Effects of β - κ -casein (*CSN2-CSN3*) haplotypes, β -lactoglobulin (*BLG*) genotypes, and detailed protein composition on coagulation properties of individual milk of Simmental cows

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ABSTRACT

The aim of this study was to investigate the effects of *CSN2-CSN3* (β - κ -casein) haplotypes, *BLG* (β -lactoglobulin) genotypes, content of milk protein fractions, and protein composition on coagulation properties of milk (MCP). Rennet coagulation time (RCT) and curd firmness (a_{30}) were measured using a computerized renneting meter, and the contents of major milk protein fractions were quantified by reversed-phase HPLC in individual milk samples of 2,167 Simmental cows. Cow genotypes at *CSN2*, *CSN3*, and *BLG* were ascertained by reversed-phase HPLC, and *CSN2-CSN3* haplotype probabilities were estimated for each cow. Phenotypes for MCP were regressed on *CSN2-CSN3* haplotype probabilities using linear models that also included the effects of herd-test-day, parity, days in milk, pH, somatic cell score, renneting meter sensor, sire of the cow, *BLG* genotype, and content of major protein fractions or, alternatively, protein composition. When the statistical model did not account for protein fraction contents or protein composition, haplotypes carrying *CSN3* B were associated with shorter RCT and greater a_{30} compared with those carrying *CSN3* A. Haplotypes carrying *CSN2* B had the effect of decreasing RCT and increasing a_{30} relative to haplotype A²A. When effects of protein fractions content or protein composition were added to the model, no difference across haplotypes due to *CSN3* and *CSN2* alleles was observed for MCP, with the exception of the effect of *CSN2* B on RCT, which remained markedly favorable. Hence, the effect of *CSN3* B on MCP is related to a variation in protein composition caused by the allele-specific expression of κ -casein, rather than to a direct role of the protein variant on the coagulation process. In addition, the favorable effect exerted by *CSN2* B on a_{30} was caused by the increased β -casein content in milk. Conversely, *CSN2* B is likely to exert a direct genetic effect on

RCT, which does not depend upon variation of β -casein content associated with *CSN2* B. Increased RCT was observed for milk yielded by *BLG* BB cows, even when models accounted for protein composition. Rennet clotting time was favorably affected by κ -casein content and percentage of κ -casein to total casein, whereas a_{30} increased when contents and percentages of β -CN and κ -CN increased. Changes of milk protein composition and allele frequency at casein and whey protein genes affect variation of MCP.

Key words: casein haplotype, *BLG* genotype, milk protein composition, coagulation property

INTRODUCTION

Coagulation ability of milk is influenced by several factors including genetic polymorphisms of milk proteins (Schaar et al., 1985; Mayer et al., 1997; Ikonen et al., 1999b). Rennet clotting time (RCT) and curd firmness (a_{30}) have also been affected by milk protein composition (Wedholm et al., 2006; Jōudo et al., 2008). Previous studies investigated the effects of milk protein polymorphisms on coagulation properties (Buchberger and Dovč, 2000) and on protein composition (Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010). Because simultaneous assessment of all major milk protein contents is difficult and time-consuming when the number of samples is large, the effect of protein composition on milk coagulation properties (MCP) has rarely been investigated, and existing studies have been carried out on bulk milk samples (Walsh et al., 1998) or on limited numbers of animals (Marziali and Ng-Kwai-Hang, 1986; Wedholm et al., 2006).

Significant associations between *CSN3* (κ -CN) B and enhanced MCP (van den Berg et al., 1992; Lodes et al., 1996b; Walsh et al., 1998) have been reported. These studies did not clarify whether detected associations between MCP and genotypes at milk protein loci were attributable to different structural properties of protein variants or to variation of protein fraction content or protein composition or both related to genetic polymorphisms of milk proteins. Because overexpression of *CSN3*

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B compared with *CSN3* A has been reported (Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010), effects of *CSN3* on MCP might result from differences in CN content of milk or κ -CN to total CN ratio (i.e., CN composition) across genotypes. Other factors, such as differences in micelle structure (van Eenennaam and Medrano, 1991; Lodes et al., 1996a) or in the structure or molecular properties of the curd, may also contribute to the observed variation of MCP across κ -CN variants. No study has investigated the effects of *CSN3* genotypes on MCP while accounting for variation in milk protein fraction contents or protein composition using a large sample of animals. Moreover, estimation of these effects is difficult for some cattle breeds because of the low frequency of specific genotypes or haplotypes. Compared with other cattle populations, where frequency of some alleles (e.g., *CSN3* B or *CSN2* B) is very high or very low, the Simmental breed exhibits more balanced allele frequencies at the *CSN2*, *CSN3*, and *BLG* loci and a moderate frequency of *CSN2* B (Bonfatti et al., 2010). The aim of this study was to estimate the effects of CN haplotypes, *BLG* genotypes, protein fraction contents, and protein composition on MCP in Italian Simmental cows.

MATERIALS AND METHODS

Animals and Data

Details on milk sample collection and preservation, measures of protein fraction contents and protein composition, assessment of genotypes at *CSN2*, *CSN3*, and *BLG*, and computation of *CSN2-CSN3* haplotype probabilities can be found in the companion paper by Bonfatti et al. (2010). Briefly, individual milk samples of 2,167 Simmental cows, reared in 47 commercial herds in the north of Italy, were collected from November 2007 to December 2008. Samples (1 per animal) were collected during the morning or evening milking of a test-day. Herd and test-day effects were confounded because all cows of a herd were sampled on the same test-day. The Italian Simmental Cattle Breeders Association (ANAPRI, Udine, Italy) supplied milk yield records and pedigree information for all sampled cows.

