Genetic variation and effects of candidate-gene polymorphisms on coagulation properties, curd firmness modeling and acidity in milk from Brown Swiss cows

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The aims of this study were to estimate the genetic variation of traditional milk coagulation properties (MCPs), milk acidity, curd firmness (CF) modeled on time (CFt; comprising: RCTeq, rennet coagulation time estimated from the equation; CFP, the asymptotic potential curd firmness; kCF, the curd firming instant rate constant; and kSR, the syneresis instant rate constant) and maximum CF traits (MCF; comprising CFmax, the maximum CF value; and tmax, the time of attainment). Furthermore, we investigated 96 single nucleotide polymorphisms (SNPs) from 54 candidate genes, testing their associations with the above-listed traits. Milk and blood samples were collected from 1271 cows (each sampled once) from 85 herds. Genotyping was performed using a custom Illumina VeraCode GoldenGate approach. A Bayesian linear animal model (including the effects of herd, days in milk, parity and additive polygenic effects) was used to estimate the genetic parameters of the studied traits. The same model with the addition of the SNP genotype effect was used for our association analysis. The heritability estimates of CFt and the MCF traits (RCTeq = 0.258; kCF = 0.230; CFmax = 0.191; tmax = 0.278) were similar to those obtained using traditional MCPs (0.187 to 0.267), except for the lower estimates for CFP (0.064) and kSR (0.077). A total of 13 of the 51 tested SNPs had relevant additive effects on at least one trait. We observed associations between MCPs and SNPs in the genes encoding ATP-binding cassette sub-family G member 2 (ABCG2), chemokine ligand 2 (CCL2), growth hormone 1 (GH1), prolactin (PRL) and toll-like receptor 2 (TLR2). Whereas, CFt and the MCF traits were associated with polymorphisms in the α-s1-casein (CSN1S1), β-casein (CSN2), GH1, oxidized low-density lipoprotein receptor 1 (OLR1), phospholipase C β1 (PLCB1), PRL and signal transducer and activator of transcription 5A (STAT5A) genes.

Keywords: milk coagulation properties, milk acidity, heritability, candidate gene, dairy cow

Imlications

Our analysis of 51 single nucleotide polymorphisms revealed that 13 were significantly associated with one or more of the tested milk traits, which included the traditional milk coagulation properties, curd firmness (CF) modeled on time (CFt), maximum CF (MCF) and acidity. These results suggest that such loci could be useful in gene-assisted selection programs aimed at improving milk technological traits in Brown Swiss cattle.

Introduction

Traditionally, animal production has focused on providing large amounts of food at a low cost. More recently, however, there has been a growing interest in the global quality and technological aspects of livestock production, especially dairy products. The fraction of milk used for cheesemaking is on the rise worldwide (International Dairy Federation, 2013), increasing the importance of the relevant milk parameters, which include cheese yield, milk coagulation properties (MCPs), and the fat and protein contents (Bittante et al., 2012). MCPs, which are important measures of the technological qualities of milk (Anniabdi et al., 1977; Bittante et al., 2012), include rennet reactivity, curd-firming capacity,
syneresis ability and whey drainage, all of which are crucial features for cheesemaking. The suitability of milk for cheesemaking is traditionally evaluated by measuring the rennet coagulation time (RCT), the time required for curd firming (k20) and the firmness (a30 or a45, depending on the testing time used), elasticity, permeability, contractility and syneresis of the curd. However, these traditional MCPs are single-point measures obtained from computerized renneting meters, and their usefulness is limited by the presence of non-coagulating samples, problems in measuring k20 for slowly coagulating milk, the low repeatability and reproducibility of k20 and the high dependency of a30 on RCT (Ikonen et al., 2004). A recent study (Bittante et al., 2012) suggested a new approach for overcoming these limitations: the authors modeled the coagulation process by using all of the point observations collected during lactodynamographic analysis and extending the duration of the test. This approach allowed the calculation of new parameters summarizing all available information: curd firmness (CF) modeled for time t (CFt) and the maximum CF (MCF) traits (Figure 1). This analysis yielded values for the traditional parameters similar to those obtained from the computerized renneting meters, while further allowing estimations to be made for samples with very late coagulation or very slow curd firming. However, no previous study has estimated the genetic parameters of CFt and MCF.

Another important trait related to cheesemaking is milk acidity, with high-pH milk typically showing a longer RCT and lower a30 and a45 values. Okigbo et al. (1985) reported that CF decreased as the pH increased, and Ikonen et al. (2004) confirmed that changes in pH can significantly affect CF. However, although these traits exhibit genetic variations (Cecchinato et al., 2011) their inclusion as breeding goals in conventional selection programs has been hampered by the high cost of the necessary phenotyping studies.

