kwai-Hang and Grosclaude, 2003).

2.2. Milk coagulation process and its measurement

The coagulation properties of milk are of great importance because they influence cheese yield and quality. Milk with favourable coagulation characteristics (short coagulation time, firm curd) is expected to give a greater quantity of cheese with a desirable composition than milk with unfavourable properties (Riddell-Lawrence and Hicks, 1989; Lucey and Kelly, 1994). Thus, much research has focused on milk coagulation parameters (Okigbo et al., 1985; Jakob and Puhan, 1992; Macheboeuf et al., 1993; Lodes et al., 1996; Ostersen et al., 1997; Ojala et al., 2005). The rennet coagulation of milk can be divided into two stages. The first stage is enzymatic hydrolysis, during which the renneting enzyme, chymosin, separates caseinomacropeptides from κ-casein, resulting in the disappearance of resistance to the aggregation of casein micelles. The second stage is aggregation, during which casein micelles are bound into a three-dimensional network that binds moisture, milk fat and other milk solids. Before aggregation can begin, about 87% of κ-casein must be enzymatically degraded (Lucey, 2002). The first stage of rennet coagulation is described by a parameter called rennet coagulation time (RCT), which is the time from the addition of rennet to milk until the beginning of coagulation. To estimate the efficiency of the second stage of rennet coagulation, different parameters for coagulation rate have been used:

– the time spent from the addition of rennet to milk or from the start of curd formation to reach curd firmness of 20 mm (Okigbo et al., 1985; Tervala et al., 1985; Lucey and Fox, 1992; Ostersen et al., 1997);
– curd firmness after a certain time: 2 · RCT (Lodes et al., 1996), 10 minutes (Tervala et al., 1985) or 15 minutes (Raynal and Remeuf, 2000) from the start of curd formation;
– curd firmness E_t, where t is the time after rennet addition (Okigbo et al., 1985; Lucey and Fox, 1992; Ostersen et al., 1997).

The methods used for detecting rennet coagulation parameters are based on the physicochemical changes that occur in milk during rennet coagulation, e.g. hydrolysis of κ-casein aggregation of rennet-altered casein micelles, changes in viscosity and elasticity (Auldist et al., 2001; O'Callaghan et al., 2002). Several systems have been used to assess milk coagulation based on a wide range of mechanical, vibrational, ultrasonic, thermal or optical instrument methods (O'Callaghan et al., 2002; Klandar et al., 2007). Measurements with the Formagraph instrument are based on the tiny forces acting on submerged pendulums when samples of coagulating milk are exposed to linear oscillations. The recorded graphs are firmness/time diagrams. Firmness is defined as a combination of viscosity and elasticity characteristics, as the curd is not damaged towards the end of the test (McMahon and Brown, 1982; Formagraph Instruction Manual). Measurements made with the Optigraph are not based on a rheological method but on an optical signal in the NIR. During a coagulation test, the light emitted through the milk gradually weakens because of changes in the micellar structure of casein. The Optigraph calculates the coagulation parameters (coagulation time, curd firmness, speed of aggregation) by means of particular feature points extracted from optical information acquired in real time (Optigraph User's Manual).

2.3. Factors affecting MCP

Variations in milk composition are the major influencing factors in the rennet coagulation properties of milk. Strong influencing factors in the rennet coagulation properties of milk are: pH (Okigbo et al., 1985; Hooydonk et al., 1986; Ostersen et al., 1997; Ikonen et al., 2004); calcium content (Tervala et al., 1985; Ostersen et al., 1997); protein content, including the influence of caseins (Ostersen et al., 1997; Guinee et al., 2001; Auldist et al., 2002); and casein number of milk (Wedholm et al., 2006). Other influencing factors of milk coagulation properties that are related to

composition and genetic factors of milk are age of animals (Schaar, 1984; Tyrisevä et al., 2003), stage of lactation (Okigbo et al., 1985; Davoli et al., 1990; Ostersen et al., 1997; Tyrisevä et al., 2004), composition of feeding rations (Macheboeuf et al., 1993; O'Brien et al., 1999; Guinee et al., 2001), season (Okigbo et al., 1985; O'Brien et al., 1999a), and breed (Grandison, 1986; Macheboeuf et al., 1993; Auldist et al., 2002).

Since the discovery of genetic polymorphism in β-lactoglobulin by Aschaffenburg and Drewry (1955), genetic variants have been found in all major milk proteins and many researchers from different countries have demonstrated that milk composition, milk yield and technological properties are connected with milk protein genetic variants (Jakob and Puhan, 1992; Jakob and Puhan, 1995; Ng-Kwai-Hang, 1998). Several studies have demonstrated the influence of genetic variants of milk proteins on the contents of protein and casein in milk (Buchberger and Dovč, 2000). These findings have aroused the interest of many research groups around the world because of the potential of using milk protein genes as markers to aid in selection for milk yield and quality. The majority of the reports are based on comparisons between variants of κ-Cn and β-Lg (Ng-Kwai-Hang, 1998). As the α_{S1}-Cn locus is especially monomorphic and variant B occurs in most breeds with a frequency of 95 to <99%, there are very few reports in the literature regarding relationships between genetic variants of these proteins and production traits (Jakob and Puhan, 1995; Ng-Kwai-Hang, 1998). Due to a large number of alleles occurring at the β-Cn locus and considerable variability in the experimental conditions the reports on associations between the β-Cn variants and the composition or technological properties of milk are conflicting (Jakob and Puhan, 1992; Lodes et al., 1996).

3. AIMS OF THE STUDY

The coagulation properties of milk are of great importance because they influence cheese yield and quality. Milk with favourable coagulation properties (short coagulation and curd firming times, and a firm curd) is expected to give more cheese with desirable composition than milk with unfavourable coagulation properties. In many countries it has been found that as a result of cattle breeding there has been an increase in milk production, but the coagulation properties of milk have decreased, and the number of cows in the population producing non-coagulated milk has increased. The aims of this study were:

1. To compare two rennet coagulation measurement techniques, the Formagraph and the Optigraph, and convert the electrical parameter (volts) obtained by the Optigraph to millimetres, the measurement used by the Formagraph to estimate curd firmness. This would then allow comparable analysis of results obtained by both analytical techniques (**I**).
2. To review, and provide an overview of milk rennet coagulation properties and factors affecting it among dairy cattle in Estonia (**II, III, IV, V**).
3. To find the frequencies of the genetic variants of κ-casein and β-lactoglobulin, and their relationships to milk rennet coagulation properties in Estonian dairy breeds (**II, III**).
4. Due to the nonindependent segregation of four casein genes in cattle, to examine the genetic variation of different milk proteins in milk from Estonian Native cows, and to determine the genotypic distributions and their effects on milk coagulation properties (**IV**).
5. To evaluate the variation in the content of different milk proteins among dairy cattle breeds in Estonia, and to investigate the effect of milk proteins on rennet coagulation properties (**V**).

4. MATERIAL AND METHODS

4.1. Collection of data

Milk samples were taken during the years 2001–2005 on repeated occasions (Table 2) from Põlula Research Farm, where individuals representing all dairy cattle breeds raised in Estonia were kept under similar conditions (Ots, 2006). In addition, during the year 2003 milk samples (n = 5,189) were collected every second month from 930 cows (609 Estonian Holstein and 321 Estonian Red; **III**) on six more farms, recommended by the breeding organisations. Milk samples (n = 335) were collected from 112 Estonian Native cows on six farms, recommended by the Estonian Native Cattle Breed Society and from six cows on the Põlula Research Farm, once every two months from March through November 2004 (**IV**). A total of 1,269 cows were sampled: 741 (58.4%) EHF, 378 (29.8%) ER, 126 (9.9%) EN, and 24 (1.9%) RHF.

Table 2. Basic information of samples taken from Põlula Research Farm

Period	No. of cows	No. of samples	Measured traits	Paper
2001–2002	87	2,161	RCT, K_{20}, E_{30}, pH, Ca, P, κ-Cn and β-Lg genotypes	**II**
Nov. 2002–Apr. 2004	54	516	RCT, E_{30}, pH, Ca, α_{S1}-, α_{S2}-, β- and κ-Cn and β-Lg	**V**
Feb. 2005	80	81	RCT, E_{30}, R, A_{30}	**I**

All milk samples were taken simultaneously with milk recording using in-line milk meters at two or three consecutive milkings. The afternoon (if milked three times a day), evening and morning milk samples were combined proportionately according to yield, to give one sample per cow, and were preserved with Board Spectrum Microtabs® II (D&F Control Systems, Inc., California, USA), transported at 4 °C to the laboratories and analysed the next day.

Blood samples, for detecting κ-Cn and β-Lg genotypes, were taken once from all animals (**II**, **III**). Blood was stabilized with K_3EDTA.

4.2. Laboratory analyses

The pH of milk was determined at 20 °C with a pH meter (MP 220; Mettler Toledo GmbH, Greifensee, Switzerland) before rennet coagulation analysis (**II, III, IV, V**). Milk calcium (**II, V**) and phosphorus (**II**) contents were determined using IDF standard methods (36A:1992, 42B:1990).

The milk coagulation properties were determined on the day after milking at 37 °C using either a Formagraph (Foss Electric, Hillerød, Denmark) or an Optigraph (Ysebaert, Frepillon, France). Rennet (Milase MRS 750 IMCU/ml; CSK Food Enrichment B.V., Netherlands) was diluted 1:100 (v/v), and added 0.2 ml to 10 ml milk. In the Formagraph analysis (**I, II, III, IV, V**) the milk rennet coagulation data was recorded diagrammatically (Figure 1; **II**): milk rennet coagulation time (**RCT** – time in minutes from the addition of rennet into milk to the beginning of coagulation), curd-firming time ($\mathbf{K_{20}}$ – time in minutes from the beginning of coagulation to the moment the width of the diagram was 20 mm) and curd firmness ($\mathbf{E_{30}}$ – width of the diagram in mm 30 min after the addition of rennet). If diagram width was less than 20 mm, the samples were classified as milk with poor rennet coagulation properties (NK_{20}; Figure 1b). In commercial cheese production such poorly coagulating milk would not reach the firmness needed to adequately cut the curd. For samples that did not coagulate at all, it was only possible to record curd firmness (E_{30} = 0 mm), and these samples were classified as noncoagulated milk (NCM; Figure 1c). For Optigraph testing (**I**), rennet coagulation time (R) detection was based on the maximum derivative of the signal. To determine the firmness of the curd, the Optigraph signal (A_{30}, in volts) 30 minutes after rennet addition was used.

To separate and quantify the major milk proteins: α_{S1}-, α_{S2}-, β- and κ-Cn, and β-Lg in a single run a modification of the reversed-phase high-performance liquid chromatography (RP-HPLC) methods proposed by Visser et al. (1991) and Trujillo et al. (2000) was used (**V**). For the determination of response curves for various milk proteins, the purified milk proteins (α_S-, β- and κ-Cn, and β-Lg, Sigma-Aldrich, Buchs, Switzerland) solutions in a sample buffer with known protein content were used. The HPLC 2D ChemStation Software with a ChemStation Spectral SW module was used for the chromatographic process guidance as well as processing results from analysis of milk protein fractions. Total casein content was defined as the

sum of the concentrations of α_{S1}-, α_{S2}-, β- and κ-caseins (**V**).

Genetic variants of κ-casein and β-lactoglobulin were determined by PCR-RFLP analysis (Sabre, 2003) in the Laboratory of Genetics of the Institute of Animal Sciences of the Estonian Agricultural University. The genomic DNA was extracted from blood (**II, III**).

Milk protein (α_{S1}-, β- and κ-Cn, and β-Lg) genotypes from Estonian Native cows were analysed at the Laboratory of Raw Milk, Munich University of Technology, Freising, Germany (**IV**), by an isoelectric focusing/ electrophoresis technique (Baranyi et al., 1993).

Information on the birth, calving, and pedigree of the cows and daily milk performance, milk protein, fat, and lactose contents, and somatic cell count data were obtained from the Estonian Animal Recording Centre (**I, II, III, IV, V**).

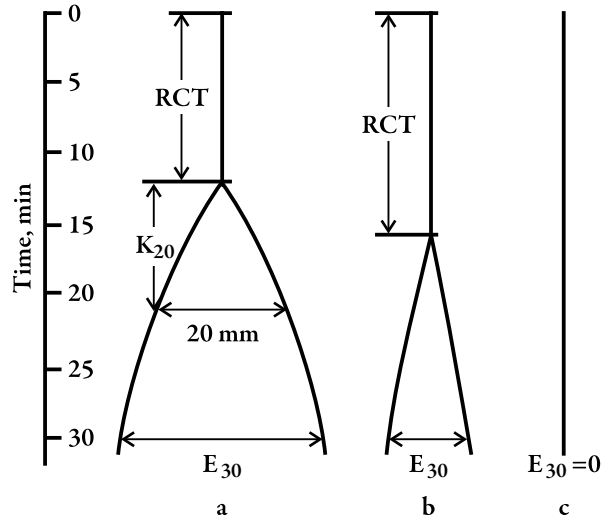

Figure 1. Diagrams (a – normally coagulated milk, b – poorly coagulated milk and c – noncoagulated milk) produced by a Formagraph, and the three milk rennet coagulation parameters (RCT – rennet coagulation time, K_{20} – curd firming time and E_{30} – curd firmness) measured from the diagrams.

4.3. Statistical analysis

To determine the regression and correlations between the rennet coagulation parameters from both methods of measurement (Formagraph and Optigraph; **I**) the results were evaluated statistically with MS Excel (Windows '98) linear regression analysis. The t-test with paired two samples for mean at 1% level was used to estimate the difference between the means of the rennet coagulation parameters obtained by the two techniques.

Different mixed linear models including both discrete and continuous effects and assuming a first-order autoregressive variance structure of the repeated measurements of the individual cow (SAS INST. Inc., 1999, 2006) were used for statistical evaluation of results (**II, III, IV, V**). The autoregressive variance structure took into account that milk samples close in time were more closely correlated than milk samples further apart.

Allele and genotype frequencies were computed by direct counts (**II, III, IV**).

5. RESULTS

5.1. A comparison of the Formagraph and Optigraph methods for determination of MCP

The milk rennet coagulation time as measured with the Optigraph showed a significantly ($F < 0.0001$) strong correlation ($r = 0.973$) with the Formagraph but was also significantly ($P < 0.0001$) lower than the results obtained with the Formagraph (Figure 2; **I**): Therefore an assisted calibration procedure was conducted to find out the best approximation for the Optigraph system parameters (default: R slope = 1 and R offset = 0). This procedure gave the following new Optigraph system parameters: R slope = 1.784 and R offset = –2.303. Based on these, the Optigraph program computed new values for rennet coagulation time that were in good accordance throughout the scale with the Formagraph results (Figure 2; **I**): the means were no longer statistically different ($P = 0.865$) and the correlation was strong ($r = 0.973$).

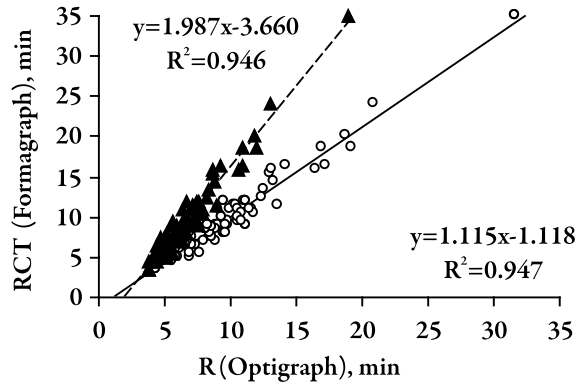

Figure 2. Relationships between Optigraph and Formagraph measurement of rennet coagulation time (before (▲) and after (○) assisted calibration).

To characterise the firmness of the curd, the distance (mm) between diagram branches (E_{30}) in the case of the Formagraph, and the signal (A_{30}) 30 min after rennet addition in the case of the Optigraph were used. There was a strong correlation between curd firmness as measured both with the Formagraph and the Optigraph (Figure 3; **I**). To study the relationship between the curd firmness parameters obtained with the Optigraph and Formagraph, three different regression equations were applied: linear, linear to zero, and polynomial. The results showed that all the coefficients in the models used were statistically significant ($P < 0.001$) and that the models described this relationship significantly ($P < 0.0001$). The best predictions throughout the scale were obtained with the following polynomial model: $E_{30} = -0.0357 \cdot A_{30}^2 + 2.8795 \cdot A_{30} - 5.2991$ ($r = 0.962$), especially for higher and lower values of curd firmness (Figure 3; **I**). Both of the linear predictions overestimated the firmness of the curd in the low and high value regions.

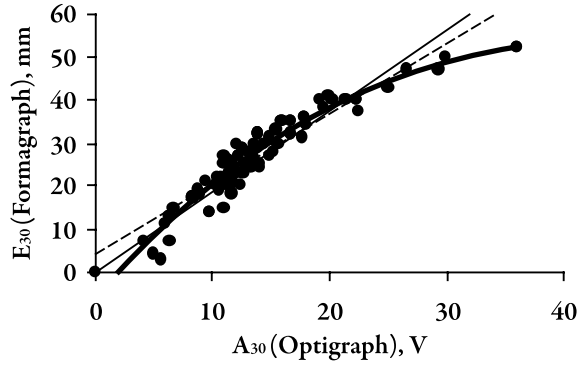

Figure 3. Relationships between Optigraph and Formagraph measurement of curd firmness (●) (linear (- - -), linear to zero (——), and quadratic (▬▬) regression trendlines).

5.2. Phenotypic variation in MCP

MCP varied on a large scale. Coefficients of variation for RCT and for E_{30} in the dataset of all original articles (**I-V**) were 41% (Table 3) and in different datasets (**I**, **II**, **IV**, **V**) ranged from 40 to 52% and from 39 to 44%, respectively. At the same time the coefficient of variation for major

milk constituents, such as fat, protein, lactose, calcium and phosphorus, was lower than 20% (Table 3).

Table 3. Descriptive statistics of studied milk traits in datasets of original articles **I-V**

Trait	Mean	SD[1]	Min	Max	CV[1], %
DMY, kg	26.9	10.12	4.5	61.8	37.7
Fat, %	4.00	0.793	1.44	8.87	19.8
Protein, %	3.47	0.392	2.33	7.13	11.3
Lactose, %	3.85	0.209	3.89	5.56	4.3
Ca, %	0.119	0.0148	0.078	0.221	12.4
P, %	0.098	0.0111	0.063	0.130	11.3
SCS	2.096	0.600	0.301	4.081	28.6
Urea, mg/l	269	82.0	0	594	30.5
pH	6.77	0.088	6.36	7.12	1.3
RCT, min	7.9	3.26	1	31.6	41.1
K_{20}, min	8.5	4.38	1	33	51.3
E_{30}, mm	30	12.4	0	61	41.0

[1] SD – standard deviation, CV – coefficient of variation (100·SD/Mean)

For 14 to 16% of milk samples (**II, III, V**) the curd formed was too soft (E_{30} < 20 mm) to be cut at the usual cutting point and these samples recorded no value for curd firming time (K_{20}). Furthermore, 3 to 6% (**II, III, IV, V**) of the milk samples did not coagulate within 30 min (E_{30} = 0 mm) and these samples also received no value for rennet coagulation time (RCT). Distribution of curd firmness of all milk samples (n = 7,880), in the datasets of original articles (**I-V**), was skewed towards the unfavourable values (Figure 4).

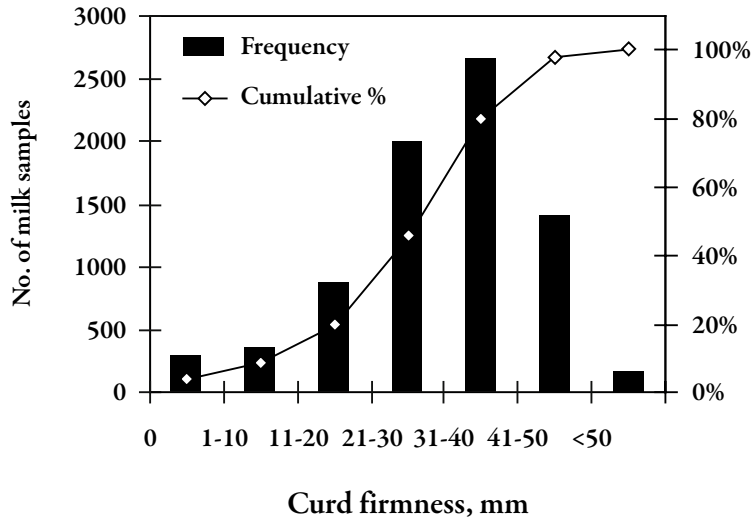

Figure 4. Distribution of values for curd firmness in datasets of original articles **I-V**.

5.3. Genotype and allele frequencies of milk proteins in Estonian dairy cattle breeds

Milk protein, κ-Cn and β-Lg, genotypes were detected for cows representing dairy cattle breeds (EHF, RHF, ER and EN) raised in Estonia (**II, III, IV**). A total of 1,040 cows were genotyped. This represented, according to the Estonian Animal Recording Centre data, ~1% of the total dairy cattle population (mean of 101,111) in Estonia in the years 2001 to 2005. Estonian Native cows (n=118) were additionally genotyped for α_{S1}- and β-Cn (**IV**). This sample represented more than 21% of the total EN cows included in the milk recording.

Most frequent genotypes for β-Lg were AB and BB and for κ-Cn AA, but among EN and ER cows the most frequent κ-Cn genotype was AB (Table 4; **III, IV**). Among cows (n=87) from the Põlula Research Farm during the years 2001-2002 the κ-Cn E allele was not detected (**II**), but when analysing larger number of animals, the E allele was found in both EHF and ER cows (Table 5; **III**). The κ-Cn E allele was not detected in EN cows sampled (**II, IV**). In α_{S1}-Cn a single genotype (α_{S1}-Cn BB) was prevalent, found in 83.9% of the studied EN cows (**IV**). Of the 16 detected aggregate genotypes, 11 genotypes occurred at a frequency above 1%, and

four of these (α_{S1}-β-κ-Cn) BB A^2A^2 AA (21.2%), BB A^1A^2 AB (16.9%), BB A^1A^2 AA (14.4%) and BB A^2A^2 AB (10.2%) were found among nearly two thirds of the analysed EN cows (**IV**).

Table 4. Genotype frequencies of κ-Cn and β-Lg in Estonian dairy breeds in datasets of original articles **I-V**

Locus/genotype	EHF	RHF	ER	EN	All
κ-Cn					
AA	0.620	0.737	0.403	0.429	0.534
AB	0.215	0.157	0.422	0.529	0.313
AE	0.126	0.053	0.048		0.087
BB	0.022	0.053	0.105	0.042	0.050
BE	0.015		0.022		0.015
EE	0.002				0.001
β-Lg					
AA	0.203	0.053	0.044	0.101	0.141
AB	0.457	0.368	0.419	0.420	0.439
BB	0.340	0.579	0.537	0.479	0.420

Table 5. Milk protein allele frequencies (%) of Estonian dairy breeds

Locus	Allele	EN* (**II, IV**)	EHF (**II, III**)	ER (**II, III**)
α_{S1}-Cn	B	92		
	C	9		
β-Cn	A^1	32		
	A^2	64		
	B	4		
κ-Cn	A	70 – 75	79 – 92	64 – 65
	B	25 – 30	8 – 14	32 – 35
	E	-	0 – 7	0 – 3
β-Lg	A	31 – 50	42 – 49	25 – 37
	B	50 – 69	51 – 58	63 – 75

* α_{S1}- and β-Cn genetic variants were detected only for EN cows in one sampling (**IV**)

5.4. Contents of milk proteins in Estonian dairy cattle breeds

Milk from the ER cows contained more β-Lg, α_{S2}- and κ-Cn than that from EHF cows (Table 6, **V**). ER milk was characterized by higher amounts of α_{S1}- and κ-Cn in casein, and the κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios were higher, but the relative content of α_{S1}-Cn in total casein was lower than those for the EHF breed. Although milk from ER cows contained more casein than milk from EHF cows, the casein number was smaller for the ER breed.

