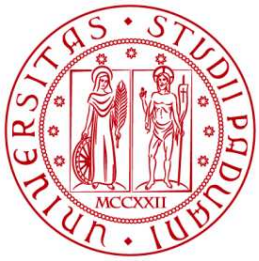

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CICLO XXII

Genetic analysis of milk protein composition and of its relationship with renneting properties of individual cow milk

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SUMMARY

Milk coagulation properties (**MCP**) are a fundamental aspect in cheese production, but un unfavorable trend over year on MCP have been observed in several countries. The cheese yield has decreased, accentuating the necessity to provide dairies with milk well suited for dairy products manufacture. During the past decades the focus of milk production has been kg's of milk protein, but total milk protein content is a poor indicator of MCP, and the lack of an appropriate high-throughput analysis for routine determination of milk coagulation is currently limiting the opportunity to improve MCP by direct selection. Milk protein composition has long been a subject of interest for worldwide dairy researchers. As a consequence, information on milk protein genotype could be utilized to improve milk protein composition and MCP trough marker assisted selection without having to phenotype large progeny groups. Considering such options, it would be desirable to gain further knowledge about effects of milk protein genetic variants on milk protein composition and on MCP.

Aims of the study were to investigate the effects of *CSN2-CSN3* haplotypes (β-κ-casein) and *BLG* (β-Lactoglobulin, **β-LG**) genotypes on milk production traits, contents of protein fractions and detailed protein composition; to investigate the effects of *CSN2-CSN3* haplotypes, *BLG* genotypes, contents of milk protein fractions and protein composition on MCP; to investigate the effect exerted by the relative ratio of κ-CN A to κ-CN B content on MCP and industrial cheese yield of three Italian cheese varieties. The final aim was to estimate genetic parameters of major milk protein fractions and estimate genetic and phenotypic correlation between milk protein fractions and MCP.

A new reversed-HPLC method for the separation and quantification of the most common genetic variants of bovine milk proteins was developed and validated testing linearity, repeatability, reproducibility and accuracy. Contents of major protein fractions were measured by this new method in individual milk samples of 2,167 Simmental cows. Protein composition was measured as weight percentage of each casein (**CN**) fraction to total casein (**TCN**) and as weight percentage of β-LG to total whey protein (**WH**). Genotypes at *CSN2*, *CSN3* and *BLG* loci were also assessed by HPLC and *CSN2-CSN3* haplotype probabilities were estimated for each cow. Rennet coagulation time (**RCT**) and curd firmness ($\mathbf{a_{30}}$) were measured using a computerized renneting meter.

Effects of haplotypes and *BLG* genotypes on yields were weak or trivial. Haplotypes carrying *CSN2* B and *CSN3* B exhibited greater TCN and casein number (**CI**), in comparison with all other haplotypes. Genotype BB at *BLG* was associated with increased protein, TCN and CI, when compared to genotype AA. Haplotypes including *CSN3* B were associated with greater κ-CN content and percentage. Allele *CSN2* B was associated with an increase of β-CN content, which occurred at the expense of content of α_{S1}-CN. Haplotypes including allele *CSN2* A_1 exhibited decreased β-, α_{S2}- and γ-CN concentrations and increased α_{S1}- and κ-CN contents, whereas *CSN2* I exerted positive effects on β-CN concentration, without altering other protein fractions content. Effects exerted by haplotypes on CN composition were similar to those exhibited on CN fractions contents. Allele *BLG* A increased β-LG concentration and altered the β-LG to α-Lactalbumin (**α-LA**) ratio.

When protein fractions contents or protein composition were not included in the statistical model, haplotypes carrying *CSN3* B allele exhibited shorter RCT and greater a_{30}, in comparison with those carrying *CSN3* A, and haplotypes carrying *CSN2* B allele were responsible for a noticeable decrease of RCT and for an increase of a_{30}, when compared to haplotype A^2A. When effects of protein fractions contents or of 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. Also, the favorable effect exerted by *CSN2* B on a_{30} was mediated by the increase of β-CN B in milk. Conversely, β-CN B is likely to exert a molecular effect on RCT, which does not depend upon variation of β-CN content associated to allele B.

To test if the lack of effect of κ-CN genetic variant would have been observed also on cheese yield, milks with different κ-CN A to κ-CN B content ratios were separately manufactured to produce Montasio, Asiago and Caciotta cheese. Milk was characterized by having similar composition in terms of protein, TCN, CI, CN

composition, β-CN composition and pH. Milk with the higher proportion of κ-CN B (**HIGHB**) exhibited similar coagulation properties but a higher cheese yield in all the investigated cheese in comparison with milk with a lower proportion of κ-CN B (**LOWB**). However, the increment of yield observed for HIGHB milk in Montasio cheese was ascribed to a greater fat content of HIGHB milk in comparison with LOWB milk. The probability of HIGHB milk giving a cheese yield 5 % greater than that of LOWB milk ranged from 51 to 67 % for Montasio cheese, but was lower than 21 % for Asiago and Caciotta cheeses. Thus, the ratio of κ-CN A to κ-CN B content did not relevantly affect industrial cheese yield when milks of similar CN composition were processed, and an indirect effect due to the higher κ-CN content of κ-CN B milk on cheese yield is to be suggested.

Values of heritability for α_{S1}-CN%, κ-CN% and β-CN% were similar and ranging from 0.61 to 0.70, whereas heritability of α_{S2}-CN%, γ-CN% and β-LG% were 0.28, 0.29 and 0.33, respectively. When *CSN2-CSN3* haplotype and *BLG* genotype were accounted for by the model, heritability estimates of all the protein fractions became similar suggesting that proteins synthesis is regulated by specific genes which control the overall production of milk protein. Genetic correlations among the contents of the five CN fractions and between CN fractions and WH fractions were generally low. Generally, all the CN fractions were also moderately positively correlated with WH. When data where adjusted for *CSN2-CSN3* haplotype and *BLG* genotype, genetic correlations among the contents of protein fractions markedly increased confirming that all the fractions undergone a common regulation. The content and the relative proportion of κ-CN were not genetically correlated with RCT, α_{S1}- and α_{S2}-CN were unfavourately correlated with RCT, but increasing the content of β-CN in milk would result in a shorter RCT. Stronger curds were associated with higher κ-CN and β-CN, and with lower α_{S1}-, α_{S2}-, and γ-CN contents and proportions. Results confirm the lack of favorable associations between TCN and MCP indicating that other traits, i.e. milk protein fractions, should be used for the genetic improvement of cheese-making properties.

RIASSUNTO

Le proprietà di coagulazione del latte (**MCP**) sono un aspetto fondamentale nella produzione di formaggio, tuttavia, negli ultimi anni, è stato registrato un andamento sfavorevole della coagulazione del latte in diversi Paesi. La resa in formaggio è diminuita, accentuando la necessità di fornire i caseifici con latte più adatto per la trasformazione in formaggio. Nel corso degli ultimi decenni il miglioramento genetico si è focalizzato sui kg di proteina del latte, ma il contenuto totale di proteina non sembra essere un buon indicatore delle MCP, e la mancanza di un metodo di analisi che consenta la determinazione delle MCP su larga scala attualmente limita la possibilità di migliorare le MCP attraverso una selezione diretta. La composizione proteica del latte è stato a lungo oggetto di interesse per i ricercatori di tutto il mondo. Di conseguenza, le informazioni sul genotipo delle proteine del latte potrebbero essere utilizzate per migliorare la composizione della proteina oppure nella selezione assistita da marcatori per migliorare le MCP, senza dover fenotipizzare grandi gruppi di progenie. Alla luce di tali possibilità, sarebbe auspicabile poter acquisire ulteriori conoscenze sugli effetti delle varianti genetiche delle proteine sulla composizione proteica del latte e sulle MCP.

Obiettivi di questa tesi sono stati: studiare gli effetti dell'aplotipo *CSN2-CSN3* (β-κ-caseina) e del genotipo al locus *BLG* (β-lattoglobulina, **β-LG**) su caratteri produttivi, contenuto di frazioni proteiche e composizione proteica; studiare gli effetti dell'aplotipo *CSN2-CSN3* e del genotipo al locus *BLG*, del contenuto di frazioni proteiche e della composizione proteica sulle MCP, studiare l'effetto esercitato dal rapporto relativo tra κ-CN A e B sulle MCP e sulla resa industriale in tre varietà di formaggi italiani. Inoltre, ultimo obiettivo del lavoro è stato la stima dei parametri genetici delle principali frazioni proteiche del latte e delle correlazioni genetiche e fenotipiche tra le frazioni proteiche e le MCP.

Un nuovo metodo di analisi HPLC a fase inversa per la separazione e la quantificazione delle più comuni varianti genetiche delle proteine del latte bovino è stato sviluppato e validato attraverso test di linearità, ripetibilità, riproducibilità e accuratezza. Il contenuto delle principali frazioni proteiche è stato misurato con questo nuovo metodo in campioni di latte individuale di 2,167 bovine di razza Simmental. La composizione proteica è stata espressa come percentuale in peso di ogni frazione caseinica rispetto al contenuto totale di caseina (**TCN**) e come percentuale del peso della β-LG sul totale di proteine del siero (**WH**). Il genotipo ai loci *CSN2, CSN3* e *BLG* è stato determinato tramite HPLC e le probabilità aplotipiche per gli aplotipi CSN2-CSN3 sono state stimate per ogni animale. Tempo di coagulazione (**RCT**) e consistenza del coagulo (**a_{30}**) sono stati misurati utilizzando un lattodinamografo.

Gli effetti dell'aplotipo delle caseine e del genotipo al locus *BLG* sui caratteri produttivi sono stati limitati o trascurabili. Gli aplotipi contenenti gli alleli *CSN2* B e *CSN3* B hanno mostrato valori più elevati di TCN e un indice caseinico (**CI**) superiore, rispetto a tutti gli altri aplotipi. Il genotipo BB al locus *BLG* è stato associato ad un aumento del contenuto proteico e ad un CI superiore rispetto al genotipo AA. Gli aplotipi contenenti l'allele *CSN3* B sono stati associati a contenuti e percentuali di κ-CN maggiori. L'allele *CSN2* B è risultato associato con un aumento del contenuto di β-CN, che si è verificato a scapito del contenuto di α_{S1}-CN. Gli aplotipi che includevano la variante *CSN2* A_1 hanno mostrato una diminuzione del contenuto di β-, α_{S2}- e γ-CN e un aumento del contenuto di α_{S1}- e κ-CN, mentre la variante *CSN2* I ha esercitato effetti positivi sulla concentrazione di β-CN, senza alterare il contenuto delle altre frazioni proteiche. L'allele A al locus *BLG* è stato associato ad una maggiore concentrazione di β-LG e ad un più elevato rapporto tra β-LG e α-lattoalbumina (α-LA).

Quando il contenuto delle frazioni proteiche o la composizione della proteina non erano inclusi nel modello statistico, gli aplotipi contenenti l'allele *CSN3* B erano associati ad RCT più brevi ed a_{30} maggiori, rispetto a quelli che includevano l'allele *CSN3* A, e gli aplotipi contenenti la variante *CSN2* B erano responsabili di una notevole diminuzione dei valori di RCT e per valori di a_{30} maggiori, rispetto agli aplotipi contenente la variante A^2. Quando gli effetti del contenuto delle frazioni proteiche o della composizione proteica sono stati inclusi nel modello statistico, nessuna differenza tra aplotipi riconducibile agli alleli ai loci *CSN3* e *CSN2* è stata osservata per le MCP, con l'eccezione dell'effetto della *CSN2* B su RCT, che è rimasto molto favorevole. L'effetto favorevole esercitato dall'allele *CSN2* B su a_{30} è risultato mediato dall'aumento di β-CN

B nel latte. Al contrario, la β-CN B esercita probabilmente un effetto diretto su RCT, che non dipende dalla variazione del contenuto di β-CN associato all'allele B.
Per verificare se la mancanza di effetto diretto delle varianti genetiche di κ-CN sarebbe stato osservato anche sulla resa in formaggio, latte con differenti rapporti tra κ-CN A e B sono stati lavorati separatamente per la produzione di Montasio, Asiago e Caciotta. Il latte lavorato aveva composizione simile in termini di proteina, TCN, CI, composizione caseinica, composizione della β-CN e pH simile. Il latte con la percentuale maggiore di κ-CN B (**HIGHB**) ha presentato valori di MCP simili, ma una resa superiore in tutti i tipi di formaggio esaminati, rispetto al latte con una percentuale inferiore di κ-CN B (**LOWB**). Tuttavia, l'incremento di resa osservato per il formaggio Montasio è stato attribuito a un maggior contenuto di grasso del latte HIGHB in confronto con il latte LOWB. La probabilità del latte HIGHB di dare un formaggio con una resa del 5% superiore a quella del latte LOWB variava dal 51 al 67% per il Montasio, ma è stata inferiore al 21% per Asiago e Caciotta. Il rapporto tra le varianti A e B di κ-CN non ha quindi influito in modo rilevante sulla resa casearia industriale, quando la composizione del latte era bilanciata per la composizione caseinica, ed è possibile supporre pertanto che vi sia un effetto indiretto delle varianti di κ-CN sulla resa casearia, a causa del più elevato contenuto di κ-CN associato alla variante B.
I valori di ereditabilità per α_{S1}-CN%, κ-CN% e β-CN% erano simili e variabili da 0.61 al 0.70, mentre l'ereditabilità di α_{S2}-CN%, γ-CN% e β-LG% erano 0.28, 0.29 e 0.33, rispettivamente. Quando l'effetto dell'aplotipo *CSN2*-*CSN3* e del genotipo al locus *BLG* sono stati inclusi nel modello, le stime di ereditabilità di tutte le frazioni proteiche sono divenute simili suggerendo che la sintesi di proteine del latte sia sottoposta a un controllo genetico da parte di geni specifici che controllano il livello generale di proteina del latte. Le correlazioni genetiche tra il contenuto delle 5 frazioni caseiniche e tra le frazioni caseiniche e le frazioni sieriche erano generalmente basse. In generale, tutte le frazioni caseiniche erano anche moderatamente positivamente correlata con WH, suggerendo che vi sia una regolazione generale del livello di proteina del latte che coinvolge contemporaneamente TCN e WH. Quando l'effetto dell'aplotipo *CSN2*-*CSN3* e del genotipo al locus *BLG* sono stati inclusi nel modello, le correlazioni genetiche tra i contenuti delle frazioni proteiche sono aumentate significativamente, supportando l'ipotesi che tutte le frazioni siano oggetto di una regolazione generale. Il contenuto di κ-CN del latte non è risultato essere geneticamente correlato con RCT, α_{S1}- e α_{S2}-CN hanno mostrato una correlazione sfavorevole con RCT, mentre un aumento della β-CN nel latte sarebbe a favore di RCT più brevi. Coaguli più consistenti sono stati associati ad un maggior contenuto di κ-CN e β-CN e ad un minor contenuto di α_{S1}-, α_{S2}-, e γ-CN. I risultati ottenuti confermano la mancanza di un'associazione favorevole tra TCN e MCP, sottolineando l'esigenza di utilizzare altri caratteri, come il contenuto delle frazioni proteiche, per il miglioramento genetico delle proprietà casearie del latte.

GENERAL INTRODUCTION

Trends in Milk and Cheese Production

According to the Food and Agricultural Policy Research Institute (FAPRI, 2008), world cheese production is expected to grow 22.3 % over ten years, with the US and the EU accounting for over 64 %. The expected trends in milk and cheese production in Europe from 2010 to 2017 will be stationary for milk but +10.8 % for cheese production. Within the same period, the consumption of milk is expected to decrease (-0.3 %) whereas the consumption of cheese is expected to have a marked increase (+12 %).

Besides France, Italy is the country with the largest number of locally-made cheeses, nowadays approximately counting 400 products. Dairy sector accounts for 13 % of the food industry incomes and exerts a key role for the Italian food industry on an international level, involving more than 2,000 dairy implants and transformation facilities. Italy accounts 9 % of the European milk production (11 million tons, 94 % cow milk), but 15 % of the cheese production (Pieri e Del Bravo, 2005). More than 70 % of the overall milk production (ISTAT, 2006) is used in the manufacture of cheese and 55 % of total milk is processed for the 31 PDO (Protected Designation of Origin) cheeses (Cassandro, 2003).

Improvement of Milk Coagulation Properties

Milk coagulation properties (**MCP**) are a fundamental aspect in cheese production, especially in those countries where dairy industry is based on traditional products (Cassandro et al., 2008).

A general worsening of MCP has been observed in several countries. Unfavorable trends over years on MCP, at the phenotypic level, have been evidenced by some authors (Mariani et al., 1992; Cassandro and Marusi, 1999; Sandri et al., 2001) on milk yielded in dairy herds located in traditional areas for cheese production in Italy. A comparison of the Swedish dairy milk produced within the period from 1970 to 1996 (Lindmark-Månsson et al., 2003) showed that although there was no difference in total protein concentration, the proportion of casein in total protein was significantly decreased over that period. Although there are no published data on variation of MCP over the past decades in Finland, according to observations made in Finnish dairies, the average coagulation ability of milk has been deteriorating during the past 20 to 30 years, and the ratio of cows producing non-coagulating (**NC**) milk has increased (Ikonen et al., 1999). Stagnating cheese yields despite increased total protein concentration of milk has been reported in France also (Coulon et al., 2001). Consequently, the cheese yield has decreased, accentuating the necessity to provide dairies with milk well suited for dairy products manufacture.

During the past decades the focus of milk production has been kg of milk protein, but total milk protein content is a poor indicator of MCP. In a study conducted by Ikonen et al. (2004), neither protein nor casein content of milk was found to be suitable for implementing an indirect selection aimed to improve MCP. The genetic correlation between them was almost one, indicating that the protein content reflects the casein content well. Genetic correlations between MCP and protein and casein content of milk were, however, almost zero.

Possibility for the Genetic Improvement of MCP

Genetic improvement of MCP by direct selection has been suggested (Caroli et al., 1990; Ikonen et al., 1999a; Bittante et al., 2002; Cassandro et al., 2008; Tyrisevä, 2008). This would mean that the coagulation of milk for each cow, based on its repeatability, needs to be measured at least three times during one lactation (Tyrisevä, 2008), for a proper genetic evaluation. Although new and more automated measuring techniques for MCP are emerging (Dal Zotto et al., 2008), this is still a very laborious task. Therefore, the lack of an appropriate high-throughput analysis for routine determination of MCP is currently limiting this opportunity.

Genetic variants of milk proteins have been shown to be associated with the protein composition and thereby with the technological properties of milk (Buchberger and Dovč, 2000). Milk protein composition has long been a subject of interest for worldwide dairy researchers, although examples of practical implementations are scarce. Lack of simple routine analyses to measure i.e. casein content in milk, is one major factor limiting progress in this direction. As a consequence, information on milk protein genotype could be utilized to improve milk protein composition and MCP trough marker assisted selection without having to phenotype large progeny groups. Moreover, the finding of candidate genes for non-coagulation of milk (Tyrisevä et al., 2008) may present new possibilities for genetic selection regarding the improvement of MCP. Considering such options, it would be desirable to gain further knowledge about effects of milk protein genetic variants on milk protein composition and on MCP.

Milk Protein

Milk protein is a complex group of peptides in which over 200 different molecules have been characterized (Ng-Kwai-Hang, 2002). Bovine milk generally contains about 3.5 % protein, of which approximately 80 % are caseins and 20 % are whey proteins, traditionally divided according to their solubility at pH 4.6.

Milk caseins are fundamental in cheese making process because they form the gel network that entraps the other constituents of cheese. Casein consists of α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN in approximate proportions 4:1:4:1. γ-CNs are different C-terminal segments of β-CN obtained after

proteolytic cleavage by the enzyme plasmin. Moreover, post-translational modifications such as phosphorylation, glycosylation, disulphide bonding, proteolysis, and the existence of genetic variants, cause further diversity within the casein group (Ng-Kwai-Hang, 2002).

Polar and apolar regions on the casein peptide chains are not uniformly distributed, giving them an amphiphilic structure. This, in addition to their proline and phosphate content, constitutes the basis for the ability of caseins to form micelles. The concentrations of calcium generally found in milk would cause precipitation of α_{S1}-CN, α_{S2}-CN, and β-CN, which are calcium-sensitive proteins, by calcium binding to their phosphoserine residues. The κ-CN, however, is soluble in calcium and stabilizes the other caseins in a colloidal state (Farrell et al., 2006). The κ-CN is found in several glycosylated forms where the C-terminal part (the caseinomacropeptide) contains varying numbers of O-glucosidic linked residues (Farrell et al., 2004).

In milk about 95 % of the caseins are aggregated in colloidal structures (Farrell et al., 2006) and several theories of the casein micelle structure have been proposed. Although there is no unanimously accepted model, there are some general properties that are commonly recognized. These include the notion of partly hydrophobic caseins being stabilized by κ-CN predominantly located near the micelle surface. The hydrophilic and negatively charged C-terminal end of κ-CN protrudes from the micelle, which is open and porous, to form a hairy layer that, by steric and electrostatic repulsion, prevents any further submicelle aggregation and also micelle flocculation (Walstra, 1999). Micelle stability is suggested to be maintained by excess of hydrophobic attraction over electrostatic repulsion (Farrell et al., 2006). Containing only one phosphoserine residue, micelle growth is limited by κ-CN acting as a dead-end capping unit, which becomes part of the micelle surface structure (Horne, 1998). The stabilizing function of κ-CN and its role in micelle growth makes it a key protein in determining micelle size (Dalgleish et al., 1989; Donnelly et al., 1984; Risso et al., 2007) and also some functional properties.

The three main whey proteins are β-LG, α-LA and blood serum albumin (**BSA**), representing approximately 50, 20 and 10 % of total whey proteins, respectively. The remaining part comprises immunoglobulins (**Ig**) and trace amounts of several other proteins, including enzymes and growth factors.

Most whey proteins are globular with organized secondary and tertiary structure, which, in contrast to the caseins, make them sensitive to heat denaturation at temperatures above 60 °C. A reactive thiol group is exposed at heat denaturation of β-LG, which forms disulphide-thiol interchanges with other β-LG molecules as well as with κ-CN (Creamer et al., 2004). α-LA is important in the biosynthesis of lactose being a sub-component in the lactose synthetase complex (Ng-Kwai-Hang, 2002). Whereas α-LA and β-LG are synthesized in the mammary gland, BSA and Ig are components of blood serum.

Genetic Polymorphism of Milk Proteins

Polymorphisms have been observed for all milk proteins (Farrell et al., 2004). However, α_{S2}-CN and α-LA have been shown to be essentially monomorphic in all Western dairy breeds and variation at the α_{S1}-CN locus is rather limited (Farrell et al., 2004).

Single nucleotide substitutions in coding sequences of a gene may give rise to amino acid shifts affecting the physico-chemical properties of the protein polymorphism. But amino acidic substitutions can occur also in promoter regions, leading to variations of protein expression.

Milk protein polymorphisms play an important role in the technological quality of milk (Buchberger and Dovč, 2000) as a result of both the qualitative and quantitative protein variations. The former depend on molecular composition of the polypeptide, resulting in different chemical and physical behavior of a variant when compared to another and are the consequence of the specific amino acid variation. The latter are relate to the different capacities of expression of alleles that control the synthesis of that specific protein (Ikonen et al., 1997; Mayer et al., 1997).

Qualitative variations. The most common qualitative variations affect the net charge of the protein. For example, variant B of α_{S1}-CN carries one more negative charge than variant C via the substitution of Gly for Glu, the presence of Asp in β-LG A increase the negative charged of this variant in comparison with that of β-LG B, whereas the B variant of β-CN has one and two more net positive charges than A^1 and A^2 variants, respectively. It has been suggested that the repulsive forces between casein micelles containing variants such as α_{S1}-CN C, β-CN B and κ-CN B, in which amino acid substitution results in lower net negative charge, are decreased compared to micelles containing more negatively charged protein variants (McLean, 1986). This would thus facilitate aggregation.

In addition, κ-CN and β-CN B variants have been associated with lower electronegativity and alkaline pH, with positive effects on the stability of micelles and on their reactivity to rennet, which results in shorter clotting times and more compact curds (Mariani and Summer, 1999).

However, there are also mutations affecting not only the net charge of the protein. For example, the rare α_{S1}-CN variant A is characterized, when compared to variants B and C, by the lack of an hydrophobic region (resulting by a deletion of 13 amino acids residues), which makes it less sensitive to proteolytic degradation of chymosin and pepsin, originating a soft, friable and unsuitable clot for the production of cheese (Cocker et al., 1997).

Milk containing different genetic variants has also been shown to yield gels with an altered structure, due to different bonding and cross-linking patterns (Nuyts-Petit et al., 1997; Walsh et al., 1998).

Quantitative variations. Apart from effects of polymorphism in the coding part of the gene on the resulting protein structure, polymorphism in the non-coding regions of milk protein genes is

believed to affect protein transcription (Lum et al., 1997; Robitaille et al., 2000, 2002). It has been suggested (Martin et al., 2002) that, if polymorphisms in the promoter region of a gene are linked to polymorphisms located in the coding region, a specific protein might be characterized by an allele-specific expression.

In general, the B allele of κ-CN has been associated with a higher κ-CN concentration in milk compared to A (Lodes et al., 1997; Ikonen et al., 1997; Bobe et al., 1999; Graml and Pirchner, 2003), and also with higher total protein and casein number, which results in different MCP depending on κ-CN genotype. The influence of *CSN3* genotypes on the amount of total κ-CN in milk has important consequences on the size of the micelles also. The more uniform micelle systems are characterized by increased quantities of small micelles, which form a protein network more dense and compact. In contrast, the *CSN3* E allele has been associated with a lower κ-CN content compared to B, possibly also to A (Oloffs et al., 1992; Ikonen et al., 1997).

In several studies, κ-CN B allele has been associated with the most favorable MCP (Schaar, 1984; van den Berg et al., 1992; Ikonen et al., 1997), whereas κ-CN A has been associated with longer coagulation times and softer curds. Poorest MCP has been ascribed to the κ-CN E allele (Oloffs et al., 1992; Ikonen et al., 1999a). These effects of the different variants with MCP of milk are also found regarding cheese yield (Schaar et al., 1985; van den Berg et al., 1992; Walsh et al., 1995; Walsh et al., 1998).

Cows carrying the β-CN B allele have been reported to produce milk with increased total protein and β-CN concentrations (McLean et al., 1984; Ng-Kwai-Hang et al., 1986) and it has been shown that the amount of protein and casein decrease in the order $A^1A^1 > A^1A^2 > A^2A^2$ (Ng-Kwai-Hang et al., 1986; Jakob & Puhan, 1994). The B allele of β-CN has been linked to an improved coagulation compared to the A variants. Higher protein recovery in cheese has been reported for β-CN A^1A^1 compared to A^1A^2 (Marziali & Ng-Kwai-Hang, 1986), and for β-CN A^2B compared to β-casein A^2A^2 (Mayer et al., 1997). Finally, the B variant of β-LG has been shown to be expressed at a markedly lower level in milk compared to the A variant, with a concomitant increase in casein number (Lodes et al., 1997; Lundén et al., 1997).

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AIMS

The general aim of this thesis was to gain further knowledge about the association of milk protein polymorphisms with detailed milk protein composition, and about the effect exerted by genetic variants at milk protein loci and protein composition on milk coagulation and cheese yield. The main aim was to clarify if effects exerted by milk protein polymorphisms on renneting ability of milk and cheese yield are due to specific biochemical properties of protein genetic variants or to variations of the relative ratios between protein fractions associated with the presence of specific polymorphisms.

Specific aims were to:

- Develop a method for the quantification of the major milk protein fractions and for the determination of their most common genetic variants (Chapter 1);

- Study the effect of casein haplotypes and β-LG genotype on detailed protein composition of individual milk of Simmental cows (Chapter 2);

- Study the effects of casein haplotypes and β-LG genotype together with the detailed protein composition of milk on milk coagulation properties of individual milk of Simmental cows (Chapter 3);

- Assess the effect of different ratios between κ-CN A and B protein genetic variants in bulk milk on the cheese yield obtained in industrial scale cheese-making experiments (Chapter 4);

- Estimate genetic parameters for milk protein fractions contents, protein composition and milk coagulation properties (Chapter 5).

Chapter 1.

Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants

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ABSTRACT

A new RP-HPLC method for the separation and quantification of the most common genetic variants of bovine milk proteins is described. A reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, 3.5 µm, 300 Å, 150×4.6 I.D.) was used. All the most common casein and whey protein genetic variants, including β-$CN_{I,}$ were detected and separated simultaneously in less than 40 min, with the exception of α_{S1}-CN B and C variants. Purified protein genetic variants were employed in calibration and showed different absorbances at 214 nm. The procedure was developed using 40 raw individual milk samples of cows belonging to four different breeds and certified skim milk powder BCR-063R. Method validation consisted in testing linearity, repeatability, reproducibility and accuracy. A linear relationship (R^2>0.99) between the concentration of proteins and peaks area was observed over the concentration range, with low detection limits. Repeatability and reproducibility were satisfactory for both retention times and peak areas. The RSD of peak areas ranged from 0.92 to 4.32% within analytical day and from 0.85 to 9.52% across analytical days. The recoveries, calculated using mixtures of samples previously quantified, ranged from 98.1 to 103.7%.

INTRODUCTION

Bovine milk protein has been widely studied because of its relationships with composition, nutritional and technological properties of milk. Lactoproteins include caseins (CNs) and whey proteins which differ in their solubility behavior at pH 4.6. Caseins account for nearly 80% of total milk protein and include α_{S1}-, α_{S2}-, β- and κ-CN fractions, in the relative proportion of 4:1:4:1. Whey proteins include α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), in the approximate proportion of 1:3, together with bovine serum albumin (BSA) and immunoglobulins (Igs) as minor

constituents (Farrell et al., 2004). For CN, β-Lg and α-La, more than 60 genetic variants have been identified, albeit in western bovine breeds few polymorphisms occur exclusively or are strongly predominant (Farrell et al., 2004). As genetic variants of CNs and whey proteins influence many properties of milk that are crucial in the cheese-making process, the development of analytical methods for the separation and quantification of variants of milk proteins within single protein fractions is of interest. As an example, the κ-CN_{BB} genotype seems to be associated with an increased protein and CN content, reduced rennet coagulation time, enhanced cheese curd firmness and overall cheese yield (Marziali and Ng-Kwai-Hang, 1986a,b; Buchberger and Dovč, 2000). Also, milk protein polymorphisms have been reported to be associated to variation in protein composition of milk (Bobe et al., 1999) which is an important factor for the profitability of the dairy industry (Pabst, 1994). Because genes that encode milk CNs and whey proteins are autosomal genes exhibiting Mendelian inheritance, selection of cows for desired proteins variants is feasible and milk protein polymorphisms might be used in gene-assisted selection programs for altering composition and technological properties of milk. Despite DNA-based genotyping of animals is available for large scale applications, it can not provide any information on milk protein composition. Moreover, the quantification of relative ratios for milk protein fractions, or even variants, is of interest for milk quality payment systems.