In recent years, due to their genomic abundance and typing flexibility, single nucleotide polymorphisms (SNPs) have been used to determine the genetic background of underlying quantitative traits and analyzed for their possible use in marker-assisted selection. Thus, researchers could potentially overcome the phenotyping-cost issues by identifying SNPs that reflect linkage with milk quality and MCPs. These may then be integrated into selection programs.

In a previous study (Cecchinato et al., 2014), we analyzed 96 SNPs in 54 candidate genes within a population of Brown Swiss cows. We retained 51 polymorphic SNPs (in 37 candidate genes) for an association analysis with milk yield, composition, milk urea nitrogen content and somatic cell score. Here, we extend our previous work by performing an association analysis between the identified polymorphic SNPs and the traditional and new technological traits of milk (i.e., MCPs, CF, the MCF traits and acidity) in the same population, in order to increase our knowledge regarding the mutations responsible for individual differences related to milk technological traits. More specifically, we herein sought to estimate the variance components and heritabilities of the traditional MCPs, the new parameters (CFt modeling and the MCF traits) and milk pH, and to investigate their associations with 51 SNPs from 37 candidate genes in Brown Swiss cows.

**Material and methods**

**Field data**

The present study is part of the Cowplus Project described by Cipolat-Gotet et al. (2012). Briefly, a total of 1271 Brown Swiss cows from 85 herds located in Trento Province (Italy) were sampled once. Details on the milk sampling procedure, cows, herds and pedigree were previously reported (Cecchinato et al., 2014).

**Analysis of milk acidity and MCPs**

Before MCP analysis, pH was measured using a Crison Basic 25 electrode (Crison, Barcelona, Spain). MCPs were obtained using two mechanical lactodynamographs (Formagraph; Foss Electric A/S, Hillerød, Denmark) according to the procedure described by Cipolat-Gotet et al. (2012). Each individual milk sample (10 ml) was heated to 35°C and mixed with 200 µl of a 1.2% (w/v) rennet solution (Hansen Standard 215 with 80 ± 5% chymosin and 20 ± 5% pepsin; Pacovis Amrein AG, Bern, Switzerland) diluted in distilled water to yield 0.051 IMCU x ml\(^{-1}\). The observation period lasted 90 min, beginning immediately after the addition of rennet. The instrument recorded the width (in mm) of the oscillatory graph every 15 s during testing and directly provided the traditional MCP traits: RCT (min), defined as the time from enzyme addition to milk gelation; k20 (min), defined as the time from gelation to that at which the width of the graph reached 20 mm; and the widths of the graph at 30 min (a30, mm), from rennet addition (measuring the extent of CF).
Modeling the CF of individual milk samples

CF was measured every 15 s for 90 min, for a total of 360 recorded CF values per sub-sample. The four-parameter equation proposed by Bittante et al. (2013) for modeling an extended observation of CF was used to analyze the data. The four-parameter model is given as follows:

\[ \text{CF}_t = \text{CF}_P \times (1 - e^{-k_{CF} \times (t - t_{max})}) \times e^{-k_{SR} \times (t - t_{RCT})} \]

where \( \text{CF}_t \) is the CF at time \( t \) (mm), \( \text{CF}_P \) the asymptotic potential maximum value of CF (mm), \( k_{CF} \) the curd firming instant rate constant (% × min\(^{-1}\)), \( k_{SR} \) the curd syneresis instant rate constant (% × min\(^{-1}\)) and \( t_{RCT} \) the rennet coagulation time (min). Thus, CF represents a function of the maximum asymptotic CF (\( \text{CF}_P \)) and the processes of curd firming and syneresis (represented by the two rate constants). The first constant, \( k_{CF} \), is assumed to increase CF toward its potential asymptotic value (\( \text{CF}_P \)), while \( k_{SR} \) is assumed to reduce CF toward zero due to the expulsion of whey and the free floating of curd. After gelation, \( k_{CF} \) prevails over \( k_{SR} \), and CF increases until the values of the two opposing phenomena are equal and a maximum value (\( \text{CF}_{max} \)) is achieved at time \( t_{max} \). Thereafter, the curve descends toward a null asymptotic value (Figure 1). This model uses all available information to estimate the four parameters, and these non-single-point measurements are less interdependent than the traditional MCPs. Moreover, this method allows us to estimate the traditional parameters of RCT, \( k_{CD} \) and \( a_{CD} \) as well as \( \text{CF}_{max} \) and \( t_{max} \). The \( \text{CF}_t \) observations obtained for each sub-sample were fitted with curvilinear regressions using the non-linear procedure (PROC NLIN) of SAS (SAS Institute Inc., Cary, NC, USA). The parameters of each individual equation were estimated by employing the Marquardt iterative method (350 iterations and a \( 10^{-3} \) level of convergence).