The contents of all the studied milk proteins and the relative contents of single caseins in total casein were significantly influenced by the sampling month, but the effect of pasturage was significant only for contents of total casein, α_{S1}- and β-Cn, casein number, and the α_{S2}-Cn ratio in total casein (Table 6, **V**). The effect of month of lactation was not significant only for the α_{S2}-Cn:Cn ratio. A significant effect of parity was found for the content of β-Cn and β-Lg, and on the relative contents of α_{S1}- and β-Cn.

Table 6. Least square mean concentrations (g L^{-1}) and the relative contents of analyzed milk proteins and the significances (P values) of different influences on the contents of proteins (V)

	EHF (n = 331)	RHF (n = 56)	ER (n = 129)	P value				
				Breed	Lact. month	Parity	Sampling month	Pasturage
α_{S1}-Cn	9.756	10.010	9.847	n.s.	***	n.s.	**	**
α_{S2}-Cn	1.899 [a]	1.925 [b]	2.037 [a,b]	***	***	n.s.	**	n.s.
β-Cn	13.884	14.236	14.223	*	***	*	***	***
κ-Cn	3.764 [a]	3.994	4.175 [a]	***	***	n.s.	***	n.s.
β-Lg	3.714 [a]	3.837	3.872 [a]	*	***	**	***	n.s.
α_{S1}-Cn:Cn	0.333 [a]	0.332	0.326 [a]	**	***	*	**	n.s.
α_{S2}-Cn:Cn	0.065	0.065	0.067	n.s.	n.s.	n.s.	**	**
β-Cn:Cn	0.474	0.474	0.470	n.s.	*	**	*	n.s.
κ-Cn:Cn	0.127 [a]	0.131	0.137 [a]	**	***	n.s.	*	n.s.
κ-Cn:β-Cn	0.269 [a]	0.277	0.291 [a]	**	***	n.s.	*	n.s.
κ-Cn:α_S-Cn	0.322 [a]	0.332	0.350 [a]	**	***	n.s.	**	n.s.
Protein	33.7 [a,b]	35.6 [a]	35.5 [b]	**	***	n.s.	n.s.	n.s.
Casein	29.33 [a]	30.13	30.27 [a]	*	***	n.s.	***	**
Casein number	0.834 [a]	0.810	0.819 [a]	*	***	n.s.	***	***

[a,b] Least square means within rows sharing a common superscript differ significantly (P < 0.05)
*** P < 0.001; ** P < 0.01; * P < 0.05; n.s. P ≥ 0.05

5.5. Investigated factors affecting MCP

5.5.1. Stage of lactation and major milk constituents

Effect of **parity** on rennet coagulation parameters was not clearly expressed. Parity did not have a significant effect on the studied milk rennet coagulation parameters among EN cows (**IV**) but it had an effect on curd firming time and curd firmness, when the other breeds were involved (**II**). There were more noncoagulated and poorly coagulated milk samples in the first parity when milk protein content was lowest. Milk formed a firmer curd in the second to fourth parity when milk fat and protein contents were higher (**IV**).

MCP were significantly influenced by **month of lactation** (**II, IV**). The poorest MCP were observed during midlactation (Figure 5). The percentages of noncoagulated and poorly coagulated milk were at their lowest at the beginning of lactation and clearly at their highest during midlactation. Milk fat and protein contents decreased over the first two or three months of lactation and then started to increase again during midlactation when the coagulation properties were at their poorest.

Milk rennet coagulation parameters correlated significantly with **major milk compositional characteristics**, such as fat, protein, lactose, calcium and phosphorus contents, and milk pH. Rennet coagulation time shortened and curds became firmer with an increase in the contents of milk fat, protein, lactose, and calcium, and with a decrease in milk pH (**II, V**). Firmness of the curd correlated most strongly with milk calcium (0.273 to 0.425; **II, V**), phosphorus (0.399; **II**) and protein contents (0.310 to 0.349; **II, V**). Rennet coagulation time had a stronger correlation with milk pH (0.386 to 0.432), and calcium (−0.206 to −0.312) content (**II, V**). Somatic cell score did not have a clearly expressed effect on the rennet coagulation properties of milk (**II, V**).

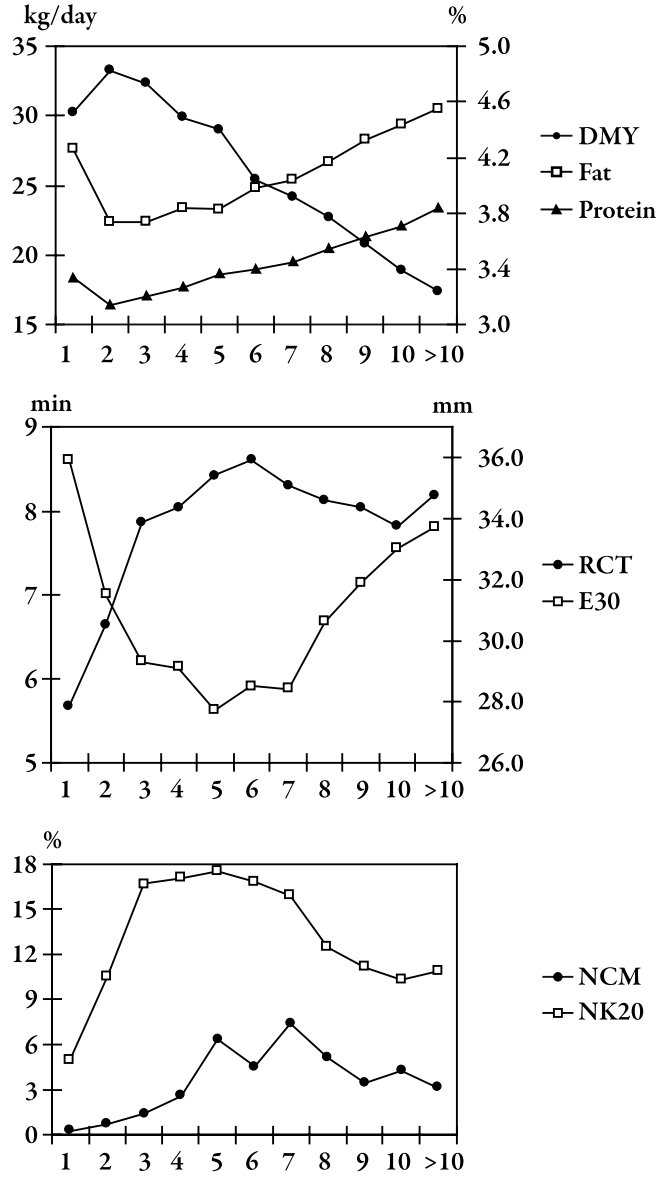

Figure 5. Estimates of effect of lactation month on milk traits and percentages of noncoagulated (*NCM*; E_{30} = 0 mm) and poorly coagulated (*NK20*; 0<E_{30}<20 mm) milk samples within respective lactation month in datasets of original articles **I-V**.

5.5.2. Breed

Breed had a significant effect on MCP (**II, V**). Improved MCP were recorded for ER and EN cows, compared to EHF and RHF cows (Figure 6; **II, IV, V**). The percentage of milk samples with insufficient curd firmness ($0<E_{30}<20$ mm) was higher among RHF (17.8 and 21.1%; **II, V**) and EHF (16.6 and 15%; **II, V**) cows, and lower among EN (0.8 or 7.5%; **II, IV**) and ER (13.9 and 14.5%; **II, V**) cows. Also, the percentages of noncoagulated milk samples for ER (3.6 and 2.1%; **II, V**) and EN (0 and 5.7%; **II, V**) cows were lower than those for EHF (5.0 and 2.4%; **II, V**) and RHF (7.7 and 7.0%; **II, V**) cows. Among all cows (n=1,269), in the datasets of the original articles (**I-V**), percentages of cows that provided noncoagulated milk samples at least at once during lactation were 52% in RHF, 42% in EHF, 32% in ER and 22% in EN.

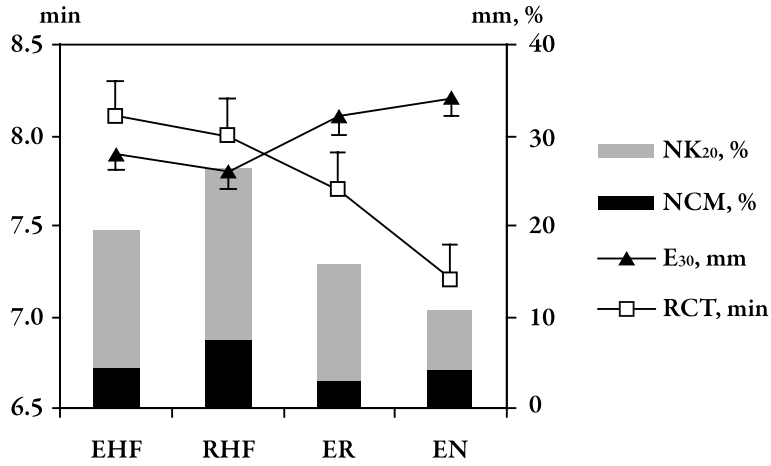

Figure 6. Milk coagulation parameters (with standard errors (⊥)) for different breeds in the datasets of the original articles **I-V**.

5.5.3. Milk protein polymorphism

MCP were significantly ($P < 0.0001$) influenced by the κ-Cn genetic variants in the case of large number of animals and samples in the dataset (**III**), but when the number of animals sampled was smaller, the effect of

κ-Cn on RCT turned out to be insignificant (**II**). MCP were better for the κ-Cn BB and worse for the κ-Cn AA, AE, and EE genetic variants (Figure 7; **II, III**). κ-Cn BB also exhibited the lowest percentage of NCM and samples that did not reach 20 mm curd firmness 30 min after enzyme addition. Moreover, in our studies milks with κ-Cn AB and BB variants contained more protein (3.48 to 3.58% and 3.50 to 3.70%, respectively) than means of all milks sampled (3.46 to 3.53%; **II, III**).

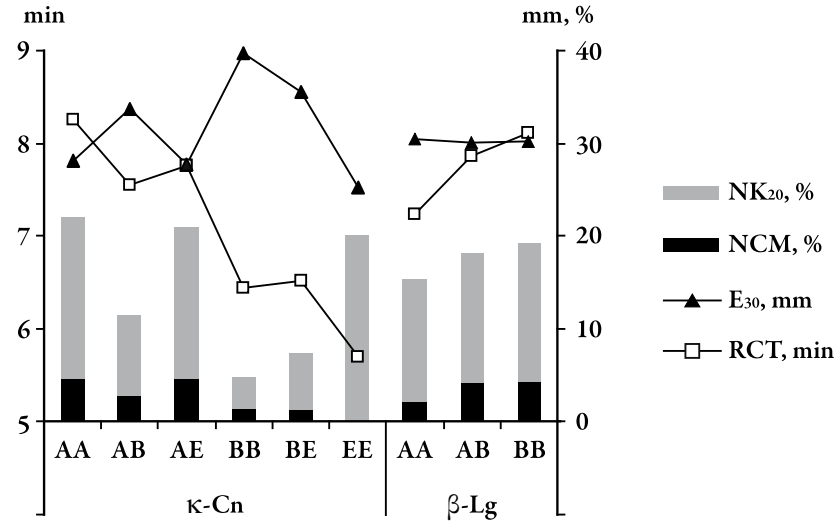

Figure 7. Milk coagulation parameters for different κ-casein and β-lactoglobulin genotypes (adapted from Paper **III**, Table 2).

An overall effect of β-Lg genetic variants on the MCP was not clearly identified. Milk rennet coagulation time exhibited a tendency to be shorter, and percentages of noncoagulated milk samples and samples with poor coagulation properties (NK_{20}) were lower for the β-Lg AA genotype (**II, III**). For the EN breed the β-Lg genotype had a significant effect on curd firmness. β-Lg AB had a significantly softer curd than BB, and the percentages of noncoagulated and poorly coagulated milk samples were the highest. Among EN cows no noncoagulated milk samples were observed for β-Lg AA (**IV**).

The aggregate α_{S1}-β-κ-Cn genotype was found to have a significant ($P < 0.05$) overall effect on MCP among EN cows (**IV**). Two aggregate casein genotypes, CC A^2A^2 AB and BC A^1A^2 BB, significantly differed from others, to a large extent, in terms of better curd firmness (Figure 8), but they occurred at low frequency (0.8 and 2.5%, respectively).

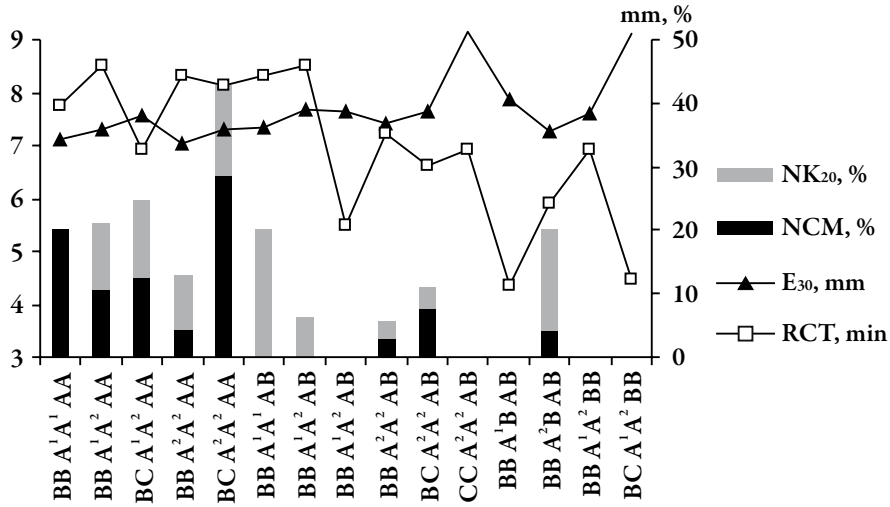

Figure 8. Effects of aggregate α_{S1}-β-κ-casein genotypes on MCP (adapted from Paper **IV**, Table 6).

Among frequent aggregate genotypes, better rennet coagulation parameters were observed for BB A^1A^2 BB. Among aggregate genotypes possessing the same β- and κ-Cn genotype, there was a tendency for the combinations with α_{S1}-BC or CC genotypes to have a shorter milk rennet coagulation time and to form firmer curd than those for combinations with α_{S1}-BB. Within aggregate casein genotypes with α_{S1}-Cn BB and κ-Cn AB, milk from cows possessing β-Cn A^1A^1 or A^1A^2 had significantly longer rennet coagulation time than the cows with β-Cn A^2B, but the curd showed a tendency to be firmer and no noncoagulated milks were observed (**IV**). Among EN cows 78.9% of all noncoagulated milk samples originated from cows possessing the κ-Cn AA genotype. Among aggregate genotypes with κ-Cn AB, noncoagulated milks were observed in combinations with BB A^2A^2, BC A^2A^2, and BB A^2B (α_{S1}- and β-Cn genotypes, respectively). No noncoagulated milk samples were observed for κ-Cn BB, α_{S1}-Cn CC, or β-Cn A^1B genotypes (α_{S1}-Cn CC and β-Cn A^1B were represented only by one cow; **IV**).

5.5.4. Milk protein composition

Milk rennet coagulation parameters were significantly influenced by α_{S1}-Cn, β-Cn and β-Lg contents, and casein number. The content of the κ-Cn and κ-Cn:β-Cn ratio had a significant effect on curd firmness and the proportion of α_{S1}-Cn in total casein had a significant effect on RCT.

Rennet coagulation time shortened, and a firmer curd was formed, in parallel with increasing content of milk protein, primarily with the contents of the studied milk protein fractions including total casein and casein number. A stronger curd was formed when the proportions of α_{S2}-Cn and β-Cn in total casein were smaller or the κ-Cn:Cn, κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios were higher.

The contents of all studied milk proteins were significantly lower for noncoagulated (E_{30} = 0 mm) and poorly coagulated (E_{30} = 1...19 mm) milks as compared to normally and well coagulated milks (E_{30} ≥ 20 mm; Figure 9, **V**). The relative amount of casein in total protein was higher in milks that formed a stronger curd. The relative content of α_{S1}-Cn in total casein did not significantly vary in the different rennet coagulation classes. Noncoagulated milks, compared to well coagulated milks, had a higher content of α_{S2}-Cn and a lower content of κ-Cn in total casein, and the κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios were also lower. The β-Cn ratio in total casein was higher for noncoagulated and poorly coagulated milks than that for well coagulated milks.

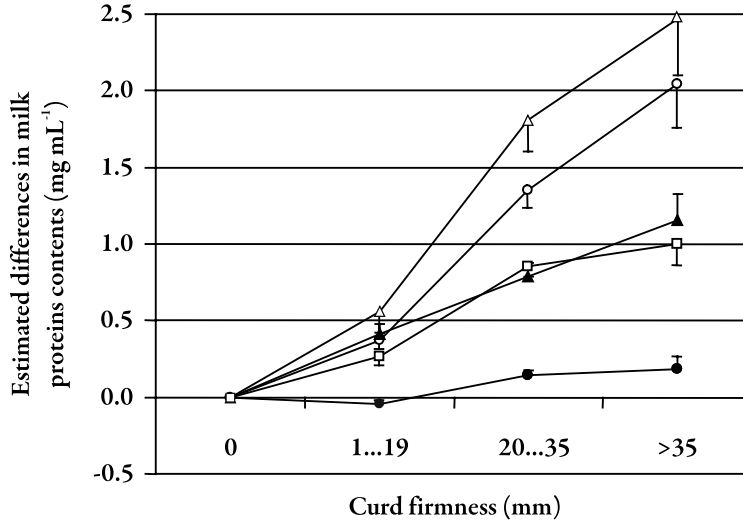

Figure 9. Estimated differences (with standard errors (⊥)) in α_{S1}-Cn (○), α_{S2}-Cn (●), β-Cn (△), κ-Cn (▲) and β-Lg (□) contents for the curd firmness classes (E_{30} = 0 mm was class of comparison) (V).

6. DISCUSSION

6.1. Measurement of rennet coagulation

The coagulum for cheesemaking needs to be cut when the gel has been become sufficiently firm to ensure the highest possible cheese yield and good quality. As the curd cutting point occurs some time later than the gelation point, it is necessary to measure the firmness of the curd as it forms and up to the point where it is ready to synerese. In addition, measurement of rennet coagulation has been used in research concerning the understanding of the mechanisms underlying milk coagulation and the factors influencing it. Various systems to measure milk rennet coagulation ability have been developed (O'Callaghan et al., 2002; Klandar et al., 2007). In our laboratory, until 2005, the Formagraph was used. This method was also commonly in use elsewhere (Ng-Kwai-Hang et al., 1989; Ostersen et al., 1997; Mayer et al., 1997; Ikonen et al., 1999; Auldist et al., 2002). This method recorded the drag force on a suspended slowly moving body. While the instrument itself, and primarily the light-sensitive photopaper used for recording the data was no longer manufactured, we had to replace it with the Optigraph, based on the optical signal in the NIR. We observed strong correlations between rennet coagulation parameters measured with both methods, but there were significant differences between the results obtained with each method. As the scale used for evaluation of curd firmness was based on Formagraph measurement, we needed to ensure that the results obtained with the Optigraph were equalised to the same scale. Such comparison of methods to assess milk rennet coagulation has also been made by other research groups (Hemar et al., 2004; Castillo et al., 2006; Klandar et al., 2007), and in most cases the Formagraph (as used by ourselves) or a lowamplitude dynamic oscillatory rheometer have been used as reference technique.

After the assisted calibration procedure, carried out for determination of the new Optigraph system parameters, new values for rennet coagulation time were estimated. These were in good accordance throughout the scale with the Formagraph results and the means were no longer statistically different. The best predictions throughout the scale for the curd firmness parameters, obtained with the Optigraph and Formagraph, were obtained with the polynomial model. Applying the new Optigraph system parameters and polynomial model ensured that the results obtained by both techniques,

the Formagraph and the Optigraph, were comparable and allowed accurate data interpretation (simultaneous use in analyses) in studies using either method.

6.2. Effect of milk composition on MCP

Besides the well known lactational variation in the main components of milk, variation has also been observed in renneting properties. Milk coagulation properties were at their best at the beginning of lactation, deteriorated quickly during the first three months of lactation, were worst during midlactation, and then improved during the second part of lactation. Guinee (2003) has reviewed lactational and seasonal changes in milk composition and associated variations in rennet coagulation properties. However, in our study lactational patterns in the content of milk major components did not precisely follow these in terms of coagulation properties (Figure 5). The values for milk fat and protein contents were lowest in the second month of lactation and already began to increase during midlactation, when coagulation properties were at their poorest. Similar results, regarding the change in the renneting properties of milk, have been described by Kreuzer et al. (1996), Ostersen et al. (1997) and Ikonen et al. (1999, 2004). Okigbo et al. (1985) and Davoli et al. (1990), however, have reported that the renneting properties of milk become worse at the end of lactation; whereas Schaar (1984) and Lodes et al. (1996) have found that the lactation stage has no effect on coagulation of milk. Moreover, Coulon et al. (1998) have stated that at the end of lactation the rennet coagulation time is longer and the curd firmness is stronger, but these changes are not significantly plausible. Kreuzer et al. (1996) and Ostersen et al. (1997) considered that one of the reasons for these different results, regarding the effect of the month of lactation on milk renneting properties, might be different feeding levels and ignoring this factor in these investigations. An important role of feeding level has also been confirmed by Lucey and Fox (1992), Macheboeuf et al. (1993), and Ostersen et al. (1997) who stated that a low feeding level resulted in poor renneting properties and higher proteolysis level at the end of lactation. One of the reasons for the deterioration of milk rennet coagulation properties at the end of lactation is increased plasmin-derived proteolytic activity. (Okigbo et al., 1985; Bastian et al., 1991; Ostersen et al., 1997; Nicholas et al., 2002). Plasmin hydrolyzes β-casein, generating several γ-caseins and proteose peptones (Kelly and McSweeney, 2003). Milk γ-casein content is especially high

at the end of lactation in herds with restricted diets and with low daily milk yields (O'Keeffe et al., 1982; O'Brien et al., 1999). Ostersen et al. (1997) found that an adequate nutritional state of the cows could ensure a low degree of milk proteolysis and good renneting properties at the end of lactation. These results agree with our study (Kübarsepp et al., 2003) where cows were fed *ad libitum* with well-balanced rations and good MCP were achieved at the end of lactation.

