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ANALYSIS OF MILK PROTEIN COMPOSITION IN CROSSBRED AND PUREBRED DAIRY COWS

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SUMMARY

Milk proteins have been widely studied in the last fifty years because of their great importance and strong relationship with milk quality and milk coagulation properties (MCP). The general interest in dairy science gave the possibility to achieve many important scientific results, such as polymorphism identification concerning this kind of molecules and differences among species and breeds. Studies which considered effects of milk protein polymorphisms on milk characteristics and milk properties started since 1970 in such countries like Italy, where more than 70% of milk is used in the cheese-making industry and transformed in typical products, which play a key role for the economic valorization, like the Protected Designation of Origin Products.

The importance of this kind of topics and the possibility to improve milk and the cheese-making processes brought to a strong change concerning breeding value aims and selection strategies employed in dairy science. In the last decades many farms focused on high milk production animals, but this sort of management took to a worsening of health and functional characteristics of the animals, such as fitness, robustness, longevity and fertility, as well as genetic variability. Today situation get different, because an acceptable compromise was found among farm productivity and milk quality; greater economic value was given to typical products and local breeds were protected, because strong linked to the environmental preservation, local tradition, and historical tradition conservation; moreover crossbreeding schemes are employed, because they carry out very appreciable animal performances, but breed merits are kept and at the same time some very important effect, like heterosis, are exploited. Considering such options and lack of studies in literature, it would be desirable to gain further knowledge on the effects of some sources of variation on milk protein fraction and milk protein component.

Aims of the study were to find and develop a new reverse phase HPLC method able to quantify and identify the most common milk protein fractions; to investigate the sources of variation on milk protein fractions and composition of individual milk samples collected in dairy herds which employed crossbreeding schemes; to investigate the sources of variation on milk protein fractions and composition of individual milk samples collected in multi-breed dairy herds located in mountainous areas.

A new reverse phase HPLC method for the identification and quantification of the most common protein fractions and genetic variants of bovine milk was developed, including some minor components, like lactoferrin. The method was validated by testing linearity and repeatability. For genetic variants identification, milk of DNA-genotyped animals was used to find peaks and to associate them with the known genotype of animals. Calibrations with a coefficient of determination higher than 0.99 were obtained for every single genetic protein variant, nevertheless commercial standards for every variant were not available.

Method was after employed for individual milk samples collected in crossbred dairy herds located in the north of Italy and to compare pure Holstein with different bull semen of Alpine European and Nordic breeds, like Montbeliarde, Brown Swiss and Swedish Red. It came out that milk protein fractions, are influenced by the stage of lactation and parity; breed effect was significant especially for κ -CN, α -LA and β -Lg, which are linked. Among crossbred

combinations, Brown Swiss and Montbeliarde crosses showed high significance, because of the selection strategy or the natural presence of the B variant of the κ -CN, respectively; B variant is very important in the cheese making industry because animals carrying AB or BB genotype show a higher milk casein content and also a higher milk quality.

Method was after employed for individual milk samples collected in herds where two breeds were attended at least; six breeds were employed, three high-production and three dual-purpose: Holstein-Friesian, Brown Swiss, Jersey and Gray Alpine, Simmental and Rendena, respectively. Four types of dairy herd were considered in this study: modern herd type, traditional herd type with silage use, traditional herd type without grazing use and traditional original herd type. Effect of stage of lactation and parity influenced milk protein fractions; among breeds, Jersey showed a higher milk protein content than the other breeds; in Gray Alpine a new genetic κ -CN variant was detected using our reverse phase HPLC method, but not identified, so further investigation is required to confirm this aspect, employing other techniques maybe coupled with HPLC. Traditional dairy herd type with silage use was the best strategy with the highest milk protein content, although it showed the highest somatic cell content; modern herd type gave unexpectedly inferior performances and traditional herd without grazing was instead the worse concerning milk protein content.

RIASSUNTO

Negli ultimi cinquant'anni le proteine del latte sono state oggetto di ricerca approfondita, date le loro caratteristiche e l'importanza ad esse attribuita per quanto riguarda qualità e capacità di coagulazione del latte. L'interesse creatosi ha reso possibile il raggiungimento di diversi risultati di grande rilevanza scientifica, come l'identificazione di polimorfismi a livello di queste molecole e di differenze anche piuttosto accentuate tra diverse specie e razze bovine. Gli studi che hanno considerato gli effetti dei polimorfismi proteici sulle caratteristiche e proprietà del latte hanno trovato ampio spazio in Italia, già a partire dai primi anni '70, dove gran parte del latte viene usato nell'industria lattiero-casearia e dove è fondamentale l'ottimizzazione della produzione di prodotti trasformati e prodotti tipici, tra i quali figurano anche i Prodotti a Denominazione di Origine Protetta.

In zootecnia la rilevanza di tali argomenti e la possibilità di migliorare considerevolmente il latte e il suo processo di trasformazione hanno portato un profondo cambiamento per quanto riguarda gli obiettivi e le strategie di selezione e miglioramento genetico degli animali da reddito; nel corso degli ultimi anni molte aziende del settore si sono concentrate su animali altamente produttivi in termini di quantità di latte prodotto, ma tale management ha portato purtroppo ad un netto peggioramento della salute e delle caratteristiche funzionali degli animali, quali la fitness, la rusticità, la longevità e la fertilità, oltre alla perdita di preziosa variabilità genetica. Oggi la situazione è diversa, in quanto si è cercato di trovare un compromesso accettabile tra produttività degli allevamenti e qualità di prodotto, investendo economicamente nella valorizzazione di prodotti tipici e tutelando razze meno produttive, ma fondamentali per esempio nel contesto rurale, ampiamente diffuso in Italia, proprio per le loro caratteristiche di adattabilità e rusticità, oltre ad essere utili per la salvaguarda del territorio e della tradizione; oppure utilizzando gli schemi di incrocio tra razze, che riescono a garantire ottime performance degli animali, mantenendo le caratteristiche di razza e contemporaneamente sfruttano alcuni effetti di grande utilità, come l'eterosi. Alla luce di tali cambiamenti, e data ancora la scarsità di informazioni presente in letteratura, è auspicabile poter continuare ad acquisire preziose informazioni sugli effetti di alcune fonti di variazione sulla componente proteica del latte.

Obiettivi della tesi sono stati: sviluppare e validare un nuovo metodo HPLC in fase inversa atto a identificare e quantificare le frazioni proteiche più comuni del latte bovino; studiare gli effetti di alcune fonti di variazioni sulle frazioni proteiche individuate con tale metodica di latte bovino individuale proveniente da allevamenti che si servissero degli schemi di incrocio al loro interno; studiare gli effetti di alcune fonti di variazione sulle frazioni proteiche di latte bovino individuale proveniente da allevamenti montani multi-razza.

Un nuovo metodo di analisi HPLC in fase inversa è stato sviluppato e validato per consentire l'identificazione e la contemporanea quantificazione delle più comuni frazioni proteiche, comprese le loro varianti genetiche, presenti nel latte bovino, oltre a componenti minori poco conosciute ma di grande interesse, come la lattoferrina. Tale nuova metodica è stata sottoposta a test di linearità e ripetibilità. Per l'identificazione delle varianti è stato utilizzato latte proveniente da animali precedentemente genotipizzati, in modo da riconoscere i picchi a

livello cromatografico ed associarli al corretto genotipo della data proteina presa in esame. E' risultato possibile ottenere le calibrazioni corrette con un coefficiente di determinazione superiore a 0.99 per tutte le singole varianti genetiche delle frazioni proteiche, sebbene non fossero disponibili standard commerciali per varianti singole.

Tale metodo è stato poi applicato per l'analisi di latte individuale proveniente da allevamenti del nord Italia che utilizzavano lo schema di incrocio di prima e seconda generazione tra razza pura Holstein e tre differenti semi di tori del nord Europa e dell'arco Alpino, quali razza Montbèliarde, Brown Swiss e Rossa Svedese. Dalla prova è emerso che le frazioni proteiche del latte sono influenzate da alcune fonti di variazione come lo stadio di lattazione e l'ordine di parto; la razza in vece influenza in particolar modo κ -CN, α -La e β -Lg, frazioni proteiche tra loro intimamente associate. Tra gli schemi di incrocio, hanno presentato un'alta significatività le combinazioni con la razza Bruna e la Montbeliarde, la cui selezione o la presenza naturale rispettivamente dell'allele B della κ -CN è di grande interesse per l'industria di trasformazione poichè porta ad un latte di qualità maggiore, dato il contenuto più alto di caseina totale.

Tale metodo è stato in ultimo applicato per l'analisi di latte individuale proveniente da aziende che allevassero contemporaneamente almeno due delle sei razze scelte per la prova, tra specializzate e a duplice attitudine, cioè razza Holstein-Friesian, Brown Swiss, Jersey e Grigia Alpina, Pezzata Rossa e Rendena rispettivamente. Nella prova sono state considerate anche quattro diverse tipologie aziendali, cioè l'allevamento di tipo moderno, di tipo tradizionale con l'utilizzo di insilati, di tipo tradizionale senza la malga estiva, di tipo tradizionale originale.

Anche in questo caso stadio di lattazione ed ordine di parto hanno influito notevolmente sulle frazioni proteiche oggetto di studio; tra razze, la Jersey si è distinta notevolmente dalle altre, proprio perché caratterizzata naturalmente da un latte di contenuto proteico notevolmente alto. Nella razza Grigia è stata inoltre identificata tramite cromatografia un'ulteriore probabile variante della κ -CN, anche se sarà necessario raccogliere maggiori informazioni servendosi anche di tecniche complementari o accoppiate all'HPLC. La tipologia aziendale di tipo tradizionale con uso di insilati è risultata essere la strategia migliore, dato il contenuto proteico molto alto riscontrato nel latte, anche se è risultata la strategia con anche il più alto numero di cellule somatiche; l'allevamento moderno inaspettatamente non ha dato le performances migliori, mentre l'allevamento tradizionale senza uso della malga è risultato il peggiore per quanto riguarda la componente proteica del latte.

GENERAL INTRODUCTION

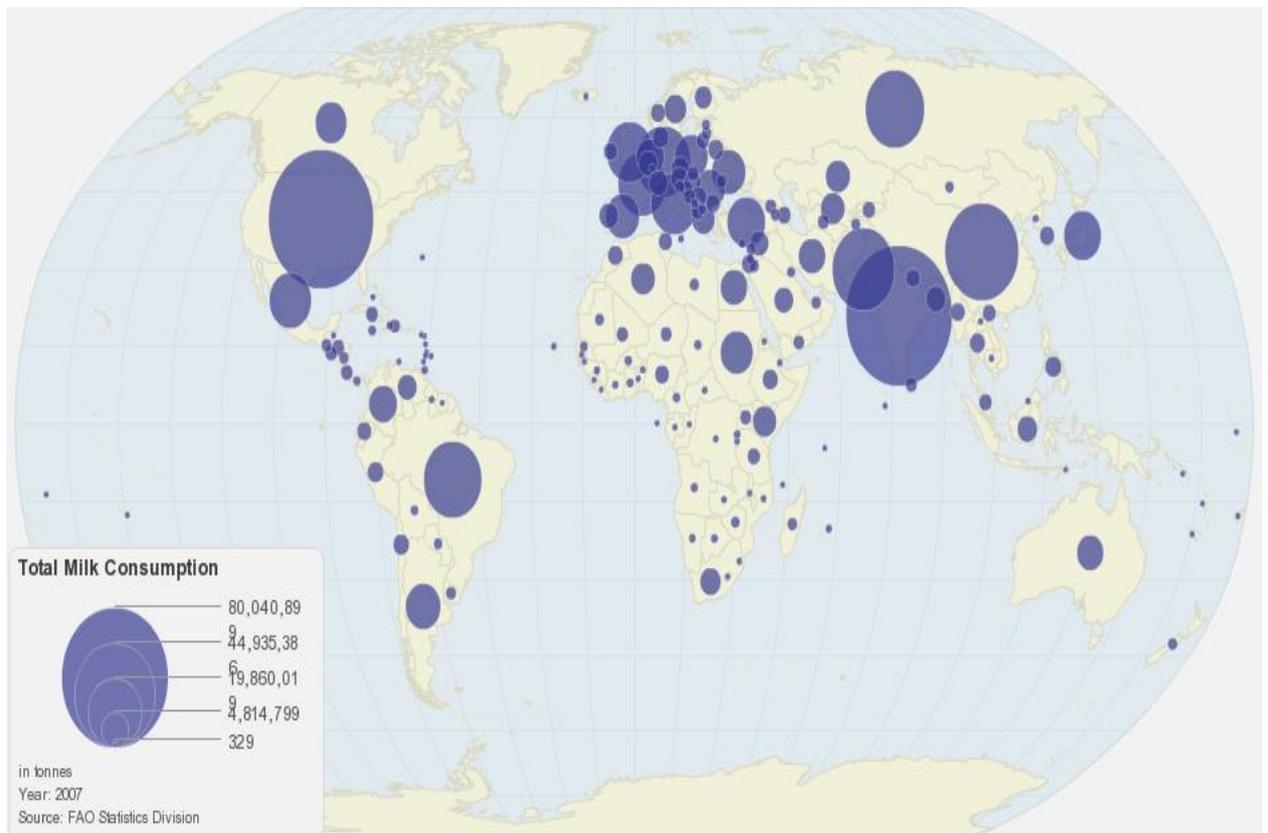
Global milk production and milk consumption

Global food consumption patterns are undergoing change, especially in large parts of the developing world, where growth and urbanization became both to increasing levels of overall food intake; this carried out changes in composition of food consumption, with growing shares of high-value products and of food of animal origin in particular. Growth of dietary energy intake in the developing countries has been followed by a rapid change in diet composition, in which consumption of livestock products has been increased.

Many factors played a key role in worldwide consumption changing of livestock products. An important factor has been urbanization; in cities, peoples typically consume more food, such as pre-cooked and convenient foods (Gerosa et Skoet, 2012). Moreover other social and cultural factors have a large influence locally, like cultural preferences, natural resources attended or not in the countries, relative costs and prices of different food commodities, intolerances (Dong, 2006).

Looking to milk production patterns, developing country growth in demand for and consumption of milk has been followed by an increasing production, while production in the rest of the developed countries such as Europe and America has grown only slowly; as explained in Figure 1, different regions contribute to vary different degrees in overall milk production; South Asia pole plays a fundamental role in milk production, which shows a continuous and sustained growth. Today India alone is responsible for almost a third of developing country production. China, Latin America and Russia showed a strong increase of milk production, but with an expanding production that seem to by slower than South Asia. Cow milk dominates global milk production (85% of global production), but however milk from other animals and species is very important in some countries and regions. In South Asia (44%), buffaloes are the most important source of milk; they make a great contribution to total production also in East Asia, especially China and in North Africa. On the other hand, contribution of milk from goat (3%), sheep (1.5%) and camels (0.2%) is limited, but these kinds of milk are relative significant in sub-saharian Africa and North Africa (FAO report, 2012).

Figure 1. Total milk consumption



Changes in the production systems

There are many differences between developed and developing countries; in most cases, in developing countries milk is produced in a traditional scale system with little or absent mechanization or technological innovations. In addition, adverse conditions for milk production play an important role, such as high ambient temperature and humidity; environmental characteristics imply a stronger adaptation of the animals and in many cases a reduction in the expression of the full genetic potential of the cows. Moreover difficult access to high-quality feed is a limiting factor. So finally an efficient management and housing systems is required to optimize milk production and preserve health status of the animals (McDowell, 1981).

Most developed countries have seen increasing herd size and higher milk production per cow; many farmers adopted technological innovations to improve productivity, which often require high capital and therefore bigger herds to be profitable. At the same time, feeding get on

different strategies, like employment of feed concentrates to reach the higher yields (Gerosa and Skoet, 2012).

European milk production

Milk production systems vary across Europe, ranging from lowland to mountain and from extensive to intensive (European Commission, 2000). In the last years progressive intensification increased environmental impacts and rigorous policy planning are required to ensure a good evaluation of the sustainability of milk production system (Ming-Jia Yan et al., 2011). The EU is a major player in the world dairy sector; about 90% of the milk produced is for domestic consumption of dairy products. Compared to year 2011, milk production has increased of +0.6% in year 2012, because of the great export demand in some countries, such as Poland, Denmark and Germany (European Commission, 2012). According to the food and Agricultural Policy Research Institute (FAPRI, 2011), dairy market analysis outlooks from 2011 to 2020 show that milk production will continue to increasing in the next 10 years at the average growth rate equal to the one of the past decade. The majority of the growth is expected to come from developing countries where increasing incomes and westernization of diets results in strong demand for dairy products; in Europe the expected trends in milk and cheese production will be +7% for milk and +10% for cheese production. Italy is with France the country with the highest number of locally-made cheeses, counting about 400 different products; in Italy more than 70% of the overall milk production is used in the cheese making industry, and almost 50% of total milk is used for PDO cheeses (Protected Designation of Origin) production (Cassandro et al., 2003).

Aims of dairy industry

Farm animals have been undergoing human-managed selection ever since their original domestication. Initially, selection was probably limited to docility and manageability, but aims of selection and animal breeding changed rapidly in conjunction with human society and requirements. In the last 60 years breeding programs focused on the genetic improvement of production traits, such as milk yield and production. Selection was based initially on animal phenotype, and just experience gave guidelines to obtain offspring with a superior phenotype with few measurements. With the advent of quantitative genetics in the middle of 20th century, a great advance in selection practice occurred, including optimized breeding schemes based on intense bull dam selection, systematic progeny testing of bulls, and large amounts of

data (Sørensen et al., 2008) but as results, animal production industry came under dramatic changes (Oltenacu and Broom, 2010). Concerning dairy industry, a typical example is given by the Holstein dairy breed. Holstein was imported from north America in the late 1800s, initially with live animals followed by semen and embryos. Exports were mainly to EU member countries, especially Italy, the Netherlands, Germany and France. This because it became worldwide that Holsteins gave higher milk yields than most breeds.

After that, in many countries yield per cow has more than doubled in the last 40 years, because of the rapid progress in genetics and management. This kind of strategy brought to long-terms problems which occur most likely in high-producing animals. As consequence a progressive loss of genetic variability and a deterioration of functional traits, like productive life, fertility and longevity occurred. Diseases, such as uterine infections and other disorders which can give reproductive problems, increased, because of metabolic stress associated with milk production. Calving interval increased from <13.0 months to > 14.5 months and number of inseminations per conception from 2.0 to > 3.5 (Lucy, 2001).

Inbreeding results from the mating of related individuals is also increasing, with negative effects on animal welfare; the most unfavorable effects are however inbreeding depression, including an increase in the incidence on abnormalities caused by recessive alleles, like lethal diseases such as bovine leukocyte adhesion deficiency (BLAD) and complex vertebral malformations (CVM), loss of genetic variance and random drift in the population mean. Values of many traits are reduced, such as fitness, general health status and fertility (Thompson et al., 2000).

Finally breeding organizations implemented strategies designed to maintain genetic variability and prevent the increase of inbreeding in the dairy population. For example, including health, fertility and other fitness traits in the breeding objectives along with production traits, because of the antagonistic genetic correlations between them, considering genotype by environmental interactions, taking advantage of the molecular genetics tools already available (Mark, 2004; Miglior et al., 2005).

Today crossbreeding for example may help to guarantee these kind of needs; crossbreeding is the mating of individuals from different lines, breeds, or populations. The reasons to implement breeding programs with this kind of strategy are firstly to utilize different additive genetic levels between breeds to generate offspring with better economic ability caused by new combinations of additive genetic components, and secondary to exploit heterosis, which its expression carry out animals more robust and economically efficient compared with the parental breeds (Mäki-Tanila, 2007). Initially crossbreeding was used for a long time in beef

cattle, pig and poultry production systems, but less used in dairy cattle, except New Zealand, because of the low reproductive rate within dairy cattle (Heins, 2007).

Technological aspects of milk

After the detection of β -Lg polymorphism discovered by Aschaffenburg and Drewry (1957) fifty years ago, scientific interest increased in dairy research; one of the most important effects of the milk protein polymorphisms on traits with economic interest is their strong relationship with milk composition and cheese making properties of milk, defined as milk coagulation properties (MCP). Because of the strong importance of milk in such countries, like Italy, where a great part of whole milk production is used in the cheese making industry, milk coagulation properties (MCP) were widely studied until today. Coagulation properties of milk influence cheese-making ability, cheese yield and quality (Ng-Kwai-Hang et al., 1989; Johnson et al., 2001; Bittante et al., 2012).

As explained by Joudu et al. (2008) rennet coagulation parameters are significantly influenced by α_{s1} -CN, β -Cn and β -Lg contents and by casein number. Rennet coagulation time is reduced and curd firmness is higher, along with increasing contents of the studied milk protein fractions, including total casein and casein number.

At the same time large variability exists among MCP and other factors, such as calcium, pH (Storry et al., 1983; Najera et al., 2003), age of the animal, stage of lactation, composition of ration, season and breed, although results reported in literature are contradictory (Ostensen et al., 1997; Auldust et al., 2002; Hansen et al., 2006; Wedholm et al., 2006).