Blood sampling, DNA extraction, SNP selection and genotyping

Blood sampling, DNA extraction and SNP selection and genotyping were performed as previously described (Cecchinato et al., 2014). Briefly, DNA was extracted using a DNeasy® 96 Blood & Tissue Kit (Qiagen, Hilden, Germany) from 100 μl of individual whole blood, quantified using the QBit system (Invitrogen, Carlsbad, CA, USA) and assessed for integrity by 1% agarose gel electrophoresis. Owing to the scarcity of information regarding the association of individual genes with milk coagulation traits, other genes believed to influence MCP-correlated milk characteristics were considered as candidate genes. Out of 113 SNPs initially selected we chose the 89 SNPs with the best Illumina designability rank scores and seven SNPs with scores between 0.5 and 0.6. The 96 selected SNPs, which are located in 54 genes and span 22 chromosomes, were genotyped with the GoldenGate system (Illumina, San Diego, CA, USA). We retained 51 polymorphic SNPs (in 37 candidate genes) for the association analysis (Cecchinato et al., 2014).

Statistical analysis

Genetic variations in MCPs, CF, the MCF traits and acidity were investigated with the following mixed linear animal model:

\[ y_{ijkl} = \mu + \text{DIM}_i + \text{Parity}_j + h_k + a_l + \epsilon_{ijkl} \]

(1)

where \( y_{ijkl} \) is the phenotypic record for the analyzed trait; \( \text{DIM}_i \), the effect of the \( i^{th} \) class of days in milk (\( \text{DIM}_i \); \( i = 1 \) to 10; 30 days for each class, with class 1 being <30 days and class 10 being >300 days); \( \text{Parity}_j \), the effect of the \( j^{th} \) parity of the cow (\( j = 1 \) to 5 or more); \( h_k \), the effect of the \( k^{th} \) herd (\( k = 1 \) to 85); \( a_l \), the infinitesimal genetic effect of individual \( l \) and \( \epsilon_{ijkl} \) the random residual term. For \( a_{30d} \), the renneting meter sensor of the lactodynamograph (10 levels) was included as an additional systematic effect, as previously reported by Cecchinato et al. (2013).

The association studies for all investigated genes were carried out using a Bayesian methodology. The following mixed linear animal model was used:

\[ y_{ijkl} = \mu + \text{DIM}_i + \text{Parity}_j + h_k + a_l + x_{lm} + \epsilon_{ijkl} \]

(2)

where \( y_{ijkl} \) is the phenotypic record for the analyzed trait; \( \text{DIM}_i \), \( \text{Parity}_j \), \( h_k \) and \( a_l \) as are described in model 1; \( x_{lm} \) (0,1,2) reflects the number of copies of the minor allele at the \( m^{th} \) SNP of subject \( l \); \( \beta_m \) is the additive genetic effect of the \( m^{th} \) SNP; and \( \epsilon_{ijkl} \) the random residual term.

For the univariate analyses, bounded uniform priors were used for all environmental variables, and \( a \) and \( h \) were assumed \( a \) priori to be independent and normally distributed, as:

\[ a \mid \sigma^2_a \sim N(0, \sigma^2_a) \]
\[ h \mid \sigma^2_h \sim N(0, \sigma^2_h) \]

where \( \sigma^2 \) is the known additive genetic relationship matrix and \( \sigma^2 \) is the identity matrix. The pedigree file included information on 8845 animals; there were 1326 sires, 264 of which had progeny (between 2 and 80 daughters) with records in the data set.

The marginal posterior distributions of all parameters were obtained using a Gibbs sampler running with a single chain of 1 000 000 points; the first 5 000 were discarded as burn-in, as previously tested by Raftery and Lewis (1992). Samples were saved every 100 iterations. Owing to autocorrelations between successive samples, convergence was tested using Geweke’s Z-criterion (Geweke, 1992). Monte Carlo sampling errors and the effective sample size were computed using the time-series procedures described by Geyer (1992). The parameters of concern were the dispersion parameters and the additive effects of SNPs, as defined by Falconer and Mackay (1996). The posterior mean was used as a point estimate for the parameter of concern. The lower and upper bounds of the 95% highest posterior probability density regions (HPD95) for each additive effect were estimated from the Gibbs samples. For all traits, the model was fitted to separately estimate the contribution of each SNP (i.e., the
model was run 51 times/trait). A SNP was considered as having a relevant effect on the trait when the posterior means of the additive effect did not include 0 in the HPD95 interval. Moreover, as suggested by Ramirez et al. (2013), we computed PPN0, which was the posterior probability of the estimated effect of being <0 for negative effects or >0 for positive effects. Only relevant SNPs are presented in the tables. The genetic variance explained by a given SNP (\(\sigma^2\)) was calculated from the estimated genotypic effects and the observed genotypic frequencies. The results are expressed as the percentage of the total additive genetic variance obtained from model 1 without the genotypic effect.