Protein, a major component in most cheeses, has a significant influence on the MCP (Guinee, 2003). Likewise our results showed a significant effect of milk protein content on MCP, except in dataset II. But in the model used in statistical analysis in article II the effects of milk protein and calcium contents were analysed concurrently. Since association between milk calcium and protein contents was stronger than their correlations with curd firmness, the effect of milk protein on curd firmness was described by the effect of the calcium content. This is also shown by the fact that if the effect of calcium content was excluded from the model, the effect of milk protein became significant without altering the other results. The increase in milk protein content resulted in a shortened time for coagulation and curd firming, whereas the formed curd was firmer at cutting (**II, V**). Results of the paper **V** showed that all the studied milk protein contents, except α_{S2}-Cn, influenced the coagulation parameters of milk. However, the major milk components were correlated significantly with either, or both, RCT and $E_{30.}$ The concentrations of the major milk components (as indicated by r^2) resulted in variations, up to a maximum of 12%, in these parameters, with two exceptions. The pH resulted in a 19% variation in RCT, and calcium content resulted in an 18% variation in E_{30}. Moreover, all the studied milk proteins' contents, except α_{S2}-Cn, resulted in a variation of rennet coagulation time of up to 17% and a variation of curd firmness of as high as 41%. But Ikonen et al. (2004) found that phenotypic correlations among the coagulation parameters and milk protein and casein contents was very low ($R<0.1$), and the correlations between milk coagulation characteristics and milk pH were also less than 0.3. The results obtained from this study are similar to the results from Auldist et al. (2002) indicating that the effect of casein and κ-Cn contents on coagulation parameters is larger, but the effect of total milk protein content is smaller. However, the findings of Auldist et al. (2004) differed from ours in that their study did not find any significant effect of contents of milk proteins on RCT. A lower content of κ-Cn and its proportion in

relation to α_S-Cn and β-Cn, in poorly coagulated milks, have also been observed by Wedholm et al. (2006). An increased rate of β-Cn in total casein has a negative effect on the rennet coagulation properties of milk. St-Gelais and Hache (2005) also obtained similar results; they described the poorer milk coagulation properties of milk enriched with β-Cn powder. Dalgleish (1992) proposes a significant effect of κ-Cn content on milk coagulation properties, that an increase in κ-Cn concentration leads to a higher number of smaller casein micelles, which form a firmer curd than would be formed by larger micelles. In contrast, an increase in the ratio of β-Cn in total casein leads to the formation of larger micelles and the deterioration of the rennet coagulation properties of milk.

6.3. Genetic factors affecting MCP

We observed similar effects of casein genotypes on coagulation properties (**II, III, IV**) to those reported by several research groups (Jakob and Puhan, 1992; Van den Berg et al., 1992; Ikonen and Ojala, 1995; Lodes et al., 1996; Ng-Kwai-Hang, 1998; Buchberger and Dovč 2000; Hallen et al., 2007). The favourable effect of κ-Cn B on the renneting properties of milk has been confirmed in several studies, as reviewed Jacob and Puhan (1992) and Ng-Kwai-Hang (1998). Reviewing the results of different studies, Ng-Kwai-Hang (1998) found that, comparing the κ-Cn B variant with the A variant, the decrease in coagulation time ranged between 10−40%, and the increase in curd firmness was within the range of 20−140%. The positive effect of κ-Cn B may be partly due to the higher fat and protein, primarily casein, contents in milk containing this variant (Ng-Kwai-Hang, 1998; Ikonen et al., 1999a). In our study (**II**), we also found that milks with κ-Cn AB and BB variants contained more protein and fat than the mean of all milks. Milk from cows having the κ-Cn AB and BB genotypes was, throughout the lactation, more suitable for cheese making than the mean of all milks (Kübarsepp, 2004). The effect of β-Lg genotypes on MCP was not so clearly expressed; among EN cows we observed a significant effect on curd firmness (**IV**), but among other breeds the effect on MCP was not significant (**III**) or only the RCT was significantly affected (**II**). Milk coagulation time was the shortest, and percentages of noncoagulated milk samples and samples with poor coagulation properties (NK_{20}) were lower, for the β-Lg AA genotype (**II**). Our results are similar to those reported by Ikonen and Ojala (1995) in Finland. Milk coagulation time was the shortest for the β-Lg AA genotype in the Finnish Ayrshire whereas the

β-Lg genotypes had no significant effect on any renneting trait in the Finnish Friesian (**II**).

Due to the close linkage of four casein genes in chromosome 6 within a region of about 250kb in cattle (Rijnkels, 2002), segregation of the α_S-Cn, b-Cn, and κ-Cn variants occurs nonindependently (Aleandri et al., 1990; Eenennaam and Medrano, 1991). Ikonen et al. (1999a) reported that a possible reason for the conflicting results for the effect of single casein genotypes on milk production traits can be the close linkage of casein genes. They suggested the use of casein aggregate genotypes as a more appropriate way to estimate the effect of casein polymorphism than the use of individual casein genotypes. Studying the co-effect of β- and κ-Cn genotypes on milk rennet coagulation properties we found that, within κ-Cn AA genotype, both milk rennet coagulation time and curd firmness did not significantly differ between β-Cn genotypes. Within the κ-Cn AB genotype, milk from cows possessing β-Cn A^1A^1 had longer rennet coagulation time than the cows with β-Cn A^2A^2 or A^2B, and their curd firmness was lower than that of cows with β-Cn A^1A^2 (**IV**).

A predominance of α_{S1}-Cn B (or its monomorphism) has also been observed in the common dairy breeds in Europe (Tervala et al., 1983; Ikonen et al., 1996; Lunden et al., 1997; Erhard et al., 1998; Lien et al., 1999). The same predominant variants in κ- and β-Cn loci have been found in most dairy cattle: κ-Cn A, except for Finncattle, Jersey and Brown Swiss, where the B-allele is widespread, and alleles A^1 and A^2 at β-Cn (Tervala et al., 1983; Ikonen et al., 1996; Freyer et al., 1999). The frequencies of β-Lg A and B alleles were similar in Estonian dairy breeds and in the dairy breeds of adjacent countries (Bech and Kristiansen, 1990; Velmala et al., 1993; Ikonen et al., 1996; Lunden et al., 1997). The most frequent κ-Cn genetic variant for EHF and RHF was AA while for EN and ER the most frequents variant was AB. Favourable κ-Cn BB and AB genetic variants were most frequently associated with the β-Lg BB variant, and unfavourable AE, EE, and BE κ-Cn variants with the β-Lg AB variant (**III**). Of the 16 detected aggregate genotypes (**IV**), 11 genotypes occurred at a frequency above 1%, and four of these (α_{s1}-β-κ-Cn) BB A^2A^2 AA (21.2%), BB A^1A^2 AB (16.9%), BB A^1A^2 AA (14.4%) and BB A^2A^2 AB (10.2%), were found among nearly two thirds of the analysed EN cows. α_{s1}-Cn BC and CC genotype combinations occurred only with β-Cn A^1A^2 and A^2A^2. Similar aggregate genotypes have been to be frequent in the Swedish Red and White and

Swedish Holstein breeds (Lunden et al., 1997). Our results (**IV**), regarding the casein allele frequencies, further support the genetic relationship of EN with Western Finncattle, as revealed by DNA microsatellites in a recent analysis (Tapio et al., 2006). A comparison of milk protein allele frequencies between EN and the breeds used for improvement (Finncattle, Danish Jersey) revealed similarities between breeds. The most frequent alleles of the three respective casein loci in EN cows' milk were as follows: α_{s1}-Cn B (91.5%), β-Cn A^2 (64.4%), and κ-Cn A (69.5%). The observed difference between EN and Finncattle in the frequency of β-Lg variants in the current study probably results from genetic material introduced into EN by Jersey bulls and/or, on a smaller scale, Holstein and/or red breeds. At the same time, being in one genetic cluster with old indigenous breeds, the EN breed showed a very similar distribution of milk protein aggregate genotypes to those of the common commercial dairy breeds.

A comparison of milk protein allele frequencies between the EN breed and the other dairy breeds raised in Estonia showed that EN's frequency of the favourable κ-Cn B allele resembled that of ER, but was higher than for EHF. The unfavourable κ-Cn E allele was found both among the commercial breeds EHF and ER, but not among EN cows in the current sampling. Results of earlier studies in Estonia (Toome, 1972; Orasson, 2000) about allele frequencies of κ-Cn indicate that the κ-Cn B allele frequency was considerably decreased in the Estonian Holstein cows. The frequency of the κ-Cn B allele among ER and EN has remained at the same level (Kübarsepp et al., 2004; **II**).

Differences in milk coagulation properties between breeds may be due to differences in milk composition that are attributable to variation in the genome. Several earlier studies (Tervala et al., 1983; Macheboeuf et al., 1993; Auldist et al., 2002) associated better renneting properties among native breeds compared with the Holstein with a higher frequency of κ-Cn B allele. A positive effect of this allele was also shown in our studies on EN cows, which also showed a comparatively high frequency of the allele (**II, IV**). Milk from EN cows has been found to form a stronger curd (E_{30} = 33 mm) than milk from the other Estonian breeds, EHF (E_{30} = 27.6 mm) and ER (E_{30} = 31.1 mm). Also, the percentage of poorly coagulated and noncoagulated milk samples (E_{30} < 20 mm) was lowest for EN (13.1%), while the percentages for EHF and ER were 19.5 and 17.5%, respectively (**II**).

6.4. Possibilities of genetic improvement of MCP

6.4.1. Selection for MCP

Improvement in MCP is possible by selecting breeding animals directly for these traits. There is a wide variation in MCP (**I, II, III, IV, V**). An essential part of this variation is genetic, as indicated by initial heritability estimates from the Estonian Bio-Competence Centre of Healthy Dairy Products for curd firmness (0.73), and for coagulation time (0.55) among EHF, recently found by Pärna et al. (2006). Ikonen et al. (1999, 2004) and Tyrisevä (2002) have also found high heritability estimates for rennet coagulation parameters (for E_{30} 0.31 to 0.40 and for RCT 0.22 to 0.36). Furthermore, the wide variation in the proportion of daughters producing non-coagulated milk between the sires (0 to 47%) suggested that noncoagulation of milk is partly caused by additive genetic factors (Ikonen et al., 2004). Our studies (**II**) have revealed that 14.5% of Estonian cows (in some herds 39%) produce non-coagulated milk at least once during their lactation history.

Examining the daughters' milk production and milk coagulation properties provides the information necessary for genetic evaluation of the test and proven bulls and determination of the MCP improvers of their daughters, thus increasing cheese-making efficiency. Comparing bulls by their daughters' milk coagulation properties, RCT and E_{30} indicated significant ($P < 0.0001$) differences (Pärna et al., 2006). Frequent use of bulls, having daughters with poor MCP, may have an undesirable impact on the genetic potential of the milk coagulation properties in the entire Estonian Holstein population. Occurrence of bulls with favourable milk coagulation properties in the Estonian Holstein population is a prerequisite for genetic improvement of these traits.

Selection for MCP requires reliable, routine measurement of MCP for breeding animals in the population. According to data from the Estonian Animal Recording Centre (Eesti Jõudluskontrolli Aastaraamat, 2006) in Estonia there were 108,900 dairy cows in the national herd, of which 91.5% are included in the milk recording data. Due to the high repeatability estimates of MCP Ikonen (2000) inferred that three measurements of MCP per lactation and per cow would give breeding value estimates with sufficient accuracy.

Use of the previous milk coagulation measure, the Formagraph, for such routine measurement of MCP in milk recording is unsuitable due to the low capacity and large requirement for manual work. Furthermore, the production of the instrument and its accessories has been stopped. A new instrument, the Optigraph, was chosen to improve the automatization of the measurement process. The good approximations for rennet coagulation parameters indicate that the two techniques for detecting milk coagulation properties are comparable and that the data obtained by these methods allow for data interpretation in studies using either method (**I**).

Evaluation of MCP for all cows in milk recording is too time-consuming (analysis of 10 milk samples takes about 45 minutes). Measurement of MCP for a limited number of animals, e.g., bull dams, daughters of test and proven bulls could be used to improve the MCP in the population. This work has begun within the framework of the Bio-Competence Centre of Healthy Dairy Products.

6.4.2. Selection for associated characteristics

Milk protein polymorphism
To apply the genetic information into a breeding programme, genotyping of milk protein genotypes should be carried out for all young bullsselected for future breeding. Selection to increase the positively affecting allele frequencies in one casein locus has to be combined with the avoidance of undesirable alleles in closely linked loci. If the total population size is relatively small, it is reasonable to use individual mating and applying genotyping of potential sires as the genetic evaluation of sires is complicated when the number of daughters is small.

Dairy traits
The contents of all the studied milk proteins were lower for noncoagulating and poorly coagulating (E_{30} < 20 mm) milks than those for well-coagulating milks. We found that those cows in the dataset that produced noncoagulating milk at least once during lactation, had no differences in milk protein content in comparison with other cows, but they had a significantly lower casein number ($P < 0.0001$), casein ($P < 0.0001$), and κ-Cn contents ($P < 0.0001$). Similary to Ikonen et al. (2004), our results showed that if farmers select animals that produce milk with higher protein contents, then better coagulation properties of milk could be achieved

but also the number of animals producing noncoagulating milk could be increased (Figure 10).

Ikonen et al. (2004) suggested the selection of low somatic cell count for genetic improvement of rennet coagulation properties of milk and for the reduction of the occurrence of noncoagulating milk. Our study was unable to confirm these propositions because SCS did not have a statistically significant effect on the rennet coagulation properties of milk; there were no differences in SCS among the rennet coagulation classes, and cows producing noncoagulating milk at least once did not have a different SCS from other cows in the study.

The database, formed with the evaluation of MCP within the framework of the Bio-Competence Centre of Healthy Dairy Products, allowed adjustment of the markers that could be used for selection for improving the milk rennet coagulation.

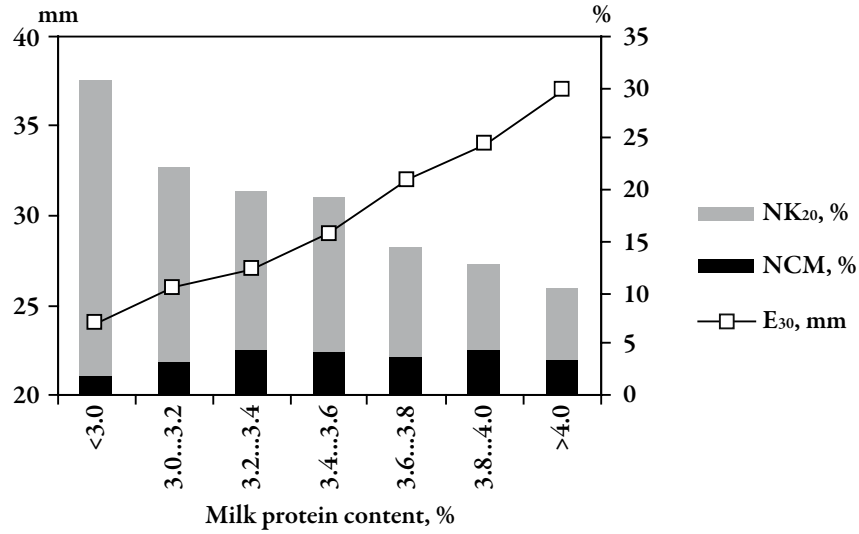

Figure 10. Effect of milk protein content on MCP.

7. CONCLUSIONS

1. We found a strong correlation between the rennet coagulation properties of milk measured with the Formagraph and the Optigraph. Applying the Optigraph system parameters: R slope = 1.784 and R offset = –2.303, the values for rennet coagulation time were in good accordance throughout the scale with the Formagraph results. The best approximation of curd firmness, determined by the Optigraph, gives a quadratic function ($E_{30} = -0.0357 \cdot A_{30}^2 + 2.8795 \cdot A_{30} - 5.2991$) that allows analytical comparison of curd firmness measured by both techniques, the Formagraph and the Optigraph (**I**).

2. A wide variance was observed both in the composition and in the rennet coagulation properties of the milk samples used in these studies (**I, II, IV, V**). Percentages of noncoagulated and poorly coagulated (E_{30} < 20 mm) milks ranged from 3 to 6%, and from 14 to 16%, respectively (**II, III, IV, V**). Milk rennet coagulation properties were influenced by stage of lactation (**II, IV**), breed (**II**), milk composition (**II, IV, V**), and milk protein genotypes (**II, III, IV**), but results from the effects of parity and udder health, characterised by somatic cell count, were not clear (**II, IV**).

3. Milk coagulation properties were at their best at the beginning of lactation, deteriorated quickly during the first three months of lactation, were worst during midlactation, and subsequently improved during the second part of lactation (**II, IV**).

4. All measured rennet coagulation parameters were significantly better for the κ-Cn BB, and worse for the κ-Cn AA, AE, and EE genotypes. κ-Cn BB also exhibited the lowest percentage of noncoagulated milk samples and samples that did not reach K_{20} 30 min after enzyme addition. Milk from cows having κ-Cn AB and BB genotypes were, throughout the lactation, more suitable for cheese making than the mean of all milks. The effect of β-Lg genetic variants on rennet coagulation parameters was not clearly identified (**II, III**).

5. The most frequent κ-Cn genotype among EHF and RHF cows was AA, and among ER and EN cows AB genotype. For β-Lg AB and BB genotypes were more frequent. Cows of the Estonian Native and

Estonian Red breeds, giving milk with better coagulation properties, had higher frequencies of the κ-Cn B allele. The frequency of the κ-Cn B allele, associated with better coagulation properties, has considerably decreased in Estonian Holstein cows (**II, III**). Estonian Native cattle showed a relatively high frequency of the favourable κ-Cn B allele, although predominantly in heterozygote combination with the A allele, whereas no unfavourable κ-Cn E alleles were detected in EN in the current study (**IV**).

6. Among Estonian Native cattle breed we found 16 aggregate casein genotypes (α_{s1}-, β-, κ-caseins), of which four – namely, BB A^2A^2 AA (21.2%), BB A^1A^2 AB (16.9%), BB A^1A^2 AA (14.4%), and BB A^2A^2 AB (10.2%) – occurred among nearly two-thirds of the analysed cows. Aggregate casein genotype had a significant overall effect on rennet coagulation parameters. Better rennet coagulation properties were found for aggregate casein genotypes CC A^2A^2 AB and BC A^1A^2 BB, among frequent genotypes for BB A^1A^2 AB. On the other hand, favourable aggregate casein genotypes (containing κ-Cn BB, α_{s1}-Cn BC or CC genotype) for improving the conversion of milk protein into cheese were rarely observed in EN. Noncoagulated milk originated mainly from cows possessing κ-Cn AA genotype. (**IV**).

7. Our study showed significant effects of sampling month and month of lactation on the content of the studied milk proteins and the relative content of caseins in total casein. Milk protein, casein, α_{S2}-Cn, κ-Cn, and β-Lg content, and the relative contents of α_{S1}- and κ-Cn in total casein were higher in milk from cows of the Estonian Red breed than those in milk of the Estonian Holstein breed. Higher contents of milk protein, casein and all the studied protein fractions, and the casein number reduced the rennet coagulation time and formed a firmer curd. Milk formed a firmer curd when the proportion of α_{S2}-Cn and β-Cn in total casein was smaller, or the proportion of κ-Cn in total casein was higher. In addition, a higher proportion of κ-Cn with respect to α_{S1}-Cn and β-Cn assisted in forming a firmer curd (**V**).

8. Better MCP among ER and EN cows, compared to EHF and RHF cows, are probably partly explainable by a higher frequency of the κ-casein B allele, associated with better coagulation properties (**II, III, IV**), and ER cows also recorded higher contents of milk proteins, compared to EHF cows (**V**).

9. In order to apply the genetic information obtained from this study in breeding programmes, we need to conduct additional determination of milk protein genotypes for breeding bulls and bull dams. It is necessary to increase the allele frequencies with a positive effect and to avoid unfavourable alleles in closely linked loci (**II, III, IV**). Selection for associated milk compositional characteristics (such as milk protein or casein contents, or κ-Cn genotype) needs additional study. The database, formed with evaluation of MCP within the framework of the Bio-Competence Centre of Healthy Dairy Products, gives a good foundation for the design and carrying out future research in this area. Further information about the factors causing the noncoagulation of milk needs to be discovered.

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SUMMARY IN ESTONIAN

PIIMA VALGULISE KOOSTISE JA GENEETILISE POLÜMORFISMI MÕJU PIIMA LAAPUMISOMADUSTELE

Piimal, millest valmistatakse juustu, peavad lisaks muudele kvaliteedinäitajatele olema head laapumisomadused (lühike laapumisaeg ja tugev kalgend), sest halvasti kalgenduvast piimast ei lähe osa kaseiini juustu koostisse, vaid jääb kaona vadakusse, põhjustades juustutööstustele suurt majanduslikku kahju, kuna juustu väljatulek väheneb ning kvaliteet halveneb. Paljudes arenenud veisekasvatusega riikides on leitud, et intensiivse aretustöö tulemusena on piimatoodang suurenenud, kuid piima laapumisomadused halvenenud. Samuti on suurenenud nende lehmade osakaal, kes võivad vähemalt korra laktatsiooni jooksul anda mittelaapuvat piima. Eestis läbiviidud varasemad (2000–2002) uuringud näitavad, et 8–9% piimaproovidest ei kalgendu üldse ning täiendavalt 17–20% kalgenduvad halvasti. Mõnes karjas võib vähemalt kord laktatsioonil mittelaapunud piimaproovi andvate lehmade osakaal küündida meie andmetel kuni 39%ni. Antud uurimistöö moodustab ühe osa suuremast uurimusest, mille käigus selgitatakse näitajad, mille alusel on võimalik välja töötada võtted juustutootmiseks sobiliku piima saamiseks, ning suurendada selle abil piimatööstuse konkurentsivõimet.

Töö käigus selgitati erinevate tegurite mõju piima laapumisnäitajatele (artiklid **II, III, IV, V**), leiti κ-kaseiini ja β-laktoglobuliini geneetiliste variantide esinemissagedus Eestis kasvatatavatel piimaveise tõugudel ning uuriti nende variantide mõju piima laapumisnäitajatele (**II, III**). Et nelja kaseiini geenid on aheldatud, siis uuriti eesti maatõu baasil piimavalkude geneetilist variatsiooni ja kaseiini agregaatgenotüüpide mõju piima laapumisnäitajatele (**IV**). Võrreldi eesti piimaveisetõugude piima valgulist koostist ning selgitati selle mõju piima laapumisnäitajatele (**V**). Seoses varem laialdaselt kasutatud piima laapumisnäitajaid mõõtnud seadme Formograaf väljavahetamisega Optigraafi vastu, leiti sobivaimad sisendid uuele seadmele ning lähendid, mis võimaldavad kasutada mõlema seadmega mõõdetud näitajaid üheaegselt andmeanalüüsis (**I**).

1. Piima laapumisnäitajate võrdlusmõõtmisel Optigraafi ja Formograafiga selgus, et mõlema seadmega mõõdetud laapumisajad korreleerusid tugevalt, kuid Optigraafiga mõõtes saadi oluliselt väiksemad väärtused.