Contents of α_{S1} -CN, α_{S2} -CN, β -CN, γ -CN, κ -CN, β -LG, and α -LA and genotypes of cows at *CSN2*, *CSN3*, and *BLG* was assessed by reversed-phase HPLC using the method proposed by Bonfatti et al. (2008). Because it was not possible to discriminate *CSN1S1* (α_{S1} -CN) alleles by reversed-phase HPLC, allele frequencies at *CSN1S1* were preliminarily investigated by direct DNA sequencing (Bonfatti et al., 2010) using 200 randomly chosen samples. Because the frequency of *CSN1S1* C

was <6%, sequencing was not extended to all samples and *CSN1S1* was not considered in the definition of CN haplotypes. With such a limited frequency, effects of haplotypes carrying *CSN1S1* C would have been estimated with limited reliability.

Protein Composition

After quantification of milk protein fractions by HPLC, total CN content was defined as the sum of α_{S1} -CN, α_{S2} -CN, β -CN, γ -CN, and κ -CN contents. Total whey protein content was calculated as the sum of α -LA and β -LG contents. Total protein content was expressed as the sum of CN and whey protein content. Protein composition (i.e., relative content of protein fractions) was measured as percentage of α_{S1} -CN, α_{S2} -CN, β -CN, γ -CN, and κ -CN to total CN and as percentage of β -LG to total whey protein. These measures of protein composition were preferred over those quantifying the relative content of each fraction in total protein because they ensured that variation in casein number associated with some haplotypes did not affect estimation of effects.

Analysis of MCP

Measures of MCP of individual milk samples were obtained using a computerized renneting meter (model CRM-48, Polo Trade, Monselice, Italy) within 3 h after sample collection. The principle of the computerized renneting meter is based on the swing of a pendulum driven by an electromagnetic field. During the milk coagulation process, differences in the electromagnetic field are recorded. After the addition of the clotting enzyme, coagulation takes place and the swing of the pendulum becomes smaller: the greater the extent of coagulation, the smaller the pendulum swing. The analysis produces a diagram as reported by Dal Zotto et al. (2008).

Milk samples (10 mL) were heated at 35°C, and 200 μ L of rennet (Hansen standard 160 with 80% chymosin and 20% of pepsin, Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% in distilled water was added to milk. Measurement of MCP ended within 31 min after the addition of the clotting enzyme to samples. This analysis provided measurements of RCT (the time interval in minutes from the addition of the clotting enzyme to the beginning of the coagulation process) and a_{30} (the width, in millimeters, of the diagram at 31 min after the addition of rennet, which is a measure of curd firmness). Samples that did not coagulate within 31 min were classified as noncoagulating milk. These samples were not considered in the statistical analysis

because of missing information on RCT. Measures of pH (pH-Burette 24, Crison, Barcelona, Spain) were obtained before measurement of MCP.

Statistical Analysis

The effects of CN haplotypes and *BLG* genotypes on phenotypic variation of MCP were estimated using 4 linear mixed models with the MIXED procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). The first model (model M1) was

$$y_{ijklmnopq} = \text{HTD}_i + P_j + \text{DIM}_k + \text{SCS}_l + S_m + \text{pH}_n + \sum_{a=1}^h \beta_a \text{PR}_{a:ijklmnopq} + \text{BLG}_o + \text{sire}_p + e_{ijklmnopq},$$

where $y_{ijklmnopq}$ is a measure of RCT or a_{30} ; HTD_i is the fixed effect of herd-test-day i ($i = 1, \dots, 47$); P_j is the fixed effect of parity j of the cow ($j = 1$: first parity, $j = 2$: second parity, $j = 3$: third parity, $j = 4$: fourth and later parities); DIM_k is the fixed effect of DIM class k (12 classes of 30-d intervals, with the exception of the last class, which included samples collected at DIM 330 or greater); SCS_l is the fixed effect of class l of SCS, computed as $\log_2(\text{SCC} \times 10^3) + 3$ (6 classes of 1-point intervals, with the exception of the last class, which included records with $\text{SCS} \geq 5$); S_m is the fixed effect of the renneting meter sensor m ($m = 1, \dots, 10$); pH_n is the fixed effect of pH class n (12 classes of 0.02-point intervals, except for the first class, which included records with $\text{pH} \leq 6.62$ and the last class, which included records with $\text{pH} \geq 6.82$); h is the total number of haplotypes evaluated ($h = 8$); $\text{PR}_{a:ijklmnopq}$ is the probability that the cow carried haplotype a ($a = 1, \dots, 8$), expressed as the expected number of copies of the haplotype carried by the animal; β_a is the regression coefficient on the probability of haplotype a ; BLG_o is the fixed effect of *BLG* genotype o ($o = 1$: AA, $o = 2$: AB, $o = 3$: BB); sire_p is the random effect of the sire of the cow ($p = 1, \dots, 210$) assumed to follow a normal distribution with $\text{sire}_p \sim N(\mathbf{0}, \mathbf{A}\sigma_s^2)$, where σ_s^2 is the sire variance and $\mathbf{A}$ is the additive relationship matrix among sires; and $e_{ijklmnopq}$ is a random residual assumed to follow a normal distribution with $e_{ijklmnopq} \sim N(0, \sigma_e^2)$, where σ_e^2 is the residual variance.

Milk coagulation occurs through an enzymatic reaction and coagulation properties are markedly affected by acidity of milk that can be more or less close to the optimum for rennet enzyme. Because this study aimed to investigate the role of protein composition in milk coagulation, the effect of milk pH class was included in the statistical models to estimate effects of protein

composition on MCP while accounting for variation in enzyme efficiency related to milk acidity.