Intra-herd heritability, which was computed without considering the SNP effect in the model, was defined as:

\[
h^2 = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_e}
\]

where \(\sigma^2_a\) and \(\sigma^2_e\) are the additive genetic and residual variances, respectively.

Results and discussion

Descriptive statistics

The descriptive statistics for the investigated traits are reported in Table 1. A comprehensive discussion of the phenotypic variations among MCP traits was previously published by Cipolat-Goet et al. (2012). In the present work, the average values for RCT and \(k_{20}\) were 19.95 and 5.36 min, respectively, whereas the average value for \(a_{30}\) was 30.09 mm. Notably, we were able to obtain RCT and \(k_{20}\) values for all samples because we prolonged the observation time to 90 min after rennet addition. The presence of late-coagulating samples in our analysis helps explain why our point estimates of the genetic variance component for RCT values are higher than those in the previous report (Bittante et al., 2012). The new parameters of CF modeled at time \(t\) had average values of 20.79 min, 54.46 mm, 12.45% \(\times 10^{-3}\) and 1.38% \(\times 10^{-3}\) for \(R_{CTeq}, CFP, k_{CF}\) and \(k_{SR}\), respectively. On average, \(CF_{max}\) was 37.08 mm and \(t_{max}\) was 40.68 min. The pH values obtained in the present work were similar to those reported by Ikonen et al. (2004) and Bonfatti et al. (2011).

Variance components and heritability

The point estimates and features of the marginal posterior densities for the additive genetic variances and heritabilities of the considered traits (without considering SNP effects) are reported in Table 2. The genetic variances for the MCP traits were 7.335, 2.22 and 22.107 mm² for RCT, \(k_{20}\) and \(a_{30}\), respectively. The corresponding estimates of intra-herd heritability were 0.267, 0.227 and 0.187, respectively. The (co)variance components and heritability estimates for the MCPs were reported from the same data set by Cecchinato et al. (2013). However, no previous study has considered these components and estimates for the new CFt parameters and \(CF_{max}\). Here, we found that the genetic variances for \(CF_t\) parameters were 6.639 mm², 12.377, 6.274 and 0.024 mm² for \(R_{CTeq}, CFP, k_{CF}\) and \(k_{SR}\), respectively. For the same traits, the heritability estimates were 0.258, 0.064, 0.230 and 0.077, respectively. The estimated additive genetic variances for the MCP traits were 8.069 mm² for \(CF_{max}\) and 25.164 min² for \(t_{max}\), while the corresponding estimates of heritability were 0.191 and 0.278, respectively. With the exception of the low estimates obtained for \(CF_P\) and \(k_{SR}\), the new CF modeling traits showed heritability values between 0.191 and 0.278, which were similar to those of the traditional MCPs found for this data set (0.187 to 0.267).

The point estimate of the genetic variance component for \(\text{pH}\) was 0.001, which was comparable to that obtained by Cecchinato et al. (2011) using a different data set of the same breed. As expected, the intra-herd heritability obtained in the present study (0.333) was higher than the previous across-herd estimate (Cecchinato et al., 2011).

Association analysis with traditional MCPs

The features of the marginal posterior densities of the additive effects for the relevant SNPs with respect to MCPs are reported in Table 3. The marginal posterior distributions of the additive effects were approximately normal. A total of 14 tested SNPs located in 13 genes (two SNPs belonged to CSN2) were significantly associated with at least one of the cheesemaking-related traits or milk acidity. Figure 2 shows a map of the relevant relationships between the traits and candidate genes.

Considering the traditional MCPs, seven SNPs were found to affect the coagulation traits. RCT was positively associated with CSN2 rs43703011 (A v. C = + 1.26 min; PPN0 = 0.994;
Table 2: Features of marginal posterior densities of additive genetic ($\sigma_a^2$), herd ($\sigma_h^2$) and residual ($\sigma_e^2$) variance and heritability for milk coagulation properties (MCPs), parameters of curd firmness modeling on time t (CFt), maximum curd firmness traits (MCF) and acidity

