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# UDDER HEALTH IN DAIRY CATTLE: ASSOCIATION WITH MILK COMPOSITION, CHEESE-MAKING TRAITS, AND BLOOD SERUM PROTEINS


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*“Science is a voyage of discovery, and*

*beyond each horizon there is another.”*

Francis Hitching, "The Neck of the Giraffe"

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## SUMMARY

The main objective of this PhD thesis was to study the association between udder health [focusing on subclinical cases of bovine mastitis identified by somatic cell count (SCC) and bacteriological analyses] and several milk quality and technological traits related to the cheese-making process, and blood serum proteins, as possible immune response indicators.

To achieve our goal, the work was splitted in 4 chapters. Two datasets were used: for the 1st chapter, milk samples from 1,271 Brown Swiss cows from 85 herds were used. In the subsequent 3 chapters, milk and blood samples were collected from 1,508 dairy specialized and dual-purpose cows of 6 different breeds (Holstein Friesian, Brown Swiss, Jersey, Simmental, Rendena and Grey Alpine) housed in 41 multi-breed herds.

The aim of the 1st chapter was to determine the effects of very low to very high SCC on milk yield, composition, coagulation properties [including traditional milk coagulation properties (MCP) and new curd firming model parameters ($CF_t$)], cheese yield (CY) and recovery of milk nutrients in the curd (REC) at the individual cow level. The objective of the 2nd chapter was to investigate the association between blood serum proteins [i.e., total protein, albumin, globulin and the ratio of albumin-to-globulin (A:G)] and milk SCC. Since several factors should be considered to appropriately interpret serum proteins concentration in blood, we explored the effect of herd productivity (defined according to the average net energy of milk yielded daily by the cows), breed, and individual cow factors (i.e., stage of lactation and parity) on blood traits. In chapters 3 and 4, pathogen-specific information was included in the analysis to gain a better understanding of the specific changes in the traits previously investigated. Subclinical cases of mastitis were confirmed by bacteriological analysis and multiplex-PCR assays. In particular, in the 3rd chapter we investigated the association between pathogen-specific cases of subclinical mastitis and several milk quality and technological traits (i.e., milk yield, composition, detailed protein profile, coagulation properties and

cheese-related traits). Based on the results of the 2nd chapter, the 4th chapter studied the association between pathogen-specific cases of subclinical mastitis and blood serum proteins, that in chapter 2 showed a correlation with SCC in milk.

Results of chapter 1 confirmed the negative effect of high SCC on milk yield, composition, MCP, $CF_t$, CY and REC traits. As somatic cell score (SCS) increased, a linear loss of milk production and variations in milk composition (e.g., casein-to-protein ratio, lactose and pH) were observed. These changes decreased the quality and clotting ability of the processed milk, which showed a slower coagulation time and a weaker curd firmness. This, in turn, affected the cheese processing (as confirmed by reductions in the CY and the recovery of milk nutrients in the curd). Our findings showed nonlinear trends for some milk traits with respect to SCS, highlighting the negative effect of very low SCC on some milk technological traits.

Our 2nd chapter showed that cows in high producing herds had greater serum albumin concentrations. Breed differences in serum protein profile could be associated with individual genetic variation and could also be explained by the different selective breeding programs to which breeds have been subjected. Changes in blood serum proteins were observed throughout the entire lactation and according to the parity order. Linear relationships between blood serum proteins and SCS confirmed the importance of SCC as an indicator of mammary gland inflammation. Moreover, our results highlighted the potential use of blood serum proteins as indicators of immune response of the mammary gland to infections and their analysis represents a possible initial screening test to identify animals which need further clinical investigations. Such non-genetic factors affecting variation in blood serum proteins should also be considered in future genetics/genomics investigations.

Results of the 3rd chapter revealed that compared with normal milk, all culture-positive samples and culture-negative samples with medium to high SCC presented significant

variations in the casein-to-protein ratio and lactose content. Given that no differences were observed comparing milk infected by contagious, environmental and opportunistic pathogens, our findings suggested an effect of inflammation rather than infection. The greatest impairment in milk yield and composition, clotting ability and cheese production was observed for milk samples with the highest SCC (i.e., culture-positive samples where contagious pathogens were recovered, and culture-negative samples with high SCC), revealing a discrepancy between inflammatory status and bacteriological results, and thus confirming the important role of SCC as udder health indicator. Culture-negative samples with high SCC were possibly undergoing a strong inflammatory response and pathogens could not be isolated because engulfed by macrophages.

In the 4$^{th}$ chapter, culture-negative samples with high milk SCC, which we hypothesized to be infected by contagious bacteria engulfed by neutrophils, and milk samples infected by contagious and environmental bacteria were associated with greater globulin content (and lower A:G) in blood. In accordance with the results in chapter 3 for milk traits, variation in blood serum proteins seemed to be associated with inflammation rather than infection, as globulin significantly increased in the blood of cows whose milk samples had the highest SCC, independently from intramammary infection pathogens.

# RIASSUNTO

L’obiettivo principale di questa tesi di dottorato è stato quello di studiare l'associazione tra stato sanitario della mammella [con particolare riferimento a casi subclinici di mastite bovina identificati attraverso conta delle cellule somatiche (SCC) e analisi batteriologica] e una serie di caratteri qualitativi e tecnologici del latte legati al processo di caseificazione, e le proteine del siero, quali possibili indicatori di risposta immunitaria dell’animale.

Per raggiungere tale obiettivo, il lavoro è stato suddiviso in 4 capitoli. Due diversi datasets sono stati utilizzati: nel 1° capitolo sono stati utilizzati campioni di latte raccolti da 1,271 bovine di razza Bruna provenienti da 85 allevamenti. Nei successivi 3 capitoli, i campioni di latte e di sangue sono stati raccolti da 1,508 bovine da latte e a doppia attitudine di 6 diverse razze (Frisona, Bruna, Jersey, Pezzata Rossa, Rendena e Grigio Alpina) provenienti da 41 allevamenti multi-razza.

Nel 1° capitolo di questa tesi sono stati analizzati, a livello individuale, gli effetti di un contenuto variabile di SCC nel latte (da molto basso a molto alto) sulla produzione di latte, la composizione chimica, le proprietà di coagulazione [includendo le proprietà di coagulazione tradizionali (MCP) e nuovi parametri modellizzati di consistenza della cagliata ($CF_t$)], la resa casearia (CY) e il recupero di nutrienti nella cagliata (REC). Lo scopo del 2° capitolo è stato quello di studiare l'associazione tra proteine del siero [proteine totali, albumina, globulina e il rapporto tra albumina e globulina (A:G)] e SCC nel latte. Tuttavia, per interpretare in modo appropriato la concentrazione delle proteine nel sangue, devono essere presi in considerazione diversi fattori. Pertanto, è stato valutato l'effetto del livello produttivo dell’allevamento (definito sulla base dell'energia netta del latte prodotta in media giornalmente dalle bovine), della razza, dello stadio di lattazione e dell’ordine di parto sulle proteine ematiche. Nei capitoli 3 e 4, sono state incluse nelle analisi informazioni a livello patogeno-specifico allo

scopo di acquisire una migliore comprensione dei cambiamenti precedentemente osservati nei caratteri tecnologici e di qualità del latte e nei parametri ematici esaminati. I casi subclinici di mastite sono stati confermati attraverso analisi batteriologica e saggi PCR in multiplex. In particolare, l'obiettivo del 3° capitolo è stato quello di studiare l'associazione tra i casi di mastite subclinica a livello patogeno-specifico e i diversi caratteri qualitativi e tecnologici del latte (produzione, composizione chimica, profilo proteico dettagliato, proprietà di coagulazione e caratteri legati al processo di caseificazione). Sulla base dei risultati ottenuti nel capitolo 2, nel 4° capitolo è stata valutata l'associazione tra i casi di mastite subclinica a livello patogeno-specifico e le proteine del siero, che nel capitolo 2 erano risultate correlate alle SCC nel latte.

I risultati ottenuti nel capitolo 1 hanno confermato l'effetto negativo di un alto contenuto di SCC sulla produzione di latte, la composizione e i caratteri MCP, $CF_t$, CY e REC. All'aumentare del punteggio di cellule somatiche (SCS), sono state osservate una diminuzione lineare della quantità di latte prodotto e alcune variazioni nella composizione (in particolare nel rapporto tra caseina e proteina, nel contenuto di lattosio e nel pH). Questi cambiamenti hanno causato una riduzione della qualità e dell'attitudine casearia del latte trasformato, caratterizzato da una coagulazione più lenta e una ridotta consistenza del coagulo. Di conseguenza, tali variazioni hanno avuto ripercussioni negative sul processo di caseificazione, ovvero ridotta resa in formaggio e minor recupero di nutrienti nella cagliata. I risultati ottenuti hanno inoltre evidenziato andamenti non lineari per alcuni caratteri del latte rispetto ad SCS, mettendo in evidenza l'effetto negativo di un contenuto molto basso di SCC su alcuni caratteri tecnologici del latte.

Nel 2° capitolo è stato dimostrato che le bovine allevate in allevamenti ad alta produttività presentavano una maggiore concentrazione di albumina sierica. Le differenze nel profilo proteico osservate tra le diverse razze potrebbero essere associate alla variazione

genetica individuale e ai diversi programmi di selezione a cui tali razze sono state sottoposte. Variazioni del contenuto di proteine ematiche sono state riportate all'avanzare della lattazione e a seconda dell'ordine di parto. Le relazioni lineari tra proteine del siero e SCS hanno confermato l'importanza delle SCC come indicatore di infiammazione della mammella. I risultati ottenuti hanno evidenziato inoltre il potenziale uso delle proteine del siero come indicatori di risposta immunitaria della ghiandola mammaria alle infezioni e la loro analisi rappresenta un possibile test iniziale di screening per identificare animali che hanno bisogno di ulteriori indagini cliniche. Tali fonti di variazione non-genetiche delle proteine del siero dovrebbero essere prese in considerazione anche in future analisi genetiche e genomiche.

I risultati del 3° capitolo hanno mostrato che, rispetto al latte di bovine sane, tutti i campioni di latte risultati positivi all'esame batteriologico e i campioni che non hanno evidenziato crescita batterica ma con un contenuto medio-alto di SCC presentavano significative variazioni nel rapporto tra caseina e proteina, nonchè nel contenuto di lattosio. Poiché non sono state osservate differenze significative confrontando latte infetto da patogeni contagiosi, ambientali e opportunisti, i risultati ottenuti hanno evidenziato un deterioramento del latte dovuto alla risposta infiammatoria dell'animale piuttosto che all'infezione stessa. Un peggioramento più pronunciato per quanto riguarda produzione e composizione chimica del latte, attitudine alla coagulazione e resa in formaggio è stato osservato per i campioni di latte con il più alto contenuto di SCC (ovvero i campioni infettati da patogeni contagiosi e i campioni risultati negativi all'esame batteriologico ma con un alto contenuto di SCC). Questo ha rivelato una discrepanza tra stato infiammatorio e risultati batteriologici, confermando così il ruolo importante delle SCC quale indicatore dello stato di salute della mammella. É possibile che, nei campioni risultati negativi all'esame batteriologico ma con un alto contenuto di SCC, una risposta infiammatoria intensa abbia impedito l'isolamento degli agenti patogeni in quanto internalizzati dai macrofagi.

Nel capitolo 4, i campioni che non hanno evidenziato crescita batterica ma con un alto contenuto di SCC, che abbiamo ipotizzato essere infettati da batteri contagiosi internalizzati dai neutrofili, e i campioni di latte infettati da batteri contagiosi e ambientali sono risultati associati a un maggior contenuto di globulina (e a un valore inferiore del rapporto A:G) nel sangue. In accordo con i risultati relativi ai caratteri del latte ottenuti nel capitolo 3, la variazione del profilo proteico del sangue sembra essere associata al processo infiammatorio piuttosto che all'infezione. Infatti è stata osservata una concentrazione elevata di globulina nel sangue di bovine il cui latte presentava un contenuto elevato di SCC, indipendentemente dal tipo di patogeno causa di infezione.

# GENERAL INTRODUCTION

Over the last decades, genetic pressure on production traits (e.g., milk yield) in breeding programs (Miglior et al., 2005) has led to an unfavourable decline of cow health, fertility, longevity, and conformation traits (Oltenacu and Broom, 2010). Moreover, negative genetic correlations exist between milk yield and incidence of production diseases (e.g., mastitis, ketosis) (Ingvartsen et al., 2003).

Bovine mastitis is considered one of the costliest disease of dairy herds and has a detrimental impact on farm economics due to: reduced animal welfare, temporary or permanent loss in milk production, reduced milk quality and value, high treatment and labor costs, low longevity and fertility, and premature culling (Seegers et al., 2003; Halasa et al., 2007; Viguier et al., 2009). Halasa and colleagues (2009) estimated for a herd of 100 dairy cows an average annual net cost of mastitis, due to 4 simulated pathogens combined, of about 4,900 €. Therefore, udder health has become a critical production issue for dairy farmers (Schukken et al., 2003).