A variety of methods are employed to analyze milk protein fractions: electrophoretic techniques (Ng-Kwai-Hang and Kroeker, 1984; Kim and Jimenez-Florez., 1994) and isoelectric focusing (IEF) (Kim and Jimenez-Florez., 1994), high-performance liquid chromatography (HPLC) by ion-exchange (Hollar et al., 1991), hydrophobic interactions (Bramanti et al., 2002) and reversed-phase methods (Visser et al., 1991; Bobe et al., 1998; Bordin et al., 2001; Veloso et al., 2002), and, more recently, capillary electrophoresis and capillary zone electrophoresis (Recio et al., 1997; Fairise and Cayot, 1998; Miralles et al., 2001; Ferreira and Caçote, 2003), mass spectrometry (Miralles et al., 2003) or combinations of them (Mollé and Léonil, 2005). In particular, high performance liquid chromatography allows rapid and automated analysis, characterized by good separations, high resolutions and accuracy and reproducible results.

Previous investigations on the application of HPLC methods to the analysis of milk protein have been focused on the quantification of protein fractions using bulk-tank milk and employing protein commercial standards. Those methods lack the capacity to quantify single milk protein genetic variants within fraction, with the only exception of β-Lg. As protein composition might be involved in future breeding programs for dairy cattle populations aiming to enhance technological properties of milk, the development of methods for the quantification of single protein fractions or variants in individual milk samples is of interest and UV absorbance of each genetic variant should be assessed.

In this study, an RP-HPLC method for simultaneous qualitative and quantitative analysis of most common genetic variants of bovine caseins and whey proteins was optimized and validated. Our method was developed improving the results obtained by Bordin et al. (2001) allowing a 20 min lower analytical time, and with the use of purified genetic variants in the calibration phase to assess the response of each protein genetic variant. The validation of the method has been performed by testing the linearity, the precision (repeatability and reproducibility) and the accuracy.

MATERIALS AND METHODS

Reagents, Standards and Samples

Guanidine hydrochloride (GdnHCl) (lot G-4505, purity>99%) and Bis-Tris Buffer (lot B-9754, >98%) were supplied by Sigma (Sigma Aldrich, St. Louis, MO, USA). DL-dithiothreitol (lot 43817, >98%) was from Fluka (Buchs, Switzerland). Purified major proteins from bovine milk were also purchased from Sigma and their purity checked by electrophoresis or by polyacrilamide gel electrophoresis: κ-CN (lot C-0406, >80%), α-CN (lot C-6780, >70%), β-CN (lot C-6905, >90%), α-La (lot L-5385 type I, ~85%), β-Lg_B (lot L-8005, >90%) and β-Lg_A (lot L-7880, >90%). Ultra pure water (Milli-Q Plus System, >18.3 MΩ cm) was obtained in the laboratory.

Bovine individual milk and blood samples for 10 Holstein Friesian, 10 Brown Swiss, 10 Jersey and 10 Italian Simmental cows were collected directly in dairy herds. Preservative (Bronopol, 2-bromo-2-nitropropan-1,3-diol) was immediately added to raw milk samples at 0.6:100 (v/v) to prevent microbial growth and aliquots containing 0.8 ml of milk were frozen at -20°C. Milk samples were prepared following the method proposed by Bobe et al. (1998). No preliminary separation or precipitation procedures of the casein fraction were required.

HPLC Equipment

The HPLC equipment consisted of an Agilent 1100 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump (Agilent 1100 Series, G1312A). A variable-wavelength ultraviolet detector (Agilent 1200 Series, G1314B) was also used. The equipment was controlled by the Agilent ChemStation for LC Systems software which set solvent gradient, data acquisition and data processing. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5 µm, 300 Å, 150×4.6 I.D.). A Security Guard Cartridge System (product No. AJ0-4330, Phenomenex) was used as pre-column (widepore C4 4×3.0 mm). The sample vial was kept at constant low temperature (4°C) by a liquid refrigerator (Agilent 1200 series, G1330B) and injected via an auto-sampler (Agilent 1200 series, G1329 A). An injection loop of 100 µl was used.

Chromatographic Conditions

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile.

Separations were performed with the following program: linear gradient from 33% to 35% B in 5 min (0.4% B min^{-1}), from 35% to 37% B in 4 min (0.5% B min^{-1}), from 37% to 40% B in 9 min (0.33% B min^{-1}), from 40% to 41% B in 4 min (0.25% B min^{-1}), followed by an isocratic elution at 41% B during 5.5 min, then linear gradient from 41% to 43% B in 0.5 min (4% B min^{-1}), from 43% to 45% B in 8 min (0.25% B min^{-1}) and return linearly to the starting condition in 1 min. Before inject the following sample, the column was re-equilibrated under the starting conditions for 8 min. Therefore, the total analysis time per sample was 45 min.

The flow rate was 0.5 ml/min, the column temperature was kept at 45°C and the detection was made at a wavelength of 214 nm. The injection volume consisted of 5 µl.

Purified Proteins

Pure protein genetic variants were extracted for calibration experiments because other caseins and whey proteins are present as impurities in commercial standards and commercial standards for each genetic variant are not available.

Each protein genetic variant was purified by RP-HPLC, starting from individual milk samples of DNA-genotyped animals, and then lyophilized and weighted. For this purpose, the same elution conditions were used in semi-preparative experiments by collecting the major peaks (κ-CN variants A and B, α_{S2}-CN, α_{S1}-CN, β-CN variants A^1, A^2, B, and I). α-La, β-Lg_A and β-Lg_B were used without further purification. A semi-preparative Zorbax 300SB-C8 (5 µm, 300 Å, 9.4×250 mm, Agilent Technologies) column was used. The flow rate was 2 ml/min. After lyophilisation, purified proteins were stored at -20°C.

Because of the limited amount of protein obtainable from a semi-preparative method, purified genetic variants were only used to check their response coefficients and to quantifying the content of each protein genetic variant into a milk sample. This milk sample was stored at -196°C and used as standard for further analysis. The quantitative precision was improved by running this milk sample at the beginning of each analytical day to adjust for column-to-column and day-to-day variation.

DNA Genotyping

To ensure a higher variability of genotypes, individual milk and blood samples collected from Holstein Friesian, Brown Swiss, Jersey and Italian Simmental cows were used to check the correspondence between genotyping results provided by DNA-based techniques and RP-HPLC.

DNA was extracted from leukocytes by standard protocols and genotyped by direct sequencing. Primers and sequences are reported by Chessa et al. (2007).

Validation

Ten individual milk samples from Italian Simmental cows were used in the validation tests as this breed exhibits more balanced allelic frequencies when compared with Holstein Friesian, Brown Swiss and Jersey. Moreover, Italian Simmental shows a rather high occurrence of β-CN_I allele (Jann et al., 2002, 2004).

Before calibration, linearity was tested by running the same sample at 5, 10, 20, 40 and 80 µl in triplicate. The precision of the method was then evaluated by estimating the repeatability and reproducibility. Areas under all peaks on the chromatogram were used to validate the method.

For the validation procedure, 10 samples were analyzed daily, repeating the analysis of the same sequence over 4 days (sample injection volume = 5 µl). The reproducibility was calculated as the RSD of peaks area and elution times across days. The repeatability was studied by running 10 consecutive replications of the same sample and calculating the RSD for peaks area and elution times (sample injection volume = 5 µl). All the aliquots employed in validation were stored at -196 °C to prevent enzymatic lysis.

The accuracy was determined by quantifying each genetic variant in two samples and by repeating the quantification on different mixtures of them (at 75, 50 and 25%). Each mixture was analyzed in duplicate. The percentage recovery rate was calculated using the experimental response values and values provided by the calibration curves for the same quantity of analyte. Student's *t*-test was performed to assess whether the recovery rate differed statistically from 100% at $P < 0.05$.

The external standard method was used to calibrate the chromatographic system for protein quantifications. For this purpose, standard solutions consisting of purified protein genetic variants were used. Each solution was analyzed in duplicate.

Calibration curves were computed for each protein genetic variant by estimating parameters of the linear regression of the peak area on the amount injected, at increasing injection volume (5, 10, 20, 40 and 80 µl).

Certified Reference Material BCR-063R Skim Milk Powder

BCR-063R skim milk powder was supplied by the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium). The certified total Nitrogen content was 62.3±0.8 mg/g. Its 'true protein' content was calculated using the average conversion factor of 6.38 (Fox and McSweeney, 2003). The estimated protein concentration was therefore 39.75±1.02 g/100g. Three aliquots of BCR-063R were weighted, suspended and analyzed in duplicate.

RESULTS AND DISCUSSION

Separation

In this study, HPLC conditions were optimized for mobile phase composition, gradient, operating temperature and flow-rate. Retention times of the major eluted peaks coincided with retention times of standard protein fractions. It was therefore ascertained that proteins eluted in the following order: κ-CN, α_{S2}-CN, α_{S1}-CN, β-CN, α-La and β-Lg. It is worth to note that commercial standards were available for the mixture α-CN only, but not for α_{S2}-CN and α_{S1}-CN fractions and that the chromatographic profiles showed the contamination of the standards due to the presence of traces of other milk proteins.

The identification of peaks of genetic variants of milk proteins was confirmed by comparison with commercial standards that consisted of purified genetic variants (β-Lg variants A and B) or by comparison with chromatograms of individual milk samples of DNA-genotyped animals (Fig. 1).

For α_{S2}-CN, assignment was made on the basis of α_{S2}/α_{S1}-CN ratio, α_{S2}-CN being 10-12% of total protein (Walstra and Jenness. 1984).

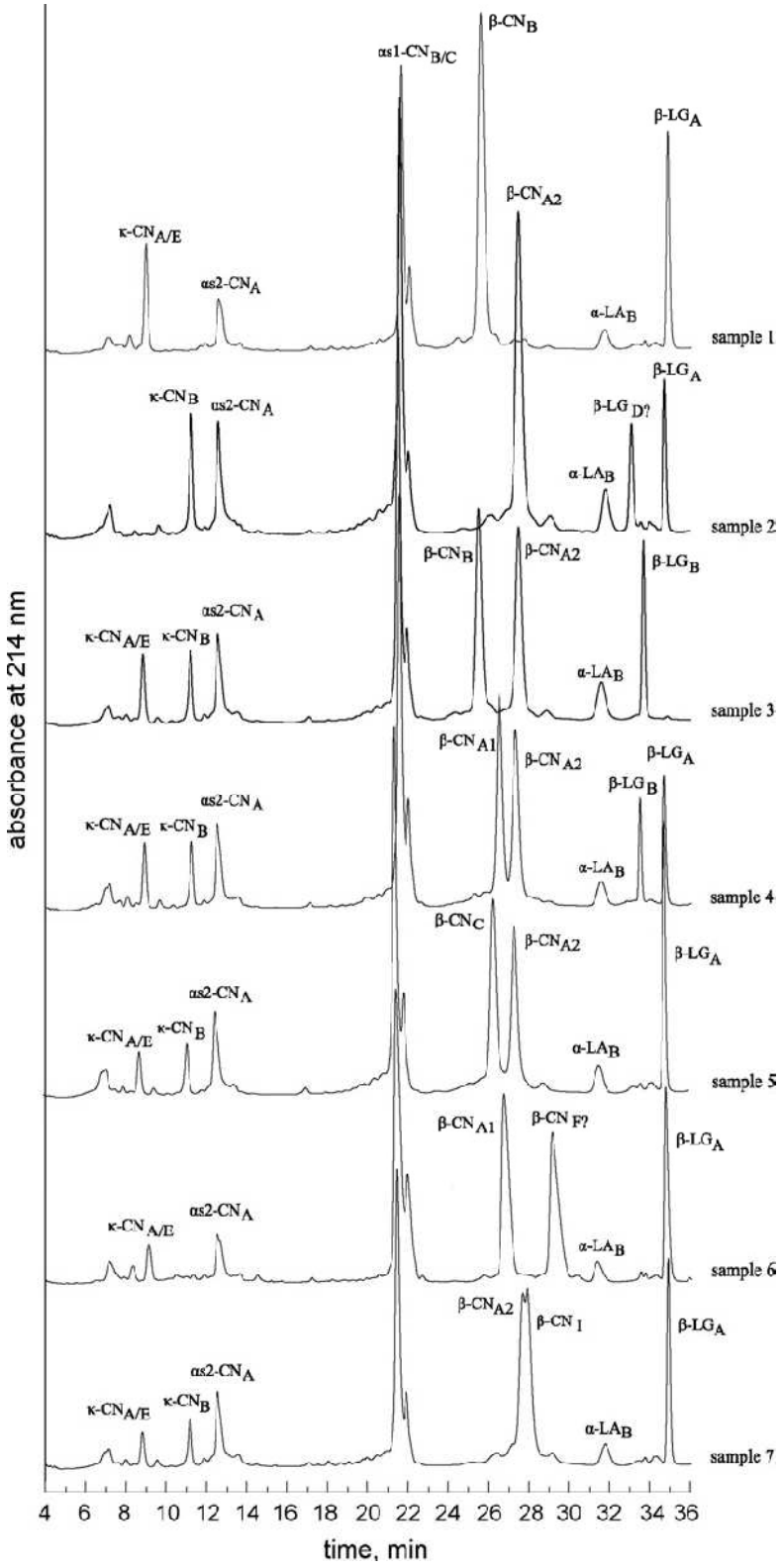
κ-CN eluted as several distinct peaks which consisted of glycosylated and unglycosylated forms of κ-CN_A and κ-CN_B (Farrell et al., 2001). Chromatograms from animals carrying different κ-CN genetic variants are reported in Fig. 2. The profile of the different forms of κ-CN was well resolved and it was very similar to those obtained by Visser et al. (1991). The main genetic variants A and B (glycol-free form A and B), together with their glycosylated forms were detectable. Glycosylated forms belonging to variant A and variant B were identified as the peaks that were not visible in κ-CN_{BB} and κ-CN_{AA} animals, respectively.

Under the conditions used, κ-CN_A and the infrequent variant κ-CN_E coeluted, but κ-$CN_{A/E}$ and κ-CN_B were perfectly resolved. κ-CN_E is an uncommon variant which has been detected in Italian Brown Swiss and Holstein Friesian cattle populations and exhibits a high frequency (~30%) in the Finnish Ayrshire (Formaggioni et al., 1999).

As previously reported by Bordin et al. (2001), α_{S2}-CN multiple peaks and shoulders were probably caused by the partial separation of differently phosphorylated forms of α_{S2}-CN (Farrell et al., 2004). Resolution between κ-CN_B and α_{S2}-CN was higher than 2.4, ensuring a complete separation.

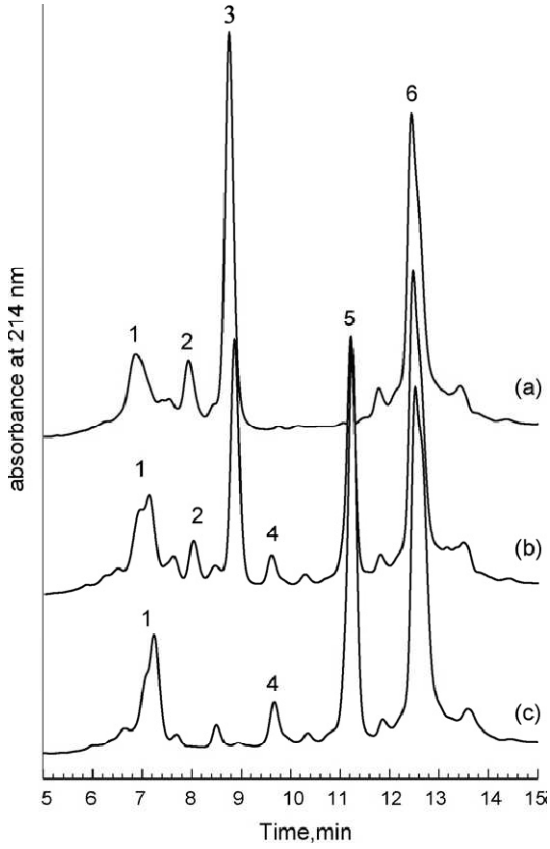
Separation of variants B and C of α_{S1}-CN was not feasible with the current method or by any other published RP-HPLC method (Visser et al., 1991; Bobe et al., 1998; Bordin et al., 2001). For homozygous animals, these two variants gave rise to a double peak, which is likely to occur for a difference in the degree of post-translational phosphorylation (Farrell et al., 2004). No individuals carrying α_{S1}-CN_A or α_{S1}-CN_D were found in the group of sampled animals and assessment of elution times for these two variants was not feasible.

Figure 1. Chromatograms relative to individual milk samples (samples 1-7) obtained using the optimized elution condition: Zorbax 300SB-C8 RP (Agilent Technologies); linear gradient from 33% to 35% B in 5 min (0.4% B min^{-1}), from 35% to 37% B in 4 min (0.5% B min^{-1}), from 37% to 40% B in 9 min (0.33% B min^{-1}), from 40% to 41% B in 4 min (0.25% B min^{-1}), followed by an isocratic elution at 41% B during 5.5 min, then linear gradient from 41% to 43% B in 0.5 min (4% B min^{-1}), from 43% to 45% B in 8 min (0.25% B min^{-1}) and return linearly to the starting condition in 1 min, where solvent A consisted of 0.1% trifluoroacetic acid (TFA) in ultra pure water and solvent B was 0.1% TFA in acetonitrile; flow rate 0.5 ml/min at 45°C; UV detection at 214 nm.



For A^1, A^2 and B variants of β-CN, all peaks were well resolved. The most difficult separation, the one between A^1 and A^2 variants, was achieved by an isocratic elution at 41% of solvent B. The resolution between these two peaks exceeded 1.15, which is considered satisfactory in chromatographic separations. As reported by Bobe et al. (1998), the C variant, which is a rare variant, partially coeluted with A^1 variant, albeit differences in peak shape allowed detection of heterozygous individuals CA^1. Peak for variant C was well resolved and recognizable in heterozygous individuals in absence of A^1 variant. Variant I of β-CN was detectable albeit it partially coeluted with A^2 variant. Hence, our method ensured the discrimination between homozygous I and heterozygous IA^2 individuals. To our knowledge, this is achieved for the first time at protein level. Up to date, the detection of genetic polymorphisms of milk proteins has been mainly achieved through various electrophoretic techniques, but these methods are restricted to separation of proteins differing in net charge caused by amino acid substitution. Differences between β-CN A^2 and I variants are caused by a neutral amino acid exchange and will not be detectable by electrophoretic techniques at protein level (Jann et al., 2002). Likely, the peak eluting at approximately 29.5 min is attributable to $\beta\text{-CN}_F$ (Visser et al., 1995).

Figure 2. Chromatograms relative to individual milk samples of DNA-genotyped cows: (a) $\kappa\text{-CN}_{AA}$, (b) $\kappa\text{-CN}_{AB}$ and (c) $\kappa\text{-CN}_{BB}$; peak 1: glycosylated form κ-CN variant A and B; peak 2: glycosylated form κ-CN variant A; peak 3: unglycosylated form κ-CN variant A; peak 4: glycosylated form κ-CN variant B; peak 5: unglycosylated form κ-CN variant B; peak 6: α_{S2}-CN.



Under the conditions used and as reported for other RP-HPLC methods that analyzed whole milk (Bobe et al., 1998), γ-CNs (proteolytic products of β-CNs) partially coeluted with β-CN. The same absorption coefficient for γ-CNs and the respective β-CN variants were assumed. Detection of γ-CNs was made after comparison with chromatograms of commercial standards incubated with plasmin at time 0 and after 20, 60 and 120 min (Bastian and Brown, 1996). Plasmin action affected resolution between β-CN_{A1} and β-CN_{A2} as it gave rise to a proteolytic peak just between them. Moreover, plasmin activity caused a decrease of resolution between κ-CN and α_{S2}-CN by increasing the background noise (data not shown).

α-LA eluted after β-CNs group and it is monomorphic in variant B in all western breeds (Formaggioni et al., 1999). For β-Lg, variant B eluted before variant A. As reported by Bordin et al. (2001), β-Lg_A exhibited a minor peak followed by a main one and the relative proportion between the size of the peaks can be considered as an indicator of the extent of proteolysis occurred. A third β-Lg genetic variant, likely β-Lg_D (Formaggioni et al., 1999), eluted immediately before B variant in samples belonging to Simmental cows.

On the basis of comparisons with separate injections of standards, it was observed that BSA eluted just before β-Lg_B whereas Igs coeluted with α_{S2}-CN. However, both BSA and Igs were not detectable when analyzing individual milk samples.

There was full agreement between HPLC-inferred genotypes and genotyping results provided by DNA-based techniques, with the only exceptions of chromatographic discriminations between κ-CN A and E and between α_{S1}-CN B and C. In some cases, there were uncertainties in the assignment of genotypes by DNA-based analysis when β-CN_I was present.

Detection wavelength at 214 nm was preferred over 220 and 280 nm because it improved the baseline (less noise), the peak efficiency and the resolution of the protein peaks. Temperature exerted a significant effect on the resolution between milk proteins. In particular, increasing the column temperature from 45 to 50 °C improved the resolution between all β-CN variants, mostly the one between β-CN_{A1} and β-CN_{A2}, whereas κ-CN_B and α_{S2}-CN coeluted. Whey proteins were badly resolved and tended to coelute with β-CNs when temperature was higher than 45 °C. These results suggest the use of a temperature gradient to improve the overall separation. In agreement with results by Bordin et al. (2001), temperature had no effect on the resolution of κ-CN variants.

Differently to other investigations using long-chain columns (Visser et al., 1991; Groen et al., 1994), no carryover effect caused by column adsorption was observed in this study with the exception of the carryover ascribed to proteins retained in the pre-column. To prevent the worsening of separation caused by enzymatic lysis of proteins, milk samples should be stored at -20°C immediately after collection and analyzed within few days. In addiction to preservative, enzymatic inhibitors might be used to limit plasmin activity.

Quantification

All former RP-HPLC quantifications of milk proteins have been based on the use of bulk-tank milk. In addition, calibration curves have been derived from parameters of regressions computed for whole protein fractions by using commercial standards which contain a number of genetic variants per fraction. When quantification by RP-HPLC is performed for individual milk samples, response factors for single variants have to be considered because, for homozygous animals, a whole protein fraction consists of a single variant.

Linearity

From 5 to 80 µl of injection volume, the relation between peak area and injected amount of protein variant was linear ($R^2 > 0.99$; data not shown). Parameters of calibration curves are reported in Table 1. As a consequence of small errors in the integration phase, intercepts of regression equations were statistically different from zero at $P < 0.05$ for most protein variants with the exception of whey proteins, α_{S2}-CN and κ-CN_A. For all peaks, there was a very tight relationship between the amount of protein and the detector response as indicated by R^2 values that exceeded 0.99.

Table 1. Parameters of regression equations for calibration curves, response factors, and limit of detection (LOD) for single protein genetic variants or protein fractions[1].

Protein	Intercept ± SE	Slope ± SE	R^2	Response ± SD (µg /area) $\cdot 10^3$	LOD (µg)	Injected amount (µg)	Theoretical plates ($N \cdot 10^3$)
κ-CN_A	1.29 ± 18.23	1169.6 ± 5.01	0.9999	0.86 ± 0.01	0.5	3.5 – 56.4	7.83
κ-CN_B	-77.3 ± 16.74	923.8 ± 4.67	0.9999	1.16 ± 0.06	0.6	3.5 – 55.6	10.22
α_{S2}-CN	-35.8 ± 37.15	788.3 ± 7.91	0.9999	1.37 ± 0.15	0.7	2.3 – 37.2	2.16
α_{S1}-CN	-138.3 ± 52.88	766.3 ± 7.73	0.9998	1.44 ± 0.17	0.7	6.6 – 106.0	15.57
β-CN_B	-150.1 ± 25.86	693.6 ± 3.99	0.9999	1.62 ± 0.19	0.9	6.3 – 100.4	22.24
β-CN_{A1}	-209.3 ± 32.35	1013.2 ± 4.88	0.9999	1.13 ± 0.18	0.6	6.4 – 102.8	23.02
β-CN_{A2}	-186.2 ± 52.04	1140.1 ± 7.88	0.9999	0.97 ± 0.11	0.5	6.4 – 102.4	21.23
β-CN_I	-263.4 ± 46.67	924.6 ± 7.17	0.9998	1.25 ± 0.15	0.7	6.3 – 100.4	34.12
α-La	-122.7 ± 73.56	1004.2 ± 9.67	0.9974	1.14 ± 0.10	0.6	1.1 – 17.6	20.62
β-Lg_B	-11.2 ± 9.63	755.9 ± 3.01	0.9999	1.34 ± 0.02	0.7	3.1 – 49.6	120.69
β-Lg_A	7.3 ± 37.77	1066.4 ± 9.31	0.9999	0.93 ± 0.01	0.5	3.6 – 56.8	153.28

[1] Separated solutions of purified protein genetic variants injected at volume of 5, 10, 20, 40 and 80 µl in duplicate.
LOD = 10×(3×SD) where SD is the standard deviation of the background noise.
For computation of the number of theoretical plates, peak width at the baseline was obtained by tangential lines drawn at half-height; for α_{S2}-CN, peak width was calculated including all major and minor peaks.

At a wavelength of 214 nm, response factors were of different magnitude for different protein variants. Conversely, absorbance coefficients were homogeneous at 280 nm (data not shown). At 280 nm the absorbance of proteins is almost exclusively due to the aromatic amino acids content,

which is the same for all variants within a protein fraction, whereas at 214 nm absorbance coefficients also depend upon the secondary structure of the protein (Rosenheck and Doty, 1961). Because variation of response across variants is large, specific coefficients have to be used when working with individual milk samples.

For milk samples used in the validation procedure, CN content was nearly 87% of total protein. Within caseins, α_{S1}-CN was less than 50% and β-CN roughly one third of total CN, while κ-CN and α_{S2}-CN accounted for about 10% of total CN. These results are in agreement with average content values for bovine milk reported in the literature (Walstra and Jenness. 1984; Fox and McSweeney, 2003). Because non-protein nitrogen, proteose-peptones and minor constituents of whey protein were not detectable, the CN content of samples used in our study was greater than the average CN content of fresh milk reported in other investigations.

Table 2. Relative standard deviation of retention times and peak areas for milk proteins fractions or genetic variants obtained in the analysis of repeatability and reproducibility.

Protein	Repeatability[1]		Reproducibility[2]		
	Retention time R.S.D. (%)	Area R.S.D. (%)	Retention time R.S.D. (%)	Area R.S.D. (%)	Samples (*n*)
κ-CN_A	0.51	1.53	2.01	1.17	7
κ-CN_B	0.32	0.92	1.41	1.21	6
α_{S2}-CN	0.15	4.32	0.71	9.52	10
α_{S1}-CN	0.14	1.68	0.63	0.85	10
β-CN_B	-	-	0.87	2.78	2
β-CN_{A1}	0.28	2.14	0.90	2.59	5
β-CN_{A2}	0.29	1.73	0.91	2.12	9
β-CN_I	-	-	0.87	2.45	1
α-La	0.23	1.82	0.70	6.54	10
β-Lg_B	0.12	1.30	0.31	5.34	7
β-Lg_A	0.12	1.08	0.28	4.22	9

[1] Ten aliquots of the same individual raw milk sample were injected consecutively.
[2] A sequence of 10 individual raw milk samples was injected over 4 days.

Repeatability and Reproducibility

Values of RSD for retention times and peak areas obtained in the analysis of repeatability and reproducibility are presented in Table 2. All RSD values were similar to those reported in literature for within- and between-days variation (Bobe et al., 1998; Bordin et al., 2001). Results indicate that the precision (repeatability and reproducibility) of the method was acceptable. The RSD values for retention times were below 0.51% within analytical day (repeatability) and below 2.01% across analytical days (reproducibility). Values of RSD for peak areas were below 4.32% within day and below 6.54% across days with the exception of RSD for α_{S2}-CN. Likely, α_{S2}-CN response was

markedly affected by pre-column conditions, as roughly a half of the injected amount was retained at this level. Pre-column conditions might have also affected the reproducibility of quantification of whey proteins. Thus, a frequent guard-cartridge turnover is advisable. In addition, a blank injection might be used after each sample run. In our study, a blank injection, consisting of 1 part of buffer and 3 parts of solvent (4.5 M of GdnHCl in TFA:ACN:$H_2$0,1:100:900) improved the precision of the method (data not shown). The "cleaning run" was an isocratic elution at 45% solvent B over 5 min with a flow rate of 0.8 ml/ml. The re-equilibrium was introduced after each cleaning and not at the end of the analytical run. Thus, including the cleaning run, the total analysis time per sample was 46 min.

Accuracy

Recovery studies were carried out to determine the accuracy of the method (Table 3). Recoveries ranged from 98.9% for α_{S2}-CN content to 103.4% for β-Lg_B content and were close to 100% for all other proteins. Results of Student's *t*-test indicated that recovery rates were not significantly different from 100% at $P < 0.05$.

Table 3. Results of the analysis of accuracy[1].

Protein	Recovery rate (%)	R.S.D. (%)	*t*-test ($t_{(0.05,2)} = \pm 4.30$)
κ-CN_A	100.27	4.31	0.15
κ-CN_B	103.13	4.37	1.70
α_{S2}-CN	98.10	3.88	-1.22
α_{S1}-CN	100.93	1.85	1.23
β-CN_{A1}	103.66	4.33	2.00
β-CN_{A2}	100.69	1.78	0.93
α-La	102.54	3.08	1.97
β-Lg_B	103.36	3.89	2.05
β-Lg_A	101.16	1.56	1.80

[1]Mixtures of two raw milk samples were obtained following relative proportions of 75, 50 and 25%. Mixtures and whole samples were analysed in duplicate and recovery rates were calculated using expected areas provided by calibration curves and observed areas.

Certified Reference Material BCR-063R Skim Milk Powder

Due to spray drying treatment, milk powders showed chromatograms of poorer quality in comparison with those obtained from fresh milk. The RP-HPLC method estimated total protein content of BCR-063R to be 38.07±0.67 g/100g. The estimated content was in good agreement with the one reported by the manufacturer which was 39.75±1.02 g/100g. As expected, the total protein content was slightly underestimated because the amount of non-proteic-nitrogen was not measurable by RP-HPLC. Total casein of BCR-063R was 93.33±0.13% of the estimated total

protein, with α_{S1}-CN and β-CN accounting for roughly 38, and 43% of total protein, respectively, and κ-CN and α_{S2}-CN accounting for 9.5% of total protein. In agreement with results reported by Bordin et al. (2001) for powder milk samples, whey proteins content of BCR-063R measured by our method was lower, as a consequence of heat sensitivity of these proteins, than the average content of whey proteins of raw milk.

CONCLUSIONS

In this study, a new RP-HPLC method for separation and quantification of milk protein genetic variants was developed. The method allows the separation of all milk protein fractions and genetic variants in one run with very high resolutions. It allows a decrease of 20 min of the total run time if compared to the method proposed by Bordin et al. (2001). In addition, it ensures a precise quantification of most common genetic variants based on specific response factors and it might be a useful tool for studies aiming at measurement of milk protein composition.

In conclusion, this procedure is suitable for routine analysis of raw individual and bulk-tank milk samples as no complex sample preparation prior to injection is required and the total analysis time per sample is considerably short if considering the great amount of information that the method can provide.