<table>
<thead>
<tr>
<th>Trait</th>
<th>$\sigma_a^2$ Estimate</th>
<th>$\sigma_h^2$ Estimate</th>
<th>$\sigma_e^2$ Estimate</th>
<th>Heritability Estimate HPD95</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCT (min)</td>
<td>7.335</td>
<td>4.507</td>
<td>19.345</td>
<td>0.267; 0.12; 0.46</td>
</tr>
<tr>
<td>$k_{20}$ (min)</td>
<td>2.220</td>
<td>0.315</td>
<td>7.491</td>
<td>0.227; 0.08; 0.42</td>
</tr>
<tr>
<td>$a_{30}$ (mm)</td>
<td>22.107</td>
<td>6.740</td>
<td>95.098</td>
<td>0.187; 0.04; 0.37</td>
</tr>
<tr>
<td>CFt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCTeq (min)</td>
<td>6.639</td>
<td>4.006</td>
<td>18.549</td>
<td>0.258; 0.11; 0.44</td>
</tr>
<tr>
<td>CFmax (mm)</td>
<td>12.377</td>
<td>21.628</td>
<td>148.97</td>
<td>0.064; 0.01; 0.20</td>
</tr>
<tr>
<td>$k_{SR}$ (% $\times$ min$^{-1}$)</td>
<td>6.274</td>
<td>4.428</td>
<td>19.902</td>
<td>0.230; 0.08; 0.43</td>
</tr>
<tr>
<td>$k_{SR}$ (% $\times$ min$^{-1}$)</td>
<td>0.024</td>
<td>0.035</td>
<td>0.250</td>
<td>0.077; 0.01; 0.22</td>
</tr>
<tr>
<td>MCF</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CFmax (mm)</td>
<td>8.069</td>
<td>9.054</td>
<td>32.546</td>
<td>0.191; 0.07; 0.35</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>25.164</td>
<td>14.972</td>
<td>62.143</td>
<td>0.278; 0.11; 0.48</td>
</tr>
<tr>
<td>Acidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>0.001</td>
<td>0.004</td>
<td>0.002</td>
<td>0.333; 0.17; 0.52</td>
</tr>
</tbody>
</table>

Estimate = mean of the marginal posterior density of the parameter; HPD95 = lower and upper bound of the 95% highest posterior density region; RCT = rennet coagulation time of samples coagulating within 45 min from enzyme addition; $k_{20}$ = curd-firming time of samples reaching 20 mm of firmness within 45 min from enzyme addition; $a_{30}$ = curd firmness at 30 min after enzyme addition; RCTeq = rennet coagulation time estimated using the CF equation; CFmax = maximum curd firmness achieved within 45 min; tmax = time at achievement of CFmax

Table 3: Features of the estimated marginal posterior densities of additive effects and of the proportion of additive variance of the trait explained (Vp) for the relevant single nucleotide polymorphism (SNP)$^1$ on milk coagulation properties (MCPs), on parameters of curd firmness modeling on time t (CFt), on maximum curd firmness traits (MCF) and on acidity