Mastitis is an inflammation of the mammary gland in response to infection, resulting from the colonization of the gland by pathogenic microorganisms, mostly bacteria, entering through the teat canal (Harmon, 1994; Hogan et al., 1999). It appears in two forms, as clinical and subclinical mastitis. Clinical mastitis is defined as the production of abnormal milk, eventually accompanied by systemic signs of illness, like heat, pain and swelling (Erskine et al., 2003). Cases of clinical mastitis can be easily detected by palpating and visually checking the udder, and by observing foremilk in the stripping before the attachment of the milking unit. Conversely, cows with subclinical mastitis cannot be identified only by visual inspection, as no visible changes in milk and udder appearance occur, hence it represents a hidden treat for healthy animals (Nyman et al., 2014). In both cases, milk from infected cows presents alterations in yield and composition and it is characterized by increased somatic cell

count (**SCC**) (Harmon, 1994). Since the number of cells in milk is associated with the inflammatory status of the mammary gland, SCC is recognized as the international standard indicator of udder health and milk quality (Harmon, 2001). Moreover, it plays a key role in the dairy industry as it affects the price paid for milk, influencing premium payments and penalties applied above predefined thresholds (Duarte et al., 2015). For example, in the European Community, a bulk milk SCC threshold of 400,000 cells/mL has been established for milk destined for human consumption.

In general, milk cellular components have been described for the first time by Prescott and Breed in 1910, referring to a rise in milk "body cells" of mastitic cows as a result of exfoliation of epithelial cells. By the 1960's, the term "body" has been replaced with "somatic". Nowadays, it is well-established that most of the cells found in milk are leukocytes. The presence of cellular components in milk is an important mechanism of defense of the mammary gland. The immune response consists of an innate response, directed at preventing the spread of bacteria, and an adaptive pathogen-specific response (Schukken et al., 2011). The innate defense system includes physical barriers such as the teat sphincter, chemical barriers like keratin of the teat canal and lactoferrin, and also leukocytes. When bacteria colonize the mammary gland and establish an infection, the inflammatory response is triggered, accompanied by the recruitment of white blood cells from the bloodstream. In uninfected glands, SCC is generally low (< 100,000 cells/mL) and macrophages are the predominant cell type. In infected glands, the number of somatic cells increases and the proportion of cell types changes, with neutrophils consisting up to 95% of the total count (Kehrli and Shuster, 1994). A threshold of SCC greater than 200,000 cells/mL is usually considered to identify cows with subclinical infection (Dohoo and Leslie, 1991).

The pathogenesis and mechanisms of the mammary immune response have been extensively described in literature (Burton and Erskine, 2003; Sordillo, 2005; Oviedo-Boyso

et al., 2007). Briefly, once inside the teat cistern, bacteria release toxins and the contact between bacteria and somatic and epithelial cells induces the activation of the innate immune system (Wellnitz and Bruckmaier, 2012). Pathogen recognition, which is the first step of the immune response, is realized through the binding between specific receptors, like the cytokines toll-like receptors (**TLRs**) and CD14, and conserved bacterial molecules called pathogen-associated molecular pattern (**PAMPs**) (Schukken et al., 2011). Toll-like receptors recognize lipopolysaccharide (**LPS**) as PAMP associated with gram-negative bacterial infections and lipothecoic acid (**LTA**) as PAMP associated with gram-positive bacteria (Rainard and Riollet, 2006). At the same time, the resident leukocytes population releases several molecules, like tumor necrosis factor alpha (**TNF-α**) and interleukin 8 (**IL-8**), inducing the synthesis of acute phase proteins (Viguier et al., 2009). After pathogen recognition, bacterial growth is inhibited through the recruitment of leukocytes from the bloodstream to the mammary tissue. Neutrophils release proteases which destroy the bacteria, but also some of the epithelial cells, resulting in decreased milk production. Moreover, several enzymes related to inflammation, like lactate dehydrogenase (**LDH**) and N-acetyl-β-D-glucosaminidase (**NAGase**), are released (Viguier et al., 2009). Neutrophils are eliminated by apoptosis or ingested by macrophages. Dead epithelial cells and leukocytes are secreted into the milk, resulting in high SCC. If the infection persists, the blood-milk barrier is damaged and extracellular components, such as sodium and chloride, might leak and alter the ion concentration in milk (Kitchen, 1981).

Although the increase in milk SCC is commonly used to detect an inflammation of the mammary gland, mastitis can be caused by a wide number of microorganisms and different immune responses have been observed according to the infectious agent (Bannerman et al., 2004; Lahouassa et al., 2007; Petzl et al., 2008). Mastitis-causing bacteria are usually classified as contagious or environmental on the basis of the reservoir and mode of

transmission (Makovec and Ruegg, 2003). Contagious bacteria can be found in infected udders and can be transmitted among cows or among quarters in the same animal by contact with infected milk. This group includes *Streptococcus agalactiae*, *Staphylococcus aureus, Corynebacterium bovis* and *Mycoplasma* spp. Contagious pathogens are mostly responsible for chronic and subclinical infections, with periodic clinical episodes (Harmon, 1994). Environmental bacteria are present in the animal's surroundings (e.g., bedding, soil and manure) and the transmission occurs by teat contamination (Oviedo-Boyso et al., 2007). The major environmental pathogens are gram-negative coliforms, like *Escherichia coli* and *Klebsiella* spp., environmental streptococci, like *Streptococcus dysgalactiae* and *Streptococcus uberis*, and enterococci. It has been reported that approximately 70 to 80% of coliform infections and about 50% of environmental streptococcal infections show clinical symptoms of disease (Harmon, 1994). Coagulase-negative staphylococci (**CNS**) are opportunistic pathogens commonly found in teat skin, which can cause mild subclinical udder inflammation (Schukken et al., 2009b).

Differences in the innate immune response of the mammary gland have been observed comparing 2 of the most prevalent pathogens causing mastitis, *E. coli* and *Staph. aureus* (Wellnitz and Bruckmaier, 2012). More precisely, *E. coli*, that is an environmental gram-negative pathogen mostly associated with short duration acute infections (Oliver et al., 2011), causes a strong reaction of the immune system, with a rapid and intense increase in SCC induced by LPS (Bannerman et al., 2004) and the secretion of several components (e.g., TNF-α, lactoferrin and lysozyme). Instead, *Staph. aureus*, a contagious gram-positive bacterium associated with less severe but long term subclinical and chronic infections (Riollet et al., 2001), induces moderate and delayed SCC increase and a limited cytokine response (Bannerman et al., 2004).

Apart from the negative effect on a cow's health, bovine mastitis has a detrimental effect on raw milk yield and composition and dairy products yield and quality (Auldist and Hubble, 1998; Le Maréchal et al., 2011). The release of bacterial toxins and the secretion of enzymes during the inflammatory response damage the mammary gland tissue, inhibiting the biosynthesis of fat, protein, lactose and thereby decreasing milk production (Kitchen, 1981; Harmon, 1994). In addition, casein content in milk, which is the main component involved in the cheese-making process, decreases as a result of an increased proteinases-mediated degradation (Haenlein et al., 1973; Urech et al., 1999). Furthermore, the changes in the permeability of the blood-milk barrier result in leakage of lactose and minerals (Shuster et al., 1991; Auldist et al., 1995), thus affecting milk pH (Batavani et al., 2007). Alteration in the chemical composition of milk with high SCC, and in particular variation in casein content and milk pH, negatively affects milk clotting ability, curd formation, cheese yield and quality (Grandison and Ford, 1986; Barbano et al., 1991; Klei et al., 1998; Vianna et al., 2008; Summer et al., 2015).

However, even if cheese production represents a major use of milk and an important income for dairy industry, information on the effect of SCC variation on the cheese-making process is still scarce and further studies are required (Le Maréchal et al., 2011). Cheese-making is a complex process with many interrelated factors being involved. Apart from the protein fractions and fat in the milk and the technological traits related to coagulation, more traits, such as the amount of cheese obtained from a given amount of milk, are important and have been recently investigated. For instance, the traditional milk coagulation properties (**MCP**) developed by Annibaldi et al. (1977) present some limitations due to the presence of late-coagulating and noncoagulating milk and the dependence between 2 out of the 3 measured traits [i.e., rennet coagulation time (**RCT**) and the measure of curd firmness after 30 minutes from rennet addition ($\mathbf{a_{30}}$)] (Bittante et al., 2011). To overcome these limitations, the

prolongation of the testing time and the modeling of new curd firmness traits (**$CF_t$**) using all available information have recently been suggested (Bittante et al., 2013). Moreover, although collection and analysis of individual milk samples is expensive and time-consuming in comparison to the use of bulk milk, research at cow level should be carried out. In this way, it would be possible to explore individual sources of variation of cheese-related traits linked with the animal and use this information to possibly include these traits as breeding goals in dairy cows. In this perspective, a model cheese-manufacturing procedure that mimics all phases of cheese production has recently been developed by Cipolat-Gotet et al. (2013) to analyze cheese yield (**CY**) and recovery of nutrients in the curd (**REC**) of individual milk samples.

Differences in SCC variation (de Haas et al., 2002), milk production (Coulon et al., 2002; Gröhn et al., 2004; Schukken et al. 2009a), composition (Coulon et al., 2002; Leitner et al. 2006; Chaneton et al., 2008) and milk clotting ability (Leitner et al., 2006; Merin et al., 2008; Fleminger et al., 2011) have been reported, according to the infectious agent. However, the current scientific knowledge is scarce and mostly based on quarter-level analysis performed on relatively small sample sizes for a small number of traits related to the technological properties of milk. Thus, further investigations are needed to better understand the specific changes in milk during pathogen-specific cases of mastitis.

The best method to identify intramammary infections (**IMI**) in cows is to perform bacteriological culturing or PCR analysis (Nyman et al., 2014). Nevertheless, these methods are expensive and time-consuming, hence they are not applicable as routine tests. Instead, other indirect udder health indicators are used to identify the inflammatory status of the mammary gland and eventually select cows for subsequent bacteriological analysis (Pyörälä, 2003). Besides SCC, which is the international standard indicator of udder inflammation, possible diagnostic tools include: detection of variation in milk electrical conductivity (Jensen

and Knudsen, 1991; Norberg et al., 2004), measuring of the levels of inflammation-related enzymes (e.g., NAGase and LDH) (Chagunda et al., 2006; Nyman et al., 2014), detection of inflammation-related biomarkers, like the acute phase proteins haptoglobin (**Hp**) and serum amyloid A (**SAA**) (Petersen et al., 2004; Eckersall and Bell, 2010). Among all, the analysis of the concentration of blood serum proteins represents a developing area of clinical biochemistry for disease diagnosis (Eckersall, 2008), as it provides valuable information for assessing infection, inflammation and trauma in animals (Murata et al., 2004). For instance, the ratio of albumin-to-globulin (**A:G**), which is used to identify dysproteinaemia (Eckersall, 2008), has been proposed as useful marker to assess immune status of the cow (Piccinini et al., 2004). However, variation in blood serum proteins has not been fully exploited (e.g., possible differences among breeds) and information is lacking on their relationships with other udder health indicators (e.g., SCC and bacteriological results) and with milk production, composition and cheese-related traits.

## AIMS OF THE THESIS

The main purpose of this PhD thesis was to investigate the association between udder health (focusing on subclinical cases of bovine mastitis identified by SCC and bacteriological analyses) and several milk quality and technological traits (i.e., composition, detailed protein profile, and technological traits related to the cheese-making process), and other immune response indicators (i.e., blood serum proteins).

To achieve our goal, the work was splitted in 4 chapters and 2 different datasets were used: for the 1st chapter, milk samples from 1,271 Brown Swiss cows from 85 herds were used. In the subsequent 3 chapters, milk and blood samples were collected from 1,508 dairy specialized and dual-purpose cows of 6 different breeds housed in 41 multi-breed herds.

In particular, the objectives were to:

- Determine the effects of very low to very high SCC on milk yield, composition, coagulation properties (including traditional MCP and new curd firming model parameters), cheese yield and recovery of milk nutrients in the curd at the individual cow level in dairy cows (CHAPTER 1).
- Explore the effect of herd productivity, breed, and individual cow factors (i.e., stage of lactation and parity) on blood serum proteins and investigate association between blood serum proteins and SCC in specialized and dual-purpose cows (CHAPTER 2).
- Study the association between pathogen-specific cases of subclinical mastitis and milk yield, composition, detailed milk protein profile and cheese-making traits in specialized and dual-purpose cows (CHAPTER 3).
- Assess the association between pathogen-specific cases of subclinical mastitis and blood serum proteins in specialized and dual-purpose cows (CHAPTER 4).