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Chapter 2.

Effects of *CSN2-CSN3* haplotype and *BLG* genotype on milk production traits, contents of protein fractions and detailed protein composition 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 and *BLG* (β-Lactoglobulin) genotypes on milk production traits, contents of protein fractions and detailed protein composition of individual milk of Simmental cows. Contents of major protein fractions were measured by reversed-phase HPLC in individual milk samples of 2,167 cows. Protein composition was measured as weight percentage of each casein fraction to total casein and as weight percentage of β-Lactoglobulin (**β-LG**) to total whey protein. Genotypes at *CSN2*, *CSN3* and *BLG* loci were also assessed by HPLC and *CSN2-CSN3* haplotype probabilities were estimated for each cow. Traits were analyzed with a linear model including the fixed effects of herd-test day, parity, days in milk and somatic cell score class, linear regressions on haplotype probabilities, *BLG* genotype and the random effect of the sire of the cow. Effects of haplotypes and *BLG* genotypes on yields were weak or trivial. Haplotypes carrying *CSN2* B and *CSN3* B exhibited greater casein (**CN**) content and casein number, in comparison with all other haplotypes, but no differences in whey protein content. Genotype BB at *BLG* was associated to increased protein and CN contents and casein number when compared to genotype AA. Haplotypes including *CSN3* B were associated with greater κ-CN content and κ-CN to total CN ratio compared with those carrying the A variant. The increase of κ-CN to total CN ratio was coupled with decreased α_{S1}- and γ-CN to total CN ratios. Allele *CSN2* B was associated with an increase of β-CN content, which occurred at the expense of content of α_{S1}-CN. Haplotypes including allele *CSN2* A^1 exhibited decreased β-, α_{S2}- and γ-CN concentrations and increased α_{S1}- and κ-CN contents, whereas *CSN2* I exerted positive effects on β-CN concentration, without altering other protein fractions content. Effects exerted by haplotypes on CN composition were similar to those exhibited on CN fractions contents. Allele *BLG* A increased β-LG concentration and altered the β-LG to α-Lactalbumin ratio. The higher

relative concentration of β-LG associated with the A variant was compensated by a lower concentration of all other milk proteins, in particular of β-CN and α_{S1}-CN. Estimated additive genetic variance for investigated traits ranged from 14 to 39 % of total variance. Increasing the frequency of specific genotypes or haplotypes might be an effective way for altering milk protein composition.

INTRODUCTION

Most research concerning milk protein polymorphisms focused on the associations of *CSN3* (κ-CN) and *BLG* (β-LG) polymorphisms with milk production traits, coagulation time, curd firmness and cheese yield. Results for the association between milk protein genetic variants and production traits are inconsistent across studies (Braunschweig et al., 2000; Ikonen et al., 2001; Boettcher et al., 2004a), but the B allele of *CSN3* has been consistently reported to be associated with improved milk clotting properties (Schaar, 1984) and increased cheese yields (Schaar et al., 1985; Caroli et al., 2000).

Milk coagulation properties have been reported to be influenced also by protein composition (Wedholm et al., 2006; Jõudo et al., 2008). A few studies investigated the role of milk protein polymorphisms on protein composition and focused mostly on *CSN3* and *BLG* loci. Significant effects of A and B alleles at *BLG* and *CSN3* on the concentration of β-LG, κ-CN, and total casein in milk have been reported (Lunden et al., 1997; Mayer et al., 1997; Robitaille et al., 2002) and genetically linked polymorphisms in the non-coding regions of these genes are assumed to affect transcription of proteins (Martin et al., 2002). Variation in casein composition has been reported also for *CSN2* (β-CN) variants (Heck et al., 2009), but knowledge on the effects exerted by *CSN2* B allele is scarce because of its limited frequency in all previously investigated cattle populations.

The effects of polymorphisms at milk protein loci on all major protein fractions concentrations or relative ratios have been investigated using limited numbers of animals (Bobe et al., 1999; Hallén et al., 2008), because of the difficulty of quantifying all major milk proteins simultaneously, and genotypes rather than haplotypes effects have been considered in all those studies. Genes encoding α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN are located on chromosome 6, within a region of about 250 kb (Ferretti et al., 1990; Threadgill and Womack, 1990), making the estimation of the effects of single CN genes difficult (Lien et al., 1995). For this, evaluation of the effects of casein haplotypes should be preferred to the estimation of genotypes effects (Ikonen et al., 2001; Boettcher et al., 2004a). Recently, for the first time, the effects exerted by casein haplotypes on milk protein composition have been investigated using a large sample of Dutch Holstein Friesian cows (Heck et al., 2009).

With few exceptions (Bobe et al., 1999; Heck et al., 2009), estimation of milk protein

polymorphisms effects on detailed protein composition has been performed using ordinary least squares procedures and effects due to polygenes have been ignored. As a consequence, estimated effects of milk protein loci might be biased (Kennedy et al., 1992).

Despite a wide spreading of the breed, only few studies have been carried out on the variation of milk protein composition in Simmental cattle (Pérez-Rodríguez et al, 1998; Amigo et al., 2001; Graml and Pirchner, 2003). The Simmental breed exhibits more balanced allele frequencies within each locus if compared with other breeds where frequency of some alleles (e.g., *CSN3* B or *CSN2* B) is very high or very low. This facilitates the estimation of haplotype and genotype effects. Moreover, this breed exhibits a moderate presence of allele *CSN2* I whose effects have never been reported previously.

The aim of this study was to estimate the effects exerted by casein haplotypes and *BLG* genotypes on milk production traits, protein fractions concentrations and detailed milk protein composition in individual milk samples of Italian Simmental cows.

MATERIALS AND METHODS

Animals and Milk Sampling

Individual milk samples of 2,167 Simmental cows, distributed across 47 commercial herds in the north of Italy, were collected from November 2007 to December 2008. Milk sampling occurred once per animal, during the morning or evening milking, concurrently with the monthly milk recording of the herd. Herd and test-day effects were confounded because all cows of a herd were sampled in the same test day.

Milk was added with preservative (Bronopol, 0.6:100 vol/vol) immediately after collection, to prevent microbial growth, and stored at –40°C until reversed-phase (RP-) HPLC analysis, to prevent enzymatic proteolysis. Procedures used in the national milk recording program for the assessment of fat and protein contents are based on mid-infrared spectroscopy and a Fourier-transformed interferogram (MilkoScan FT 6000, Foss Elecrtic, Hillerød, Denmark). Milk yield records and pedigree information were supplied by the Italian Simmental cattle breeders association (ANAPRI, Udine, Italy).

Milk Protein Composition and Genotyping

Contents of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN, κ-CN, β-LG, and α-LA of individual milk samples were measured using the RP-HPLC method proposed by Bonfatti et al. (2008). Genotypes of cows for *CSN2*, *CSN3*, and *BLG*, were also derived by RP-HPLC. Briefly, the method ensures the separation of all major casein and whey protein fractions in one run, with very high resolutions, and provides

the quantification of A[1], A[2], B and I variants of β-CN, A and B variants of κ-CN, and A, B and D variants of β-LG. When analyzing individual milk samples of homozygous animals, a whole protein fraction consists of a single genetic variant, and quantification performed by RP-HPLC can be affected by the response factor of the specific protein form. Since purified protein genetic variants were used to develop specific calibration equations, the method used in this study was suitable for the quantification of protein fractions contents in individual milk samples. A detailed description of the RP-HPLC technique used in this study can be found in Bonfatti et al. (2008).

Table 1. Descriptive statistics for milk production traits, protein fractions contents and protein composition (n = 2,167)

Trait	Mean	SD	Minimum	Maximum
Milk yield, kg/d	26.18	7.44	7.00	50.40
Fat yield, kg/d	1.02	0.32	0.22	2.62
Protein yield, kg/d	0.92	0.23	0.27	1.70
Fat, %	3.92	0.72	1.36	6.98
Protein, %	3.56	0.37	2.50	5.12
SCS[1]	2.67	1.95	-2.05	10.34
Protein measured by reversed-phase HPLC[2]				
Protein, g/L	38.78	4.54	19.58	59.16
Casein, g/L	33.88	3.40	16.92	51.76
Whey protein, g/L	4.91	0.71	2.58	7.63
Casein number, %	87.35	1.11	84.20	91.26
Protein fractions content[3], g/L				
α_{S1}-CN	11.38	1.41	5.82	17.38
α_{S2}-CN	4.23	0.74	1.74	7.24
β-CN	12.99	2.02	5.99	22.83
γ-CN	1.62	0.45	0.23	4.51
κ-CN	3.66	0.85	1.29	6.84
α-LA	1.25	0.23	0.53	2.10
β-LG	3.66	0.61	1.76	6.13
Protein composition[4], %				
α_{S1}-CN%	35.61	2.65	28.00	45.46
α_{S2}-CN%	12.11	1.60	5.39	18.36
β-CN%	37.14	2.90	27.31	46.02
γ-CN%	4.68	1.33	0.77	12.56
κ-CN%	10.46	1.95	4.70	17.57
β-LG%	74.49	3.79	61.25	87.38

[1]$SCS = \log_2(SCC \times 10^3) + 3$;

[2]Casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA ; protein = casein + whey protein; casein number = (casein/protein) × 100;

[3]Contents of all protein fractions were measured by reversed-phase HPLC on skimmed milk;

[4]α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content.

Because of the very low frequency of *BLG* D, records of cows carrying this allele were not considered in the statistical analysis. The RP-HPLC method did not allow the discrimination between *CSN1S1* (α_{S1}-CN) alleles. Because the frequency of the C allele at *CSN1S1* has been reported to range from 0.08 to 0.11 for Simmental cattle (Pérez-Rodríguez et al, 1998; Jann et al., 2004), suitability of genotyping based on alternative techniques was carefully evaluated in terms of benefits and disadvantages. This involved the assessment of genotypes at *CSN1S1* in a random sample of 200 cows by direct DNA sequencing. Portion of exon 17 of *CSN1S1* (allele B and C discriminating fragment) was amplified by standard PCR using primers Csn1S1_ex17f 5'-TGGATGCCTATCCATCTGGT-3' and Csn1S1_ex17r 5'-CACTGCTCCACATGTTCCTG-3'. Amplicons (333bp) were directly sequenced using Csn1S1_ex17f primer on a CEQ8000 sequencer (BeckmanCoulter, Fullerton, CA, USA). Because of the very low frequency (less than 6%) of the *CSN1S1* C allele, genotyping through direct DNA sequencing was not extended to all milk samples, and *CSN1S1* locus was not considered when computing haplotypes probabilities. With such a limited frequency, haplotypes carrying the C allele at *CSN1S1* would have been included in the group of "rare" haplotypes or would have obtained a trivial haplotype probability, thus limiting the reliability of the estimated haplotype effects and jeopardizing expected advantages arising from the gain of additional gene information. Furthermore, a very limited bias is to be expected when neglecting *CSN1S1* contribution to haplotype definition.

Frequency of *CSN3* E allele, which cannot be differentiated from *CSN3* A by RP-HPLC, is known to be quite low for the Simmental breed and ranges from 4%, for Czech Simmental (Matějíček et al., 2008), to 0% for Italian Simmental (Jann et al., 2004), albeit the allele has been detected in other cattle breeds (Ikonen et al., 1999a; Heck et al., 2009). Due to its low frequency, the incidence of *CSN3* E was not assessed by direct DNA sequencing. Thus, the estimated effects for *CSN3* A might be slightly biased being the combined effects of *CSN3* A and E variants.

Despite the partial co-elution of β-CN I with A^2 variant, the double peak of heterozygous A^2I samples exhibited a typical shape that was easily recognizable. Also, heterozygous A^1I and BI milks exhibited a characteristic shape of the peaks valley so that genotyping errors were unlikely. Shifts of the chromatogram were rare and usually homogeneous in the whole β-CN elution interval. More likely, genotyping errors occurred for A^2A^2 and II samples.

Fraction γ-CN consisted of a number of minor peaks eluting close to β-CN, or partially co-eluting with it. Although constituted by multiple peaks characterized by low resolutions, total γ-CN exhibited good repeatability and reproducibility with values similar to those obtained by Bonfatti et al. (2008) for the other protein fractions, and can be used as an indicator of the extent of proteolysis occurred in the milk sample.

Traits Definition

For proteins quantified by RP-HPLC, total casein (**TCN**, g/L) was computed as the sum of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN and κ-CN contents of milk. Total whey protein (**WH**, g/L) was calculated as the sum of α-LA and β-LG contents. Total protein (**PRT**, g/L) was expressed as the sum of casein and whey protein contents. Casein number (**CI**, %) was calculated as the percentage ratio of TCN to PRT. Protein composition (i.e., relative contents of protein fractions) was computed as weight percentage ratio of α_{S1}-CN (**α_{S1}-CN%**, %), α_{S2}-CN (**α_{S2}-CN%**, %), β-CN (**β-CN%**, %), γ-CN (**γ-CN%**, %), and κ-CN (**κ-CN%**, %) to TCN and as the percentage ratio of β-LG to WH (**β-LG%,** %). When protein fractions are expressed as percentage ratios of PRT, for a certain haplotype associated with increased CI, the percentage ratios of all casein fractions are expected to increase whereas those of whey fractions is expected to decrease. Expressing protein composition as percentage ratios of the TCN or total WH ensures that estimations of effects will not be affected by simultaneous variations in CI.

Computation of Haplotypes Probabilities

Sires families of limited size occur rather commonly in the Italian Simmental population. For 60% of the sires, the family size was between 2 and 5 daughters, for 17.2% was between 6 and 20, and for 10.4% between 21 and 50 daughters. Only 3% of the sires had more than 50 daughters. Because sire genotypes were unknown, but also to avoid problems related to the attribution of haplotypes in families of very small size that would have markedly reduced the size of the dataset, we estimated haplotypes probabilities. Cows (2,016 animals) originated by sires having at least 2 genotyped daughters were used to estimate *CSN2-CSN3* haplotypes probabilities. Two animals had genotypes that were incompatible with observed haplotypes in their families and were discarded. The probability of haplotypes inherited by each daughter was estimated using the method proposed by Boettcher et al. (2004b).

Computation of haplotypes probabilities was carried out by assuming no recombination events across CN genes. The expected number of copies of each possible haplotype carried by an animal was indirectly derived from the estimated probabilities. To test the null hypothesis that genotypes at one locus were independent upon genotypes at another locus (i.e., to test the hypothesis of linkage equilibrium between loci), GENEPOP Version 4.0 was used. The program creates contingency tables for all pairs of loci in each sample and then performs a probability test for each table using a Monte Carlo Markov chain method. Hardy Weinberg equilibrium was also verified for each locus by the Monte Carlo Markov chain method. Values of r^2 for the haplotype *CSN3-BLG* were computed following the method proposed by Hill and Robertson (1968) and Zhao et al. (2005), and used by Heck et al. (2009).

Statistical Analysis

Somatic cell score (**SCS**) was computed as $\log_2(\text{SCC} \times 10^3) + 3$. The effects of casein haplotypes and *BLG* genotypes on phenotypic variation of traits of concern were estimated using a linear mixed model with the MIXED procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC). The mixed model was:

$$y_{ijklmno} = HTD_i + P_j + DIM_k + SCS_l + \sum_{s=1}^{h} \beta_s PR_{s:ijklmno} + BLG_m + sire_n + e_{ijklmno}$$

where $y_{ijklmno}$ is a measure for a trait; HTD_i is the fixed effect of herd-test day i (i = 1, …, 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 SCS class l (6 classes of 1-point intervals, with the exception of the last class which included records with SCS $\geq$ 5); h is the total number of haplotypes evaluated (h = 8); $PR_{s:ijklmno}$ is the probability that the cow carried haplotype s, expressed as the expected number of copies of haplotype s carried by the animal; β_s is the regression coefficient on the probability of haplotype s; BLG_m is the fixed effect of *BLG* genotype m (m = 1: AA, m = 2: AB, m = 3: BB); $sire_n$ is the random effect of the sire of the cow (n = 1, …, 211) assumed to follow a normal distribution with $sire_n \sim N(\mathbf{0}, \sigma_s^2)$, where σ_s^2 is the sire variance; and $e_{ijklmno}$ is a random residual assumed to follow a normal distribution with $e_{ijklmno} \sim N(\mathbf{0}, \sigma_e^2)$, where σ_e^2 is the residual variance.

A probability (ranging from 0 to 1) was assigned to each possible haplotype carried by an animal. For example, if the only possible haplotype couple was composed by two copies of the same haplotype, that haplotype gained a final value of 2 because the animal was expected to carry 2 copies of that haplotype, each one with a probability of 1.

Hence, the difference between the estimated regression coefficients for two haplotypes of interest provides a quantification of 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 for only one haplotype, 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., 2004a). Only additive effects of haplotypes were estimated and dominance effects were not considered. Sire effects were included in the model to account for variation due to polygenes underlying the investigated traits. All analyses and hypothesis testing were carried out using the MIXED procedure of SAS.

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics for the investigated traits are reported in Table 1. The difference between average protein content assessed in the milk recording program and average protein content measured by RP-HPLC was large. This must be ascribed to the skimming of milk prior to chromatographic analysis and to the specific gravity of milk: protein content assessed by HPLC was measured in weight per volume whereas protein content quantified in the milk recording program was measured in weight per weight. After adjusting for skimming and specific gravity of milk, averages of the two traits were comparable. However, since protein content provided by mid-infrared spectroscopy consisted in a prediction of the content of crude protein (including the NPN fraction), higher values for this trait are to be expected in comparison with protein content measured by RP-HPLC (which do not include NPN and minor protein fractions). Nevertheless, protein content measured by HPLC was constantly greater than that recorded in the milk recording program. This is attributable to more favorable preservation conditions guaranteed for sample aliquots analyzed by HPLC than for samples collected in the milk recording program. Differences between measures of milk protein content provided by RP-HPLC and those obtained in the milk recording program increased in summer, when preservation of samples is more critical (data not shown).

Table 2. Genotype and allele frequencies for *CSN2* (β-CN), *CSN3* (κ-CN), and *BLG* (β-LG) loci (n = 2,167)

Locus	Genotype frequency		Allele frequency	
	Genotype	Frequency	Allele	Frequency
CSN2	A^2A^2	0.362	A^2	0.596
	A^1A^2	0.224	A^1	0.188
	A^2B	0.177	B	0.158
	A^1B	0.065	I	0.058
	A^2I	0.064		
	A^1A^1	0.036		
	BB	0.025		
	BI	0.022		
	A^1I	0.019		
	II	0.006		
CSN3	AA	0.438	A	0.652
	AB	0.432	B	0.348
	BB	0.130		
BLG	AB	0.466	A	0.543
	AA	0.306	B	0.449
	BB	0.213	D	0.008
	AD	0.008		
	BD	0.007		

As a consequence of lack of NPN and minor serum proteins, high values of CI, ranging from 84 to 91%, were observed. This was in agreement with results reported by Heck et al. (2009), who used capillary electrophoresis for the analysis of individual milk samples of Dutch Holstein Friesian cows.

Alleles and Haplotype Frequencies

Frequencies of genotypes and alleles, obtained by gene counting, for *CSN2*, *CSN3* and *BLG* are presented in Table 2. To our knowledge, it is the first time that these frequencies are computed investigating a large sample of Simmental cows. Four alleles (A^1, A^2, B and I) for *CSN2*, 2 alleles (A and B) for *CSN3* and 3 alleles (A, B and D) for *BLG* were present in the sampled population.

Conversely to other breeds in which some alleles at CN loci have very high or very low frequencies, rather balanced allelic frequencies were detected at all investigated loci and mostly at *CSN2* where B allele showed a moderate frequency (0.16). With the only exception of *CSN2* I, allelic frequencies were in good agreement with those reported by Jann et al. (2004) for a sample of Simmental cows of limited size. Almost 30 % of animals carried at least one copy of *CSN2* B allele and the frequency of *CSN3* BB animals (n = 282) was rather high. Within the studied loci, only *CSN2* genotype frequencies were in Hardy-Weinberg equilibrium ($P > 0.05$).

Table 3. Frequency of *CSN2-CSN3* haplotypes (n = 2,016)

Haplotype	Frequency
A^2A	0.387
A^2B	0.207
A^1A	0.128
A^1B	0.059
BA	0.119
BB	0.040
IA	0.016
IB	0.043

Similarly to results for other breeds (Ikonen et al., 2001; Boettcher et al., 2004a), linkage disequilibrium between *CSN2* and *CSN3* loci has been detected ($P < 0.001$). Following haplotype reconstruction, 8 *CNS2-CSN3* haplotypes, including all combinations between *CNS2* and *CSN3* alleles, were identified. Haplotypes were assigned with a probability of 1 to 1,493 cows (i.e., 74% of cows). Haplotype frequencies (Table 3) were calculated as the weighted mean of the haplotype probabilities obtained, thus all the animals have been included in the computing. The most recurrent *CNS2-CSN3* haplotype was A^2A which had a frequency of 39%. Haplotypes carrying allele *CSN2* B had a moderate frequency whereas the rarest haplotype, exhibiting a frequency of 1.6%, was IA. Linkage disequilibrium was detected ($P < 0.001$) also between *CSN3* and *BLG*, which are located in different chromosomes, and might be due to random drift. To quantify linkage disequilibrium, the correlation coefficient (r^2) of the *CSN3* and *BLG* variants, as described by Hill and Robertson (1968), was computed. The overall r^2 for the haplotype *CSN3-BLG* was below 0.006 and showed that the haplotypes AB and BA had a higher frequency than the frequency expected by random mating. However, since *CSN3* A and *BLG* B had the opposite effect,

the bias due to the exclusion of *BLG* from the haplotype is expected to be low. Moreover, both casein haplotype and *BLG* locus were simultaneously included in all the statistical analysis.

Effect of Casein Haplotypes and BLG Genotypes on Milk Production Traits

With the only exception of the effect of *CSN3* on protein content, results concerning the effect of casein polymorphisms on milk production traits are not consistent across studies and breeds (Braunschweig et al., 2000; Ikonen et al., 2001; Boettcher et al., 2004a). Estimates of the effects exerted by casein haplotypes and *BLG* genotypes on milk production traits are reported in Table 4 and 5, respectively. All estimates are expressed in standard deviation units of the trait to make across-traits comparisons of the magnitude of effects feasible. Milk yield, fat and protein contents and fat and protein yields were affected neither by casein haplotypes nor by *BLG* genotypes, with few exceptions. In comparison with haplotype A^2A, a slight increase ($P < 0.05$) of fat and protein content and protein yield was observed in presence of haplotype A^1A, and a slight increase ($P < 0.05$) of protein content was associated with haplotypes A^2B and IB. These results are not in agreement with those obtained by Boettcher et al. (2004a) for Italian Holstein Friesian and Brown Swiss cows. Bovenhuis et al. (1992) suggested that associations between casein alleles and milk production traits are attributable to effects of linked loci rather than to direct effects of the protein loci themselves. Under this assumption, associations between milk protein genes and linked QTLs might be different across breeds and sire families and might explain inconsistencies across studies.

Similarly to CN loci, results for the effect of *BLG* genotypes on milk production traits are not consistent across studies. Some authors (Lunden et al., 1997; Ojala et al., 1997; Heck et al., 2009) reported no association between *BLG* genotype and milk production traits whereas Ikonen et al. (1999a) reported that genotype AA exerted favorable effects on milk and protein yield and genotype BB increased milk fat content. In our study, genotype BB did not significantly affect ($P > 0.05$) milk production and protein yield.

In a study concerning the effect of genetic merit on protein composition, Bobe et al. (2007) concluded that there were small differences in milk protein composition of cows of different genetic merit for milk yield and that, as a consequence, prolonged selection for increased yield is not expected to affect composition of milk protein if selection is only for a few generations.

Estimated genetic correlations between protein yield and concentrations of the major milk proteins have been reported to be low or very low, indicating that selection for protein yield is expected to exert negligible effects on protein composition (Shopen et al., 2009). However, selection to enhance protein percentage is expected to result in a slight increase in κ-CN% while decreasing α_{S1}-CN% and α-LA%, and selection for increased milk yield is expected to exert the opposite effect (Shopen et al., 2009).

Table 4. Estimated effects (± SE) of *CSN2-CSN3* haplotypes compared to haplotype A^2A on yield (kg/d) and content (%) of milk protein and fat (magnitude of effects is expressed in SD units of traits)[1]

Haplotype	Milk yield	Protein yield	Fat yield	Protein content	Fat content
A^2A	0	0	0	0	0
A^1A	-0.022 ± 0.041	-0.003 ± 0.045	0.046 ± 0.047	0.093 ± 0.042*	0.110 ± 0.051*
BA	0.070 ± 0.043	0.069 ± 0.047	0.097 ± 0.049†	0.007 ± 0.044	0.035 ± 0.054
IA	-0.143 ± 0.134	-0.124 ± 0.148	0.044 ± 0.155	0.098 ± 0.134	0.185 ± 0.167
A^1B	-0.090 ± 0.059	-0.082 ± 0.065	-0.016 ± 0.068	0.064 ± 0.060	0.074 ± 0.074
A^2B	-0.043 ± 0.035	-0.016 ± 0.039	0.003 ± 0.041	0.090 ± 0.037*	0.069 ± 0.045
BB	-0.060 ± 0.077	-0.051 ± 0.084	-0.026 ± 0.088	0.051 ± 0.078	0.094 ± 0.095
IB	0.029 ± 0.068	0.103 ± 0.075	0.001 ± 0.078	0.208 ± 0.069**	-0.043 ± 0.085

[1]All measures have been collected in the national milk recording program and measures of protein yield and milk protein content are derived from mid infrared predictions;
$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 5. Estimated effects (± SE) of *BLG* genotypes compared to genotype AA on yield (kg/d) and content (%) of milk protein and fat (magnitude of effects is expressed in SD units of traits)[1]

Genotype[2]	Milk yield	Protein yield	Fat yield	Protein content	Fat content
AA	0	0	0	0	0
AB	0.007 ± 0.037	0.011 ± 0.041	0.009 ± 0.043	-0.002 ± 0.038	0.043 ± 0.046
BB	0.091 ± 0.047†	0.090 ± 0.052†	0.069 ± 0.054	-0.005 ± 0.049	0.008 ± 0.060
Additive deviation	0.046 ± 0.023†	0.045 ± 0.026†	0.034 ± 0.027	-0.003 ± 0.024	0.004 ± 0.030
Dominance deviation	-0.039 ± 0.032	-0.034 ± 0.036	-0.025 ± 0.037	0.001 ± 0.032	0.038 ± 0.040

[1]All measures have been collected in the national milk recording program and measures of protein yield and milk protein content are derived from mid infrared predictions;
[2]Additive 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.
$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effects of Casein Haplotypes on Protein, Casein, Whey Protein and Casein Number

Estimates of CN haplotypes effects on PRT, TCN, WH and CI are reported in Table 6. Conversely to milk production traits, CN haplotypes exerted significant effects on PRT and TCN content measured by RP-HPLC. Estimated haplotypes effects on PRT are closely related to those obtained for TCN because these two variables were tightly associated (r = 0.99, $P < 0.001$). Differences in TCN were ascribed to both *CSN2* and *CSN3* alleles. Haplotypes carrying *CSN3* B exhibited a greater TCN and CI and no difference in WH in comparison with haplotypes carrying *CSN3* A, but the effect of *CSN3* B on TCN was not consistent across haplotypes. As an example, haplotypes A^1B and A^1A exhibited no significant difference in TCN content. In particular, marked differences in TCN and CI due to *CSN3* variants were observed in association with *CSN2* A^2 and B. Also haplotypes carrying allele *CSN2* B showed a greater TCN but similar WH in comparison with

haplotypes including alternative *CSN2* alleles. Conversely, Bobe et al. (1999) reported no significant effect of milk protein genotypes on total protein content measured by RP-HPLC.

Table 6. Estimated effects (± SE) of *CSN2*-*CSN3* haplotypes compared to haplotype A^2A on contents (g/L) of protein, casein, whey protein and casein number (%) measured by reversed-phase HPLC (magnitude of effects is expressed in SD units of traits)[1]

Haplotype	Protein	Casein	Whey protein	Casein number
A^2A	0	0	0	0
A^1A	-0.063 ± 0.044	-0.056 ± 0.044	-0.086 ± 0.043*	0.056 ± 0.044
BA	0.354 ± 0.047***	0.401 ± 0.047***	0.009 ± 0.046	0.467 ± 0.047***
IA	0.183 ± 0.140	0.223 ± 0.140	-0.081 ± 0.137	0.432 ± 0.138*
A^1B	0.009 ± 0.063	0.034 ± 0.064	-0.130 ± 0.063*	0.238 ± 0.063***
A^2B	0.219 ± 0.039***	0.252 ± 0.039***	-0.014 ± 0.038	0.316 ± 0.038***
BB	0.645 ± 0.081***	0.747 ± 0.082***	-0.078 ± 0.080	0.964 ± 0.081***
IB	0.485 ± 0.072***	0.528 ± 0.073***	0.133 ± 0.071†	0.430 ± 0.072***

[1]Protein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN + β-LG + α-LA; casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA; casein number = (casein/protein) × 100;
$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effects of Casein Haplotypes on Contents of Protein Fractions

Estimates of the effects of CN haplotypes on contents of milk protein fractions are reported in Table 7. Casein haplotypes exerted a marked effect on contents of all CN fractions. In agreement with Hallén et al. (2008), alleles at *CSN2* and *CSN3* affected the expression of the protein fraction which the locus encodes for and had limited effects on whey protein fractions. Only a few studies investigated milk protein composition, mainly in relation to effects of CN genotypes (Ng-Kwai-Hang et al., 1987; Bobe et al., 1999; Hallén et al., 2008) whereas effects exerted by CN haplotypes on milk protein composition have been investigated only by Heck et al. (2009). In that study, only the glyco-free κ-CN content was assessed because the glycosylated form was not detectable. Because Coolbear et al. (1996) showed that the κ-CN B variant is highly glycosylated when compared with the A variant, the effect of *CSN3* B on total content of κ-CN might have been underestimated in the study by Heck et al. (2009).

In our study, the increased CN content ($P < 0.001$) due to the presence in the haplotype of *CSN3* B in comparison with *CSN3* A was exclusively determined by a significant increase in κ-CN content, with no effects on contents of other protein fractions. Despite several authors (Van Eenennaam and Medrano, 1991; Robitaille and Petitclerc, 2000; Vachon et al., 2004) reported an allele-specific protein expression for *CSN3* locus, others (Ehrmann et al., 1997; Hallén et al., 2008) found no significant differences between expressions of allele A and B at *CSN3*. As possible explanation of these inconsistencies, Robitaille and Petitclerc (2000) suggested that a differential expression might result from polymorphisms in the non-coding region of the gene.