Pärast uute sisendite (R tõus = 1,784 ja R nihe = –2,303) rakendamist langesid mõlema seadmega mõõdetud piima laapumisajad kogu skaala ulatuses hästi kokku. NIR spektromeetrial baseeruva Optigraafiga leitud kalgendi tugevuse elektrilise näitaja konverteerimiseks varem kasutusel olnud pikkusühikuteks (Formograafil leiti kalgendi tugevus diagrammi harude vahelise kaugusena millimeetrites) saadi parim lähend, kui kasutati ruutfunktsiooni $E_{30} = -0{,}0357 \cdot A_{30}^2 + 2{,}8795 \cdot A_{30} - 5{,}2991$, kus E_{30} on kalgendi tugevus millimeetrites ja A_{30} on Optigraafil leitud kalgendi tugevus voltides. Leitud Optigraafi sisendid ja lähend kalgendi tugevuse ümberarvutamiseks võimaldavad võrrelda laapumisnäitajaid, mis on saadud erinevate mõõteriistadega, ja nende samaaegset kasutamist andmeanalüüsis (**I**).

2. Nii piima laapumisomadused kui ka koostiskomponentide sisaldused varieerusid suures ulatuses (**I, II, IV, V**). Mittelaapunud piimaproove oli 3–6% ja lõikamiseks ebapiisavalt tugeva kalgendiga proove 14–16% (**II, III, IV, V**). Piima laapumisnäitajaid mõjutasid oluliselt laktatsioonijärk (**II, IV**), tõug (**II**), piima koostis (**II, IV, V**) ja piimavalkude genotüübid (**II, III, IV**). Laktatsiooni ja somaatiliste rakkude arvu mõju ei avaldunud nii selgelt (**II, IV**).

3. Piima laapumisomadused olid parimad laktatsiooni alguses ja halvimad laktatsiooni keskel. Laktatsiooni teisel poolel piima laapumisomadused paranesid (**II, IV**).

4. Piima laapumisnäitajad olid paremad κ-kaseiini BB-genotüübiga lehmadel. Halvemad piima laapumisomadused kaasnesid κ-kaseiini AA-, AE- ja EE-genotüüpidega. κ-kaseiini AB- ja BB-genotüüpidega lehmade piim oli kogu laktatsiooni jooksul keskmisest paremate laapumisnäitajatega. β-laktoglobuliini geneetilised variandid ei avaldanud olulist mõju piima laapumisnäitajatele (**II, III**).

5. Kõige sagedamini esines EHF ja RHF tõugu lehmadel κ-kaseiini AA- ning ER ja EN tõugu lehmadel AB-genotüüpi. Sagedasemad β-laktoglobuliini genotüübid olid AB ning BB. Eesti maatõugu ja eesti punast tõugu lehmadel esines κ-kaseiini B-alleeli sagedamini ning ka nende piima laapumisnäitajad olid paremad kui eesti holsteini tõugu lehmadel. Võrreldes varasemate (1972. a) Eestis läbiviidud uuringutega on piima paremate laapumisomadustega seostatava κ-kaseiini B-alleeli

esinemissagedus eesti holsteini tõugu lehmadel märgatavalt vähenenud (**II, III**). Kuigi eesti maatõugu lehmadel oli piima laapumise seisukohalt soodsa κ-kaseiini B-alleeli esinemissagedus suhteliselt suur, esines see alleel enamjaolt heterosügootses kombinatsioonis A-alleeliga. Samas ei leitud eesti maatõugu lehmadel ebasoodsat E-alleeli, mida esines teistel Eestis kasvatatavatel piimaveise tõugudel (**II, III, IV**).

6. Eesti maatõugu lehmadel (n = 118) leiti 16 kaseiinide (α_{S1}-, β-, κ-kaseiin) agregaatgenotüüpi, millest neli – BB A^2A^2 AA (21,2%), BB A^1A^2 AB (16,9%), BB A^1A^2 AA (14,4%) ja BB A^2A^2 AB (10,2%) – esines peaaegu kahel kolmandikul loomadel. Agregaatgenotüüp avaldas olulist mõju piima laapumisnäitajatele. Paremini laapus nende lehmade piim, kellel esines kaseiinide agregaatgenotüüp CC A^2A^2 AB või BC A^1A^2 BB. Võrreldes sagedamini esinevate agregaatgenotüüpidega lehmi, ilmnes, et piima paremad laapumisomadused kaasnesid BB A^1A^2 AB-genotüübiga. Piimavalgu juustuks konverteerimist soodustavaid κ-kaseiini BB- ja α_{S1}-kaseiini BC- või CC-genotüüpe sisaldavaid agregaatgenotüüpe leiti eesti maatõugu lehmadel harva. Enamik mittelaapunud piimaproove saadi lehmadelt, kelle kaseiini agregaatgenotüüp sisaldas κ-kaseiini AA-genotüüpi (**IV**).

7. Piima peamiste valkude (α_{S1}-, α_{S2}-, β- ja κ-kaseiin, β-laktoglobuliin) sisaldust ja kaseiinide osatähtsust kogu kaseiinis mõjutasid oluliselt nii laktatsiooni- kui ka kalendrikuu. Võrreldes eesti holsteini tõugu lehmadega sisaldas eesti punast tõugu lehmade piim oluliselt rohkem valku, kaseiini, α_{S2}-kaseiini ja κ-kaseiini ning β-laktoglobuliini, samuti oli α_{S1}- ja κ-kaseiini osakaal kogu kaseiinis suurem. Piima laapumisaeg oli lühem ning moodustus tugevam kalgend, kui piim sisaldas rohkem valku, kaseiini, sh üksikuid peamisi piimavalkusid, ning kaseiini ja valgu suhe oli suurem. Tugevama kalgendi moodustamist soodustasid väiksem α_{S2}- ja β-kaseiini või suurem κ-kaseiini osakaal kogu kaseiinis ning suurem κ-kaseiini suhe α_{S1}- ja β-kaseiini (**V**).

8. Eesti punast ja maatõugu lehmade piima paremad laapumisomadused võrreldes holsteini tõugu lehmadega on osaliselt põhjendatavad paremate laapumisomadustega seostuva κ-kaseiini B-alleeli suurema esinemissagedusega (**II, III, IV**) ning eesti punasel tõul ka suurema piimavalkude sisaldusega (**V**).

9. Selleks, et kasutada selle töö käigus saadud geneetilist informatsiooni aretusprogrammides, tuleks määrata piimavalkude genotüübid nii aretuspullidel kui ka potentsiaalsetel pulliemadel. See on vajalik, et suurendada paremate piima laapumisomadustega seostunud alleelide esinemissagedust, samas vältides ebasoodsate alleelide esinemissageduse suurenemist (**II, III, IV**). Piima laapumisomadustega assotsieeruvate koostisnäitajate (nagu näiteks piima valgu- või kaseiinisisaldus) sobivus kasutamaks neid aretusprogrammides piima laapumisomaduste parandamiseks, vajab täiendavat uurimist. Sellealasteks uuringuteks annab hea eelduse OÜ Tervislike Piimatoodete Biotehnoloogiate Arenduskeskuse raames loodav andmebaas. Edasiste uuringutega tuleks välja selgitada ka piima mittelaapuvuse ja laktatsiooni keskel laapumisomaduste halvenemise põhjused.

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I

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ORIGINAL ARTICLE

A comparison of the methods for determination of the rennet coagulation properties of milk

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Abstract
The objective of the present study was to compare two methods for measuring the rennet coagulation properties of milk: the Formagraph, and the Optigraph. The parameters used to describe rennet coagulation of 81 milk samples were rennet coagulation time (RCT, Formagraph, and R, Optigraph) and curd firmness 30 min after the addition of rennet (E_{30}, Formagraph, and A_{30}, Optigraph).

New Optigraph system recipes were found: R slope = 1.784 and R offset = −2.303. Using the above Optigraph system recipes, no statistically significant differences were found in rennet coagulation time measurements in either of the studied methods. Regarding curd firmness, the best approximation to describe the relationship between the methods was polynomial: $E_{30} = -0.0357 \star A_{30}^2 + 2.8795 \star A_{30} - 5.2991$ ($R^2 = 0.925$). Such strong correlations indicate that the two techniques for detecting milk coagulation properties are comparable and that the data obtained by these methods allow for data interpretation in studies using either method.

Keywords: *Coagulation parameters, Formagraph, Optigraph.*

Introduction

The coagulation properties of milk are of great importance because they influence cheese yield and quality. Milk with favourable coagulation characteristics (short coagulation time, firm curd) is expected to give higher cheese yield with a desirable composition than milk with unfavourable properties (Riddell-Lawrence & Hicks, 1989; Lucey & Kelly, 1994). Thus, many researches have focused on milk coagulation parameters (Okigbo et al., 1985; Jakob & Puhan, 1992; Macheboeuf et al., 1993; Lodes et al., 1996; Ostersen et al., 1997; Ojala et al., 2005). The methods used for detecting these are based on the physicochemical changes that occur in milk during rennet coagulation (O'Callaghan et al., 2002). The rennet coagulation of milk can be divided into two stages. The first stage is enzymatic hydrolysis, during which the renneting enzyme, chymosin, separates caseinomacropeptides from κ-casein, resulting in the disappearance of resistance to the aggregation of casein micelles. The second stage is aggregation, during which casein micelles are bound into a three-dimensional network that binds moisture, milk fat, and other milk solids. Before aggregation can begin, about 87% of κ-casein must be enzymatically degraded (Lucey, 2002). The first stage of rennet coagulation is described by a parameter called rennet coagulation time (RCT), which is the time from the addition of rennet to milk until the beginning of coagulation. To estimate the efficiency of the second stage of rennet coagulation different parameters for coagulation rate have been used:

1) the time from the addition of rennet to milk or from the start of curd formation until reaching a curd firmness of 20 mm observed by Formagraph method (Okigbo et al., 1985; Tervala et al., 1985; Lucey & Fox, 1992; Ostersen et al., 1997); 2) curd firmness after a certain time: 2 * RCT (Lodes et al., 1996), 10 min (Tervala et al., 1985) or 15 min (Raynal & Remeuf, 2000) from the start of curd formation; 3) curd firmness E_t, where t is the time

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after rennet addition (Okigbo et al., 1985; Lucey & Fox, 1992; Ostersen et al., 1997).

Measurements with the Formagraph instrument are based on the tiny forces exerted by pendulums when samples of coagulating milk are exposed to linear oscillations. The recorded graphs are firmness/time diagrams. Firmness is defined as a combination of viscosity and elasticity characteristics, as the curd is not damaged towards the end of the test (Formagraph Instruction Manual). Measurements made with the Optigraph are not based on a rheological method but on an optical signal in the NIR. During a coagulation test, the light emitted through the milk gradually weakens because of changes in the micellar structure of casein. The Optigraph calculates the coagulation parameters (coagulation time, curd firmness, speed of aggregation) by means of particular feature points extracted from optical information acquired in real time (Optigraph User's Manual).

The Formagraph has been used for years to determine milk renneting properties. Now that the manufacture of both the apparatus and its photographic paper has been terminated, it is necessary to replace the Formagraph by a modern device. The Optigraph is a new instrument developed by INRA with a different kind of operating principle. The aim of the current study was to compare these two rennet coagulation measurement techniques and find possibilities to convert the electrical parameters obtained by the Optigraph to millimetres, which were used in the past to estimate curd firmness.

Materials and methods

Cows and sampling

Milk samples ($n = 81$) were collected in February 2005 from Polula Research Farm, where all the dairy cattle breeds raised in Estonia are included: Estonian Holstein, Red-and-White Holstein, Estonian Red and Estonian Native. The sampled cows represented different lactations and stages of lactation (1–5 parity and 8–478 days after calving). Samples were collected using in-line milk meters at three consecutive milkings. The lunchtime, evening and morning milk samples were combined proportionately according to yield, to provide one sample per cow. In addition, one sample was taken from the bulk tank. The samples were preserved with Board Spectrum Microtabs® II (D&F Control Systems, Inc., California, USA), transported at 4°C to the laboratories and analysed the next day. All cows were kept and fed identically *ad libitum* on a well-balanced TMR (during days 10–150 of lactation, the ration dry matter contained 12 MJ/kg metabolizable energy (ME), 105 g/kg metabolizable protein (MP), and at least 130 g/kg crude fibre, and from day 151 until the end of lactation, the ration dry matter contained 11 MJ/kg of ME, 95 g/kg of MP, and at least 150 g/kg crude fibre).

Laboratory analyses

The contents of milk fat, protein, and lactose were measured at the Milk Analysis Laboratory of the Animal Recording Centre, using an automated infrared milk analyser (System 4000; Foss Electric, Hillerød, Denmark). Milk pH was measured at 25°C with a pH meter (MP 220; Mettler Toledo GmbH, Greifensee, Switzerland) immediately before the coagulation measurements.

Prior to the assessment of rennet coagulation properties, the samples were adjusted to the renneting temperature (35°C). The rennet (Milase MRS 750 IMCU/ml; CSK Food Enrichment B.V., Netherlands) used in both analyses (Formagraph and Optigraph) was diluted 1:100 (v/v) with distilled water and added 0.2 ml to 10 ml milk.

Milk coagulation properties were determined at 35°C on the next day after milkings. Determinations with the Formagraph (Foss Electric, Hillerød, Denmark) were made at MTT Food Research, and with the Optigraph (Ysebaert, Frepillon, France) at the Laboratory of Milk Quality (Institute of Veterinary Medicine and Animal Sciences, Department of Animal Nutrition) of the Estonian Agricultural University. Two milk coagulation parameters were measured from the Formagraph diagrams: RCT – rennet coagulation time, the time in min from rennet addition to milk until the beginning of coagulation; and E_{30} – curd firmness, the width of the diagram in mm 30 min after rennet addition. In the case of non-coagulated milk $E_{30} = 0$.

For Optigraph testing, the intensity of light emission was adjusted automatically before each analysis to obtain a constant reception of 1 volt. Because coagulation time (R) is not a configurable feature point in Optigraph analysis, its detection was based on the maximum derivative of the signal. To determine the firmness of the curd, the Optigraph signal (A_{30}, in volts) 30 min after rennet addition was used.

Statistical analysis

The results were evaluated statistically with MS Excel (Windows 1998) linear regression analysis to determine the regression and correlations between the rennet coagulation parameters from both methods of measurement. The *t*-test with paired two samples for mean at 1% level was used to

Table I. Means, ranges and standard deviations (SD) for milk composition and rennet coagulation parameters.

	Mean	Min.	Max.	SD
Fat,%	3.94	2.70	8.08	0.790
Protein,%	3.41	2.56	4.62	0.456
Lactose,%	4.81	4.38	5.18	0.174
Formagraph				
RCT, min	9.5	3.5	35	4.95
E_{30}, mm	26.3	0	52	10.34
Optigraph				
$R_{initial}$, min	6.63	3.73	19.00	2.423
R, min	9.53	4.36	31.59	4.320
A_{30}, V	13.72	0	35.98	5.855

estimate the difference between the means of the rennet coagulation parameters obtained by the two techniques.

Results and discussion

By selecting milk from different breeds and stages of lactation we managed to obtain samples with a marked variation in both milk composition and in milk coagulation properties (Table I). The samples thus gave a sufficient basis for comparing the two techniques for milk coagulation measurement.

The milk rennet coagulation time ($R_{initial}$) measured with the Optigraph showed significantly ($p < 0.0001$) strong correlation ($r = 0.973$; Figure 1) with the Formagraph (RCT) but was also significantly ($p < 0.0001$) lower than the results obtained with the Formagraph (Table I, Figure 1). We therefore conducted an assisted calibration procedure (special procedure in Optigraph software for determination of a correct coagulation time) to find the best approximation for Optigraph system recipes (default: R slope = 1 and R offset = 0). This procedure gave the following new Optigraph system recipes: R slope = 1.784 and R offset = −2.303. Based on

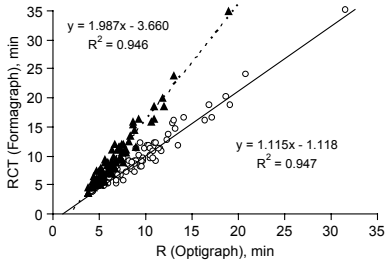

Figure 1. Relationships between Optigraph and Formagraph measurement of rennet coagulation time (before (▲) and after (○) assisted calibration).

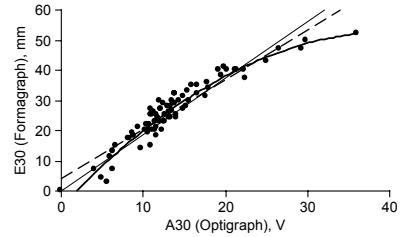

Figure 2. Relationships between Optigraph and Formagraph measurement of curd firmness (trendlines of linear (⟵ ⟶), linear to zero (⟵), and quadratic (⟵) regressions).

these, the Optigraph program computed new values for rennet coagulation time (R) that were in good accordance throughout the scale with the Formagraph results (Table I, Figure 1): the means were no longer statistically different ($p = 0.865$) and the correlation was strong ($r = 0.973$).

To characterize the firmness of the curd, we used the distance (mm) between diagram branches (E_{30}) in the case of the Formagraph, and the signal (A_{30}) 30 min after rennet addition in the case of the Optigraph. A strong correlation between curd firmness measured with the Formagraph and the Optigraph was observed and calculated (Figure 2). To study the relationship between the curd firmness parameters obtained with the Optigraph and Formagraph, we applied three different regression equations: linear ($E_{30} = 1.6587 * A_{30} + 3.5299$; $r = 0.9388$), linear to zero ($E_{30} = 1.8767 * A_{30}$; $r = 0.9292$), and polynomial ($E_{30} = -0.0357 * A_{30}^2 + 2.8795 * A_{30} - 5.2991$; $r = 0.9617$). The results showed that all the coefficients in the used models were statistically significant ($p < 0.001$) and that the models described this relationship significantly ($p < 0.0001$). The best predictions throughout the scale were obtained with the polynomial model,

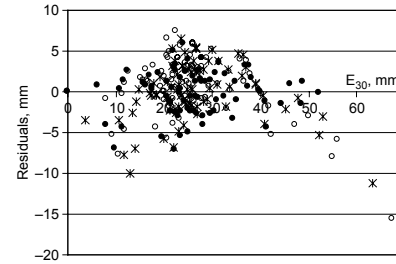

Figure 3. Residuals plot of predictions of curd firmness (residuals of linear (*), linear to zero (○), and quadratic (●) regressions).

especially for higher and lower values of curd firmness (Figures 2 and 3). Both of the linear predictions overestimated the firmness of the curd in the region of low and high values.

Conclusions

A wide variance was observed both in the composition and in the rennet coagulation properties of the milk samples used in this study. The study compared two measuring systems applied to determine the coagulation properties of milk. We found a strong correlation between the rennet coagulation properties of milk measured with the Formagraph and the Optigraph. The best approximation of curd firmness, determined by the Optigraph, gives a quadratic function that allows a comparison (simultaneous use in analyses) of curd firmness measured by both techniques, the Formagraph and the Optigraph.

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II

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Effect of κ-casein and β-lactoglobulin genotypes on the milk rennet coagulation properties

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Abstract. Purpose of this study was to find connections between milk renneting properties of dairy breeds in Estonia and the genetic variants of κ-casein and β-lactoglobulin. Milk (n = 2161) and blood (n = 87) samples were taken from Põlula Research Farm where all dairy cattle breeds are represented: Estonian Holstein (EHF) – 45 cows, Red-and-White Holstein (RHF) – 12 cows, Estonian Red (EPK) – 26 cows and Estonian Native (EN) – 4 cows) raised in Estonia. Milk samples were analysed for fat, protein, calcium, and phosphorus contents, somatic cell count, and rennet coagulation parameters. Rennet coagulation properties of milk from cows of four experimental groups were higher in EK group. No noncoagulated milk samples were observed in this group. Estonian Red breed has the second-best rennet coagulation properties of milk. Percentage of noncoagulated milk samples in the group of EPK (3.6%) was lower than in the groups of EHF and RHF (percentage of noncoagulated milk samples 5.0% and 7.7%, respectively). All measured rennet coagulation parameters were significantly better for the κ-casein BB and worse for the κ-casein AA genotype. κ-Cn BB exhibited also the lowest percentage of noncoagulated milk samples and samples that did not reach K_{20} 30 min after enzyme addition. β-Lg genotypes had no significant effect on milk rennet coagulation parameters, but it was possible to observe tendencies that milk rennet coagulation time was the shortest and the percentages of noncoagulated milk samples and samples with poor coagulation properties (NK_{20}) were lower for the β-Lg AA genotype. Better milk rennet coagulation properties among native breeds are explicable with a higher frequency of κ-Cn B allele. The frequency of κ-Cn B allele has been decreased among Estonian Holstein cows.

Key words: milk coagulation properties, milk proteins, κ-casein, β-lactoglobulin, genetic variants

INTRODUCTION

Since the discovery of genetic polymorphism in β-lactoglobulin by Aschaffenburg and Drewry (1955), genetic variants have been found in all major milk proteins, and many researchers from different countries have demonstrated that milk composition, milk yield and technological properties are connected with milk protein genetic variants (Jakob & Puhan, 1992; Jakob & Puhan, 1995; Ng-Kwai-Hang, 1998). Several studies have demonstrated the influence of genetic variants of milk proteins on the contents of protein and casein in milk (Buchberger & Dovč, 2000). These findings have aroused the interest of many research groups around the world because of the

potential using of milk protein genes as markers to aid in the selection for milk yield and quality.

The coagulation properties of milk are of great importance as they influence cheese yield and quality. Milk with favourable coagulation properties (short coagulation and curd firming times, and a firm curd) is expected to give more cheese with desirable composition than milk with unfavourable properties. In many countries it has been found that, as a result of the breeding of cows, there has been an increase in milk production, however, the coagulation properties of milk have decreased, and the number of cows in the population producing non-coagulated milk has increased (Malossini et al., 1996; Tyriseva et al., 2003). The majority of the reports are based on comparisons between the variants of κ-casein and β-lactoglobulin (Ng-Kwai-Hang, 1998). As the α_{s1}-casein locus is especially monomorphic and variant B occurs in most breeds with a frequency of 95…< 99%, there is practically no report in the literature regarding relationships between genetic variants of these protein and production traits (Jakob & Puhan, 1995; Ng-Kwai-Hang, 1998). Due to a large number of alleles occurring at β-casein locus and a considerable variability of the experimental conditions, the reports on association between β-Cn variant and the composition and technological properties of milk are conflicting (Jakob & Puhan, 1992; Lodes et al., 1996).

About 29% of the milk produced in Estonia is used for cheese production, and for the production of similar cheeses 1 kg more milk than in Europe is needed in Estonia. To improve the efficiency of cheese production, it is necessary to identify technologies and strategies that provide improvement of raw milk rennet coagulation properties. The purpose of this research, carried out on Põlula Research Farm, was to find connections between the milk rennet coagulation properties of dairy breeds in Estonia and the genetic variants of κ-casein and β-lactoglobulin.