A probability (ranging from 0 to 1) was assigned to each possible haplotype carried by an animal. For example, if 2 copies of the same haplotype composed the only possible haplotype pair, that haplotype gained a final value of 2 because the animal was expected to carry 2 copies of the haplotype, each one with a probability of 1. The difference between the regression coefficients for 2 haplotypes of interest estimates the expected change in the phenotype of a given trait when a copy of the second haplotype replaces a copy of the first haplotype. Likewise, in the hypothetical situation in which 2 animals differ by one haplotype only, the difference between the estimated coefficients would correspond to the expected difference in the phenotype of the 2 animals for a given trait (Boettcher et al., 2004). Only additive effects of haplotypes were estimated and dominance effects were not considered.

To assess whether there was an interaction between alleles at *CSN2* and *CSN3*, hypothesis tests for linear combinations of the regression coefficients on haplotype probabilities were carried out. To evaluate the effects of CN haplotypes and *BLG* genotypes while accounting for variation in content of protein fractions or in protein composition, alternative models were considered. In the second model (model M2), the linear effects due to variation in content (g/L) of α_{S1-} , α_{S2-} , β -, and κ -CN, and β -LG were added to effects in M1. In the third model (model M3), the linear effects due to variation in total protein content (g/L) of milk and in content (g/L) of protein fractions were added to effects accounted for by M1. The last model (model M4) included linear regressions on percentage of α_{S1-} , α_{S2-} , β -, and κ -CN to CN and of β -LG to total whey protein. In models M2, M3, and M4, additional effects were included as covariates.

In these models, variation in protein composition and its effects on MCP were accounted for in 2 ways: by including linear regressions on content (g/L) of protein fractions and of total milk protein (model M3) and by including linear regressions on percentages (i.e., relative content) of CN fractions to CN and of β -LG to total whey protein (model M4). In model M3, effects of content of protein fractions were estimated at a constant total milk protein content and, for this, are comparable to effects caused by variation in the relative content of CN fractions and β -LG. This model aimed to estimate haplotype effects on MCP while taking into account possible effects of haplotypes on variation of total milk protein content.

Sire effects were included in the model to account for variation due to polygenes underlying the investigated traits. Although there were multiple daughter records

Table 1. Estimated effects of *CSN2-CSN3* (β - κ -CN) haplotypes on rennet clotting time (min $\pm$ SE) obtained using different statistical models

Haplotype	Statistical model ¹			
	M1	M2	M3	M4
A ² A	0	0	0	0
A ¹ A	-0.646 $\pm$ 0.200**	-0.372 $\pm$ 0.213†	-0.030 $\pm$ 0.234	-0.225 $\pm$ 0.232
BA	-2.442 $\pm$ 0.209***	-2.474 $\pm$ 0.332***	-2.610 $\pm$ 0.334***	-2.409 $\pm$ 0.334***
IA	0.100 $\pm$ 0.626	0.111 $\pm$ 0.623	0.081 $\pm$ 0.623	0.162 $\pm$ 0.623
A ¹ B	-1.339 $\pm$ 0.289***	-0.065 $\pm$ 0.387	0.415 $\pm$ 0.412	0.259 $\pm$ 0.419
A ² B	-0.822 $\pm$ 0.174***	0.108 $\pm$ 0.265	0.180 $\pm$ 0.266	0.221 $\pm$ 0.270
BB	-3.036 $\pm$ 0.359***	-1.990 $\pm$ 0.522***	-2.023 $\pm$ 0.523***	-1.888 $\pm$ 0.509***
IB	-1.128 $\pm$ 0.325***	-0.402 $\pm$ 0.392	-0.247 $\pm$ 0.393	-0.242 $\pm$ 0.390

¹Model M1 included the fixed effects of herd-test-day, parity, DIM, SCS and pH class, renneting meter sensor, *BLG* (β -LG) genotype, regressions on *CSN2-CSN3* haplotype probabilities, and the random effect of the sire of the cow. In addition, model M2 included the regressions on α_{S1-} , α_{S2-} , β -, κ -CN, and β -LG contents (g/L); model M3 included the regressions on contents (g/L) of α_{S1-} , α_{S2-} , β -, κ -CN, and β -LG, and total protein; and model M4 included the regressions on percentages of α_{S1-} , α_{S2-} , β -, κ -CN to CN and of β -LG to total whey protein. In models M2, M3, and M4, these additional effects were included as covariates. Estimates quantify the expected change of rennet clotting time due to the replacement of 1 copy of haplotype A²A with 1 copy of another haplotype.

† $P < 0.10$; ** $P < 0.01$; *** $P < 0.001$.

per sire taken at different stages of lactation, a random regression model was not applicable because most sire families were of limited size.

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics for contents of protein fractions and protein composition, as well as genotype, allele, and haplotype frequencies for data used in this study, can be found in Bonfatti et al. (2010). In total, 6.3% of samples were noncoagulating milk, which was in agreement with literature findings, indicating that presence of noncoagulating samples varied across cattle breeds: 13.2% (Ikonen et al., 2004) and 8.6% (Tyrisevä et al., 2004) in Finnish Ayrshire, 1.3% in Finnish Holstein (Tyrisevä et al., 2004), 9.7% in Italian Holstein-Friesian (Cassandro et al., 2008) and 4% in Italian Brown Swiss (Cecchinato et al., 2009). These samples were excluded from the statistical analysis because RCT was unknown. Mean (SD) of RCT and a_{30} for samples that coagulated was 16.5 (4.6) min and 29.1 (7.5) mm, respectively. Measures of RCT and a_{30} ranged from 4 to 30.1 min and from 5 to 46 mm, respectively. Because standard procedures for calibration of the computerized renneting meter are lacking, comparison of average RCT and a_{30} obtained in this study with those reported in other investigations is not appropriate.

Effects of CN Haplotypes on MCP

Estimated effects of CN haplotypes on MCP are reported in Tables 1 and 2 for RCT and a_{30} , respectively.