Table 7. Estimated effects (± SE) of *CSN2-CSN3* haplotypes compared to haplotype A^2A on contents (g/L) of milk protein fractions (magnitude of effects is expressed in SD units of traits)

Haplotype	α_{S1}-CN	α_{S2}-CN	β-CN	γ-CN	κ-CN	α-LA	β-LG
A^2A	0	0	0	0	0	0	0
A^1A	$0.240 \pm 0.048^{***}$	$-0.173 \pm 0.044^{***}$	$-0.106 \pm 0.038^{**}$	$-0.811 \pm 0.045^{***}$	$0.173 \pm 0.032^{***}$	$-0.219 \pm 0.045^{***}$	-0.021 ± 0.042
BA	$-0.323 \pm 0.051^{***}$	$-0.091 \pm 0.047^{\dagger}$	$1.009 \pm 0.040^{***}$	$-0.095 \pm 0.048^{*}$	$0.154 \pm 0.034^{***}$	-0.001 ± 0.047	0.010 ± 0.045
IA	0.082 ± 0.153	0.092 ± 0.138	$0.269 \pm 0.121^{*}$	$0.188 \pm 0.150^{***}$	0.089 ± 0.100	-0.165 ± 0.142	-0.037 ± 0.132
A^1B	0.011 ± 0.070	$-0.325 \pm 0.063^{***}$	$-0.106 \pm 0.055^{\dagger}$	$-1.088 \pm 0.066^{***}$	$1.242 \pm 0.046^{***}$	$-0.165 \pm 0.064^{*}$	-0.090 ± 0.061
A^2B	0.007 ± 0.042	-0.011 ± 0.038	0.040 ± 0.033	$0.081 \pm 0.040^{*}$	$1.049 \pm 0.028^{***}$	$-0.136 \pm 0.039^{**}$	0.033 ± 0.037
BB	$-0.325 \pm 0.089^{***}$	$-0.147 \pm 0.081^{\dagger}$	$1.208 \pm 0.070^{***}$	-0.120 ± 0.086	$1.372 \pm 0.058^{***}$	-0.109 ± 0.082	-0.050 ± 0.078
IB	-0.066 ± 0.079	$0.437 \pm 0.072^{***}$	$0.512 \pm 0.063^{***}$	$-0.235 \pm 0.076^{**}$	$1.123 \pm 0.052^{***}$	0.033 ± 0.073	$0.141 \pm 0.069^{*}$

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 8. Estimated effects (± SE) of *CSN2-CSN3* haplotypes compared to haplotype A^2A on composition of casein and of whey protein (magnitude of effects is expressed in SD units of traits)[1]

Haplotype	α_{S1}CN%	α_{S2}CN%	β-CN%	γ-CN%	κ-CN%	β-LG%
A^2A	0	0	0	0	0	0
A^1A	$0.197 \pm 0.011^{***}$	$-0.121 \pm 0.029^{***}$	$-0.054 \pm 0.010^{***}$	$-0.593 \pm 0.036^{***}$	$0.119 \pm 0.013^{***}$	$-0.032 \pm 0.006^{***}$
BA	$-0.438 \pm 0.012^{***}$	$-0.289 \pm 0.031^{***}$	$0.499 \pm 0.011^{***}$	$-0.200 \pm 0.037^{***}$	-0.009 ± 0.014	$0.294 \pm 0.007^{***}$
IA	$-0.087 \pm 0.035^{*}$	-0.072 ± 0.092	$0.077 \pm 0.033^{*}$	0.040 ± 0.116	0.012 ± 0.042	$0.045 \pm 0.020^{*}$
A^1B	-0.027 ± 0.016	$-0.300 \pm 0.042^{***}$	$-0.090 \pm 0.015^{***}$	$-0.799 \pm 0.051^{***}$	$0.817 \pm 0.019^{***}$	$-0.053 \pm 0.009^{***}$
A^2B	$-0.149 \pm 0.010^{***}$	$-0.153 \pm 0.026^{***}$	$-0.094 \pm 0.009^{***}$	-0.026 ± 0.031	$0.597 \pm 0.012^{***}$	$-0.056 \pm 0.005^{***}$
BB	$-0.614 \pm 0.021^{***}$	$-0.508 \pm 0.054^{***}$	$0.440 \pm 0.019^{***}$	$-0.311 \pm 0.067^{***}$	$0.637 \pm 0.025^{***}$	$0.260 \pm 0.011^{***}$
IB	$-0.346 \pm 0.018^{***}$	0.067 ± 0.048	$0.096 \pm 0.017^{***}$	$-0.332 \pm 0.059^{***}$	$0.536 \pm 0.022^{***}$	$0.056 \pm 0.010^{***}$

[1] α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content;

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

An allele-specific differential expression was reported also for *CSN2*. In agreement with previous investigations (Ng-Kwai-Hang et al., 1987; Hallén et al., 2008), our study clearly indicates that allele *CSN2* B is associated with an increased β-CN content and effects of alternative *CSN2* alleles are significant but of smaller magnitude than the one exerted by allele B. The increased expression of β-CN observed for haplotypes carrying *CSN2* B was associated to a decrease of α_{S1}-CN and, to a lesser extent, of α_{S2}-CN. Effects of *CSN2* B were favorable also for content of κ-CN whereas those on WH content were trivial.

In comparison with haplotypes including allele *CSN2* A^2, haplotypes containing allele A^1 showed lower β- and α_{S2}-CN contents and greater α_{S1}- and κ-CN contents, but a major effect exerted by A^1 allele was a noticeable reduction of γ-CN content. Effects of *CSN2* I have never been studied before and knowledge about its role for protein composition has never been reported. From results obtained in this study, *CSN2* I seems to exert positive effects on the expression of β-CN, when compared with the most frequent alleles, without altering contents of other protein fractions.

Casein haplotypes exerted limited effects on whey proteins contents. Bobe et al. (1999) reported that *CSN3* B did not affect α-LA concentration whereas Ng-Kwai-Hang et al. (1987) and Heck et al. (2009) observed a slightly decrease of α-LA associated with this allele. A moderate positive effect of haplotype IB on β-LG was observed, which partially agree with results obtained by Bobe et al. (1999), who reported a slightly increase of the ratio of β-LG to total protein in relation to *CSN3* B.

Effect of Casein Haplotypes on Protein Composition

Effects of CN haplotypes on protein composition are reported in Table 8. Several studies (Lunden et al., 1997; Mayer et al., 1997; Robitaille et al., 2002) reported a significant effect of *CSN3* alleles on the ratio of κ-CN to total casein and a significant association of allele B with increased κ-CN%. As expected, all haplotypes carrying *CSN3* B increased κ-CN% ($P < 0.001$) in comparison with those carrying *CSN3* A. This result is consistent across studies (Van Eenennam and Medrano, 1991; Bobe et al., 1999; Heck et al., 2009). Similarly to results obtained for contents of milk protein fractions, the marked increase of κ-CN% associated with *CSN3* B occurred at the expense of all other casein fractions, with the exception of β-CN%. A marked decrease of α_{S1}-CN relative content in CN, associated with *CSN3* B variant, has been reported also in other studies (Bobe et al., 1999; Hallén et al., 2008; Heck et al., 2009). The inverse relationship between κ-CN% and α_{S1}-CN% supports the hypothesis that specific proteins compete for expression in the mammary gland (McClenaghan et al., 1995).

With respect to *CSN2* alleles, they exhibited different effects on protein composition both in magnitude and in sign. As an example, haplotypes including *CSN2* B were associated with increased β-CN% in comparison with alternative haplotypes. As observed for protein fractions

contents, the increased β-CN% associated with haplotypes carrying *CSN2* B occurred mostly at the expense of α_{S1}-CN% and, to a lesser extent, of α_{S2}-CN%, without altering relative content of κ-CN. Fractions β-CN and α_{S1}-CN seem to undergo a competitive synthesis and their relative contents in CN were inversely related (r = -0.51; $P < 0.001$). Consequently, haplotypes which influenced positively β-CN% exerted opposite effects on α_{S1}-CN%. Likely, milk proteins, in particular α_{S1}- and β-CN, are co-regulated. According to Bobe et al. (1999), a limited pool of transcriptional factors or amino acids or both might explain how a gene sequence can influence the synthesis of other abundant milk proteins, primarily α_{S1}-CN. In opposition to results obtained for proteins contents, haplotypes including *CSN2* B were also associated with a variation of whey protein composition, namely an increased relative content of β-LG.

In agreement with Heck et al. (2009), haplotypes carrying allele *CSN2* A^1 were associated with greater α_{S1}-CN% and κ-CN% and with lower α_{S2}-CN% and β-CN% in comparison with haplotypes carrying *CSN2* A^2. In addition, a marked decrease of γ-CN% was observed for haplotypes carrying *CSN2* A^1 allele.

Also the effects exerted by *CSN2* I on both contents and composition of milk proteins were very similar, with a slightly positive effect on the relative content of β-CN, compared with alleles A^1 and A^2. Even in this case, the increase in β-CN% was associated with a decrease of α_{S1}-CN%.

Conversely with the findings obtained for whey fractions contents, whey composition was significantly affected by casein haplotypes, but the strongest effect was attributable to *CSN2* B, as reported above.

Effect of BLG Genotypes on Contents of Protein Fractions and on Protein Composition

Effects of *BLG* genotypes on contents of protein fractions and on protein composition are reported in Table 9. Genotypes at *BLG* significantly influenced contents of protein fractions and protein composition. In comparison with genotype AA, genotype BB was associated with increased PRT and TCN and with decreased WH in milk (all $P < 0.001$). As a consequence, allele *BLG* B was also responsible for an increase of CI ($P < 0.001$). These results are in agreement with those reported by Braunschweig et al. (2000). Hallén et al. (2008) reported a negative association between *BLG* B and PRT whereas Bobe et al. (1999) found no effect of *BLG* genotypes on PRT. Inconsistencies of results across studies might be partly attributed to different breeds and to the limited size of investigated samples used in some studies.

In comparison with *BLG* A, allele B at *BLG* influenced contents of all protein fractions (all $P < 0.05$) and increased β-LG% ($P < 0.001$), by regulating the relative amount of β-LG in WH, but exerted trivial effects on CN composition. The large effect of *BLG* genotypes on β-LG content of milk has been previously attributed to differences in the expression of the whey protein caused

by polymorphisms in the promoter region of the gene which is physically linked to *BLG* (Wagner et al., 1994; Lum et al., 1997; Folch et al., 1999). Differences in the stability of mRNA derived from A and B alleles have been also suggested (Heck et al., 2009) as a possible explanation for variation of β-LG content across *BLG* genotypes. Heck et al. (2009) reported that *BLG* genotypes explained 90% of total genetic variation of relative concentration of β-LG in milk protein.

Table 9. Estimated effects (± SE) of *BLG* genotypes compared to genotype AA on protein, casein, and whey protein contents (g/L), casein number (%), protein fractions contents (g/L) and protein composition (%) measured by reversed-phase HPLC (magnitude of effect is expressed in SD units of traits)[1]

Trait	Genotype		Additive deviation	Dominance deviation
	AB	BB		
Protein[2]	0.085 ± 0.039*	0.191 ± 0.052***	0.095 ± 0.026***	-0.010 ± 0.034
Casein[3]	0.131 ± 0.039***	0.314 ± 0.052***	0.157 ± 0.026***	-0.026 ± 0.034
Whey protein[4]	-0.188 ± 0.039***	-0.540 ± 0.051***	-0.270 ± 0.026***	0.082 ± 0.033*
Casein number[5]	0.442 ± 0.039***	1.183 ± 0.052***	0.591 ± 0.026***	-0.149 ± 0.034***
Prot. fract. contents				
α_{S1}-CN	0.158 ± 0.043***	0.361 ± 0.057***	0.180 ± 0.028***	-0.023 ± 0.037
α_{S2}-CN	0.076 ± 0.039†	0.140 ±0.052**	0.070 ±0.026**	0.006 ±0.034
β-CN	0.072 ± 0.034*	0.227 ± 0.044***	0.114 ± 0.022***	-0.041 ± 0.029
γ-CN	0.092 ± 0.042*	0.075 ± 0.052	0.038 ± 0.026	0.054 ± 0.036
κ-CN	0.072 ± 0.028*	0.187 ± 0.037***	0.094 ± 0.019***	-0.022 ± 0.024
α-LA	-0.018 ± 0.040	0.345 ± 0.052***	0.172 ± 0.026***	-0.191 ± 0.034***
β-LG	-0.216 ± 0.037***	-0.764 ± 0.050***	-0.382 ± 0.025***	0.166 ± 0.032***
Protein composition[6]				
α_{S1}-CN%	0.037 ± 0.026*	0.072 ± 0.035*	0.036 ± 0.017*	0.002 ± 0.023
α_{S2}-CN%	-0.018 ± 0.041*	-0.095 ± 0.055†	-0.047 ± 0.028†	0.029 ± 0.036
β-CN%	-0.054 ± 0.027*	-0.015 ± 0.035	-0.008 ± 0.018	-0.047 ± 0.023*
γ-CN%	0.035 ± 0.043	-0.053 ± 0.055	-0.027 ± 0.027	0.061 ± 0.037†
κ-CN%	0.022 ± 0.023	0.046 ± 0.031	0.023 ± 0.015	-0.001 ± 0.020
β-LG%	-0.171 ± 0.037***	-1.040 ± 0.049***	-0.520 ± 0.024***	0.349 ± 0.032***

[1]Additive 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;

[2]Protein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN + β-LG + α-LA;

[3]Casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN;

[4]Whey protein = β-LG + α-LA ;

[5]Casein number = (casein/protein) × 100;

[6]α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content.;

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Consistently, Bobe et al. (1999) concluded that *BLG* genotypes regulate almost solely the proportion of β-LG in total milk protein. Significant dominance effects were estimated in our study

in relation to both content and composition of whey protein whereas these effects were trivial for CN fractions contents and CN composition.Less investigated is the effect of *BLG* genotypes on relative concentrations of individual CN fractions and use of different breeds might partly explain inconsistencies of results. In our study, genotypes at *BLG* affected TCN and CI, with no effect on CN composition with the exception of a very small increase of α_{S1}-CN% ($P < 0.05$) and decrease of α_{S2}-CN% ($P < 0.1$) associated with genotype BB. Previous investigations (Ng-Kwai-Hang et al., 1987; Heck et al., 2009) reported that the increased relative concentration of β-LG associated with A variant was counterbalanced by a decreased relative content of all other fractions in milk protein, especially β-CN and α_{S1}-CN. Likewise, Bobe et al. (1999) concluded that the A allele at *BLG* increased the proportion of β-LG at the expense of α_{S1}-CN and β-CN relative concentrations. For all those studies, these results were partly expected because concentrations of casein fractions were expressed as weight percentages of total milk protein, albeit variation of casein composition due to *BLG* alleles remains noticeable. Mechanisms causing the effect of *BLG* locus on relative contents of the other milk proteins are currently unknown.

Based on these results, breeding practices aimed to increase the frequency of *BLG* B in the Simmental cattle population are expected to affect the composition of whey protein and to increase CN content with trivial effects on casein composition. As discussed by Heck et al. (2009), a lack of change of casein composition ensures that selection for *BLG* B allele does not affect cheese quality and, therefore, is beneficial for increasing cheese yield with no negative effects on cheese properties.

Contribution of Additive Genetic Variance to Total Variance of Protein Fractions Contents

Estimates of additive genetic variances (data not presented in tables) indicate that the polygenic background of the cow affects variation of protein fractions contents, even when effects of casein haplotypes and *BLG* genotypes are accounted for by the statistical model. Estimated additive genetic variance was 38 and 39 % of total variance, for α_{S2}-CN and β-LG contents, respectively. The effect of the sire of the cow on variation of γ-CN content was trivial and ratios of additive genetic variance to total variance observed for β-CN and α-LA contents were low, ranging from 14 to 16 %. For all other protein fractions contents, additive genetic variance to total variance ranged from 21 % to 32 %. Results for protein composition were similar to those obtained for protein contents. Although genetic variation of protein fractions contents and protein composition is greatly attributable to casein haplotypes and *BLG* genotypes, the effect of the polygenic background of the animal is noteworthy. Besides milk protein genes, other loci are responsible of variation of protein fractions contents and protein composition. Estimation of genetic parameters for milk protein fractions contents and composition will be the objective of future investigations.

CONCLUSIONS

Our results indicate that *CSN2-CSN3* haplotypes and *BLG* genotypes play an important role for variation of milk protein fractions contents and milk protein composition. This suggests that increasing the frequency of specific genotypes or haplotypes might be an effective way for altering milk protein composition. The marked effects of casein haplotypes and *BLG* genotypes on protein composition, together with the lack of effects on milk production traits, ensure that altering milk protein composition does not interfere with yield traits. In addition to milk protein loci, polygenes affect phenotypic variation of concentrations of milk protein fractions and their relative contents in total milk protein. Genetic variation of protein composition might be exploited in breeding programs aiming to enhance milk renneting properties and cheese yield or to obtain milk with increased contents of specific protein fractions. Currently, knowledge concerning the effects of milk protein composition, casein haplotypes and *BLG* genotypes on the technological properties of milk, as well as availability of estimated genetic parameters for protein composition, is scarce and requires specific investigations.

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Chapter 3.

Effects of *CSN2-CSN3* haplotype, *BLG* genotype 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, contents of milk protein fractions and protein composition on coagulation properties of milk (**MCP**). Rennet coagulation time (**RCT**) and curd firmness ($\mathbf{a_{30}}$) were measured using a computerized renneting meter and contents of major milk protein fractions were quantified by reversed-phase HPLC in individual milk of 2,167 Simmental cows. Cow genotype at *CSN2*, *CSN3*, and *BLG* loci was assessed by HPLC. Haplotype probabilities were estimated for each cow. Phenotypes for MCP were regressed on these probabilities using linear models that included the effects of herd-test day, parity, days in milk, pH, somatic cell score, renneting meter sensor, sire of the cow, and, contents of major protein fractions or, alternatively, protein composition. When protein fractions contents or protein composition were not included in the statistical model, haplotypes carrying *CSN3* B allele exhibited shorter RCT and greater a_{30}, in comparison with those carrying *CSN3* A, and haplotypes carrying *CSN2* B allele were responsible for a noticeable decrease of RCT and for an increase of a_{30}, when compared to haplotype A^2A. When effects of protein fractions contents or of 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 exerted by κ-casein (**κ-CN**) B on MCP is related to a variation of protein composition caused by the allele-specific expression of this protein fraction, rather than to a direct role of the protein variant on the coagulation process. Also, the favorable effect exerted by *CSN2* B on a_{30} was mediated by the

increase of β-casein (**β-CN**) B in milk. Conversely, β-CN B is likely to exert a molecular effect on RCT, which does not depend upon variation of β-CN content associated to allele B. An 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-CN content and percentage on total casein, whereas a_{30} increased when contents and percentages of both β-CN and κ-CN increased. Changes in milk protein composition exert correlated effects on MCP and changes in frequency of specific allelic variants at CN and whey protein loci might be beneficial or detrimental for MCP.

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 ($\mathbf{a_{30}}$) have been reported to be affected also by milk protein composition (Wedholm et al., 2006; Jõudu et al., 2008). Previous studies investigated the effects of milk protein polymorphisms either on coagulation properties (Buchberger and Dovč, 2000) or on protein composition (Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2009). Because simultaneous assessment of all major milk proteins contents is difficult and time-consuming when the amount of samples is large, the effect of protein composition on milk coagulation properties (**MCP**) has been scarcely 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 allele and improved milk coagulation properties (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 fractions contents or protein composition related to genetic polymorphisms of milk proteins or both. As an over-expression of *CSN3* B in comparison with *CSN3* A has been reported (Hallèn et al., 2008; Heck et al., 2009; Bonfatti et al., 2009), effects of *CSN3* on MCP might result from differences in casein content of milk or in κ-CN to total CN ratio (i.e., casein composition) across genotypes. Other factors, like differences in micelle structure (van Eenennam and Medrano, 1991; Lodes et al., 1996a) or differences in the structure or molecular properties of the curd, may also contribute to the observed variation of MCP associated to different κ-CN variants. No study investigated effects of *CSN3* genotypes on MCP while accounting for milk protein fractions contents or protein composition or both using a large sample of animals. Moreover, estimation of these effects is troublesome for some cattle breeds due to the low frequency of specific genotypes or

haplotypes. In comparison 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 exhibit more balanced allele frequencies at *CSN2*, *CSN3*, and *BLG* loci and is characterized by a moderate frequency of *CSN2* B (Bonfatti et al., 2009). The aim of this study was to estimate the effects of CN haplotypes, *BLG* genotypes, protein fractions contents and protein composition on MCP of individual milk of Italian Simmental cows.

MATERIALS AND METHODS

Animals and Data

Details on milk samples collection and preservation, measures of protein fractions contents and protein composition, assessment of genotypes at *CSN2*, *CSN3*, and *BLG* and computation of *CSN2*-*CSN3* haplotype probabilities can be found in Bonfatti et al. (2009). Briefly, individual milk samples were collected from 2,167 Simmental cows from 47 commercial herds in Italy. Samples (1 per animal) were collected during the morning or the evening milking of a test day. Herd and test-day effects were confounded because all cows of a herd were sampled in the same test day. Milk yield records and pedigree information for sampled cows were supplied by the Italian Simmental Cattle Breeders Association (ANAPRI, Udine, Italy).

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* were assessed by reversed-phase HPLC using the method proposed by Bonfatti et al. (2008). More indications about the method used are reported also by Bonfatti et al. (2009).

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., 2009) using 200 randomly-chosen samples. Because of the frequency of *CSN1S1* C allele was lower than 6 %, sequencing was not extended to all samples and *CSN1S1* was not considered in the definition of CN haplotypes.

With such a limited frequency, haplotypes carrying the C allele at *CSN1S1* would have been included in the group of “rare” haplotypes or would have obtained a trivial haplotype probability, thus limiting the reliability of the estimated haplotype effects and jeopardizing expected advantages arising from the gain of additional gene information. Furthermore, a very limited bias is to be expected when neglecting *CSN1S1* contribution to haplotype definition.

Due to its low frequency in Simmental cattle (Jann et al., 2004; Matějíček et al., 2008), the incidence of *CSN3* E, which cannot be differentiated from *CSN3* A by RP-HPLC, was not assessed. Thus, the estimated effects for *CSN3* A might be slightly biased being the combined effects of *CSN3* A and E variants.

Protein Composition

For proteins quantified by RP-HPLC, total casein (**TCN**, g/L) was computed as the sum of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN and κ-CN contents of milk. Total whey protein (**WH**, g/L) was calculated as the sum of α-LA and β-LG contents. Total protein (**PRT**, g/L) was expressed as the sum of casein and whey protein contents. Casein number (**CI**, %) was calculated as the percentage ratio of TCN to PRT. Protein composition (i.e., relative contents of protein fractions) was computed as weight percentage ratio of α_{S1}-CN (**α_{S1}-CN%**, %), α_{S2}-CN (**α_{S2}-CN%**, %), β-CN (**β-CN%**, %), γ-CN (**γ-CN%**, %), and κ-CN (**κ-CN%**, %) to TCN and as the percentage ratio of β-LG to WH (**β-LG%,** %).

Analysis of Milk Coagulation Properties

Measures of MCP of individual milk samples were obtained by using the Computerized Renneting Meter (CRM-48, Polo Trade, Monselice, Italy) within 3 h after sample collection. The principle of the Computerized Renneting Meter is based on the control of the oscillation, which is driven by an electromagnetic field, created by a swinging pendulum immerged into the milk container. A survey system measures differences in the electromagnetic field due to milk coagulation: 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 preheated at 35 °C, and 200 μL of rennet (Hansen standard 160, 80 % chymosin, 1:14,900, Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6 % (vol/vol) in distilled water was added to milk. Measurement of MCP ended within 31 min after rennet addition 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). Non-coagulating milk was defined as milk that did not begin to coagulate within 31 min after the addition of the clotting enzyme. These samples were not considered in the statistical analysis because of missing information on coagulation time. Measures of pH (pH-Burette 24, Crison) were obtained before measurement of MCP.

Statistical Analysis

The effects of casein 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:

$$y_{ijklmnopq} = \mathrm{HTD}_i + \mathrm{P}_j + \mathrm{DIM}_k + \mathrm{SCS}_l + \mathrm{S}_m + \mathrm{pH}_n + \sum_{a=1}^{h} \beta_a \mathrm{PR}_{a:ijklmnopq} + \mathrm{BLG}_o + \mathrm{sire}_p + \mathrm{e}_{ijklmnopq}$$

where $y_{ijklmnopq}$ is an observation on RCT or a_{30}; HTD_i is the fixed effect of herd-test day i (i = 1, …, 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 SCS class l (6 classes of 1-point intervals, with the exception of the last class which included records with SCS ≥ 5); S_m is the fixed effect of the renneting meter sensor m (m = 1, …, 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 pH ≤ 6.62, and the last class, which included records with pH ≥ 6.82); h is the total number of haplotypes evaluated (h = 8); $PR_{a:ijklmnopq}$ is the probability that the cow carried haplotype a (a = 1, …, 8), expressed as the expected number of copies of haplotype a 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, …, 210) assumed to follow a normal distribution with $sire_p \sim N(\mathbf{0}, \sigma_s^2)$, where σ_s^2 is the sire variance; and $e_{ijklmnopq}$ is a random residual assumed to follow a normal distribution with $e_{ijklmnopq} \sim N(\mathbf{0}, \sigma_e^2)$, where σ_e^2 is the residual variance.

A probability (ranging from 0 to 1) was assigned to each possible haplotype carried by an animal. For example, if the only possible haplotype couple was composed by two copies of the same haplotype, that haplotype gained a final value of 2 because the animal was expected to carry 2 copies of that haplotype, each one with a probability of 1.

Hence, the difference between the estimated regression coefficients for two haplotypes of interest provides a quantification of the expected change in the phenotype of a given trait when a copy of the first haplotype is replaced by a copy of the second haplotype. Likewise, in the hypothetical situation in which 2 animals differ for only one haplotype, 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., 2004a). Only additive effects of haplotypes were estimated and dominance effects were not considered.

To evaluate the effects of casein haplotypes and *BLG* genotypes while accounting for contents of protein fractions or for protein composition, alternative models were considered. For the second model (model M2), the linear effects due to contents (g/L) of α_{S1}-, α_{S2}-, β-, κ-CN, and β-LG were added to the effects included in M1. In the third model (model M3) the linear effects due to total protein content of milk, were added to those of M2. Because effects of protein fractions contents are evaluated at the same level of total protein content, model M3 is a model that in fact accounts for protein composition. The last model (model M4) accounted for composition of CN and whey protein and included regressions on α_{S1}-, α_{S2}-, β-, κ-CN, expressed as weight percentages of total

CN, and on β-LG, expressed as weight percentage of total whey protein. In M2, M3 and M4, all additional effects were included as covariates.

Sire effects were included in the model to account for variation due to polygenes underlying the investigated traits. All analyses and hypothesis testing were carried out using the MIXED procedure of SAS.

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. (2009). In total, 6.3 % of samples were non-coagulating milk, which was in accordance with the literature, where the presence of non-coagulating samples varied in relation to cattle breeds: 13.2 % (Ikonen et al., 2004) and 8.6 % (Tyrisevä et al., 2004) in Finnish Ayrshire, 1.3 % in the 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 CRM 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 Milk Coagulation Properties

Estimated effects exerted by CN haplotypes on MCP are reported in Tables 1 and 2 for RCT and a_{30}, respectively. When detailed protein composition was not included in the model (model M1), *CSN3* exhibited a marked effect on MCP. Haplotypes carrying *CSN3* B allele were associated ($P < 0.001$) with lower measures of RCT and greater a_{30} in comparison with those carrying *CSN3* A allele. This is in agreement with results obtained in several studies on *CSN3* genotypes (Van den Berg et al., 1992; Walsh et al., 1995; Lodes et al., 1996a).

When effects of protein fractions contents or of protein composition (models M2, M3 and M4) were accounted for by the model, no significant difference across haplotypes attributable to *CSN3* alleles 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 due to CN composition was accounted for in the statistical analysis, though 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 this allele and not to a direct effect of the specific protein genetic variant on the coagulation process. Likely, the decreased micelle size related to κ-CN B is also to be attributed to the increased content of κ-CN associated to *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). As a consequence, shortened RCT exhibited by κ-CN B milk in comparison with κ-CN A milk might be the outcome of a lower critical level of κ-CN hydrolysis required for the onset of gelation whereas increased a_{30} is likely attributable to a smaller micelle size allowing for more compact arrangements of para-CN micelles and for an increased number of inter-micellar bonds per unit of surface area (Horne et al., 1998). Because structural changes occurring in κ-CN B in comparison with κ-CN A are all located in the C-terminal part of the protein (Ile_{136} and Ala_{148}), which is split off during the enzymatic coagulation phase, these changes are unlike to have effects on the aggregation process and on MCP. When associated to *CSN2* I allele, the effect of *CSN3* B variant on a_{30} was more favorable than when the allele was associated to other *CSN2* alleles. This marked effect was partly reduced when the model accounted for protein composition, but the interaction between *CSN3* B and *CSN2* I still induced a significant increase in a_{30}.

Table 1. Estimated effects (min) ± SE of *CSN2-CSN3* haplotypes on rennet clotting time obtained using different statistical models[1]

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

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected effects on rennet clotting time exerted by the replacement of 1 copy of haplotype A^2A with 1 copy of another haplotype.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

With model M1, *CSN2* alleles exhibited significant effects on MCP. Haplotypes carrying the B variant were responsible for a noticeable decrease of RCT (ranging from -2.44 to -3.04 min, in comparison with haplotype A^2A) and for an increase of a_{30} (ranging from 1.85 to 4.54 mm, in comparison with haplotype A^2A). The effect of *CSN2* B on RCT remained markedly favorable when accounting for contents of protein fractions or protein composition, and an interaction between *CSN2* B and *CSN3* A, which decreased RCT, was observed. The reduced magnitude of the effect of haplotype BB observed with models M2, M3, and M4, in comparison with that obtained with model M1, is to be ascribed to the adjustment for the effect exerted by *CSN3* B variant on κ-CN content. Different allelic-specific expressions have been reported for β-CN also. Allele *CSN2* B has been associated with an increase of β-CN content in milk (Bonfatti et al., 2009) and is likely to exert a specific molecular effect on RCT, which does not depend upon variation of β-CN content associated with this allele. The increased net charge of this protein variant, exhibiting one or two additional net positive charges compared to A^1 or A^2 variants, respectively, for the presence of two positively charged amino acids residues (Arg_{122} and His_{67}), is a possible explanation for direct effects of β-CN B on MCP. Repulsive forces between casein micelles containing variants like β-CN B, in which amino acid substitution results in lower negative charge, seem to be weaker in comparison to micelles containing more negatively charged protein variants (McLean, 1986). As a consequence, a greater proportion of β-CN B into casein micelles facilitate aggregation.

Table 2. Estimated effects (mm) ± SE of *CSN2-CSN3* haplotypes on curd firmness obtained using different statistical models[1]

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

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected effects on curd firmness exerted by the replacement of 1 copy of haplotype A^2A with 1 copy of another haplotype.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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

The A^1 variant, compared to the most common A^2, exerted a positive effect on both RCT and a_{30}, in particular when associated with κ-CN A. When effects due to associated variation in CN composition were accounted for by the model, this favorable interaction effect was somewhat reduced because estimation was performed at the same level of κ-CN% (Heck et al., 2009; Bonfatti et al., 2009). Results obtained in this study for the effect of *CSN2* on MCP partially agree with those reported by Marziali and Ng-Kwai-Hang (1986), who concluded that, after adjustments for concentration of major milk protein fractions, MCP were not different across β-CN phenotypes. That study compared A^1 and A^2 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 of a moderate frequency of this allele in some dairy cattle breeds such as Italian Holstein Friesian and Jersey (Jann et al., 2002). Although frequency of haplotypes containing this allele was low, estimated effects indicate that *CSN2* I has no influence on RCT, when compared to A^2, and affects a_{30} unfavorably ($P < 0.001$) when associated with κ-CN A. Likewise, variant I might exert, when associated to κ-CN A in the haplotype, a negative effect on variation of a_{30}, but relevant biochemical mechanisms are currently unknown.