# CHAPTER 1.

## The nonlinear effect of somatic cell count on milk composition, coagulation properties, curd firmness modeling, cheese yield and curd nutrient recovery

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## ABSTRACT

The aim of this study was to investigate the relationships between somatic cell count (SCC) in milk and several milk technological traits at the individual cow level. In particular, we determined the effects of very low to very high SCC on traits related to (1) milk yield and composition; (2) coagulation properties, including the traditional milk coagulation properties (MCP) and the new curd firming model parameters; and (3) cheese yield and recovery of milk nutrients in the curd (or loss in the whey). Milk samples from 1,271 Brown Swiss cows from 85 herds were used. Nine coagulation traits were measured: 3 traditional MCP [rennet coagulation time (RCT, min), curd firming rate ($k_{20}$, min), and curd firmness after 30 min ($a_{30}$, mm)] and 6 new curd firming and syneresis traits [potential asymptotic curd firmness at infinite time ($CF_P$, mm), curd firming instant rate constant ($k_{CF}$, % × $min^{-1}$), syneresis instant rate constant ($k_{SR}$, % × $min^{-1}$), rennet coagulation time estimated using the equation ($RCT_{eq}$, min), maximum curd firmness achieved within 45 min ($CF_{max}$, mm), and time at achievement of $CF_{max}$ ($t_{max}$, min)]. The observed cheese-making traits included 3 cheese yield traits (%$CY_{CURD}$, %$CY_{SOLIDS}$, and %$CY_{WATER}$, which represented the weights of curd, total solids and water, respectively, as a % of the weight of the processed milk) and 4 nutrient recoveries in the curd ($REC_{FAT}$, $REC_{PROTEIN}$, $REC_{SOLIDS}$, and $REC_{ENERGY}$, which each represented the percentage ratio between the nutrient in the curd and milk). Data were analyzed using a linear mixed model with the fixed effects of days in milk, parity, somatic cell score (SCS), and the random effect of the herd-date. Somatic cell score had strong influences on casein number and lactose, and also affected pH; these were traits characterized by a quadratic pattern of the data. The results also showed a negative linear relationship between SCS and milk yield. Somatic cell score influenced almost all of the tested coagulation traits (both traditional and modeled), with the exceptions of $k_{20}$, $CF_P$, and $k_{SR}$. Gelation was delayed when the SCS decreased (slightly) and when it increased (strongly) with respect to a value of 2, as confirmed by the

quadratic patterns observed for both RCT and $RCT_{eq}$. The SCS effect on $a_{30}$ showed a quadratic pattern almost opposite to that observed for RCT. With respect to the $CF_t$ parameters, $k_{CF}$ decreased linearly as SCS increased, resulting in a linear decrease of $CF_{max}$ and a quadratic pattern for $t_{max}$. Milk SCS attained significance for $\%CY_{CURD}$, $\%CY_{WATER}$ and $REC_{PROTEIN}$. As the SCS increased beyond 3, we observed a progressive quadratic decrease of the water retained in the curd ($\%CY_{WATER}$), which caused a parallel decrease in $\%CY_{CURD.}$ With respect to $REC_{PROTEIN}$, the negative effect of SCS was nearly linear. Recovery of fat and (consequently) $REC_{ENERGY}$ was characterized by a more evident quadratic trend, with the most favorable values associated with an intermediate SCS. Together, our results confirmed that high SCS has a negative effect on milk composition and technological traits, highlighting the nonlinear trends of some traits across the different classes of SCS. Moreover, we report that a very low SCS has a negative effect on some technological traits of milk.

**Key words**: somatic cell count, milk coagulation property, curd firming, cheese yield, whey loss

## INTRODUCTION

The consumption of milk and dairy products is growing worldwide (International Dairy Federation, 2013), making increased milk production a key dairy breeding goal in recent decades (VanRaden, 2004; Miglior et al., 2005). However, selection for higher milk production has led to deteriorations of milk quality and cow welfare (Oltenacu and Broom, 2010). For instance, unfavorable genetic correlations between milk yield and diseases (e.g., mastitis and ketosis) have been reported (Ingvartsen et al., 2003). Bovine mastitis is one of the most economically important diseases in dairy herds; the consequent high SCC, which is measured as a standard indicator trait of udder health and milk quality, reduces the price paid for milk (Seegers et al., 2003; Viguier et al., 2009). Moreover, milk with a high cell count is reported to have lower casein (Haenlein et al., 1973; Auldist and Hubble, 1998) and lactose (Kitchen, 1981; Auldist and Hubble, 1998) contents, due to increased proteinase-mediated degradation and decreased biosynthesis, respectively. The influence of SCC on the fat concentration is more controversial; although some authors (Harmon, 1994; Schallibaum et al., 2001) found lower values due to reduced synthetic activity of the mammary gland, others observed a higher fat content due to a reduced milk volume (Shuster et al., 1991; Bruckmaier et al., 2004).

Alterations in the chemical composition of high-SCC milk makes it less suitable for consumption and cheese processing, with the latter issue reflecting slower coagulation, weak consistency of the curd and reduced cheese yield (Barbano et al., 1991; Auldist and Hubble, 1998). The technological quality of the milk used in cheese-making is commonly evaluated by measuring milk coagulation properties (**MCP**; Annibaldi et al., 1977; McMahon and Brown, 1982) with computerized renneting meters. The 3 traditional parameters that define the clotting ability of milk, and that can be measured by mechanical lactodynamograph (Formagraph; Foss Electric A/S; Hillerød, Denmark), are rennet coagulation time (**RCT**,

min), curd-firming time (**$k_{20}$**, min) and firmness of the curd at 30 minutes after the addition of rennet (**$a_{30}$**, mm). An association between elevated SCC and an increase in RCT has been observed by different authors (Ng-Kwai-Hang et al., 1989; Barlowska et al., 2009).

The large majority of relevant published studies have included SCC as a linear covariate in the model, or compared the results obtained using milk with "normal" versus "high" SCC. In these studies, when several classes of SCC were used, they normally do not include classes <100,000 cells/mL, so the detailed effects of very low to very high SCC on milk technological traits have not been fully studied.

Recent studies introduced the strategy of prolonging the observation time and modeling curd firmness (**CF**) using new time (**$CF_t$**) parameters (Bittante, 2011; Cipolat-Gotet et al., 2012; Bittante et al., 2013). Milk coagulation properties are of interest for 2 main reasons: first, they have technological value for optimizing the cheese-making process and predicting possible abnormalities both during the process and in the final product; and second, they may be used to indirectly predict cheese yield (**CY**) through their relationships with losses of fines in whey and with moisture retained in the curd. This second aspect is important because MCP are relatively easy to measure in multiple samples at the laboratory level, whereas direct measurements of CY and nutrient recovery traits are expensive and time-consuming.

Given the complexity of cheese making, the fat and protein contents of milk have frequently been used as proxies for measuring CY. However, the efficiency of the cheese-making process is better defined by the recoveries of milk components in the curd and their losses in the whey (Banks, 2007). More recently, percentage cheese yield (**%CY**) and nutrient recovery (**REC**) of individual milk samples have been analyzed using a model cheese-making procedure developed by Cipolat-Gotet et al. (2013), which mimics all phases of cheese

production. However, there is little information available regarding the relationships between technological traits of milk and SCC.

Even if bulk tank milk is used for cheese production, information at the cow level might be useful in order to include milk technological traits as breeding goals in dairy cows. Moreover, the individual variation, which is higher compared to the one of bulk samples, helps to clarify the relationships between milk SCC and cheese-making traits. Therefore, the aim of this study was to elucidate the relationship between SCC and milk quality and technological traits at the individual cow level. In particular, we performed a detailed investigation of the effects of a range of SCC (from very low to very high) on (1) the milk yield and composition (i.e., fat, protein, casein, casein number, lactose, urea and pH); (2) the coagulation properties (traditional MCP and the new CF model parameters); and (3) the cheese yield and recovery of milk nutrients in the curd and loss in the whey.

## MATERIALS AND METHODS

### *Milk Samples Collection*

This study is part of the Cowability-Cowplus Projects, which were described in detail by Cipolat-Gotet et al. (2013) and Cecchinato et al. (2013). Briefly, individual milk samples of 1,271 Brown Swiss cows were collected once from 85 herds (a maximum of 15 cows/herd, 1 or 2 herds per week, 13 mo in total) located in Trento province, northern Italy. The relevant environmental conditions were described in detail by Sturaro et al. (2013). The milk samples (one per cow) were collected during the evening milking. After collection, each sample was divided into 2 subsamples, which were refrigerated (4°C, without preservative). One subsample (50 mL) was transferred to the Milk Quality Laboratory of the Breeders Federation of Trento Province (Trento, Italy) for milk composition analysis. The other (2,000 mL) was transferred to the Cheese-Making Laboratory of the Department of Agronomy, Food, Natural

Resources, Animals and Environment (DAFNAE) at the University of Padova (Legnaro, Padova, Italy) for MCP analysis and model cheese making. Data on herds and cows were provided by the Breeders Federation of Trento Province and the Superbrown Consortium (Italy).

### *Analysis of Quality Traits, MCP, CF Modeling, Cheese Yield, and Whey Losses*

***Milk Composition Traits*.** In the Milk Quality Laboratory in Trento (Italy), each milk subsample was analyzed within 20 h of milking for fat, protein, casein, lactose (%), and urea (mg/100 g) using a Milkoscan FT6000 (Foss Electric A/S), calibrated according to the following reference methods: fat (ISO, 2010b; ISO1211|IDF 1; gravimetric method, Rose-Gottlieb); protein (ISO, 2014; ISO 8968–1|IDF 20-1; titrimetric method, Kjeldahl); casein (ISO, 2004a; ISO 17997-1|IDF 29; titrimetric method, Kjeldahl); lactose (ISO, 2002; ISO 5765-1|IDF 79-1; enzymatic method); urea (ISO, 2004b; ISO 14637|IDF 195; differential pH method); TS (ISO, 2010a; ISO 6731|IDF 21; determination of TS content). Ten reference milk samples per month (Italian Breeders Association, Roma, Italy) were used for adjusting calibrations, and 1 repeated sample every 50 analyzed samples was used to control repeatability of analyses.

Milk pH, adjusted for sample temperature, was measured using a Crison Basic 25 electrode (Crison Instruments SA, Barcelona, Spain). Each SCC was obtained with a Fossomatic FC counter (Foss Electric A/S) and log-transformed to an SCS (Ali and Shook, 1980). Bacterial count was not measured on individual milk samples.

***Traditional MCP.*** In the cheese-making laboratory of the University of Padova (Italy), within 20 h of milking, the time from rennet addition to milk gelation (RCT, min), curd-firming rate (min to a curd firmness of 20 mm; $k_{20}$) and curd firmness after 30 minutes from rennet addition ($a_{30}$, in mm) were determined using a mechanical lactodynamograph

(Formagraph; Foss Electric A/S). The duration of the test was extended to 90 min, so that almost all (99.7%) of the milk samples coagulated and yielded $k_{20}$ values. The details of the experimental conditions (e.g., temperature of the milk samples, concentration and type of rennet) were as reported in Cipolat-Gotet et al. (2012).

***New Curd Firming and Syneresis Traits.*** For each milk sample, a total of 360 curd firmness values were recorded (1 every 15 s for 90 min). The new parameters of curd firmness modeled on time t ($CF_t$) were estimated using the 4-parameter model proposed by Bittante et al. (2013):

$$CF_t = CF_P \times (1 - e^{-k_{CF} \times (t - RCTeq)}) \times e^{-k_{SR} \times (t - RCTeq)}$$

where $CF_t$ (mm) is the curd firmness at time t; $\mathbf{CF_P}$ (mm) is the maximum asymptotic curd firmness; $\mathbf{k_{CF}}$ (%×min$^{-1}$) is the curd-firming instant rate constant; $\mathbf{k_{SR}}$ (%×min$^{-1}$) is the curd syneresis instant rate constant; and $\mathbf{RCT_{eq}}$ (min) is the rennet coagulation time. Moreover, 2 additional traits related to the maximum curd firmness (**MCF**) were calculated: the maximum $CF_t$ value ($\mathbf{CF_{max}}$, mm) and time at $CF_{max}$ ($\mathbf{t_{max}}$, min).

***Individual Cheese Yield and Curd Nutrient Recovery.*** These phenotypes were obtained through a model cheese-making procedure performed on a milk subsample (1,500 mL) from each individual cow. Cheese yield was assessed using 7 components that formed 2 groups of traits: (1) 3 %CY traits that expressed the weights of the fresh curd ($\mathbf{\%CY_{CURD}}$), the curd DM ($\mathbf{\%CY_{SOLIDS}}$), and the water retained in the curd ($\mathbf{\%CY_{WATER}}$) as percentages of the weight of the processed milk; and (2) 4 REC traits representing the proportions of milk nutrients and energy retained in the curd ($\mathbf{REC_{SOLIDS}}$, $\mathbf{REC_{FAT}}$, $\mathbf{REC_{PROTEIN}}$ and $\mathbf{REC_{ENERGY}}$, calculated as the percentage ratios between a given component in the curd versus the processed milk). The energy within the curd was calculated as the difference between the energy in the milk and the whey (NRC, 2001). A detailed description of the individual model cheese-making procedure used to obtain the phenotypes analyzed in this

study, as well as the relevant sources of phenotypic variation, can be found in Cipolat-Gotet et al. (2013). Finally, all analyses (milk composition, coagulation and cheese-making traits) were performed within 20 h from milk collection.