MATERIALS AND METHODS

Data. Milk samples (n = 2161) were collected twice a month at the same time as animal recording and preserved with Bronopol (Board Spectrum Microtabs®II, D&F Control Systems, Inc., California, USA) during the years 2001 and 2002 from 87 cows of Põlula Research Farm (RF), divided into four experimental groups, formed on a basis of dairy breeds raised in Estonia: Estonian Holstein (EHF – 45 cows), Red-and-White Holstein (RHF – 12 cows), Estonian Red (EPK – 26 cows), and Estonian Native (EK – 4 cows). Blood samples for detecting the κ-casein and β-lactoglobulin genotypes were taken from all cows (n = 87) during the years 2001–2002.

Keeping and feeding conditions were same for all cows on Põlula RF. The cows were fed identically *ad libitum* on a well-balanced totally mixed rations (during 10–150 day of lactation ration dry matter contained 12 MJ kg^{-1} metabolizable energy (ME), 105 g kg^{-1} metabolizable protein (MP), at least 130 g kg^{-1} crude fibre and from the 151st day of lactation until the end of lactation ration dry matter contained 11 MJ kg^{-1} of ME, 95 g kg^{-1} of MP (at least 150 g kg^{-1} crude fibre).

Laboratory analyses. The pH of milk was determined (MP 220; Mettler Toledo GmbH, Greifensee, Switzerland) at the room temperature before the rennet coagulation analysis. Milk calcium and phosphorus contents were determined once a month during

the year 2001 by using the IDF standard methods (36A:1992, 42B:1990) at the Dairy Laboratory of the Institute of Animal Science. Daily milk performance, milk protein and fat contents, and somatic cell count (SCC) data were received from Estonian Animal Recording Centre.

The milk rennet coagulation properties were determined on the next day after milking at 37°C using a Formagraph (Foss Electric, Hillerød, Denmark) at the Dairy Laboratory of the Institute of Animal Science. Rennet (Maxiren®, DSM, Heerlen, The Netherlands) was diluted 1:100 (v/v), and 0.2 ml rennet solution was added to 10 ml milk. The milk samples were allowed to coagulate for 30 minutes as in dairy industries curd is usually cut 30 min after the addition of rennet to the milk. The three milk coagulation parameters were measured from the diagrams (Fig. 1): milk coagulation time (RCT – time in minutes from the addition of rennet to milk to the beginning of coagulation), curd-firming time (K_{20} – time in minutes from the beginning of coagulation to the moment the width of the diagram was 20 mm) and firmness of the curd (E_{30} – width of the diagram in mm, 30 min after the addition of rennet). If the width of the diagram was less than 20 mm (Fig. 1b) it was impossible to measure the curd firming time and these milk samples were classified as milk with poor rennet coagulation properties (NK_{20}). In cheese production, these poorly coagulating samples would not reach the firmness needed to be able to properly cut the curd. For samples that do not coagulate at all (Fig. 1c), it was possible to record only curd firmness ($E_{30} = 0$), and these samples were classified as noncoagulated milk (NCM).

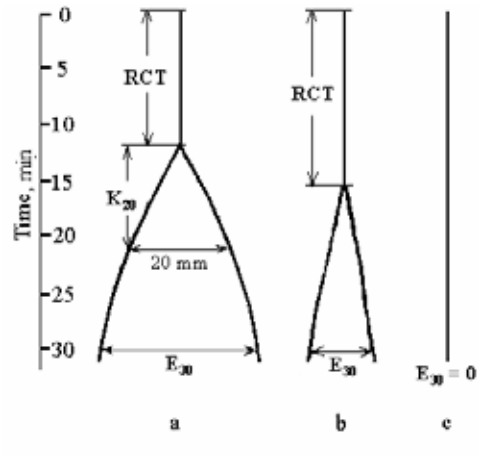

Fig. 1. Diagrams (a – normally coagulated milk, b – poorly coagulated milk and c – noncoagulated milk) produced by a Formagraph, and the three milk rennet coagulation parameters (RCT – rennet coagulation time, K_{20} – curd firming time and E_{30} – curd firmness) measured from the diagrams.

The genetic variants of κ-casein and β-lactoglobulin were determined by PCR-RFLP analysis (Sabre, 2003) at the Laboratory of Genetics of the Institute of Animal Science. The genomic DNA was extracted from blood.

Statistical analysis. Milk samples taken during the first 5 days after calving and samples from cows that were sampled only once were excluded from the statistical analysis. The data used for statistical analysis consisted of 2,161 milk samples collected from 87 cows (Table 1). More than two-thirds of the cows (n = 63) were sampled during two lactations. For somatic cell count, it was impossible to obtain normal distribution and thus, these values were logarithmically transformed and called somatic cell score (SCS). Results were evaluated statistically by using the mixed linear model including both discrete and continuous effects and assuming a first-order autoregressive variance structure of the repeated measurements of an individual cow (SAS INST. Inc., 1991). The autoregressive variance structure took into account that milk samples close in time were more closely correlated than milk samples further apart. In order to estimate the effects of different factors on milk coagulation parameters the following model ($P < 0.001$) was assumed:

$$Y_{ijklmno} = \mu + group_i + parity_j + month_k + Cn_l + Lg_m + b_1 * protein_{ijklmno} + b_2 * Ca_{ijklmno} + b_3 * pH_{ijklmno} + b_4 * SCS_{ijklmno} + e_{ijklmno},$$ where

$Y_{ijklmno}$ – rennet coagulation parameters (RCT, K_{20}, E_{30}),
μ – general mean,
$group_i$ – fixed effect of trial group (breed), $i \in \{EHF, RHF, EPK, EK\}$,
$parity_j$ – fixed effect of parity, $j \in \{1, 2\}$,
$month_k$ – fixed effect of month of lactation $k \in \{1, 2,..., 11\}$ (1…10 months of lactation included 30 days and all days after the 301th day of lactation formed the 11th month of lactation),
Cn_l – fixed effect of κ-casein genotype class, $l \in \{1, 2, 3\}$
Lg_m – fixed effect of β-lactoglobulin genotype class, $m \in \{1, 2, 3\}$
$protein_{ijklmno}$ – milk protein content,
$Ca_{ijklmno}$ – milk calcium content,
$pH_{ijklmno}$ – milk pH,
$SCS_{ijklmno}$ – somatic cell score (logarithmically transformed milk SCC),
b_1, b_2, b_3, b_4 – linear regression coefficients,
$e_{ijklnmno}$ – random residual effect including the effect of repeated measurements of the cows.

Pearson's correlation coefficients were used for describing the linear association between the various traits. To test differences between milk protein genotypes, milk compositional and daily milk yield data were examined for statistical significance by using the two tailed paired *t*-test (Table 4).

RESULTS AND DISCUSSION

Factors affecting milk rennet coagulation properties

All measured milk rennet coagulation parameters (RCT, K_{20}, E_{30}) were significantly influenced by the month of lactation, pH, and milk calcium content (Table 2). Curd-firming time and firmness of the curd were significantly influenced also by parity, somatic cell count and κ-Cn genotype. Milk protein content affected the curd-firming time, β-Lg genotype affected the rennet coagulation time and the experimental group (breed) had an effect on firmness of the curd.

The correlations between different rennet coagulation parameters were clear (Table 3). In the present study, the strongest negative correlation between rennet coagulation parameters and milk production traits was observed between curd-firming time and milk protein content (r = –0.445). The strongest positive correlation was found between firmness of the curd and milk protein content (r = 0.310). The increase of calcium, phosphorus and fat contents in the milk resulted in a shortened time for rennet coagulation and curd firming, whereas the formed curd was firmer at cutting. The rennet coagulation time prolonged since milk pH increased (r = 0.386).

A significant effect of milk protein and calcium content on milk rennet coagulation properties is described also by Tervala et al. (1985) and Kübarsepp et al., (2003). The calcium content of milk had a significant effect on all the rennet coagulation parameters (Table 2) as calcium directly participated in the process of rennet coagulation. During the rennet coagulation process, calcium forms bonds with *para*-casein (product of the action of rennet on casein), resulting in increased aggregation and firmer curd (Lucey, 2002).

An increase of pH in milk contributes to formation of colloidal calcium compounds and a decrease in the dissolved Ca content (Keogh et al., 1982), and increases plasmin activity with a resultant increase in proteolysis (Donnelly & Barry, 1983). The studies on the effect of milk pH on renneting properties are characterised by a large variability. According to the data by Macheboeuf et al. (1993) and Lodes et al. (1996), the milk rennet coagulation properties deteriorate in parallel with rising pH. Grandison et al. (1984) and Ostersen et al. (1997) found that milk pH affects predominantly only the rennet coagulation time. Our data (Tables 2 and 3) indicated that pH influenced statistically significantly all the studied rennet coagulation parameters, most of all the rennet coagulation time of milk (r = 0.386).

Rennet coagulation properties and milk production traits for different milk protein genotypes

All measured rennet coagulation parameters were significantly better for the κ-casein BB and worse for the κ-casein AA genotype (Table 4). κ-Cn BB exhibited also the lowest percentage of noncoagulated milk samples and samples that did not reach K_{20} 30 min after enzyme addition. β-Lg genotypes had no significant effect on milk coagulation parameters: only milk coagulation time was the shortest and the percentages of noncoagulated milk samples and samples with poor coagulation properties (NK_{20}) were lower for the β-Lg AA genotype. Our results are similar to those reported by Ikonen & Ojala (1995) in Finland. Milk coagulation time was the shortest for the β-Lg AA genotype in the Finnish Ayrshire whereas the β-Lg genotypes had no significant effect on any renneting trait in the Finnish Frisian.

Table 1. Means and variations for the studied traits.

Trait	n	Mean	SD	min	max
RCT, min	2,058	8.07	3.19	1	23
K_{20}, min	1,731	8.57	4.47	1	24
E_{30}, mm	2,161	28.98	12.70	0	59
Daily milk yield, kg	2,161	29.8	8.66	4.5	61.8
Fat content, %	2,161	3.79	0.77	1.93	8.87
Protein content, %	2,161	3.53	0.40	2.33	7.13
Log SCC	2,161	2.13	0.59	0.60	4.08
pH	2,161	6.77	0.09	6.38	7.12
Calcium content, %	1,129	0.122	0.017	0.078	0.213
Phosphorus content, %	389	0.098	0.013	0.040	0.141

Table 2. Significance (P) of factors influencing milk renneting properties.

Trait	RCT, min	K_{20}, min	E_{30}, mm
Experimental group (breed)	0.1099	0.0792	0.0026
Parity	0.5585	0.0476	0.0319
Month of lactation	0.0041	<0.0001	<0.0001
κ-Cn genotype	0.2168	0.0284	0.0386
β-Lg genotype	0.0092	0.8456	0.5128
Protein content, %	0.0545	0.0054	0.8801
Calcium content, %	<0.0001	0.0004	<0.0001
pH	<0.0001	<0.0001	<0.0001
SCS	0.6085	0.0320	0.0004

Significant differences between κ-casein and β-lactoglobulin genotypes were found in protein and phosphorus contents (AA<AB<BB; $P < 0.05$). κ-Cn and β-Lg genotypes had no significant effect on milk yield, fat and calcium content, pH and somatic cell count. Only κ-Cn AA contained less fat and κ-Cn BB less calcium, and had lower pH than other genotypes.

The favourable effect of κ-Cn B on the renneting properties of milk has also been confirmed in several studies (Jacob & Puhan, 1992). Reviewing results of different studies, Ng-Kwai-Hang (1998) found that comparing κ-Cn B variant with A variant, the decrease in coagulation time was ranging between 10–40%, and the increase in curd firmness was within a range of 20–140%. The positive effect of κ-Cn B may be partly due to higher fat, and protein, primarily casein, contents in milk having this variant (Ng-Kwai-Hang, 1998; Ikonen et al., 1999).

Table 3. Correlations between the studied coagulation and milk production traits parameters.

Trait	RCT, min	K_{20}, min	E_{30}, mm
Month of lactation	-0.003	-0.227*	0.199*
Daily milk yield, kg	0.123*	0.300*	-0.246*
Fat, %	-0.214*	-0.305*	0.295*
Protein, %	-0.040	-0.445*	0.310*
SCS	0.111*	0.008	-0.037
pH	0.386*	0.144*	-0.146*
Ca, %	-0.206*	-0.291*	0.273*
P, %	-0.072	-0.287*	0.399*
RCT, min	1	0.556*	-0.692*
K_{20}, min		1	-0.803*

$P < 0.001$

Table 4. Least square means of milk coagulation parameters, and mean milk production traits for different κ-casein and β-lactoglobulin genotypes.

	κ-casein			β-lactoglobulin		
	AA	AB	BB	AA	AB	BB
No. of animals	62	21	4	16	42	29
No. of milk samples	1461	580	120	396	1081	684
RCT, min	8.03[a]	7.78[a]	6.77	6.79	7.61	8.18
K_{20}, min	9.12	8.13	6.51	7.92[a]	8.07[a]	7.77[a]
E_{30}, mm	29.5	31.6	37.5	34.1[a]	32.6[a]	31.9[a]
[1]NCM, %	5.54	3.62	0.83	2.77	4.72	5.99
[1]NK_{20}, %	17.73	10.86	4.17	12.59	14.63	17.40
Milk yield,	30.1[a]	29.4[a]	26.7[a]	29.7[a]	30.1[a]	29.2
Fat, %	3.76	3.81[a]	3.94[a]	3.67[a]	3.80[a,b]	3.82[b]
Protein, %	3.49	3.58	3.70	3.40	3.53	3.60
Log SCC	2.096[a]	2.195[a]	2.205[a]	2.123[a]	2.088[a]	2.191
pH	6.77[a]	6.77[a]	6.76	6.77[a]	6.77[a]	6.77[a]
Ca, %	0.1219[a]	0.1230[a]	0.1180	0.1198[a]	0.1227[b]	0.1223[a,b]
P, %	0.0964[a]	0.1000[a]	0.1009	0.0916	0.0977	0.1001

[1] percentage of noncoagulated milk samples (NCM) and samples that did not reach K_{20} 30 min after enzyme addition (NK_{20}) from samples of respective κ-casein or β-lactoglobulin variant

[a,b] means with the same superscripts in the same row inside of κ-casein or β-lactoglobulin genotypes are not significantly different ($P > 0.05$)

Table 5. Milk rennet coagulation parameters for different breeds in the trial.

		The Whole Farm	EHF	RHF	EPK	EK
Number of cows		87	45	12	26	4
RCT, min	n	2058	974	347	619	118
	$\bar{x}$	8.1	8.2	8.0	8.2	6.9
	SD	3.2	3.1	3.4	3.3	2.1
K_{20}, min	n	1731	804	280	530	117
	$\bar{x}$	8.6	9.4	9.1	7.5	6.3
	SD	4.4	4.5	4.6	4.1	3.5
E_{30}, mm	n	2161	1025	376	642	118
	$\bar{x}$	28.9	27.6	26.0	31.1	38.7
	SD	12.7	11.9	12.9	13.2	9.2

Table 6. Allele frequencies of κ-casein and β-lactoglobulin in earlier and present studies in Estonia.

Year of study	Breed	n (κ-Cn/ β-Lg)	Allele frequencies			
			κ-Cn		β-Lg	
			A	B	A	B
1972*	EHF	114 / 2033	0.69	0.31	0.47	0.53
	EPK	86 / 710	0.71	0.29	0.10	0.90
2000*	EHF	632	0.96	0.04	0.69	0.31
Present	EHF	45	0.92	0.08	0.49	0.51
	RHF	12	0.92	0.08	0.29	0.71
	EPK	26	0.65	0.35	0.37	0.63
	EK	4	0.75	0.25	0.50	0.50
	Farm	87	0.83	0.17	0.43	0.57

* Results of Toome (1972) and Orasson (2000)

Milk rennet coagulation parameters, κ-casein and β-lactoglobulin alleele frequencies for different breeds

From the studied milk samples (n = 2161), 2,058 samples coagulated, and it was possible to record curd firming time in 1,731 cases (Table 5). From the studied milk samples, 103 samples (4.8%) did not coagulate. At least one noncoagulated milk sample appears in 34 cows (39% from cows in the trial).

Rennet coagulation properties of milk from cows of the five experimental groups were higher in EK group (RCT = 6.9 min; K_{20} = 6.3 min; E_{30} = 38.7 mm). No noncoagulated milk samples were observed in this group. Estonian Red breed has the second-best coagulation properties of milk. The percentage of noncoagulated milk

samples in the group of EPK (3.6%) was lower than in the groups of Estonian Holstein and Red-and-White Holstein (percentage of noncoagulated milk samples 5.0% and 7.7%, respectively). It has to be mentioned that with the percentage of cows given, at least one noncoagulated milk sample in the group of Estonian Holstein breed was lower than in the groups of Estonian Red and Red-and-White Holstein. The number of cows with noncoagulated milk samples was, at least once during lactation, 17 (38%) in the group of EHF, 11 (42%) in EPK and 6 (50%) in RHF.

Several earlier studies (Tervala, et al. 1983; Macheboeuf, et al. 1993; Auldist, et al. 2002) asserted better renneting properties among native breeds, compared with the Holstein breed. Differences between breeds in milk coagulation properties may result from differences in milk composition derived from the genotype. Better milk coagulation properties among native breeds have been explained by the mentioned studies with a higher frequency of κ-Cn B allele. On Põlula Research Farm, the frequencies of κ-Cn A and B allele were 0.83 and 0.17, respectively, and these of β-Lg A and B allele 0.43 and 0.57, respectively (Table 6). Results of earlier studies in Estonia (Toome, 1972; Orasson, 2000) about allele frequencies of κ-Cn and β-Lg are presented in Table 6. The results of the present study indicate that the κ-Cn B allele frequency was considerably decreased in the Estonian Holstein cows. The frequency of κ-Cn B allele among local (red) breeds (EPK and EK) has remained at the same level.

CONCLUSIONS

Significant differences were found in renneting properties between κ-casein genotypes (AA<AB<BB). β-lactoglobulin had a significant effect only on milk coagulation time, having the shortest time for the β-Lg AA genotype.

Cows of Estonian Native and Estonian Red breeds giving milk with better coagulation properties have higher frequencies of κ-casein B allele. The frequency of κ-casein B allele, associated with better coagulation properties, has been considerably decreased in the Estonian Holstein cows.

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III

Kübarsepp, I., Henno, M., Pärna, E., Viinalass, H., Sabre, D. 2006. FREQUENCIES OF K-CN AND β-LG GENETIC VARIANTS AMONG ESTONIAN CATTLE BREEDS AND THEIR EFFECT ON THE MILK RENNETING PROPERTIES. Proceedings Of The 8th World Congress On Genetics Applied To Livestock Production, Belo Horizonte, Brazil, August 13-18, 2006, Communication No 01 - 65. http://www.wcgalp8.org.br/wcgalp8/articles/paper/1_66-1731.pdf

FREQUENCIES OF κ-Cn AND β-Lg GENETIC VARIANTS AMONG ESTONIAN CATTLE BREEDS AND THEIR EFFECT ON THE MILK RENNETING PROPERTIES

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INTRODUCTION

Since the discovery of genetic polymorphism in β-lactoglobulin by Aschaffenburg and Drewry (1955), genetic variants have been found in all major milk proteins and many researchers from different countries have demonstrated that milk composition, milk yield and technological properties are connected with milk protein genetic variants. Several studies have demonstrated the influence of genetic variants of milk proteins on the contents of protein and casein in milk (Buchberger, Dovč 2000). These findings have aroused the interest of many research groups around the world because of the potential using of milk protein genes as markers to aid in the selection for milk yield and quality. The majority of the reports are based on comparisons between variants of κ-casein and β-lactoglobulin (Ng-Kwai-Hang 1998). The α_{s1}-casein locus is especially monomorphic and variant B occurs in most breeds with frequency of 95…<99% (Ng-Kwai-Hang 1998). Due to a large number of alleles occurring at β-casein locus the reports on association between β-Cn variant and the composition and technological properties of milk are conflicting (Jakob, Puhan 1992). About 29 % of the milk produced in Estonia is used for cheese production. To improve efficiency of cheese production is necessary to identify strategies provide improvement of raw milk rennet coagulation properties. The purpose of this research was finding the frequencies of the genetic variants of κ-casein and β-lactoglobulin and their connections between milk rennet coagulation properties of dairy breeds in Estonia.

MATERIAL AND METHODS

Milk samples (n=5189) were collected over monthly during the year 2003 from 930 cows (609 Estonian Holstein (EHF), and 321 Estonian Red (ER)). Cows were selected from 7 farms, suggested by the animal breeding organisations.

Genetic variants of κ-casein and β-lactoglobulin were determined by PCR-RFLP analysis. The genomic DNA was extracted from blood.

The milk coagulation properties were determined on the next day after milking at 37°C using a Formagraph (Foss Electric, Hillerød, Denmark). Rennet (Milase MRS 750 IMCU/ml; CSK Food Enrichment B.V., Netherlands) was diluted 1:100 (v/v), and added 0.2 ml to 10 ml milk. The two milk coagulation parameters were measured from the diagrams: milk coagulation time (**RCT** – time in minutes from the addition of rennet into milk up to the beginning of coagulation), and firmness of the curd ($\mathbf{E_{30}}$ – width of the diagram in mm 30 min after the addition of rennet) Milk protein content data were received from Estonian Animal Recording Centre.

Results were evaluated statistically using the following mixed linear model including both discrete and continuous effects and random regression part corresponding to individual cows (SAS Inst. Inc., 2006):

$$Y_{ijklmn} = \mu + breed_i + farm_j + Cn_k + Lg_l + b_1 * protein_{ijklmn} + b_2 * month_{ijklmn} + b_3 * month^2_{ijklmn} + a_m + b_{2m} * month_{ijklmn} + b_{3m} * month^2_{ijklmn} + e_{ijklmn},$$

where Y_{ijklmn} – rennet coagulation parameters (RCT, E_{30}); μ – general mean; $breed_i$ – fixed effect of breed, i ∈ {EHF, EPK}; $farm_j$ – fixed effect of farm, j ∈ {1, 2, …, 7}; Cn_k – fixed effect of κ-casein genotype class, k ∈ {1, 2, …, 6}; Lg_l – fixed effect of β-lactoglobulin genotype class, l ∈ {1, 2, 3}; $protein_{ijklmn}$ – milk protein content; $month_{ijklmn}$ – fixed effect of month of lactation (1…10 months of lactation included 30 days and all days after 301. day of lactation formed 11. month of lactation); b_1, b_2, b_3 – fixed regression coefficients; a_m – random animal effect, m ∈ {1, 2, …, 930}; b_{2m}, b_{3m} – random regression coefficients; $e_{ijklnmn}$ – random residual effect.

RESULTS AND DISCUSSION

Milk protein, κ-casein (κ-Cn) and β-lactoglobulin (β-Lg), genetic variants were detected for 930 cows, which formed according to Estonian Animal Recording Centre data ~1% from dairy cattle population (n = 101 785) in Estonia in year 2003. Most frequent genetic variant for κ-Cn was AA and for β-Lg AB and BB (Table 1). Favourable κ-Cn BB and AB genetic variants were most frequently associated with β-Lg BB variant and unfavourable AE, EE, and BE κ-Cn variants with β-Lg AB variant.