When effects of milk protein composition and major protein fraction contents were not accounted for by the model (model M1), *CSN3* exhibited a marked effect on MCP. Haplotypes carrying *CSN3* B were associated ($P < 0.001$) with shorter RCT and greater a_{30} compared with those carrying *CSN3* A. This result is in agreement with results obtained in several studies that investigated *CSN3* genotype effects (van den Berg et al., 1992; Lodes et al., 1996a; Walsh et al., 1998).

When the statistical model accounted for variation in protein fractions content or protein composition (models M2, M3 and M4), no difference ($P > 0.05$) across haplotypes attributable to *CSN3* was observed for RCT and a_{30} . Likewise, Marziali and Ng-Kwai-Hang (1986) reported that estimated effects of *CSN3* genotypes on MCP were trivial when variation caused by CN composition was accounted for in the statistical analysis, although reliability of their results was, to some extent, jeopardized by the limited size of the investigated sample. Our findings indicate that the favorable effects exerted by *CSN3* B on MCP are to be attributed to modifications of protein composition caused by the differential expression of the allele and not to a direct effect of the specific protein genetic variant on the coagulation process. Likely, the decreased CN micelle size associated with κ -CN B is also to be attributed to the increased content of κ -CN associated with *CSN3* B. A greater κ -CN content ensures a wider hydrophilic surface area and, as a consequence, a greater area of the micelle surface (Niki et al., 1994; Walsh et al., 1998). Therefore, the shortened RCT exhibited by κ -CN B milk compared with κ -CN A milk might be the outcome of a lower critical level of κ -CN hydrolysis required for the onset of gelation. The increased a_{30} associated

Table 2. Estimated effects of *CSN2-CSN3* (β - κ -CN) haplotypes on curd firmness (mm $\pm$ SE) obtained using different statistical models

Haplotype	Statistical model ¹			
	M1	M2	M3	M4
A ² A	0	0	0	0
A ¹ A	0.747 $\pm$ 0.352*	0.606 $\pm$ 0.363†	0.013 $\pm$ 0.399	-0.438 $\pm$ 0.405
BA	1.848 $\pm$ 0.368***	-0.131 $\pm$ 0.570	-0.082 $\pm$ 0.572	0.858 $\pm$ 0.585
IA	-1.320 $\pm$ 1.116	-1.891 $\pm$ 1.074†	-1.797 $\pm$ 1.071†	-1.448 $\pm$ 1.098
A ¹ B	2.196 $\pm$ 0.511***	0.272 $\pm$ 0.661	-0.595 $\pm$ 0.703	-1.355 $\pm$ 0.732†
A ² B	1.832 $\pm$ 0.307***	0.101 $\pm$ 0.453	-0.044 $\pm$ 0.453	0.049 $\pm$ 0.471
BB	4.540 $\pm$ 0.638***	0.184 $\pm$ 0.898	0.223 $\pm$ 0.895	1.471 $\pm$ 0.894
IB	3.163 $\pm$ 0.575***	0.632 $\pm$ 0.670	0.397 $\pm$ 0.671	0.969 $\pm$ 0.681

¹Model M1 included the fixed effects of herd-test-day, parity, DIM, SCS and pH class, renneting meter sensor, *BLG* (β -LG) genotype, regressions on *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2 included the regressions on α_{S1^-} , α_{S2^-} , β -, κ -CN, and β -LG contents (g/L); model M3 included the regressions on contents (g/L) of α_{S1^-} , α_{S2^-} , β -, κ -CN, and β -LG, and total protein; and model M4 included the regressions on percentages of α_{S1^-} , α_{S2^-} , β -, κ -CN to CN and of β -LG to total whey protein. In models M2, M3, and M4, these additional effects were included as covariates. Estimates quantify the expected change of curd firmness due to the replacement of 1 copy of haplotype A²A with 1 copy of another haplotype.

† $P < 0.10$; * $P < 0.05$; *** $P < 0.001$.

with *CSN3* B is likely attributable to a smaller micelle size allowing for more compact arrangements of para-CN micelles and an increased number of intermicellar bonds per unit of surface area (Horne, 1998). Because structural changes occurring in κ -CN B compared with κ -CN A are all located in the C-terminal part of the protein (Ile₁₃₆ and Ala₁₄₈), which is split off during the enzymatic coagulation phase, these changes are unlikely to have effects on the aggregation process and MCP. The effect of *CSN3* B on a_{30} was more favorable when associated with *CSN2* I than when associated with other *CSN2* alleles. This effect was smaller when the model accounted for protein composition, but it still induced a significant increase in a_{30} .

In model M1, *CSN2* exhibited important effects on MCP. Haplotypes carrying *CSN2* B had favorable effects on RCT (ranging from -2.44 to -3.04 min) and a_{30} (ranging from 1.85 to 4.54 mm) compared with haplotype A²A. The effect of *CSN2* B on RCT remained markedly favorable when accounting for variation in content of protein fractions or protein composition, and an interaction between *CSN2* B and *CSN3* A, which decreased RCT, was observed. The effects of haplotype BB estimated with models M2, M3, and M4 were smaller than those obtained with model M1. This difference is to be ascribed to the accounting of variation in κ -CN content associated with *CSN3* alleles. Differential expression has also been reported for *CSN2* alleles. Allele *CSN2* B has been associated with increased β -CN content in milk (Bonfatti et al., 2010), but it is likely to exert a direct effect on RCT, which does not depend upon variation in β -CN content. Presence of 2 positively charged amino acid residues (Arg₁₂₂ and His₆₇) and the resulting increased net charge of β -CN B, which

exhibits 1 or 2 additional net positive charges compared with β -CN A¹ or A², respectively, are possible causes for direct effects of *CSN2* B on MCP. Compared with CN micelles containing more negatively charged protein variants, repulsive forces between micelles containing β -CN B, in which amino acid substitution results in a decreased negative charge, seem to be weaker (McLean et al., 1984). Consequently, a greater proportion of β -CN B in CN micelles facilitates aggregation.