***Statistical Analysis***

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with the following linear model:

$$y_{ijklm} = \mu + DIM_i + Parity_j + SCS_k + Herd\text{-}date_l + e_{ijklm},$$

where $y_{ijklm}$ is the observed trait; $\mu$ is the overall mean; $DIM_i$ is the fixed effect of the $i$th class of days in milk [$i$ = 6 classes: class 1 ≤ 60 (n = 178); 60 < class 2 ≤ 120 (n = 265); 120 < class 3 ≤ 180 (n = 226); 180 < class 4 ≤ 240 (n = 167); 240 < class 5 ≤ 300 (n = 176); class 6 > 300 (n = 188)]; $Parity_j$ is the fixed effect of the $j$th parity [$j$ = 1 (n = 368); 2 (n = 362); 3 (n = 201); 4 (n = 144); ≥ 5 (n = 186)]; $SCS_k$ is the fixed effect of the $k$th class of SCS [$k$ = 1 to 7; class 1 ≤ 0.66 (n = 131); 0.66 < class 2 ≤ 1.59 (n = 193); 1.59 < class 3 ≤ 2.52 (n = 215); 2.52 < class 4 ≤ 3.45 (n = 224); 3.45 < class 5 ≤ 4.38 (n = 210); 4.38 < class 6 ≤ 5.31 (n = 137); class 7 > 5.31 (n = 147)]; $Herd\text{-}date_l$ is the random effect of the $l$th herd-date ($l$ = 1 to 85); and $e_{ijklm}$ is the random residual. For the studied coagulation properties, cheese yield traits and nutrient recoveries in curd, the pendulum/$vat_m$ effect was added to the above-described model as the fixed effect of the $m$th pendulum ($m$ = 1 to 10) for coagulation properties or the $m$th number of the vat or waterbath ($m$ = 1 to 15) for cheese yield and nutrient recoveries. Herd-date and residuals were assumed to be normally distributed with a mean of zero and variances of $\sigma_h^2$ and $\sigma_e^2$, respectively. The proportion of variance explained by herd-test date was calculated by dividing the corresponding variance component by the total variance.

Polynomial contrasts ($P < 0.05$) were estimated to look at the response curve of the data for the SCS effect; the first-order comparisons measured linear relationships, while the second- and third-order comparisons measured quadratic and cubic relationships, respectively.

## RESULTS

### *Descriptive Statistics*

As all the investigated traits were characterized by normal distributions (Cipolat-Gotet et al., 2012, 2013), only the 1st and 99th percentiles are provided in Table 1. The milk yield of the Brown Swiss cows reared in the different dairy farming systems averaged 24.2 kg/d and showed a large variability (CV = 31.7%). Among the milk composition traits, casein number and pH showed the lowest variabilities, with CV of 1.65% and 1.19%, respectively. The other milk composition traits confirmed the good quality that characterizes milk of Brown Swiss cows and presented intermediate variabilities (from 3.7% for lactose to 31.4% for milk urea).

The SCS ranged from -0.47 (1st percentile) to 7.77 (99th percentile), corresponding to SCC of 9,000 and 2,722,000 cells/mL, respectively. Both SCS and SCC exhibited very high variabilities.

The mean values for RCT and $k_{20}$ were 19.9 min and 5.6 min, respectively, whereas $a_{30}$ averaged 29.6 mm. In the present work, the $CF_t$ parameters had mean values as follows: 20.9 min for the coagulation time calculated for each milk sample on the basis of all 360 data points ($RCT_{eq}$); 54.6 mm for the asymptotic potential curd firmness theoretically achievable at infinite time in absence of curd syneresis ($CF_P$); 12.6%×min$^{-1}$ for the instant rate constant of curd firming ($k_{CF}$), leading the $CF_t$ curve toward a value of $CF_P$ shortly after RCT; and 1.39%×min$^{-1}$ for the instant rate constant of syneresis, leading $CF_t$ toward zero over an

extended duration ($k_{SR}$). On average, the maximum CF value ($CF_{max}$) was 36.5 mm, and it was achieved ($t_{max}$) 41.5 min after rennet addition.

The mean $\%CY_{CURD,}$ which corresponded to the sum of $\%CY_{SOLIDS}$ and $\%CY_{WATER}$, was 15%. The curd nutrient recovery was, on average, close to the mean casein number for protein, almost 90% for fat, slightly more than half for total solids, and about two-thirds for milk energy (Table 1).

***Sources of Variation among Quality Traits***

The results of our ANOVA for the milk yield and composition traits are summarized in Table 2. The proportion of variance explained by herd-test date was more than 70% for urea; approximately 50% for milk production, casein number and pH; 20% for fat, protein, and casein contents and fat:protein ratio; and slightly more than 10% for lactose and SCS. As expected, DIM and parity effects played important roles in explaining the variation of almost all considered traits, with the exceptions of the fat, fat:protein and urea traits, for which parity had a negligible effect. Detailed information on the effect of DIM and parity on the investigated traits was reported by Cipolat-Gotet et al. (2012, 2013) and Bittante et al. (2015). The influence of DIM and parity was tested also on milk SCS (Table 2) and the results showed an increase in SCS with advancing age and stage of lactation (data not shown).

In the present study, we focused on the effect of SCS, which had strong influences on casein number and lactose, and also affected pH and urea. The results of the polynomial contrasts are reported in Table 2. The least squares means (**LSM**) and the corresponding standard errors of the milk yield and composition traits for which SCS had significant effects are presented in Figure 1, together with the responding curve of the data across the different classes of SCS (according to the obtained significant linear or quadratic contrasts). Even though the ANOVA did not reveal a significant effect of SCS on milk yield (Table 2), the

contrasts showed a negative linear relationship ($P < 0.05$) between these 2 traits (Figure 1a). Milk fat, protein, and casein contents were not affected by milk SCS. Urea exhibited an erratic trend across the SCS classes and was characterized by a high standard error of the means (Figure 1d). The casein number and lactose showed clear quadratic patterns: their LSM remained relatively constant across the first 3 classes of SCS, and then decreased almost linearly as the SCS increased beyond 2 (Figures 1b and 1c). Finally, although the LSM differences for milk pH were modest, decreases in lactose and the casein number were accompanied by decreases in milk acidity (Figure 1e).

***Sources of Variation among MCP and CF Modeling***

The results from our statistical analysis of the various coagulation properties are summarized in Table 3. Notably, the proportion of variance explained by herd-test date was lower for the coagulation properties than for the quality traits and much lower than for milk yield; the values for coagulation properties ranged between ~ 4% and 16%, with the exception of $t_{max}$ (22%). As expected, DIM had strong influences on both the traditional MCP and the new $CF_t$ model parameters. Parity was significant ($P < 0.05$) in explaining the variation of only RCT among the traditional traits, but it was significant for all of the $CF_t$ traits (with the exception of $CF_P$).

The effect of pendulum, which was the only effect related to instrument repeatability, was confirmed to play important roles in explaining the variation of all traits except for RCT and $RCT_{eq}$. Somatic cell score influenced almost all of the tested coagulation traits, both traditional and modeled, with the only exceptions being $k_{20}$, $CF_P$ and $k_{SR}$.

The LSM of the coagulation traits affected by SCS are reported in Figure 2. The most rapid gelation was exhibited by milk samples with an SCS in the class centered on a value of 2, both when expressed as single point trait (RCT) or as a $CF_t$ equation parameter ($RCT_{eq}$).

Gelation was delayed when SCS decreased (slightly) or increased (strongly) with respect to the class centered on an SCS of 2, as confirmed by the quadratic patterns observed for both RCT and $RCT_{eq}$. Considering the curd firming process, within the traditional MCP, SCS did not show any significant effect on $k_{20}$, whereas the effect on $a_{30}$ seemed to be opposite to that seen for RCT (Figure 2b). In the case of the $CF_t$ parameters, $CF_P$ and $k_{SR}$ were not affected, but $k_{CF}$ decreased linearly as SCS increased (Figure 2d). This yielded a linear decrease of $CF_{max}$ (Figure 2e) and a quadratic pattern for $t_{max}$, which exhibited a relevant delay only when SCS exceeded 3 (Figure 2f).

***Sources of Variation among Cheese Yield and Whey Losses***

Table 4 shows the roles of the considered effects in explaining variations of the cheese yield traits and milk nutrient recovery in the curd. For these traits, the proportion of variance explained by herd-test date (20% to 40%) was much greater than that seen for the coagulation and curd firming traits. As expected, DIM was the most important source of variation affecting all of the studied traits. Parity had a strong influence on $\%CY_{WATER}$ and (consequently) $\%CY_{CURD}$, and affected $REC_{PROTEIN}$. Unlike the coagulation and curd firming traits, the effects of the instruments used (waterbaths and individual vats) were negligible in explaining the variations of the cheese yield and curd recovery traits. Similar to parity, SCS attained significance exclusively for $\%CY_{CURD}$, $\%CY_{WATER}$ and $REC_{PROTEIN}$.

Figure 3 depicts the LSM results for the cheesemaking-related traits across the different classes of SCS. All of the affected traits showed nonlinear effects for SCS. As SCS increased beyond 3, there was a progressive decrease of the water retained in the curd (decreasing $\%CY_{WATER}$; Figure 3b), which caused a parallel decrease in $\%CY_{CURD}$ (Figure 3a). For $REC_{PROTEIN}$, the negative effect of SCS was almost linear (Figure 3c). In contrast, $REC_{FAT}$ (Figure 3d) and (consequently) $REC_{ENERGY}$ (Figure 3e) showed more evident

quadratic trends, with the most favorable values being observed for milk with an intermediate SCS content, whereas lower values were seen for milk samples with smaller and greater SCS.

## DISCUSSION

### *SCS Affects the Variation of Milk Yield and Composition*

An infection in the udder triggers an inflammatory response characterized by recruitment of white cells from the bloodstream and altered secretion of various molecules (Wellnitz and Bruckmaier, 2012). Because cell numbers in milk are associated with inflammation, SCC is recognized as the international standard indicator of udder health and milk quality (Sharif and Muhammad, 2008). Bovine mastitis increases the milk SCC, decreasing milk production and changing the composition of the milk (Kitchen, 1981; Le Maréchal et al., 2011). Inflammation damages the mammary gland tissue, inhibiting the biosynthesis of fat, protein and lactose, and thereby decreasing the milk yield (Harmon, 1994; Schallibaum, 2001). In the present study, we observed a negative linear relationship between milk production and the classes of SCS (Figure 1a), although the numeric pattern seemed slightly curvilinear. This trend agrees with the results obtained by Koldeweij et al. (1999), who found a linear relationship between $\log_{10}$(SCC) and test-day milk yield. The same authors evaluated quadratic and cubic effects, but found that they contributed little to the overall fit of the models. The linear relationship predicts that milk production will decrease by 2.04 kg for each unit increase in $\log_{10}$(SCC), leading to a 10-fold increase in SCC. This corresponds to a decrease of 0.6 kg/d of milk per unit of increase in $\log_2$(SCC), which corresponds to a 2-fold increase in SCC. Our estimate (-0.18 kg/d/unit) is much smaller, even in the interval between SCS values of 2 and 5 (-0.5 kg/d). However, it should be noted that we excluded any cow that showed signs of clinical mastitis. Previously, the relationships between repeated records of milk yield and the SCS of 33,453 first-lactation Norwegian Red cows

were described by de los Campos et al. (2006) using equations that considered possible recursive or simultaneous effects between traits. Those authors found evidence that SCS has a negative effect on milk yield (the infection effect, for which SCS is a standard indirect indicator), with an increase of 1 unit of SCS decreasing milk yield by about 1.1 kg/d; however, they did not find any reciprocal effect of milk production on SCS (dilution effect). Similar results were obtained by Wu et al. (2007), who used a Bayesian approach, compared with the maximum likelihood method used by de los Campos et al. (2006).

The lactose level in milk decreases during mastitis not only because of lower biosynthesis, but also because membrane permeability increases, allowing lactose to leak from the milk into the blood (Shuster et al., 1991); consequently, the milk contents of some minerals increase. As shown in Figure 1c, we observed that an increased SCC was accompanied by a nonlinear reduction in the lactose content, with lactose values remaining relatively constant in milk samples with SCS <2, but decreasing almost linearly above this value. The association between a high SCC and a decreased lactose concentration is well documented (Kitchen, 1981; Auldist et al., 1995; Wickstrom et al., 2009), and it has been proposed that lactose could potentially be used to monitor udder health (Pyörälä, 2003). However, most of the existing reports have used a 2-level evaluation (low vs. high) to examine the effect of SCC on milk yield and composition; far fewer studies have examined response curves across different classes of SCC. Bruckmaier et al. (2004) observed lower lactose concentrations (43.8 vs. 48.1 g/L) in infected quarters (logSCC/mL >6) compared to healthy contralateral quarters (logSCC/mL <5.2), which was consistent with the previous findings of other authors (Fox et al., 1985; Harmon, 1994). As lactose content decreases, the concentrations of certain minerals (sodium and chloride) increase to maintain the osmotic equilibrium (Batavani et al., 2007). Moreover, lower values of casein might be observed in high SCC milk as a result of plasmin- and somatic-cell-protease-mediated activity against

caseins, especially $\alpha_{s1}$- and β-casein (Urech et al., 1999). The changes in the ionic environment and the degradation of casein due to a higher enzymatic activity (Verdi et al., 1987; Franceschi et al., 2003) are responsible for the rise in milk pH observed during mastitis. This increase seems to follow a nonlinear quadratic trend (Figure 1e), with constant LSM values found for the first 4 classes of SCS, followed by moderate increases in classes 5, 6 and 7. Batavani et al. (2007) compared milk samples collected from healthy quarters and quarters with subclinical mastitis (defined as a leukocyte count higher than 500,000 cells/mL) and found that the pH of mastitic milk was significantly higher than that of healthy milk (6.69 vs. 6.59, respectively; $P < 0.01$). Vianna et al. (2008) investigated the effect of low (<200,000 cells/mL) and high (>700,000 cells/mL) SCC on raw milk composition and observed higher pH (6.85 vs. 6.76) and lower lactose values (4.53% *vs*. 4.69%) in high SCC milk. The effect of mastitis on the total protein content is controversial (Le Maréchal et al., 2011), but drastic changes clearly occur in the protein profile (Urech et al., 1999). The proteolysis of casein and the decreased synthesis of the major whey proteins, β-LG and α-LA, are balanced by increases in the contents of BSA and immunoglobulin via leakage through the blood-milk barrier (Haenlein et al., 1973). Thus, the total protein content in milk may not be significantly influenced by high SCC (Munro et al., 1984). In the present study, the casein and protein contents were not affected by the SCS class, but the casein:protein ratio was affected (Table 2). In fact, the casein number was influenced by high SCC, and showed a nonlinear quadratic pattern similar to that observed for the lactose content (Figure 1b). Geary et al. (2013) performed a meta-analysis on the data available in the literature and estimated the relationships between SCC and milk composition traits. A total of 32 published articles, mostly based on 2- or 3-class (low, intermediate and high SCC) evaluations, were included in the analysis. Significant ($P < 0.01$) negative linear relationships were found between SCS and lactose content, and between SCS and casein number. Quadratic and cubic effects were also

tested in the random regression models, but they were not significant. Coulon et al. (1998) reported that the decrease in casein number became significant when SCC >200,000 cells/mL; this value corresponds to our SCS class 4, which is the class in which we observed the casein number begin to decrease as SCS increased (Figure 1b). Notably, an SCC >200,000 cells/mL is considered the threshold value for detecting subclinical mastitis at the individual cow level (Guidry, 1985).