Many strategies were employed to improve animal selection and milk quality, although practical implementation possibilities are scarce. Genetic variation can be detected by different protein identification techniques, such as electrophoretic and isoelectrophoretic methodologies, capillary electrophoresis and chromatography (Caroli et al., 2009); detection and quantification of genetic milk protein variants is possible through other techniques such as near infrared spectroscopy (Laporte et al., 1998; Ferrand et al., 2012; Marinoni et al., 2013) and predictions of MIR spectra of milk contents were used as indicator traits in breeding programs (Cecchinato et al., 2009; De Marchi et al., 2010). At the DNA level, new techniques were discovered and employed to investigate protein variants at the genome level, like PCR-RFLP, direct sequencing, genotyping microarray, microsatellites (Damiani et al., 1990; Chessa et al., 2007; Dalvit et al., 2008). Despite of great importance giving to MCP, a general worsening of them has been observed in several countries; this trend was evidenced by some authors (Mariani et al., 1992; Cassandro and Morusi, 2001; Sandri et al., 2001) and

some studies evidenced the presence of candidate genes for non-coagulating milk (Tyrisev et al., 2008).

Milk proteins

The major constituents of milk are either directly synthesized and secreted from the mammary epithelial cells into the alveolar lumen or transported across the epithelial barrier from other sources (Mather, 2011). Bovine milk contains several 200 different compounds, mostly at trace levels. These constituents can be classified into four categories:

- ✚ Organ- and species-specific constituents (lipids and principal proteins)
- ✚ Organ- but non species-specific constituents (lactose)
- ✚ Species- but non organ-specific constituents (some whey proteins, like WPs)
- ✚ Neither organ- nor species-specific constituents, although the profile and concentrations are species specific (water, salts, vitamins)

Bovine milk is typically made of 87% water, 3,9% fat, 3,25% protein, and 5,5% of other molecules, like lactose. Of the 3,25% protein, approximately 80% is composed of caseins (Walstra and Jenness, 1984; Jeng et al., 1997; Holland et al., 2010). Caseins are the most important protein group of almost mammalian species; they are characterized by a stable calcium phosphate binding which determines micelles complexes, large colloidal particles with diameter ranging from <50 nm to >500 nm and an average diameter of approximately 200 nm as measured by dynamic light scattering (Frederiksen et al., 2011); they precipitate at PH 4.6 at 20° C by acidification from raw skim milk (Farrell et al., 2004); casein composition affects micelles structure, aggregation, enzyme activity and, above all, the milk processing in dairy industry and cheese making process (Bramanti et al., 2002). Briefly, during renneting, which is the moment of rennet addition to milk, two phases divide the process: firstly κ -CN is cleaved by rennet enzymes such as the endopeptidase chymosin between the residue Phe105 and Met106; thereby, caseinomacropeptide (CMP), which is the highly hydrophilic part of the C-terminal of κ -CN, is released into the whey, with casein micelles destabilization; the secondary phase is non-enzymatic: the destabilized casein micelles start a calcium-dependent spontaneous aggregation, resulting in the curd or coagulum (Green and Morant, 1981; Fox and McSweeney, 1998; Sandra et al., 2007).

Bovine milk contains four major caseins: α_{s1} -, α_{s2} -, β - and κ -caseins; the concentration of those proteins is ca. 10, 2.6, 9.3 and 3.3 mg/mL. γ -CNs are different C-terminal segments of β -CN derived from proteolytic cleavage by the enzyme plasmin. Polar and apolar regions on

the casein peptide chains are not uniformly distributed, giving them amphiphilic structure; moreover proline and phosphate contents bring to the ability of caseins to form micelles.

The α_{S1} -CN family, which comprehends almost the 40% of the CN fraction, consists of one major and one minor component. Both proteins are single-chain polypeptides with the same amino acid sequence (Mercier et al., 1971; Grosclaude et al., 1973) and differ just on their phosphorylation degree. The α_{S2} -CN, which represents the 10% of the CN fraction in milk, consists of 2 major and other minor components attending many levels of post-translational phosphorylation. The α_{S2} -CN is the most hydrophilic of all caseins. β -CN, almost the 45% of total CN fraction and the most hydrophobic, is quite complex because of the action of the native milk protease plasmin (Eigel et al., 1984). κ -CN family consists of a major carbohydrate-free components and of 6 minor components, which represent degrees of phosphorylation and glycosilation (Doi et al., 1979). Mollè and Lèonil (1995) found 14 glycosilated forms. The points of attachment of the oligosaccharide chains was detected at 131, 133 or 135 of Thr residues (Jollès et al., 1973).

The remaining protein components are the whey proteins; whey is composed by α -lactalbumin, β -lactoglobulin, serum albumin, immunoglobulins and other proteins, with an amount of ca. 2, 10, 1, 2 and 2% respectively (O'Donnell et al., 2004). β -Lactoglobulin is the major protein in whey and affect properties of milk markedly; another feature is the ability to bind molecules like palmitic acid, vitamin D, retinol (vitamin A), which is essential in mammalian growth (Sawyer, 2003). α -LA, approximately attended at concentration of 1.2 to 1.5 g/L has a specific physiological function in the mammary gland, because interacts to form the lactose synthase complex. Its concentration in milk decreases near the end of lactation, and it seem to be correlated with the decline of lactose concentration.

Serum albumin (SA), Immunoglobulins (Ig) and Lactoferrin (Lf) are attended in low concentration in bovine milk. SA plays an important role in the transport, metabolism and distribution of ligands; bovine SA in milk is physically and immunologically identical to blood SA. Ig group, formed by IgG, IgA and IgM, are attended in milk and above all in colostrums, and give immunity to calf. Finally Lf, member of specific iron-binding proteins, is attended in milk at a concentration between 20 and 200 mg/L (Plate et al., 2006). Concentration varies and increases in response to inflammation or infection (Ashwell et al., 2004).

Most whey proteins are globular with secondary and tertiary structure, which make them sensitive to heat denaturation at more than 60°C of temperature, despite of caseins (Farrell et al., 2004).

Milk protein polymorphism

Polymorphism has been observed for all milk proteins. Concerning Caseins, B variant is the predominant variant of the α S₁-CN in *Bos taurus*; variant C in *Bos indicus* and variant D in some breeds of France (Mariani and Russo, 1975). 3 new genetic variants were identified; variant F was found in German black and white cattle, variant G in Italian Brown cow, and variant A, which was found in Holstein-Friesian, Red Holstein and German Red cattle (Farrell et al., 2004). α S₂-CN and α -LA have been shown to be essentially monomorphic in all Western dairy breeds.

The κ -CN genetic variants are designed as A and B variant; the A variant seems to be predominant in most dairy breeds, excepted the Jersey cattle (Ng-Kwai-Hang et al., 2003). B variant is naturally predominant in such breeds like Montbeliarde or it was objective of selection in such breeds like Brown Swiss, because its strong relationship with improved milk clotting properties and higher cheese yield. Other variants have been detected, such as E, which is related with unfavorable milk clotting properties, C and D variant. Concerning β -CN, the most common variants are B, A1 and A2 variants. Other variants have been reported by other works, like I, F, H a G variant. Concerning whey, β -Lg attends two genetic variants, A and B, at high frequency in most cow breeds, and the presence of one or the other affects milk properties, partially because the A variant is expressed at higher level than the B variant, or the C variant (Hill et al., 1996). A new variant was detected, called variant C (Paterson et al., 1995).

Milk protein polymorphisms play a key role in the technological properties and quality of milk; different chemical and physical behavior and specific amino acid variations bring to protein variation and to a different allele expression which control protein synthesis (Bonfatti et al., 2010). Moreover several studies focused on the 6 structural genes that code milk proteins (Caroli et al., 2009). Because casein loci are closely linked on bovine chromosome 6, alleles of the different casein are in linkage disequilibrium; moreover, because of this, aggregate casein genotypes has to be considered and evaluated (Hallen et al., 2008). Genotypes of milk proteins, particularly of κ -CN and β -Lg, were found to affect milk composition. Genetically linked mutations in the non-coding regions of κ -CN and β -Lg are assumed to affect the transcription of κ -CN and β -Lg, respectively (Bobe et al., 2004). Generally, B allele of κ -CN has been associated with a higher κ -CN concentration in milk, compared to A and also with higher total protein, it is assumed that B variant is particularly favorable to milk coagulation and syneresis (Ikonen et al., 1997). On the other hand, A allele was associated with longer coagulation time and softer curd firmness.

B allele of β -CN seems to be related with higher total β -CN concentration, and an epistatic effect of the β -CN casein locus on κ -CN content was detected (Graml and Pirchner, 2003). Hallen et al (2008) found that the A^1 variant of β -CN was at higher concentration than the A^2 variant in milk from heterozygous A^1A^2 cows but no significance was found when analyzing κ -CN genotype. Furthermore polymorphism in the promoter region of αS_2 -CN was found to be associated with varying contents of αS -CN and β -CN. B allele of β -CN has been linked to a better coagulation compared to the A variant; finally, as explained before, B variant of β -Lg seem to be less expressed than the A variant; moreover A variant is strongly associated with a greater relative concentration of β -LG and a lesser concentration of the other protein fractions (McLean et al., 1984; Ng-Kwai-Hang et al., 1986; Lunden et al., 1996; Huang et al., 2012).

REFERENCES

- Aschaffenburg, R and J. Drewry. 1957. Genetics of the β -lactoglobulins of cow's milk. *Nature (Lond.)*. 180:376-378.
- Ashwell, M. S., D. W. Heyen, T. S. Sonstegard, C. P. Van Tassel, Y. DA, P. M. Van Raden, M. Ron, J. I. Weller, and H. A. Lewin. 2004. Detection of quantitative trait loci affecting milk production, health, and reproductive traits in Holstein cattle. *J. Dairy Sci.* 87: 468-475.
- Auldist, M. J., K. A. Johnston, N. J. White, W. P. Fitzsimons, and M. J. Boland. 2004. A comparison of the composition, coagulation characteristics and cheese making capacity of milk from Friesian and Jersey dairy cows. *J Dairy Res.* 71:51-57.
- Auldist, M., C. Mullins, B. O'Brien, B. T. O'Kennedy, and T. Guinee. 2002. Effect of cow breed on milk coagulation properties. *Milch-wissenschaft* 57:140-143.
- Bittante, G., M. Penasa, and A. Cecchinato. 2012. Invited review: Genetics and modeling of milk coagulation properties. *J. Dairy Sci.* 95:6843-6870.
- Bobé G., A. E. G. Freeman, G. L. Lindberg, and D. C. Beitz. 2004. Milk protein genotypes explain variation of milk protein composition. *Anim. Ind. Report ASL R1901*.
- Bramanti, E., C. Sortino, and G. Raspi. 2002. New chromatographic method for separation and determination of denatured α_{s1} -, α_{s2} -, β - and κ -casein by hydrophobic interaction chromatography. *J. Chromatogr A.* 958:157-166.
- Caroli, A. M., S. Chessa, and G. J. Erhardt. 2009. *Invited review*: Milk protein polymorphisms in cattle: effect on animal breeding and human nutrition. *J Dairy Sci.* 92:5335-5352.
- Cassandro, M. 2003. Status of milk production and market in Italy. *Agricolturae Conspectus Scientificus*. Vol. 68(2), pp. 65-69, ISSN: 1331-7768.
- Cecchinato, A., M. De Marchi, L. Gallo, and G. Bittante. 2009. Mid-infrared spectroscopy predictions as indicator traits in breeding programs for enhanced coagulation properties of milk. *J Dairy Sci.* 92: 5304-5313.
- Chessa, F., F Chiatti, G. Ceriotti, A. Caroli, C. Consolandi, G. Pagnacco, and B. Castiglioni. 2007. Development of a SNP genotyping microarray platform for the identification of bovine milk protein genetic polymorphisms, *J Dairy Sci.* 90:451-464.
- Dalvit, C., M. De Marchi, R. Dal Zotto, E. Zanetti, T. Meuwissen, and M. Cassandro. 2008. Genetic characterization of the Burlina cattle breed using microsatellites markers. *J. Animal Breed. Genet.* 125:137-144.

- Damiani, G., L. Ferretti, G. Rognoni, and V. Sgaramella. 1990. Restriction fragment length polymorphism analysis of the kappa-casein locus in cattle. *Anim. Genet.* 21:107-114.
- De Marchi, M., V. Bonfatti, A. Cecchinato, G. Di Martino, and P. Carnier. 2010. Prediction of protein composition of individual cow milk using mid-infrared spectroscopy. *Ital. J. Anim. Sci.* Vol 8 (Suppl.2) 399-401.
- Doi, H., F. Ibuki, and M. Kanamori. 1979. Heterogeneity of reduced bovine κ -casein. *J. Dairy Sci.* 62:195-203.
- Dong, F., 2006. The outlook for Asian dairy markets: The role of demographics, income and prices. *Food Pol.* 31:260-271.
- Eigel, W. N., J. E. Butler, C. A. Ernstrom, H. M. Farrell, Jr., V. R. Harwalkar, R. Jenness, and R. M. Whitney. 1984. Nomenclature of proteins of cow's milk: fifth revision. *J Dairy Sci.* 67:1599-1631.
- European Commission, 2012.
- FAO (Food and Agriculture Organisation), 2012. Milk availability. Trends in production and demand and medium-term outlook.
- FAPRI (Food and Agricultural Policy Research Institute), 2011. Agricultural commodity markets outlook 2011-2020. A comparative analysis.
- Farrell H. M., Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk-sixth revision. *J. Dairy Sci.* 87:1641-1674.
- Ferrand, M., G. Miranda, H. Larroque, O. Leray, S. Guisnel, F. Lahalle, M. Brochard, and P. Martin. 2012. Determination of protein composition in milk by mid-infrared spectrometry. *ICAR Tech Se.* 16:41-45.
- Fox, P. F., and P. L. H. McSweeney. 1998. *Dairy Chemistry and Biochemistry*. Blackie Academic and Professional. London, UK.
- Frederiksen, P. D., K. K. Andersen, M. Hammershøj, H. D. Poulsen, J. Sørensen, M. Backman, K. B. Qvist, and L. B. Larsen, 2011. Composition and effect of blending of noncoagulating, poorly coagulating, and well-coagulating bovine milk from individual Danish Holstein cows. *J Dairy Sci.* 94:4787-4799.
- Gerosa, S., and J. Skoet. 2012. Milk availability. Trends in production and demand and medium-term outlook. FAO working paper.
- Graml, R., and F. Pirchner. 2003. Effects of milk protein loci on content of their proteins. *Arch. Tierz. Dummerdorf* 46:331-340.

- Green, M. L., and S. V. Morant. 1981. Mechanism of aggregation of casein micelles in rennet-treated milk. *J. Dairy Res.* 48:57-63.
- Grosclaude, F., M. F. Mahè, and B. Ribadeau-Dumas. 1973. Structure primaire de la caseine α_{s1} -et de la caseine β -bovine. *Eur. J. Biochem.* 40:323-324.
- Hallèn, E., A. Wedholm, A. Andrèn, and A. Lùnden. 2008. Effect of β -CN, κ -CN and β -lactoglobulin genotypes on concentration of milk protein variants. *J. Anim. Breed. Genet.* 125:119-129.
- Hansen, J. V., N. C. Friggens, and S. Højsgaard. 2006. The influence of breed and parity on milk yield, and milk yield acceleration curves. *Livest. Sci.* 104:53-62.
- Heins, B. J. 2007. Impact of an old technology on profitable dairying in the 21st century. *Petersen Symposium.* Pp.7-19.
- Hill, J. P., M. J. Boland, L. K. Creamer, S. G. Anema, D. E. Otter, G. R. Paterson, R. Lowe, R. L. Motion, and W. C. Thresher. 1996. Effect of bovine β -lactoglobulin phenotype on then properties of β -lactoglobulin, milk composition, and dairy products. *ACS symposium, ser. 650, Macromolecular Interactions in food technology*, pp. 281-294.
- Holland B., S. Rahimi Yadzi, G. Ion Titapiccolo, and M. Corredig. 2010. Short communication: separation and quantification of caseins and casein macropeptides using ion-exchange chromatography. *J. Dairy Sci.* 93:893-900.
- Huang W., F. Peñagaricano, K. R. Ahmad, J. A. Lucey, K. A. Weigel, and H. Khatib. 2012. Association between milk protein gene variants and protein composition traits in dairy cattle. *J. Dairy Sci.* 95:440-449.
- Ikonen, T., M. Ojala, and E. L. Syvaöja. 1997. Effects of composite casein and β -lactoglobulin genotypes on renneting properties and composition of bovine milk by assuming an animal model. *Agric. Food Sci. Finland.* 6:283-294.
- Jeng, S.-Y., T. G. Bleck, M. B. Wheeler, and R. Jemenez-Flores. 1997. Characterization and partial purification of Bovine α -Lactalbumin and β -Casein produced in milk of transgenic mice. *J. Dairy Sci.* 80:3167-3176.
- Jollès, J., A.-M. Fiat, C. Alais, and P. Jollès. 1973. Comparative study of cow and sheep κ -caseino-glycopeptides: determination of the N-terminal sequences with a sequencer and the location of the sugars. *FEBS Lett.* 30:173-176.
- Johnson, M. E., C. M. Chen, and J. J. Jaeggi. 2001. Effect of rennet coagulation time on composition, yield, and quality of reduced-fat Cheddar cheese. *J. Dairy Sci.* 84:1027-1033.

- Jõudu I., M. Henno, T. Kaart, T. Püssa, and O. Kärt. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *Int. Dairy J.* 18:964-967.
- Laporte, M. F., R. Martel, and P. Paquin. 1998. The near-infrared optic probe for monitoring rennet coagulation in cow's milk. *Int. Dairy J.* 8:659-666.
- Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: where will it end?. *J. Dairy Sci.* 84:1277-1293.
- Lundèn A., M. Nilsson, and L. Janson. 1997. Marked effect of β -lactoglobulin polymorphism on the ratio of casein to total protein in milk. *J. Dairy Sci.* 80:2996-3005.
- Mäki-Tanila, A. 2007. An overview on quantitative and genomic tools for utilizing dominance genetic variation in improving animal production. *Agric. Food Sci.* 16:188-198.
- Mariani, P., p. Monatti, and S. Sandri. 1992. Contenuto di urea, pH, acidità titolabile e caratteristiche di coagulazione del latte di singoli allevamenti. *Ind. Latte* 28:2996-3005.
- Marinoni, L., L. Monti, S. Barzagli, and B. de la Roza-Delgado. 2013. Quantification of casein fractions and of some of their genetic variants in phosphate buffer by near infrared spectroscopy. *J. Near Infrared Spectrosc.* 21:385-394.
- Mark, T. 2004. Applied genetic evaluations for production and functional traits in dairy cattle. *J. Dairy Sci.* 87:2641-2652.
- Mather, I. H., 2000. A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *J. Dairy Sci.* 83: 203-247.
- McDowell, R. E. 1981. Limitations for dairy production in developing countries. *J. Dairy Sci.* 64:2463-2475.
- Mclean D. M., E. R. Graham, R. W. Ponzoni, and H. A. McKenzie. 1984. Effects of milk protein genetic variants on milk yield and composition. *J. Dairy Res.* 51:531-546.
- Mercier, J.-C., F. Grosclaude, and B. Ribadeau-Dumas. 1971. Structure primaire de la casein α_{s1} bovine. Sequence complete. *Eur. J Biochem.* 23:41-51.
- Miglior, F., B. L. Miur, and B. J. Van Doormaal. 2005. Selection indices in Holstein cattle of various countries. *J. Dairy Sci.* 88:1255-1263.
- Ming-Jia, Y., J. Humphreys, and N. M. Holden. 2011. An evaluation of cycle assessment of European milk production. *J. Environmental Manag.* 92:372-379.