The favorable effect of *CSN2* B on a_{30} observed with model M1 was not confirmed by models accounting for protein fraction contents or protein composition (models M2, M3, and M4), indicating that effect of *CSN2* B originated from the increased β -CN content of milk.

With model M1, an interaction effect on MCP, involving *CSN2* and *CSN3* genes, was detected. In particular, *CSN2* A¹, relative to the more common A², exerted a positive effect on both RCT and a_{30} when associated with *CSN3* A. When effects due to associated variation in CN composition were accounted for by the model, this interaction effect was somewhat reduced because MCP were adjusted for the proportion of κ -CN in CN (Heck et al., 2009; Bonfatti et al., 2010).

Results obtained in this study for the effect of *CSN2* on MCP partly agree with those reported by Marziali and Ng-Kwai-Hang (1986), who concluded that, after adjustments for concentration of major milk protein fractions, MCP did not differ across β -CN phenotypes. That study compared A¹ and A² alleles only because no animal carried *CSN2* B.

Effects exerted by *CSN2* I on MCP have never been investigated in previous studies and they might be of interest because *CSN2* I is common in other dairy cattle populations such as Italian Holstein-Friesian and Jersey

Table 3. Estimated additive and dominance deviations at *BLG* (β -LG) locus for rennet clotting time (min $\pm$ SE) and curd firmness (mm $\pm$ SE) obtained using different statistical models

Trait ²	Statistical model ¹			
	M1	M2	M3	M4
Rennet clotting time				
Additive deviation	0.517 $\pm$ 0.116***	0.706 $\pm$ 0.142***	0.674 $\pm$ 0.142***	0.414 $\pm$ 0.129**
Dominance deviation	-0.151 $\pm$ 0.152	-0.079 $\pm$ 0.154	-0.099 $\pm$ 0.154	-0.224 $\pm$ 0.156
Curd firmness				
Additive deviation	0.384 $\pm$ 0.203†	-0.330 $\pm$ 0.242	-0.260 $\pm$ 0.242	0.584 $\pm$ 0.225**
Dominance deviation	0.490 $\pm$ 0.271†	0.255 $\pm$ 0.265	0.292 $\pm$ 0.264	0.569 $\pm$ 0.274*

¹Model M1 included the fixed effects of herd-test-day, parity, DIM, SCS and pH class, renneting meter sensor, *BLG* (β -LG) genotype, regressions on *CSN2-CSN3* (β - κ -CN) haplotype probabilities and the random effect of the sire of the cow. In addition, model M2 included the regressions on α_{S1-} , α_{S2-} , β -, κ -CN, and β -LG contents (g/L); model M3 included the regressions on contents (g/L) of α_{S1-} , α_{S2-} , β -, κ -CN, and β -LG, and total protein; and model M4 included the regressions on percentages of α_{S1-} , α_{S2-} , β -, κ -CN to CN and of β -LG to total whey protein. In models M2, M3, and M4, these additional effects were included as covariates.

²Additive deviation was computed as $0.5 \times (\hat{\beta}_{BLG BB} + \hat{\beta}_{BLG AA})$, where $\hat{\beta}_{BLG BB}$ and $\hat{\beta}_{BLG AA}$ are model solutions for *BLG* BB and AA genotypes, respectively; dominance deviation was computed as $\hat{\beta}_{BLG AB} - 0.5 \times (\hat{\beta}_{BLG BB} + \hat{\beta}_{BLG AA})$, where $\hat{\beta}_{BLG AB}$ is the model solution for *BLG* AB genotype.

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Jann et al., 2002). Although frequency of haplotypes containing *CSN2* I was low, estimated effects indicate that the allele had no influence on RCT when compared with A², but affected a₃₀ unfavorably ($P < 0.001$) when associated with *CSN3* A. Biochemical mechanisms responsible for this effect are currently unknown.

A favorable effect of *CSN1S1* C on MCP has been reported (Buchberger and Dovč, 2000). Hence, neglecting *CSN1S1* in CN haplotype definition might have slightly biased the haplotype effect estimate. Also, estimated effects of *CSN3* A might be slightly biased because they are the combined effects of *CSN3* A and E.

Effect of *BLG* Genotypes on MCP

Estimated additive and dominance deviations at *BLG* for RCT and a₃₀ are reported in Table 3. As expected, the magnitude of these deviations was small relative to the effects of CN genes because whey proteins are not directly involved in the milk coagulation process. In agreement with Ikonen et al. (1999a), additive deviations were positive for RCT indicating that *BLG* B was associated with increased RCT, even when models accounted for detailed protein composition. Causes for the unfavorable effect of *BLG* B on RCT are currently unknown. In the literature, results for the effect of *BLG* genotypes on RCT are controversial: some authors detected an effect of *BLG* genotypes on RCT (Ikonen et al., 1999a; Ng-Kwai-Hang et al., 2002; Kübarsepp et al., 2005), whereas others reported no effect (Pagnacco and Caroli, 1987; Ikonen et al., 1997). It has been suggested (Lunden et al., 1997; Bobe et al., 1999) that the favorable effect of *BLG* B on RCT detected in some studies

might be ascribed to the increased total CN content and CN number associated with *BLG* B. Nevertheless, inconsistent results have been obtained for the association between RCT and CN content or casein number (Ikonen et al., 2004), which might explain controversial reports for the effects of *BLG* genotypes on RCT.