***SCS Affects Variation in Milk Coagulation and Curd Firming Pattern***

Although cheese production is a major use of milk, relatively few studies have examined the effect of SCC on the cheese-making process or the properties of the produced cheese (Le Maréchal et al., 2011). Nonetheless, a high milk SCC is generally recognized to affect not only milk composition, but also the technological traits related to clotting ability and cheese processing (Barbano et al., 1991; Auldist and Hubble, 1998). As variation in milk pH could strongly affect both traditional MCP and the $CF_t$ parameters (Stocco et al., 2015), the main effect of SCS on these traits could reflect increases in pH caused by subclinical mastitis. Higher pH, in association with elevated SCC, negatively affects the cheese-making ability of milk by decreasing the activities of clotting-related enzymes (Swaisgood, 1982). Moreover, alterations in casein may also affect milk coagulation, as proteose-peptones released during the degradation of casein seem to have negative effects on clotting time, curd firmness and curd formation (Le Maréchal et al., 2011). In the present study, the increased alkalinity and lower casein numbers of milk samples characterized by SCS >3 were responsible for a prolonged coagulation time (RCT, Figure 2a; $RCT_{eq}$, Figure 2c), and a weaker coagulum ($a_{30}$, Figure 2b) with reduced syneresis; moreover, a nonlinear quadratic relationship was observed between MCP and SCS. Associations between elevated SCC and reduced MCP (e.g., longer rennet clotting time and lower curd firmness) have been described

in literature (Munro et al., 1984; Rogers and Mitchell, 1994; Klei et al., 1998). Ng-Kwai-Hang et al. (1989) reported that gelation was delayed (+5 min) as milk SCC increased from 100,000 to 600,000 cells/mL. Pellegrini et al. (1994) found that the coagulation time of ewe milk increased ~ 5 min between class 1 (SCC <100,000 cells/mL) and class 4 (SCC >500,000 cells/mL). In a recent paper, Vasquez et al. (2014) reported a positive (unfavorable) linear relationship between SCC and RCT, with the latter increasing by 0.9 min (54 s) for each increase in SCC of 100,000 cells/mL. It should be noted that the effect of clinical or subclinical mastitis is not fully represented in the traditional MCP because these tests are often limited to 30 min. Certain relevant factors (e.g., an increased SCC) can delay gelation time, thereby increasing the incidence of samples that fail to coagulate within the test duration [called noncoagulating (NC) milk, as reviewed by Bittante et al. (2012)].

The newer strategy of prolonging observation time and modeling all available information allows the estimation of more informative parameters. In the present study, the test was prolonged to 90 min, which allowed almost all of the samples to coagulate, as previously described (Cipolat-Gotet et al., 2012). Moreover, the modeling of all 360 data points available for each milk sample increased the repeatability of the trait measures. The traditional (RCT; Figure 2a) and modeled ($RCT_{eq}$; Figure 2c) gelation times both showed clear nonlinear patterns, with the best results yielded by milk samples of the SCS class centered on the value of 2 (corresponding to a SCC of 50,000 cells/mL). The high-SCS-related delay in gelation time was consistent with previous reports (Barbano et al., 1991; Auldist and Hubble, 1998). However, we also observed that gelation was delayed in milk samples with very low SCS (<2).

It is difficult to directly compare the present work with prior studies that used linear covariates, assessed only 2 or 3 classes of SCC, or (when more classes were examined) tested distributions from milk with higher SCC than examined in the present study. For example,

Politis and Ng-Kwai-Hang (1988) reported that RCT values remained relatively constant for classes with 100,000 to 500,000 somatic cells/mL, and thereafter increased as the SCC increased to over 1,000,000 cells/mL. However, the authors did not report any SCC class below 100,000 cells/mL. Regarding the variation of traditional RCT with increasing SCS, our results are consistent with those of Toffanin et al. (2012), although they used bulk milk samples and did not test the data for nonlinearity.

The published reports regarding the effect of SCC on the other traditional MCP are more variable. This is also due to the biases induced on $a_{30}$ trait by NC samples, which has often been excluded from data analyses or assumed to have a 0 value, or on the $k_{20}$ trait, which presents a much higher (compared with noncoagulating samples) frequency of samples not reaching 20 mm of curd firmness within the usual 30-min time limit (Bittante et al., 2012). Because of this problem, many studies do not consider $k_{20}$. Here, prolonging the test duration allowed us to record $a_{30}$ and $k_{20}$ values for almost all of the analyzed samples. Our results showed that SCS did not have a significant effect on $k_{20}$, whereas its effect on $a_{30}$ was characterized by a pattern (Figure 2b) opposite than that observed for RCT (Figure 2a). This confirms the strong correlation of these 2 traits and emphasizes that little information is gained from the latter when the former is known.

Considering the new model-based curd firming traits, SCS did not affect potential curd firmness ($CF_P$) or the syneresis rate constant of curd ($k_{SR}$; the velocity at which whey is expelled from the curd). It did, however, exert an almost linear negative effect on the curd firming instant rate constant ($k_{CF}$, Figure 2d), which was reflected by the similar negative effect on maximum curd firmness ($CF_{max}$, Figure 2e) and the curvilinear effect on the time at which $CF_{max}$ was achieved ($t_{max}$, Figure 2f).

***SCS Affects Variation of Cheese Yield and Milk Nutrient Recovery in Curd or Loss in Whey***

Milk coagulation properties are important for 2 main reasons: (1) they are technologically valuable for optimizing the cheese-making process and predicting possible abnormalities during the process and in the final product; and (2) they may be used to indirectly predict cheese yield, especially with respect to fines losses in whey and the moisture retained in the curd. This second aspect is important because the MCP are relatively easy to measure in multiple samples at the laboratory level, whereas direct measurements of the %CY and REC traits are expensive and time-consuming.

The experimental results about the possibility of indirect prediction of cheese yield are controversial. Some authors failed to find significant differences in cheese yield between milk with good versus poor clotting abilities (Ikonen et al., 1999; Bonfatti et al., 2014), whereas others found that better MCP were associated with higher cheese yield and increased recovery of milk protein and fat in the curd (Aleandri et al., 1989; Malacarne et al., 2006). Recent work performed by Cecchinato and Bittante (2016) on a large dataset revealed weak relationships between traditional MCP and the %CY and REC traits but stronger correlations with the parameters depicting the latter portion of the $CF_t$ curve (especially $CF_P$ and $k_{SR}$).

In studies involving a small number of experimental cheese-making sessions, some authors reported that the cheese yield decreased as the SCC increased (Ali et al., 1980; Munro et al., 1984; Barbano et al., 1991). In particular, high SCC was shown to reduce the yields of cottage (Vianna et al., 2008), Parmigiano Reggiano (Summer et al., 2015) and Cheddar (Grandison and Ford, 1986; Auldist et al., 1996; Marino et al., 2005) cheeses, whereas it seemed not to affect Prato cheese (Mazal et al., 2007; Vianna et al., 2008) or Mozzarella (Andreatta et al., 2007). However most of these studies used bulk milk, enrolled a limited

number of samples to compare low versus high SCC, and did not consider the patterns across different classes of SCS.

In the present study, we observed a longer coagulation time and weaker curd firmness at the time of cutting; this likely reflected the impaired acidification of mastitic milk, which leads to a greater loss of nutrients in the whey (Figures 3c-e). Moreover, the mean value of $REC_{PROTEIN}$ (78.1%, Table 1) was, as expected, close to the mean value of the casein number (77.1%, Table 1), and the 2 traits showed similar, almost linear patterns with increasing SCS. The total variation induced by SCS on $REC_{PROTEIN}$ (Figure 3c) was 4-fold its effect on casein number (Figure 1c), indicating that the negative effect of somatic cells in milk is not limited to modification of the casein:total protein ratio, but also involves a loss of casein in the whey. In fact, although the nitrogen content remains constant, less casein is incorporated in the reticulum of the curd during cheese-making (Ali et al., 1990), decreasing the curd tension at the time of the cutting (Yun et al., 1982), reducing the cheese yield, and triggering a greater nutrient loss (Bynum and Olson, 1982). Positive relationships between SCC and protein losses into the whey were also reported by Politis and Ng-Kwai-Hang (1988) and Barbano et al. (1991).

Unlike the recovery of protein, the recoveries of milk fat ($REC_{FAT}$, Figure 3d) and (consequently) milk energy ($REC_{ENERGY}$, Figure 3e) showed evident nonlinear quadratic trends, with the highest recoveries yielded by the milk samples with intermediate SCS and slightly lower values for milk samples with both smaller and greater SCS. Summer et al. (2015) reported that high SCC has a negative effect on fat recovery in cheese due increased lipolysis. In contrast, no change in the loss of fat into the whey was reported by Politis and Ng-Kwai-Hang (1988), Mazal et al. (2007) or Vianna et al. (2008).

Increasing SCS was associated with a decrease in the water retained in the curd ($\%CY_{WATER}$, Figure 3b), especially for SCS <3, yielding a parallel decrease of $\%CY_{CURD}$

(Figure 3a). Interestingly, both high and low SCC appeared to affect some of the technological traits of milk. For example, milk belonging to SCS classes 1 and 2 (corresponding to SCC <38,000 cells/mL) was characterized by slower coagulation (Figure 2a), lower curd firmness (Figure 2b), and lower recoveries of fat (Figure 3d) and energy (Figure 3e) in the curd compared with milk of the intermediate classes of SCS. When we represented the equations using the LSM of the new curd firming traits (Figure 4), we detected differences in curd firmness between SCS classes 1, 3 and 7, with the best results obtained from milk belonging to the third class. Low SCC (<200,000 cells/mL) was previously reported as a possible risk factor for clinical mastitis (Suriyasathaporn et al., 2000), and might be associated with a reduced immune response to infections. The milk of healthy cows contains a resident leukocyte population that plays a key role in innate defense, and cows with very low milk SCC were reported to exhibit a less efficient response to IMI (Wellnitz et al., 2010). Thus, we speculate that some of the cows belonging to SCS class 1 had deficits in their immune responses and were affected by an undetectable mastitic event that had slight effects on milk yield (Figure 1a), casein number (Figure 1b) and lactose content (Figure 1c). This could explain why these values were lower for class 1 than for classes 2 and 3. Moreover, the pH was slightly higher in class 1 milk compared to the intermediate classes, worsening the technological traits of this low-SCC milk.

## CONCLUSIONS

The results of the present study confirmed the negative effect of high SCC (indicator of mammary gland inflammation) on milk yield, milk composition, milk coagulation properties and cheese-related traits. This study offers new insights into the relationships between the aforementioned variables, and explored the response curve of data obtained from individual milk samples across different classes of SCS. Our results show nonlinear trends for some milk

composition and technological traits with respect to SCS. This is the first report to test the relationships between SCS and the new technological traits [i.e., curd firming traits, syneresis traits, individual cheese yield (%CY) and curd nutrient recoveries (REC)]. As SCS increased, we observed a linear loss of milk production and several changes in milk composition. These variations decreased the quality and clotting ability of the milk, which showed slower coagulation and weaker curd firmness. This consequently decreased the cheese processing and cheese-making properties (as shown by reductions in the cheese yield and recoveries of nutrients in the curd). Further studies will be required to clarify the relationships between results obtained analysing individual and bulk samples, and the nonlinear relationships between SCS and milk technological traits observed in this study should be considered in genetic analysis. New studies are needed to explore the negative effect of low SCC on milk technological traits, as milk of SCS classes 1 and 2 showed slower coagulation, lower curd firmness and lower nutrient recoveries in the curd. Moreover, as mastitis may be caused by different pathogens that raise different immune responses, such variations should be considered in future investigations of how mastitis affects milk quality and technological traits.