Table 1. Frequencies of κ-casein and β-lactoglobulin genetic variants

b-Lg \ k-Cn	AA	AB	AE	BB	BE	EE	Totally
AA	0.076	0.046	0.010	0.006	0.003	-	0.142
AB	0.247	0.116	0.047	0.020	0.011	0.001	0.443
BB	0.223	0.124	0.042	0.024	0.003	-	0.415
Totally	0.546	0.286	0.099	0.051	0.017	0.001	1.000

Both measured rennet coagulation parameters were significantly ($p<0.0001$) influenced by the κ-casein genetic variants and better for the κ-casein BB and worse for the κ-casein AA, AE, and EE genetic variants (Table 2). κ-Cn BB exhibited also the lowest percentage of noncoagulated milk samples and samples that did not reach 20 mm curd firmness 30 min after enzyme addition. The favourable effect of κ-Cn B on the renneting properties of milk has also been confirmed in several studies (Jacob, Puhan 1995). The positive effect of κ-Cn B may be partly due to higher fat, and protein, primarily casein, contents in milk containing this variant (Ng-Kwai-Hang 1998; Ikonen *et al.* 1999). In our study also milks with κ-Cn AB and BB variants contined more protein (protein content was 3.48 and 3.50%, respectively) than milks on the average (3.46%).

β-Lg genetic variants effect on rennet coagulation parameters was not significant. Rennet coagulation time was shorter and percentages of noncoagulated milk samples was lower for the β-Lg AA genotype. Our results are similar to those reported by Ikonen and Ojala (1995) in Finland. Milk coagulation time was the shortest for the β-Lg AA genotype in the Finnish Ayrshire whereas the β-Lg genotypes had no significant effect on any renneting trait in the Finnish Frisian.

Table 2. Milk coagulation parameters (LSM±SE) for different κ-casein and β-lactoglobulin genetic variants

Genetic variant		n	RCT	E_{30}	NCM[1]	NK_{20}[1]
κ-Cn	AA	3070	$8.02\pm0.13^{a,b}$	$28.4\pm0.44^{a,b}$	4.04	17.10
	AB	1359	$7.06\pm0.15^{a,c}$	$35.0\pm0.53^{a,c}$	2.57	7.43
	AE	453	7.71 ± 0.25^{c}	$28.1\pm0.87^{c,d,e}$	4.42	16.11
	BB	220	$5.92\pm0.34^{a,c}$	$39.7\pm1.18^{a,d}$	1.36	1.82
	BE	82	6.66 ± 0.54^{b}	$35.6\pm1.89^{b,e}$	1.22	6.10
	EE	5	5.78±2.23	30.0±7.77		20.00
β-Lg	AA	887	6.72±0.44	32.4±1.52	1.92	13.53
	AB	2402	6.78±0.39	32.8±1.38	3.79	13.11
	BB	1900	7.06±0.41	33.2±1.41	3.89	14.42

[1] percentage of noncoagulated milk samples (NCM) and samples that did not reach 20 mm curd firmness 30 min after enzyme addition (NK_{20}) from samples of respective κ-casein or β-lactoglobulin genetic variant

[a,b,c,d,e] means with the same superscripts in the same column inside of κ-casein or β-lactoglobulin genetic variants are significantly different ($p<0.05$)

Table 3. Allele frequencies of κ-casein and β-lactoglobulin in Estonian dairy breeds according to different studies

Year of study	Breed	n (κ-Cn/β-Lg)	κ-Cn			β-Lg	
			A	B	E	A	B
1972	EHF	114 / 2033	0.693	0.307	-	0.465	0.535
	ER	86 / 710	0.709	0.291	-	0.103	0.897
2000	EHF	632	0.956	0.044	-	0.688	0.312
Present	EHF	609	0.790	0.138	0.072	0.421	0.579
	ER	321	0.642	0.324	0.034	0.254	0.746
	All	930	0.739	0.202	0.059	0.363	0.637

Several earlier studies (Tervala, *et al.* 1983; Macheboeuf, *et al.* 1993; Auldist, *et al.* 2002) asserted better renneting properties among native breeds, comparing with Holstein breed. We found that milk from Estonian Red cows has significantly ($P<0.05$) better coagulation properties than milk from Estonian Holstein cows. Differences between breeds in milk coagulation properties may result from the differences in milk composition derived from genotype. Mentioned studies explain better milk coagulation properties among native breeds with higher frequency of κ-Cn B allele.

In our study the frequencies of κ-Cn A, B, and E allele were 0.739, 0.202 and 0.059, respectively and β-Lg A and B allele 0.363 and 0.637, respectively (Table 3) and B allele. Results of earlier studies in Estonia (Toome 1972; Orasson 2000) about allele frequencies of κ-Cn and β-Lg indicate that the κ-Cn B allele frequency was considerably decreased in the Estonian Holstein cows. Frequency of κ-Cn B allele among local red breed (EPK) has remained on the same level. In earlier studies have not been detected presence of κ-Cn unfavorable E allele.

CONCLUSION

Most frequent genetic variant for κ-Cn was AA and for β-Lg AB and BB. All measured rennet coagulation parameters were significantly better for the κ-casein BB and worse for the κ-

casein AA, AE, and EE genotypes. β-Lg genetic variants had no significant effect on rennet coagulation parameters. Percentages of noncoagulated milk samples was lower for the β-Lg AA genotype. Frequency of κ-casein B allele, associated with better coagulation properties, has been considerably decreased in the Estonian Holstein cows. In earlier studies in Estonia have not been detected presence of unfavorable κ-Cn E allele.

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IV

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MILK PROTEIN GENOTYPES AND MILK COAGULATION
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Milk protein genotypes and milk coagulation properties of Estonian Native cattle

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The genetic variation of α_{s1}-, β- and κ-caseins and b-lactoglobulin was determined and their effects on the rennet coagulation properties were examined using 335 milk samples from 118 Estonian Native (EN) cows. We found 16 aggregate casein genotypes (α_{s1}-, β-, κ-caseins), of which four – namely, BB A^2A^2 AA (21.2%), BB A^1A^2 AB (16.9%), BB A^1A^2 AA (14.4%), and BB A^2A^2 AB (10.2%) – occurred among nearly two-thirds of the analysed cows. Aggregate casein genotype had a significant overall effect on rennet coagulation parameters. Better rennet coagulation properties were found for aggregate casein genotypes CC A^2A^2 AB and BC A^1A^2 BB, among frequent genotypes for BB A^1A^2 AB. Of the cattle breeds raised in Estonia, milk from EN had the best coagulation properties and highest frequency of favourable κ-Cn B allele.

Key-words: Estonian Native cattle, milk protein polymorphism, coagulation properties

Introduction

Up until the 19th century, the Estonian farmers raised indigenous cattle. Breeding of Estonian Native (EN) cattle was started in 1910 when the West-Finnish breed was accepted as a breeding component. In 1956–1961 and 1989–1992, Jersey bulls were used to reduce the level of inbreeding in EN cattle. Swedish Red Polled bulls were employed in the late 1990s to modify the breed composition of the local cattle population (Kalamees 2004).

Estonian Native cattle are typically yellow-whitish red and hornless, with a medium wide chest and strong legs and hooves. Adult males weigh

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on average 700 kg and females 436 kg, and their wither height is 134 cm and 128 cm, respectively. The breed is characterised by good longevity, adaptation to the local conditions, easy calving, and low feed consumption per production unit. Average milk yield per cow per lactation was 4524 kg in 2005, i.e. lower than of other breeds in Estonia, whereas their milk fat (4.59%) and protein (3.44%) content were the highest.

According to the Estonian Agricultural Registers and Information Board, the total EN cattle population at the beginning of 2005 was 1,525 head including crossbreds (752 cows, 554 female calves, 143 young bulls and 76 bulls), of which 538 EN cows (including 420 purebreds) from 167 farms were included in milk recording. The share of native cattle has not decreased but remained at the same level, constituting about 0.5% of the total cattle population in Estonia. Due to its small population size, the Estonian Native cattle was categorised as an endangered breed by FAO in 1993. At present the breed has the risk status of an endangered-maintained breed (World Watch List for Domestic Animal Diversity 2000).

Since the most sustainable conservation strategy is to promote self-supporting, productive populations, it would be beneficial to establish a well-functioning selection programme for the breed. Genetic improvement could concentrate on maintaining or increasing the profitability of production in traits for which the breed still possesses a competitive edge (Toro and Mäki-Tanila 1999). Suitability of milk for cheese production could be one such trait. A preliminary comparison of milk coagulation properties among Estonian dairy breeds in an earlier study showed certain advantages of milk from EN cows, despite the limited number of EN cows in the study (Kübarsepp et al. 2005a). Moreover, once its suitability for cheese production is confirmed, milk from EN cows can be used for the production of Protected Denomination of Origin (PDO) cheeses. The PDO cheese-making process requires milk with good renneting properties from specific (local) breeds. Bertoni et al. (2005) found that the PDO cheeses have gained increasing value, not only in economic but also in cultural terms, particularly in some European countries. Besides showing certain organoleptic characteristics, these cheeses also represent a production system that is traditional and environmentally friendly.

The objective of this study was to examine the genetic variation of different milk proteins in milk from EN cows, and to determine the genotypic distributions and their effects on milk coagulation properties. To this end, we studied the rennet coagulation properties of milk among EN cows to assess its suitability for cheese production, and thereby to increase public interest in the breed and offer EN breeders better opportunities to maintain their EN herds.

Materials and methods

Cows and sampling

Milk samples (n=335) were collected from 112 cows on six farms recommended by the Estonian Native Cattle Breed Society, once every two months from March through November 2004, and from 6 cows on the Põlula Research Farm once a month throughout 2004. The sample represents more than 21% of the total EN cows included in milk recording.

Samples were taken simultaneously with the monthly milk recording using in-line milk meters at two consecutive milkings, and preserved with Bronopol® for an analysis of milk composition and renneting. Samples for determing milk protein genotypes were collected from the cows in March 2004, preserved with sodium azide and transported without delay to Freising, Germany.

Laboratory analyses

Concentrations of fat and protein were measured from each milk sample at the Milk Analysis Laboratory of Estonian Animal Recording Centre using an automated infrared milk analyser (System 4000, Foss Electric).

Milk protein genotypes (α_{s1}-casein, β-casein, κ-casein and β-lactoglobulin) were analysed at the Laboratory of Raw Milk, Munich University of Technology, Freising, Germany, by an isoelectric focusing/electrophoresis technique (Baranyi et al. 1993).

Milk rennet coagulation properties were determined on the day after milking at the Laboratory of Milk Quality, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, by a Formagraph method (Kübarsepp et al. 2005b). Two milk coagulation parameters were measured: milk coagulation time (RCT = time in minutes from rennet addition to milk until the beginning of coagulation) and curd firmness (E_{30} = diagram width in mm 30 min after rennet addition). If diagram width was less than 20 mm, the samples were classified as milk with poor rennet coagulation properties (NK_{20}). In commercial cheese production such poorly coagulating milk would not reach the firmness needed to properly cut the curd. For samples that did not coagulate at all, it was only possible to record curd firmness (E_{30}=0), and these samples were classified as noncoagulated milk (NCM).

Statistical analysis

The statistical analysis was performed on a dataset of altogether 335 milk samples from 118 Estonian Native cows (Table 1).

Information on the birth, calving and pedigree of the cows was obtained from the Estonian Animal Recording Centre. The pedigree data used for statistical analysis covered two to four generations, amounting to a total of 606 animals in the pedigree file. The cows in the dataset had calved 1 to 12 times, and parity was grouped into four classes: 1, 2, 3 to 4, and ≥ 5 parities. Lactation stage was grouped into 11 classes of 30-day intervals, except for the last class, which covered the days from the 301st day after calving to the end of lactation. Rennet coagulation time was logarithmically transformed to obtain a normal distribution.

Results were evaluated statistically using a general linear mixed model assuming a first-order autoregressive variance structure of repeated measurements from the individual cows (SAS Inst. Inc. 2006). In order to estimate the effects of different factors on the milk coagulation, compositional parameters, the following models were used:

$$y_{ijklmof} = \mu + parity_i + lactmonth_j + \alpha\beta\kappa\_Cn_k + \beta\_Lg_l + a_o + pe_f + \varepsilon_{ijklmof},$$

where: $y_{ijklmnop}$ = milk coagulation (log RCT, E_{30}), production (daily milk yield) or compositional trait (milk fat and protein contents), μ = general mean, $parity_i$ = fixed effect of parity class i (i = 1 to 4), $lactmonth_j$ = fixed effect of month of lactation j (j = 1 to 11), $\alpha\beta\kappa\_Cn_k$ = fixed effect of aggregate α_{s1}-, β- and κ-Cn genotypes k (k = 1 to 15), β_Lg_l = fixed effect of β-Lg genotype l, (l = 1 to 3); a_o = random additive genetic effect of animal o, $N(0, A\sigma^2_a)$; pe_f = random permanent environmental effect of farm f, $N(0, I\sigma^2_{pe})$; ε = random residual effect with spatial power covariance structure, N(0, R).

Interaction of genotypes at the α-, β- and κ-Cn

Table 1. Descriptive statistics of daily milk production, compositional and rennet coagulation parameters RCT in 118 Estonian Native cows.

	DMY[a], kg	Fat, %	Protein, %	RCT, min	log RCT	E_{30}, mm
Mean	15.8	4.67	3.57	7.3	0.83	33.0
SD[b]	6.34	0.870	0.422	3.05	0.169	12.96
Min	3.6	2.20	2.50	2.5	0.40	0
Max	39.5	7.25	4.72	23.0	1.36	57.0
Count	335	335	335	316	316	335

[a]Daily milk yield, [b]Standard deviation

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loci were considered because of their close genetic linkage.

The basic genetic variability of milk proteins was analysed by applying the Arlequine software package for population genetics (Excoffier et al. 2005). An estimation of gene diversities – expected (H_E) and observed (H_O) heterozygosity – and a probability test for detecting genotypic deviations from Hardy-Weinberg equilibrium were performed. Genotypic disequilibrium was tested under the null hypothesis (genotypes at one locus are independent from those at another locus). A Markov chain method was used to obtain P-value estimates using the Genepop computer program (Raymond and Russout 1995).

Allele and genotype frequencies were computed by direct counts.

Results

Effects of systematic environmental factors on studied traits

Parity did not have any significant overall effect on the studied milk rennet coagulation parameters, daily milk yield (DMY), and milk protein and fat content (Table 2). However, compared to the later parities, there were more noncoagulated and poorly coagulated milk samples in the first parity when milk protein content was lowest. Milk formed the firmer curd in the second to fourth parity when milk fat and protein contents were higher. Daily milk yield exhibited a tendency to improve with increasing number of lactation.

Lactation month had a significant effect on both the studied rennet coagulation traits ($P<0.001$) as well as on DMY and milk protein and fat content ($P<0.0001$). Milk coagulation properties were at their best in a very early stage and curd firmness also improved in the second half of lactation (Fig. 1). The proportions of noncoagulated and poorly coagulated milk were at their lowest at the beginning of lactation and clearly at their highest during midlactation. Daily milk yield declined during lactation. Also, milk fat and protein content decreased over the first three or four months of lactation and then started to increase again during midlactation when the coagulation properties were at their poorest. Milk fat and protein content rose steeply in the second part of lactation.

Genetic variability of milk proteins

All of the analysed proteins showed genetic polymorphism. Two to three alleles per locus were detected by isoelectrophoretic separation of milk (Table

Table 2. Estimates ±SE of effect of parity on studied traits (zero refers to class of comparison) and percentages of noncoagulated (NCM) and poorly (E_{30}<20 mm) coagulated (NK_{20}) milk samples of all samples in respective parity class.

Trait	Parity				P value
	1	2	3–4	≥5	
Number of samples	125	63	106	40	
Daily milk yield, kg	0^{a}	0.72 ± 1.03^{ab}	1.50 ± 0.88^{b}	2.20 ± 1.06^{b}	0.1605
Fat, %	0^{a}	0.50 ± 0.19^{b}	0.31 ± 0.16^{b}	0.19 ± 0.19^{ab}	0.0665
Protein, %	0^{a}	0.13 ± 0.09^{a}	0.07 ± 0.08^{a}	0.05 ± 0.09^{a}	0.5461
log RCT	0^{a}	-0.02 ± 0.04^{ab}	-0.06 ± 0.03^{b}	-0.02 ± 0.04^{ab}	0.2798
$^{1}E_{30}$, mm	0^{ab}	0.92 ± 2.32^{a}	0.98 ± 1.97^{a}	-3.47 ± 2.31^{b}	0.2321
NCM, %	10.4	6.3	1.9	–	
NK_{20}, %	10.4	6.3	5.6	5.0	

[1]Estimates of curd firmness of coagulating (E_{30}>0 mm) milk samples

[a,b]Estimates within row with differing letters in superscript are significantly different ($P<0.05$)

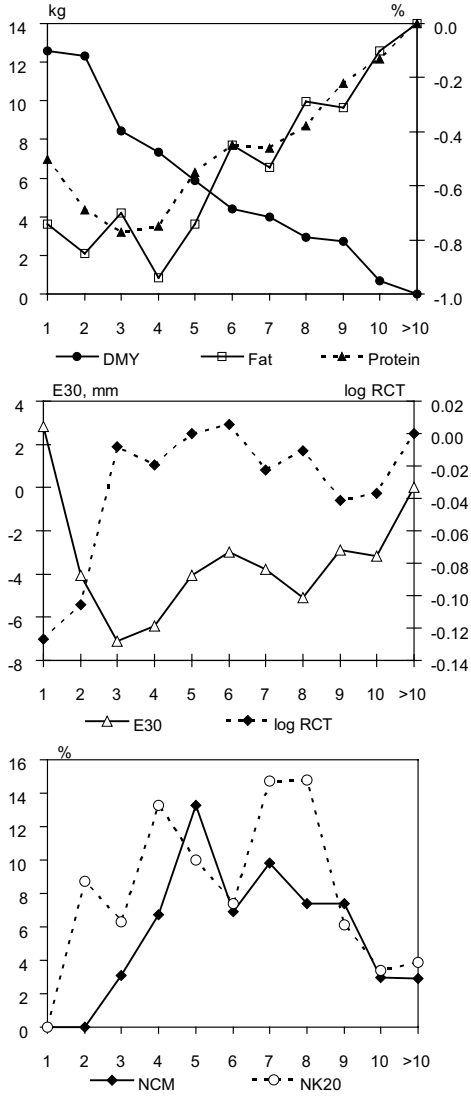

Figure 1. Estimates of effect of lactation month on milk traits (log RCT – logarithmically transformed rennet coagulation time, E30 – curd firmness of coagulating milk samples, DMY – daily milk yield, Fat – milk fat content, Protein – milk protein content) and percentages of noncoagulated (NCM; E_{30}=0 mm) and poorly coagulated (NK20; 0 mm<E_{30}<20 mm) milk samples within respective lactation month. Zero refers to class of comparison.

3). Milk protein gene diversities varied between H_O=0.152 (α_{s1}-Cn) and 0.525 (β-Cn), the average heterozygosity being 0.374. Allele frequencies ranged from 0.038 (β-Cn B allele) to 0.915 (α_{s1}-Cn B allele) as shown in Table 4. The κ-Cn E allele was not detected in EN in current sampling.

In α_{s1}-Cn a single genotype (α_{s1}-Cn BB) was prevalent, found in 83.9% of the studied EN cows (Table 5). Also β-Cn A^2A^2 (42.4%) and κ-Cn AB (52.5%) were frequent genotypes. Of the 16 detected aggregate genotypes, 11 genotypes occurred in more than one individual, and four of these (α_{s1}-β-κ-Cn) BB A^2A^2 AA (21.2%), BB A^1A^2 AB (16.9%), BB A^1A^2 AA (14.4%) and BBA^2A^2AB (10.2%) were found among nearly two thirds of the analysed cows. β-lactoglobulin genotype AA, AB and BB frequencies were 9.9, 42.2 and 47.9%, respectively. The frequencies of heterozygotes were consistent with the observed allele frequencies (Table 3), except for κ-Cn which showed a significant deviation from Hardy-Weinberg equilibrium, with an increased frequency of heterozygotes from both homozygote genotypes.

Among the three possible pairs of casein loci we found disequilibrium between β-Cn and κ-Cn at a significance level of 0.05, where the κ-Cn BB genotype combined with β-Cn genotypes containing A^1 as well as A^2, but was never observed with the homozygote A^2A^2 (Table 5). On the other hand, κ-Cn AA was most frequently found in combination with β-Cn A^2A^2. The α_{s1}-Cn BC genotype combinations occurred only with β-Cn A^1A^2 and A^2A^2. The rare α_{s1}-Cn CC genotype was observed only in one individual, in combination with β-Cn A^2A^2.

Table 3. Number of detected alleles, expected (H_E) and observed (H_O) heterozygosity of milk proteins.

Locus	No. of detected alleles	H_E	H_O
α_{S1}-Cn	2	0.164	0.152
β-Cn	3	0.485	0.449
κ-Cn	2	0.426	0.525*
β-Lg	2	0.441	0.424

*Significant (P=0.0055) heterozygosity excess

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Table 4. Milk protein allele frequencies of Estonian dairy breeds and of breeds used for genetic improvement of Estonian Native cattle (number of cows are given in brackets).

Locus	Allele	Estonian Native (118)	Western Finncattle[a] (41)	Danish Jersey[a] (32)	Estonian Holstein[b] (609)	Estonian Red breed[b] (321)
α_{s1}-Cn	B	0.915	0.939	0.781		
	C	0.085	0.061	0.219		
β-Cn	A^1	0.318	0.293	0.094		
	A^2	0.644	0.671	0.688		
	B	0.038	0.037	0.219		
κ-Cn	A	0.695	0.671	0.512	0.790	0.642
	B	0.305	0.305	0.488	0.138	0.324
	E	–	0.024	–	0.072	0.034
β-Lg	A	0.314	0.098	0.463	0.421	0.254
	B	0.686	0.902	0.537	0.579	0.746

[a]Lien et al. (1999), [b]Kübarsepp et al. (2006).

Table 5. Frequencies of casein genotypes in 118 Estonian Native cows.

κ-Cn	α_{S1}-Cn	β-Cn					Total
		A^1A^1	A^1A^2	A^2A^2	A^2B	A^1B	
AA	BB	0.025	0.144	0.212			0.381
	BC		0.017	0.034			0.051
Total AA		0.025	0.161	0.246			0.432
AB	BB	0.093	0.169	0.102	0.068	0.008	0.441
	BC		0.008	0.068			0.076
	CC			0.008			0.008
Total AB		0.093	0.178	0.178	0.068	0.008	0.525
BB	BB	0.008	0.008				0.017
	BC		0.025				0.025
Total BB		0.008	0.034				0.042
Total		0.127	0.373	0.424	0.068	0.008	1.000

Effect of milk protein genotypes on rennet coagulation properties

Aggregate casein genotype was found to have a significant ($P<0.05$) overall effect on both of the studied rennet coagulation parameters. The β-Lg genotype had a significant effect on curd firmness (Table 6).