<table>
<thead>
<tr>
<th>Trait</th>
<th>Gene</th>
<th>Allele</th>
<th>Estimate</th>
<th>HPD95</th>
<th>PPN0</th>
<th>Vp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RCT (min)</td>
<td>CSN2 rs43703011</td>
<td>A v. C</td>
<td>1.26</td>
<td>0.25; 2.25</td>
<td>0.994</td>
<td>7.67</td>
</tr>
<tr>
<td>RCT (min)</td>
<td>GHI1 rs41923484</td>
<td>C v. G</td>
<td>0.75</td>
<td>0.02; 1.45</td>
<td>0.978</td>
<td>2.72</td>
</tr>
<tr>
<td>$k_{20}$ (min)</td>
<td>CSN2 rs43703011</td>
<td>A v. C</td>
<td>1.12</td>
<td>0.49; 1.76</td>
<td>0.997</td>
<td>20.01</td>
</tr>
<tr>
<td>$k_{20}$ (min)</td>
<td>CSN2 rs43703013</td>
<td>G v. C</td>
<td>–0.66</td>
<td>–1.29; –0.03</td>
<td>0.981</td>
<td>5.27</td>
</tr>
<tr>
<td>RCTeq (min)</td>
<td>ABCG2 rs41577686</td>
<td>T v. G</td>
<td>0.45</td>
<td>0.08; 0.80</td>
<td>0.994</td>
<td>4.55</td>
</tr>
<tr>
<td>CFmax (mm)</td>
<td>PRL rs109428015</td>
<td>T v. C</td>
<td>0.01</td>
<td>0.19; 1.43</td>
<td>0.996</td>
<td>10.78</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>PLCB1 rs41624761</td>
<td>T v. C</td>
<td>–0.88</td>
<td>–1.82; 0.00</td>
<td>0.978</td>
<td>8.90</td>
</tr>
<tr>
<td>$a_{30}$ (mm)</td>
<td>CCL2 rs41255713</td>
<td>C v. T</td>
<td>1.67</td>
<td>0.04; 3.30</td>
<td>0.976</td>
<td>4.47</td>
</tr>
<tr>
<td>CFt</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RCTeq (min)</td>
<td>CSN2 rs43703011</td>
<td>A v. C</td>
<td>0.88</td>
<td>0.04; 1.76</td>
<td>0.923</td>
<td>3.74</td>
</tr>
<tr>
<td>RCTeq (min)</td>
<td>GHI1 rs41923484</td>
<td>C v. G</td>
<td>0.65</td>
<td>0.06; 1.25</td>
<td>0.981</td>
<td>2.04</td>
</tr>
<tr>
<td>RCTeq (min)</td>
<td>OLR1 rs13362924</td>
<td>A v. C</td>
<td>–2.49</td>
<td>–4.09; –0.84</td>
<td>0.965</td>
<td>15.21</td>
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<tr>
<td>CFmax (mm)</td>
<td>CSN15 rs109817504</td>
<td>A v. G</td>
<td>–7.07</td>
<td>–11.34; –2.88</td>
<td>0.962</td>
<td>72.69</td>
</tr>
<tr>
<td>CFmax (mm)</td>
<td>STAT5A rs109578101</td>
<td>T v. C</td>
<td>–5.26</td>
<td>–9.46; –1.04</td>
<td>0.974</td>
<td>43.84</td>
</tr>
<tr>
<td>$k_{CF}$ (% $\times$ min$^{-1}$)</td>
<td>PLCB1 rs41624761</td>
<td>T v. C</td>
<td>–0.51</td>
<td>–1.01; –0.02</td>
<td>0.975</td>
<td>5.16</td>
</tr>
<tr>
<td>$k_{SR}$ (% $\times$ min$^{-1}$)</td>
<td>PRL rs109428015</td>
<td>T v. C</td>
<td>–0.0008</td>
<td>–0.001; –0.00001</td>
<td>0.921</td>
<td>9.73</td>
</tr>
<tr>
<td>MCF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tmax (min)</td>
<td>OLR1 rs13362924</td>
<td>A v. C</td>
<td>1.69</td>
<td>0.21; 3.14</td>
<td>0.923</td>
<td>4.14</td>
</tr>
<tr>
<td>$a_{30}$ (mm)</td>
<td>OLR1 rs13362924</td>
<td>A v. C</td>
<td>3.71</td>
<td>0.23; 7.19</td>
<td>0.984</td>
<td>9.85</td>
</tr>
<tr>
<td>Acidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>SCDF rs136334180</td>
<td>A v. G</td>
<td>–0.005</td>
<td>–0.01; 0.00</td>
<td>0.983</td>
<td>1.25</td>
</tr>
<tr>
<td>pH</td>
<td>GRLF1 rs41572288</td>
<td>T v. C</td>
<td>0.006</td>
<td>0.00; 0.01</td>
<td>0.982</td>
<td>1.80</td>
</tr>
<tr>
<td>pH</td>
<td>LIPF rs110137537</td>
<td>A v. C</td>
<td>–0.007</td>
<td>–0.01; 0.00</td>
<td>0.976</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Estimate = mean of the marginal posterior density of the parameter; HPD95 = lower and upper bound of the 95% highest posterior density region; PPN0 = the posterior probability of the additive effect to be over or below zero; Vp (%) = proportion of genetic variance explained by each SNP; RCT = rennet coagulation time of samples coagulating within 45 min from enzyme addition; $k_{20}$ = curd-firming time of samples reaching 20 mm of firmness within 45 min from enzyme addition; $a_{30}$ = curd firmness at 30 min after enzyme addition; RCTeq = rennet coagulation time estimated using the CF equation; CFmax = maximum curd firmness achieved within 45 min; tmax = time at achievement of CFmax