- Mollè, D., and J. Léonil. 1995. Heterogeneity of the bovine κ -casein caseino-macropeptide, resolved by liquid chromatography on-line with electrospray ionization mass spectrometry. *J. Chromatogr. A.* 708:223-230.
- Najera, A. I., M. De Renobales, and L. J. R. Barron. 2003. Effects of pH, temperature, CaCl₂ and enzyme concentrations on the rennet-clotting properties of milk: a multifactorial study. *Food Chem.* 80:345-352.
- Ng-Kwai-Hang, K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1986. Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *J. Dairy Sci.* 69:22-26.
- Ng-Kwai-Hang, K. F., I. Politis, R. I. Cue, and A. S. Marziali. 1989. Correlation between coagulation properties of milk and cheese yielding capacity and cheese composition. *Can. Inst. Food Sci. Technol.* 22:291-294.
- O'Donnell, R., J. W. Holland, H. C. Deeth, and P. Alewood. 2004. Milk Proteomics. *Int. Dairy J.* 14:1013-1023.
- Oltenucu, P. A., and D. M. Broom. 2010. The impact of genetic selection for increased milk yield on the welfare of dairy cows. *Anim. Welf.* 19:39-49.
- Ostensen S., J. Foldager, and J. E. Hermansen. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *J. Dairy Res.* 64:207-219.
- Paterson, G. R., J. P. Hill, and D. E. Otter. 1995. Separation of β -lactoglobulin A, B and C variants of bovine whey using capillary zone electrophoresis. *J. Chromatogr. A* 700:105-110.
- Plate, K., S. Beutel, H. Buchholz, W. Demmer, S. Fischer-Frühholz, O. Reif, R. Ulber, and T. Scheper. 2006. Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. *J. Chromatogr. A.* 1117:81-86.
- Sandra, S., M. Alexander, and D. G. Dalgleish. 2007. The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *J. Colloid Interface Sci.* 308:364-373.
- Sandri, S., F. Tosi, P. Mariani, P. Vecchia, M. Malacarne, and A. Summer. 2001. Observations on the trend of the main dairy-characteristics of Parmigiano-Reggiano cheese milk during the years 1990-2001. *Animali della Facoltà di Medicina Veterinaria, Università degli Studi di Parma, vol XXI, 235-247.*

- Sawyer, L. 2003. β -lactoglobulin. *Advanced Dairy Chemistry*. Vol. 1 Proteins, pp. 319-386. Ed. Kluwer Academic/Plenum Publisher, New York, NY.
- Sørensen, M. K. E. Norberg, J. Pedersen, and L. G. Christensen. 2008. *Invited review: Crossbreeding in dairy cattle: a Danish perspective*. *J. Dairy Sci.* 91:4116-4128.
- Storry, E. J., A. S. Grandison, D. Millard, A. J. Owen, and G. D. Ford. 1983. Chemical composition and coagulating properties of renneted milks from different breeds and species of ruminants. *J. Dairy Res.* 50: 215-229.
- Thompson, J. R., R. W. Everett, and N. L. Hammerschmidt. 2000. Effects of inbreeding on production and survival in Holsteins. *J. Dairy Sci.* 83:1856-1864.
- Tyrisevä, A. M., K. Elo, A. Kuusipuro, V. Vilva, I. Jänönen, H. Karjalainen, T. Ikonen, and M. Ojala. 2008. Chromosomal regions underlying noncoagulation of milk in Finnish Ayrshire cows. *Genetics* 180(2), 1211-1220.
- Walstra, P. 1999. Casein sub-micelles: do they exist?. *Int. Dairy J.* 9(3-9):189-192.
- Wedholm, A., L. B. Larsen, H. Lindmark-Månsson, H. Karlsson, and A. Andréén. 2006. Effect of protein composition on the cheesemaking properties of milk from individual dairy cows. *J. Dairy Sci.* 89:3296-3305.

AIMS

The general aim of the thesis was to gain further knowledge about milk protein fraction and milk protein composition in crossbred and purebred dairy cows. The effects of some sources of variation were investigated to better understand changes and relationships between milk proteins and milk proportion considering different crossbreeding combinations and different dairy managements in multi-breeds dairy herds; moreover it was needed to clarify the specific properties of protein fractions and their genetic variants on the productive performances of the animals.

Specific aims were to:

- Develop a reverse phase HPLC method for the identification and quantification of the most common milk protein fractions and their genetic variants (Chapter 1)
- Investigate the sources of variation on milk protein fractions and composition of individual milk samples collected in dairy herds which employed crossbreeding schemes (Chapter 2)
- Investigate the sources of variation on milk protein fractions and composition of individual milk samples collected in multi-breed dairy herds located in mountainous areas (Chapter 3)

CHAPTER 1.

Detection and quantification of α_{S1} -, α_{S2} -, β -, κ -casein, α -lactalbumin, β -lactoglobulin and lactoferrin in bovine milk by reverse-phase high-performance liquid chromatography

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SUMMARY: Bovine milk proteins has been widely studied because of the strong association and relationship with composition and technological properties of milk. Cow's milk quality is very important, above all in such countries like Italy, where about 70% of whole milk production is used in cheese-making industry. A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to identify and quantify rapidly the most common genetic variants of bovine milk proteins, included lactoferrin. A reverse-phase analytical column C8 (Aeris WIDEPORÉ XB-C8, Phenomenex, 3,6 μm , 300 \AA , 250 x 2,1 I.D.) was used for the analysis. All the most common casein (CN) and whey protein genetic variants were detected and separated simultaneously in less than 20 min; purified bovine milk protein genetic variants were employed in calibration. A linear relationship ($R^2 > 0.99\%$) between concentration and peak areas of individual milk protein variants was observed.

Key words: bovine casein, whey protein, lactoferrin, genetic variants, HPLC

AIM – Several methods were employed to analyze milk protein fractions, such as electrophoretic techniques (Veloso et al., 2002), proteomic approaches (Jensen et al., 2012) isoelectring focusing (IEF) (Strange et al., 1992), ion-exchange HPLC chromatography (Holland et al., 2010) or HPLC chromatography coupled with mass spectrometry (Bonizzi et al., 2009; Mollè et al., 2009). Previous investigations focused on the separation of bovine milk protein fraction, overlooking the quantification of single milk protein genetic variants with few exceptions (Bonfatti et al., 2008); furthermore very few studies focused on other minor components, like lactoferrin (Palmano et al., 2001) and BSA, and generally are time consuming. The aim of this study was to develop an RP-HPLC method able to identify and quantify the single genetic variants of protein fractions, included some minor components

such as lactoferrin, improving the run time analysis of chromatograms, even so ensuring good separation of protein fractions and high resolution. Validation of the method includes testing linearity.

MATERIALS AND METHODS – The present study is part of a larger project aimed at the study of relationships between quality and technological traits of milk of Brown Swiss cows (Bittante, 2012; Bittante et al., 2011a, b, and 2013; Cecchinato et al., 2009, 2011, 2012a, b; Cipolat et al., 2012; Macciotta et al., 2012; Maurmayr et al., 2011).

Guanidine hydrochloride (GdnHCl) (lot G-4505, purity >99%), Bis-tris Buffer (lot B-9754, >98%), Trifluoroacetic acid (lot T-6508, >99%), sodium citrate (lot 71498, >99%) and DL-Dithiothreitol (lot 43817, >99%) were supplied by Sigma (Sigma-Aldrich, St. Louis, MO, USA). Purified major protein from bovine milk were purchased from Sigma: 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%), β -Lg_A (lot L-7880, >90%) and lactoferrin (lot L-9507, >85%). Ultra pure water (Milli-Q System, >18,2 M Ω cm) was obtained in the laboratory. Individual and bulk bovine milk was collected directly in dairy herds. Preservative (Bronopol, 2-bromo-2-nitropropan-1,3-diol) was added to raw milk samples to prevent microbial growth and 2 aliquots for each sample containing 1 ml of milk were frozen at -20°C during milk collection and transferred at -80°C in the laboratory since the HPLC analysis was performed. Milk samples were prepared following the method of Bobe et al. (1998). No preliminary separation or precipitation procedures of the casein fraction was required.

The HPLC equipment consisted of an Agilent 1260 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump (Agilent 1260 Series, G1311B). A Diode Array Detector (Agilent 1260 Series, DAD VL+, G1315C) was used. The equipment was controlled by the Agilent Chemstation for Lc System software which sets solvent gradient, data acquisition and data processing. Separation was performed on a reversed-phase analytical column C8 (Aeris WIDEPORÉ XB-C8, Phenomenex) with a large pore core-shell packing (3,6 μ m, 300Å, 250 x 2,1 I.D.). A Security Guard ULTRA Cartridge System (product No. AJ0-8785, Phenomenex) was used as pre-column (UHPLC WIDEPORÉ C8, 2,1 mm I.D.). Sample vials were kept at low constant temperature (4°C) and injected via an auto-sampler (Agilent 1100 Series, G1313A).

After comparing different chromatographic conditions, the followed was adopted to optimize analytical quality and time required: a) gradient elution was carried out with a mixture of two

solvents: solvent A consisted of 94.9% water, 5.0% acetonitrile and 0,1% trifluoroacetic acid and Solvent B consisted of 0,1% TFA in acetonitrile; b) separation of bovine protein fraction was performed with the following program: linear gradient from 20 to 29 % B in 0.5 min, from 29 to 33% B in 5.5 min, from 33 to 36% B in 6 min, from 36 to 45% B in 6 min and return linearly to the starting condition in 1 min; c) the column was re-equilibrated under starting conditions for 3 min, before inject the following sample and the total analysis time per sample was 22 min; d) the flow rate was 0.5 ml/min; e) the column temperature was kept at 70°C; f) the detection was made at a wavelength of 214 nm; and g) the injection volume consisted of 2 μ l.

Concerning standard solutions, single-fraction mother solutions were prepared by dissolving, respectively, 5 mg of purified α -CN, 2,5 mg of purified κ -CN, 4 mg of purified β -CN, 1.5 mg of purified lactoferrin, 1 mg of purified α -La, 2 mg of both purified β -Lg A and B in 0.75 ml of GndHcl solution; then, a set of five decreasing concentration solutions was obtained by each single mother solution by applying the dilution scheme reported in table 1.

TABLE 1: Dilution scheme and composition of the casein fraction concentration standards

Standard level	Dilution (ml)	Fraction concentration (mg)						
		k-CN	α_s -CN ^a	β -CN	Lfe	α -La	β -Lg A	β -Lg B
5	0.75	2.5	5	4	1.5	1	2	2
4	1.5	1.87	3.75	3	1.12	0.75	1.5	1.5
3	2.25	1.40	2.81	2.25	0.84	0.56	1.12	1.12
2	3	1.05	2.10	1.68	0.63	0.42	0.84	0.84
1	3.75	0.79	1.58	1.26	0.47	0.31	0.63	0.63

a: for quantification it was applied the 4:1 proportion between α_{s1} and α_{s2} fractions

The resulting standard solutions were analyzed in order to construct the α_{s1-s2} , β and κ -CN, lactoferrin, α -La, β -LG_{A-B} calibration curves. Since α_{s1} and α_{s2} are not available as single proteins, the corresponding values were calculated from the α_s applying the 4:1 proportion known for cow milk (Bonizzi et al., 2009). Before calibration, linearity was tested by running the same sample at the five dilution points in triplicate. Areas under peaks of the chromatogram were used to validate the method. Concerning validation procedure, 30 individual milk samples were analyzed and every sample was run two times repeating the analysis of the same sequence (sample injection=2 μ l). The external standard method was used for the calibration of the chromatographic system for the protein quantification. Calibration curves were computed for each protein genetic variant by applying simple linear

regression of the peak area on the amount injected, at decreasing injection volume. Results are reported in Table 2.

TABLE 2: Parameters of regression equation curves for single protein genetic variants or protein fraction^a

STANDARD	MEAN	DS	b	a	RDS ^b	R ²
K-CN A	763.4	399.80	583.41	-126.30	49.91	0.99
K-CN B	665.4	289.05	420.56	24.5	42.18	0.99
α S2-CN 1	658.23	347.24	252	-109.62	51.54	0.99
α S2-CN 2	624.48	377.88	276.20	-217.11	33.24	0.99
α -S1-CN B	4517.04	2722.79	1992.43	-1553.88	203.72	0.99
α -S1-CN C	1873.38	1107.25	809.47	-593.06	95.85	0.99
Lactoferrin	3446.56	1594.40	3885.93	-101.29	91.46	0.99
β -CN B	513.22	321.50	293.06	-205.54	25.86	0.99
β -CN -A1	2425.84	1329.91	1215.76	-555.94	35.57	0.99
β -CN -A2	4469.56	2031.04	1854.79	-79.51	106.65	0.99
α - LA	2146.29	1167.42	4279.76	-461.79	76.49	0.99
β -Lg A	1887.22	1206.06	2189.63	-799.89	137.18	0.99
β -Lg B	2682.68	1679.44	3053.04	-1073.78	71.83	0.99

a Separated solutions of purified protein genetic variants at different concentration in duplicate

b Residual standard deviation

RESULTS AND DISCUSSION – All major peaks and chromatograms are reported in Figure 1.

HPLC conditions were optimized for mobile phase conditions, gradient, flow rate and temperature. Retention times of the major eluted peaks coincided with the retention times of the major milk proteins present in standards. It was ascertained that proteins eluted following this order: κ -CN, α_{S2} -CN, α_{S1} -CN, lactoferrin, α -La, β -CN and β -Lg.

The identification of peaks of genetic variants was confirmed comparing them with commercial standards which consisted of purified genetic variants (just for β - Lg variants are available, A and B respectively) or comparing them with chromatograms of individual milk samples of DNA-genotyped animals. The κ -CN eluted as three distinct peaks which consisted of glycosylated and unglycosylated forms of κ -CN_A and κ -CN_B. Chromatograms with a different κ -CN genetic variants were well resolved, variant A and B are evident, although the infrequent variant κ -CN_E coeluted with the A variant using the current method; κ -CN_E variant is a uncommon variant which exhibits a high frequency in the Finnish Ayrshire (Ikonen et al., 1996), but not in Brown Swiss or Holstein Friesian breeds (Bittante et al. 2012). The genetic

variants of this protein fraction showed an effect also on cheese yield and quality (Alipanah and Kalashnikova, 2007; Bonfatti et al., 2011). Between κ -CN_{A/E} and κ -CN_B peaks eluted α_{S2} -CN, which consisted of two major peaks; multiple peaks and shoulders of α_{S2} -CN are probably caused by the partial separation of many phosphorylated forms of α_{S2} -CN (Bordin et al., 2001).

Separation of α_{S1} variant B and C was enough feasible with the current method (the height of the inflection point between the two peaks is respectively the fifth part of the first peak B variant and the second part of the second peak C variant). In the current analysis it was not found an animal carrying α_{S1} -CN A or α_{S1} -CN D variants. Separation of lactoferrin was well resolved, retention time and peak area is the same for the sample of unknown amount and the standard used for calibration curves, although peak area of lactoferrin is very small because of the very low amount of this iron-binding protein in bovine milk (20-200 mg/L) (Farrell et al., 2004; Plate et al., 2006). α -La eluted after α_{S1} -CN and it showed monomorphic peak in variant B in all samples analyzed.

Concerning β -CN, B, A1 and A2 variants eluted after α -La and all peaks were identified and well resolved. The C variant coeluted with A1 variant and it was not detectable with the current method; the A3 variant, which coeluted with the A2 variant was detected with the current method; there is another variant that eluted between A1 and A2 variant which is not named because of their absence in milk samples of DNA-genotyped animals that were used for the identification and detection of the major milk protein genetic variants during the validation procedure of the method.

For β -Lg, variant B eluted before variant A; this last one has got a peak followed by a main one and the proportions of the area between these two peaks can be considered as an indicator of proteolysis (Bordin et al., 2001). Comparing the injections of standards, BSA eluted between lactoferrin and α -La, and Ig eluted at the end of the chromatographic run, after β -Lg. It was not possible to detect and quantify both BSA and Ig during the analysis of milk samples, although peaks were better resolved and visible analyzing colostrums; however colostrum seems to need a different preparation or a different method development to detect other proteins, such as BSA and Ig, which are in low proportion in milk, but in high level at the beginning of the lactation (Farrell et al., 2004).

Concerning quantification, calibration curves have been derived from parameters of simple linear regression computed for whole protein fraction by using commercial standards. Considering all the five-point calibration setting used for each standard at five different

dilutions, the relation between peak area and injected amount of protein variant was linear ($R^2 > 0.99\%$).

Concerning milk sample, CN content was nearly 84% of total protein. Within caseins, α_s -CN was ca. 42% of the total casein fraction, whereas k-CN was 19% and β -CN was 37% of total casein fraction, whereas the β -Lg was 84% of the total whey protein. The CN content of samples as a proportion of total protein content in our study was greater than the casein index reported by other studies because non-protein nitrogen, proteose-peptones and minor constituents of whey protein cannot be quantified with this method.

FIGURE 1: Chromatograms relative to individual raw milk samples (samples 1-5) obtained using the optimized elution condition describe in matherials and method section

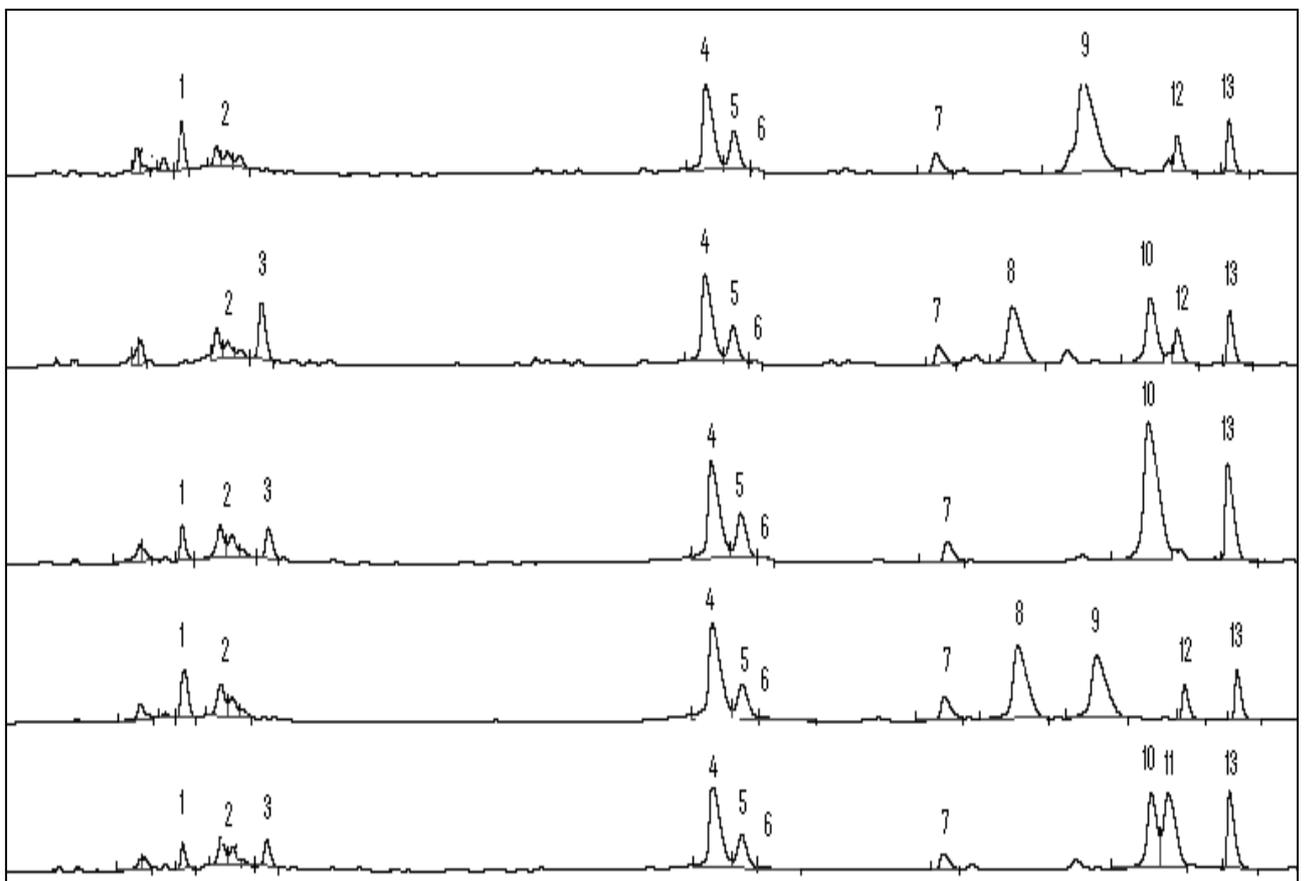


Fig. 1. Chromatograms of different individual milk samples; no 1: k-CN A; no 2: α_{s2} -CN ; no 3: k-CN B; no 4: α_{s1} -CN B; no 5: α_{s1} -CN C; no 6: Lactoferrin; no 7: α -La; no 8: β -CN B; no 9: β -CN A1; no 10: β -CN A2; no 11: β -CN A3; no 12: β -Lg B; no 13: β -Lg A

CONCLUSIONS - A RP-HPLC method was developed to identify and quantify the most common milk protein genetic variants of cows. The method guaranteed the detection and the quantification of all major milk protein fractions, included some minor components like lactoferrin, and their main genetic variants in one fast run with good resolution. It allows a run time equal or lower than halved if compared to methods proposed earlier.

In conclusion this method can be applied to analysis of raw individual and bulk milk samples; preparation procedure is very easy and fast and the total analysis time per sample is short (22 min) considering the amount of information that could be collected: concentration of different protein fractions of milk, separately for each main genetic variants, and thus also genotype of animals for the gene codifying for protein fractions can be derived. This methodology can favor new understanding on the complex relationships among protein fractions and variants, milk quality, milk coagulation, cheese yield and quality.