The estimated additive deviation at *BLG* was slightly positive for a₃₀ when the statistical model (model M1) did not account for protein fraction contents or when effects due to variation in CN and whey protein composition were accounted for (model M4). In agreement with previous studies reporting positive effects of *BLG* B on CN content (Lunden et al., 1997) and cheese yield (Schaar et al., 1985; van den Berg et al., 1992; Wedholm et al., 2006), this result might be ascribed to the increased CN content associated with *BLG* B. Results obtained in this study with models accounting for variation in protein fraction concentration (model M2) or total protein content (model M3) support this hypothesis.

Results of our study partly agree with Marziali and Ng-Kwai-Hang (1986), who reported decreased RCT and enhanced a₃₀ at cutting for β -LG AA milk relative to β -LG AB and BB phenotypes. In that study, the statistical model accounted for CN content, indicating that effects exerted by *BLG* genotypes on MCP were not interpretable as indirect effects because of variation in milk protein content.

Except for the estimate for a₃₀ obtained with models M1 and M4, dominance deviations were trivial, indicating no significant deviations of *BLG* heterozygous genotypes from the mean of homozygous genotypes.

Table 4. Estimated effects of major protein fractions content (models M2 and M3) or percentage (model M4) to total casein (α_{S1} -, α_{S2} -, β -, and κ -CN) and to total whey protein (β -LG) for rennet clotting time (min·SD⁻¹ ± SE) obtained using different statistical models

Protein fraction	Statistical model ¹		
	M2	M3	M4
α_{S1} -CN	0.129 ± 0.188	-1.291 ± 0.374***	-0.474 ± 0.320
α_{S2} -CN	0.341 ± 0.125**	-0.311 ± 0.220	0.006 ± 0.109
β -CN	0.160 ± 0.220	-1.284 ± 0.458**	-0.319 ± 0.219
κ -CN	-0.888 ± 0.192***	-1.636 ± 0.286***	-1.050 ± 0.224***
β -LG	0.311 ± 0.159†	-0.167 ± 0.210	-0.262 ± 0.109*

¹All models included the fixed effects of herd-test-day, parity, DIM, SCS and pH class, renneting meter sensor, *BLG* (β -LG) genotype, regressions on *CSN2-CSN3* (β - κ -CN) haplotype probabilities, and the random effect of the sire of the cow. In addition, model M2 included the regressions on α_{S1} -, α_{S2} -, β -, κ -CN, and β -LG content (g/L); model M3 included the regressions on α_{S1} -, α_{S2} -, β -, κ -CN, β -LG, and total protein content (g/L); and model M4 included the regressions on percentages of α_{S1} -, α_{S2} -, β -, and κ -CN to CN and of β -LG to total whey protein. Estimates quantify the expected change of rennet clotting time due to a 1-SD change in the content or percentage of a specific protein fraction.

† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Contribution of Additive Genetic Variance to Total Variance of MCP

Although estimation of genetic parameters for MCP was not a specific aim of the investigation, estimated additive genetic variances indicate that MCP are moderately influenced by polygenic effects. Depending upon the statistical model, estimates of additive genetic variance for RCT and a_{30} ranged from 16.8 to 18.4% and from 9.9 to 11.5% of total variance, respectively. Estimation of genetic parameters for MCP, protein fraction contents, and protein composition will be the objective of future investigations.

Effects of Major Protein Fraction Contents and Protein Composition on MCP

Estimated regression coefficients for the effects of protein fractions content (models M2 and M3) or protein composition (model M4) on RCT and a_{30} are reported in Tables 4 and 5, respectively. These estimates quantify the expected change of RCT or a_{30} occurring when the concentration of a specific protein fraction or its relative content (in casein for CN fractions or in whey protein for β -LG) increases by 1 SD. In the literature, studies on the role of major protein fraction contents and milk protein composition in MCP variation are scarce. In our study, RCT was favorably affected ($P < 0.001$) by increased κ -CN content and κ -CN proportion in CN, whereas a_{30} increased when amounts of κ -CN and β -CN increased both in milk and in CN.

Previous studies (Yun et al., 1982; Storry et al., 1983) reported that RCT was related to proportions of α_S -CN and β -CN in CN and that curd tension significantly increased with β -CN fortification, suggesting an essential role of β -CN for curd hardening. Recently, Van Hekken

and Holsinger (2000) and St-Gelais and Haché (2005) observed that changes in α_S -CN to β -CN content ratio reflect an alteration in the gelation properties of milk. Wedholm et al. (2006) reported that κ -CN content and its ratio to α_{S1} - and β -CN content were significantly lower in poorly coagulating or noncoagulating milk than in milk exhibiting optimal coagulation properties, but, consistent with results of Jōudo et al. (2008), they observed no effect of β -CN content on MCP. Similar to previous reports (McLean et al., 1984; van den Berg et al., 1992; Jōudo et al., 2008), our results indicate that κ -CN concentration plays a role as a source of variation of a_{30} . A possible explanation is that milk with increased κ -CN content exhibits shortened RCT (van den Berg et al., 1992), which leaves more time for curd firming and, as a consequence, enhances a_{30} at cutting. In addition, κ -CN content has been associated with increased CN retention in curd during chymosin-induced coagulation. The effect of κ -CN concentration on CN loss into whey is likely attributable to the association between increased κ -CN content and decreased CN micelle size (Donnelly et al., 1984; Dalgleish et al., 1989). Milk containing small micelles produces gels that better entrap milk constituents compared with milk containing larger micelles, reducing CN losses into whey (Niki et al., 1994; Walsh et al., 1998).