Two aggregate casein genotypes, CC A^2A^2 AB and BC A^1A^2 BB, distinctly differed from others in significantly better milk rennet coagulation properties, but they occurred in few animals (one and three cows, respectively). Among the more frequent aggregate genotypes, there was a tendency of firmer curd formed in milk from cows carrying the BB A^1A^2 BB genotype. Among aggregate genotypes possessing the same β- and κ-Cn genotype, there was a tendency for the combinations with α_{s1}-BC or CC genotypes to have shorter milk rennet coagulation time and to form firmer curd than that for combinations with α_{s1}-BB. Within aggregate casein genotypes with α_{s1}-Cn BB and κ-Cn AB, milk from cows possessing β-Cn A^1A^1 or A^1A^2 had significantly longer rennet coagulation time than the cows with β-Cn A^2B, but curd showed the tendency to be firmer and no

noncoagulated milks were observed (Table 6).

Among β-Lg genotypes, β-Lg AB had a significantly softer curd than BB, and the percentages of noncoagulated and poorly coagulated milk samples were the highest (Table 6).

No noncoagulated milk samples were observed in association with β-Lg AA, κ-Cn BB, α_{s1}-Cn CC, or β-Cn A^1B (α_{s1}-Cn CC and β-Cn A^1B were represented only by one animal), whereas 78.9% of all noncoagulated milk samples originated from cows possessing κ-Cn AA genotype (Table 6).

The overall effect of aggregate casein and β-Lg genotypes on daily milk yield, milk fat and protein content was not significant.

Discussion

The results show that stage of lactation had significant effect on the rennet coagulation properties of milk. The variation in coagulation properties with lactation month showed a clear pattern, with the poorest coagulation properties in mid lactation. The results of the current study and our previous studies (Kübarsepp et al. 2005a) are consistent with those reported by Davoli et al. (1990), Tyrisevä et al. (2004), and Ikonen et al. (2004). The present results also showed that milk coagulation characteristics varied between parities, although the overall effect of parity was not significant. Percentages of noncoagulated and poorly coagulated milk samples diminished with increasing parity number. The literature contains contradictory

Table 6. Estimates ±SE of effects of aggregate casein and β-Lg genotypes on milk coagulation parameters (zero refers to class of comparison) and percentages of noncoagulated (NCM) and poorly (NK_{20}) coagulated milk samples within respective genotype

Aggregate (α_{s1}-, β-, κ-) casein genotype	Number of cows/samples	log RCT	$^1E_{30}$, mm	NCM %	NK_{20}, %
BB A^1A^1 AA	3/5	–0.03±0.10^{abc}	0.64±5.95^{ab}	20.0	
BB A^1A^2 AA	17/47	0.01±0.04^{a}	2.33±2.43^{ab}	10.6	10.6
BC A^1A^2 AA	2/8	–0.08±0.09^{abc}	4.48±5.16^{abc}	12.5	12.5
BB A^2A^2 AA	25/94	0^{a}	0^{a}	4.3	8.5
BC A^2A^2 AA	4/14	–0.01±0.08^{ac}	2.24±4.27^{abc}	28.6	14.3
BB A^1A^1 AB	11/20	0.00±0.05^{a}	2.55±3.05^{ab}	–	20.0
BB A^1A^2 AB	20/32	0.01±0.04^{a}	5.26±2.60^{b}	–	6.25
BC A^1A^2 AB	1/4	–0.18±0.12^{abc}	5.17±6.68^{abcd}	–	–
BB A^2A^2 AB	12/34	–0.06±0.05^{abc}	3.21±2.91^{ab}	2.9	2.9
BC A^2A^2 AB	8/27	–0.10±0.05^{abc}	4.98±3.04^{ab}	7.4	3.7
CC A^2A^2 AB	1/10	–0.08±0.04^{b}	17.54±5.98^{cd}	–	–
BB A^1B AB	1/4	–0.28±0.11^{abc}	6.98±7.10^{abcd}	–	–
BB A^2B AB	8/25	–0.15±0.13^{bc}	1.99±2.92^{ab}	4.0	16.0
BB A^1A^2 BB	1/4	–0.08±0.13^{abc}	4.82±6.80^{abcd}	–	–
BC A^1A^2 BB	3/6	–0.27±0.09^{b}	17.21±5.17^{d}	–	–
P value		0.0341	0.0417		
β-Lg AA	12/38	0.05±0.05^{a}	–3.18±2.91^{ab}	–	5.26
AB	49/142	0.01±0.03^{a}	–4.39±1.62^{a}	8.45	11.27
BB	56/154	0^{a}	0^{b}	4.55	4.55
P value		0.5761	0.0293		

[1]Estimates of curd firmness of coagulating milk samples (E_{30}>0 mm)

[a,b,c,d]Estimates within milk coagulation trait and aggregate casein or β-Lg genotype with differing letters in superscript are significantly different (P<0.05)

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results about the influence of parity on milk rennet coagulation properties. Schaar (1984) found a favourable effect of increasing parity number on coagulation properties, but in some other studies parity had either no significant effect (Davoli et al. 1990, Ikonen et al. 2004, Tyrisevä, et al. 2004) or coagulation properties were deteriorating with increasing number of lactation (Tyrisevä et al. 2003).

In genetic terms, the Estonian Native belongs to the Nordic cattle breeds, with a close relationship to Western Finncattle, as revealed by DNA microsatellites in a recent analysis (Tapio et al. 2006).

Our results regarding the casein allele frequencies further support the genetic relationship of EN with Western Finncattle. The observed difference between EN and Finncattle in the frequency of β-Lg variants in the current study probably results from genetic material introduced into EN by Jersey bulls and/or, on a smaller scale, Holstein and/or red breeds. Despite belonging to one genetic cluster with other old indigenous breeds (Tapio et al. 2006), the EN breed showed very similar distribution of milk protein aggregate genotypes with common commercial dairy breeds (Eenennaam and Medrano 1991, Lien et al. 1999).

A comparison of milk protein allele frequencies between EN and the breeds used for improvement (Finncattle, Danish Jersey) revealed similarities between breeds. A predominance of α_{s1}-Cn B (or its monomorphism) has also been observed in the common dairy breeds in Europe (Tervala et al. 1983, Ikonen et al. 1996, Lundén et al. 1997, Erhard et al. 1998, Lien et al. 1999). The same predominant variants in κ- and β-Cn loci have been found in most dairy cattle: κ-Cn A, except for Finncattle, Jersey and Brown Swiss, where B-allele is widespread, and alleles A^1 and A^2 at β-Cn (Tervala et al. 1983, Ikonen et al.1996, Freyer et al. 1999). The frequencies of β-Lg A and B alleles were similar in EN and in Jersey as well as in the dairy breeds of adjacent countries (Bech and Kristiansen 1990, Velmala et al. 1993, Ikonen et al.1996, Lundén et al. 1997). A comparison of milk protein allele frequencies between the EN breed and the other dairy breeds raised in Estonia, namely the Estonian Holstein (EHF) and Estonian Red (EPK), showed that EN's frequency of favourable κ-Cn B allele resembled that of EPK, but was higher than for EHF (Kübarsepp et al. 2005a). Unfavourable κ-Cn E allele (Jakob and Puhan 1992, Buchberger and Dovč 2000) was found both among the commercial breeds EHF and EPK (Kübarsepp et al. 2006), but not among EN cows in the current sampling.

Most of the genotypes in our sample followed the Hardy-Weinberg equilibrium. Only one protein, κ-Cn, displayed significantly higher observed heterozygosity than would have been expected by the allele frequencies. The excess of heterozygote κ-Cn genotypes probably reflects the occurrence of individuals with crossbred ancestors in the study. Freyer et al. (1999) presumed that the heterozygous κ-Cn genotype might have a heterotic effect on the milk yield. The occurrence of linkage disequilibrium between alleles at the different casein loci in our data indicates a relatively recent introduction of genetic material carrying specific casein haplotypes. The most common aggregate genotype was the homozygous combination BB A^2A^2 AA, reflecting a high frequency of the haplotype BA^2A in the breed.

We observed similar effects of casein genotypes on coagulation properties to those reported for other breeds by several research groups (Jakob and Puhan 1992, Van den Berg et al. 1992, Ikonen and Ojala 1995, Lodes et al. 1996, Ng-Kwai-Hang 1998, Buchberger and Dovč 2000).

Due to the close linkage of four Cn genes in chromosome 6 within a region of about 250kb in cattle (Rijnkels 2002) segregation of the α_{sn}-Cn, b-Cn, and κ-Cn variants occurs nonindependently (Aleandri et al. 1990, Eenennaam and Medrano 1991). Because of this close linkage of Cn genes, the use of casein aggregate genotypes is a more appropriate way to estimate the effect of Cn polymorphism on milk production traits than the use of individual Cn genotypes (Ikonen et al. 1999). Aggregate genotypes, similar to those of Estonian Native breed, have been frequent in Swedish Red and White and in Swedish Holstein breed (Lundén et al. 1997). Aggregate casein genotype had statistically significant ($P<0.05$) effect on curd firmness and rennet coagulation time. Most noncoagulated milk samples originated from cows possessing κ-Cn AA genotype.

Although the number of cows sampled was a considerable as a proportion (>21%) of the EN population, the size of the data was statistically speaking

small (Table 6). Although the overall effect of aggregate genotypes on milk rennet coagulation characteristics was significant the differences between genotypes were mostly not significant probably due to the high standard error values resulting from the small number of animals and samples representing each genotype. As the number of animals in the study was relatively small, the statistical analysis described by Hallén et al. (2007) was also carried out (results not shown). The results were no different however, and it was not possible to verify the superiority of any casein locus.

According to Kübarsepp et al. (2005a) milk from EN cows form a stronger curd (E_{30} = 33 mm) than milk from the other Estonian breed, EHF (E_{30} = 27.6 mm) and EPK (E_{30} = 31.1 mm). Also the percentage of poorly coagulated and noncoagulated milk samples (E_{30}<20 mm) was lowest for EN, 13.1%, while the percentages for EHF and EPK were 19.5 and 17.5%, respectively (Kübarsepp et al. 2005a). Several earlier studies (Tervala et al. 1983, Macheboeuf et al. 1993, Auldist et al. 2002) also asserted better renneting properties among native breeds as compared with the Holstein. Differences in milk coagulation properties between breeds may be due to differences in milk composition that is attributable to variation in other parts of genome. The studies mentioned above associated the better milk coagulation properties among native breeds with a higher frequency of κ-Cn B allele. A positive effect of this allele was shown also in the present study on EN cows, which also showed a comparatively high frequency of the allele.

Conclusions

Our present findings confirm previously observed relationships between genetic milk protein variants and milk properties for cheese-making. In contrast to common commercial dairy cattle breeds, Estonian Native cattle breed showed a relatively high frequency of the favourable κ-Cn B allele, although predominantly in heterozygote combination with the A allele, whereas no unfavourable κ-Cn E alleles were detected in EN in current study. On the other hand, favourable aggregate casein genotypes (containing κ-Cn BB, α_{s1}-Cn BC or CC genotype) for improving the conversion of milk protein into cheese were rarely observed in EN. Noncoagulated milk originated mainly from cows possessing κ-Cn AA genotype. If we compare the milk coagulation properties among the cattle breeds raised in Estonia, based on our current and previous results, the best milk for cheese-making comes from Estonian Native cattle.

In order to apply the genetic information obtained from this study in EN breeding programmes, we need to conduct additional determination of milk protein genotypes for all breeding bulls. This is necessary to increase the allele frequencies with a positive effect and to avoid unfavourable alleles in closely linked loci.

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Short communication

The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows

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ABSTRACT

The objective of this study was to investigate the effect of milk proteins on rennet coagulation properties. Milk samples ($n = 516$) were taken from 54 cows once a month during one full lactation period and analysed for rennet coagulation properties, and for contents of α_{S1}-, α_{S2}-, β- and κ-casein, and β-lactoglobulin. The contents of the analysed milk proteins and the relative contents of different caseins (Cn) in total casein were significantly influenced by sampling month, breed, and the month of lactation. An increase in milk protein, casein, casein fractions, and the casein number decreased the rennet coagulation time of milk and increased curd firmness. Milk formed a firmer curd when the relative content of α_{S1}- and β-Cn in total casein was lower, or the relative content of κ-Cn in total casein was higher. Higher values of κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios had a positive effect on curd firmness.

1. Introduction

In many milk-producing countries, a large proportion of milk production is destined for cheese production. Coagulating properties of milk influence cheese-making ability, cheese yield, and its quality (Johnson, Cen, & Jaeggi, 2001; Ng-Kwai-Hang, Politis, Cue, & Marziali, 1989). At the same time there is a large variability in the rennet coagulation properties of milk. Milk coagulation properties are influenced by several factors such as age of animals, stage of lactation, composition of ration, season, and breed (Auldist, Mullins, O'Brien, O'Kennedy, & Guinee, 2002; Guinee, Mulholland, O'Brien, & Murphy, 2001; Ostersen, Foldager, & Hermansen, 1997; Tyrisevä, Vahlsten, Ruottinen, & Ojala, 2004). Variations in milk composition are the major influencing factors in the rennet coagulation properties of milk (Auldist et al., 2002; Ikonen, Morry, Tyrisevä, Routtinen, & Ojala, 2004; Wedholm, Larsen, Lindmark-Månssom, Karlsson, & Andrén, 2006). In addition, the genetic polymorphism of milk proteins is related to the composition and technological properties of milk (Buchberger & Dovč, 2000; Ng-Kwai-Hang, 1998).

The objectives of this study were to evaluate the variation in content of milk proteins among dairy cattle breeds raised in Estonia and to investigate the effect of milk proteins on rennet coagulation properties. This study involves a more extensive analysis to ascertain specific markers that could be used to identify milk suitable for cheese-making and thereby provide economic advantage to the dairy industry.

2. Materials and methods

2.1. Cows and sampling

Monthly milk samples ($n = 516$) were obtained from 54 cows (15 Estonian Red – ER, 5 Red-and-White Holstein – RHF, 34 Estonian Holstein – EHF) from a Põlula, Estonia research farm during a single lactation period between November 2002 and April 2004. The cows were fed identically ad libitum total mixed ration and milked three times a day. Milk samples were collected simultaneously, with animal recording using in-line milk meters at three consecutive milkings.

2.2. Laboratory analysis

Rennet coagulation properties of milk were determined on the day after milking at the Laboratory of Milk Quality (Estonian University of Life Sciences) using the Formagraph method as described by Kübarsepp, Henno, Kärt, and Tupasela (2005). Two milk coagulation parameters, rennet coagulation time (RCT = time, in min, from rennet addition until the beginning of coagulation) and curd firmness (E_{30} = diagram width, in mm, 30 min after rennet addition) were measured. The pH of the milk was determined using a pH meter (MP 220; Mettler Toledo GmbH, Greifensee, Switzerland) at room temperature before rennet coagulation analysis. The calcium content of milk was determined using the International Dairy Federation Standard method 36A (IDF, 1992).

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A modification of the reversed-phase high-performance liquid chromatography (RP-HPLC) method proposed by Visser, Slangen, and Rollema (1991) and Trujillo, Casals, and Guamis (2000) was used to separate and quantify the major milk proteins: α_{S1}-, α_{S2}-, β- and κ-casein (Cn) and β-Lg in a single run. Proteins were separated at 35 °C on a Jupiter300 reversed-phase column (5 μm particle, C18, 300 Å porosity, 150 × 2.0 mm I.D., Phenomenex, Torrance, CA, USA) with Security Guard Cartridge System (Widepore C18 ODS, 4 × 2.0 mm I.D., Phenomenex) as a guard column. The following program was used: a linear gradient from 30% to 40% B in 10 min, then from 40% to 50% B in 15 min at a flow rate of 0.2 mL min^{-1}, where eluent A was composed of 1 mL L^{-1} trifluoroacetic acid (TFA) in ultra pure water, and eluent B was 1 mL L^{-1} TFA in acetonitrile. Peak detection was with a UV–Vis diode array detector at 220 nm. For the determination of response curves for various milk proteins, the purified milk proteins (α_S-, β- and κ-Cn and β-Lg, Sigma–Aldrich, Buchs, Switzerland) solutions in a sample buffer (Visser et al., 1991) with known protein content were used. Total casein content was defined as the sum of the concentrations of α_{S1}-, α_{S2}-, β- and κ-Cn. In some analyses the sum of the concentrations of α_{S1}- and α_{S2}-Cn was used, labelled α_S-Cn. Milk protein, fat, lactose contents, and somatic cell count (SCC) data were obtained from the Estonian Animal Recording Centre.

2.3. Statistical analysis

A statistical analysis was performed on a dataset of all 516 milk samples from 54 cows. The cows were distributed into three classes according to parity: first lactation (1; $n = 23$), second lactation (2; $n = 13$), and third lactation (3; $n = 18$). Lactation stage was grouped into 11 classes of 30-day intervals, except for the last class, which covered the days from the 301st day after calving to the end of lactation. All the breeds were represented within each parity and lactation class. According to differing curd firmness values, the milk samples were divided into four classes: non-coagulated milk ($E_{30} = 0$ mm; $n = 15$), poorly coagulated milk (0 mm $< E_{30} <$ 20 mm; $n = 82$), fairly coagulated milk (20 mm $\leq E_{30} <$ 35 mm; $n = 231$), and milk with good rennet coagulation properties ($E_{30} \geq 35$ mm; $n = 188$). To obtain a normal distribution, SCCs were transformed to a natural logarithm of SCC and called somatic cell score (SCS).

Pearson correlation analysis was carried out to study the associations between milk rennet coagulation and compositional parameters. To study the factors influencing milk protein contents, and to estimate milk protein contents for different rennet coagulation classes, the general linear model (version 9.1.3, SAS Inst. Inc., Cary, NC, USA, 2006) incorporating fixed effects of breed, parity, calendar (sampling) month or season (pasture, indoor), month of lactation and rennet coagulation class was used. To study the effect of milk proteins on rennet coagulation parameters, the same model described above was used, only the fixed effect of rennet coagulation class was replaced by the effect of content of α_{S1}-, α_{S2}-, β- and κ-Cn and β-Lg, the relative content of α_{S1}-, α_{S2}-, β- and κ-Cn in total casein, or the casein number (casein:protein), ratio of κ-Cn:β-Cn and ratio of κ-Cn:α_S-Cn. In all models a first-order autoregressive variance structure of repeated measurements from individual cows was assumed.

3. Results and discussion

3.1. Factors influencing the content of milk proteins

The contents of all the studied milk proteins, except total protein content, and the relative contents of single caseins in total casein were significantly influenced by the sampling month, but the effect of pasturage was significant only for total casein, α_{S1}- and β-Cn, casein number, and the α_{S2}-Cn ratio in total casein (Table 1). The effect of the month of lactation was significant for all proteins excepting the α_{S2}-Cn:Cn ratio. A significant effect of parity was found on the contents of β-Cn and β-Lg and on the relative contents of α_{S1}- and β-Cn (Table 1). Reported results in the literature on the effect of season, parity and month of lactation are contradictory (Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1987; Ostersen et al., 1997; Rampilli, Caroli, Bolla, & Pirlo, 1988; Wedholm, Hallen et al., 2006). These contradictory results may have arisen from differences in experimental design, for example the number of animals, feeding conditions, sampling frequency and period.

The effect of breed was significant for all the studied milk protein fraction contents, except for α_{S1}-Cn and on the proportions of α_{S1}- and κ-Cn in total casein (Table 1). Milk from the ER breed contained more β-Lg, α_{S2}- and κ-Cn and the κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios were higher, but the relative content of α_{S1}-Cn in total casein was lower than those for the EHF breed. Although milk from the ER cows contained more casein than milk from the EHF cows, the casein number was smaller for the ER breed. The contents of individual milk proteins and ratios of individual caseins in total casein that apply to Estonian cattle breeds have also been found to be applicable for the Jersey breed (Auldist, Johnson, White, Fitzsimons, & Boland, 2004), for the Swedish Red-and-White breed, and for the Swedish Holstein breed (Wedholm, Hallen et al., 2006). However, studies on Holstein Friesian cows in

Table 1
Least square mean concentrations (g L^{-1}), the relative contents of analysed milk proteins, and the significance of different effects on the protein contents[a]

	EHF ($n = 331$)	RHF ($n = 56$)	ER ($n = 129$)	Significance				
				Breed	Lact. month	Parity	Sampling month	Pasturage
α_{S1}-Cn	9.756^{a}	10.010^{a}	9.847^{a}	n.s.	***	n.s.	**	**
α_{S2}-Cn	1.899^{a}	1.925^{a}	2.037^{b}	***	***	n.s.	**	n.s.
β-Cn	13.884^{a}	14.236^{a}	14.223^{a}	*	***	*	***	***
κ-Cn	3.764^{a}	3.994^{a,b}	4.175^{b}	***	***	n.s.	***	n.s.
β-Lg	3.714^{a}	3.837^{a,b}	3.872^{b}	*	***	**	***	n.s.
α_{S1}-Cn:Cn	0.333^{a}	0.332^{a,b}	0.326^{b}	**	***	*	**	n.s.
α_{S2}-Cn:Cn	0.065^{a}	0.065^{a}	0.067^{a}	n.s.	n.s.	n.s.	**	**
β-Cn:Cn	0.474^{a}	0.474^{a}	0.470^{a}	n.s.	*	**	*	n.s.
κ-Cn:Cn	0.127^{a}	0.131^{a,b}	0.137^{b}	**	***	n.s.	*	n.s.
κ-Cn:β-Cn	0.269^{a}	0.277^{a,b}	0.291^{b}	**	***	n.s.	*	n.s.
κ-Cn:α_S-Cn	0.322^{a}	0.332^{a,b}	0.350^{b}	**	***	n.s.	**	n.s.
Protein	33.7^{a}	35.6^{b}	35.5^{b}	**	***	n.s.	n.s.	n.s.
Casein	29.33^{a}	30.13^{a,b}	30.27^{b}	*	***	n.s.	***	**
Casein number	0.834^{a}	0.810^{a,b}	0.819^{b}	*	***	n.s.	***	***

[a] Parameters are defined in Section 2. Values within rows with differing superscript letters are significantly different ($P < 0.05$). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s.: $P \geq 0.05$.

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the USA (Ng-Kwai-Hang et al., 1987) and Danish Holstein cows (Ostersen et al., 1997) showed a higher content of α_S-Cn and lower contents of β-Cn and κ-Cn than any other breeds studied in this article.

3.2. Effect of milk protein content on rennet coagulation properties

Rennet coagulation parameters of milk were significantly influenced by α_{S1}-Cn, β-Cn, and β-Lg contents and by casein number (Table 2). The content of the κ-Cn and κ-Cn:β-Cn ratio had a significant effect on curd firmness, and the proportion of α_{S1}-Cn in total casein had a significant effect on RCT.