$^1$SNP were considered having a relevant effect on the trait when the posterior means of the additive effect did not include 0 in the HPD interval.
Regarding other genes, the \(CSN3\) rs110870535 G allele was fixed and no C variant was present in our data set. In addition, the \(CSN3\) rs43703015 C allele, which distinguishes the B and C variants from the A-derived alleles, had a lower frequency compared with the T allele. Thus, even if the other \(CSN3\) loci failed during the analysis (possibly because these SNPs are too close together, compromising primer hybridization), the frequency of the B allele in our population could be estimated at about 0.78. This was even higher than that found by Chessa et al. (2013), who reported it increasing from 0.64 to 0.71. This indicates that selection is rapidly increasing the frequency of the B allele. Indeed, the B allele is included in the total economic index used for selection of the Italian Brown Swiss population, and its effect may therefore already be incorporated in our analysis. The \(GH1\) rs41923484 C allele was also found to be associated with a decrease in casein content in the same data set (Cecchinato et al., 2014), potentially explaining the negative relationship with RCT. The curd-firming time (\(k_{20}\)) yielded the most associations, including those with \(CSN2\) rs43703011 (A v. C = +1.12 min; PPN0 = 0.997; \(V_s = 20.01\%\)), \(CSN2\) rs43703013 (G v. C = −0.66 min; PPN0 = 0.981; \(V_s = 5.27\%\)), \(ABCG2\) rs41577868 (T v. G = +0.45 min; PPN0 = 0.994; \(V_s = 4.55\%\)), \(PRL\) rs109428015 (T v. C = +0.81 min; PPN0 = 0.996; \(V_s = 10.78\%\)) and \(TLR2\) rs43706434 (A v. G = −0.88 min; PPN0 = 0.978; \(V_s = 8.90\%\)). As noted above for RCT, the \(CSN2\) rs43703011 A allele was found to be responsible for the deterioration of \(k_{20}\) (+1.12 min; PPN0 = 0.997). Considering that the \(CSN2\) rs43703013 C allele (a marker of the B/C variants) is positively associated with \(k_{20}\) (−0.66 min, PPN0 = 0.98), we propose that the \(A^1\) variant could be responsible for the observed worsening of \(k_{20}\).

The \(ABCG2\) protein facilitates the transport of hydrophobic substances across cellular membranes, and the encoding gene has been associated with mammary epithelial cell proliferation. Given that mammary cell numbers are positively correlated with milk production, researchers have speculated that functional variations in \(ABCG2\) may affect milk production (Wei et al., 2012). However, a SNP in this gene was not found to affect milk production traits in the same population studied in the present paper (Cecchinato et al., 2014). This suggests that the effect of such SNP on \(k_{20}\) could be due to the transportation of MCP-modulating metabolites or possible linkage with a yet-unknown SNP. The first hypothesis is supported by the observation that \(ABCG2\), which is highly synthesized and up-regulated in lactating bovine mammary tissue, is also present in the milk fat globule membrane; there, it plays an essential role in promoting the secretion of important milk constituents through a yet-unidentified mechanism (Bionaz and Loor, 2008).

The \(PRL\) rs109428015 and \(TLR2\) rs43706434 were also found to be important in explaining the variation of \(k_{20}\). \(TLR2\) is a receptor for cell-wall components of gram-positive bacteria; it recognizes a highly diverse set of pathogen-associated motifs and triggers differential intracellular signaling pathways, thereby playing an important role in an individual's...
resistance against infection, including mastitis (Fleminger et al., 2011). In a comparative study of casein hydrolysis and its effect on clotting parameters in milk from cows that were sub-clinically infected with four major udder pathogens (Staphylococcus aureus, Escherichia coli, Streptococcus dysgalactiae and Staphylococcus chromogenes), Leitner et al. (2006) found that milk from infected glands possessed inferior clotting parameters. Since TRL2 is known to protect casein against hydrolysis by bacterial enzymes, the association of TLR2 with a better milk clotting aptitude could reflect increased protection of caseins by the A variant of TLR2.

The a30 value was influenced only by CCL2 rs41255713. The posterior distribution of the additive effect showed that the C allele increased CF by 1.67 mm (PPN0 = 0.976), with the SNP explaining 4.47% of the additive genetic variance. CCL2 belongs to the chemokine family; together with its receptor, CCL2 contributes to the trafficking of leukocytes to the mammary gland (Nishimura, 2003) and may play important roles in the host immune response during acute and chronic intramammary infections. Thus, the association of CCL2 with better coagulation could reflect higher infection responses and increased protection of milk components.

**Association analysis with CF modeling parameters**

The effects mapped in Figure 2 show that (except for RCTeq) the genes affecting the CF parameters and CFmax differ from those that influence the traditional MCPs. In the case of RCTeq, our results confirmed its significant associations with GH1 rs41923484 and CSN2 rs43703011. The C allele of GH1 rs41923484 increased the considered parameter by +0.65 min (PPN0 = 0.981; Va = 2.04%), while the A allele of CSN2 rs43703011 increased it by +0.88 min (PPN0 = 0.923; Va = 3.74%). Interestingly, RCTeq was significantly associated with a SNP that was not associated with the traditional one. For OLR1 rs133629324, the estimated substitution effect of the A allele was −2.49 min (PPN0 = 0.962; Va = 15.21%). ORL1 encodes a low-density lipoprotein receptor belonging to the C-type lectin superfamily. This gene is regulated through the cyclic adenosine monophosphate receptor belonging to the C-type lectin superfamily. Its receptor, CCL2 contributes to the trafficking of leukocytes to the mammary gland (Nishimura, 2003) and may play important roles in the host immune response during acute and chronic intramammary infections. Thus, the association of CCL2 with better coagulation could reflect higher infection responses and increased protection of milk components.