## ACKNOWLEDGMENTS

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## TABLES AND FIGURES

**Table 1.** Descriptive statistics of single test-day milk yield, composition, traditional milk coagulation properties (MCP), curd firming, cheese yield (%CY) and curd nutrient recovery (REC)[1]

| Trait[2] | N[3] | Mean | SD | P1 | P99 |
|---|---|---|---|---|---|
| Milk yield, kg/d | 1,233 | 24.2 | 7.7 | 8.5 | 44.8 |
| Milk composition | | | | | |
| Fat, % | 1,242 | 4.20 | 0.65 | 2.71 | 5.86 |
| Protein, % | 1,250 | 3.70 | 0.42 | 2.86 | 4.70 |
| Fat:Protein | 1,239 | 1.14 | 0.18 | 0.74 | 1.62 |
| Casein, % | 1,250 | 2.89 | 0.32 | 2.26 | 3.65 |
| Casein number, % | 1,246 | 77.1 | 1.3 | 73.9 | 79.9 |
| Lactose, % | 1,246 | 4.86 | 0.18 | 4.36 | 5.22 |
| Urea, mg/100g | 1,252 | 25.9 | 8.1 | 9.0 | 45.2 |
| pH | 1,248 | 6.64 | 0.08 | 6.45 | 6.83 |
| SCC, $10^3$/mL | 1,257 | 252 | 615 | 9 | 2,722 |
| SCS, units | 1,257 | 2.98 | 1.86 | -0.47 | 7.77 |
| Traditional MCP | | | | | |
| RCT, min | 1,253 | 19.9 | 5.7 | 10.3 | 38.3 |
| $k_{20}$, min | 1,241 | 5.62 | 3.59 | 2.00 | 19.30 |
| $a_{30}$, mm | 1,192 | 29.6 | 11.0 | 0.7 | 50.8 |
| Curd firming | | | | | |
| $RCT_{eq}$, min | 1,250 | 20.9 | 6.4 | 11.1 | 41.1 |
| $CF_p$, mm | 1,141 | 54.6 | 13.9 | 26.1 | 97.9 |
| $k_{CF}$, %×min$^{-1}$ | 1,155 | 12.6 | 5.7 | 2.4 | 28.4 |
| $k_{SR}$, %×min$^{-1}$ | 1,153 | 1.39 | 0.56 | 0.15 | 2.95 |
| $CF_{max}$, mm | 1,248 | 36.5 | 7.3 | 18.5 | 53.4 |
| $t_{max}$, min | 1,226 | 41.5 | 12.0 | 22.4 | 81.4 |
| Cheese yield (%CY) | | | | | |
| $\%CY_{CURD}$ | 1,247 | 15.0 | 1.9 | 11.0 | 19.6 |
| $\%CY_{SOLIDS}$ | 1,238 | 7.22 | 0.94 | 5.37 | 9.93 |
| $\%CY_{WATER}$ | 1,241 | 7.80 | 1.28 | 5.04 | 11.23 |
| Nutrient recovery (REC) | | | | | |
| $REC_{PROTEIN}$, % | 1,242 | 78.1 | 2.4 | 72.4 | 83.3 |
| $REC_{FAT}$, % | 1,231 | 89.9 | 3.6 | 78.7 | 95.9 |
| $REC_{SOLIDS}$, % | 1,244 | 52.1 | 3.6 | 44.1 | 60.9 |
| $REC_{ENERGY}$, % | 1,231 | 67.3 | 3.3 | 59.2 | 75.1 |

[1]P1 = 1$^{st}$ percentile; P99 = 99$^{th}$ percentile.

[2]SCS = $\log_2$ (SCC/100,000) + 3; RCT = rennet coagulation time; $k_{20}$ = curd firming rate as min to a curd firmness of 20 mm; $a_{30}$ = curd firmness after 30 min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using the equation; $CF_P$ = potential asymptotic curd firmness at infinite time; $k_{CF}$ = curd firming instant rate constant; $k_{SR}$ = syneresis instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$; $\%CY_{CURD}$ = fresh cheese yield; $\%CY_{SOLIDS}$ = total solids cheese yield; $\%CY_{WATER}$ = water entrapped in the curd; $REC_{PROTEIN}$, % = protein retention; $REC_{FAT}$, % = fat retention; $REC_{SOLIDS}$, % = total solids retention; $REC_{ENERGY}$, % = energy retention.
[3]Number of samples.

**Table 2.** Results from ANOVA (*F*-value and significance) for single test-day milk yield and composition traits

| Trait | DIM | Parity | SCS[1] | SCS Contrasts | | | Herd-date,[2] % | RMSE[3] |
|---|---|---|---|---|---|---|---|---|
| | | | | Linear | Quad. | Cubic | | |
| Milk yield, kg/d | 118.4*** | 35.7*** | 1.5 | 4.2* | 0.1 | 3.7 | 48.9 | 4.67 |
| Milk composition | | | | | | | | |
| Fat, % | 14.4*** | 0.3 | 1.0 | 0.7 | 0.4 | 0.1 | 19.8 | 0.57 |
| Protein, % | 175.1*** | 8.2*** | 0.4 | 0.9 | 0.2 | 0.2 | 21.3 | 0.27 |
| Fat:Protein | 12.3*** | 2.3 | 0.6 | 0.5 | 0.0 | 0.0 | 17.0 | 0.16 |
| Casein, % | 167.4*** | 12.0*** | 0.6 | 0.5 | 1.1 | 0.1 | 22.1 | 0.21 |
| Casein number, % | 4.5*** | 14.0*** | 13.5*** | 60.5*** | 8.1** | 4.3* | 57.1 | 0.79 |
| Lactose, % | 11.4*** | 21.2*** | 38.4*** | 198.6*** | 17.1*** | 1.2 | 10.5 | 0.14 |
| Urea, mg/100g | 4.9*** | 0.5 | 2.5* | 2.6 | 0.1 | 0.0 | 72.9 | 4.26 |
| pH | 5.5*** | 6.4*** | 3.6** | 12.4*** | 4.4* | 0.7 | 50.8 | 0.06 |
| SCS[1], units | 26.8*** | 16.7*** | - | - | - | - | 11.3 | 1.62 |

[1] SCS = $\log_2$ (SCC/100,000) + 3.

[2] Herd-date effect expressed as proportion of variance explained by herd-test date calculated by dividing the corresponding variance component by the total variance.

[3] RMSE = root mean square error.

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

**Table 3.** Results from ANOVA (*F*-value and significance) for traditional milk coagulation properties (MCP) and curd firming traits

| Trait[1] | DIM | Parity | Pendulum[2] | SCS[3] | SCS Contrasts | | | Herd-date,[4] % | RMSE[5] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Linear | Quad. | Cubic | | |
| Traditional MCP | | | | | | | | | |
| RCT, min | 25.7*** | 2.9* | 1.0 | 6.7*** | 15.5*** | 12.1*** | 1.3 | 14.6 | 4.95 |
| $k_{20}$, min | 4.6*** | 0.4 | 2.3** | 1.0 | 1.0 | 1.6 | 0.0 | 4.3 | 3.51 |
| $a_{30}$, mm | 9.0*** | 2.0 | 2.4** | 2.6* | 5.1* | 4.6* | 0.3 | 6.4 | 10.28 |
| Curd firming | | | | | | | | | |
| $RCT_{eq}$, min | 20.6*** | 2.9* | 0.7 | 5.2*** | 11.7*** | 10.1** | 0.1 | 11.1 | 5.68 |
| $CF_p$, mm | 18.8*** | 1.7 | 8.3*** | 0.7 | 0.0 | 0.0 | 1.8 | 11.9 | 12.13 |
| $k_{CF}$, %×min$^{-1}$ | 15.9*** | 3.3* | 2.9*** | 2.3* | 4.0* | 1.2 | 0.1 | 13.8 | 5.00 |
| $k_{SR}$, %×min$^{-1}$ | 2.4* | 5.0*** | 18.4*** | 2.0 | 3.1 | 2.6 | 1.8 | 15.6 | 0.47 |
| $CF_{max}$, mm | 20.1*** | 4.7*** | 5.1*** | 2.7* | 9.7** | 2.6 | 0.8 | 21.9 | 6.02 |
| $t_{max}$, min | 14.7*** | 5.2*** | 4.3*** | 3.0** | 7.3** | 5.7* | 0.3 | 15.0 | 10.49 |

[1]RCT = rennet coagulation time; $k_{20}$ = curd firming rate as time (min) to a curd firmness of 20 mm; $a_{30}$ = curd firmness after 30 min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using the equation; $CF_P$ = potential asymptotic curd firmness at infinite time; $k_{CF}$ = curd firming instant rate constant; $k_{SR}$ = syneresis instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$.

[2]Pendulum = measuring unit of the coagulation meter.

[3]SCS = $\log_2$ (SCC/100,000) + 3.

[4]Herd-date effect expressed as proportion of variance explained by herd-test date calculated by dividing the corresponding variance component by the total variance.

[5]RMSE = root mean square error.

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

**Table 4.** Results from ANOVA (*F*-value and significance) for cheese yield (%CY) and milk nutrient recovery in curd (REC)

| Trait[1] | DIM | Parity | Vat/WB[2] | SCS[3] | SCS Contrasts | | | Herd-date,[4] % | RMSE[5] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Linear | Quad. | Cubic | | |
| Cheese yield (%CY) | | | | | | | | | |
| $\%CY_{CURD}$ | 53.2*** | 4.1** | 1.2 | 3.0** | 9.1** | 6.4* | 0.0 | 29.8 | 1.41 |
| $\%CY_{SOLIDS}$ | 39.8*** | 1.2 | 1.6 | 1.1 | 0.2 | 3.5 | 0.1 | 19.7 | 0.76 |
| $\%CY_{WATER}$ | 37.0*** | 5.6*** | 1.3 | 4.2*** | 16.5*** | 5.0* | 0.6 | 41.5 | 0.90 |
| Nutrient recovery (REC) | | | | | | | | | |
| $REC_{PROTEIN}$, % | 8.0*** | 11.1*** | 1.2 | 22.1*** | 121.5*** | 2.8 | 0.0 | 32.2 | 1.86 |
| $REC_{FAT}$, % | 15.4*** | 0.2 | 1.3 | 1.9 | 0.5 | 6.4* | 0.3 | 31.1 | 2.92 |
| $REC_{SOLIDS}$, % | 33.8*** | 0.5 | 1.7 | 1.3 | 1.7 | 2.2 | 0.8 | 20.1 | 2.92 |
| $REC_{ENERGY}$, % | 9.5*** | 0.2 | 1.7 | 1.5 | 0.9 | 5.5* | 0.8 | 20.2 | 2.89 |

[1]$\%CY_{CURD}$ = fresh cheese yield; $\%CY_{SOLIDS}$ = total solids cheese yield; $\%CY_{WATER}$ = water entrapped in the curd; $REC_{PROTEIN}$, % = protein retention; $REC_{FAT}$, % = fat retention; $REC_{SOLIDS}$, % = total solids retention; $REC_{ENERGY}$, % = energy retention.

[2]WB = water bath.

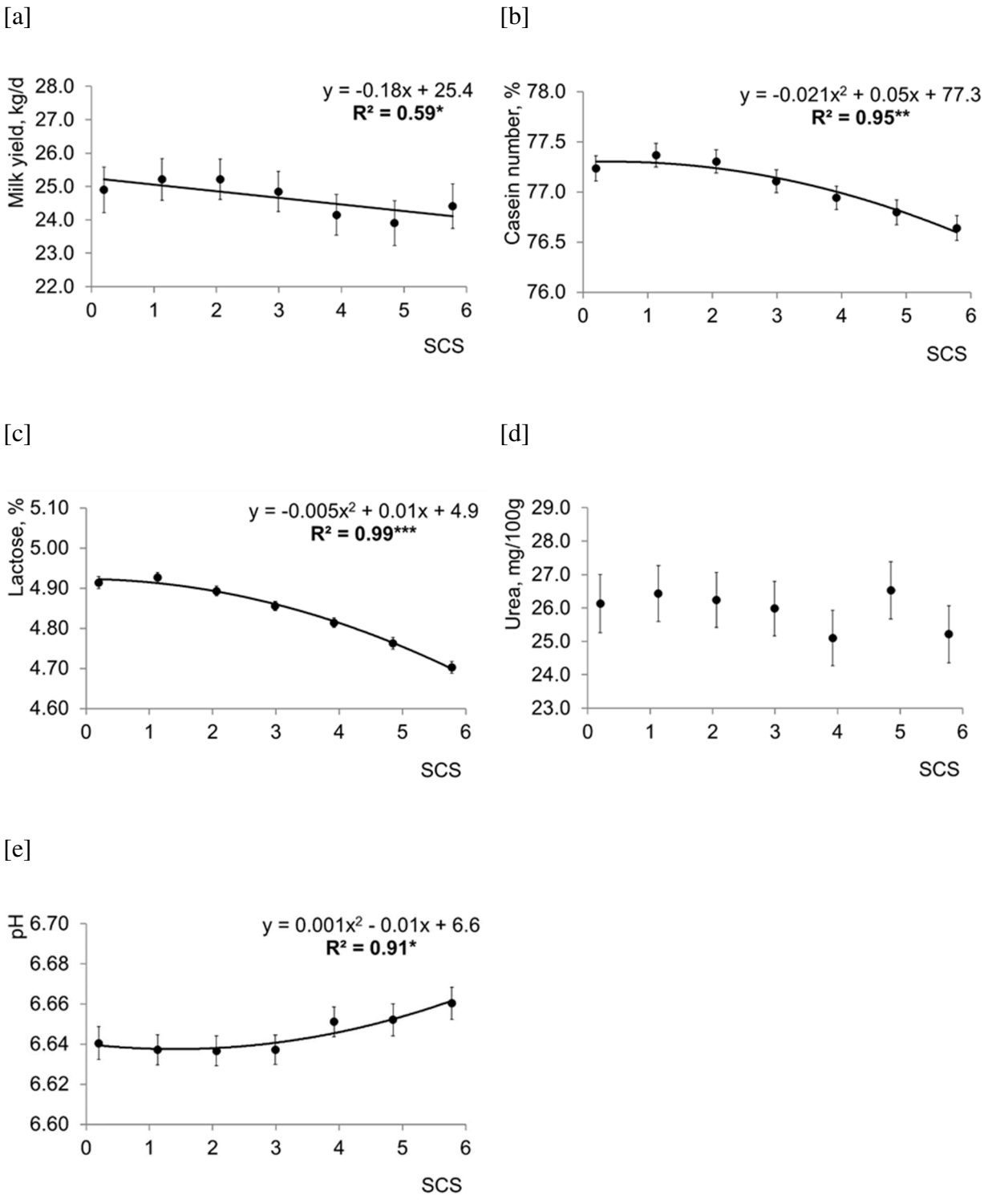
[3]SCS = $\log_2$ (SCC/100,000) + 3.