Effect of BLG Genotypes on Milk Coagulation Properties

Effects of *BLG* genotypes on RCT and a_{30}, as well as estimates of additive and dominance deviations, are reported in Table 3 and 4, respectively. As expected, magnitude of these effects was relatively small when compared to that of effects of CN genes because whey proteins are not directly involved in the milk coagulation process. In agreement with Ikonen et al. (1999a), an increased RCT was observed for milk yielded by *BLG* BB cows, even when models accounted for detailed protein composition. Explanation for the unfavorable effect of β-LG B variant on RCT is 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 other studies 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 variant on RCT estimated in a number of studies might be ascribed to increased total casein content and casein number associated with *BLG* B. Nevertheless, inconsistent results have been obtained on the association between casein content or casein number and RCT (Ikonen et al., 2004), and this might explain controversial reports on effects of *BLG* genotypes and RCT.

Table 3. Estimated effects (min) ± SE of *BLG* genotypes compared to genotype AA on rennet clotting time obtained using different statistical models[1]

Genotype[2]	Statistical model			
	M1	M2	M3	M4
AA	0	0	0	0
AB	$0.667 \pm 0.176^{***}$	$0.785 \pm 0.183^{***}$	$0.773 \pm 0.183^{***}$	$0.638 \pm 0.176^{***}$
BB	$1.033 \pm 0.231^{***}$	$1.411 \pm 0.284^{***}$	$1.348 \pm 0.284^{***}$	$0.827 \pm 0.259^{**}$
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 ± 0.152	-0.079 ± 0.154	-0.099 ± 0.154	-0.224 ± 0.156

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected effects on rennet clotting time exerted by the replacement of 1 copy of haplotype A^2A with 1 copy of another haplotype;

[2]Additive 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.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 4. Estimated effects (mm) ± SE of *BLG* genotypes compared to genotype AA on curd firmness obtained using different statistical models[1]

Genotype[2]	Statistical model			
	M1	M2	M3	M4
AA	0	0	0	0
AB	-0.106 ± 0.312	$-0.585 \pm 0.314^{\dagger}$	$-0.551 \pm 0.313^{\dagger}$	0.014 ± 0.308
BB	$0.767 \pm 0.407^{\dagger}$	-0.660 ± 0.484	-0.519 ± 0.484	$1.167 \pm 0.450^{**}$
Additive deviation	$0.384 \pm 0.203^{\dagger}$	-0.330 ± 0.242	-0.260 ± 0.242	$0.584 \pm 0.225^{**}$
Dominance deviation	$0.490 \pm 0.271^{\dagger}$	0.255 ± 0.265	0.292 ± 0.264	$0.569 \pm 0.274^{*}$

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected effects on curd firmness exerted by the replacement of 1 copy of haplotype A^2A with 1 copy of another haplotype;

[2]Additive 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.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 5. Estimated effects (min·SD^{-1}) ± SE of major protein fractions content or percentage on total casein (α_{S1}-, α_{S2}-, β-, κ-CN) or on total whey protein (β-LG) for rennet clotting time obtained using different statistical models[1]

Protein fraction	Statistical model		
	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*

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected change of rennet clotting time due to a variation of 1 SD in the content or percentage of a specific protein fraction.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 6. Estimated effects (mm·SD^{-1}) ± SE of major protein fractions content or percentage on total casein (α_{S1}-, α_{S2}-, β-, κ-CN) or on total whey protein (β-LG) for curd firmness obtained using different statistical models[1]

Protein fraction	Statistical model		
	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**

[1]Model M1 included the fixed effect of herd-test day, parity, DIM, SCS and pH class, renneting meter sensor, BLG genotype, the regression of *CSN2-CSN3* haplotype probabilities and the random effect of the sire of the cow. In addition, model M2, included 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 content, and model M4 included the regressions on α_{S1}-, α_{S2}-, β-, κ-CN expressed as percentages on total casein, and on β-LG expressed as percentage on total whey protein. In M2, M3 and M4, all additional effects were included as covariates. Estimates correspond to the expected change of curd firmness due to a variation of 1 SD in the content or percentage of a specific protein fraction.

$^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

A slightly positive effect on a_{30} was estimated for allele B at *BLG* when the statistical model (model M1) did not account for protein fractions contents or when effects due to variation of CN and whey protein composition where 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 this genetic variant. Results from models estimating non significant effects ($P > 0.10$) of *BLG* genotypes on a_{30} while adjusting for variation of protein fractions concentrations (model M2) and total protein content (model M3) support this hypothesis.

These results partially agree with Marziali and Ng-Kwai-Hang (1986) who reported shorter mean RCT for phenotypes AA than for phenotypes AB and BB and enhanced a_{30} at cutting for phenotypes AA in comparison with phenotypes AB and BB. In that study, the statistical model accounted for CN content, indicating that effects exerted by β-LG genotypes on MCP are not interpretable as indirect effects due to altered milk protein content.

Contribution of Additive Genetic Variance to Total Variance of Milk Coagulation Properties

Although estimation of genetic parameters for MCP is not a specific aim of the investigation, estimates of additive genetic variance obtained in our study indicate that MCP are moderately influenced by polygenic effects. Depending on the statistical model (M1-4), estimates of additive genetic variance for RCT and a_{30} ranged from 16.8 to 18.4% and from 9.9 to 11.5%, respectively, of total variance. Estimation of genetic parameters for MCP, protein fraction contents and protein composition will be the objective of future investigations.

Effects of Major Protein Fractions Contents and Protein Composition on MCP

Estimated regression coefficients for the effects of protein fractions contents (model M2 and M3) or protein composition (model M4) on RCT and a_{30} are reported in Table 5 and 6, 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 CN for CN fractions or in whey protein for β-LG) increases of 1 SD. In literature, studies on the role of major protein fractions contents and milk protein composition for variation of MCP are scarce. In our study, RCT was favorably affected ($P < 0.001$) by increased κ-CN content and κ-CN proportion on total CN whereas a_{30} increased when amounts of both κ-CN and β-CN in milk and in CN increased.

Previous studies (Yun et al., 1982; Storry et al., 1983) reported that RCT was related to proportions of α_S- and β-CN in CN and that curd tension increased significantly 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 contents ratio

reflect alteration in the gelation properties of milk. Wedholm et al. (2006) reported that the concentration of κ-CN in milk and ratios of this fraction to α_{S1}- and β-CN were significantly lower in poorly-coagulating or non-coagulating milk than in milk exhibiting optimal coagulation ability, but, consistently with Jõudo et al. (2008), they observed no effect of β-CN content on MCP. Similarly to previous reports (McLean, 1986; van den Berg et al., 1992; Jõudu 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, and enhances a_{30} at cutting. In addition, κ-CN content has been also associated with increased casein retention in curd during chymosin-induced coagulation. The effect of κ-CN concentration on casein losses into whey may partly be explained by the association between increased κ-CN content and small casein micelle size (Dalgleish et al., 1989; Donnelly et al., 1984). Milk containing small-sized micelles originates gels with an improved structure, which may increase the ability to entrap milk constituents possibly reducing casein losses into whey (Niki et al., 1994; Walsh et al., 1998).

CONCLUSIONS

This study indicates that previously observed differences across CN genotypes in coagulating properties of milk may have been mostly due to differences in contents of major protein fractions and in detailed protein composition, that are known to be associated with milk protein genotypes. Factors expected to affect milk composition are expected to affect processing properties of milk also. Although further research is needed to investigate the effect exerted by milk protein composition and casein haplotypes on cheese yield, results obtained in this study suggest that changes in milk protein composition might exert correlated effects on milk coagulation properties and that changes in frequency of specific allelic variants at CN and whey protein loci might be beneficial or detrimental for coagulation properties of milk. Genetic parameters for contents 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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Chapter 4.

The effect of the relative κ-CN B content in bulk milk on Montasio, Asiago, and Caciotta cheese yield using milks of similar protein composition

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ABSTRACT

The aim of this study was to investigate the effect exerted by the relative ratio of κ-CN A to κ-CN B content on milk coagulation properties and industrial cheese yield of three Italian cheese varieties (Montasio, Asiago, and Caciotta). Twenty-four cheese-making experiments were carried out in two industrial and in one artisanal scale dairy plants. Detailed protein composition of bulk milk of 380 herds conferring to these dairies was analyzed by reversed-phase HPLC (RP-HPLC). In order to obtain experimental milks with different κ-CN A to κ-CN B content ratios, herds were selected on the basis of their milk protein composition and κ-CN genetic composition. Milk of selected groups of herds was separately collected and processed. For each cheese-making trial in each factory, amounts of milk, ranging from 2,000 to 6,000 kg, with different κ-CN A to κ-CN B content ratios were separately manufactured. Each vat contained milk collected from at least 4 dairy herds. Cheese-yields at different ripening times were recorded. A difference of about 20 % for the κ-CN A to κ-CN B content ratio was obtained experimentally in each dairy plant. Milk was characterized by having similar composition in terms of protein, casein, casein number, casein composition, β-CN composition and pH. Milk with the higher proportion of κ-CN B (**HIGHB**) exhibited similar coagulation properties but a higher cheese yield in all the investigated cheese in comparison with milk with a lower proportion of κ-CN B (**LOWB**). However, the increment of yield observed for HIGHB milk in Montasio cheese was ascribed to a greater fat content of HIGHB milk in comparison with LOWB milk. The probability of HIGHB milk giving a cheese yield 5 % greater than that of LOWB milk ranged from 51 to 67 % for Montasio cheese, but was lower than 21 % for Asiago and Caciotta cheeses. The ratio of κ-CN A to κ-CN B content did not relevantly affect industrial cheese yield when milks of similar casein composition were processed. Thus, an indirect effect due to the higher κ-CN content of κ-CN B milk on cheese yield is to be suggested.

INTRODUCTION

Cheese yield (**CY**) plays a fundamental role in the profitability of dairy industry and it is influenced by many factors, such as casein (**CN**) content and milk protein polymorphisms. The effect of the *CSN3* (κ-CN) B genetic variant has generated considerable interest because, when compared to the A variant, it has been associated with improved milk coagulation properties (**MCP**) (Van den Berg et al., 1992; Lodes et al., 1996b; Walsh et al., 1998a,b) and increased CY in a range of cheese varieties such as Cheddar, Mozzarella, Parmigiano-Reggiano, Svecia, Gouda (Buchberger and Dovč, 2000). Also, studies investigating Cheddar cheese-making have shown that milk produced by homozygous *CSN3* BB animals exhibited higher fat recoveries into cheese, which was likely associated with the finer gel structure of κ-CN BB milk (Walsh et al., 1998b). Because the two amino acid substitutions differentiating κ-CN A and B variants are not close to the site of action of rennet during the initial phase of coagulation, a direct effect of this substitution on the enzymatic process can be excluded (Glosclaude, 1988).

Several causes have been considered to explain the enhanced properties of κ-CN BB milk. They involved the different net charge of the two protein variants, the content of citric acid in milk, a difference in the degree of glycosylation, and a greater homogeneity of the size of the micelles in κ-CN BB milks (Grosclaude, 1988). Finally, these properties have been partly attributed to greater overall CN content associated with κ-CN B (Jakob and Puhan, 1992; Walsh et al., 1995) in comparison with κ-CN A milk.

To date, most studies have been pilot-scale cheese-making studies or have been using bulk milk of genotyped cows (Buchberger and Dovč, 2000) in which total CN content (**TCN**) or contents of other protein fractions were unknown or unlike across compared milks. Genotypes at *CSN3* have been associated with differences in κ-CN content of milk (Heck et al., 2009; Bonfatti et al., 2009b), which are responsible for a greater number of small micelle in κ-CN BB milk, resulting in more compact curds (Horne et al., 1998). In addition, a marked effect of CN composition and *CSN2* (β-CN) genotypes on MCP has been recently reported (Bonfatti et al., 2009a). All these factors may contribute to observed variation in CY and MCP occurring when milks exhibiting different κ-CN variants content are processed. Literature studies have not clarified whether detected associations between CY and specific genotypes at *CSN3* were attributable to effects exerted by different structural properties of κ-CN protein variants or were largely due to modifications of CN composition of milk.

The aim of this study was to investigate the effect exerted by the ratio of κ-CN A to κ-CN B bulk milk content on industrial CY of Montasio (**MO**), Asiago (**AS**), and Caciotta (**CA**) cheeses when CN content and composition and contents of β-CN variants were alike in bulk milk.

MATERIALS AND METHODS

Data Collection

A total of 24 cheese-making trials were carried out in three commercial dairies located in the north of Italy: one artisanal plant, manufacturing MO, and two industrial plants, one manufacturing AS and one manufacturing CA. The cheese-making trials were carried out from January to June 2009. An exploratory sampling of bulk milk of all herds (13 herds for MO, 125 for AS and 242 for CA) supplying the three dairies involved in the study was initially performed to assess across-herds variation of detailed protein composition. After the initial assessment, sampling of herds selected to supply milk used in experimental cheese making trials was replicated few days before each cheese making experiment. This was performed to ensure that characteristics of experimental milk were consistent with the desired ones. Two samples were collected in each herd, stored in portable chilling devices immediately after collection, frozen at -20°C, and then transferred to the milk laboratory of the Department of Animal Science (Padova, Italy) to measure protein composition and MCP and to the Milk quality Laboratory of Veneto Agricoltura (Thiene, Italy) to measure fat content by infrared analysis with a Milko-Scan FT120 (Foss Electric, Hillerød, Denmark).

Analysis of Protein Composition

Contents of α_{S1}-, α_{S2}-, βγ-, κ-CN, β-LG and α-LA were measured in herd bulk milk samples, vat-milk and in cheese-whey samples, by reversed-phase HPLC (RP-HPLC), following the method proposed by Bonfatti et al. (2008). The method allows the separation and quantification of major CN and whey protein fractions and of their most common genetic variants. Contents of κ-CN A and B, of β-LG A and B, and of β-CN A^1, A^2 and B were quantified. The content of each genetic variant was quantified through the use of specific calibration equations as estimated by Bonfatti et al. (2008). γ-CN elutes in the interval of elution of the F variant of β-CN. For this reason, the peak eluting in this interval (**γ-CN/β-CN$_F$**) was considered to be composed by γ-CN and β-CN and the total content of γβ-CN was given. Total CN (**TCN**) content was defined as the sum of α_{S1}-CN, α_{S2}-CN, γβ-CN, and κ-CN content. Total whey protein content (**WH**) was calculated as the sum of α-LA and β-LG contents. Total protein content (**PRT**) was expressed as the sum of TCN and WH. Casein number (**CI**) was calculated as the ratio of TCN to PRT. Casein composition (i.e., relative contents of CN fractions) was expressed as the percentage ratio of α_{S1}-CN (**α_{S1}-CN%**), α_{S2}-CN (**α_{S2}-CN%**), γβ-CN (**γβ-CN%**), and κ-CN (**κ-CN**%) contents to TCN. The glycosylated portion of κ-CN (**glico-κ-CN**) was also quantified and expressed as a percentage ratio of the total κ-CN content in milk. The ratio of κ-CN A to κ-CN content (**κ-CN$_A$%**) was expressed as the percentage ratio of glyco-free κ-CN A content to the total content of glyco-free κ-CN.

Milk Coagulation Properties

Measures of MCP of bulk-milk and vat-milk samples were obtained by using the Computerized Renneting Meter (CRM-48, Polo Trade, Monselice, Italy), within few hours after samples collection. Samples (10 mL) were preheated at 35 °C and added with 200 μL of rennet (Hansen standard 160, 80 % chymosin, 1:14900, Pacovis Amrein AG, Bern, Switzerland) 1.6 % vol/vol in distilled water. Measures of rennet clotting time (RCT, min), curd firming time (K_{20}, min) and curd firmness (a_{30}, mm) were obtained. Traits definition and details on used methods can be found in Dal Zotto et al. (2008). Non coagulating milks (i.e., milk that did not coagulated within 31 min since rennet addition) obtained no value for RCT and K_{20}. Measures of pH were carried out (pH-Burette 24, Crison) prior to rennet coagulation analysis.

Manufacture of Montasio Cheese

Montasio cheese is a Protected Designation of Origin (PDO) product (Reg. CEE n. 1107/96), a geographical definition that includes the entire territory of Friuli-Venezia Giulia region and the whole province of Belluno and Treviso and a fraction of Padova and Venice provinces. It is a semi-hard pressed cheese, cylindrical, 6–10 cm height and with an average weight ranging from 5 to 9 kg, with a diameter of 30–40 cm. The crust is smooth and elastic. The paste is slightly straw-coloured and has small uniform holes.

Weekly and over a period of 4 weeks, two experimental milks (HIGHB and LOWB), differing for the relative ratio of κ-CN A to κ-CN B content (HIGHB: κ-CN_A% = 40 %; LOWB: κ-CN_A% = 60 %) were processed consecutively on the same day and using the same vat. Each experimental milk derived by the blending of bulk milk of a minimum of 4 herds. The volume of processed milk ranged from 2,000 to 3,000 L, but was similar for the two experimental milks within day of processing. Immediately after pasteurization (71.5 °C for 20 s), milk was loaded into the vat (4,000 L-capacity) and heated at 37 °C. When milk reached 37 °C, a starter (strain ST Mesophilic and Thermophilic; Lyofast ST 073, 0.5 U for every 100 L of milk) and a natural milk starter (2.5–3.3 L for every 100 L vat milk) were added. The latter derived from naturally acidified milk collected on the preceding day of the day of cheese making and its addition aimed to enrich the milk with lactic acid bacteria. After 35 min since the starter addition, rennet was added to milk (natural calf rennet powder, 3.6 g for every 100 L milk, 1:125,000, 95 % chymosin, 5 % pepsin). During the rest period after the addition of rennet, the exact volume of milk in the vat was measured through the use of a dip-stick. The coagulation process began within 16–18 min since the addition of rennet. The curd was broken up into small granules. The first cut of the curd was made 6 min after curd formation and a second cut was made after removing 15 % of the whey contained in the vat and after a 10-min healing period. After the second cutting, the curd was cooked for 22 min at 47 °C. During cooking,

the curd was agitated continuously and, finally, curd was drained off the vat to form the moulds. Moulds were pressed for 35–40 minutes and then, after a gradual cooling (1 d) and drying, the wheels of cheese were placed into brine (20 % salt) for 24 h. Temperature of the ripening rooms was kept at a 9–11 °C, and relative humidity around 80 %. Moulds were turned on the planks of the ripening rooms every 2 weeks. At d 30 of ripening, moulds were scraped with a steel plate and at d 50 they were oiled with linseed oil.

Manufacture of Asiago Cheese

Asiago cheese is a PDO product (Reg. CEE n. 1107/96). The officially-recognized production region for Asiago cheese includes the entire provinces of Vicenza and Trento, and two areas in the provinces of Treviso and Padova, all located in the north-east of Italy. It is a cylindrical soft cheese, with a thin and elastic crust. The mould is 11–15 cm height and its weight ranges from 8 to 12 kg. The paste has small and scattered holes.

Two experimental milks (HIGHB and LOWB), differing for the relative ratio of κ-CN A to κ-CN B content (HIGHB: κ-CN_A% = 60 %; LOWB: κ-CN_A% = 75 %), were processed within a single vat (11,000 L volume) on three consecutive days, over two weeks, in order to obtain two replicates for each experimental milk. The volume of processed milk in each trial was 5,500 L. Each experimental milk derived by the blending of bulk milk of a minimum of five herds. After pasteurization (72 °C for 18 s), milk was loaded in the vat and starter culture (like AP 100 thermophilic lactic cultures, CHR Hansen, 200 U per 110 q of milk diluted in 2 L of water) was added when the temperature of milk reached 37 °C. The addition of liquid calf rennet (1:10,000, 80 % chymosin, 20 % pepsin) occurred 35 min after the starter addition. During the rest period after the addition of rennet, the exact volume of milk in the vat was measured through the use of a dip-stick. Curd formation occurred after 17–18 min and the curd was cut after 30 min since rennet addition to milk. The healing time after cutting was 12 min. After healing, whey was partially drained off the vat and the curd was cooked for 20 min at 42 °C. Finally, curd was drained off the vat to form the moulds. Moulds were pressed for 2 h and, after a drying period of 72 h, were transferred into brine. Moulds were kept into brine for 48 h and then transferred in the ripening rooms. During the 20-d period of ripening, moulds were kept at 10 °C and 80 % relative humidity.

Manufacture of Caciotta Cheese

Caciotta cheese is a soft short-maturing cheese, with small-sized and spherical-cylindrical moulds. This cheese is produced in northern Italy with standardized technologies and on a semi-industrial scale. Ripening lasts few weeks. Paste is slightly pigmented, compact, uniform or slightly eyes-scattered.

Two experimental milks (HIGHB and LOWB), differing for the relative ratio of κ-CN A to κ-CN B content (HIGHB: κ-CN_A% = 45 %; LOWB: κ-CN_A% = 65 %), were processed simultaneously. Each experimental milk was processed into three industrial vats of equal volume (1,900 L). The experimental cheese making experiment was repeated on three consecutive days over a week, for two weeks. Each experimental milk derived by the blending of bulk milk of at least 8 herds. Immediately after pasteurization (72 °C for 16 s), milk was loaded in the vats (the volume of milk was measured automatically at loading) and heated at 41 °C. At this temperature, a starter (strain TB1 Mesophilic yeasts and lactobacilli) was added. Salt (12.5 kg/vat) was introduced immediately before the addition of rennet (1:12,000 chymosin 75 %, pepsin 25 %, 50 mL per 100 L of milk), which was added after 35 min since the starter addition to milk.

Curd was formed within 14–15 min and cutting of the curd occurred after 25 min since rennet addition. After cutting, the curd was drained off the vat and moulds were formed. After 1 h of pressing, moulds were kept into brine (17 °BE, 9 °C) for 2 d, then moulds were ripened for 5 d at 3 °C and kept, during the following 5 d, at 5 °C, with ventilation to allow crust formation.

Analysis of Vat-milk and Cheese-whey

Analysis of milk composition was carried out twice for milk sampled from the vat before the adding of the starter and for the cheese-whey collected after curd extraction from the vat.

The following traits were measured: pH with a pH-meter, titratable acidity with 0.25 M NaOH using the Soxhlet-Henkel method (Anon, 1963) and fat by infrared analysis with a Milko-Scan FT120 (Foss Electric, Hillerød, Denmark). Analysis of protein composition was carried out by RP-HPLC (Bonfatti et al., 2008). Milk coagulation properties were also measured in duplicate (CRM-48, Polo Trade, Monselice, Italy) on vat-milk.

Cheese Yield Recording and Analysis of Cheese

For all cheeses, the first measure of CY (**CY1**) was recorded after the brine period at d 2 or 3 for MO and CA or AS, respectively. Cheese yield at the end of the ripening period (**CY2**) was also measured. At the end of the ripening period, which lasted 60, 20, or 10 d for MO, AS, or CA, respectively, three moulds per experimental milk in each day of processing were randomly sampled. Cheese samples were analyzed for moisture by vacuum oven at 100 °C (method 926.08; AOAC, 2003), fat by the Mojonnier method (method 933.05; AOAC, 2003), ash using a muffle furnace at 550 °C (method 935.42; AOAC, 2000), and total protein by macro-Kjeldahl (method 2001.14; AOAC, 2002). As κ-CN variants have been reported (Walsh et al., 1998a) to exert trivial effects on primary and secondary proteolysis during ripening or on the grading scores awarded for acceptability, cheese quality was not evaluated.

Statistical Analysis

Variation of composition of vat-milk and cheese-whey, MCP of vat-milk, CY at different ageing periods, and moisture, protein and fat content of manufactured cheese attributable to the effects of experimental milk (i.e., the effects of the ratio of κ-CN A to κ-CN B content) was investigated through Bayesian analyses. A difficulty when comparing CY of different experimental groups is due to the small sample size achievable in this type of studies. Moreover, relevance of effects is often difficult to evaluate. A Bayesian statistical approach performing numerical integration through Monte-Carlo Markov Chain (MCMC) procedures allows the use of marginal posterior probability densities for treatment means comparisons. Thus, the probability for a treatment mean of being greater or lower than the one of another treatment group, or the probability of a relevant difference across treatments can be obtained. These procedures ensure high flexibility for the interpretation of results.

As differences in the ratio of κ-CN A to total κ-CN content across processed milks were different for MO, AS, and CA cheese making trials, separated statistical analyses were performed for each type of manufactured cheese. Models included the effect of the experimental milk (2 levels: HIGHB and LOWB) and the effect of the day of cheese making (4 levels for MO, 2 levels for AS, and 6 levels for CA). Prior distributions for both effects were assumed to be uniform. A unique Gibbs chain of 100,000 iterations was run for each analysis, with a burn-in of 10,000 iterations. Samples were saved every 10 iterations. Convergence was tested for each chain using the Z criterion of Geweke (Geweke, 1992). Monte Carlo standard errors were also calculated (Sorensen and Gianola, 2002). The posterior median was used as a point estimate of parameters of concern. Features of the marginal posterior distribution for the difference between means of experimental milks were obtained. Lower and upper bounds of the 95 % posterior probability interval (**HPD**) for the mean of experimental milks and for the difference between means of experimental milks were obtained from the estimated marginal densities. The probability of similarity between experimental milks was also calculated for each trait and was expressed as the probability of the percentage difference between means being in the range from -8 to +8 %, for milk composition and MCP, and from -5 to +5 % for cheese composition.

The ranges of similarity for milk and cheese composition were established considering the variability due to sampling and analytical procedures. The probability of HIGHB having a higher cheese-yield than LOWB was also calculated, as well as the probability of the increase in cheese-yield of HIGHB being higher than 5 % in comparison with LOWB. Without considering milk composition, a 5 % increase has been the minimum value of the difference between BB and AA milk reported in literature (Buchberger and Dovč, 2000), with few exceptions (Schaar et al., 1985; Rahali and Menard, 1991).

RESULTS AND DISCUSSION

Protein Composition and Milk Coagulation Properties of Bulk Milk Samples

Descriptive statistics for protein composition and coagulation properties of bulk milk from all 380 dairy herds supplying the three dairies involved in the study are reported in Table 1. The high value of average protein content of milk is to be ascribed to the skimming of milk prior to chromatographic analysis.

Exploratory sampling included all herds with no distinction of breed and herd size, thus resulting in a large variability of protein fractions and protein genetic variants contents in bulk milk. Composition of bulk milk yielded by a limited number of animals is essentially determined by characteristics of these animals and also less common genetic variants at the population level may be prevalent in bulk milk of small herds.

Genetic composition of κ-CN showed a large variability. Relative content of κ-CN A in total κ-CN (κ-CN_A%) ranged from 4.1 to 89.0 %. Overall, content of κ-CN A was slightly greater than that of κ-CN B. Also the β-CN fraction exhibited a great variability of genetic composition (i.e., relative ratios between genetic variants) with a large prevalence of variant A^2 (50.2 % on total β-CN). This was expected as a consequence of the high frequency of this allele in many Italian dairy cattle breeds (Comin et al., 2008; Bonfatti et al., 2009b). However, bulk milk of sampled herds exhibited also a rather high content of β-CN A^1. Variant B of β-CN was also detected in milk of several herds, with an average relative content of 16.28 % on total β-CN. The rather high content of this variant, not common for the Holstein Friesian breed, may be attributable to the presence of Brown Swiss or Simmental cows in the sampled herds.

Whey protein composition showed a slight predominance (52.54 %) of β-LG B over β-LG A. This result was expected, due to the slightly higher frequency of variant B, in respect to A variant, in Holstein Friesian and Simmental, and its high frequency in Brown Swiss cows (Formaggioni et al., 1999).

Of the investigated bulk milk samples, 7.4 % coagulated within 15 min since rennet addition and exhibited a rather short RCT, 43.9 % coagulated in the interval from 15 to 20 min, which is considered an optimal RCT range, and 33.9 % coagulated after 20, but before 25 min. The proportion of milk not suitable for processing (RCT > 25 min) was 14.7 %.

Within this category of milk, a great proportion of non-coagulating milk (6.8 % of all samples) was observed. The high incidence of non-coagulating milk and averages values for RCT and a_{30} (20 min and 24 mm, respectively) confirm the poor coagulation ability of milk in Italy, which has been reported in previous studies (Mariani et al., 1992; Sandri et al., 2001).

Table 1. Descriptive statistics for protein composition and coagulation properties of bulk-milk samples (n = 380)

Item[1]	Mean	SD	Minimum	Maximum
Protein, g/L	40.57	2.85	29.97	52.77
Casein, g/L	35.38	2.51	25.72	46.33
Whey protein, g/L	5.19	0.46	4.02	6.75
Casein number,%	87.21	0.67	85.45	91.01
Protein fractions content, g/L				
α_{S1}-CN	13.52	1.01	10.50	18.18
α_{S2}-CN	4.35	0.47	2.80	5.99
βγ-CN	14.08	1.05	9.94	18.25
κ-CN	3.43	0.40	2.33	5.36
α-LA	1.17	0.14	0.78	1.53
β-LG	4.02	0.41	3.02	5.85
Casein genetic variants content, g/L				
β-CN_B	0.82	0.22	0.41	2.66
β-CN_{A1}	3.86	1.27	0.85	8.65
β-CN_{A2}	7.08	1.62	0.98	14.73
γ-CN/β-CN_F	0.82	0.22	0.41	2.66
κ-CN_A	1.03	0.27	0.10	1.79
κ-CN_B	0.82	0.41	0.17	3.15
β-LG_A	1.92	0.58	0.13	5.30
β-LG_B	2.10	0.49	0.42	4.11
Casein composition, %				
α_{S1}-CN%	38.21	1.24	33.08	42.95
α_{S2}-CN%	12.30	0.91	9.55	16.58
β-CN%	39.80	1.20	35.15	43.96
κ-CN%	9.69	0.81	7.77	15.58
κ-CN composition, %				
κ-CN_A%	56.98	16.02	4.11	89.00
Glyco-κ-CN	45.94	3.69	34.95	60.30
β-CN composition, %				
β-CN_{A1}%	27.62	9.45	5.95	71.52
β-CN_{A2}%	50.21	10.58	8.14	83.00
β-CN_B%	16.28	8.29	2.77	54.95
γ-CN/β-CN_F%	5.89	1.70	2.31	20.62
Milk coagulation properties				
RCT, min	20.00	3.56	7.08	29.57
K_{20}, min	2.97	2.10	0.00	8.95
a_{30}, mm	24.07	7.31	3.00	41.00

[1] Protein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN + β-LG + α-LA; casein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN; casein number = casein/protein×100; κ-CN_A% = κ-CN_A/(κ-CN_A + κ-CN_B) × 100; whey protein = β-LG + α-LA; Milk coagulation properties referred only to coagulating samples (n = 354); RCT = rennet clotting time; K_{20} = curd firming time; a_{30} = curd firmness.