CONCLUSIONS

This study suggests that previously observed variation across CN genotypes in coagulating properties of milk might be largely attributed to differences in content of major protein fractions and protein composition that are known to be associated with milk protein genotypes. Factors affecting content of protein fractions and milk protein composition are also expected to affect

Table 5. Estimated effects of major protein fractions content (models M2 and M3) or percentage (model M4) to total casein (α_{S1} -, α_{S2} -, β -, and κ -CN) and to total whey protein (β -LG) for curd firmness (mm·SD⁻¹ ± SE) obtained using different statistical models

Protein fraction	Statistical model ¹		
	M2	M3	M4
α_{S1} -CN	0.245 ± 0.321	2.210 ± 0.641***	2.704 ± 0.563***
α_{S2} -CN	-0.120 ± 0.214	0.976 ± 0.376**	0.767 ± 0.192***
β -CN	1.799 ± 0.380***	3.980 ± 0.785***	2.724 ± 0.383***
κ -CN	1.644 ± 0.329***	2.933 ± 0.491***	3.063 ± 0.395***
β -LG	-0.700 ± 0.273*	0.137 ± 0.360	0.495 ± 0.191**

¹All models included the fixed effects of herd-test-day, parity, DIM, SCS and pH class, renneting meter sensor, *BLG* (β -LG) genotype, regressions on *CSN2-CSN3* (β - κ -CN) haplotype probabilities, and the random effect of the sire of the cow. In addition, model M2 included the regressions on α_{S1} -, α_{S2} -, β -, κ -CN, and β -LG content (g/L); model M3 included the regressions on α_{S1} -, α_{S2} -, β -, κ -CN, β -LG, and total protein content (g/L); and model M4 included the regressions on percentages of α_{S1} -, α_{S2} -, β -, and κ -CN to CN and of β -LG to total whey protein. Estimates quantify the expected change of curd firmness due to a 1-SD change in the content or percentage of a specific protein fraction.
P* < 0.05; *P* < 0.01; ****P* < 0.001.

processing properties of milk. Although further research is needed to investigate the effect exerted by milk protein composition and CN haplotypes on cheese yield, results obtained in this study suggest that changes in milk protein composition and allele frequency at CN and whey protein loci exert effects on milk coagulation properties. Genetic parameters for content of milk protein fractions and protein composition, as well as their relationships with breeding goal traits of dairy cattle populations, need to be investigated in further studies.

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REFERENCES

Bobe, G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1999. Effect of milk protein genotypes on milk protein composition and its genetic parameter estimates. *J. Dairy Sci.* 82:2797–2804.
Boettcher, P. J., A. Caroli, A. Stella, S. Chessa, E. Budelli, F. Canavesi, S. Ghiroldi, and G. Pagnacco. 2004. Effects of casein haplotypes on milk production traits in Italian Holstein and Brown Swiss cattle. *J. Dairy Sci.* 87:4311–4317.
Bonfatti, V., G. Di Martino, A. Cecchinato, D. Vicario, and P. Carnier. 2010. Effects of β - κ -casein (*CSN2-CSN3*) haplotypes and β -lactoglobulin (*BLG*) genotypes on milk production traits, protein fraction contents, and protein composition of individual milk of Simmental cows. *J. Dairy Sci.* 93:3797–3808.
Bonfatti, V., L. Grigoletto, A. Cecchinato, L. Gallo, and P. Carnier. 2008. Validation of a new reversed-phase high-performance liquid chromatography method for separation and identification of bovine milk protein genetic variants. *J. Chromatogr. A* 1195:101–106.
Buchberger, J., and P. Dovč. 2000. Lactoprotein genetic variants in cattle and cheese making ability. *Food Technol. Biotechnol.* 38(Suppl. 2):91–98.

Cassandro, M., A. Comin, M. Ojala, R. Dal Zotto, M. De Marchi, L. Gallo, P. Carnier, and G. Bittante. 2008. Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. *J. Dairy Sci.* 91:371–376.
Cecchinato, A., M. De Marchi, L. Gallo, G. Bittante, and P. Carnier. 2009. Mid-infrared spectroscopy predictions as indicator traits in breeding programs for enhanced coagulation properties of milk. *J. Dairy Sci.* 92:5304–5313.
Dal Zotto, R., M. De Marchi, A. Cecchinato, M. Penasa, M. Cassandro, P. Carnier, L. Gallo, and G. Bittante. 2008. Reproducibility and repeatability of measures of milk coagulation properties and predictive ability of mid-infrared reflectance spectroscopy. *J. Dairy Sci.* 91:4103–4112.
Dalglish, D. G., D. S. Horne, and A. J. R. Law. 1989. Size-related differences in bovine casein micelles. *Biochim. Biophys. Acta* 991(Suppl. 3):383–387.
Donnelly, W. J., G. P. McNeill, W. Buchheim, and T. C. A. McGann. 1984. A comprehensive study of the relationship between size and protein-composition in natural bovine casein micelles. *Biochim. Biophys. Acta* 789(Suppl. 2):136–143.
Hallén, E., A. Wedholm, A. Andrén, and A. Lundén. 2008. Effect of β -casein, κ -casein and β -lactoglobulin genotypes on concentration of milk protein variants. *J. Anim. Breed. Genet.* 17:791–799.
Heck, J. M. L., A. Schennink, H. J. F. van Valenberg, H. Bovenhuis, M. H. P. W. Visker, J. A. M. van Arendonk, and A. C. M. van Hooijdonk. 2009. Effects of milk protein variants on the protein composition of bovine milk. *J. Dairy Sci.* 92:1192–1202.
Horne, D. S. 1998. Casein interactions: Casting light on the black boxes, the structure in dairy products. *Int. Dairy J.* 8(Suppl. 3):171–177.
Ikonen, T., K. Ahlfors, R. Kempe, M. Ojala, and O. Ruottinen. 1999a. Genetic parameters for the milk coagulation properties and prevalence of non-coagulating milk in Finnish dairy cows. *J. Dairy Sci.* 82:205–214.
Ikonen, T., S. Morri, A. M. Tyrisevä, O. Ruottinen, and M. Ojala. 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content, and pH of milk. *J. Dairy Sci.* 87:458–467.
Ikonen, T., M. Ojala, and O. Ruottinen. 1999b. Associations between milk protein polymorphism and first lactation milk production traits in Finnish Ayrshire cows. *J. Dairy Sci.* 82:1026–1033.
Ikonen, T., M. Ojala, and E. L. Syväoja. 1997. Effects of composite casein and β -lactoglobulin genotypes on renneting properties and composition of bovine milk by assuming an animal model. *Agric. Food Sci. Finl.* 6:283–294.
Jann, O., G. Ceriotti, A. Caroli, and G. Erhardt. 2002. A new variant in exon VII of bovine β -casein gene (*CSN2*) and its distribution