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REFERENCES:

- Alipanah, M., Kalashnikova, L.A. (2007). Influence of k-casein genetic variant on cheese making ability. *J Anim Vet Advances* 6: 855-857
- Bittante G. (2012). Modeling rennet coagulation time and curd firmness of milk. *J Dairy Sci* 94: 5821-5832
- Bittante G., Cecchinato A., Cologna N., Penasa M., Tiezzi F., and De Marchi M. (2011a). Factors affecting the incidence of first-quality wheels of Trentingrana cheese. *J Dairy Sci* 94: 3700-3707
- Bittante G., Cologna N., Cecchinato A., De Marchi M., Penasa M., Tiezzi F., Endrizzi I., and Gasperi F. (2011b). Monitoring of sensory attributes used in the quality payment system of Trentingrana cheese. *J Dairy Sci* 94: 5699-5709
- Bittante G., Contiero B., Cecchinato A. (2013). Prolonged observation and modelling of milk coagulation, curd firming, and syneresis. *Int Dairy J* 29: 115-123
- Bittante G., Penasa M., and Cecchinato A. (2012). Invited review: Genetics and modeling of milk coagulation properties. *J Dairy Sci* 95: 6843-6870
- Bobe G., Beitz D. C., Freeman A. E., Lindberg G. L. (1998). Separation and quantification of bovine milk proteins by reversed-phase High Performance Liquid Chromatography. *J Agric Food Chem* 46: 458-463
- Bonfatti V., Cecchinato A., Di Martino G., De Marchi M., Gallo L., Carnier P. (2011) Effect of k-casein on Montasio, Asiago and Caciotta cheese yield using milk of similar protein composition. *J Dairy Sci* 94: 602-613
- Bonfatti V., Grigoletto L., Cecchinato A., Gallo L., Carnier P. (2008). Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *J Chromatogr A* 1195: 101-106
- Bonizzi I., Buffoni J. N., Feligini M. (2009). Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. *J Chromatogr* 1216: 165-168

- Bordin G., Cordeiro Raposo F., de la Calle B., Rodriguez A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. *J Chromatogr A* 928: 63-76
- Cecchinato A., Cipolat-Gotet C., Casellas J., Penasa M., Rossoni A., and Bittante G. (2012a). Genetic analysis of rennet coagulation time, curd-firming rate, and curd firmness assessed on an extended testing period using mechanical and near-infrared instruments. *J Dairy Sci* 96: 50-62
- Cecchinato A., De Marchi M., Gallo L., Bittante G., and Carnier P. (2009). Mid-infrared spectroscopy predictions as indicator traits in breeding programs for enhanced coagulation properties of milk. *J Dairy Sci* 92: 5304–5313
- Cecchinato A., Penasa M., De Marchi M., Gallo L., Bittante G., and Carnier P. (2011). Genetic parameters of coagulation properties, milk yield, quality, and acidity estimated using coagulating and noncoagulating milk information in Brown Swiss and Holstein-Friesian cows. *J Dairy Sci* 94: 4214-4219
- Cecchinato A., Ribeca C., Maurmayr A., Penasa M., De Marchi M., Macciotta N. P. P., Mele M., Secchiari P., Pagnacco G., and Bittante G. (2012b). Short communication: Effects of β -lactoglobulin, stearoyl-coenzyme A desaturase 1, and sterol regulatory element binding protein gene allelic variants on milk production, composition, acidity and coagulation properties of Brown Swiss cows. *J Dairy Sci* 95: 450-454
- Cipolat-Gotet C., Cecchinato A., De Marchi M., Penasa M., and Bittante G. (2012). Comparison between mechanical and near-infrared optical methods for assessing milk coagulation properties. *J Dairy Sci* 95: 6806–6819
- Farrell H. M., Jimenez-Flores R., Bleck G. T., Brown E. M., Butler J. E., Creamer L. K., Hicks C. L., Hollar C. M., Ng-Kwai-Hang K. F., Swaisgood H. E. (2004). Nomenclature of the proteins of Cows' milk – Sixth Revision. *J Dairy Sci* 87: 1641-1674
- Holland B., Rahimi Yazdi S., Ion Titapiccolo G., Corredig M. (2010). Short communication: separation and quantification of caseins and casein macropeptide using ion-exchange chromatography. *J Dairy Sci* 93: 893-900

- Ikonen T., Ruottinen O., Erhardt G., Ojala M. (1996). Allele frequencies of the major milk proteins in the Finnish Ayrshire and detection of a new K-casein variant. *Anim Genet* 27: 179-181
- Jensen H. B., Holland J. W., Poulsen N. A., Larsen L. B. (2012). Milk protein genetic variants and isoforms identified in bovine milk representing extremes in coagulation properties. *J Dairy Sci* 95: 2891-2903
- Macciotta N.P.P., Cecchinato A., Mele M., and Bittante G. (2012). Use of multivariate factor analysis to define new indicator variables for milk composition and coagulation properties in Brown Swiss cows. *J Dairy Sci* 2012: 7346-7354
- Maurmayr A., Ribeca C., Cecchinato A., Penasa M., De Marchi M., and Bittante G. (2011). Effects of Stearoyl-CoA Desaturase 1 and Sterol Regulatory Element Binding Protein gene polymorphism on milk production, composition and coagulation properties of individual milk of Brown Swiss cows. *Agric Conspectus Scientificus* 76: 235-237
- Palmano K. P., Elgar D. F. (2002). Detection and quantification of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *J Chromatogr A* 947: 307-311
- Plate K., Beutel S., Buchholz H., Demmer W., Fischer-Frühholz S., Reif O., Ulber R., Scheper T. (2006). Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. *J chromatogr A* 1117: 81-86
- Strange, E. D., Malin E: L., Van Hekken D. L., Basch J. J. (1992). Chromatographic and electrophoretic methods used for analysis of milk proteins. *J Chromatogr A* 624: 81-102
- Veloso A. C. A., Teixeira N., Ferreira I. M. P. L. V.O. (2002). Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis Detection of milk adulterations. *J Chromatogr A* 967: 209-218
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CHAPTER 2.

Effect of days in milk, parity and crossbred combinations of purebred Holsteins and first/second-generation crossbred cows from Swedish Red, Montbéliarde and Brown Swiss bulls on milk protein fractions and composition

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ABSTRACT

The aim of this study was to investigate the effect of some environmental source of variation and of crossbreeding on the proportion and amount of the most common milk protein fractions, included some minor components like lactoferrin. A total of 505 individual milk samples were collected during evening milking (2 to 3 sampling days per herd) in three commercial farms located in Northern Italy. The three farms have followed crossbreeding programs on part of their cows, even if most of the animals were purebred Holstein (H). The basic scheme was a three-breed rotational based on the use of Swedish Red (S) semen on H cows ($S \times H$), Montbeliarde (M) semen on $S \times H$ cows [$M \times (S \times H)$], and again H semen on $M \times (S \times H)$. In all herds, a smaller proportion of purebred H were mated inverting the breed order ($M \times H$ and $S \times (M \times H)$) or using Brown Swiss (B) bulls ($B \times H$), and then M bulls ($M \times (B \times H)$). Milk samples were analyzed by reverse-phase high performance liquid chromatography and protein fractions amount (g/L) and proportion (% of total true protein) were obtained. Traits were analyzed by using a linear model including the fixed effects of herd-test-day, parity, days in milk and breed combination. The traits considered in this work showed generally normal distribution, with the exception of lactoferrin which showed an asymmetrical distribution. All milk protein fractions were influenced by the stage of lactation and parity; the increase of protein concentration during lactation is due especially to β -CN, lactoferrin and β -Lg. The greater protein content of heifers's milk is due to an increase of all

casein fractions and partly to α -LA. The higher protein content of crossbred cows respect to purebred Holsteins was due especially to an increase of κ -CN, α -LA, and β -LG. The three-way crossbreds differed from two-way crossbred only for a greater proportion of α -LA of their milk. Within the three-way crossbred cows, the S sired ones yielded milk with a smaller content and proportion of β -LG respect to the M sired cows and, consequently, by a higher proportion of caseins respect to whey proteins.

Key words: crossbreeding, casein fractions, whey proteins, lactoferrin, Swedish Red, Montbeliarde.

INTRODUCTION

Bovine milk contains almost ~3.5 % protein, but this level varies substantially with breed, individuality, health and nutritional status of the animal. Almost 80% is composed of caseins, corresponding to 2.5–2.8%, w/v (Holland et al., 2010). Caseins are the most important proteins as for quantitative, technological and nutritional aspects. The major proteins belonging to this group are usually referred to as α_{s1} -, α_{s2} -, β - and κ -fractions (Bonizzi et al., 2009) characterized by a stable calcium phosphate binding which determines micelles complexes (Farrell et al., 2004). It is demonstrated that genetic, environmental and management factors represent sources of variability in the milk protein content (Martin et al., 2002, Summer et al., 2003). Whey proteins are described as the group of milk protein that remain soluble in serum or whey after precipitation of milk casein at pH 4.6; β -Lactoglobulin (β -Lg), α -Lactoalbumin (α -LA), serum albumin, Immunoglobulins, Lactoferrin (Lf), and proteose-peptone fractions have been considered the major characterized components of whey (Ng-Kwai-Hang et al., 2003).

Casein are responsible of curd formation after rennet addition and consequently of cheese yield; milk was designed to coagulate in the neonatal stomach, which is achieved

through limited proteolysis of the micelle-stabilizing protein, κ -CN, by a specific gastric proteinase, chymosin (Fox, 2011). Despite the lower economic importance, also whey proteins have peculiar nutritional and physiological properties; BSA is a leakage protein from blood, while IgG is transported selectively from blood into colostrums and gives passive immunity on the young of species who do not receive Ig *in utero*. Finally, few studied, lactoferrin, an iron-binding protein with antimicrobial and antifungal activity. So, modification of content, proportion and daily yield of milk protein fractions can be very important in nutritional, technological and economic terms according to the destination of milk and of the characteristics of the market.

The genetic polymorphism within the caseins and the whey proteins has been demonstrated and it determines the improvement or the worsening of some characteristics of milk, like curd-firming capacity, syneresis ability, whey drainage and cheese making properties (Bittante et al., 2012; Cecchinato et al., 2011 ; Jensen et al., 2012). In literature several works investigated associations of milk protein polymorphisms with protein composition and cheese making properties of milk (Bonfatti et al., 2010, Mayer et al., 1997; Wedholm et al., 2006; Bonfatti et al., 2010; Cecchinato et al., 2011; Bittante et al., 2012).

Breeding for milk protein genotypes has been suggested as a practical way to selection and to change the composition of the milk protein fraction; Selection and crossbreeding are the two strategies that can be employed to alter genetically the yield of milk and its components (Lopez-Villalobos et al., 2000). In the past, in most countries the high selection pressure was focused just to improve productivity of the animals and this kind of strategy brought as consequence to a progressive decrease milk quality traits and a deterioration of functional traits. Success of selection for milk production has contributed to the spreading of the Holstein breed around the world, but now are known the heavy consequences of this diffusion on important traits like milk quality, productive life, fertility and longevity. Today different selection indices, breeding value estimation techniques, and mating schemes ensure

a better management of dairy cows and their genetic richness, including milk quality and functional traits such as fertility, health, calving ease, and longevity. Recently, crossbreeding gained great attention because it may help to overcome most of these problems, in fact it brings about new combinations of additive genetic components, expression of heterosis, more economical efficiency and a better strength of crossbred animals compared with the parental breeds (Van Raden, 2003, Sorensen et al., 2008).

Crossbreeding of pure Holstein (**H**) with other breeds is a useful strategy to improve functional and productive traits of animals; Heins et al., (2006) reported that Scandinavian Red (**S**) and Montbeliarde (**M**) sires induced a fat plus protein daily production amounts 2.2 and 3.8% lower than purebred H, but survival to 150 d in the first lactation was 5% higher compared with pure H, and also calving difficulty and stillbirth rates were significantly reduced (Heins et al., 2006, Heins et al., 2012).

Several studies considered pure breeds employed in crossbreeding schemes for functional traits, like conception rate, days open, and productive life, and for productive traits such as milk yield, fat, protein, and somatic cell score (Bryant et al., 2007; Prendiville et al., 2010; Heins et al., 2012) but knowledge on the effect of crossbreeding on content, proportion (and daily yield) of milk protein fractions is very scarce.

The aim of this work was to analyze the milk protein fractions content, proportion (and daily yield) of individual milk samples of purebred Holsteins, of two-way crossbred cows with B (**B**×**H**), M (**M**×**H**) and S (**S**×**H**) bulls and of three-way crossbred cows with the same breeds [**M**× (**B**×**H**); **M**×(**S**×**H**); and **S**×(**M**×**H**)] and of estimating the effects of herd/test day, parity, stage of lactation and breed combination on these traits.

MATERIALS AND METHODS

Animals and milk sampling

Individual milk samples of purebred H cows ($n = 159$), and of crossbred cows reared in three herds located in the north of Italy, were collected from January to February 2013. The herds were following a common crossbreeding scheme starting from purebred H cows. The basic scheme was a three-breed rotational based on the use of Swedish Red (S) semen on H cows (S×H), Montbeliarde (M) semen on S×H cows [M×(S×H)], and again H semen on M×(S×H). In all herds, a smaller proportion of purebred H were mated inverting the breed order (M×H and S×(M×H)) or using Brown Swiss (B) bulls (B×H), and then M bulls (M×(B×H)). The crossbreeding scheme is represented in Figure 1 together with the number of cows sampled for each breed combination.

The sampled cows represented different stages of lactation (6 classes of days in milk; DIM) and parities (1-4). Milk sampling occurred once per animal, during evening milking. Management of herds was in accordance with the rules established by EU for producing the Protected Designation of Origin (PDO) cheese Parmigiano-Reggiano, and thus had similar management conditions: silage, pasture and fresh herbage were not allowed, and the rations (fed as a total mixed diet) were based on dry roughage, concentrates and added water.

Preervative (Bronopol, 0.6:100 v/v, Sigma-Aldrich, St. Louis, MO) was added in eppendorfes (1.5 ml) the day before sample collection to prevent microbial growth; during samples collection milk was immediately transferred at -20 C° and after stored at -80 C° in the Laboratory of the Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE) of the University of Padova (Legnaro, Padova, Italy) since the RP-HPLC analysis was performed

Milk quality trait analyses and protein fractions detection by RP-HPLC

Individual milk samples were analyzed for fat, protein, casein contents using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count values were obtained from the Fossomatic FC counter (Foss, Hillerød, Denmark) and were then converted to SCS by means of logarithm transformation (Ali and Shook, 1980).

Contents of α_{S1} -CN, α_{S2} -CN, β -CN, κ -CN, β -LG, α -LA and LF of individual milk samples were measured using the RP-HPLC method proposed by Maurmayr et al. (2013).

Briefly, the method provides to separate the caseins and whey proteins in one run, and to quantify content of A and B variants of κ -CN, content of A1, A2, A3, B, I variants of β -CN, content of A and B variants of β -LG, and content of α -LA and lactoferrin, even so ensuring a rapid analysis (less than 20 minutes) and a good resolution of peaks. Single mother solution of purified proteins of commercial standards were employed to develop specific calibration curves; standards are a mixture of milk protein genetic variants and for this reason single calibration equation were set for every genetic variant. The content of different protein genotypes of each protein fraction were summed to obtain the total content of that fraction. Since α_{S1} -CN and α_{S2} -CN are not available as single proteins, the corresponding values were calculated from the α_S applying the 4:1 proportion known for cow milk (Bonizzi et al., 2009). The γ -CN fraction, consisting of proteolytic products of β -CN, was not detectable with the current method, multiple peaks were visible on the chromatographic run but it was not possible to detect and quantify certainly. For protein quantification by the current RP-HPLC method, total casein content (Casein, g/L) was obtained as the sum of glycosylated and unglycosylated forms of κ -CN, α_S -CN, and β -CN content in milk; total whey proteins content (g/L) was computed as the sum of β -LG, α -LA and Lf content.

Total true protein content (PRT, g/L) was obtained summing up casein and whey proteins contents. The proportion of protein fractions were expressed as percentage on PRT,

calculating κ -CN, glycosylated and unglycosylated forms of κ -CN, α_{s1} -CN, α_{s2} -CN, β -CN, α -LA, β -LG and LF on PRT content.

Statistical analysis

Data were analyzed by the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) according to the following linear model:

$$y_{ijklm} = \mu + \text{HTD}_i + \text{Parity}_j + \text{DIM}_k + \text{Breed}_l + e_{ijklm}$$

where y_{ijkl} is the measure of a trait; μ is the general mean of the model; HTD_i is the fixed effect of herd-test day i ($i = 1, \dots, 7$), Parity_j is the fixed effect of parity j of the cow ($j = 1$: first parity, $j = 2$: second parity, $j = 3$: third and later parities), DIM_k is the fixed effect of DIM class k (5 classes of 60-d interval, with the exception of the last class, which included samples collected at DIM 240 days or greater), Breed_l is the fixed effect of l th breed combination ($l = 1$ to 7) and e_{ijklm} is a random residual assumed to follow a normal distribution with $e_{ijkl} \sim N(0, \sigma_e^2)$, where σ_e^2 is the residual variance. Orthogonal contrasts were estimated between LSM of traits for the effect of DIM: a) linear component; and b) quadratic component, between LSM of traits for the effect of parity: a) first-parity vs. second- and later-parity cows; and b) second-parity vs. third- and later-parity cows, and between LSM of traits for the effect of breed combination: a) the effect of crossbreeding (H vs. all crossbred cows); b) the effect of generation (first-generation vs. second-generation crosses); c) the effect of S sires in the first-generation cross (S×H vs. M×H + B×H); d) the comparison of Alpine breeds sire in the first-generation cross (M×H vs. B×H); e) the comparison of S sires and M sires in the second-generation cross (S×MH vs. M×SH + M×BH); and f) the effect of maternal grand-sire breed in second-generation crosses with M sire (M×SH vs. M×BH).

RESULTS AND DISCUSSION

Milk quality traits and protein fractions amount

Descriptive statistics for the investigated traits are reported in Tab.1

The content of milk protein fractions obtained by RP-HPLC showed differences between the protein content assessed in the national milk recording program.

This could be probably explained because different methodologies and kind of analysis give often different results ; in literature milk proteins has been widely studied considering many investigation approaches, like liquid chromatography (Bobe et al., 1998; Holland et al., 2010) isoelectring focusing (Strange et al., 1992) electrophoretic techniques (Veloso et al., 2002) proteomic approaches (Jensen et al., 2012) and often results are in disagreement; kind of sample preparation, conservation options and type of technique influence attended composition and average of the substances, in this case different protein content amount has to be expected. Moreover protein content detected by RP-HPLC is greater than content and casein index reported in other studies because the method is not able to provide NPN and minor components amount, such as BSA and Ig.

On the other hand, sample conditions seem to play an important role; more favorable conservation of samples after herd collection ensure a good condition of milk for HPLC analysis than milk collected in the milk recording program (Bonfatti et al., 2010).

Milk protein fraction detection

Two variants were detected for κ -CN: A and B variants; variant E coeluted closely with variant A. Five variants were detected for β -CN: B, A1, A2, A3 and a fifth variant called "X" because of its absence in the DNA-genotyped animals pool used for the method validation; for β -Lg, 2 variants were identified: A and B variants; in our population, concerning α S1-CN, the two variants B and C seem to be fixed in the population.

No polymorphism was detected for α -LA; several studies confirmed the presence of genetic variants for this kind of whey protein (Farrell et al., 2004), but in our study any animal carried alleles. No variability was observed for Lf, although further investigations are required for this kind of protein, because of their absence in literature.

Concerning β -Lg, it is important to note that it is associated with κ -CN; particularly, the two genotypes are not linked because they are on separate chromosomes in cattle, but different alleles combinations carry out differences in milk protein composition; especially, the A variant of β -LG increases the proportion of κ -CN and generally has a favorable effect on protein yield (in agreement with Bobe et al., 1999); moreover breeds like pure Holstein have a higher frequency of A allele of κ -CN, and this variant gives a lower content of total casein; on the other hand, the B variant of β -LG increased the proportion of BLG and fat content in total milk (Tsiaras et al., 2005).

Herd test day effect

Estimated effect of herd test day, Dim and parity on milk protein fractions (g/L) are reported in Tab. 3. As expected, HTD was highly significant for every single protein fractions.

Days in milk and Parity effects

All protein fractions, except κ -CN and α S2-CN, seem to be influenced by lactation stage; in fact, at the begin of lactation, the amount of proteins in milk is low, probably because body weight of cows tend to decrease in the first period (90 d), such as protein and fat; this because the body energy changes through lactation, and it have to be noted that the greatest energy mobilization occurred in the first 2 wk of lactation (Koenen et al., 2001; Friggens et al., 2007).

In the second phase, after the peak of lactation and just until the dry period, milk production starts to decrease, but at the same time fat, protein and body weight increase, as result of the energy balance change (Pirlo et al., 2000; Heins et al., 2006).

Protein fractions have the same trend; this is not true for κ -CN and α S2-CN, because both seem to follow more closely the trend of the lactation curve; other works confirm that these two protein fractions are strictly associated because the B variant of κ -CN is associate with a greater relative concentration of α S2-CN and total κ -CN (Heck et al., 2009). Results obtained for protein composition are different, in fact significance has been observed just for caseins and Lf; the 4 casein genes are closely linked and organized in a casein locus located in bovine chromosome 6; this can partially explain the relation existing between the casein fractions on milk protein composition, but it is difficult to determine whether the effect of a casein variant is specific to another casein fraction or is maybe an effect of another casein gene, close linked.

Considering parity effect, *F and P values* for casein fractions are statistically significant, but nor for whey protein fractions and total whey; Reported results in the literature are contradictory; Joudu et al., (2008) found that parity effect on milk protein content was significant on β -LG. Contradictions may have arisen from differences in experimental design, technique employed for milk analysis, number of animals, feeding conditions. In our study casein fractions showed a decrease depending on the parity level; this is in accordance with other studies, as lactation's number influences milk protein content. Pluriparous animals attend a higher milk production, but milk quality get worse, because protein average decreases (Hansen et al., 2006). Results obtained for protein composition (%) are different; significance was observed just for κ -CN, β -CN and β -LG content.