Composition of Experimental Milks

Inferences were made from features of marginal posterior distributions of the differences between experimental milks. MCMC chains converged in all cases and Monte Carlo standard errors were very small (lower than 0.004, data not reported in table).

In general, the range of variation of κ-CN_A% across experimental milks was smaller in comparison with that observed in the exploratory sampling. This was due to the need of keeping other characteristics similar across experimental milks when bulk milks were blended. Exclusive blending of milk from most extreme herds for milk κ-CN_A% was not feasible because those herds exhibited also the most extreme total κ-CN contents and casein number and it would have led to experimental milks with large differences in terms of protein composition. Nevertheless, the range of the κ-CN A to κ-CN B content ratio for experimental milks was approximately equal to 1 κ-CN_A% SD in the sampled bulk milks and, for nearly half of sampled herds, κ-CN_A% was within the interval of experimental milks κ-CN_A%.

For fat content and protein composition, features of the marginal posterior density for the experimental milk means and for the difference between means are reported in Table 2, 3, and 4, for MO, AS and CA trials, respectively. The main advantage of using Bayesian techniques when compared with classical statistics is the use of estimated posterior probability densities, which permits greater accuracy about the evaluations of the uncertainty in the estimated parameters and allows the construction of several types of probability intervals to test hypotheses. For example, in Table 2, 3, and 4, almost all differences between means of HIGHB and LOWB milk for protein composition had a HPD interval that included zero. From a classical point of view, this will lead to the conclusion that there are no significant differences between milks. However, this interval is only an indication of the accuracy of the measurement of the difference across milks. To answer the question of whether milks are different or not in relation to a characteristic of concern, the probability of difference (or similarity) needs to be investigated.

Montasio cheese milks

For MO cheese, in HIGHB milk the relative content of κ-CN A was approximately 40 %, whereas it was 60 % in LOWB milk. Because of large variability of fat content over time, equal contents of fat across experimental milks were not obtained and HIGHB milk exhibited a greater fat content (+13 %) in comparison with LOWB milk. Moreover, HIGHB milk was also characterized by a slightly higher content of α_{S1}-CN and κ-CN, which increased also overall CN and PRT content. The proportions of CN fractions on TCN were comparable across experimental milks, but a lower β-CN B to total β-CN content ratio, counterbalanced by a higher proportion of β-CN A^2, was observed in HIGHB milk. Allele B of *CSN*2 is reported to have positive effects on MCP (Buchberger and Dovč, 2000; Wedholm et al., 2006; Bonfatti et al., 2009a), and a different incidence of this variant in milk

is expected to affect coagulation ability and possibly CY. Although there was a relevant difference in the proportion of β-CN_B% on total β-CN, the content of β-CN B was similar for the two experimental milks. As content of κ-CN B variant is associated to that of β-LG B, β-LG genetic composition was unbalanced between the two experimental milk, with HIGHB milk exhibiting a higher content of β-LG B over the A variant. Literature results for the effect of β-LG variants on MCP and CY are conflicting. No significant differences between CY or MCP for milks of different phenotypes for β-LG have been reported (Marziali and Ng-Kwai-Hang, 1986a), but in most studies (Lodes et al., 1996b; Schaar et al., 1985; van den Berg et al., 1992) β-LG B, for its increased CN to PRT content ratio in comparison with β-LG A, has been associated with increased CY. In our study, HIGHB and LOWB milks had very similar CI and HIGHB milk (which was expected to give an increased CY) had the lowest content of β-LG B.

Asiago cheese milks

Two experimental milks were processed to investigate the effect of κ-CN composition on CY of AS cheese. No relevant differences of contents and composition were observed between HIGHB and LOWB milks. Experimental milks were characterized by having similar contents of fat and TCN, CN composition (α_{S1}-, α_{S2}-, β-, and κ-CN to TCN content ratios), and WH composition (β-LG and α-LA to WH content ratios). Contents of all genetic variants for major CN fractions were also similar, with the only expected exception of κ-CN A and κ-CN B contents. Like for MO trials, an increased proportion of β-LG B was observed for HIGHB milk. For the manufacturing of AS cheese, the difference for the relative content of κ-CN A across experimental milks was rather limited (κ-CN_A% was 61 and 75 % of total κ-CN for HIGHB and LOWB milks, respectively). This was mainly due to the large amount of milk required for processing (a minimum of 5,500 L) which reduced variation of κ-CN_A% and did not permit to obtain experimental milks with larger differences in genetic composition of κ-CN.

Caciotta cheese milks.

For the composition of vat-milk of CA manufacture, a difference between the two experimental milks of approximately 20 % in the relative proportion of κ-CN A in κ-CN content was obtained. HIGHB milk exhibited a slightly greater amount of PRT content, due to increased CN and WH contents of HIGHB milk when compared to LOWB milk, but these differences did not appear to be relevant. Within CN fractions, HIGHB milk had a greater content of κ-CN (+8 % in comparison with LOWB milk). However, the relative proportion of κ-CN to TCN was very similar in both milks. HIGHB milk exhibited also a greater content of β-CN B and A^2, and a smaller amount of β-CN A^1 in comparison with LOWB milk. The relative proportions of CN fractions were well balanced, but with a higher content of β-CN and an increased percentage ratio of β-CN to TCN content for HIGHB milk.

Table 2. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to milk and protein composition for Montasio cheese production[1]

	Experimental milk		Difference between milks (%)		
Item	HIGHB	LOWB	Median	HPD95%	*P*
Fat, %	4.38	3.90	13.05	-5.48 – 35.71	26
Protein, g/L	40.94	38.83	5.69	-3.38 – 14.81	79
Casein, g/L	35.82	33.80	6.23	-2.58 – 14.17	75
Whey protein, g/L	5.12	5.04	1.85	-14.21 – 20.10	75
Casein number, %	87.25	87.49	0.28	-1.03 – 1.66	100
Protein fractions content, g/L					
α_{S1}-CN	13.35	12.37	8.55	-1.31 – 19.84	42
α_{S2}-CN	4.63	4.63	-0.13	-9.05 – 9.52	92
βγ-CN	14.09	13.33	5.80	-0.69 – 12.63	83
κ-CN	3.76	3.47	9.25	-3.09 – 22.71	37
α-LA	1.23	1.27	-3.28	-25.94 – 22.85	64
β-LG	3.89	3.78	3.25	-10.34 – 17.05	77
Casein genetic variants content, g/L					
$\beta\text{-CN}_B$	2.54	2.60	-1.79	-21.58 – 18.95	68
$\beta\text{-CN}_{A1}$	3.39	3.34	1.68	-8.72 – 12.99	85
$\beta\text{-CN}_{A2}$	7.44	6.64	13.65	-1.47 – 28.79	12
$\gamma\text{-CN}/\beta\text{-CN}_F$	0.72	0.76	-5.09	-29.89 – 25.12	45
$\kappa\text{-CN}_A$	0.80	1.12	-31.40	-56.35 – -7.88	0
$\kappa\text{-CN}_B$	1.24	0.72	78.34	15.00 – 165.50	0
$\beta\text{-LG}_A$	1.50	2.40	-41.37	-73.06 – -10.60	0
$\beta\text{-LG}_B$	2.39	1.38	76.89	-4.106 – 175.30	0
Casein composition, %					
α_{S1}-CN%	37.26	36.56	1.98	-1.46 – 5.32	100
α_{S2}-CN%	12.92	13.72	-5.50	-12.03 – 1.30	85
βγ-CN%	39.32	39.46	-0.36	-3.19 – 2.34	100
κ-CN%	10.49	10.25	2.37	-1.76 – 6.81	100
κ-CN composition, %					
$\kappa\text{-CN}_A$%	39.23	60.51	-36.30	-52.93 – -16.68	0
Glyco κ-CN	45.45	47.09	-3.40	-8.13 – 0.70	98
βγ-CN composition, %					
$\beta\text{-CN}_{A1}$%	24.12	25.02	-3.62	-14.22 – 7.65	79
$\beta\text{-CN}_{A2}$%	52.74	49.76	6.61	-3.34 – 16.80	67
$\beta\text{-CN}_B$%	18.05	19.53	-6.07	-27.24 – 14.73	55
$\gamma\text{-CN}/\beta\text{-CN}_F$%	5.08	5.71	-10.50	-34.29 – 13.52	33

[1] Protein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN + β-LG + α-LA; casein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN; whey protein = β-LG + α-LA; $\kappa\text{-CN}_A\% = \kappa\text{-CN}_A/(\kappa\text{-CN}_A + \kappa\text{-CN}_B) \times 100$; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; *P* = probability of (HIGHB - LOWB)/LOWB × 100 being within ±8%.

Table 3. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to milk and protein composition for Asiago cheese production[1]

	Experimental milk		Difference between milks (%)		
Item	HIGHB	LOWB	Median	HPD95%	*P*
Fat, %	3.94	3.87	1.93	-2.07 – 6.57	99
Protein, g/L	39.29	40.08	4.39	-6.77 – 9.63	94
Casein, g/L	34.10	34.64	-1.57	-7.17 – 4.09	98
Whey protein, g/L	5.17	5.43	-4.77	-14.28 – 4.49	81
Casein number, %	86.83	86.45	0.44	-0.29 – 1.16	100
Protein fractions content, g/L					
α_{S1}-CN	12.95	13.22	3.94	-7.61 – 3.75	98
α_{S2}-CN	4.11	4.26	-3.43	-8.57 – 1.84	96
βγ-CN	13.66	13.91	-1.72	-8.56 – 4.97	96
κ-CN	3.38	3.26	3.68	-5.46 – 13.36	83
α-LA	1.28	1.41	-8.59	-20.17 – 3.47	43
β-LG	3.89	4.03	-3.36	-13.50 – 7.38	83
Casein genetic variants content, g/L					
$\beta\text{-CN}_B$	1.49	1.54	-3.27	-15.60 – 10.38	74
$\beta\text{-CN}_{A1}$	4.07	3.93	3.59	-4.92 – 12.47	85
$\beta\text{-CN}_{A2}$	7.21	7.66	-5.93	-16.65 – 4.71	69
$\gamma\text{-CN}/\beta\text{-CN}_F$	0.89	0.77	16.25	-19.74 – 56.71	23
$\kappa\text{-CN}_A$	1.07	1.24	-13.32	-26.58 – 1.08	16
$\kappa\text{-CN}_B$	0.68	0.40	68.77	-14.01 – 191.20	2
$\beta\text{-LG}_A$	1.76	2.13	-17.44	-30.13 – -3.48	5
$\beta\text{-LG}_B$	2.13	1.90	12.18	-4.81 – 29.13	25
Casein composition, %					
α_{S1}-CN%	37.98	38.18	-0.52	-1.22 – 0.29	100
α_{S2}-CN%	12.05	12.29	-1.94	-7.50 – 2.88	98
βγ-CN%	40.07	40.14	-0.17	-1.61 – 1.22	100
κ-CN%	9.90	9.40	5.36	-3.04 – 13.71	77
κ-CN composition, %					
$\kappa\text{-CN}_A$%	61.36	75.38	-18.63	-33.37 – -1.42	7
Glyco κ-CN	48.27	49.74	-2.85	-7.19 – 2.03	98
βγ-CN composition, %					
$\beta\text{-CN}_{A1}$%	29.74	28.19	5.49	-2.78 – 13.38	78
$\beta\text{-CN}_{A2}$%	52.70	55.06	-4.29	-11.18 – 3.30	87
$\beta\text{-CN}_B$%	10.95	11.14	-1.71	-14.37 – 11.69	79
$\gamma\text{-CN}/\beta\text{-CN}_F$%	6.61	5.60	18.09	-19.30 – 59.17	21

[1] Protein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN + β-LG + α-LA; casein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN; whey protein = β-LG + α-LA; $\kappa\text{-CN}_A\% = \kappa\text{-CN}_A/(\kappa\text{-CN}_A + \kappa\text{-CN}_B) \times 100$; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; *P* = probability of (HIGHB - LOWB)/LOWB × 100 being within ±8%.

Table 4. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to milk and protein composition for Caciotta cheese production[1]

	Experimental milk		Difference between milks (%)		
Item[1]	HIGHB	LOWB	Median	HPD95%	*P*
Fat, %	3.62	3.62	0.20	-4.98 – 5.77	100
Protein, g/L	39.46	37.62	4.93	-0.44 – 11.27	90
Casein, g/L	34.48	32.90	4.89	-0.60 – 10.63	91
Whey protein, g/L	4.97	4.73	5.04	-1.47 – 12.00	86
Casein number, %	87.40	87.44	-0.04	-0.46 – 0.36	100
Protein fractions content, g/L					
α_{S1}-CN	12.69	12.17	4.09	-0.54 – 8.815	95
α_{S2}-CN	4.58	4.32	6.50	-2.27 – 14.80	69
βγ-CN	13.66	13.08	4.43	-1.41 – 10.55	92
κ-CN	3.58	3.30	8.65	0.79 – 16.99	41
α-LA	1.29	1.20	5.40	-2.70 – 13.90	80
β-LG	3.71	3.53	4.89	-2.11 – 11.80	86
Casein genetic variants content, g/L					
β-CN_B	2.24	1.99	14.68	2.20 – 28.45	8
β-CN_{A1}	3.66	4.04	-8.59	-17.63 – 1.87	44
β-CN_{A2}	6.93	6.25	10.97	-1.16 – 23.61	26
γ-CN/β-CN_F	0.82	0.80	2.71	-7.59 – 13.95	85
κ-CN_A	0.89	1.10	-17.44	-28.07 – -6.82	3
κ-CN_B	0.99	0.59	78.74	15.18 – 191.60	0
β-LG_A	1.61	1.76	-7.85	-16.53 – 1.22	51
β-LG_B	2.09	1.77	18.71	8.28 – 29.61	2
Casein composition, %					
α_{S1}-CN%	36.78	36.70	-0.62	-2.35 – 1.01	100
α_{S2}-CN%	13.26	13.16	0.81	-3.40 – 5.34	99
βγ-CN%	39.58	39.76	-0.48	-1.18 – 0.28	100
κ-CN%	10.37	10.05	3.36	-0.11 – 6.89	99
κ-CN composition, %					
κ-CN_A%	47.84	65.37	-24.51	-34.33 – -14.82	1
Glyco κ-CN	47.50	48.94	-2.95	-6.80 – 1.25	99
βγ-CN composition, %					
β-CN_{A1}%	26.92	30.89	-11.90	-22.40 – -1.62	17
β-CN_{A2}%	50.68	47.74	6.15	-0.27 – 12.94	75
β-CN_B%	16.32	15.20	8.70	0.17 – 17.67	38
γ-CN/β-CN_F%	6.06	6.16	-1.67	-11.89 – 8.31	90

[1] Protein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN + β-LG + α-LA; casein = α_{S1}-CN + α_{S2}-CN + βγ-CN + κ-CN; whey protein = β-LG + α-LA; κ-CN_A% = κ-CN_A/(κ-CN_A + κ-CN_B) × 100; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; *P* = probability of (HIGHB - LOWB)/LOWB × 100 being within ±8%.

Whey Composition

No significant difference for pH and for contents of protein fractions was observed in cheese-whey from LOWB and HIGHB milks (data not reported in tables). Also dry matter, ash, protein and fat contents did not vary between milks and exhibited a probability of similarity of at least 67 %. Conversely, other authors reported higher levels of fat in the κ-CN AA cheese whey for Cheddar (Graham et al., 1986; Walsh et al., 1995, 1998b), Gouda (van der Berg et al., 1992) and Mozzarella (Walsh et al., 1998a). This finding was attributed to finer gel networks and to the resulting smaller porosity of gels derived from of k-CN BB milks causing an improved fat retention. This effect was likely reduced when milks with similar κ-CN contents were processed.

Milk Coagulation Properties

Posterior medians and HPD for the effect of experimental milk on MCP are reported in Table 5. The probability of similarity between MCP of HIGHB and LOWB milks is also reported. A slightly higher titratable acidity was observed in LOWB milks in comparison with HIGHB, even if pH was similar in both types of milk.

In general, differences between HIGHB and LOWB milks observed for MCP were not relevant. Although slight variations of MCP can be observed, they were not in favour of HIGHB milk. As MCP are strictly related to pH of milk, a lacking difference in pH across experimental milks is expected to have limited the difference in RCT between HIGHB and LOWB milk.

In a previous study (Walsh et al., 1998b) conducted using milks with similar contents of CN, κ-CN BB milk had shorter RCT, and higher a_{30} when compared with κ-CN AA milk. It has been suggested (Horne et al., 1996) that the shorter RCT of κ-CN BB milk probably results from a lower critical level of κ-CN hydrolysis required for the onset of gelation. However, this property might be ascribed to the increased κ-CN content of BB milk. Because, for a given CN content, the curd firming rate has been reported to be inversely proportional to the cube of the micelle diameter (Horne et al., 1996) and the micelle size is smaller when κ-CN content is greater, the higher curd firming rate for κ-CN BB milk might be attributable to the smaller micelle size of this milk, which allows for more numerous intermicellar bonds and for a more compact arrangement of the curd (Horne et al., 1996). In agreement with our results, when the statistical analysis of MCP data have been performed accounting for protein fractions contents or protein composition, no significant effect of *CSN3* alleles was observed for RCT and a_{30} (Marziali and Ng-Kwai-Hang, 1986b; Bonfatti et al., 2009a). Our results seem to confirm that the favorable effects exerted by *CSN3* B on MCP are to be entirely attributable to modifications of protein composition caused by the differential expression of this allele and not to a direct effect of the specific protein genetic variant on the coagulation process.

Table 5. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to acidity and milk coagulation properties for Montasio, Asiago and Caciotta cheese production[1]

Item	Experimental milk		Difference between milks (%)		
	HIGHB	LOWB	Median	HPD95%	*P*
Montasio					
pH	6.74	6.73	0.18	-1.31 – 1.68	100
SH°	3.22	3.37	-4.69	-19.64 – 11.36	34
RCT, min	21.80	21.24	2.73	-12.09 – 18.86	41
K_{20}, min	2.33	2.43	-3.67	-30.96 – 30.88	26
a_{30}, min	37.13	36.93	0.50	-14.66 – 15.60	49
Asiago					
pH	6.91	6.91	-0.08	-1.59 – 1.49	100
SH°	2.79	3.27	-14.08	-42.70 – 17.78	24
RCT, min	24.42	24.60	-0.71	-21.54 – 22.40	57
K_{20}, min	3.47	2.99	16.26	-54.1 – 128.00	17
a_{30}, min	27.92	30.19	-7.36	-48.52 – 44.27	27
Caciotta					
pH	6.76	6.78	-0.19	-1.82 – 1.29	100
SH°	3.24	3.30	-1.71	-15.70 – 13.47	74
RCT, min	21.93	22.07	-0.59	-31.59 – 33.75	43
K_{20}, min	2.65	2.44	8.47	-54.74 – 96.30	25
a_{30}, min	36.42	35.50	2.79	-26.53 – 32.28	46

[1]RCT = rennet clotting time; K_{20} = curd firming time; a_{30} = curd firmness; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; *P* = probability of (HIGHB - LOWB)/LOWB × 100 being within ±8%.

Cheese Yield

Posterior medians and HPD for the effect of experimental milk on CY are reported in Table 6. The probability that HIGHB milk gave a greater CY in comparison with LOWB milk is also reported, together with the probability that the increase in CY for HIGHB milk was greater than 5 % of CY for LOWB milk.

All HPD intervals estimated for CY1 and CY2 and for all trials included zero. However, mean CY1 and CY2 for HIGHB milk were higher than corresponding means for LOWB milk in MO, AS and CA, with a probability of at least 70 %. Literature values reported that BB milk is responsible for a 5 % minimum increase of CY in comparison with AA milk. Hence, the probability of obtaining a 5 % greater CY when manufacturing HIGHB milk in comparison with LOWB milk was calculated. Considering a 5 % increase in CY as a relevant difference across experimental milks, the probability of a relevant difference markedly decreased. Only in MO cheese trials, HIGHB milk exhibited CY means greater than 5 % of those provided by LOWB milk with a rather high probability (67 and 51 % for CY1 and CY2, respectively). However, the increased CY observed for HIGHB milk in MO trials was ascribed to its greater fat content in comparison with LOWB milk.

Regression analyses reported by Marziali and Ng-Kwai-Hang (1986a) indicated that for a 1 %-increase in milk fat content a 1.07 %-increase in CY is to be expected. For a similar increase in milk CN content a 1.59 %-increase in CY is likely to occur. For MO cheese, HIGHB milk had a fat content that was 13 % higher than that of LOWB milk and the probability of the difference being higher than 8 % was nearly 75 %. When data were adjusted for fat content, the increase of CY of HIGHB milk, compared to LOWB milk, was smaller than 1 %, and the probability of obtaining a relevant difference of CY across experimental milks was trivial (data not reported in table).

Although a slightly higher CY was observed for HIGHB milks in comparison with LOWB milks, no relevant effects on CY due to the different relative ratio between κ-CN A and B genetic variants were found on industrial scale cheese-making, within the range of milk κ-CN_A% considered. When milk composition was comparable across experimental milks, as for AS and CA cheese-making trials, the probability that HIGHB milk gave a 5 % higher CY than that obtained with LOWB milk was lower than 21 %. Likely, the slightly higher CY associated with HIGHB milk is to be attributed to its slightly higher TCN or κ-CN content.

Table 6. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to cheese yield after brine (CY1) and final cheese yield (CY2) for Montasio, Asiago and Caciotta cheese

Item[1]	Experimental milk		Difference between milks (%)			
	HIGHB	LOWB	Median	HPD95%	$P > 0$	$P > 5$
Montasio						
CY1	12.12	11.44	5.89	-0.16 – 11.89	98	67
CY2	10.70	10.15	5.47	-1.13 – 11.73	85	51
Asiago						
CY1	11.59	11.44	1.28	-0.76 – 3.23	92	0
CY2	10.85	10.81	0.42	-1.36 – 2.39	70	0
Caciotta						
CY1	12.84	12.54	2.34	-3.06 – 8.10	83	14
CY2	11.71	11.39	2.89	-3.20 – 9.71	87	21

[1] CY1was measured at 2 d for Montasio and Caciotta and 3 d for Asiago cheese; CY2 was measured for Montasio, Asiago and Caciotta at 60, 20 and 10 d, respectively; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; $P > 0$: probability of (HIGHB - LOWB)/LOWB × 100 being greater than 0; $P > 5$: probability of (HIGHB - LOWB)/LOWB × 100 being greater than 5%.

In most investigations involving Parmigiano-Reggiano, Cheddar or other cheese varieties, higher yields of cheese, ranging from 5 to 10 %, were found in favour of κ-CN BB compared with κ-CN AA milk (Buchberger and Dovč, 2000). For example, the moisture-adjusted Cheddar CY from κ-CN BB milk was 8.2 % higher than that from κ-CN AA milk, whereas, for Mozzarella cheese, the

moisture-adjusted CY was 12 % higher in κ-CN BB milk (Walsh et al., 1998a,b).

In contrast to the majority of results, Schaar (1986) obtained no significant effect of *CSN*3 polymorphism on CY of Swedish cheese Svecia and he explained the inconsistency of results in comparison with those obtained for Parmigiano-Reggiano by Morini et al. (1982) by the fact that the cooking temperature when manufacturing Parmigiano-Reggiano is higher (55 °C instead of 41 °C for Svecia), thus supporting the syneresis and promoting the retention of fat in cheese. However, temperature for Cheddar cheese-making was 31 °C in the experiment of Walsh et al. (1998b), thus this inconsistency seems not to be ascribed to temperature.

Table 7. Median and 95 % high posterior density interval (HPD95%) for the difference between means of experimental milk (low and high κ-CN A to B content ratio) in relation to cheese composition of Montasio and Caciotta cheese at the end of the ripening period[1]

Item	Experimental milk		Difference between milks (%)		
	HIGHB	LOWB	Median	HPD95%	*P*
Montasio					
DM	63.46	63.72	-0.41	-2.57 – 1.85	100
Protein, %DM	37.51	38.33	-2.11	-4.95 – 0.51	93
Fat, %DM	50.05	49.70	0.70	-3.43 – 4.99	93
Ash, %DM	6.25	6.28	-0.53	-5.55 – 4.97	86
Caciotta					
DM	56.70	56.50	0.37	-3.78 – 4.70	94
Protein, %DM	37.25	37.13	0.34	-2.00 – 2.67	100
Fat, %DM	49.80	51.48	-3.26	-6.71 – 0.29	79
Ash, %DM	6.06	6.37	-4.83	-13.74 – 5.20	44

[1] The ripening period was 60 d for Montasio and 10 d for Caciotta; DM = dry matter; HIGHB = milk containing a low κ-CN A to κ-CN B content ratio; LOWB = milk containing a high κ-CN A to κ-CN B content ratio; difference between milk was computed as (HIGHB - LOWB)/LOWB × 100; *P* : probability of (HIGHB - LOWB)/LOWB × 100 being within ±5%.

However, up to date, most studies investigated the effect of κ-CN genetic variants on CY processing milk of cows grouped on the basis of their *CSN*3 genotypes, without analyzing the CN composition of milk (Van den Berg et al. 1992, Ng-Kwai-Hang and Grousclaude, 1994, Lodes et al., 1996a). The only exception is the work of Marziali and Ng-Kwai-Hang (1986a). In that study, a significant effect of *CSN*3 genotype on CY of 283 individual milk samples was observed, even when accounting for protein composition, but a different CY was detected between AB and BB genotypes and not between AA and BB genotypes. In addition, the content of the two variants in milk was not quantified in that study. Several studies (Hallén et al., 2008; Heck et al., 2009) reported that milk produced by heterozygous animals contains intermediate amounts of both variants when compared to homozygous animals and the effect observed in the study by Marziali and Ng-Kwai-Hang (1986a) seems to be not attributable to the difference in the relative ratio of

genetic variants contents.

In the literature, there is little documented evidence on differences between κ-CN AA and BB milks containing similar CN levels in relation to rennet coagulation and cheese-making properties of milk. Using small milk volumes and a limited number of samples, Walsh et al. (1998b) reported that the *CSN*3 genotype had a significant effect on Cheddar CY obtained from milks with similar contents of total CN. Although overall CN content did not differ significantly across milks of different *CSN*3 genotypes, content of κ-CN was not quantified in the study of Walsh et al. (1998b) and possible effects of different protein compositions associated with *CSN*3 genotypes cannot be discarded. In that study, the average micelle size of κ-CN B bulk milk was significantly smaller than that of the κ-CN A milk (Lodes et al., 1996b; Walsh et al., 1998b) and a smaller average micelle size has been attributed to the greater κ-CN content associated with κ-CN B variant (Lodes et al., 1996a).

The increased raw and moisture-adjusted CY of κ-CN BB milk in comparison with AA milk has been attributed to its higher fat retention, a feature associated with its finer gel structure and its higher fat-retaining ability (Walsh et al., 1998b), which can be ascribed to a greater content of total κ-CN. When Cheddar CY was adjusted for levels of protein and fat, no significant difference was observed across different *CSN*3 genotype milks (Walsh et al., 1998b), whereas, for Mozzarella cheese, yield from κ-CN BB milk remained significantly higher than that from κ-CN AA milk (Walsh et al., 1998b). In both those studies the increase in CY associated with processing of κ-CN BB milk was nearly 5 %. In all previous studies, milk containing alternatively κ-CN A or B was used, thus ensuring a grater variation in κ-CN genetic composition. In our case, the limited effect of the relative ratio between κ-CN A and B variants might be due in part to the fact that the difference in κ-CN genetic composition of experimental milks was too small to determine a relevant difference in CY.

Cheese Composition

Posterior median and HPD for the effect of experimental milk on MO and CA cheese composition are reported in Table 7. Cheese composition did not vary across experimental milks in MO trials, but a variation in the ratio of protein to fat content in CA cheese has been observed. In particular there was a decrease in fat content counterbalanced by an increase of protein content in HIGHB cheese in comparison with LOWB cheese. In two experiments based on the manufacture of Gouda cheese, a slightly higher conversion of total nitrogen of milk into cheese nitrogen was observed for κ-CN BB milk in comparison with κ-CN AA (van den Berg et al., 1992). However, also a better recovery of fat into cheese manufactured using κ-CN BB milks has been observed in several studies (Walsh et al., 1995, 1998b). Thus, the effects of κ-CN genetic variants on cheese composition still remains unclear.

CONCLUSIONS

This study showed that effects of genetic variants A and B at *CSN*3 on cheese yield are limited when milks with similar protein composition and contents are processed. Hence, it is likely that the positive universally-recognized effect of κ-CN B is almost exclusively due to the mediated effect of the increased κ-CN content associated with κ-CN B milk, rather than to a direct effect of this variant on the efficiency of the coagulation process. Further evidence might be provided by experimental trials processing milk with similar κ-CN A to κ-CN B content ratio, but exhibiting different levels of κ-CN to total casein content ratio. Finally, similarly to what experimented in this study, cheese-making trials might be conducted to investigate the effect exerted by a variation in the content of total β-CN and of its genetic variants in milk on cheese yield. Also this protein fraction exhibits allele-specific expressions, in particular with the B variant, and its effect on cheese yields needs to be investigated more accurately. A major critical issue for industrial cheese-making trials is the volume of milk required, which markedly limit the number of experimental samples. Methodologies that make feasible the assessment of cheese yield using reduced volumes of milk should be developed and implemented.

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Chapter 5.

Estimation of genetic parameters for detailed milk protein composition and coagulation properties in Simmental breed

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ABSTRACT

The aim of this study was to estimate genetic parameters of major milk protein fractions and estimate genetic and phenotypic correlations between milk protein fractions and coagulation properties. Contents of α_{S1}-, α_{S2}-, β-, γ-, and κ-casein (**CN**), β-Lactoglobulin (β-LG), and α-Lactalbumin (α-LA) were measured by reversed-phase HPLC in individual milk samples of 2,167 Simmental cows. Values of estimated heritability ranged from 0.11 to 0.52, for the content of α-LA and κ-CN, respectively. The highest values were those of κ-CN and β-CN which synthesis is mainly controlled by *CSN3* and *CSN2* loci. Values of heritability for α_{S1}-CN%, κ-CN% and β-CN% were similar and ranged from 0.61 to 0.70, whereas heritability of α_{S2}-CN%, γ-CN% and β-LG% were 0.28, 0.29 and 0.33, respectively. When *CSN2-CSN3* haplotype and *BLG* genotype are accounted for by the model, heritability estimates of all the protein fractions became similar suggesting that proteins synthesis is regulated by genes which control the overall production of milk protein. Genetic correlations among the contents of the five CN fractions and between CN fractions and whey protein (**WH**) fractions were generally low. Increasing the level of total CN (**TCN**) would increase the proportion of β-CN and κ-CN while decreasing the others. Generally, all the CN fractions were also moderately positively correlated with WH. When data where adjusted for *CSN2-CSN3* haplotype and *BLG* genotype, genetic correlations among the contents of protein fractions markedly increased confirming that all the fractions undergone a common regulation. The content and the relative proportion of κ-CN were not genetically correlated with rennet coagulation time (**RCT**), α_{S1}- and α_{S2}-CN were unfavourately correlated with RCT, but increasing the content of β-CN in milk would result in a shorter RCT. Stronger curds were associated with higher κ-CN and β-CN, and with lower α_{S1}-, α_{S2}-, and γ-CN contents and proportions. Results confirm the lack of favorable associations between TCN and MCP indicating that other traits, i.e. milk protein fractions, should be used for the genetic improvement of cheese-making properties.