- among European cattle breeds. *J. Anim. Breed. Genet.* 119:65–68.
- Jõudo, I., M. Henno, T. Kaart, T. Püssa, and O. Kärt. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *Int. Dairy J.* 18:964–967.
- Kübarssepp, I., M. Henno, H. Viinalass, and D. Sabre. 2005. Effect of κ -casein and β -lactoglobulin genotypes on the milk renneting coagulation properties. *Agron. Res.* 3(Suppl. 1):55–64.
- Lodes, A., J. Buchberger, J. Aumann, and H. Klostermeyer. 1996a. The influence of genetic variants of milk proteins on the compositional and technological properties of milk: 1. Casein micelle size and the content of non-glycosylated kappa-casein. *Milchwissenschaft* 51:368–373.
- Lodes, A., J. Buchberger, J. Aumann, and H. Klostermeyer. 1996b. The influence of genetic variants of milk proteins on the compositional and technological properties of milk: 2. Rennet coagulation time and firmness of the rennet curd. *Milchwissenschaft* 51:543–547.
- Lundén, A., M. Nilsson, and L. Janson. 1997. Marked effect of betalactoglobulin polymorphism on the ratio of casein to total protein in milk. *J. Dairy Sci.* 80:2996–3005.
- Marziali, A. S., and K. F. Ng-Kwai-Hang. 1986. Effects of milk composition and genetic polymorphism on coagulation properties of milk. *J. Dairy Sci.* 69:1793–1798.
- Mayer, H. K., M. Ortner, E. Tschager, and W. Ginzinger. 1997. Composite milk protein phenotypes in relation to composition and cheesemaking properties of milk. *Int. Dairy J.* 7:305–310.
- McLean, D. M., E. R. Bruce Graham, and R. W. Ponzoni. 1984. Effects of milk protein genetic variants on milk yield and composition. *J. Dairy Res.* 51:531–546.
- Ng-Kwai-Hang, K. F., C. Dodds, M. J. Boland, and M. J. Auldist. 2002. The influence of genetic variants of β -lactoglobulin on gelation speed and firmness of rennet curd. *Milchwissenschaft* 57(Suppl. 5):267–269.
- Niki, R., K. Royama, Y. Sano, and K. Nishinari. 1994. Rheological study on the rennet-induced gelation of casein micelles with different size. *Polym. Gels Netw.* 2(Suppl. 2):105–118.
- Pagnacco, G., and A. Caroli. 1987. Effect of casein and β -lactoglobulin genotypes on renneting properties of milks. *J. Dairy Res.* 54(Suppl. 4):479–485.
- Schaar, J., B. Hansson, and H. E. Pettersson. 1985. Effects of genetic variants of κ -casein and β -lactoglobulin on cheesemaking. *J. Dairy Res.* 52:429–437.
- St-Gelais, D., and S. Haché. 2005. Effect of β -casein concentration in cheese milk on rennet coagulation properties, cheese composition and cheese ripening. *Food Res. Int.* 38:523–531.
- Storry, E. J., A. S. Grandison, D. Millard, A. J. Owen, and G. D. Ford. 1983. Chemical composition and coagulating properties of renneted milks from different breeds and species of ruminant. *J. Dairy Res.* 50:215–229.
- Tyrisevä, A.-M., T. Vahlsten, O. Ruottinen, and M. Ojala. 2004. Noncoagulation of milk in Finnish Ayrshire and Holstein-Friesian cows and effect of herds on milk coagulation ability. *J. Dairy Sci.* 87:3958–3966.
- van den Berg, G., J. T. M. Escher, P. J. de Koning, and H. Bovenhuis. 1992. Genetic polymorphism of κ -casein and β -lactoglobulin in relation to milk composition and processing properties. *Neth. Milk Dairy J.* 46:145–168.
- Van Eenennaam, A. L., and J. F. Medrano. 1991. Differences in allelic protein expression in the milk of heterozygous kappa-casein cows. *J. Dairy Sci.* 74:1491–1496.
- Van Hekken, D. L., and V. H. Holsinger. 2000. Use of cold microfiltration to produce unique β -casein enriched milk gels. *Lait* 80:69–76.
- Walsh, C. D., T. P. Guinee, W. D. Reville, D. Harrington, J. J. Murphy, B. T. O’Kennedy, and R. J. FitzGerald. 1998. Influence of kappa-casein genetic variant on rennet gel microstructure, cheddar cheesemaking properties and casein micelle size. *Int. Dairy J.* 8:707–714.
- Wedholm, A., L. B. Larsen, H. Lindmark-Månsson, A. H. Karlsson, and A. Andrén. 2006. Effect of protein composition on the cheesemaking properties of milk from individual dairy cows. *J. Dairy Sci.* 89:3296–3305.
- Yun, S. E., K. Ohmiya, and S. Shimizu. 1982. Role of β -casein in milk curdling. *Agric. Biol. Chem.* 46(Suppl. 2):443–449.