Breed effect

Results for milk protein fractions tested by crossbred effect are shown in Tab. 3. P values were significant ($P < 0.05$) for κ -CN, α -LA and BLG; B variant of κ -CN is associated with a lower relative concentration of α -LA; our results are in agreement with other studies (Heck et al., 2009), but other studies reported no effect on this kind of whey protein (Bobe et al., 1999). As explained before, protein composition changes considering different genotype combinations; animals selected for B variant of κ -CN carry out a more appreciable quality of milk, because B variant increases the total casein content; this is associated with a lower concentration of other proteins, such as α -LA and α S1-CN.

On the other hand, breeds like pure Holstein, which is not selected for these traits, have different proportions of casein and whey proteins, because κ -CN content is lower and consequently average of other proteins, like β -LG and α -LA is higher (Demeter et al., 2010). Results obtained for protein composition (%) are similar, with a statistical difference for TCN and WH too; genetic variants of protein fractions are associated with the protein composition of milk (Heck et al., 2009).

Contrasts for parity, Dim and breed

Contrasts were tested to analyze statistical differences of parity, DIM and crossbred combinations on milk protein fractions. Results of contrasts are reposted in Tab. 5.

Differences for the first type of contrast were significant for all casein and whey protein fractions, except Lf.

Concerning the second type of contrast, just κ -CN showed significant differences ($P < 0.05$).

Linear and quadratic contrasts were applied to analyze differences of DIM. Linear contrast were significant for all protein fractions (g/L), except κ -CN and α -LA, and for protein composition (%), except β -CN. Quadratic contrasts were significant just for κ -CN and lf.

As reported by other works, κ -CN genotype affects milk yield and milk protein composition (Ng-Kwai-Hang et al., 1984); in our study, κ -CN showed a similar trend to those of lactation curve; this is not in agreement with other studies, where α S and κ -CN decreased during lactation, while β -CN increased (Ostensen et al., 1997); contradictions may be explained by the conservation status of milk and health level of cows; proteolysis in milk is very moderate in high producers animals, but especially high in very low yielding cows; probably κ -CN is more resistant to natural proteolysis and generally the nutritional state of animals influence proteolytic activity.

Lf has a similar behavior, such as κ -CN; in literature just a few investigations focused on Lf and its physiological role in dairy cows; results in our study suggested first of all that distribution of Lf is slightly asymmetrical (Leclerq et al., 2009). Moreover our results are in agreement with other studies where a significant correlation between Lf concentration in milk and stage of lactation, fat and protein percentage and SCS was found (Krol et al., 2013; cheng et al., 2009; Arnould et al., 2009), this may suggest that Lf content could be potentially used as mastitis indicator (Harmon et al., 1976).

Contrasts for crossbred combinations are shown in Tab. 6.

Purebred vs crossbred contrast were significant for κ -CN ($P<0.01$), LA ($P<0.05$), Lg ($P<0.01$), total casein ($P<0.05$) and total whey ($P<0.05$). Concerning different crossbred combinations, just Sx(MxH) vs Mx(SxH)+Mx(BxH) was significant for Lg ($P<0.05$), casein ($P<0.05$) and whey ($P<0.05$).

Selective breeding can change milk protein composition to improve milk quality and milk coagulation properties; consequently purebreds like Holstein and Brown Swiss has been widely selected for some traits, like milk production and B variant of κ -CN, respectively.

Several studies focused on the relationship between protein composition, milk production and milk protein variants (Demeter et al., 2010; Ojala et al., 1997; Bobe et al., 1999; Heck et al., 2009). Cheese yield increases because of casein concentration and cheese making properties

like milk coagulation time and curd firmness depend on casein composition (Wedholm et al., 2009; Robitaille et al., 2002).

Moreover in some breeds, like pure Holstein, A and B variants of β -LG and A1 and A2 variants of β -CN occur frequently (Boettcher et al., 2004). A2 variant of β -CN is also associated with higher protein yield (Heck et al., 2009).

Because casein loci are closely linked on bovine chromosome 6, alleles of the different casein are in linkage disequilibrium; moreover, because of this, aggregate casein genotypes should be considered when estimating genotypes effects (Hallen et al., 2008).

In this study, purebreds like pure Holstein and Brown Swiss present different protein fractions amount, above all for κ -CN, α -LA and β -LG. Pure Holstein, not selected for B variant of κ -CN, has higher frequency of AB and AA genotypes for κ -CN; this combination affects whey composition of milk, because A variant of κ -CN is correlated to a lower amount of α -LA; high A variant frequency for β -LG is strongly associated with a greater relative concentration of β -LG and a lesser concentration of the other protein fractions; our results are in accordance with other investigations (McClean et al., 1984; Ng-Kwai-Hang et al., 1986; Lunden et al., 1996; Huang et al., 2012).

Concerning F1 and F2 generations, contrasts were significant just for α -LA; the protein amount is higher in the F2 generation than in F1 generation. This could be explained partially by the different genotypes combinations in crossbred cows and by the additive genetic effect.

Differenced between Sx(MxH) vs Mx(sxH) and Mx(BxH) combinations are significant for β -LG ($P < 0.05$), casein ($P < 0.05$) and whey ($P < 0.05$). A partial explanation of differences between these crossbreeds may be given by the cross-scheme itself; crossbreeding interest has been grown for its potential role in dairy industry, to improve functional and productive traits of animals; several studies reported the heterosis contribution on important traits like milk production, protein and fat percentage (McAllister et al., 1994; McAllister, 2002; Fohrman et al., 1954). Moreover Montbeliarde has naturally high frequency of b variant

of κ -CN (Ikonen et al., 1999); our results suggest that genotypes interactions and crosscombinations between Montbeliarde and Brown Swiss and Holstein influence milk composition, above all whey.

CONCLUSIONS

In conclusion, we herein described a RP-HPLC method able to identify and quantify the most common protein fractions amount of individual milk of different crossbred combinations. This method guarantee a good resolution of peaks, genotypes of animals, content of milk proteins and genetic variants too.

The possibility to analyze the complete milk protein profile just in one chromatographic run gave us a very detailed evaluation of milk characteristics and milk quality traits, such as genotypes and amount of milk protein and genetic variants. Protein composition determines the nutritional value and the technological properties of milk and this indicates that selection for specific variants can be a useful tool in selecting for animals with a desired protein composition.

Our results show that protein fractions and protein composition vary by stage of lactation, general health of the animals and number of lactations, although both traits show different significativity; important factors like cheese yield and cheese properties increases with casein concentration and casein composition; casein and whey amount itself increases or decreases depending on the protein variants attended or not in milk, because a relation exist among their, above all κ -CN seem to be close related to α S-CN, α -LA and β -LG content.

Crossbred combination and generally crossbreeding can be a very useful strategy to improve animal selection, to keep breeds with more appreciable genetic richness, depending on the intended use of milk. Productivity of pure Holstein could be kept if coupled with Alpine European breeds, especially Montbeliarde and Brown Swiss.

Finally, our study showed the relevance of crossbreeding and the importance to improve analytical methodologies to find and better understand the relationship existing among milk protein fractions, composition and genetic variants association on milk quality and cheese making properties.

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REFERENCES

- Arnould V. M.-R., H. Soyeurt, N. Gengler, F. G. Colinet, M. V. Georges, C. Bertozzi, D. Portetelle, and R. Renaville. 2009. Genetic analysis of lactoferrin content in bovine milk. *J. Dairy Sci.* 92:2151-2158.
- Bittante, G., M. Penasa, and A. Cecchinato. 2012. Invited review: Genetics and modeling of milk coagulation properties. *J. Dairy Sci.* 95:6843-6870.
- Bobe, G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1998. Separation and quantification of bovine milk proteins by reversed phase high performance liquid chromatography. *J. Agric. Food Chem.* 46:458-463.
- Bobe G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1999. Effect of milk protein genotypes on milk protein composition and its genetic parameters estimates. *J. Dairy. Sci.* 82:2797-2804.
- Bobe G., A. E. G. Freeman, G. L. Lindberg, and D. C. Beitz. 2004. Milk protein genotypes explain variation of milk protein composition. *Anim. Ind. Report ASL R1901.*
- Boettcher P. J., A. Caroli, A. Stella, S. Chessa, E. Budelli, F. Canavesi, S. Ghiroldi, and G. Pagnacco. 2004. Effects of casein haplotypes on milk production traits in italian Holstein and Brown Swiss Cattle. *J. Dairy Sci.* 87:4311-4317.
- Bonfatti V., G. Di Martino, A. Cecchinato, D. Vicario, and P. Carnier. 2010. Effects of β - κ -casein (CSN2-CSN3) haplotypes and β -lactoglobulin (BLG) genotypes on milk production traits and detailed protein composition of individual milk of Simmental cows. *J. Dairy Sci.* 93:3797-3808.
- Bonizzi I., J. N. Buffoni, and M. Feligini. 2009. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. *J. Chromatogr. A.* 1216:165-168.
- Bryant J. R., N. Lopez-Villalobos, J. E. Pryce, C. W. Holmes, D. L. Johnson, and D. J. Garrick. 2007. Short communication: effect of environment on the expression of breed and heterosis effects for production traits. *J. Dairy Sci.* 90:1548-1553.
- Cecchinato, A., M. Penasa, M. De Marchi, L. Gallo, G. Bittante, and P. Carnier. 2011. Genetic parameters of coagulation properties, milk yield, quality, and acidity estimated using coagulating and noncoagulating milk information in Brown Swiss and Holstein-Friesian cows. *J Dairy Sci.* 94:4214-4219.

- Cheng J. B., J. Q. Wang, D. P. Bu, G. L. Liu, C. G. Zhang, H. Y. Wei, L. Y. Zhou, and J. Z. Wang. Factors affecting the lactoferrin concentration in bovine milk. *J. Dairy Sci.* 91:970-976.
- Dechow C. D., G. W. Rogers, J. B. Cooper, M. I. Phelps, and A. L. Mosholder. 2007. Milk, fat, protein, somatic cell score, and days open among Holstein, Brown Swiss, and their crosses. *J. Dairy Sci.* 90:3542-3549.
- Demeter, R. M., K. Markiewicz, J. A. M. Arendonk, and H. Bovenhuis. 2010. Relationship between milk protein composition, milk protein variants, and cow fertility traits in Dutch Holstein-Friesian cattle. *J. Dairy Sci.* 93:5495-5502.
- Farrell H. M., Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk-sixth revision. *J. Dairy Sci.* 87:1641-1674.
- Fuhrman, R. H., R. E. McDowell, C. A. Matthews, and R. A. Hilder. 1954. A crossbreeding experiment with dairy cattle. *Tech Bull*, 1074. USDA, Washington, DC.
- Friggens N. C., P. Berg, P. Theilgaard, I. R. Korsgaard, K. L. Ingvarsen, P. Løvendahl, and J. Jensen. 2007. Breed and parity effects on energy balance profiles through lactation: evidence of genetically driven body energy change. *J. Dairy Sci.* 90:5291-5305.
- Gallo L., P. Carnier, M. Cassandro, R. Mantovani, I. Bailoni, B. Contiero, and G. Bittante. 1996. Change in body condition score of Holstein cows affected by parity and mature equivalent milk yield. *J. Dairy Sci.* 79:1009-1015.
- Hallèn E., A. Wedholm, A. Andrèn, and A. Lundèn. 2007. Effect of β -casein, κ -casein and β -lactoglobulin genotypes on concentration of milk protein variants. *J. Anim. Breed. Genet.* 125:119-129.
- Hansen, J. V., N. C. Friggens, and S. Højsgaard. 2006. The influence of breed and parity on milk yield, and milk yield acceleration curves. *Livest. Sci.* 104:53-62.
- Harmon, R. J., F. L. Schanbacher, L. C. Ferguson, and K. L. Smith. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and α -lactalbumin during acute experimental and natural coliform mastitis in cows. *Infect. Immun.* 13:533-542.
- Heck, J. M. L., A. Schennink, H. J. F. van Valenberg, H. Bovenhuis, M. H. P. W. Visker, J. A. M. van Arendonk, and A. C. M. van Hooijdonk. 2009. Effects of milk protein variants on the protein composition of bovine milk. *J. Dairy Sci.* 92:1192-1202.
- Heins B. J., L. B. Hansen, and A. J. Seykora. 2006. Production of pure Holstein versus crossbreds of Holstein with Normande, Monbeliarde, and Scandinavian Red. 2006. *J. Dairy Sci.* 89:2799-2804.

- Heins B. J., L. B. Hansen, A. J. Seykora, D. G. Johnson, J. G. Linn, J. E. Romano, and A. R. Hazel. 2008. Crossbreds of Jersey x Holstein compared with pure Holsteins for production, fertility, and body and udder measurements during first lactation. *J. Dairy Sci.* 91:1270-1278.
- Heins B. J., and L. B. Hansen. 2012. Short communication: fertility, somatic cell score, and production of Normande x Holstein, Montbéliarde x Holstein, and Scandinavian Red x Holstein crossbreds versus pure Holsteins during their first 5 lactations. *J. Dairy Sci.* 95:918-924.
- Holland B., S. Rahimi Yadzi, G. Ion Titapiccolo, and M. Corredig. 2010. Short communication: separation and quantification of caseins and casein macropeptides using ion-exchange chromatography. *J. Dairy Sci.* 93:893-900.
- Holt, C., J. A. Carver, H. Ecroyd and D. C. Thorn. 2013. Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *J Dairy Sci.* 96:1-20.
- Huang W., F. Peñagaricano, K. R. Ahmad, J. A. Lucey, K. A. Weigel, and H. Khatib. 2012. Association between milk protein gene variants and protein composition traits in dairy cattle.
- Ikonen T., K. Ahlfors, R. Kempe, M. Ojala, and O. Ruottinen. 1998. Genetic parameters the milk coagulation properties and prevalence of noncoagulating milk in Finnish dairy cows. *J. Dairy. Sci.* 82:205-214.
- Jensen, H. B., J. W. Holland, N. A. Poulsen, and I. B. Larsen. 2012. Milk protein genetic variants and isoforms identified in bovine milk representing extremes in coagulation properties. *J. Dairy Sci.* 95:2891-2903.
- Jõudu I., M. Henno, T. Kaart, T. Püssa, and O. Kärt. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *Int. Dairy J.* 18:964-967.
- Koenen, E. P. C., R. F. Veerkamp, P. Dobbelaar, and G. De Jong. 2001. Genetic analysis of body condition score of lactating Dutch Holstein and red-and-white heifers. *J. Dairy Sci.* 84:1265-1270.
- Król J., A. Brodziak, Z. Litwińczuk, and A. Litwińczuk. 2013. Effect of age and stage of lactation on whey protein content in milk of cows of different breeds. *Pol. J. Vet. Sci.* 16:395-397.

- Leclercq, G., N. Gengler, H. Soyeurt, C. Bastin. 2013. Genetic variability of the mid-infrared prediction of lactoferrin content in milk for Walloon Holstein first-parity cows. *Livest. Sci.* 151:158-162.
- Lopez-Villalobos N., D. J. Garrick, C. W. Holmes, H. T. Blair, and R. J. Spelman. 2000. Effects of selection and crossbreeding strategies on industry profit in the New Zealand dairy industry. *J. Dairy Sci.* 83:164-172.
- Lundèn A., M. Nilsson, and L. Janson. 1997. Marked effect of β -lactoglobulin polymorphism on the ratio of casein to total protein in milk. *J. Dairy Sci.* 80:2996-3005.
- Martin, B., D. Pomies, P. Pradel, I. Verdier-Metz, and B. Remond. 2009. Yield and sensory properties of cheese made with milk from Holstein or Montbeliarde cows milked twice or once daily. *J. Dairy Sci.* 92:4730-4737.
- Maurmayr A., A. Checchinato, L. Grigoletto, and G. Bittante. 2013. Detection and quantification of α S₁-, α S₂-, β -, κ -casein, α -Lactalbumin, β -lactoglobulin and lactoferrin in bovine milk by reverse-phase high-performance liquid chromatography. *Agric. Conspec. Sci.* 78:201-205.
- Mayer H. K. 2005. Milk species identification in cheese varieties using electrophoretic, chromatographic and PCR techniques. *Int. Dairy J.* 15:995-604.
- McAllister, A. J., A. J. Lee, T. R. Batra, and C. Y. Lin. 1994. The influence of additive and nonaddictive gene action on lifetime yields and profitability of dairy cattle. *J. Dairy Sci.* 77:2400-2414.
- McAllister, A. J. 2002. Is crossbreeding the answer to questions of dairy breed utilization?. *J. Dairy Sci.* 85:2352-2357.
- McClean D. M., E. R. Graham, R. W. Ponzoni, and H. A. McKenzie. 1984. Effects of milk protein genetic variants on milk yield and composition. *J. Dairy Res.* 51:531-546.
- Ng-Kwai-Hang K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1984. Association of genetic variants of casein and milk serum proteins with milk, fat, and protein production by dairy cattle. *J. Dairy Sci.* 67:835-840.
- Ng-Kwai-Hang, K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1986. Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *J. Dairy Sci.* 69:22-26.
- Ojala M., R. T. Famula, and J. F. Medrano. 1997. Effects of milk protein genotypes on the variation for milk production traits of Holstein and Jersey cows in California. *J. Dairy Sci.* 80:1776-1996.

- Ostensen S., J. Foldager, and J. E. Hermansen. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *J. Dairy Res.* 64:207-219.
- Pirlo G., F. Miglior, and M. Speroni. 2000. Effect of age at first calving on production traits and on difference between milk yield returns and rearing costs in Italian Holstein. *J. Dairy Sci.* 83:603-608.
- Prendiville R., K. M. Pierce, and F. Buckley. 2010. A comparison between Holstein-Friesian and Jersey dairy cows and their F₁ cross with regard to milk yield, somatic cell score, mastitis, and milking characteristics under grazing conditions. *J. Dairy Sci.* 93:2741-2750.
- Robitaille, G., M. Britten, J. Morisset, and D. Petitclerc. 2002. Quantitative analysis of β -lactoglobulin A and B genetic variants in milk of cows β -lactoglobulin AB throughout lactation. *J. Dairy Res.* 69:651-654.
- Sørensen, M. K., E. Norberg, J. Pedersen, and L. G. Christensen. 2008. Invited review: crossbreeding in dairy cattle: a Danish perspective. *J. Dairy Sci.* 91:4116-4128.
- Strange, E. D., Malin E. L., Van Hekken D. L., Basch J. J. (1992). Chromatographic and electrophoretic methods used for analysis of milk proteins. *J. Chromatogr. A* 624: 81-102.
- Summer, A., P. Franceschi, A. Bollini, P. Formaggioni, F. Tosi, and P. Mariani. 2003. Seasonal variations of milk characteristics and cheesemaking losses on the manufacture of Parmigiano-Reggiano cheese. *Vet. Res. Commun.* 27(Suppl.1):663-666.
- Tsiaras, A. M., G. G. Bargouli, G. Banos, and C. M. Boscus. 2005. Effect of Kappa-Casein and Beta-Lactoglobulin loci on milk production traits and reproductive performance of Holstein cows. *J. Dairy Sci.* 88:327-334
- VanRaden P. M., and A. H. Sanders. 2003. Economic merit of crossbred and purebred US dairy cattle. *J. Dairy Sci.* 86:1036-1044.
- Veloso A. C. A., Teixeira N., Ferreira I. M. P. L. V.O. (2002). Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis. Detection of milk adulterations. *J. Chromatogr. A.* 967:209-218.

Wedholm A., L. B. Larsen, H. Lindmark-Månsson, A. H. Karlsson, and A. Andr en. 2006.
Effect of protein composition on the cheese making properties of milk from individual
dairy cows. *J. Dairy Sci.* 89:3296-3305.

Table 1. Descriptive statistics for protein composition and milk quality traits of individual samples of purebred and crossbred cows (n = 505)¹

Trait ²		Mean	SD	P1 ¹	P99
Caseins ,	g/L	35.13	4.83	24.32	46.73
	%	83.10	2.96	89.89	70.33
κ-CN	g/L	4.49	0.84	2.52	6.62
	%	10.65	1.52	6.94	14.07
α-S ₂ -CN	g/L	3.42	0.81	1.96	5.49
	%	8.06	1.40	5.13	11.54
α-S ₁ -CN	g/L	11.64	1.55	8.00	15.39
	%	27.60	1.91	22.81	32.00
β-CN	g/L	13.09	2.14	7.15	17.36
	%	30.94	2.91	20.67	36.93
Whey proteins	g/L	7.10	1.35	4.24	10.02
	%	16.90	2.97	29.67	10.11
Lf	g/L	0.11	0.06	0.03	0.27
	%	0.26	0.14	0.07	0.63
α-LA	g/L	1.01	0.21	0.39	1.45
	%	2.40	0.45	1.15	3.36
β-Lg	g/L	5.97	1.28	3.37	8.90
	%	14.22	2.93	8.45	23.06
Milk quality traits					
fat	%	4.09	0.86	2.09	6.36
protein	%	3.71	0.30	2.94	4.41
SCS	units	2.56	1.84	-0.47	7.43
Milk yield	kg/d	31.82	9.95	11.78	55.20

¹P1 = first percentile; P99 = 99th percentile.