INTRODUCTION

Milk protein polymorphisms and protein composition are known to be responsible for the variation of milk coagulation properties (**MCP**) (Wedholm et al., 2006; Jõudo et al., 2008; Bonfatti et al., 2009a,b) and the relative concentrations of the major protein fractions is the primary cause of the effect exerted by protein polymorphisms on the variation of MCP (Bonfatti et al., 2009a). For this reason, milk protein composition might play an important role in the profitability of the dairy industry.

Genetic variation of MCP measures has been reported by many authors (Ikonen et al., 1999; Cassandro et al. 2008; Cecchinato et al., 2009) and improvement of MCP based on selective breeding has been proposed (Ikonen et al., 2004). In addition, marker-assisted selection through the use of milk protein genes has been also suggested (Wedholm et al., 2006; Hallén et al., 2008; Heck et al., 2009). To evaluate the possibility of altering milk protein composition through selective breeding, but also to evaluate the possible effects of implementing a marker-assisted selection based on milk protein polymorphisms, genetic parameters of milk protein fractions contents and genetic correlations between protein fractions contents and MCP measures need to be investigated.

Only a few studies have estimated the magnitude of the genetic variation of milk proteins (Kroeker et al., 1985; Ikonen et al., 1997; Bobe et al., 1999; Graml and Pirchner, 2003) because of the analytical difficulties of quantifying the major bovine milk proteins simultaneously on a large sample size. Only one study (Schopen et al., 2009) reported the estimated genetic correlations among protein fractions in Dutch Holstein Friesian. Genetic correlations between protein fractions and MCP measures have never been reported before.

The aims of this study were to investigate genetic variation of milk protein fractions contents, detailed protein composition and MCP, and to infer genetic correlations among protein fractions, in a population of Simmental cows. The genetic correlations between milk protein fractions contents and MCP was estimated as well.

MATERIALS AND METHODS

Animals and Milk Sampling

Individual milk samples of 2,167 Simmental cows, distributed across 47 commercial herds in the north of Italy, were collected from November 2007 to December 2008. Individual milk samples (one per animal) were collected during the morning or evening milking of a test day. Milk was added with preservative (Bronopol, 0.6:100 vol/vol) immediately after collection, to prevent microbial growth, and stored at –40°C until reversed-phase (RP-) HPLC analysis, to prevent

enzymatic proteolysis. Pedigree information was supplied by the Italian Simmental Cattle Breeders Association (ANAPRI, Udine, Italy) and included all known ancestors of sampled cows.

Milk Protein Composition and Genotyping

Contents of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN, κ-CN, β-LG, and α-LA were measured using the RP-HPLC method proposed by Bonfatti et al. (2008). Genotypes of cows for *CSN2*, *CSN3*, and *BLG* loci, were also derived by RP-HPLC. The method provides the quantification of A^1, A^2, B and I variants of β-CN, A and B variants of κ-CN, and A, B and D variants of β-LG. A detailed description of the RP-HPLC technique used in this study can be found in Bonfatti et al. (2008) and more indications are reported by Bonfatti et al. (2009b).

Milk coagulation properties

Rennet coagulation time (**RCT**) and curd firmness ($\mathbf{a_{30}}$) of individual milk samples were obtained by using the Computerized Renneting Meter (CRM-48, Polo Trade, Monselice, Italy) within 3 h after sample collection. The description of the instrument and the details of the method used have been reported by Dal Zotto et al. (2008) and Bonfatti et al. (2009a). Samples that did not coagulate within 31 min were classified as non-coagulating milk. Measures of pH (pH-Burette 24, Crison) were obtained before measurement of MCP.

Traits Definition

For proteins quantified by RP-HPLC, total casein (**TCN**, g/L) was computed as the sum of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN and κ-CN contents of milk. Total whey protein (**WH**, g/L) was calculated as the sum of α-LA and β-LG contents. Total protein (**PRT**, g/L) was expressed as the sum of TCN and WH contents. Protein composition (i.e., relative contents of protein fractions) was computed as weight percentage ratio of α_{S1}-CN (**α_{S1}-CN%**, %), α_{S2}-CN (**α_{S2}-CN%**, %), β-CN (**β-CN%**, %), γ-CN (**γ-CN%**, %), and κ-CN (**κ-CN%**, %) to TCN and as the percentage ratio of β-LG to WH (**β-LG%,** %).

Computation of Haplotypes Probabilities

The probability for each *CSN2-CSN3* possible haplotype inherited by each daughter was estimated using the method proposed by Boettcher et al. (2004). Computation of haplotype probabilities was carried out by assuming no recombination events between CN genes. The expected number of copies of each haplotype carried by each animal can be indirectly derived by the estimated probabilities. More details on the haplotype probabilities estimation can be found also in Bonfatti et al. (2009b).

Statistical analysis

The model of analysis for all traits included the infinitesimal genetic effect of each animal (a_i), as well as some non genetic sources of variation: the effects of herd-test day (47 levels); parity of the cow (4 levels); DIM (12 classes of 30-d intervals, with the exception of the last class, which included samples collected at DIM 330 or greater). An alternative model included also the genetic effect of *CSN2-CSN3* haplotype as regressions on haplotype probabilities, and *BLG* genotype (AA, AB, BB). Note that we assumed an additive model following in part the standard model of Falconer and Mackay (1996).

Estimation of (Co)variance Components and Genetic Parameters. Estimation of (co)variance components for the contents of protein fractions, protein composition, and MCP was performed through bivariate Bayesian analyses. The general model, in matrix notation, can be written as:

$$\begin{bmatrix}\mathbf{y_1}\\ \mathbf{y_2}\end{bmatrix}=\begin{bmatrix}\mathbf{X_1} & \mathbf{0}\\ \mathbf{0} & \mathbf{X_2}\end{bmatrix}\cdot\begin{bmatrix}\boldsymbol{\beta}_1\\ \boldsymbol{\beta}_2\end{bmatrix}+\begin{bmatrix}\mathbf{U_1} & \mathbf{0}\\ \mathbf{0} & \mathbf{U_2}\end{bmatrix}\cdot\begin{bmatrix}\mathbf{q_1}\\ \mathbf{q_2}\end{bmatrix}+\begin{bmatrix}\mathbf{Z_1} & \mathbf{0}\\ \mathbf{0} & \mathbf{Z_2}\end{bmatrix}\cdot\begin{bmatrix}\mathbf{a_1}\\ \mathbf{a_2}\end{bmatrix}+\begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix}$$

where $\mathbf{y_1}$ and $\mathbf{y}_2$ are vectors of phenotypic records for trait 1 and 2, respectively; $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ are vectors of non genetic effects and of the genetic effects of *CSN2-CSN3* haplotype and *BLG* genotype; $\mathbf{X_1}$ and $\mathbf{X_2}$, are known incidence matrices relating effects in $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ to $\mathbf{y_1}$ and $\mathbf{y_2}$, respectively; $\mathbf{q}_1$ and $\mathbf{q}_2$ are vectors of herd effects considered to be normally distributed as $[\mathbf{q_1} \quad \mathbf{q_2}]' \sim N(\mathbf{0}, \mathbf{Q}\otimes\mathbf{I})$ where $\mathbf{Q}$ is the (co)variance matrix between herd effects for trait 1 and 2; $\mathbf{U_1}$, and $\mathbf{U_2}$, are known incidence matrices relating herd effects in $\mathbf{q_1}$ and $\mathbf{q_2}$ to $\mathbf{y_1}$ and $\mathbf{y_2}$, respectively; $\mathbf{a}_1$ and $\mathbf{a}_2$ are vectors of additive genetic effects of animals assumed to follow a multivariate normal distribution with $[\mathbf{a_1} \quad \mathbf{a_2}]' \sim N(\mathbf{0}, \mathbf{G_0}\otimes\mathbf{A})$, where $\mathbf{G_0}$ is the (co)variance matrix between animal effects, and $\mathbf{A}$ is the numerator of Wright's relationship matrix; $\mathbf{Z_1}$, and $\mathbf{Z_2}$ are known incidence matrices relating additive genetic effects in $\mathbf{a_1}$ and $\mathbf{a}_2$ to $\mathbf{y_1}$ and $\mathbf{y_2}$, respectively; $\mathbf{e}_1$ and $\mathbf{e}_2$ are vectors of residual effects assumed to follow a multivariate normal distribution with $[\mathbf{e_1} \quad \mathbf{e_2}]' \sim N(\mathbf{0}, \mathbf{R_0}\otimes\mathbf{I})$, where $\mathbf{R_0}$ is the (co)variance between residual effects for trait 1 and 2. Prior distributions for effects in $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ were assumed to be uniform whereas those for herds, and additive genetic effects were inverted Wishart distributions. Marginal posterior distributions of parameters of concern (i.e., covariance components, heritabilities and correlations) were estimated by performing numerical integration through the Gibbs sampler, as implemented in the program TM by Legarra et al. (2007, available on request from the author at andres.legarra@toulouse.inra.fr). A unique Gibbs chain of 5,000,000 iterations was run for each bivariate analysis. Samples were saved every 250 iterations. The effective length of the burn-in

period was calculated following the methods of Raftery and Lewis (1992), respectively. The posterior median was used as a point estimate of parameters of concern. Lower and upper bounds of the highest posterior density interval with 95 % probability (**HPD**) for the heritability, and correlations were obtained from the estimated marginal densities.

Estimated heritability was defined as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_h^2 + \sigma_e^2}$$

where σ_a^2 is the additive genetic variance, σ_h^2 is the herd variance and σ_e^2 is the residual variance. Genetic correlations were estimated as:

$$r_a = \frac{\sigma_{a1,a2}}{\sigma_{a1} \cdot \sigma_{a2}}$$

where $\sigma_{a1,a2}$ is the additive genetic covariance between trait 1 and 2, and σ_{a1} and σ_{a2} are the additive genetic standard deviation of trait 1 and 2, respectively. Phenotypic correlations were computed as:

$$r_p = \frac{\sigma_{p1,p2}}{\sigma_{p1} \cdot \sigma_{p2}}$$

where $\sigma_{p1,p2}$ is the phenotypic covariance between trait 1 and 2, and σ_{p1} and σ_{p2} are the phenotypic standard deviation of trait 1 and 2, respectively.

RESULTS AND DISCUSSION

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. (2009b). Descriptive statistics for MCP can be found in Bonfatti et al. (2009a).

Heritability of Contents of Protein Fractions

Point estimates (median of the marginal posterior density of the parameter) of heritability for contents of protein fractions, protein composition and MCP are reported in Table 1. The median of the marginal posterior distribution was used as a point estimate of all the parameters of concern because in its loss function errors are considered according to their value and not to their square or other transformations, and because it is invariant to transformations. However, median, mean and mode were coincident, as posterior distributions for all the traits investigated were almost symmetrical.

Table 1. Features of the marginal posterior densities for the heritability of milk protein fractions and protein composition[1].

	Complete genome[2]			Polygenic background[3]		
Trait[4]	h^2	HPD95%	σ_a	h^2	HPD95%	σ_a
Casein, g/L	0.23	0.14 – 0.33	1.77	0.18	0.10 – 0.29	1.49
Whey protein, g/L	0.29	0.22 – 0.37	0.35	0.20	0.12 – 0.29	0.28
Protein fractions content, g/L						
α_{S1}-CN	0.23	0.16 – 0.30	0.65	0.17	0.08 – 0.26	0.53
α_{S2}-CN	0.22	0.16 – 0.29	0.33	0.19	0.12 – 0.28	0.30
β-CN	0.46	0.37 – 0.55	1.25	0.18	0.09 – 0.27	0.61
γ-CN	0.25	0.19 – 0.33	0.22	0.04	0.01 – 0.09	0.07
κ-CN	0.52	0.42 – 0.63	0.59	0.26	0.15 – 0.38	0.51
α-LA	0.11	0.05 – 0.17	0.08	0.09	0.04 – 0.15	0.07
β-LG	0.38	0.30 – 0.46	0.32	0.24	0.16 – 0.40	0.24
Protein composition, %						
α_{S1}-CN%	0.66	0.58 – 0.73	2.01	0.28	0.17 – 0.38	0.81
α_{S2}-CN%	0.28	0.22 – 0.35	0.87	0.24	0.15 – 0.33	0.78
β-CN%	0.70	0.58 – 0.81	2.30	0.18	0.10 – 0.28	0.64
γ-CN%	0.18	0.12 – 0.25	0.57	0.05	0.02 – 0.11	0.28
κ-CN%	0.61	0.53 – 0.71	1.54	0.23	0.14 – 0.33	0.53
β-LG%	0.33	0.24 – 0.43	2.19	0.19	0.11 – 0.27	1.49
Milk coagulation properties						
RCT, min	0.29	0.19 – 0.39	2.43	0.23	0.13 – 0.33	2.06
a_{30}, mm	0.12	0.03 – 0.21	3.04	0.10	0.03 – 0.18	2.16
pH	0.18	0.10 – 0.26	0.03	0.17	0.09 – 0.25	0.02

[1] h^2 = median of the marginal posterior density of the heritability; HPD95% = 95 % high posterior density interval; σ_a = median of the marginal posterior density of the additive genetic standard deviation;

[2] Model did not included information on *CSN2-CSN3* haplotype and *BLG* genotype;

[3] Model included regressions on *CSN2-CSN3* haplotype probabilities and *BLG* genotype;

[4] Contents of all protein fractions were measured by reversed-phase HPLC on skimmed milk; casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA ; protein = casein + whey protein; casein number = (casein/protein) × 100; α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content; RCT = rennet coagulation time; a_{30} = curd firmness.

Heritability estimates for the protein fraction contents ranged from 0.11 to 0.52, for the content of α-LA and κ-CN, respectively. The highest values of heritability were those of κ-CN and β-CN which synthesis is mainly controlled by *CSN3* and *CSN2* loci. The content of β-LG, which is known to be affected for a large extent by *BLG* locus, exhibited a value of heritability of 0.38.

In most studies (Bobe et al., 1999; Schopen et al., 2009) heritability estimates have been reported only for the relative proportion of protein fractions on total protein. Graml and Pirchner (2003) reported heritability estimates for the contents of protein fractions in Fleckvieh and Braunvieh cattle for roughly 2,000 cows per breed, combining heritability estimates derived from a sire model and a daughter to dam regression for both breeds.

The heritability estimates were similar to those obtained by Graml and Pirchner (2003), with the

exception of κ-CN and β-CN fractions which values were significantly lower than the ones reported in this study. These differences could be due to the combination of different methods of variance component estimation and different breeds used.

The content of α-LA get the lower value of heritability. This can be in part explained by the lower accuracy of its quantification by RP-HPLC in respect to that of the other protein fractions, which likely increased the error variance. Despite some difficulties of quantification due to the multiple peaks and their limited resolution, heritability estimated for total γ-CN content was similar to that obtained for TCN, WH, α_{S1}-CN, and α_{S2}-CN. With the only exception of α-LA, all the heritability estimates were higher than 0.10 with a probability greater than 94 % (data not reported in table).

The high values of heritabilities indicate that it might be possible to increase the contents of specific protein fractions in milk through breeding strategies. The only limitation could be due to the difficult to collect phenotypic records for these traits. Interestingly, κ-CN and β-CN resulted more heritable than TCN, indicating that selection for increasing these two protein fractions would be more effective than selection for increasing the overall level of CN of milk.

Heritability of Protein Composition

Point estimates (posterior medians) and HPD of the heritability for the proportions of protein fractions are reported in Table 1. Values of the posterior median of the heritability for α_{S1}-CN%, κ-CN% and β-CN% were similar and ranging from 0.61 to 0.70. This confirms the results obtained in previous works (Bobe et al., 1999; Bonfatti et al., 2009b) on the effect exerted by CN haplotypes on protein composition. In those studies, a tight relation between the relative proportion of these three protein fractions on TCN depending on the CN haplotype was observed.

Heritability of α_{S2}-CN% was only moderate (0.28). Conversely, Schopen et al. (2009) found a heritability estimate of α_{S2}-CN% markedly higher in comparison with our result and similar to the values obtained for α_{S1}-CN% and κ-CN%.

As suggested by Schopen et al. (2009), heritability estimates might differ across studies depending on the accuracy of the analytical methods used to quantify the contents of milk protein fractions. For example, Kroeker et al. (1985), using PAGE combined with densitometry on a dataset of over 11,000 test-day records, obtained heritability values for protein composition not significantly different from zero. When compared to other methods, RP-HPLC, like capillary zone electrophoresis, is a tool characterized by high repeatability and reproducibility (Bonfatti et al., 2008), resulting in a marked decrease of the random error variance. In the study by Schopen et al. (2009), protein fractions contents have been quantified by the method proposed by Heck et al. (2008) and only the glyco-free portion of the total κ-CN content could be quantified, because the glycosylated form, roughly a half of the total κ-CN content, co-eluted with β-CN. This might in part

explain the marked difference between values of heritability of β-CN obtained in that study and our estimates. β-CN exhibited a markedly higher heritability (0.70), in comparison with the value (0.25) obtained by Schopen et al. (2009). Estimates of heritability of κ-CN are more similar, but a lower value has been found by Schopen et al. (2009). However, also differences of sample size, breeds or allele frequencies can contribute to the inconsistencies in heritability estimates observed across studies.

As WH is composed by β-LG and α-LA, they sum up to 100%. Consequently, they obtained the same value of heritability and results for α-LA have been omitted. β-LG% exhibited only a moderate heritability (0.33) in comparison with that obtained for α_{S1}-CN%, κ-CN% and β-CN%, and had a value markedly lower than that obtained by Schopen et al. (2009), but in line with the estimates reported by Bobe et al. (1999). Differences in the extent of the allele-differential expression of heterozygous animals across population have been reported (Ng-Kwai-Hang et al., 1998) and inconsistencies in the heritability estimates for β-LG% across studies might be partially explained by the possible different extent of the effect of the *BLG* locus in determining the expression of the β-LG genetic variants across populations. Heck et al. (2008) and Bobe et al. (1999) reported that the relative contents of β-LG in milk depend almost exclusively on *BLG* genotypes, in Dutch and US Holstein Friesian, respectively, but, in a previous study conducted on Simmental cows (Bonfatti et al., 2009b), a smaller effect of the allele-specific expression of β-LG have been observed, in comparison with that reported in those studies.

Proportion of the Genetic Variation explained by CSN2-CSN3 Haplotype and BLG Genotype

The relevance of *CSN2-CSN3* haplotype and *BLG* genotype in explaining the variation of milk protein fractions was also investigated (Table 1). Results showed that milk protein genes account for a large part of the genetic variation of milk protein composition. Accounting for milk protein loci reduced considerably the polygenic additive genetic variance of α_{S1}-CN, β-CN, κ-CN and β-LG contents and relative proportions.

In agreement with Bobe et al. (1999), who found that *CSN3* and *BLG* genotypes explained a significant part of the genetic control of α_{S1}-CN, the genetic variance of α_{S1}-CN% was strongly reduced when model accounted for *CSN2-CSN3* haplotype and *BLG* genotype. Like for κ-CN%, milk protein loci explained roughly the 60 % of the heritability of α_{S1}-CN%. Conversely, Schopen et al. (2009) reported that genotypes at *CSN3* and *BLG* loci had no effect on the polygenic additive genetic variance for α_{S1}-CN. Casein haplotype explained more than 70 % of the genetic variation of β-CN%. In both those studies, the genetic control of β-LG fraction was nearly complete by *BLG* genotypes. Conversely, our results indicate that, despite a strong effect exerted by *BLG* genotype on the expression of β-LG%, the residual genetic variance remains noteworthy, indicating that other

genes, besides those considered, are responsible of the genetic variation of this protein fraction. Good candidates seem to be the polymorphisms at *BLG* promoter region (Martin et al., 2002).

Interestingly, when *CSN2-CSN3* haplotype and *BLG* genotype are accounted for in the model, heritability estimates of all the protein fractions, but also heritabilities obtained for the contents of protein fractions and their relative proportions on TCN or WH, became similar. This suggests that milk proteins synthesis undergone a genetic control by specific genes (CNs and *BLG* loci) but also by genes which control the overall production of milk protein and are responsible for one-third to almost the totality of the genetic variation, depending on the protein fraction.

While the mutations described above can dramatically affect the levels of expression of the genes in which they are located, effects on TCN or WH were less pronounced. One hypothesis is that when expression of one CN gene is downregulated, the others can be upregulated to compensate (Lerouxet al. 2003).

Inconsistencies across studies on the proportion of the genetic variation of milk proteins contents explained by milk protein loci might be due to different extent of the linkage disequilibrium between milk protein loci and polymorphisms located in their promoter region. Bovenhuis et al. (1992) suggested that the conflicting results of the effects of mutations in CN genes might be due to linkage between mutations in different CNs, as well as the different statistical models used in the analyses. They proposed a multigene model as an alternative to single-gene models. As mutations with effects on quantitative traits can occur in exons, introns promoters, and other regulatory sequences (Hoogendoorn et al. 2003), it is possible that the functional mutation(s) will not be within the set of mutations (such as single-nucleotide polymorphisms, SNPs) genotyped in the data set.

Heritability of Milk Coagulation Properties

Heritability estimates for RCT, a_{30} and pH are reported in Table 1. Estimated heritability for RCT and pH was similar to previous reports (Ikonen et al., 1999, 2004; Tyrisevä et al., 2004; Cassandro et al., 2008). Heritability estimate for a_{30} measures obtained in this study were similar to those obtained by Tyrisevä et al. (2004) from a sample of Finnish Ayrshire and Holstein-Friesian cows, and Cassandro et al. (2008) in Holstein-Friesian, but lower than estimates reported by other authors. Ikonen et al. (1999) reported estimates of heritability for a_{30} of 0.40, whereas Ikonen et al. (2004) and Cecchinato et al. (2009) obtained estimates of heritability for a_{30} ranging from 0.22 to 0.39 and from 0.22 to 0.27, respectively. Differences in breeds and statistical models might explain some of these inconsistencies.

The relevance of *CSN2-CSN3* haplotype and *BLG* genotype in explaining the variation of MCP and pH was also investigated showing that milk protein genes did not affect the genetic variation of pH, but accounted for 15 % and 30 % of the additive genetic variance of RCT and a_{30}, respectively.

Table 2. Median and 95 % highest posterior density interval (in parenthesis) of the marginal posterior density of additive genetic (above diagonal) and phenotypic (below diagonal) correlations between contents (g/L) of milk protein fractions[1]

Protein fraction	TCN	WH	α_{S1}-CN	α_{S2}-CN	β-CN	γ-CN	κ-CN	α-LA	β-LG
TCN		0.42 (0.19, 0.61)	0.41 (0.13, 0.63)	0.43 (0.18, 0.64)	0.80 (0.71, 0.88)	0.51 (0.22, 0.75)	0.45 (0.24, 0.64)	0.29 (-0.05, 0.60)	0.38 (0.16, 0.57)
WH	0.67 (0.63, 0.71)		0.26 (-0.01, 0.48)	0.43 (0.23, 0.61)	0.26 (0.05, 0.44)	0.46 (0.22, 0.69)	0.05 (-0.17, 0.26)	0.33 (0.04, 0.55)	0.98 (0.96, 0.99)
α_{S1}-CN	0.80 (0.77, 0.82)	0.61 (0.56, 0.66)		0.22 (-0.06, 0.49)	-0.07 (-0.37, 0.20)	-0.14 (-0.42, 0.17)	0.12 (-0.14, 0.36)	0.09 (-0.28, 0.42)	0.26 (0.15, 0.54)
α_{S2}-CN	0.60 (0.55, 0.66)	0.56 (0.49, 0.63)	0.43 (0.35, 0.50)		0.14 (-0.09, 0.36)	0.50 (0.24, 0.73)	0.11 (-0.13, 0.34)	0.38 (0.09, 0.64)	0.35 (0.15, 0.54)
β-CN	0.86 (0.84, 0.87)	0.52 (0.47, 0.58)	0.53 (0.49, 0.58)	0.37 (0.31, 0.44)		0.39 (0.16, 0.61)	0.08 (-0.13, 0.28)	0.14 (-0.16, 0.42)	0.25 (0.06, 0.42)
γ-CN	0.12 (0.05, 0.18)	-0.02 (-0.10, 0.05)	-0.06 (-0.12, 0.00)	0.08 (0.00, 0.16)	-0.01 (-0.07, 0.05)		0.08 (-0.15, 0.33)	0.50 (0.21, 0.77)	0.36 (0.12, 0.59)
κ-CN	0.55 (0.50, 0.60)	0.27 (0.21, 0.34)	0.30 (0.24, 0.36)	0.27 (0.20, 0.33)	0.30 (0.24, 0.36)	0.04 (-0.02, 0.10)		0.11 (-0.20, 0.41)	0.06 (-0.14, 0.26)
α-LA	0.37 (0.29, 0.45)	0.62 (0.56, 0.69)	0.27 (0.17, 0.35)	0.38 (0.33, 0.54)	0.31 (0.23, 0.38)	-0.08 (-0.16, 0.00)	0.18 (0.11, 0.26)		0.10 (-0.18, 0.37)
β-LG	0.65 (0.61, 0.69)	0.94 (0.93, 0.95)	0.62 (0.58, 0.67)	0.49 (0.42, 0.56)	0.50 (0.45, 0.55)	0.01 (-0.06, 0.08)	0.25 (0.19, 0.32)	0.31 (0.22, 0.40)	

[1] TCN = total casein, computed as the sum of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN and κ-CN; WH = total whey protein, computed as the sum of β-LG and α-LA.

Table 3. Median and 95 % highest posterior density interval (in parenthesis) of the marginal posterior density of additive genetic (above diagonal) and phenotypic (below diagonal) correlations between total casein (TCN) and whey protein (WH) content, and proportions (%) of milk protein fractions[1]

Protein fraction	TCN	WH	α_{S1}-CN%	α_{S2}-CN%	β-CN%	γ-CN%	κ-CN%	β-LG%
TCN			-0.48	-0.23	0.39	0.17	0.15	0.15
			(-0.68, -0.28)	(-0.49, 0.04)	(0.18, 0.59)	(-0.18, 0.51)	(-0.10, 0.38)	(-0.11, 0.40)
WH			-0.10	0.14	0.04	0.36	-0.10	0.70
			(-0.28, 0.09)	(-0.08, 0.37)	(-0.15, 0.22)	(0.06, 0.65)	(-0.29, 0.11)	(0.53, 0.85)
α_{S1}-CN%	-0.17	0.00		0.04	-0.68	-0.46	-0.19	0.08
	(-0.24, -0.11)	(-0.07, 0.07)		(-0.14, 0.23)	(-0.78, -0.58)	(-0.66, -0.25)	(-0.34, -0.03)	(-0.08, 0.26)
α_{S2}-CN%	-0.03	0.18	-0.21		-0.43	0.37	-0.13	0.05
	(-0.11, 0.07)	(0.09, 0.28)	(-0.28, -0.14)		(-0.58, -0.27)	(0.09, 0.63)	(-0.32, 0.07)	(-0.27, 0.17)
β-CN%	0.23	0.06	-0.46	-0.35		0.08	-0.44	-0.00
	(0.18, 0.29)	(0.00, 0.12)	(-0.51, -0.40)	(-0.40, -0.29)		(-0.16, 0.31)	(-0.57, -0.30)	(-0.18, 0.17)
γ-CN%	-0.26	-0.27	-0.22	0.03	-0.27		-0.13	-0.03
	(-0.33, -0.20)	(-0.34, -0.21)	(-0.28, -0.16)	(-0.06, 0.11)	(-0.32, -0.22)		(-0.38, 0.14)	(-0.30, 0.25)
κ-CN%	0.09	-0.05	-0.31	-0.10	-0.36	-0.05		-0.01
	(0.02, 0.16)	(-0.13, 0.02)	(-0.36, -0.25)	(-0.18, -0.03)	(-0.41, -0.31)	(-0.11, 0.02)		(-0.21, 0.18)
β-LG%	0.12	0.13	0.16	-0.15	-0.03	0.03	-0.05	
	(0.03, 0.21)	(0.02, 0.23)	(0.08, 0.23)	(-0.25, -0.04)	(-0.09, 0.03)	(-0.04, 0.12)	(-0.13, 0.03)	

[1] α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are expressed as the percentage ratio of each casein fraction on TCN; β-LG% is expressed as the percentage ratio of β-LG on WH.

Table 4. Median and 95 % highest posterior density interval (in parenthesis) of the marginal posterior density of additive genetic (above diagonal) and phenotypic (below diagonal) correlations between contents (g/L) of milk protein fractions, obtained whit a model including the regressions on *CSN2-CSN3* haplotype probabilities and the effect of *BLG* genotype[1]

Protein fraction	TCN	WH	α_{S1}-CN	α_{S2}-CN	β-CN	γ-CN	κ-CN	α-LA	β-LG
TCN		0.68	0.91	0.47	0.95	0.23	0.75	0.19	0.70
		(0.48, 0.83)	(0.84, 0.96)	(0.20, 0.70)	(0.90, 0.98)	(-0.50, 0.80)	(0.59, 0.89)	(-0.25, 0.56)	(0.52, 0.84)
WH	0.75		0.67	0.47	0.53	0.27	0.55	0.54	0.97
	(0.71, 0.78)		(0.46, 0.84)	(0.23, 0.69)	(0.27, 0.73)	(-0.34, 0.80)	(0.31, 0.75)	(0.27, 0.76)	(0.95, 0.99)
α_{S1}-CN	0.92	0.69		0.26	0.82	0.08	0.61	0.25	0.67
	(0.91, 0.93)	(0.64, 0.73)		(-0.07, 0.56)	(0.69, 0.92)	(-0.63, 0.74)	(0.37, 0.82)	(-0.17, 0.61)	(0.47, 0.83)
α_{S2}-CN	0.62	0.59	0.45		0.30	0.35	0.31	0.32	0.44
	(0.57, 0.68)	(0.52, 0.66)	(0.37, 0.52)		(-0.02, 0.58)	(-0.24, 0.81)	(0.03, 0.57)	(-0.03, 0.62)	(0.20, 0.65)
β-CN	0.93	0.67	0.84	0.46		0.21	0.66	0.03	0.58
	(0.92, 0.94)	(0.63, 0.72)	(0.82, 0.86)	(0.39, 0.53)		(-0.47, 0.80)	(0.44, 0.84)	(-0.42, 0.43)	(0.35, 0.76)
γ-CN	0.06	-0.09	-0.02	0.00	-0.12		0.15	0.24	0.22
	(-0.01, 0.13)	(-0.17, -0.01)	(-0.08, 0.05)	(-0.09, 0.09)	(-0.19, -0.05)		(-0.50, 0.75)	(-0.53, 0.90)	(-0.51, 0.86)
κ-CN	0.66	0.52	0.51	0.40	0.51	0.08		0.12	0.58
	(0.59, 0.71)	(0.45, 0.59)	(0.43, 0.58)	(0.30, 0.48)	(0.44, 0.58)	(0.00, 0.15)		(-0.28, 0.46)	(0.36, 0.76)
α-LA	0.38	0.67	0.29	0.43	0.36	-0.16	0.34		0.32
	(0.30, 0.47)	(0.61, 0.73)	(0.19, 0.38)	(0.32, 0.53)	(0.27, 0.44)	(-0.25, -0.07)	(0.24, 0.43)		(-0.00, 0.61)
β-LG	0.76	0.94	0.73	0.54	0.67	-0.04	0.49	0.38	
	(0.73, 0.79)	(0.93, 0.95)	(0.69, 0.76)	(0.47, 0.61)	(0.63, 0.72)	(-0.12, 0.03)	(0.42, 0.56)	(0.28, 0.47)	

[1] TCN = total casein, computed as the sum of α_{S1}-CN, α_{S2}-CN, β-CN, γ-CN and κ-CN; WH = total whey protein, computed as the sum of β-LG and α-LA.