[4]Herd-date effect expressed as proportion of variance explained by herd-test date calculated by dividing the corresponding variance component by the total variance.

[5]RMSE = root mean square error.

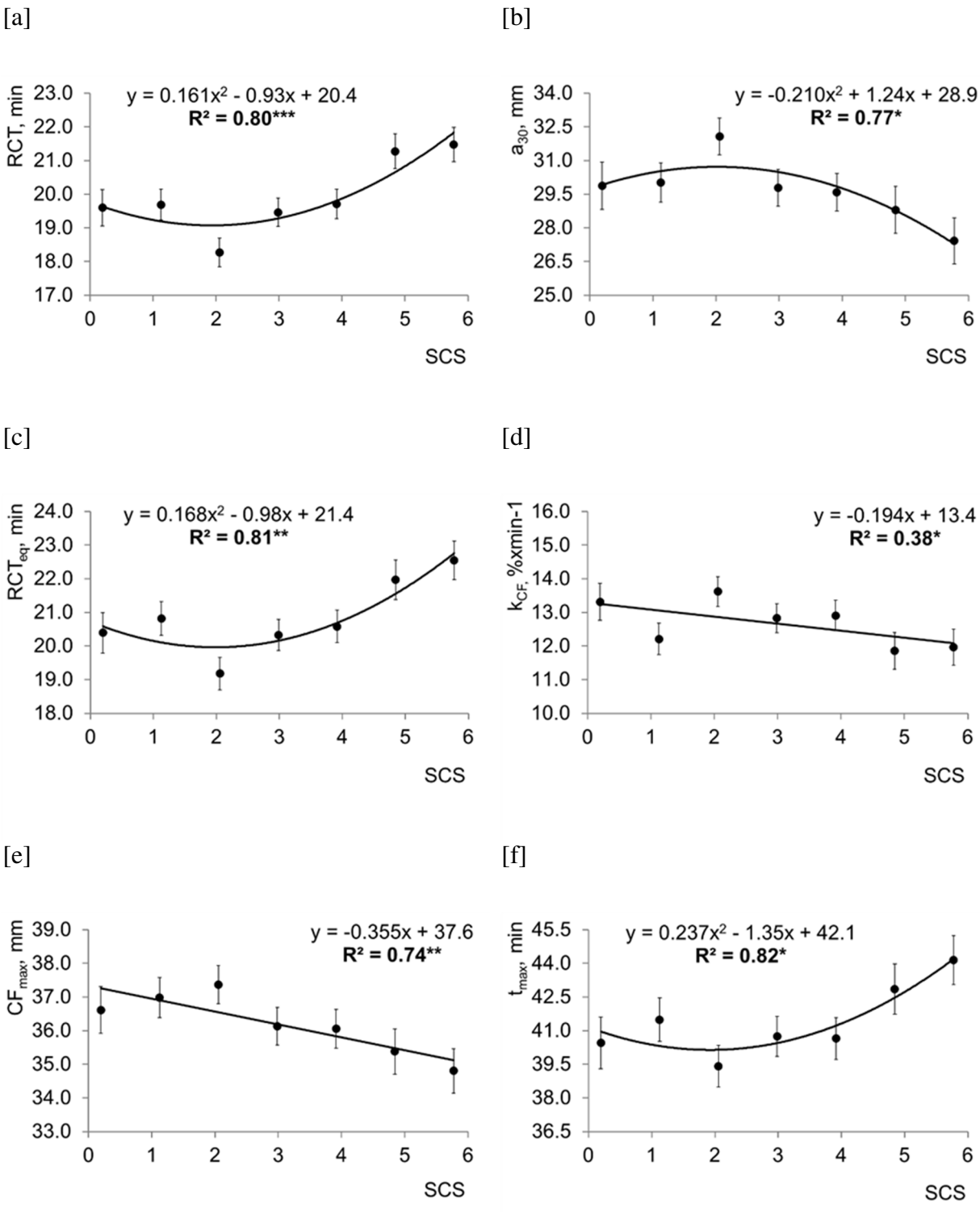
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

**Figure 1.** Least squares means of milk composition traits across SCS[1]




[1]Results of the polynomial contrasts have been reported: the response curve of the data across classes of SCS (linear or quadratic), the coefficient of determination ($R^2$) of the regression and the *P*-value of the polynomial contrasts. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars correspond to SE of LSM.

**Figure 2.** Least squares means of traditional milk coagulation property (MCP) and curd firming traits across SCS[1]

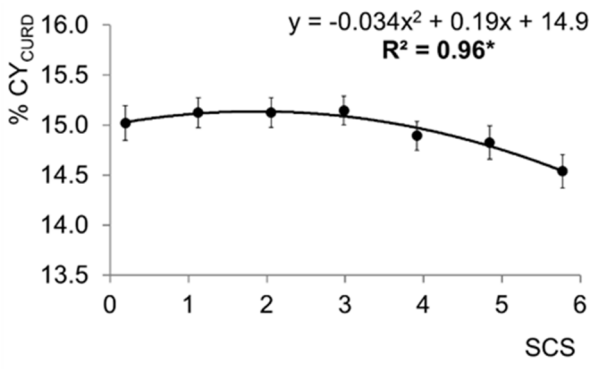



[1]Results of the polynomial contrasts have been reported: the response curve of the data across classes of SCS (linear or quadratic), the coefficient of determination ($R^2$) of the regression and the *P*-value of the polynomial contrasts. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. RCT = rennet coagulation time; $a_{30}$ = curd firmness after 30 min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using

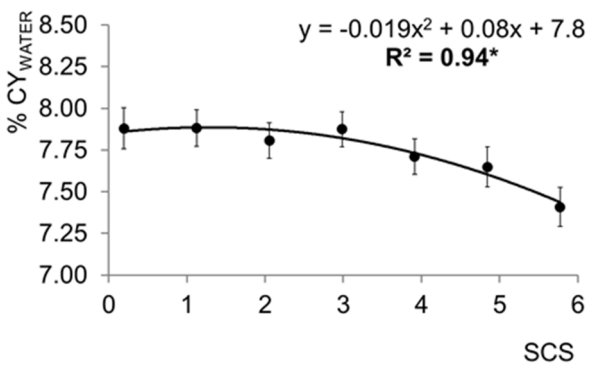
the equation; $k_{CF}$ = curd firming instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$. Error bars correspond to SE of LSM.

**Figure 3.** Least squares means of cheese yield traits (%CY) and milk nutrient recovery in curd (REC) across SCS[1]

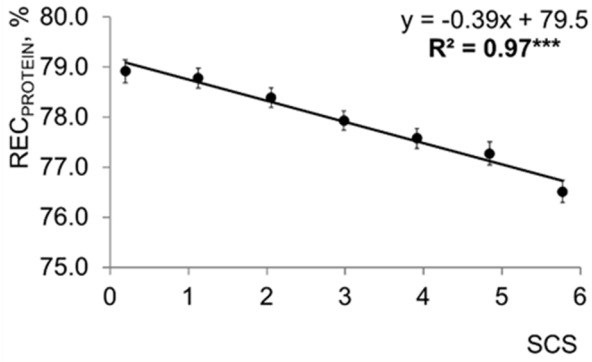
[a]



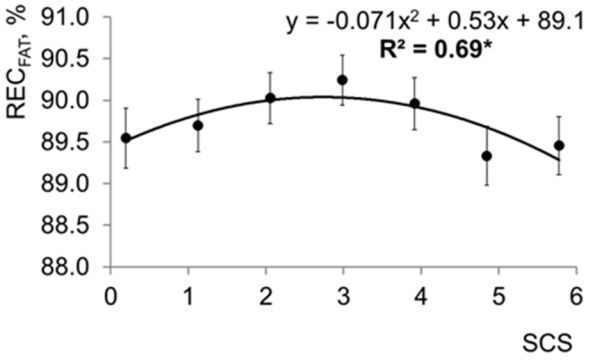

[b]



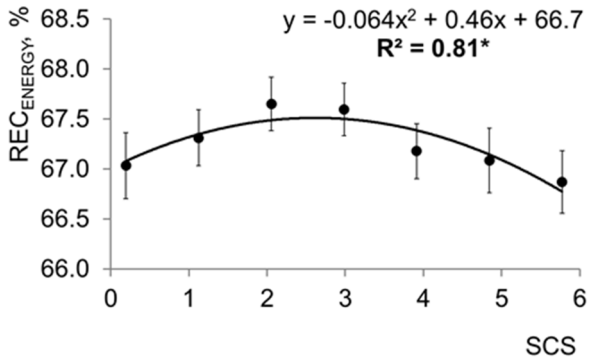

[c]




[d]

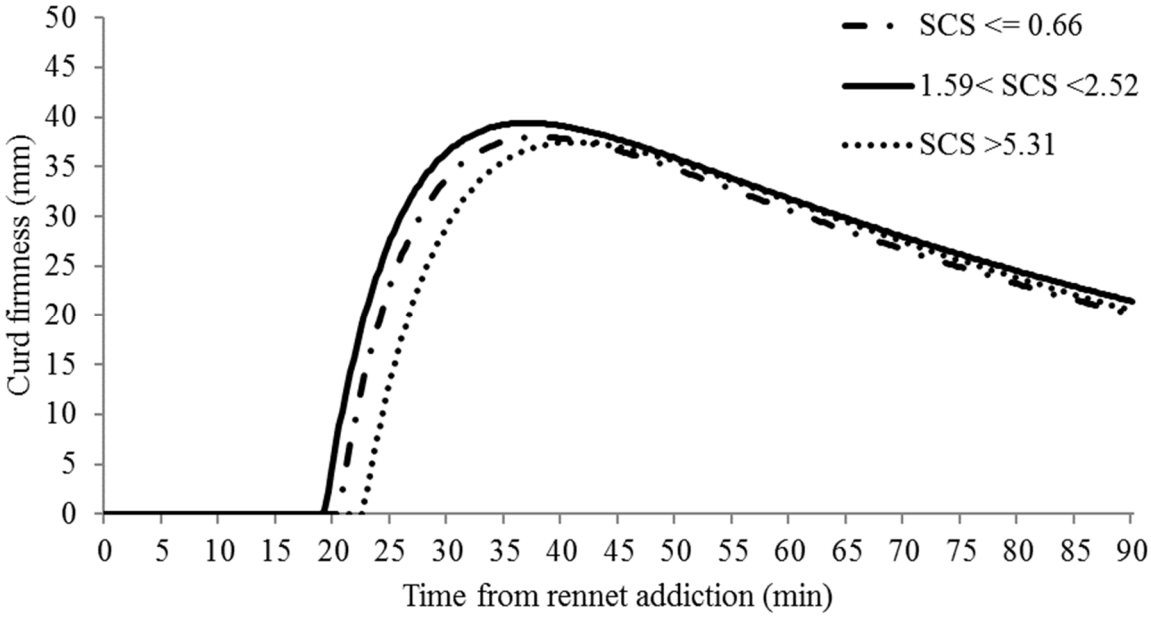



[e]




[1]Results of the polynomial contrasts have been reported: the response curve of the data across classes of SCS (linear or quadratic), the coefficient of determination ($R^2$) of the regression and the *P*-value of the polynomial contrasts. * $P < 0.05$; *** $P < 0.001$. Error bars correspond to SE of LSM.