²Contents of all protein fractions were measured by reversed-phase HPLC on skim milk. TCN = total casein content = sum of total casein fraction; WH = whey protein = sum of total whey fraction

Table 2. Results from ANOVA (*F*-value and significance) for milk protein fraction (g/L) and proportions (%)

Trait		HTD	DIM	Parity	Breed	RMSE ¹	R ²
Caseins	g/L	11.94****	17.16****	17.50****	1.24	4.14	0.29
	%	13.97****	1.26	4.12*	2.74*	2.69	0.20
κ-CN	g/L	8.69****	2.09	18.18****	2.71*	0.76	0.20
	%	9.91****	12.69****	8.23****	3.72**	1.34	0.24
α-S ₂ -CN	g/L	8.76****	1.35	7.63****	0.96	0.76	0.15
	%	6.15****	2.69*	1.00	1.51	1.35	0.10
α-S ₁ -CN	g/L	19.62****	7.83****	12.50****	1.49	1.34	0.27
	%	19.98****	11.56****	0.24	0.71	1.65	0.27
β-CN	g/L	6.34****	13.86****	21.32****	1.32	1.89	0.24
	%	4.57****	0.30	8.04****	1.25	2.80	0.10
Whey proteins	g/L	8.55****	15.05****	0.79	1.89	1.21	0.22
	%	13.97****	1.26	4.12*	2.74*	2.69	0.20
Lf	g/L	5.75****	8.91****	1.16	0.93	0.06	0.14
	%	4.00****	4.66**	0.10	1.05	0.14	0.09
α-LA	g/L	22.12****	3.33*	2.96	3.07**	0.18	0.27
	%	12.06****	1.74	0.97	2.63*	0.42	0.17
β-Lg	g/L	8.27****	13.87****	0.51	2.54*	1.15	0.22
	%	15.32****	1.72	3.92*	3.32**	2.62	0.22

¹RMSE = Root means square error; ²TCN= total casein content = sum of total casein fraction;

³WH = whey protein = sum of total whey fraction;

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

Table 3: Least squares means of milk protein fraction and milk protein composition across parities

Item		Parity			Contrast, P values	
		1	2	≥3	P1 vs P2+≥P3	P2 vs ≥P3
Caseins	g/L	37.07	34.95	33.95	***	ns
	%	83.76	82.99	82.84	**	ns
κ-CN	g/L	4.85	4.35	4.36	***	ns
	%	10.98	10.35	10.7	**	*
α-S2-CN	g/L	3.67	3.41	3.29	***	ns
	%	8.26	8.06	8.03	ns	ns
αS1-CN	g/L	12.12	11.52	11.27	***	ns
	%	27.45	27.44	27.57	ns	ns
β-CN	g/L	13.94	12.83	12.37	***	ns
	%	31.51	30.47	30.11	***	ns
Whey proteins	g/L	7.17	7.1	6.97	ns	ns
	%	16.24	17.01	17.15	**	ns
Lf	g/L	0.12	0.11	0.11	ns	ns
	%	0.27	0.26	0.26	ns	ns
α-LA	g/L	1.05	1.00	1.00	*	ns
	%	2.38	2.4	2.45	ns	ns
β-Lg	g/L	6.01	5.99	5.87	ns	ns
	%	13.59	14.35	14.44	**	ns

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

Table 4: Least squares means of milk protein fraction and milk protein composition across days in milk (DIM)

Item		DIM					Contrast	
		<60 d	60-120 d	121-180 d	181-240 d	>240 d	linear	quadratic
Caseins	g/L	33.00	33.90	35.92	36.69	37.13	***	ns
	%	83.42	83.40	83.34	83.20	82.62	*	ns
κ-CN	g/L	4.41	4.51	4.67	4.61	4.42	ns	**
	%	11.1	11.09	10.85	10.48	9.86	***	*
α-S2-CN	g/L	3.36	3.37	3.48	3.55	3.56	*	ns
	%	8.45	8.24	8.02	8.00	7.87	**	ns
αS1-CN	g/L	11.18	11.27	11.81	11.93	12.03	***	ns
	%	28.33	27.79	27.43	27.06	26.82	***	ns
β-CN	g/L	12.09	12.46	13.36	13.56	13.79	***	ns
	%	30.58	30.61	30.96	30.71	30.62	ns	ns
Whey proteins	g/L	6.53	6.70	7.09	7.33	7.79	***	ns
	%	16.58	16.60	16.66	16.80	17.38	*	ns
Lf	g/L	0.09	0.10	0.13	0.13	0.12	***	***
	%	0.22	0.25	0.29	0.30	0.26	*	**
α-LA	g/L	0.97	1.01	1.03	1.06	1.04	**	ns
	%	2.44	2.48	2.39	2.43	2.33	ns	ns
β-Lg	g/L	5.47	5.59	5.93	6.14	6.64	***	ns
	%	13.92	13.87	13.97	14.08	14.80	*	ns

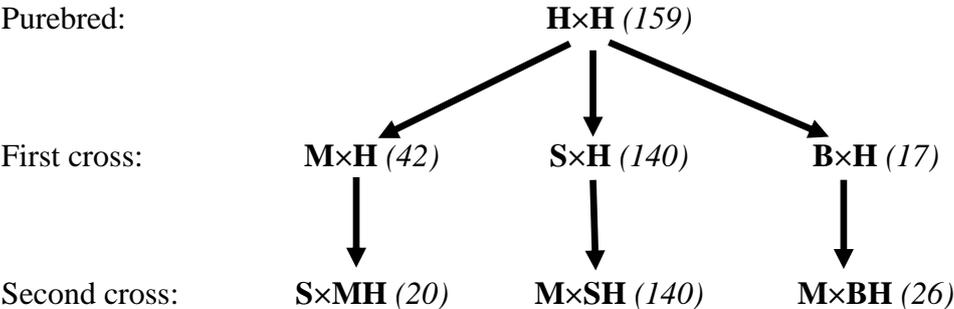
$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 5. Least squares means of milk protein fraction and milk protein composition across breed combinations

Item		Purebreds:	F1 crossbreds:			F2 crossbreds:			Contrasts:					
		H×H	S×H	M×H	B×H	S× (M×H)	M× (S×H)	M× (B×H)	Purebreds vs Crossbreds	F1 vs F2 crossbreds	S×H vs [M×H + B×H]	B×H vs M×H	S×(M×H) vs [M×(S×H)+ M×(B×H)]	M×(S×H) vs M×(B×H)
Caseins	g/L	34.79	35.88	35.87	34.89	36.11	35.33	34.43	ns	ns	ns	ns	ns	ns
	%	82.53	82.98	83.84	82.65	84.50	83.29	82.52	*	ns	ns	ns	*	ns
κ-CN	g/L	4.30	4.53	4.68	4.56	4.72	4.57	4.30	**	ns	ns	ns	ns	ns
	%	10.22	10.48	10.95	10.82	11.10	10.84	10.29	*	ns	ns	ns	ns	ns
αS2-CN	g/L	3.35	3.41	3.68	3.45	3.42	3.47	3.46	ns	ns	ns	ns	ns	ns
	%	7.90	7.88	8.52	8.07	7.99	8.14	8.28	ns	ns	ns	ns	ns	ns
αS1-CN	g/L	11.54	11.93	11.71	11.55	11.89	11.62	11.25	ns	ns	ns	ns	ns	ns
	%	27.46	27.65	27.48	27.49	27.86	27.42	27.00	ns	ns	ns	ns	ns	ns
β-CN	g/L	13.10	13.49	13.04	12.67	13.44	13.03	12.58	ns	ns	ns	ns	ns	ns
	%	31.07	31.17	30.48	29.99	31.39	30.63	30.11	ns	ns	ns	ns	ns	ns
Whey proteins	g/L	7.31	7.33	6.89	7.25	6.58	7.04	7.22	ns	ns	ns	ns	ns	ns
	%	17.46	17.01	16.15	17.34	15.49	16.70	17.47	*	ns	ns	ns	*	ns
Lf	g/L	0.12	0.12	0.11	0.10	0.12	0.11	0.11	ns	ns	ns	ns	ns	ns
	%	0.29	0.26	0.26	0.22	0.28	0.25	0.26	ns	ns	ns	ns	ns	ns
α-LA	g/L	0.98	1.02	1.00	0.97	1.08	1.07	1.02	*	ns	ns	ns	ns	ns
	%	2.32	2.36	2.33	2.32	2.53	2.53	2.48	*	*	ns	ns	ns	ns
β-Lg	g/L	6.22	6.20	5.78	6.18	5.38	5.86	6.09	*	ns	ns	ns	*	ns
	%	14.84	14.38	13.54	14.79	12.68	13.91	14.71	**	ns	ns	ns	*	ns

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

Figure 1. Crossbreeding scheme and, within brackets, number of cows sampled for milk quality, coagulation traits and individual curd firmness modeling analyses (H: Holstein; M: Montbeliarde; S: Swedish Red; B: Brown Swiss).



CHAPTER 3

Effect of breed and dairy system on cow's milk protein fraction and composition

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Effect of breed and dairy system on cow's milk protein fraction and composition

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ABSTRACT

The aim of this study was to investigate the effect of some environmental source of variation and of different dairy systems on the proportion and amount of the most common milk protein fractions, included some minor components like lactoferrin. A total of 783 individual milk samples were collected during evening milking in 26 dairy herds located northern Italy, in the Trento Province. For every farm two breeds were attended at least; six breeds were employed, three high-production and three dual-purpose: Holstein-Friesian, Brown Swiss, Jersey and Gray Alpine, Simmental and Rendena, respectively. Four types of dairy herd were considered in this study: modern herd type, traditional herd type with silage use, traditional herd type without grazing use and traditional original herd type. Milk samples were analyzed by reverse-phase high performance liquid chromatography and protein fractions amount (g/L) and proportion (% of total true protein) were obtained. Traits were analyzed by using a linear mixed model including the fixed effects of herd-test-day, herd type, herd-test-day within herd type, parity, days in milk, breed and milk yield. The traits considered in this work showed generally normal distribution, with the exception of lactoferrin which showed an asymmetrical distribution.

Within herd type affected all the tested milk protein traits; Parity was a major source of variation for all studied traits, particularly for casein content. κ -CN, α S₁-CN and β -CN, Lf and β -Lg contents, expressed as g/L, were the most influenced traits concerning stage of lactation, which although exerted a smaller effect than parity; Milk yield was less important than DIM and parity, but

nevertheless affected most of the studied traits, especially the casein content; concerning breed effect, Jersey cows showed the highest total casein and whey protein amount, and Holstein-Friesian attended the worst; in Gray Alpine a new genetic κ -CN variant was detected using our reverse phase HPLC method, but not identified, so further investigation is required.

Traditional dairy herd type with silage use was the best strategy with the highest milk protein content, although it showed the highest somatic cell content; modern herd type gave unexpectedly inferior performances and traditional herd without grazing was instead the worse concerning milk protein content.

Key words: casein fractions, whey proteins, lactoferrin, multi-breed herd, dairy system

INTRODUCTION

Dairy industry plays a very important role in Italy; more than 70 % of the whole milk production is delivered to the cheese making industry, and about the 80 % of that is used for typical italian products, or high-quality cheeses, including Protected Designation of Origin Products, such as Parmigiano-Reggiano and Grana Padano (Cassandro et al., 2003; De Marchi et al., 2008). Because of this, dairy milk is strictly associated with quality, because milk required to have great capacity to coagulate and to develop a good syneresis. Bovine milk is typically made of 87% water, 3,9% fat, 3,25% protein, and 5,5% of other molecules. Concerning milk proteins, Casein is the most important protein as for quantitative and nutritional aspects. The major proteins belonging to this group are usually referred to as α_{s1} -, α_{s2} -, β - and κ -fractions (Bonizzi et al., 2009, Farrell et al., 2004). Whey proteins are described as the group of milk protein that remain soluble in serum or whey after precipitation of milk casein at pH 4.6; β -Lg, α -LA, serum albumin, Ig, Lf, and proteose-peptone fractions have been considered the major characterized components of this fraction (Ng-Kwai-Hang et al., 2003). κ -casein and β -lactoglobulin showed to have significant effect on milk protein composition; generally the B allele of κ -casein is associated with a higher CN number, higher κ -casein concentration, and moreover higher curd firmness. The B variant of β -Lg seems to

be expressed at a lower level, compared with the A variant (Hallen et al., 2007). α -LA interacts to form the lactose synthase complex; Ig group are attended in colostrums, and give immunity; Lf, specific iron-binding proteins varies and increases in response to inflammation or infection. Polymorphism has been observed for all milk proteins and play a key role in the technological properties and quality of milk. Genotypes of milk proteins, particularly of κ -CN and β -Lg, were found to affect milk composition.

As strategies employed to improve animal value and to alter yield and composition of milk, selection and breeding programs were focused for a long time to increase productivity of animals; as consequence of this, genetic variability decreased and other functional traits, like longevity, fertility and productive life became worse (Lopez-Villalobos et al., 2000; Van Raden et al., 2003). Structure of dairy herds changed rapidly in the last decades, with a progressive concentration and specialization of animals: Holstein were imported from USA into Europe, dairy systems employed large average herd sizes, use of specialized breeds, and feed diet with high energy density, use of artificial insemination, but as consequence, management practices and animal health get worse, because product of daily energy output and duration of lactation increased, with long-term problems. The relationship existing among milk performances and health showed an unfavorable genetic correlation between milk yield and incidence of metabolic stress, ketosis, ovarian cyst, mastitis and lameness, in addition to fertility and longevity reduction and inbreeding depression (Lopez et al., 2004; Adamec et al., 2006).

Today general attention, especially in Italy, get more on milk quality and consequently on the protection of local purebreds, strong linked to the environmental preservation, local tradition and tourism, and historical tradition conservation (Corazzin et al., 2009). Sustainability of livestock farms system aims to protect and guarantee rural development; this kind of strategy has to consider farm diversification, such as resources, species and production's orientation, and some concepts such as animal welfare, biodiversity, food safety and quality, economic value (Sturaro et al., 2013).

For this reason, in the northern Italian mountain areas, milk production is focused on small farms which use pastures or grazing in summer season, and local breeds, such as Simmental, Alpine Brown, Rendena, Gray Alpine, characterized by lower milk yield and production, but better functional traits and general health status. Nevertheless, in some areas like Trento region, herds tried to get more specialized, replacing Alpine Brown with Brown Swiss and introducing other breeds like Holstein-Friesian (De Marchi et al., 2007).

Also local breeds are very useful to give information about genetic variability of dairy traits, environmental interaction, land use and farm management. Several studies focused on the importance of Italian Alpine dairy farms, above all considering milk and cheeses produced during highland grazing (Pretolani and Raffaelli, 2007; Penati et al., 2011); moreover consumers perceived differences in the sensory properties of milk and cheeses, such as taste, flavor, texture, because of the action of substances contained in some types of grass, silage and forages, like α -tocopherol, carotenoids and terpenes (Leiber et al., 2005; Bovolenta et al., 2009; Kalač et al., 2011).

On the other hand, a few studies focused on milk protein fractions and composition of local breeds (Bonfatti et al., 2010), but on our knowledge no works are available concerning these kind of traits in multi-breeds farms. So the aim of this study was to analyze individual raw milk samples of three specialized purebred cows, Holstein-Friesian, Jersey, and Brown Swiss, and of three local dual-purpose breeds, Rendena, Simmental and Gray Alpine, reared in multibreeds herds, estimating effect of herd test day, herd type, stage of lactation, parity, breed and milk yield on milk protein fractions and milk protein composition.

MATERIALS AND METHODS

Animals and milk sampling

Individual milk samples of pure Holstein-Friesian (HF, n = 180), Jersey (J, n = 47), Brown Swiss (BS, n = 269), Rendena (R, n = 103), Simmental (SI, n = 99) and Gray Alpine (GA, n = 75), reared in 26 herds located in the north of Italy (Trento and Bolzano Province) were collected from March to July 2013. Milk sampling occurred once per animal, during the evening milking. Herds considered were classified on the basis of kind of management, defining four different herd dairy systems: a) Modern (intensive herd with use of unifeed and free stall of animals; b) traditional with silage (traditional herds with silage supplementation, tied animals and alpine grazing during summer period); c) traditional no grazing (traditional herds without silage supplementation and without grazing period, tied animals) and d) traditional original (traditional herds, traditional feeding without silage, grazing period during summer, tied animals).

Preseervative (Bronopol, 0.6:100 v/v, Sigma-Aldrich, St. Louis, MO) was added in eppendorfes (1.5 ml) the day before sample collection to prevent microbial growth; during samples collection milk was immediately transferred at -20 C° and after stored at -80 C° in the Laboratory of the Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE) of the University of Padova (Legnaro, Padova, Italy) since the RP-HPLC analysis was performed. Individual milk samples were analyzed by RP-HPLC for protein fractions amount and composition; fat, protein, casein percentages were obtained using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count values were obtained from the Fossomatic FC counter (Foss, Hillerød, Denmark) and were then converted to SCS according to the formula proposed by Ali and Shook (1980): $SCS = 3 + \log_2(SCC/100,000)$. Milk pH values were recorded using a Crison Basic 25 electrode (Crison Instruments SA, Barcelona, Spain).

Protein content detection

Contents of α_{S1} -CN, α_{S2} -CN, β -CN, κ -CN, β -LG, α -LA and LF of individual milk samples were measured using the RP-HPLC method proposed by Maurmayr et al. (2013). Briefly, the method provides to separate the CN and whey protein fractions in one run, and to quantify content of A and B variants of κ -CN, content of A1, A2, A3, B, I variants of β -CN, content of A and B variants of β -LG, and content of α -LA and lactoferrin, even so ensuring a rapid analysis (less than 20 minutes) and a good resolution of peaks.

Single mother solution of purified proteins of commercial standards were employed to develop specific calibration curves; standards are a mixture of milk protein genetic variants and for this reason single calibration equation were set for every genetic variant. This method also was able to identify protein genetic variants and quantify protein fraction content using individual milk samples. Detailed description of method validation can be found in Maurmayr et al., 2013.

The RP-HPLC method was able to separate enough alleles of α_{S1} -CN fraction, allele B and C respectively; in the current analysis it was not found an animal carrying α_{S1} -CN A or α_{S1} -CN D. Since α_{S1} -CN and α_{S2} -CN are not available as single proteins, the corresponding values were calculated from the α_S applying the 4:1 proportion known for cow milk (Bonizzi et al., 2009). Concerning κ -CN, the rare E variant seems to elute very close with A variant, but it was not possible to discern certainly both variants, because no animal was found in the DNA-genotyped animal dataset carrying the E variant because of its rarity, so the peak remained unnamed.

The γ -CN fraction, consisting of proteolytic products of β -CN, was not detectable with the current method, multiple peaks were visible on the chromatographic run but it was not possible to detect and quantify certainly.

The CN content of samples as a proportion of total protein content in this study was greater than the casein index reported by other studies because non-protein nitrogen, proteose-peptones and minor constituents of whey protein cannot be quantified with this method.

Statistical Analysis

Data editing aimed to discard records with sampling or recording errors, such as protein fractions and protein contents beyond the range of mean \pm 3 standard deviation units. At the end of the editing process, 776 records were employed for the statistical analysis.

Milk protein fractions and relative proportions were analyzed using the MIXED procedure of SAS (SAS Institute, 2008) with the following linear model:

$$y_{ijklmno} = \mu + \text{herd type}_i + \text{HTD}(\text{herd type})_{i;j} + \text{Parity}_k + \text{DIM}_l + \text{breed}_m + \text{MY}_n + e_{ijklmno}$$

where $y_{ijklmno}$ is the dependent variable; μ is the general mean of the model; herd type_i is the fixed effect of herd type described above i ($i = 1, \dots, 4$), $\text{HTD}(\text{herd type})_{i;j}$ is the random effect of herd test day within the herd type ($j = 1, \dots, 26$), Parity_k is the fixed effect of parity k of the cow ($k = 1$: first parity, $k = 2$: second parity, $k = 3$: third and $k = 4$: fourth parity), DIM_l is the fixed effect of days in milk class k (10 classes of 60-d interval, with the exception of the last class, which included samples collected at DIM 600 days or greater), BREED_m is the fixed effect of breed ($l = 1, \dots, 6$) and $e_{ijklmno}$ is a random residual.

Orthogonal contrasts ($P < 0.05$) were estimated between LSM of traits for the effect of breed: a) specialized purebreds vs. local dual purpose purebreds; b) Holstein-Friesian and Brown Swiss vs. Jersey; c) Holstein-Friesian vs. Brown Swiss; d) Simmental vs. Rendena and Gray Alpine; e) Rendena vs. Gray Alpine, and between LSM of traits for the effect of herd type: a) modern vs. traditional herd types; b) traditional with silage vs. traditional without grazing and traditional original; c) traditional without grazing vs. traditional original.

RESULTS AND DISCUSSION

Protein fraction amount and proportion

Descriptive statistics for the investigated traits are summarized in Tab. 1. On average, cows produced 20.6 kg/d of milk containing 3.88% fat and 3.54% protein. The mean values of SCS were 2.93. The content of milk protein fractions obtained by RP-HPLC showed differences between the protein content assessed in the national milk recording program. This could be probably explained because kind of sample preparation, conservation options and type of technique influence attended composition and average of the substances, in this case different protein content amount has to be expected. Moreover protein content detected by RP-HPLC is greater than content and casein index reported in other studies because the method is not able to provide NPN and minor components amount, such as BSA and Ig. On the other hand, sample conditions seem to play an important role; more favorable conservation of samples after herd collection ensure a good condition of milk for HPLC analysis than milk collected in the milk recording program (Bonfatti et al., 2010).