Rennet coagulation time was reduced and a firmer curd was formed, along with increasing contents of the studied milk protein fractions including total casein and casein number (Table 3). A firmer curd was formed when the proportions of α_{S2}-Cn and β-Cn in total casein were smaller, or when the κ-Cn:Cn, κ-Cn:β-Cn, and κ-Cn:α_S-Cn ratios were larger. The major milk components were correlated more weakly with either, or both, RCT and E_{30} than caseins (except α_{S2}-Cn) and β-Lg. The concentrations of the major milk components (as indicated by R^2, calculated on the basis of Table 3) account for up to 12% of the variation in these parameters, with two exceptions: the pH resulted in a 19% variation in RCT, and calcium content resulted in an 18% variation in E_{30}. At the same time, all the studied milk proteins contents, except α_{S2}-Cn, resulted in a variation of rennet coagulation time of up to 17%, and for a variation of curd firmness up to 41%. A greater effect by casein and κ-Cn content, than by milk total protein content, on coagulation parameters has also been found by Auldist et al. (2002). But Ikonen et al. (2004) found that phenotypic correlation among the coagulation parameters and milk protein and casein contents is very low ($R < 0.1$), and the correlations between milk coagulation characteristics and milk pH were also less than 0.3. They found that if farmers select animals that produce milk with higher protein and casein contents, then not only better coagulation properties of milk could be achieved but also the number of animals producing non-coagulating milk could be increased. We found that those cows in the dataset that produced non-coagulating milk at least once during lactation ($n = 9$) had no differences in milk protein content in comparison with other cows, but they had a significantly lower casein number ($P = 4.17 \times 10^{-10}$), casein ($P = 1.38 \times 10^{-6}$), and κ-Cn content ($P = 7.54 \times 10^{-4}$). Ikonen et al. (2004) suggested the selection of low somatic cell count for genetic improvement of rennet coagulation properties of milk and for the reduction of the occurrence of non-coagulating milk. Our study was unable to confirm these propositions because SCS did not have a statistically significant effect on rennet coagulation properties of milk; there were no differences in SCS among the rennet coagulation classes, and cows producing non-coagulating milk at least once did not have a different SCS from the milk of other cows in the study.

The contents of all studied milk proteins were significantly lower for non-coagulated ($E_{30} = 0$ mm) and poorly coagulated ($E_{30} = 1 \ldots 19$ mm) milk compared with fairly and well-coagulated milk ($E_{30} \geq 20$ mm; Fig. 1). The relative amount of casein in total protein was higher in milk that formed a firmer curd (Table 4). Compared with the coagulated milk, non-coagulated milk had a higher content of α_{S2}-Cn, a lower content of κ-Cn in total casein and lower κ-Cn:β-Cn and κ-Cn:α_S-Cn ratios, which has also been observed by Wedholm, Larsen et al. (2006). An increased amount of β-Cn in total casein has a negative effect on rennet coagulation properties of milk, also confirmed by St-Gelais and Hache (2005); who reported poorer milk coagulation properties of milk enriched with β-Cn powder. A significant effect of increased κ-casein content leads to a higher number of smaller casein micelles, which form a firmer curd than would formed by larger micelles. In contrast, an increase in the ratio of β-Cn in total casein leads to the formation of larger micelles and deterioration in the rennet coagulation properties of milk (Dalgleish, 1992).

Table 2
Significance (*P*-values) of the effects of milk protein fractions on milk rennet coagulation time (RCT) and curd firmness (E_{30})

	Content					Casein number
	α_{S1}-Cn	α_{S2}-Cn	β-Cn	κ-Cn	β-Lg	
RCT (min)	<0.0001	0.1189	0.0106	0.1981	<0.0001	<0.0001
E_{30} (mm)	<0.0001	0.1797	0.0002	0.0103	<0.0001	<0.0001
	Proportion in total casein				κ-Cn:β-Cn	κ-Cn:α_S-Cn
	α_{S1}-Cn	α_{S2}-Cn	β-Cn	κ-Cn		
RCT (min)	0.0321	0.1205	0.0638	0.0882	0.9964	0.1409
E_{30} (mm)	0.5911	0.6300	0.8877	0.5149	0.0027	0.3429

Table 3
Correlation coefficients between milk rennet coagulation parameters (RCT – rennet coagulation time and E_{30} – curd firmness) and milk compositional characteristics

Traits	RCT (min)[a]	E_{30} (mm)[a]
Major milk constituents		
Fat	−0.159***	0.271***
Protein	−0.180***	0.349***
Lactose	−0.175***	0.171***
Somatic cell score	0.016	0.028
pH	0.432***	−0.306***
Calcium	−0.312***	0.425***
Protein composition		
Casein	−0.384***	0.682***
Casein number	−0.507***	0.467***
α_{S1}-Cn	−0.416***	0.638***
α_{S2}-Cn	−0.101*	0.274***
β-Cn	−0.370***	0.620***
κ-Cn	−0.209***	0.553***
β-Lg	−0.417***	0.598***
α_{S1}-Cn:Cn	−0.181***	0.101*
α_{S2}-Cn:Cn	0.211***	−0.288***
β-Cn:Cn	0.052	−0.188***
κ-Cn:Cn	0.021	0.267***
κ-Cn:β-Cn	−0.001	0.273***
κ-Cn:α_S-Cn	0.046	0.231***

[a] ***$P < 0.001$; *$P < 0.05$.

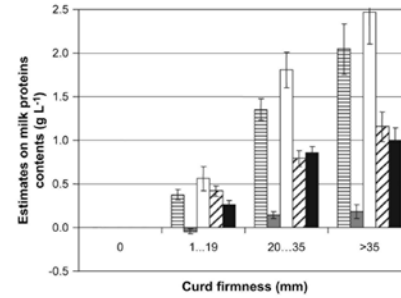

Fig. 1. Estimates of effect for curd firmness class relative to the non-coagulated milks ($E_{30} = 0$) on the content of α_{S1}-Cn (▤), α_{S2}-Cn (▩), β-Cn (□), κ-Cn (▨) and β-Lg (■). Error bars are standard errors.

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Table 4
Least square means of relative contents of milk proteins in the different rennet coagulation classes[a]

	$E_{30} = 0$ mm	$E_{30} = 1 \ldots 19$ mm	$E_{30} = 20 \ldots 35$ mm	$E_{30} > 35$ mm
No. of samples	15	83	230	188
α_{S1}-Cn:Cn	0.328^{a,b}	0.328^{a}	0.330^{a}	0.335^{b}
α_{S2}-Cn:Cn	0.071^{a}	0.065^{b}	0.065^{b}	0.062^{c}
β-Cn:Cn	0.477^{a}	0.476^{a}	0.473^{a}	0.465^{b}
κ-Cn:Cn	0.123^{a}	0.133^{b}	0.134^{b}	0.137^{c}
κ-Cn:β-Cn	0.25^{a}	0.279^{b}	0.284^{b}	0.298^{c}
κ-Cn:α_S-Cn	0.314^{a}	0.338^{b}	0.340^{b}	0.346^{b}
Casein number	0.786^{a}	0.836^{b}	0.910^{c}	0.926^{d}

[a] Parameters are defined in Section 2. Values within a row are not significantly different ($P > 0.05$) if labelled with the same superscript letter.

4. Conclusion

Our study showed the significant effects of sampling month and month of lactation on the content of the studied milk proteins and the relative content of caseins in total casein. Milk protein, casein, α_{S2}-Cn, κ-Cn, and β-Lg content, and the relative contents of α_{S1}- and κ-Cn in total casein were higher in milk from cows of the Estonian Red breed than those of the Estonian Holstein breed. Higher contents of milk protein, casein and all the studied protein fractions, and the casein number reduced the rennet coagulation time and formed a firmer curd. Milk formed a firmer curd when the proportion of α_{S2}-Cn and β-Cn in total casein was smaller, or the proportion of κ-Cn in total casein was higher. In addition, a higher proportion of κ-Cn with respect to α_{S1}-Cn and β-Cn assisted in forming a firmer curd.

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Wedholm, A., Larsen, L. B., Lindmark-Månssom, H., Karlsson, A. H., & Andrén, A. (2006). Effect of protein composition on the cheese-making properties of milk from individual dairy cows. *Journal of Dairy Science, 89*, 3296–3305.

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CURRICULUM VITAE

First name: Ivi
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Date of birth: 16 June 1970

Employment: Estonian University of Life Sciences,
Institute of Veterinary Medicine and Animal Sciences,
Department of Animal Nutrition,
Laboratory of Milk Quality Research
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Academic degree: Master's degree (MSc) in chemical biology.
Thesis: „Factors affecting milk mineral content".
Estonian Agricultural University, 2002.

Education:

2002–2008	Estonian Agricultural University / Estonian University of Life Sciences, doctoral studies
2000–2002	Estonian Agricultural University, master studies
1992–1993	University of Tartu Department of Pedagogy, profession of teacher of chemistry and mathematics
1988–1992	University of Tartu, Faculty of Physics and Chemistry, BSc in chemistry
1985–1988	Tartu 5th Secondary School
1977–1985	Tartu 8th Secondary School

Foreign languages: English, Finnish, Russian, German

Professional employment:

Since 2006	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Science, Department of Animal Nutrition, Laboratory of Milk Quality Research, researcher
2000–2005	Estonian Agricultural University Institute of Animal Sciences, Laboratory of Milk Quality Research, laboratory assistant
1994–2000	Maternity leave

1993–1994 Ülenurme Gymnasium (Ülenurme grammar school), teacher of chemistry.

Research interests:

Factors influencing milk composition. Milk proteins and their effect on milk technological properties. Milk rennet coagulation properties.

Target financed (SF) projects and grants of the Estonian Science Foundation (ESF):

2002–2006 Economical and biological modelling of sustainable strategies of animal breeding and production SF0422102s02

2003–2007 Feeding of dairy cows in different periods of energy balance SF0422595s03

2008– Cattle health and welfare - aspects of precision livestock farming SF0170165s08

2001–2004 The effect of different types of milking systems, technical parameters and methods of usage on the quality of raw milk, ESF grant No 4823

Other projects:

2000–2005 Ministry of Agriculture of Estonia „Determination of maximum milk productivity of Estonian breeds of cattle"

2004–2007 Ministry of Agriculture of Estonia „Improvement of raw milk coagulation properties and lowering somatic cell count"

2005– Bio-Competence Centre of Healthy Dairy Products, projects 1.1. „Improvement of raw milk coagulation properties" and 1.2. „Designing of milk fatty acid composition"

Professional training:

2007, 24–26.01. Winter Academy: „Teaching at university", University of Tartu

2006, Oct-Dec Estonian University of Life Science, intensive English language course for advanced students

2005, 13.12 „Composition of presentation", management centre N.O.R.T.

2005, 9–16.06.	NOVA course: „Genes and environment", Swedish University of Agricultural Sciences
2004, 20–24.09.	NOVA course: „Milk composition – functional genomics", Swedish University of Agricultural Sciences
2004, 16–27.08.	NOVA course: „The design and optimisation of animal breeding strategies" The Royal Veterinary and Agricultural University (Denmark)
2004, 24–28.05.	NOVA course: „Monitoring of quality parameters in food by non-destructive in-line measurement methods", Agricultural University of Norway
2004, April	Estimation of measurement uncertainties in chemistry laboratory. University of Tartu, Testing Centre
2003, 4–15.08.	NOVA course: „Quantitative genetics in animal breeding" University of Helsinki
2003, Jan.	Practical introduction into methods of instrumental analysis. University of Tartu, Testing Centre
2002, Jan.	Pedagogy of higher educational institutions. University of Tartu, Faculty of Education

Awards:

2003	Laureate of De'Laval research scholarship, II level
1994	Award of Estonian Ministry of Culture and Education from contest of pedagogy research

CURRICULUM VITAE

Eesnimi: Ivi
Perekonnanimi: Jõudu
Sünniaeg: 16. juuni, 1970

Töökoht: Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, söötmisosakond, piima kvaliteedi uurimise labor
Kreutzwaldi 48, Tartu 51006;
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Teaduskraad: põllumajandusteaduste magister keemilise bioloogia erialal. Väitekiri: „Mineraalained piimas ja nende sisaldust mõjutavatest teguritest". Eesti Põllumajandusülikool, 2002

Hariduskäik:

2002–2008	Eesti Põllumajandusülikool / Eesti Maaülikool, doktorantuur
2000–2002	Eesti Põllumajandusülikool, magistrantuur
1992–1993	Tartu Ülikool, pedagoogikateaduskond, keemia ja põhikooli matemaatika õpetaja
1988–1992	Tartu Ülikool, füüsika ja keemia teaduskond, diplom keemia erialal
1985–1988	Tartu 5. Keskkool
1977–1985	Tartu 8. Keskkool

Võõrkeelte oskus: inglise, soome, vene, saksa

Teenistuskäik:

alates 2006	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, söötmisosakond, piima kvaliteedi uurimise labor, erakorraline teadur
2000–2005	Eesti Põllumajandusülikool, loomakasvatuse instituut, piima kvaliteedi uurimise labor, vanemlaborant
1994–2000	lapsepuhkusel
1993–1994	Ülenurme Gümnaasium, keemia õpetaja.

Uurimistöö põhisuunad:

Piima koostist mõjutavad tegurid. Piima valguline koostis ja selle mõju tehnoloogilistele omadustele. Piima laapumisomadused.

Projektid:

2008–	Veiste tervise ja heaolu uurimine täppispidamise aspektist, SF0170165s08
2005–	OÜ Tervisliku Piima Biotehnoloogiate Arenduskeskus, projektid 1.1. „Piima valgulise koostise kujundamine" ja 1.2. „Piima rasvhappelise koostise disainimine"
2004–2007	Eesti Vabariigi Põllumajandusministeerium, „Toorpiima laapumisomaduste parandamine ja somaatiliste rakkude arvu vähendamine"
2003–2007	Lehmade söötmine erinevatel energiabilansi perioodidel, SF0422595s03
2002–2006	Loomakasvatuse produktsiooni ja aretuse säästlike strateegiate majanduslik ja bioloogiline modelleerimine, SF0422102s02
2001–2004	Lüpsisüsteemi eri tüüpide tehniliste parameetrite ja kasutusvõtete mõju toorpiima kvaliteedile, ETF grant nr 4823
2000–2005	Eesti Vabariigi Põllumajandusministeerium, „Eesti veisetõugude maksimaalse piimajõudluse väljaselgitamine"

Erialane enesetäiendus:

2007, 24.–26.1.	talveakadeemia: „Õpetamine kõrgkoolis", Tartu Ülikool
2006, okt-dets	inglise keele intentsiivkursus edasijõudnutele (kood AU-113), Eesti Maaülikooli avatud ülikool
2005, 13.12.	„Esitluse koostamine", õppekeskus N.O.R.T.
2005, 9.–16.6.	NOVA kursus: „Genes and environment", Rootsi Põllumajandusülikool
2004, 20.–24.9.	NOVA kursus: „Milk composition – functional genomics", Rootsi Põllumajandusülikool
2004, 16.–27.8.	NOVA kursus: „The design and optimisation of animal breeding strategies", Kuninglik Veterinaaria ja Põllumajanduse Ülikool (Taani)

2004, 24.–28.5.	NOVA kursus: „Monitoring of quality parameters in food by non-destructive in-line measurement methods", Norra Põllumajandusülikool
2004, aprill	Mõõtemääramatuse hindamine keemialaboris: baaskursus. Tartu Ülikool, katsekoda
2003, 4.–15.8.	NOVA kursus: „Quantitative genetics in animal breeding" Helsingi Ülikool
2003, jaan	Praktiline sissejuhatus instrumentaalanalüüsi meetoditesse. Tartu Ülikool, katsekoda
2002, jaan	Kõrgkooli pedagoogika kursus, Tartu Ülikool, haridusteaduskond

Tunnustused:

2003	De'Lavali II taseme uurimistöö stipendiumi laureaat
1994	Kultuuri- ja Haridusministeeriumi preemia kasvatusteaduslike tööde konkursil

LIST OF PUBLICATIONS

1.1. Publications indexed in the ISI Web of Science database:

Jõudu, I., Henno, M., Kaart, T., Püssa, T., Kärt, O. 2008. The effect of milk proteins' contents on the rennet coagulation properties of milk from individual dairy cows. *International Dairy Journal*, 18(9), 967-970 [in press].

Jõudu, I., Henno, M., Värv, S., Kaart, T., Kalamees, K., Kärt, O. 2007. Milk protein genotypes and milk coagulation properties of Estonian Native cattle. *Agricultural and Food Science*, 16, 222-231.

Henno, M., Ots, M., **Jõudu, I.**, Kaart, T., Kärt, O. 2008. Factors affecting the freezing point stability of milk from individual cows. *International Dairy Journal*, 18. 210-215.

Kübarsepp, I., Henno, M., Kärt, O., Tupasela, T. 2005. A comparison of the methods for determination of the rennet coagulation properties of milk. *Acta Agriculturae Scandinavica, Section A - Animal Science,* 55(4), 145-148.

1.2. Papers published in other peer-reviewed international journals with a registered code:

Rihma, E., Kärt, O., Mihhejev, K., Henno, M., **Jõudu, I.**, Kaart, T. 2007. Effect of dietary live yeast on milk yield, composition and coagulation properties in early lactation of Estonian holstein cows. *Agraarteadus*, 18(1), 37-41.

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D. 2005. Effect of k-casein and ß-lactoglobulin genotypes on the milk rennet coagulation properties. *Agronomy Research*, 3(1), 55-64.

1.3. Papers in Estonian and in other peer-reviewed research journals with a local editorial board:

Kübarsepp, I., Henno, M., Mihhejev, K., Kärt, O., Samarütel, J., Ling, K., Kaart, T. 2003. Piima laapumist mõjutavad tegurid. *Agraarteadus*, 14(2), 84-95.

Kübarsepp, I., Henno, M., Kärt, O., Kaart, T. 2002. Eesti veisetõugude piima kaltsiumi- ja fosforisisaldused ning neid mõjutavad faktorid. *Agraarteadus*, 13(3), 162-175.

Kübarsepp, I., Kärt, O., Henno, M. 2002. Söödaratsiooni kaaliumi- ja karbamiidisisalduse mõju piima mineraalainelisele koostisele. *Agraarteadus*, 13(6), 325-330.

3.2. Papers published in proceedings/books by Estonian or foreign publishers not listed in the ISI Web of Proceedings:

Kübarsepp, I. 2002. Piima mineraalainete sekretsioon. *EPMÜ Loomakasvatusinstituudi teadustöid*, 72, 31-39

Olkonen, A., Henno, M., **Kübarsepp, I.** 2002. Eestis toodetud piima ja piimatoodete kaadmiumi- ja pliisisaldusest. *EPMÜ Loomakasvatusinstituudi teadustöid*, 72, 63-69.

3.4. Papers published in the proceedings of international conferences:

Kübarsepp, I., Henno, M., Kaart, T., Pärna, E., Viinalass, H., Sabre, D. 2006. Frequencies of κ-Cn and β-Lg genetic variants among Estonian cattle breeds and their effect on the milk renneting properties. *Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, August 13-18, 2006, Belo Horizonte, Brazil*, Communication No 01 - 65.

Pärna, E., Vallas, M., Kaart, T., **Kübarsepp, I.**, Kiiman, H.; Pärna, K. 2006. Genetic improvement of milk coagulation properties. *Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, August 13-18, 2006, Belo Horizonte, Brazil*, Communication No 01 - 55.

Ots, M., Kärt, O., Henno, M., **Jõudu, I.,** Mihhejev, K., Kuusik, S., Elias, P. 2007. Effect of dietary fat sources on milk and cheese fatty acid composition. *Proceedings of the 13th Baltic Animal Breeding Conference, Pärnu, Estonia, 24-25. May, 2007.* Estonian Agricultural University, 2007, 54-58.

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D. 2006. Frequencies of k-casein and ß-lactoglobulin genotypes among cattle breeds raised in Estonia and their effect on the milk renneting properties. *Proceedings 27th IDF World Dairy Congress,* Conference CD

Kübarsepp, I., Henno, M., Buchberger, J., Biechl, Ch., Kalamees, K. 2005. Milk protein genotypes and milk production of Estonian Native breed.

Proceedings of the 11th Baltic Animal Breeding and Genetics Conference, Palanga, Lithuania, 13-14. May, 2005. Lithuanian Veterinary Academy, 2005, 47-50.

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D., Saveli, O. 2004. Effect of κ-Cn and β-Lg genetic variants on the milk renneting properties on Põlula Research Farm. *Proceedings of the 10th Baltic Animal Breeding Conference "Animal Breeding in the Baltics", Tartu, Estonia, 13-14. May, 2004.* Estonian Agricultural University, 2004, 48-53.

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D., Saveli, O. 2004. Influence of κ-casein and β-lactoglobulin genotypes on the milk coagulation properties. *Proceedings of Research for Rural Development 2004, Latvia, Jelgava, 19-22. May, 2004.* Latvia University of Agriculture, 2004, 14-19.

Kübarsepp, I., Henno, M., Mihhejev, K., Kärt, O. 2003. Factors influencing milk coagulation properties. *Proceedings of Research for Rural Development 2003, Latvia, Jelgava, 21-24. May, 2003.* Latvia University of Agriculture, 2003, 48-52.

Kübarsepp, I., Henno, M., Kärt, O., Kaart, T. 2002. Factors influencing the milk calcium and phosphorus content of dairy cattle breeds raised in Estonia. *Proceedings 26th IDF World Dairy Congress, 24-27. Sept.*, Posters CD

Kübarsepp, I., Henno, M., Kärt, O. 2002. Factors affecting milk calcium and phosphorus content. *Proceedings of Research for Rural Development 2002, Latvia, Jelgava, 22-24. May, 2002.* Latvia University of Agriculture, 2002, 78-82.

3.5. Papers published in the proceedings of Estonian conferences:

Kübarsepp, I., Henno, M., Kärt, O., Karus, A. 2001. Mineraalained piimas. *APSi Toimetised*, (14), 131-134.

5.2. Thesis published in the proceedings of international conferences:

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D., Saveli, O., Kaart, T. 2005. Influence of κ-casein and β-lactoglobulin genotypes on the milk coagulation properties. *Book of Abstracts of the 56th Annual Meeting of the European Association for Animal Production, 5-8. June, 2005, Uppsala, Sweden*, p. 259.

Kübarsepp, I., Henno, M., Mihhejev, K., Kärt, O. 2003. Milk coagulation properties of dairy cattle breeds raised in Estonia and factors influencing it. *Proceedings "Nordic Agriculture in Global Perspective", NJF's 22nd Congress, July1-4, Turku, Finland,* p. 90.

6.3. Popular science articles:

Jõudu, I. 2008. Piima koostis. *Piima kvaliteedist.* Tartu: OÜ Tervisliku Piima Biotehnoloogiate Arenduskeskus, 39-47.

Jõudu, I. 2008. Piima juustusobivus. *Piima kvaliteedist.* Tartu: OÜ Tervisliku Piima Biotehnoloogiate Arenduskeskus, 48-63.

Kübarsepp, I. 2006. Piima kaltsiumi- ja fosforisisaldust mõjutavad tegurid. *Eesti veisetõugude maksimaalse piimajõudluse väljaselgitamine*. Tartu: Triip, 170-180.

Kübarsepp, I., Henno, M., Mihhejev, K. 2006. Piima laapumist mõjutavad tegurid. *Eesti veisetõugude maksimaalse piimajõudluse väljaselgitamine*. Tartu: Triip, 181-195.

Henno, M., **Kübarsepp, I.**, Mihhejev, K., Buchberger, J., Biechl, Ch., Krause, I., Sperrer, I. 2004. Genetische varianten der Milchprotein beim Estnischen Landvieh. *Arche Nova*, 4, 12-13.

Kübarsepp, I. 2002. Piima kaltsiumisisaldus Põlula katselaudas. *Tõuloomakasvatus*, 3, 13-19.