Regarding STAT5A, the encoded protein is a member of the STAT family of transcription factors. In response to cytokines and growth factors, the receptor-associated kinases phosphorylate STAT family members, which homo- or heterodimerize, translocate to the cell nucleus, and act as transcriptional activators. STAT5A is activated by and mediates the responses of many cell ligands, including IL2, IL3, IL7 GM-CSF, erythropoietin, thrombopoietin and various growth hormones. STAT5A was first identified as a PRL-induced mammary gland factor (Gouilleux et al., 1994) and has been demonstrated to play critical roles in regulating apoptosis, promoting proliferation and enhancing cell cycle progression (Paukku and Silvennoinen, 2004). This gene was previously reported to have effects on the milk protein percentage, the milk fat percentage and embryonic survival (Khatib et al., 2008). The present study is the first to report that STAT5A appears to be associated with milk coagulation traits.

The instant rate constant, $k_{CF}$, was associated with phospholipase C β1 (PLCB1) rs41624761 (T v. C = −0.51% × min$^{-1}$; PPN0 = 0.975; Va = 5.16%). The encoded protein is responsible for hydrolyzing ~90% of the lipid phosphorus in the low- and high-density lipoprotein fractions of milk, and is known to catalyze the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate. This reaction uses calcium as a cofactor and plays important roles in the intracellular transductions of many extracellular signals. Thus, it would be interesting to further analyze the effect of this gene and verify that a functional mutation could affect minor components of milk, thereby influencing its technological properties. The analyzed mutation is located in an intronic region, and thus our result is likely to reflect linkage rather than a causative mutation.

The other instant rate constant, $k_{SR}$, was associated with PRL rs109428015 (T v. C = −0.0008% × min$^{-1}$; PPN0 = 0.921; Va = 9.73%) and ORL1 rs133629324 (A v. C = −0.003% × min$^{-1}$; PPN0 = 0.991; Va = 0.68%). The linkage of these two genes with coagulation traits was discussed above. Their association with $t_{max}$ is not surprising given that this parameter measures the time at which the positive effect of $k_{CF}$ on CF is fully compensated by the negative effect of $k_{SR}$. Here, the estimated substitution effect of the PRL rs109428015 T allele was +1.69 min (PPN0 = 0.923; Va = 4.14%), and that of the ORL1 rs133629324 A allele was +3.71 min (PPN0 = 0.984; Va = 9.85%).

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Association analysis with milk acidity

Finally, relevant associations with milk acidity were observed for SCD-1 rs136334180, GRLF rs41572288 and LIPE rs110137537. The SCD-1 rs136334180 A allele was associated with a 0.005-unit reduction in the pH value (PPN0 = 0.983); the GRLF rs41572288 T allele was associated with a 0.006-unit increase in pH (PPN0 = 0.982); and the LIPE rs110137537 A allele was associated with a 0.007-unit decrease in pH (PPN0 = 0.976). The three SNPs explained small proportions of the genetic variance in milk acidity ($V_a$ ranging from 1.25% to 1.89%). Both SCD-1 rs136334180 and GRLF rs41572288 had previously been associated with milk traits (the fat and lactose percentages, respectively) (Cecchinato et al., 2014), which explains their effects on the composition and technical properties of milk. In the future, the relationships of various coagulation properties with LIPE, which is one of the most important lipolysis-mediating genes, should be studied in greater detail.

Conclusions

The parameters of CF modeled over time (CFt) are heritable and characterized by heritability values similar to those of the traditional MCPs and generally similar to or greater than those of the milk yield and content and part of their heritability seems to be due to polymorphisms in genes analyzed in the present paper. Indeed, the results presented herein confirm some of the previously documented associations (e.g., those between CSN2 and MCPs) and a number of novel associations were identified: SNPs in ABCG2, CCL2, GH1, PRL and TLR2 were found to be associated with MCPs; SNPs in GRLF1, LIPE and SCD-1 were found to be associated with milk acidity (the fat and lactose percentages, respectively) (Cecchinato et al., 2014), which explains their effects on the composition and technical properties of milk. In the future, the relationships of various coagulation properties with LIPE, which is one of the most important lipolysis-mediating genes, should be studied in greater detail.

Acknowledgments

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References

Bionaz M and Loor JJ 2008. ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. Journal of Nutrition 138, 1019–1024.