Correlation between milk protein fraction detected by RP-HPLC and those obtained with MilkoScan FT6000 of the laboratory was 0.77 for casein and 0.76 for total protein (data not shown).

Breed and Herd frequencies

Frequencies are reported in Tab. 2. The most represented breed in the multi-breed herds sampled was the Brown Swiss with a total of 269 animals (34.80%); on the other hand Jersey frequency was the lowest, 47 animals (6.08%). Concerning the dairy systems, the traditional original herd attended 325 milking cows, the highest number of animals comparing with the other dairy systems; instead traditional herd with use of silage attended 79 milking cows, the lowest number of animals.

Sources of variation in milk protein traits

Results of ANOVA concerning protein fractions and proportions are reported in Tab. 3. HTD within herd type affected all the tested milk protein traits (except for the κ -CN proportion expressed as %); the dairy system exerted a smaller effect than HTD, above all on casein content amount, especially for κ -CN and β -CN, but not on the whey protein amount. It is important however to note that in our case effect of dairy system and collection day is confounded.

Parity was a major source of variation for all studied traits, particularly for casein content. Stage of lactation exerted a smaller effect than parity; κ -CN, α S₁-CN and β -CN, Lf and β -Lg contents, expressed as g/L, were the most influenced traits (except for α -La and α S₂-CN content). Stage of lactation plays an important role on milk composition, because the body energy changes and requirements of the animal get to be different; body weight of cows tend to decrease in the first period (90 d), such as protein and fat, also the greatest energy mobilization occurred in the first 2 week of lactation (Koenen et al., 2001; Friggens et al., 2007). In the second phase, after the peak of lactation and just until the dry period, milk production starts to decrease, but at the same time fat, protein and body weight increase, as result of the energy balance change (Pirlo et al., 2000; Heins et al., 2006). Protein fractions have the same trend, but κ -CN especially seem to follow more closely the trend of the lactation curve (Bobe et al., 1998). Considering parity effect, in fact casein and whey protein fractions showed a decrease depending on the parity level; this is in accordance with other studies, as lactation's number influences milk protein content. Pluriparous animals attend milk quality which get worse, because protein average decreases (Hansen et al., 2006).

Milk yield was less important than DIM and parity, but nevertheless affected most of the studied traits, especially the casein content, that decreases when compared with higher milk production. Milk yield affected milk composition and it depends on many factors, like animal selection and breeding programs, but also health status of the cow and feeding systems (Friggens et al., 1995; Veritè and Delaby, 2000; Brun-Lafleur et al., 2010; Dekleva et al., 2012).

Breed effect was highly significant ($P < 0.001$) for all the investigated traits, with the exception of αS_1 -CN and αS_2 -CN proportion (expressed as %).

Breed Effect for milk Protein Fraction and Composition

Least square means and significance values for the contrasts among different breeds in term of milk protein fractions (g/L) and proportion (%) are reported in Table 4.

On average, **J** cows showed the highest total casein and whey protein amount, 44.06 and 7.11 g/L respectively, comparing with the other breeds, and on the other hand **HF** attended the worst, 33.42 and 6.30 g/L respectively. **BS** cows showed a good protein level, 38.26 g/L concerning casein content and 6.92 g/L concerning whey content; as dual-purpose breeds, **GA** and **R** showed similar protein content.

Breed combinations showed that, for casein content, all types of comparison were highly significant ($P < 0.001$), with the exception of **GA** vs **R**. Concerning Whey protein content, all kind of combinations were highly significant ($P < 0.001$), except for first and second breed combination ($P < 0.05$). Moreover differences were highly significant ($P < 0.001$) concerning κ -CN, αS_1 CN, α -LA and β -Lg considering the different breed combinations.

Results could be partially explained by proportion of different protein and differences in the frequency of their genetic variants; several studies considered the relationship that exists among these kind of traits; the casein genes are closely linked and organized in a casein locus located in bovine chromosome 6, but it is difficult to determine whether the effect of a casein variant is specific to another casein fraction or is maybe an effect of another casein gene, close linked. Heck et al., 2009 found that B variant of κ -CN is associate with a greater relative concentration of total κ -CN and α -LA, but other studies are in disagreement because no effect have been reported (Bobe et al., 1999); breeds which are selected for B variant of κ -CN, like **BS**, attended a more appreciable milk quality, because of the highest casein content given by this genetic variant. On the other hand, **HF** is not selected for B variant of κ -CN, and consequently this breed showed higher frequency of

AB and AA genotypes for κ -CN; this combination affects whey composition of milk, because A variant of κ -CN is correlated to a lower total casein and α -LA amount, in accordance with other investigations (McClean et al., 1984; Ng-Kwai-Hang et al., 1986; Lunden et al., 1996; Huang et al., 2012).

J breed has naturally greater casein content, if compared with other breeds, and this characteristic could partially explain the differences between **HF** and **BS**.

Concerning dual-purpose breed combinations, literature reported that **SI** seem to exhibit more equilibrated allelic frequencies at milk protein genes (Bonfatti et al., 2010). This however give not significant variation if compared with **GA** and **R**. Considering κ -CN content, **GA** showed a higher amount of this kind of protein fraction than **R**, 4.44 and 4.13 g/L respectively. Differences are not so high, although **GA** attended a higher frequency of B variant of κ -CN than **R** (data not shown). Moreover high significance ($P < 0.001$) was observed for β -Lg content and whey protein amount; this is probably explained by the genotype frequencies attended; in fact **R** breed showed a higher frequency of B variant of β -Lg, **GA** breed instead showed a higher frequency of A variant of β -Lg. In literature is reported that concerning β -Lg, A variant is strongly associated with a greater relative concentration of β -Lg and a lesser concentration of the other major milk proteins (Lum et al., 1997; Heck et al., 2009); obviously higher β -Lg concentration carries out a higher total whey amount.

Concerning LF, on our knowledge, this is the first study in dairy science which employed a RP-HPLC method able to detect Lf with all the other milk protein fractions, in one chromatographic run.

It has been well established that iron is an essential nutrient for the growth of a lot of bacteria species and often bacteria are exposed to iron-limited conditions (Jenssen et al., 2009); in response of this, many bacteria synthesize and secrete phenolate and siderophores, which have a greater affinity for iron than lactoferrin or transferrin. More recently, it has become that some of the antimicrobial properties of lactoferrin are independent of iron-binding (Farnauld et al., 2003).

Susceptibility of bacteria was found to be dependent on their growth phase, especially the early log phase, because lactoferrin N-terminal region interact reversibly with porins of bacteria membrane or other direct interaction of the protein or its fragment with ions target (Naidu et al., 1993; Ellass-Rochard et al., 1995). In our work no differences were observed for Lf content between breeds combinations; in literature just a few investigations focused on Lf and its physiological role in dairy cows; a correlation between Lf concentration in milk and stage of lactation, fat and protein percentage and SCS was found (Krol et al., 2013; Cheng et al., 2009; Arnould et al., 2009), this may suggest that Lf content could be potentially used as mastitis indicator (Harmon et al., 1976; Farnauld and Evans, 2003). In our work just Lf amount (g/L) was significantly different between Holstein-Friesian and Brown Swiss ($P < 0.01$).

In our work, Holstein Friesian showed a higher percentage of Lf proportion and also a higher SCS than Brown Swiss (data not shown); we can also argue that animals with a high concentration of somatic cells have an increasing of Lf concentration, also Lf is positively correlated with SCS and more generally with the health status of the animals and seems to modulate the inflammatory process (Soyeurt et al., 2007); this aspect is in agreement with other works (Hagiwara et al., 2003; Chaneton et al., 2008 and 2011; Simijoki et al., 2010).

Dairy System Effect for milk Protein Fractions and Composition

Least square means and significance values for the contrasts among different dairy systems in term of milk protein fractions (g/L) and proportion (%) are reported in Table 5.

On average, traditional herd with silage use showed the highest total casein and whey protein amount, 40.52 and 7.02 g/L respectively, comparing with the other dairy systems, and on the other hand traditional herd without grazing attended the worst, 34.85 and 6.22 g/L respectively. Traditional original herd showed a good protein level, 37.70 g/L concerning casein content and 6.81 g/L concerning whey content; on the other hand modern herd showed a lower protein fraction amount and proportion than the traditional original herd.

Combinations among all dairy systems that showed high significance ($P < 0.001$) were those for the total casein content, the αS_1 -CN and the β -CN fraction amount; no significant differences were observed for αS_2 -CN, αS_1 -CN and β -LG proportions. Significance was found for whey protein content too.

Several works considered environmental interactions on productive performances of dairy cows. Nutritional management factors affecting nutritional intake are associated with **G x E** interactions; as consequence, less feed consuming or low energy diet may affect the expression of the production potential of dairy cows (Kennedy et al., 2002; Horan et al., 2005; Dekleva et al., 2012). Moreover milk production is greatly determined by dry matter intake and by the efficiency digestion of the diet; purebred like Holstein Friesian, selected for milk production since a long time, are well managed in farm systems where quantity and quality of nutrient offered can be controlled, and adjustment of the diet for cow's requirements are common practice to optimize milk production (Bargo et al., 2003; Mattiauda et al., 2013). It has to consider that increased productivity of such breeds is negative correlated with health traits and fitness; high-production breeds were also less selected for robustness and feed intake than local low-production breeds (McCharty et al., 2007; De Marchi et al., 2007).

In our work, is important to note that the best kind of dairy system strategy seem to be the traditional herd with silage use, comparing with the others dairy systems. As explained before, feed intake and ingestion capability are strictly related to animal requirements and to the morphological and physiological characteristics, and also milk production is associated with feed quality and composition. Rotational grazing during the summer period could be an economical way of feeding dairy cows, but often it is difficult to sustain maximum milk yield with high producing cows (Wu et al., 2001). Silage supplementation could be a good strategy for supporting milk production at pasture, increasing the genetic potential of breeding animals (Bovolenta et al., 2009). For these reasons, this kind of herd type gave the best performance considering milk protein fraction amount and milk protein proportion, in fact animals can better integrate their nutritional requirements and

improve the energy balance; milk composition depends also from protein attended in diet, so greater protein yield may be correlated with protein supplementation of silage; our results are in accordance also with other works (Burgess et al., 1984; Kokkonen et al., 2000). Surprisingly, Lf content was greater for this kind of herd and contrasts between the traditional original herd and the traditional herd without grazing showed high significance ($P < 0.001$); unexpectedly traditional herds with silage use presented the best protein average content in milk, but also the highest Lf amount and SCS too, possible indicator of inflammatory processes and mastitis; our results could be partially explained probably because overfeeding with protein in the diet, in this case silage supplementation, affects milk protein level and health status of the animals, with and increasing of infections susceptibility (Frank and Swensson, 2002).

On the other hand, traditional herd without any kind of feed supplementation and nutritional help for cow's requirements seem to be the worst kind of strategy in mountain area system, probably because cows cannot provide to optimize their energy balance, with a worsening of milk production and milk composition. Surprisingly, modern herd type with unifeed use did not give the best results concerning milk protein fractions.

CONCLUSIONS

In conclusion, we herein described a RP-HPLC method able to identify and quantify the most common protein fractions amount of individual milk of different crossbred combinations. This method guarantee a good resolution of peaks, content of milk proteins and genetic variants, and content of Lf too. The possibility to analyze the complete milk protein profile just in one chromatographic run gave us a very detailed evaluation of milk characteristics and milk quality traits, such as genotypes and amount of milk protein and genetic variants. Protein composition determines the nutritional value and the technological properties of milk and this indicates that selection for specific variants can be a useful tool in selecting for animals with a desired protein composition.

Our study considered multi-breed herds and our results showed that stage of lactation and parity affected milk protein fractions and milk composition; milk yield and dairy system seem to affect casein content, but no significance was found for whey protein content. Concerning breeds and dairy systems combinations, **J** showed the highest milk protein amount, comparing with the other breeds, and significant differences were found within specialized purebred cows, comparing **HF** with **BS**, and within dual-purpose local purebred cows, comparing **GA** with **R**. The most influenced protein fractions were κ -CN, α S-CN, α -LA and β -Lg, and genotype relationships were discussed.

Dairy system combinations gave interesting results; our results suggest that silage use supplementation coupled with grazing during summer can be considered the best management practice for dairy cows in mountain areas, in fact protein average was the highest, comparing with the other herd types. It has to be noted however that Lf content was the highest, including SCS. On the other hand, removing grazing and silage supplementation cannot provide to the best diet strategy and support for the cow's performances.

Environment plays a key role on genotype and on potential of productive performances of animals. Surely further investigations are required to better understand the environmental action on milk protein content and genotypes; therefore, further studies on milk protein profiles and the genetic variants of different protein fractions could better explain the differences found among breed and dairy system groups.

REFERENCES

- Arnould, V. M.-R., H. Soyeurt, N. Gengler, F. G. Colinet, M. V. Georges, C. Bertozzi, D. Portetelle, and R. Renaville. 2009. Genetic analysis of lactoferrin content in bovine milk. *J. Dairy Sci.* 92: 2151-2158.
- Adamec, V., B. G. Cassell, E. P. Smith, and R.E. Pearson. 2006. Effects of inbreeding in the dam on dystocia and stillbirths in US Holsteins. *J. Dairy Sci.* 89:307-314.
- Bargo, F., L. D. Muller, E. S. Kolver, and J. E. Delahoy. 2003. Invited review: production and digestion of supplemented dairy cows on pasture. *J. Dairy Sci.* 86:1-42.
- Bittante, G., M. Penasa, and A. Cecchinato. 2012. Invited review: Genetics and modeling of milk coagulation properties. *J. Dairy Sci.* 95:6843-6870.
- Bobe, G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1998. Separation and quantification of bovine milk proteins by reversed phase high performance liquid chromatography. *J. Agric. Food Chem.* 46:458-463.
- Bonfatti V., G. Di Martino, A. Cecchinato, D. Vicario, and P. Carnier. 2010. Effects of β - κ -casein (CSN2-CSN3) haplotypes and β -lactoglobulin (BLG) genotypes on milk production traits and detailed protein composition of individual milk of Simmental cows. *J. Dairy Sci.* 93:3797-3808
- Bonizzi I., J. N. Buffoni, and M. Feligini. 2009. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. *J. Chromatogr A.* 1216:165-168.
- Bovolenta, S., M. Corazzin, E. Saccà, F. Gasperi, F. Biasioli, and W. Ventura. 2008. Performance and cheese quality of brown cows grazing on mountain pasture fed two different levels of supplementation. *Livest. Sci.* 124:58-65.
- Brun-Lafleur, L., L. Delaby, F. Husson, and P. Faverdin. 2010. Predicting energy x protein interaction on milk yield and milk composition in dairy cows. *J. Dairy Sci.* 93: 4128-4143.
- Burgess, P. L., and J. W. G. Nicholson. 1984. Protein level in grass silage-based total mixed rations for dairy cows in mid-lactation. *Can. J. Anim. Sci.* 64:435-442.
- Cassandro, M. 2003. Status of milk production and market in Italy. *Agricolturae Conspectus Scientificus*. Vol. 68(2), pp. 65-69, ISSN: 1331-7768.

- Cecchinato, A., M. Penasa, M. De Marchi, L. Gallo, G. Bittante, and P. Carnier. 2011. Genetic parameters of coagulation properties, milk yield, quality, and acidity estimated using coagulating and noncoagulating milk information in Brown Swiss and Holstein-Friesian cows. *J Dairy Sci.* 94:4214-4219.
- Chaneton, L., J. M. Pèrez Sáez, and L. E. Bussmann. 2011. Antimicrobial activity of bovine β -Lactoglobulin against mastitis-causing bacteria. *J. Dairy Sci.* 94:138-145.
- Corazzin, M., S. Dovier, E. Bianco, and S. Bovolenta. 2009. Survey on welfare of dairy cow in tie-stalls in mountain area. *Ital. J. Anim. Sci.* 8(Suppl. 2):610-612.
- De Marchi, M., G. Bittante, R. Dal Zotto, C. Dalvit, and M. Cassandro. 2008. Effect of Holstein-Friesian and Brown Swiss Breeds on quality of milk and Cheese. *J. Dairy Sci.* 91:4092-4102.
- De Marchi, M., R. Dal Zotto, M. Cassandro, and G. Bittante. 2007. Milk coagulation ability of five dairy cattle breeds. *J Dairy Sci.* 90:3986-3992.
- Dekleva, M. W., C. D. Dechow, J. M. Daubert, W. S. Liu, G. A. Varga, S. Bauck, and B. W. Woodward. 2012. *Short communication*: interactions of milk, fat, and protein yield genotypes with herd feeding characteristics. *J. Dairy Sci.* 95:1559-1564.
- Elass-Rochard, E., A. Roseanu, D. Legrand, M. Trif, V. Salmon, C. Motas, J. Montreuil, and G. Spik. 1995. Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. *J. Biochem.* 312:1080-1091.
- Farnauld, S., and R. W. Evans. 2003. Lactoferrin – a multifunctional protein with antimicrobial properties. *Mol. Immun.* 40:395-405.
- Farrell H. M., Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk-sixth revision. *J. Dairy Sci.* 87:1641-1674.
- Frank, B., and C. Swensson. 2002. Relationship between content of crude protein in rations for dairy cows and milk yield, concentration of urea in milk and ammonia emissions. *J. Dairy Sci.* 85:1829-1838.

- Friggens N. C., P. Berg, P. Theilgaard, I. R. Korsgaard, K. L. INGvartsen, P. Løvendahl, and J. Jensen. 2007. Breed and parity effects on energy balance profiles through lactation: evidence of genetically driven body energy change. *J. Dairy Sci.* 90:5291-5305.
- Hagiwara, S.-I, K. Kawai, A. Anri, and H. Nagahata. 2003. Lactoferrin concentrations in milk from normal and subclinical mastitis cows. *J. Vet. Med. Sci.* 65:319-323.
- Hallèn E., A. Wedholm, A. Andrèn, and A. Lundèn. 2007. Effect of β -casein, κ -casein and β -lactoglobulin genotypes on concentration of milk protein variants. *J. Anim. Breed. Genet.* 125:119-129
- Hansen, J. V., N. C. Friggens, and S. Højsgaard. 2006. The influence of breed and parity on milk yield, and milk yield acceleration curves. *Livest. Sci.* 104:53-62.
- Harmon, R. J., F. L. Schanbacher, L. C. Ferguson, and K. L. Smith. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and α -lactalbumin during acute experimental and natural coliforms mastitis in cows. *Infect. Immun.* 13:533-542.
- Heck, J. M. L., A. Schennink, H. J. F. van Valenberg, H. Bovenhuis, M. H. P. W. Visker, J. A. M. van Arendonk, and A. C. M. van Hooijdonk. 2009. Effects of milk protein variants on the protein composition of bovine milk. *J. Dairy Sci.* 92:1192-1202.
- Heins B. J., L. B. Hansen, and A. J. Seykora. 2006. Production of pure Holstein versus crossbreds of Holstein with Normande, Monbeliarde, and Scandinavian Red. 2006. *J. Dairy Sci.* 89:2799-2804.
- Horan, B., P. Dillon, P. Faverdin, L. delaby, F. Buckley, and M. Rath. 2005. The interaction of strain of Holstein-Friesian cows and pasture-based feed systems on milk yield, body weight, and body condition score. *J. Dairy Sci.* 88:1231-1343.
- Huang W., F. Peñagaricano, K. R. Ahmad, J. A. Lucey, K. A. Weigel, and H. Khatib. 2012. Association between milk protein gene variants and protein composition traits in dairy cattle.
- Jennsen, H., and R. E. W. Hancock. 2009. Review: antimicrobial properties of lactoferrin. *Biochim.* 91:19-29.
- Jensen, H. B., J. W. Holland, N. A. Poulsen, and I. B. Larsen. 2012. Milk protein genetic variants and isoforms identified in bovine milk representing extremes in coagulation properties. *J. Dairy Sci.* 95:2891-2903.

- Kalač Pavel. 2011. The effects of silage feeding on some sensory and health attributes of cow's milk: a review. *Food Chem.* 125:307-317.
- Kennedy, E., M. McEvoy, J. P. Murphy, and M. O'Donovan. 2009. Effect of restricted access time to pasture on dairy cow milk production, grazing behavior, and dry matter intake. *J. Dairy Sci.* 92:168-176.
- Koenen, E. P. C., R. F. Veerkamp, P. Dobbelaar, and G. De jong. 2001. Genetic analysis of body condition score of lactating dutch Holstein and red-and-white heifers. *J. Dairy Sci.* 84:1265-1270.
- Kokkonen, T., M. Tuori, V. Leivonen, and L. Syrjälä-Qvist. 2000. Effect of silage dry matter content and rapeseed meal supplementation on dairy cows. 1. Milk production and feed utilization. *Anim. Feed Sci. Tech.* 84:213-228.
- Król J., A. Brodziak, Z. Litwińczuk, and A. Litwińczuk. 2013. Effect of age and stage of lactation on whey protein content in milk of cows of different breeds.
- Leiber, F., M. Kreuzer, H. Leuenberger, and H. R. Wettstein. 2006. Contribution of diet type and pasture conditions to the influence of high altitude grazing on intake, performance and composition and renneting properties of the milk of cows. *Anim. Res.* 55:37-53.
- Lopez, H., L. D. Satter, and M. C. Wiltbank. 2004. Relationship between level of milk production and oestrus behavior of lactating dairy cows. *Anim. Repr. Sci.* 81:209-223.
- Lopez-Villalobos N., D. J. Garrick, C. W. Holmes, H. T. Blair, and R. J. Spelman. 2000. Effects of selection and crossbreeding strategies on industry profit in the New Zealand dairy industry. *J. Dairy Sci.* 83:164-172.
- Lum, L. S., P. Dove, and J. F. Medrano. 1997. Polymorphisms of bovine beta-lactoglobulin promoter and differences in the binding affinity of activator protein -2 transcription factor. *J. Dairy Sci.* 80:1389-1397.
- Lundèn A., M. Nilsson, and L. Janson. 1997. Marked effect of β -lactoglobulin polymorphism on the ratio of casein to total protein in milk. *J. Dairy Sci.* 80:2996-3005.
- Mattiauda, D. A., S. Tamminga, M. J. Gibb, P. Soca, O. Bentancur, and P. Chilibroste. 2013. Restricting access time at pasture and time of grazing at location for Holstein dairy cows: ingestive behaviour, dry matter intake and milk production. *Livest. Sci.* 152:53-62.