Table 5. Median and 95 % highest posterior density interval (in parenthesis) of the marginal posterior density of additive genetic (above diagonal) and phenotypic (below diagonal) correlations between total casein (TCN) and whey protein (WH) content, and proportions (%) of milk protein fractions, obtained whit a model including the regressions on *CSN2-CSN3* haplotype probabilities and the effect of *BLG* genotype[1]

Protein fraction	TCN	WH	α_{S1}-CN%	α_{S2}-CN%	β-CN%	γ-CN%	κ-CN%	β-LG%
TCN			0.02	-0.14	0.18	-0.73	0.20	0.58
			(-0.31, 0.36)	(-0.44, 0.18)	(-0.19, 0.56)	(-0.99, -0.29)	(-0.14, 0.53)	(0.25, 0.86)
WH			0.08	0.05	-0.22	-0.32	0.19	0.56
			(-0.21, 0.38)	(-0.24, 0.33)	(-0.56, 0.11)	(-0.78, 0.15)	(-0.10, 0.48)	(0.31, 0.81)
α_{S1}-CN%	0.10	0.05		-0.65	-0.09	-0.45	-0.24	0.07
	(0.01, 0.18)	(-0.05, 0.14)		(-0.82, -0.47)	(-0.42, 0.25)	(-0.81, -0.02)	(-0.49, 0.03)	(-0.23, 0.36)
α_{S2}-CN%	0.02	0.18	-0.57		-0.56	0.38	-0.11	-0.15
	(-0.07, 0.11)	(0.08, 0.28)	(-0.63, -0.50)		(-0.76, -0.30)	(-0.09, 0.77)	(-0.38, 0.17)	(-0.42, 0.14)
β-CN%	0.17	0.06	0.03	-0.41		-0.23	-0.33	0.07
	(0.10, 0.24)	(-0.02, 0.13)	(-0.04, 0.11)	(-0.47, -0.33)		(-0.70, 0.32)	(-0.61, 0.01)	(-0.27, 0.43)
γ-CN%	-0.35	-0.38	-0.24	-0.04	-0.52		-0.15	-0.46
	(-0.41, -0.29)	(-0.45, -0.31)	(-0.32, -0.16)	(-0.14, 0.04)	(-0.57, -0.47)		(-0.65, 0.38)	(-0.97, 0.04)
κ-CN%	-0.06	0.00	-0.39	-0.03	-0.36	0.07		0.28
	(-0.16, 0.04)	(-0.11, 0.11)	(-0.48, -0.30)	(-0.15, 0.09)	(-0.43, -0.29)	(-0.01, 0.17)		(-0.02, 0.56)
β-LG%	0.18	0.03	0.25	-0.17	-0.02	0.05	-0.12	
	(0.08, 0.27)	(-0.08, 0.15)	(0.16, 0.35)	(-0.28, -0.05)	(-0.11, 0.06)	(-0.04, 0.14)	(-0.26, -0.01)	

[1] α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are expressed as the percentage ratio of each casein fraction on TCN; β-LG% is expressed as the percentage ratio of β-LG on WH.

Phenotypic and Genetic Correlations Among the Contents of Milk Protein Fractions

As PRT and TCN were tightly genetically and phenotypically correlated (r_a and r_p were greater than 0.98), only results for the correlation of TCN with protein fractions and MCP measures will be discussed. Phenotypic and genetic correlations among the contents of milk protein fractions are reported in Table 2. All the protein fractions were phenotypically positively correlated. Their correlation with TCN and WH approximately reflected the proportion in which they take part to the TCN and WH contents. Hence, for example, β-CN, which is quantitatively the most important CN fraction, exhibited the higher phenotypic correlation with TCN. However, in general, phenotypic correlations among CN fractions were fairly low and all positive, with the exception of those involving γ-CN which were slightly negative or close to zero. As γ-CNs are proteolytic products of the β-CN, a high content of γ-CN in milk would be expected to be counterbalanced by a lower content of β-CN and, more in general, of TCN. This can explain the lack of positive phenotypic correlation between γ-CN and all the other protein fractions. On the contrary, γ-CN exhibited positive genetic correlations with all protein fractions, with the only exception of α_{S1}-CN.

With few exceptions, genetic correlations among the contents of the five CN fractions were from very low to moderate, ranging from −0.14 to 0.50. The medians of their posterior distributions were all positive or can be considered not different from zero. The strongest genetic correlation (0.50) was between α_{S2}-CN and γ-CN. Low to moderate genetic correlation was observed also between the contents of CN fractions and the contents of WH proteins, ranging from 0.06 to 0.50. These findings show that the contents of the protein fractions are genetically rather independent, in particular the content of κ-CN in respect to all the other fractions.

It is well known that CN loci are located very closely, within a 250-kb region of chromosome 6 (Threadgill and Womack, 1990; Bevilacqua et al., 2006), and homology between the promoter region of α_{S1}-CN, α_{S2}-CN, and β-CN genes (Groenen and van der Poel, 1994) can explain possible co-regulation mechanisms. However, as suggested by Schopen et al. (2009), the generally low genetic correlations among the contents of milk protein fractions might be due to a different translation efficiency of the CNs messengers in each of the CN genes, i.e. in a differential post-transcriptional regulation, as showed by Bevilacqua et al. (2006).

Casein fractions were correlated to TCN, but with different extents. The higher genetic correlation (0.80) was between TCN and β-CN, whereas all other CN fractions were only moderately positively correlated with TCN. Generally, all the CN fractions, in particular α_{S2}-CN and γ-CN, were also moderately positively correlated with total WH, suggesting that a general regulation of the protein level in milk involves simultaneously TCN and WH. The tight genetic correlation (0.98) between WH and β-LG was in part expected because, in this study, WH was composed only by β-LG and α-LA and the latter represent only a minimal proportion of the total WH.

Only a moderate positive correlation (0.42) was observed between TCN and WH, indicating that these two heterogeneous portions of milk protein are quite independent. However, these results support the suggestion that the regulation of TCN and WH genes partially involves the same co-factors, hormones, and transcription factors (Groenen and van der Poel, 1994).

Conversely, Schopen et al. (2009) found a strong negative genetic correlation between TCN and β-LG or WH. Those authors reported that genetic correlations between TCN and WH fractions were stronger than the genetic correlations between and CN fractions. Likely, this can be explained by the strong effect of *BLG* locus on CN number found by those authors (when data have been adjusted for *BLG* genotypes, the genetic correlation between TCN and WH markedly increased). Other authors (van den Berg et al., 1992; Wedholm et al., 2006) reported a negative relationship between β-LG and TCN concentration. However, this could have been due in part also to the fact that proteins have been expressed as percentage ratios of total protein content, hence an increase of all CN fractions is somewhat expected to decrease β-LG and thus WH.

When data were adjusted for *CSN2-CSN3* haplotype and *BLG* genotype (Table 4), genetic correlations among the contents of protein fraction markedly increased. This confirms that CN and *BLG* loci contribute in creating genetic variability but suggests also that the synthesis of all the fractions undergone a common regulation. However, genetic correlations ranged from moderate to high, indicating that other genes or other factors, such as a different stability of the mRNA, might be involved in the variation of the synthesis of specific protein fractions.

Interestingly, when accounting for *CSN2-CSN3* haplotype and *BLG* genotype, the genetic correlations among α_{S1}-CN, β-CN and κ-CN changed from very low, or slightly negative, to high and positive values. In particular, the genetic correlation between α_{S1}-CN and β-CN changed from -0.07 to 0.82.

In previous studies (Bobe et al., 1999; Hallén et al., 2008; Bonfatti et al., 2009b) a competitive synthesis between these two protein fractions have been suggested. However, their generally negative correlations, together with the markedly high genetic correlation found when adjusting for CN haplotype, might be explained also by a possible close linkage of a favorable allele for the synthesis of β-CN with an allele in the promoter region of *CSN1S1* which is unfavorable for the synthesis of α_{S1}-CN (Kuss et al., 2005).

Chanat et al. (1999) have studied the transport of CNs from the endoplasmic reticulum (**ER**) to the Golgi apparatus in mammary epithelial cells. Their data suggest that for animals with a high CN content, α_{S1}-CN must interact with the other CNs for efficient transport to the Golgi. In cells that completely lack α_{S1}-CN the accumulation of β-CN (or maybe κ–β-mixtures) is observed in the ER. In the long term this causes ER stress, activates the ER-associated degradation system and impedes secretion. Preformed CN complexes of the size of the putative CN submicelles must form through

protein-protein interactions (Farrell et al., 2002) and emerge from the ER for efficient transit via secretory vesicles to the Golgi apparatus. κ-CN must either be SH capped or self associate with other CNs for the transit through the ER lumen. α_{S1}-CN can reduce aggregated species and allow the associated particles to escape the ER. This propriety of α_{S1}-CN can explain its strong correlation with β-CN and κ-CN when not accounting for CN haplotype.

Phenotypic and Genetic Correlations for Protein Composition

Posterior median and HPD intervals for the phenotypic and genetic correlations among the relative proportion of milk protein fractions are reported in Table 3. Phenotypic correlations were generally higher than genetic correlations but similar, indicating that environmental correlations are analogous to the genetic ones. However, in all the cases in which γ-CN was involved, genetic correlations were higher than the phenotypic ones, confirming results obtained for the contents of the protein fractions.

As WH is composed only by β-LG% and α-LA% and they sum up to 100%, all correlations between them and all the other fractions had the same value, but opposite direction. For this reason only the correlations of β-LG% will be discussed.

The genetic correlations for the relative contents of milk protein fractions ranged from -0.66, between α_{S1}-CN% and β-CN%, to 0.04, between α_{S1}-CN% and α_{S2}-CN%. Results of the genetic correlation between proportion of CN fractions are in contrast with those found by Schopen et al. (2009), who reported that the strongest genetic correlations were between α_{S1}-CN and α_{S2}-CN (−0.49), and between α_{S1}-CN and κ-CN (−0.56).

Selecting for TCN content of milk might alter CN composition, increasing the relative proportions of β-CN, and κ-CN in a minor extent, while decreasing α_{S1}-CN% and α_{S2}-CN%, whereas only a slightly increase of β-LG% on total WH would be expected. Casein composition seems to be completely independent from WH composition, as genetic correlations of TCN proteins with β-LG% were trivial.

When *CSN2-CSN3* haplotype and *BLG* genotype were accounted for by the model, genetic correlations between the relative proportions of protein fractions became more similar among each other and also similar to the correlations observed among the contents on protein fractions. This confirms the hypothesis of the existence of a general regulation of the protein level, in which milk protein genes act as regulator of the proportions of specific protein fractions.

A whole genome scan has been recently carried out by Schopen et al. (2009b) to detect quantitative trait loci (**QTL**) for milk protein composition of Holstein Friesian cows. The chromosomal regions most significantly related to milk protein composition were found on *Bos taurus* autosomes (**BTA**) 6, 11 and 14. The QTL on BTA6 affected α_{S1}-, α_{S2}-, β- and κ-CN. The QTL on BTA11 affected β-

LG, and the QTL on BTA14 affected protein percentage. These results was expected because CN genes and β-LG gene have been mapped on BTA6 and on BTA11, respectively (Hayes et al., 1993) and locus *DGAT1*, which is known to be associated with milk protein and fat contents (Grisart et al., 2001), has been mapped on BTA14. However, the QTL found in that study could only partially be explained by polymorphisms in milk protein genes, and several other significant QTL affecting milk protein composition were found.

Table 6. Median and 95 % high posterior density interval (HPD 95%) of the marginal posterior density of additive genetic (r_a) and phenotypic (r_p) correlations between rennet coagulation time and protein fractions contents (g/L), proportions (%) of milk protein fractions on total casein or whey protein contents, curd firmness and pH of milk

	r_a		r_p	
Trait[1]	Median	HPD 95%	Median	HPD 95%
Casein, g/L	0.20	-0.10 – 0.49	-0.08	-0.15 – 0.02
Whey protein, g/L	-0.02	-0.28 – 0.23	0.01	-0.06 – 0.08
Protein fractions content, g/L				
α_{S1}-CN	0.46	0.18 – 0.73	0.01	-0.05 – 0.07
α_{S2}-CN	0.37	0.09 – 0.65	0.03	-0.05 – 0.10
β-CN	-0.11	-0.34 – 0.14	-0.15	-0.22 – -0.09
γ-CN	0.19	-0.09 – 0.45	0.10	0.04 – 0.16
κ-CN	0.08	-0.17 – 0.33	-0.09	-0.16 – -0.03
α-LA	0.24	-0.11 – 0.56	0.01	-0.07 – 0.10
β-LG	-0.06	-0.28 – 0.19	0.01	-0.06 – 0.08
Protein composition, %				
α_{S1}-CN%	0.21	0.01 – 0.42	0.13	0.06 – 0.18
α_{S2}-CN%	0.25	-0.01 – 0.53	0.09	0.02 – 0.17
β-CN%	-0.26	-0.47 – -0.06	-0.17	-0.23 – -0.12
γ-CN%	0.14	-0.17 – 0.43	0.13	0.06 – 0.19
κ-CN%	0.02	-0.21 – 0.26	-0.06	-0.13 – -0.00
β-LG%	-0.19	-0.44 – 0.05	-0.01	-0.08 – 0.08
a_{30}	-0.78	-0.95 – -0.59	-0.70	-0.73 – -0.66
pH	0.61	0.43 – 0.79	0.47	0.40 – 0.54

[1] Contents of all protein fractions were measured by reversed-phase HPLC on skimmed milk;
Casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA ; protein = casein + whey protein; casein number = (casein/protein) × 100; a_{30} = curd firmness.
α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content.

Genetic and Phenotypic Correlations between Milk Proteins and MCP

Median and HPD interval of the marginal posterior density of additive genetic and phenotypic correlations between RCT and protein fractions contents and relative proportions are reported in Table 6. Phenotypic correlations were generally very low and lower than genetic correlations. Results confirm the lack of a positive association between RCT and TCN of milk that was reported by Ikonen et al. (1999). Although the slightly negative phenotypic correlation, the genetic

correlation between RCT and TCN was unfavorable (0.20), indicating that increasing the TCN content of milk would result in a longer RCT. This result underlines that other traits should be used as indirect breeding goals for the genetic improvement of MCP. Some protein fractions were more strongly correlated to RCT than TCN. In particular, α_{S1}- and α_{S2}-CN were genetically positively correlated with RCT, resulting in a worsening of MCP at increasing levels of these two fractions. The possible reasons of these unfavourable associations are unknown, but a study conducted by Mariani et al. (2001) on the effect of α_{S1}-CN G confirm that milk with a decreased content of α_{S1}-CN is characterized by better coagulation properties in comparison with milk having a grater α_{S1}-CN content.

Table 7. Median and 95 % high posterior density interval (HPD 95%) of the marginal posterior density of additive genetic (r_a) and phenotypic (r_p) correlations between curd firmness and protein fractions contents (g/L), proportions (%) of milk protein fractions on total casein or whey protein contents, and pH of milk

	r_a		r_p	
Trait[1]	Median	HPD 95%	Median	HPD 95%
Casein, g/L	0.27	-0.12 – 0.67	0.19	0.11 – 0.26
Whey protein, g/L	0.12	-0.23 – 0.50	0.01	-0.06 – 0.10
Protein fractions content, g/L				
α_{S1}-CN	-0.13	-0.61 – 0.32	0.12	0.05 – 0.19
α_{S2}-CN	-0.13	-0.58 – 0.32	-0.01	-0.09 – 0.07
β-CN	0.33	0.01 – 0.63	0.19	0.12 – 0.26
γ-CN	-0.10	-0.23 – 0.45	0.02	-0.05 – 0.10
κ-CN	0.43	0.13 – 0.72	0.25	0.19 – 0.31
α-LA	0.06	-0.42 – 0.57	-0.03	-0.12 – 0.06
β-LG	0.10	-0.23 – 0.45	0.02	-0.05 – 0.10
Protein composition, %				
α_{S1}-CN%	-0.31	-0.60 – -0.01	-0.09	-0.15 – -0.03
α_{S2}-CN%	-0.32	-0.73 – 0.10	-0.16	-0.23 – -0.08
β-CN%	0.17	-0.12 – 0.48	0.10	0.04 – 0.15
γ-CN%	-0.22	-0.63 – 0.25	-0.12	-0.20 – -0.05
κ-CN%	0.36	0.06 – 0.66	0.19	0.13 – 0.26
β-LG%	0.03	-0.30 – 0.42	0.04	-0.05 – 0.12
pH	-0.61	-0.87 – -0.33	-0.27	-0.36 – -0.18

[1] Contents of all protein fractions were measured by reversed-phase HPLC on skimmed milk;
Casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA ; protein = casein + whey protein; casein number = (casein/protein) × 100;
α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content.

An increased RCT was also associated with the increase of γ-CN content and relative propotion in milk. Kelly and McSweeney (2002) reported that somatic cells, the principal physiological function of which is to defend the udder from infections, contain lysosomes that release active proteolytic enzymes (i.e., elastase, collagenase, and cathepsins). Plasmin plays the main role among the

proteolytic enzymes in milk and can rapidly cleave both β-CN into γ-CN and smaller polypeptides (Auldist et al., 1996) and α_{S1}-CN and α_{S2}-CN, in a minor extent (Le Bars and Gripon, 1993; McSweeney et al., 1993). The increase in plasmin activity is the main factor responsible for impaired coagulation features and CN degradation in milk (Lucey, 1996; Srinivasan and Lucey, 2002) and leads to a reduction in cheese yield and to changes in functionality of milk protein with regard to milk coagulation (Zachos et al., 1992).

The protein fraction more favorably correlated with RCT was β-CN. The content and the relative proportion of β-CN in milk exhibited a genetic correlation with RCT of -0.11 and -0.26, respectively. Hence, phenotypically, fractions responsible for a shortening of RCT of milk were β-CN and κ-CN, but β-CN was the only protein fraction genetically affecting RCT.

Table 8. Median and 95 % high posterior density interval (HPD 95%) of the marginal posterior density of additive genetic (r_a) and phenotypic (r_p) correlations between pH of milk and protein fractions contents (g/L), and proportions (%) of milk protein fractions on total casein or whey protein contents

	r_a		r_p	
Trait[1]	Median	HPD 95%	Median	HPD 95%
Casein, g/L	0.22	-0.11 – 0.56	-0.19	-0.27 – -0.10
Whey protein, g/L	-0.00	-0.30 – 0.25	-0.16	-0.26 – -0.07
Protein fractions content, g/L				
α_{S1}-CN	0.09	-0.22 – 0.42	-0.22	-0.30 – -0.14
α_{S2}-CN	0.26	-0.07 – 0.56	-0.13	-0.24 – -0.03
β-CN	0.16	-0.09 – 0.43	-0.17	-0.25 – -0.09
γ-CN	0.15	-0.15 – 0.46	0.16	0.09 – 0.23
κ-CN	-0.00	-0.27 – 0.26	-0.07	-0.14 – 0.00
α-LA	0.25	-0.08 – 0.61	-0.11	-0.22 – 0.01
β-LG	-0.01	-0.33 – 0.18	-0.15	-0.24 – -0.06
Protein composition, %				
α_{S1}-CN%	-0.12	-0.34 – 0.11	-0.06	-0.13 – 0.01
α_{S2}-CN%	0.11	-0.18 – 0.38	-0.01	-0.12 – 0.09
β-CN%	0.09	-0.15 – 0.31	-0.07	-0.13 – 0.01
γ-CN%	0.10	-0.24 – 0.43	0.23	0.16 – 0.30
κ-CN%	-0.04	-0.29 – 0.20	0.03	-0.04 – 0.11
β-LG%	-0.21	-0.46 – 0.03	-0.02	-0.12 – 0.09

[1] Contents of all protein fractions were measured by reversed-phase HPLC on skimmed milk;
Casein = α_{S1}-CN + α_{S2}-CN + β-CN + γ-CN + κ-CN; whey protein = β-LG + α-LA ; protein = casein + whey protein; casein number = (casein/protein) × 100;
α_{S1}-CN%, α_{S2}-CN%, β-CN%, γ-CN% and κ-CN% are measured as weight percentages of total casein content; β-LG% is measured as weight percentage of total whey protein content.

Studies on the composition of micelles of different sizes have demonstrated that most, if not all, of the κ-CN is located on the surface of the micelle (Donnelly et al., 1984; Dalgleish et al., 1989). However, from the measured sized of CN micelles and the content of κ-CN in TCN, approximately

only one-third of the micellar surface can be covered by κ-CN (Dalgleish, 1998). Compositional studies of micelles suggest that both α_S-CN and β-CN may share the surface with κ-CN, but β-CN and α_S-CN have been less studied than κ-CN and their properties are not well known. However, measurements of the rate of hydrolysis of different proteins in micelles treated with tripsin suggest that β-CN is the protein nearest to the surface (Dalgleish, 1998). Moreover, it is known that β-CN dissociate from the CN micelles with cooling. When the cooled micelles are warmed and the β-CN re-associates with them, it seems unlikely that the β-CN will be able to penetrate deeply into the micelle. Thus, during re-association, the dissociated β-CN may alter the structure of the surface of the CN micelle (Dalgleish, 1998). This could partly explain the marked importance of β-CN on determining RCT.

Although there was a weak negative phenotypic correlation between κ-CN and RCT, resulting in milk with shorter RCT at increasing contents of κ-CN, the genetic correlations between both the content and the relative proportion of κ-CN and RCT were not different from zero. The lack of a favorable genetic correlation between κ-CN and RCT can be explained by the negative correlation between κ-CN% and β-CN% and by the similar correlation of κ-CN with both β-CN and α_{S1}-CN.

Results for genetic correlations between protein fractions and a_{30} (Table 7) were similar to those obtained for RCT. Genetically, weaker curds were associated with higher contents and proportions of α_{S1}-, α_{S2}-, and γ-CN and with lower contents and proportions of β-CN. But, in this case, also κ-CN was positively strongly correlated with a_{30}. The tight relation of κ-CN with a_{30} might be attributable to the finer gel network of milk with greater κ-CN content, which allow the formation of a great number of intermicellar bonds (Horne et al., 1998).

During a series of studies on casein–calcium–phosphate interactions Holt and coworkers (Holt et al., 1998a,b) discovered that the phosphopeptide fraction of β-CN could bind to and stabilize calcium–phosphate aggregates resulting in the formation of nanoclusters of a discrete size and composition; without the peptides the calcium–phosphate structures would grow randomly and precipitate. The formation of nanoclusters would drive micelle formation by randomly binding phosphoproteins causing an inverted micelle, then more proteins could coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed. Hence, β-CN seems to have a central role in the micelle formation and, possible, it might be also the responsible of the organization of the protein reticulum during curd formation. The unfavorable genetic correlation between α_{S1}-CN and MCP might be in part explained by the fact that the proportion of α_{S1}-CN in milk is negatively correlated with the relative content of β-CN.

Correlations between MCP traits and protein fractions are not expected to be greatly affected by related changing of pH of milk as, generally, correlations between protein fractions and pH were from low to very low (Table 8). Phenotypically, an increase of each CN fraction results in a

decrease of the pH of milk, with the only exception of γ-CN which are usually related to the presence of SCC and hence to milk with high pH values. Conversely, genetic correlations are generally positive, but all very low. Hence, in general, it is possible to affirm that genetic correlations between MCP and milk protein fractions are only slightly determined by related effects on pH of milk. Only the unfavourable correlation of α_{S2}-CN and α-LA with MCP might be scribed to their positive correlation (0.26 and 0.25, respectively) with pH of milk.

CONCLUSIONS

The heritability for protein fractions ranged from moderate to high and most of the genetic correlations among the major milk proteins were low. For some protein fractions, protein polymorphisms explained a considerable part of the genetic variance. However, there is still genetic variation in the rest of the genome to permit to alter protein composition by selective breeding. Selection for the total casein content of milk would not be useful for the genetic improvement of milk coagulation properties. Selection for increasing the β-CN and κ-CN contents or decreasing the content of α_{S1}-CN of milk seems to be a more attractive alternative for improving renneting ability, also because of their high heritability. The lack of rapid and automatic analytical methods which allow the quantification of milk protein fractions on a large scale limit the possibility of considering protein fractions as possible indirect traits for the improvement of milk coagulation properties through selective breeding. Altought the development of easy immunoassay is currently under investigation, marker-assisted selection for the alleles associated with the higher expression of those proteins could be a more easier strategy. However, genetic parameters between protein fractions and currently breeding goal traits must be investigated. Moreover, the effect of protein fractions in determining the non-coagulation of milk should be studied.

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GENERAL DISCUSSION AND CONCLUSIONS

Milk protein is probably the most studied food protein. However, most of the biological aspects involving milk protein fractions and coagulation ability of milk are currently unknown or are still unclear.

When studying traits such as protein fractions contents or protein composition, the investigation is complicated by a number of critical issues, which require additional care in developing research protocols and in interpreting results. The first critical issue is the lack of analytical methods able to quantify milk protein fractions content and, more in general, easily detect protein genetic variants. This is an important limit, mostly for genetic studies, in which large sample sizes are commonly needed to obtain reliable estimates of genetic parameters. No current method allows the detection of all protein genetic variants. Hence, neglected alleles can bias estimated parameters with a magnitude that is dependent upon the frequency of the allele and the extent of its effect. Moreover, the close physical linkage of casein genes on bovine chromosome 6 can produced biased estimates if models do not account for all their effects simultaneously. Information on haplotypes is difficult to obtain and estimates of the effects of rare haplotypes are likely to be unreliable.

Several factors, such as linkage with polymorphisms in the non-coding regions of casein genes (Martin et al., 2002), act on the expression of milk protein fractions. This might be responsible of inconsistent results on the effect of casein polymorphisms on protein expression or milk production traits across populations and families.

Despite the study of the physico-chemical properties of a casein variant after purification is relatively simple, the study of its effect on biological properties of milk is particularly difficult because caseins aggregate to each other forming a complex system, the micelle. The lack of knowledge about the real conformation of the casein micelle and the biological role of protein fractions, and of their genetic variants, on the coagulation process is another critical aspect of studies dealing with milk coagulation properties.

In order to provide dairies with milk well suited for the manufacture of dairy products and able to meet requirements for dairy processing, selection for specific genetic variants (gene selection) of milk proteins has been proposed. Results obtained in this study confirmed the marked effect of protein polymorphisms on the expression of their relative protein fraction. The ready availability of milk protein genotypes of animals, the tight relationship between genotypes (or haplotypes) and milk protein fraction, and the marked genetic correlation between protein composition and renneting ability make the gene-based selection a possible effective strategy for the genetic improvement of milk coagulation properties.

Our results indicate that the effect exerted by milk protein polymorphisms on coagulation properties is mainly attributable to the allele-specific expression of the proteins, which modifies milk protein composition. This allele-specific protein expression seems to be related to the presence of linkage disequilibrium between alleles at the milk protein loci and polymorphisms in the non-coding region of these genes (Gustafsson et al., 2003; Kuss et al., 2005; Keating et al., 2007). Hence, the effect exerted by protein polymorphisms is actually to be ascribed to other linked loci. In this case, selection should be based on the polymorphisms in the non-coding region, the direct responsible of the observed effect. Previous work demonstrated that the differential allele-specific accumulation of *CSN3* mRNA is not directly linked to the protein variants of κ-CN (Robitaille and Petitclerc, 2000), as in the case of other milk proteins like β-LG (Wilkins et al., 1995). Cows homozygous for a protein variant may actually be heterozygous in terms of *CSN3* expression into mRNA because of a differential allele-specific accumulation of *CSN3* mRNA. As a consequence, if the breeding goal aims to increase the content of κ-CN in milk, a selection program based on *CSN3* expression into mRNA (κ-CN%) would appear to be more appropriate than a program relying on *CSN3* protein variants (Vachon et al., 2004).

If on one side gene selection can be easily implemented, on the other side it can be affected by variation in the linkage phase between polymorphisms in the coding region and those in the non-coding region of genes. In addition, it will not exploit existing variation in genetic merit of animals of identical genotype. As a consequence, animals with an unfavorable genotype at one locus but with favorable genetic merit for content of a certain protein fraction, due to the variability of the protein expression, might be excluded from selection.

This study also highlighted the importance of considering β-casein, instead of κ-casein, as the principal gene to be used in selection programs aiming at the improvement of milk coagulation properties in Simmental cattle. The greater importance of β-casein in comparison with κ-casein reported in this study seems to be inconsistent with literature results. A possible explanation might be the fact that estimates were obtained for a population in which allele frequencies at casein loci were more balanced (not very high nor very low) and the presence of *CSN2* B was rather high when compared to that observed in previous studies. Studies on the effect exerted by *CSN2* on protein composition and coagulation properties of milk were often limited at two alleles, A^1 and A^2, whose effects are similar, if compared to the effect of B allele. Alternatively, many studies were conducted on cattle populations in which the B allele was included in the "rare" genotypes or haplotypes leading to low reliability of estimated effects or to not significant estimates as a consequence of large standard errors.

Despite the marked effect of protein genes on variation of investigated traits, there is polygenic variation in milk protein composition that might be exploited through breeding. However, to

implement breeding programs using protein composition as indicator traits for the enhancement of milk coagulation properties, protein composition should be measured on a large scale. Currently, in spite of a good repeatability of these traits (Graml and Princhner, 2003), which might limit the number of measurements of protein composition to be recorded per animal, analytical assessment is not feasible at the population level. However, the direct measurement of milk coagulation properties at the population level still remains a critical issue. At the experimental level, RP-HPLC is a reliable and useful analytical method to perform this type of analysis, albeit some alleles cannot be identified. It represents, together with capillary electrophoresis, one of the most precise methods to quantify proteins, ensuring high repeatability and reproducibility of measures. More rapid, high-throughput, automated methods need to be developed for an easier quantification of protein fractions on a large scale and, within available options, Mid Infrared Spectroscopy (MIRS) represents the most attractive one.

Other questions, such as those related to the biological role of milk proteins during the coagulation process and the effect exerted by protein composition or polymorphisms on the non-coagulation of milk, remain to be solved.

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