- Maurmayr A., A. Checchinato, L. Grigoletto, and G. Bittante. 2013. Detection and quantification of α S₁-, α S₂-, β -, κ -casein, α -Lactalbumin, β -lactoglobulin and lactoferrin in bovine milk by reverse-phase high-performance liquid chromatography. *Agric. Conspec. Sci.* 78:201-205.
- McCharty, S., B. Horan, P. Dillon, P. O'Connor, M. Rath, and L. Shalloo. 2007. Economic comparison of divergent strains of Holstein-Friesian cows in various pasture-based production systems. *J. Dairy Sci.* 90:1493-1505.
- Mclean D. M., E. R. Graham, R. W. Ponzoni, and H. A. McKenzie. 1994. Effects of milk protein genetic variants on milk yield and composition. *J. Dairy Res.* 51:531-546.
- Naidu, S. S., U. Svensson, A. R. Kishore, and A. S. Naidu. 1993. Relationship between antibacterial activity and porin binding of lactoferrin in *Escherichia coli* and *Salmonella Typhimurium*. *Antimicrob. Agents Chemother.* 37:240-245.
- Ng-Kwai-Hang K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1984. Association of genetic variants of casein and milk serum proteins with milk, fat, and protein production by dairy cattle. *J. Dairy Sci.* 67:835-840.
- Ng-Kwai-Hang, K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1986. Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *J. Dairy Sci.* 69:22-26.
- Ng-Kwai-Hang, K. F and F. Grosclaude. 2003. Genetic Polymorphism of milk proteins. *Advanced Dairy Chemistry*, Vol 1: Proteins, pp. 739-816. Edited by P. F. Fox and P. L. H. McSweeney. Springer US.
- Penati, C., P. B. M. Beretsen, A. tamburini, A. Sandrucci, and I. J. M. de Boer. 2011. Effect of abandoning highland grazing on nutrient balances and economic performance of Italian Alpine dairy farms. 2011. *Livest. Sci.* 139:142-149.
- Pirlo G., F. Miglior, and M. Speroni. 2000. Effect of age at first calving on production traits and on difference between milk yield returns and rearing costs in Italian Holstein. *J Dairy Sci.* 83:603-608.
- Pretolani, R, and R. Raffaelli. 2007. Differenziali di costo e valorizzazione delle risorse territoriali nella produzione di latte di montagna. In: Folloni, G., Cannata, G., Gorla, G., (Eds.), *Lavorare e vivere in montagna: Svantaggi strutturali e costi aggiuntivi*. Bonomia University Press, pp. 27-58.

- VanRaden P. M., and A. H. Sanders. 2003. Economic merit of crossbred and purebred US dairy cattle. *J. Dairy Sci.* 86:1036-1044
- Simijoki, H., P. Hyvönen, T. Orro, and S. Pyörälä. 2010. High concentration of human lactoferrin in milk of rhLf-transgenic cows relieves signs of bovine experimental *Staphylococcus chromogenes* intramammary infection.
- Soyeurt, H., F. G. Colinet, V. M.-R. Arnould, P. Dardenne, C. Bertozzi, R. Renaville, D. Portetelle, and N. Gengler. 2007. Genetic variability of lactoferrin content estimated by mid-infrared spectrometry in bovine milk. *J. Dairy Sci.* 90:4443-4450.
- Sturaro, E., E. Marchiori, G. Cocca, M. Penasa, M. Ramanzin, and G. Bittante. 2013. Dairy systems in mountainous areas: farm animal biodiversity, milk production and destination, and land use. *Livest. Sci.* 158: 157-168.
- Verité, R., and L. Delaby. 2000. Relation between nutrition, performances and nitrogen excretion in dairy cows. *Ann. Zootech. (Paris)* 49:217-230.
- Wu, Z., V. R. Kanneganti, L. J. Massingill, M. C. Wiltbank, R.P. Walgenbach, and L. D. Satter. 2001. Milk production of fall-calving dairy cows during summer grazing of grass-clover pasture. *J. Dairy Sci.* 84:1166-1173.

Table 1. Descriptive statistics for milk protein fractions composition (% of total protein) and content (g/L) and milk quality traits of individual samples of purebred cows (n = 773)

Trait ²	% of total protein				g/l milk			
	Mean	SD	P11	P99	Mean	SD	P1 ¹	P99
Caseins ,	84.40	2.81	74.88	93.51	36.31	6.58	23.67	53.57
κ-CN	9.93	1.95	3.37	16.24	4.29	1.18	1.29	8.39
α-S ₂ -CN	8.37	1.60	3.70	14.79	3.63	0.99	1.25	6.64
α-S ₁ -CN	27.77	2.40	15.86	34.59	11.98	2.24	6.12	19.22
β-CN	32.15	2.74	17.05	41.12	13.77	2.33	4.77	21.60
Whey proteins	15.59	2.81	6.48	25.11	6.67	1.53	3.41	10.84
Lf	0.22	0.11	0.05	0.85	0.09	0.05	0.02	0.34
α-LA	2.27	0.47	1.04	5.99	0.96	0.18	0.45	1.71
β-Lg	13.09	2.81	4.68	22.65	5.61	1.49	2.06	11.29
Milk quality traits								
Fat					38.8	8.1	14.1	61.5
Protein					35.4	5.0	26.1	49.0
SCS, units					2.93	1.90	-0.64	7.29
Milk yield, kg/d					20.61	4.01	13.20	26.72

¹P1 = first percentile; P99 = 99th percentile.

²Contents of all protein fractions were measured by reversed-phase HPLC on skim milk. Caseins = sum of total casein fraction; Whey proteins = sum of total whey fractions

Tab. 2 Breed and herd type frequencies (n=773)

Item	N	Percentage, %
Breed		
Brown swiss	269	34.80
Holstein-Friesian	180	23.39
Grey Alpine	75	9.70
Jersey	47	6.08
Simmentahl	99	12.81
Rendena	103	13.32
Dairy system		
Modern	204	26.39
traditional with silage	79	10.22
traditional without grazing	165	21.35
traditional original	325	42.04

1 Tab. 3 Results from ANOVA (*F*-value and significance) for milk protein fractions proportion (%) and content (g/L)

Trait	Herd(dairy system)	Dairy system	DIM	Parity	MY	Breed	RMSE	R ²
Milk proteins, %								
Caseins	5.87***	0.22 ns	0.80 ns	6.34***	0.23 ns	11.79***	2.49	0.27
κ-CN	1.08 ns	4.32**	6.02***	2.37ns	2.80*	24.91***	1.64	0.34
α-S ₂ -CN	2.55***	0.29 ns	2.77***	4.16***	1.10 ns	1.40 ns	1.78	0.15
α-S ₁ -CN	2.90***	0.69 ns	0.51 ns	5.61***	2.64*	3.25***	2.65	0.15
β-CN	5.47***	0.67 ns	0.67 ns	4.27***	2.92*	13.75***	2.42	0.31
Whey proteins	5.87***	0.22 ns	0.80 ns	6.34***	0.23 ns	11.79***	2.48	0.27
Lf	4.24***	0.77 ns	1.86*	1.65 ns	0.22 ns	4.91***	0.10	0.22
α-LA	6.47***	0.59 ns	6.58***	0.25 ns	1.84 ns	12.93***	0.39	0.37
β-Lg	4.72***	0.22 ns	1.03 ns	6.75***	0.21 ns	12.39***	2.51	0.26
Milk proteins, g/L								
Caseins	5.34***	4.31**	8.68***	14.58***	3.88***	28.42***	4.71	0.51
κ-CN	1.88***	7.87***	2.59***	3.17*	1.75 ns	45.86***	0.87	0.48
α-S ₂ -CN	2.66***	1.77 ns	1.01 ns	7.52***	2.06 ns	10.40***	0.98	0.28
α-S ₁ -CN	5.93***	2.23 ns	5.72***	12.73***	4.73***	14.35***	2.06	0.41
β-CN	5.77***	3.95*	8.68***	18.61***	10.35***	1.15 ns	1.79	0.44
Whey proteins	4.43***	1.73 ns	4.59***	1.24 ns	1.75 ns	10.91***	1.29	0.32
Lf	4.52***	1.06 ns	3.55***	2.58*	0.63 ns	2.01ns	0.04	0.23
α-LA	7.24***	1.03 ns	1.23 ns	10.18***	0.81 ns	9.76***	0.15	0.34
β-Lg	3.79***	1.70 ns	4.73***	1.45 ns	1.61 ns	11.58***	1.27	0.31

2 ¹RMSE = Root means square error;

3 * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001

4

Tab. 4 Least squares means of milk protein fractions composition (% of total protein) and milk protein fractions content (g/L) across breeds

Item	Breeds							Contrast, <i>P</i> values				
	specialized, S			dual-purpose, DP				S vs DP	Fr+BS vs J	FR vs BS	SI vs GR+R	R vs GR
	BS	F	J	GR	SI	R						
Caseins	%	84.6	84.0	86.2	84.3	83.3	86.3	ns	***	*	***	***
κ-CN	%	10.8	9.0	11.5	10.2	9.0	10.4	***	***	***	***	ns
α-S2-CN	%	8.6	8.1	8.8	8.3	8.1	8.5	ns	ns	ns	ns	ns
αS1-CN	%	21.0	21.0	22.0	20.0	21.1	21.0	ns	ns	*	**	ns
β-CN	%	31.4	32.9	30.7	32.2	32.1	34.5					
Whey proteins	%	15.4	16.0	13.8	15.7	16.7	13.7	ns	***	*	***	***
Lf	%	0.18	0.24	0.19	0.23	0.24	0.23	*	ns	***	ns	ns
α-LA	%	2.1	2.3	2.0	2.5	2.2	2.6	***	***	***	***	ns
β-Lg	%	13.0	13.4	11.6	13.1	14.2	10.9	ns	***	ns	***	***
Caseins	g/L	38.26	33.42	44.06	36.75	35.32	34.33	***	***	***	ns	**
κ-CN	g/L	4.85	3.60	5.85	4.44	3.79	4.13	***	***	***	***	ns
α-S2-CN	g/L	3.87	3.24	4.53	3.61	3.43	3.33	***	***	***	ns	ns
αS1-CN	g/L	12.47	11.20	14.52	11.90	12.13	10.99	***	***	***	*	*
β-CN	g/L	14.17	13.09	15.62	13.98	13.53	13.70	***	***	***	ns	ns
Whey proteins	g/L	6.92	6.30	7.11	6.84	7.03	5.46	*	*	***	***	***
Lf	g/L	0.08	0.09	0.09	0.10	0.10	0.09	ns	ns	ns	ns	ns
α-LA	g/L	0.96	0.89	1.01	1.04	0.92	1.00	*	***	***	***	ns
β-Lg	g/L	5.88	5.31	6.00	5.69	6.00	4.36	***	ns	***	***	***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

¹BS: Brown Swiss; F: Holstein-Friesian; J: Jersey; GR: Gray Alpine; SI: Simmental; R: Rendena

Tab. 5 Least squares means of milk protein fractions composition (%) and milk proteins fractions content (g/L) across dairy systems

Item	Dairy system:				Contrast, <i>P</i> values			
	Modern	Silage	Traditional No silage: No summer grazing Original		Modern vs Traditional	Silage vs No silage	Original vs No grazing	
Caseins	%	84.5	85.3	84.7	84.6	ns	ns	ns
κ-CN	%	9.8	10.6	10.3	9.8	**	*	*
α-S2-CN	%	8.6	8.3	8.3	8.4	ns	ns	ns
αS1-CN	%	28.3	28.0	27.5	27.9	ns	ns	ns
β-CN	%	31.8	32.5	32.7	32.3	***	ns	ns
Whey proteins	%	15.5	14.7	15.3	15.4	ns	ns	ns
Lf	%	0.22	0.24	0.22	0.20	ns	*	*
α-LA	%	2.3	2.1	2.4	2.3	ns	***	ns
β-Lg	%	13.0	12.3	12.7	12.9	ns	ns	ns
Caseins	g/L	35.03	40.52	34.85	37.70	***	***	***
κ-CN	g/L	4.06	5.05	4.24	4.42	***	***	ns
α-S2-CN	g/L	3.58	3.95	3.40	3.75	ns	**	***
αS1-CN	g/L	11.71	13.29	11.35	12.46	***	***	***
β-CN	g/L	13.11	15.32	13.37	14.27	***	***	***
Whey proteins	g/L	6.39	7.02	6.22	6.81	*	**	***
Lf	g/L	0.09	0.11	0.09	0.08	ns	***	ns
α-LA	g/L	0.93	0.99	0.96	1.01	***	ns	***
β-Lg	g/L	5.37	5.91	5.17	5.71	ns	**	***

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

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GENERAL DISCUSSION AND CONCLUSIONS

Milk protein is absolutely of great interest for dairy research and one of the most studied substances in food. However, many biological aspects and characteristics involving milk protein fraction, milk proportion, coagulation ability of milk and their relationship are unknown or unclear yet.

Investigation concerning milk protein fraction and milk composition is particularly difficult, and additional care has to be considered if new researches, protocols and methodological approaches are required or developed. Surely further knowledge is needed about analytical techniques employed in protein identification. No methods exist which guarantee the detection and the quantification of all milk protein fractions and their genetic variants, and often results of different kind of techniques are so different that is impossible to compare them each other; this is an important limit, above all in dairy science where genetic variants, genotypes, allele frequencies and estimation of genetic parameters for animal selection and breeding programs are fundamental aspects of research. Moreover, poor accuracy in genetic variants detection could produce bias and wrong considerations on the extent of their effects. Furthermore, casein genes are closely linked on bovine chromosome 6 and this can partially explain the relation existing between the casein fractions on milk protein composition, but it is difficult to determine whether the effect of a casein variant is specific to another casein fraction or is maybe an effect of another casein gene, and often statistical analysis cannot provide accurate estimation of genetic parameters related to milk protein genotypes and milk composition (Bobe et al., 1999).

Results obtained in this study confirmed that relationship among milk protein fractions, such as caseins and whey proteins, is really complicated and just partially explained; genetic variants could be more or less expressed, and expression of some of them influences concentration of other components. B allele of κ -CN has been widely studied because of its strong relation with cheese making process, but its concentration determines more total casein content and influences α S-CN and whey protein amount, such as α -La and β -lactoglobulin. κ -CN and β -Lg also seem to exert a significant part of the genetic control of α_{s1} -CN in total milk protein, but their effects on the proportion of other proteins in total milk proteins are minor or insignificant (Bobe et al., 2004). Moreover variation of glycosilation of κ -CN, could be more considered in future studies, because its probably influence on milk coagulation properties (Tyriseva et al., 2008).

Today selection goals are casted to improve performances of the animals, ensuring good management practices and health status too. Our results suggest that crossbreeding could be an optimal way to satisfy these requirements; crosses breeds show higher performances compared with pure breeds, although some crosses are better than other. In our study Montbeliarde and Brown Swiss crosses showed high total casein content, because of the high presence of B variant of κ -CN, [Digitare il testo]

and this aspect is an advantage in countries, like Italy, where technological properties of milk are fundamental. On the other hand Swedish Red, and related crosses with the other breeds, showed worse milk protein content, because of the high frequency of A variant of κ -CN, like pure Holstein. Nevertheless more information are needed; effect of κ -CN is known, but β -CN, especially B allele, seems to play an equally role in protein expression and milk casein concentration and its relationship has to be investigated in depth (Di Stasio and Mariani, 2000; Wedholm et al., 2006).

Milk protein fractions showed different trends concerning stage of lactation and parity effect; some milk protein decrease during the first three months of lactation and increase after this period until the dry period, like total protein, fat and body weight, but other proteins like κ -CN follow more closely the milk lactation curve and tend to decrease in the second phase.

Works focusing on Lactoferrin are still few; our RP-HPLC method is, on our knowledge, the first which could provide the quantification of this protein with all the other milk protein fractions; interest about this iron-binding protein has to grow, because many aspects are unknown yet. Firstly Lactoferrin seems to be related to iron sequestration against bacteria; secondary its distribution is asymmetrical, such as somatic cells, and it also could be considered as an indicator of mastitis or more generally inflammation processes in dairy cows. Lactoferrin is poorly influenced by other milk protein fractions and genetic variants, but further investigations are required to better understand its biological role in milk, and its correlation among other molecules, like β -Lg, Ig and BSA (Chaneton et al., 2011).

Environment plays a key role on the genotype expression on dairy traits and their interaction has to become deeper. Consideration of feeding managements are instead limited. Our results suggest that breed and dairy herd system are important effects on milk protein fractions and composition; Jersey breed attends naturally a lower A variant frequency of κ -CN compared with the other breeds, and this aspect carries out more appreciable milk casein and total protein content than other breeds, like Brown Swiss, although selected for the B allele of κ -CN. Our reverse phase HPLC method was able to identify a probably new κ -CN variant (C variant?), which frequency is higher in Gray Alpine. Relationship among this new variant and expression of other caseins or whey proteins and technological properties of milk could be better investigated in the future. Simmental breed showed the worst milk protein content, compared with the other two dual-purpose breeds, and no differences were found among Gray Alpine and Rendena.

Our results suggest that dairy herd system in mountainous areas exerted an important effect on milk protein content and composition. Curiously, modern dairy system cannot provide to the best performances on milk protein, while feed supplementation in traditional herds with silage and summer grazing gave the best results in terms of milk protein amount, although health of animals

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seems to be destabilized. Few studies exist that analyzed nutritional aspects in multi-breeds herds, considering different breeds, which carry different milk production level, and were objective of selection or not in time. Some considerations can be done: nutritional management factors affecting nutrient intake are associated with genotype and environment interaction; less feed offered to cows not capable of consuming or providing a diet with low energy concentration do not allow cows to fully express their production; local breeds should to be safeguarded, because they can optimally adapt to the strong environmental conditions. Genetic potential of large cows under restricted conditions, such as feed intake and low energy of feed, is more severely limited than the genetic potential of smaller cows (Dekleva et al., 2012).

At the experimental level, RP-HPLC is an useful and profitable method to perform this kind of analysis and to obtain good quantification of milk protein amount, although some alleles cannot be found, particularly for β -CN, because of the similar elution time of the genetic variants, and some protein fractions, such as BSA and Ig cannot be detected with this method. To obtain better results and more appreciable information about dairy traits like milk, HPLC method needs time to be optimized, possibly through different sample preparation, or kind of solvents, or column characteristics; HPLC could to be coupled with other techniques too, equally precise, rapid, and easily-automated.

Other questions, such as expression level of milk proteins , environmental effect on the potential expression of the animals, genotype effect on the protein content, remain to be solved.

REFERENCES

- Bobe G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1999. Effect of milk protein genotypes on milk protein composition and its genetic parameters estimates. *J. Dairy. Sci.* 82:2797-2804.
- Bobe G., A. E. G. Freeman, G. L. Lindberg, and D. C. Beitz. 2004. Milk protein genotypes explain variation of milk protein composition. *Anim. Ind. Report ASL R1901*.
- Chaneton, L., J. M. Pèrez Sáez, and L. E. Bussmann. 2011. Antimicrobial activity of bovine β -Lactoglobulin against mastitis-causing bacteria. *J. Dairy Sci.* 94:138-145.
- Di Stasio, L., and P. Mariani. 2000. The role of protein polymorphism in the genetic improvement of milk production. *Zoot. Nutr. Anim.* 26:69-90.
- Dekleva, M. W., C. D. Dechow, J. M. Daubert, W. S. Liu, G. A. Varga, S. Bauck, and B. W. Woodward. 2012. *Short communication*: interactions of milk, fat, and protein yield genotypes with herd feeding characteristics. *J. Dairy Sci.* 95:1559-1564.
- Tyrisevä, A. M., K. Elo, A. Kuusipuro, V. Vilva, I. Jänönen, H. Karjalainen, T. Ikonen, and M. Ojala. 2008. Chromosomal regions underlying noncoagulation of milk in Finnish Ayrshire cows. *Genetics* 180(2), 1211-1220.
- Wedholm A., L. B. Larsen, H. Lindmark-Månsson, A. H. Karlsson, and A. Andrèn. 2006. Effect of protein composition on the cheese making properties of milk from individual dairy cows. *J. Dairy Sci.* 89:3296-3305.

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