**Figure 4.** Curd firmness modeling for classes 1 (≤ 0.66), 3 (1.59-2.52) and 7 (>5.31) of SCS



# CHAPTER 2.

## Variation in blood serum proteins and association with somatic cell count in dairy cattle housed in multi-breed herds

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**ABSTRACT**

Analysis of blood serum proteins is an important tool for monitoring the health status in dairy cows and possibly represents an initial screening test to identify animals which need further clinical investigations. However, several factors should be considered to appropriately interpret blood serum protein concentrations, including environmental factors (dairy system, nutrition, climate, season), breed and individual characteristics such as stage of lactation, parity, and health. Therefore, the objectives of this study were (1) to assess the effect of herd productivity, breed, age and stage of lactation on blood serum proteins [i.e., total protein, albumin, globulin and the ratio of albumin-to-globulin (A:G)] and (2) to investigate association between blood serum proteins and somatic cell count (SCC) in dairy cattle. Milk and blood samples were collected from 1,508 cows of 6 different breeds (Holstein Friesian, Brown Swiss, Jersey, Simmental, Rendena and Grey Alpine) housed in 41 multi-breed herds. Milk samples were analyzed for composition and SCC, while blood samples were analyzed for total protein, albumin and globulin contents. Herds were classified as low or high production, according to the cow's average daily milk energy yield adjusted for breed, days in milk (DIM) and parity. Data were analyzed using a linear mixed model that included the fixed effects of DIM, parity, somatic cell score (SCS), breed, herd productivity, and the random effect of the herd-date within productivity level. Significant associations between all explanatory variables and blood serum proteins were observed. Cows in high producing herds (characterized also by larger use of concentrates in the diet) had greater albumin concentrations. Breed differences were reported for all traits, highlighting a possible genetic mechanism. The specialized dairy breed Jersey and the two dual-purpose local breeds (Grey Alpine and Rendena) had the lowest globulin content and greatest A:G. Changes in blood serum proteins were observed through lactation. Total protein reached the highest concentration during the 4$^{th}$ month of lactation. Blood albumin increased with DIM following

a quadratic pattern, while globulin decreased linearly. As a consequence, A:G increased linearly during lactation. Older cows had greater total protein and globulin contents, while albumin level seemed to be not particularly affected by age. High milk SCS was associated with greater total protein and globulin contents in blood. The rise in globulin content, together with a decrease in albumin levels, resulted in a decline in A:G as SCS of milk increased. Linear relationships between blood serum proteins and SCS (supported also by correlation estimates) confirmed the importance of SCC as an indicator of mammary gland inflammation and highlighted the potential use of blood serum proteins as indicators of immune response of the mammary gland to infections. Moreover, such non-genetic factors (i.e., DIM, parity, breed, herd productivity) must be considered to appropriately interpret blood serum proteins as animal welfare indicators and their evaluation represents an important first-step for future analysis based on the integration of metabolomics and genomic information for improving the robustness of dairy cows.

**Key words**: serum total protein, albumin, globulin, somatic cell count, dairy

## INTRODUCTION

Welfare of farm animals is of great importance for dairy farm management. The ability of cows to mount a successful immune response to infection can result in reduced treatment costs and increased milk yield and quality. The identification of new traits that are associated with improved immune function may be beneficial for improving animal health and welfare (Wagter et al., 2000). Blood components such as serum proteins, glucose, enzymes and white blood cells have been used as possible indicators of health status in dairy cows (Giuliotti et al., 2004). Albumin and globulin are the two major protein fractions in blood. Except for immunoglobulins which are produced by B-lymphocytes, blood proteins are synthesized primarily in the liver. Serum albumin is a carrier of metals, lipids and hormones and is a key contributor to maintaining the osmotic pressure of plasma and constitutes 35-50% of total protein content (Eckersall, 2008). Globulin represents a heterogeneous group of proteins, and includes carriers of insoluble molecules, antibodies and others molecules with immune function.

Physiological and pathological states can result in variation in albumin and globulin concentrations of blood (Alberghina et al., 2010). Therefore, measurement of their concentrations could be a useful tool for evaluating physiological states that affect animal welfare and possibly represents an initial screening test to identify animals which need further clinical investigations. Variations in albumin concentration could indicate impaired liver function due to inflammatory conditions (Bertoni et al., 2008; Burke et al., 2010) and concentration of total serum globulin has been suggested as indicator of the animal's immune response (Chorfi et al., 2004). In clinical pathology, great importance is placed on the ratio of albumin-to-globulin (**A:G**), as it is used to identify dysproteinaemia (Eckersall, 2008) and has been proposed as useful marker to assess immune status of the cow (Piccinini et al., 2004). Additionally, lower A:G has recently been reported in cows affected by subclinical mastitis

(Gain et al., 2015). Therefore, further investigations on the association between blood serum proteins and milk SCC, the standard indicator of mammary gland inflammation, are needed.

As blood serum proteins are characterized by species-specific variability (Irfan, 1967), separate reference values for beef and dairy cattle should be determined. Nevertheless, several factors should be considered to appropriately interpret blood serum protein concentrations, including environmental factors (dairy system, nutrition, climate, season), breed and individual characteristics such as stage of lactation, parity, and health. Reference values for blood serum proteins in cattle have been proposed based on age (Kitchenham and Rolands, 1976; Alberghina et al., 2011; Cozzi et al. 2011), stage of lactation (Rowlands et al., 1975; Cozzi et al. 2011; Piccione et al., 2011;) and season (Shaffer et al., 1981; Cozzi et al. 2011; Brscic et al., 2015). Variation in globulin concentration has been observed based on differences in methodology (venipuncture sites, time of sampling, tests of analysis) (Chorfi et al., 2004). Most previous studies were performed using only Holstein Friesian cows (Chorfi et al., 2004; Cozzi et al. 2011; Piccione et al., 2011). One study (Aeberhard et al., 2001) compared breeds but was confounded by potential herd effects as different breeds were located on different farms. Only a few studies have been performed using multi-breed dairy farms in order to assess breed differences, within herd, in blood serum proteins (Kitchenham and Rowland, 1976; Shaffer et al., 1981; Gibson et al., 1987). However, as far as we know, no previous research has investigated variation in serum protein profile in dual-purpose Simmental and Alpine local breeds in comparison with specialized dairy breeds. We hypothesized that differences in selective breeding programs may result in differences in breed-specific robustness and immune ability. To our knowledge, no previous research has been published to describe the influence of several individual cow and herd effects on proteins content in blood of dairy cows of several breeds housed in multi-breed herds.

The objectives of this paper were (1) to investigate the effect of herd productivity (defined according to the average net energy of milk yielded daily by the cows), breed, and some individual factors (i.e., stage of lactation and parity) on blood serum proteins and (2) to investigate association between blood serum proteins and SCC in dairy cows.

## MATERIALS AND METHODS

### *Data Collection*

The present study is part of the Cowplus Project, carried out by the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padova (Italy), in collaboration with the Provincial Federation of Breeders of the Autonomous Province of Trento (Italy), with the aim of studying cattle farming in mountain areas (Trentino region, north-eastern Italy). For this study, 41 herds were selected from a sample of 610 dairy farms previously investigated (Sturaro et al. 2013). Enrolled herds were multi-breed farms (with at least 2 breeds/herd) characterized by monthly milking recording system. Moreover, they were representative of the 4 different dairy farming systems of the region, previously identified by Sturaro et al. (2013): “Original Traditional, with summer pastures”, “Traditional without summer pastures”, “Traditional with silages”, and “Modern”. Traditional farms were characterized by the presence of tied animals (mostly Brown Swiss, dual-purpose Simmental and local breeds, such as Rendena and Grey Alpine) housed in old building, fed with local forages (mainly hay and concentrates). Modern farms were larger herds, where animals (mostly Holstein Friesian and Brown Swiss cows) were housed in newer buildings that included milking parlors and were fed using TMR based on silage (almost exclusively maize). Individual samples were collected from 1,508 cows of 6 different breeds, 3 of which are specialized dairy breeds: Holstein Friesian (**HF**), Brown

Swiss (**BS**) and Jersey (**JER**), while the other 3 dual-purpose breeds of Alpine origin: Simmental (**SI**) and 2 local breeds, Rendena (**REN**) and Grey Alpine (**GA**).

Cows on each farm were sampled once during the study period and only one herd per day was visited. All selected animals were clinically healthy at the time of the visit. Health status of cows was determined on the basis of rectal temperature, heart rate, respiratory profile, appetite and fecal consistency. Cows with obvious clinical diseases (e.g., retained placenta, metritis, clinical mastitis, abomasal displacement, uterine prolapse, milk fever, clinical ketosis) were excluded from the trial. During an evening milking, a milk sample (50 mL) was collected from each cow by trained technicians, and maintained at a temperature of 4°C (without preservative) until it was processed (within 24h) at the Milk Quality Laboratory of the Provincial Federation of Breeders (Trento, Italy). Blood samples were collected by the veterinarian using jugular venipuncture (VenosafeTM, Terumo Europe) with no anticoagulant additive. Information on the cows and herds was obtained from the Provincial Federation of Breeders (Trento, Italy).

***Blood Samples Analyses***

Serum separation was performed on the farm and then transported to the laboratory of the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy) at 4°C and then stored at -18°C until analysis. Serum was assessed by means of a BT1500 automated photometer analyzer (Biotecnica Instruments S.p.A., Roma, Italy) for total protein and albumin. Globulin was determined by the difference between total protein and albumin concentrations and the ratio between albumin and globulin contents was also calculated.

### *Milk Samples Analyses*

Milk samples were analyzed for fat, protein, casein, lactose (%) and urea (mg/100g) using a Milkoscan FT6000 (Foss Electric A/S, Hillerød, Denmark), calibrated according to the reference methods already reported in Bobbo et al. (2016). Somatic cell count was obtained using a Fossomatic Minor (Foss Electric A/S) and log-transformed to SCS (Ali and Shook, 1980). Milk pH was measured after sample temperature adjustment using a Crison Basic 25 electrode (Crison Instruments SA, Barcelona, Spain).

### *Definition of Herd Productivity*

Farms were classified as low or high production according to the cow's average daily milk energy yield (Tyrrell and Reid, 1965) adjusted for breed, DIM and parity. Milk energy values (kcal/kg) of each cow were converted to KJ/kg and multiplied by individual daily milk yield (kg/d) to obtain the daily milk energy production of each cow (KJ/d). To estimate least square means of the average daily milk energy production of each herd, data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) including herd, breed, parity and DIM. Farms were rankes on the basis of estimated least square means of their average daily milk energy yield, and classified as low producing or high producing based on the median (Table 1).

### *Breeds Description and Genetic Background*

In the last decades, the shift of many traditional herds (mostly characterized by presence of BS and dual-purpose breeds, such as SI and local breeds RE and GA) towards a more modern dairy system resulted in expansion of the highly specialized HF breed in Trento Province (Sturaro et al., 2013). Semen used for artificial insemination in HF cows originated mostly from Italy, Germany and USA, and 50% of the sires of AI bulls had North American origin

(Cecchinato et al., 2015). Sires of BS cows, the main breed of the area before HF expansion, had mostly Italian, Austrian, German and American origin (Cecchinato et al., 2015). The increased importation of bulls and semen from the USA lead to a replacement of the original Alpine BS with animals selected for greater milk production. Jersey, a specialized dairy breed characterized by lower milk yield but greater content of fat and protein, were obtained using semen imported mainly from USA and Denmark. Sires of dual-purpose SI cows had Italian, German-Austrian (Fleckvieh) and French (Montbeliarde) origin. Rendena and GA are local dual-purpose breeds, characterized by medium milk production, and are considered to have better conformation and functional traits (as compared to the major breeds), and greater adaptability to the mountain environment. Autochthonous breeds play an important role in the area, as they are linked with local traditions and to production of local products.

For the 3 major breeds (HF, BS and SI), Italian selection indices include milk production and quality, type traits (mainly udder traits) and functional traits (Cecchinato et al., 2015). Selection index for BS includes also the κ-casein genotype (related to technological properties of milk), while in dual-purpose breeds SI and local breeds beef traits are also considered.

***Statistical Analysis***

Data of blood serum proteins were analyzed using the SAS MIXED procedure (SAS Institute Inc., Cary, NC) using the following linear mixed model:

$$y_{ijklmno} = \mu + DIM_i + Parity_j + SCS_k + Breed_l + HP_m + HTD_n(HP)_m + e_{ijklmno}, \qquad [1]$$

where $y_{ijklmno}$ is the observed trait (blood serum proteins); $\mu$ is the overall mean; $DIM_i$ is the fixed effect of the $i$th class of days in milk ($i$ = 6 classes of 60-d intervals, from 5 ≤ class 1 ≤ 65d to class 6 > 305d); $Parity_j$ is the fixed effect of the $j$th parity ($j$ = 1 to ≥ 4); $SCS_k$ is the

fixed effect of the *k*th class of SCS, ($k$ = 1 to 7, based on half standard deviation; class 1 ≤ 0.51; 0.51 < class 2 ≤ 1.44; 1.44 < class 3 ≤ 2.37; 2.37 < class 4 ≤ 3.30; 3.30 < class 5 ≤ 4.24; 4.24 < class 6 ≤ 5.17; class 7 > 5.17); $Breed_l$ is the fixed effect of the *l*th breed ($l$ = HF, BS, JER, SI, REN and GA); $HP_m$ is the fixed effect of the *m*th herd productivity ($m$ = low or high); $HTD_n(HP)_m$ is the random effect of the *n*th herd-date ($n$ = 1 to 41) within the *m*th herd productivity; $e_{ijklmno}$ is the residual random term.

Given that only one farm per day was visited once, herd effect is combined with date of sampling and with season. Thus, a herd-date effect (**HTD**) was included in the model. Moreover, due to the hierarchical structure of the experimental design, a 2-level nested model was used. The significance of the HP effect was tested on the error line of herd-date within herd productivity, and of the effects of DIM, parity, SCS and breed on the error line of the residual variance. Herd-date and residuals were assumed to be normally distributed with a mean of zero and variance $\sigma_h^2$ and $\sigma_e^2$, respectively. Proportion of variance explained by herd-date was calculated by dividing the corresponding variance component by the total variance.

Except for herd productivity, orthogonal and polynomial contrasts ($P$ < 0.05) were estimated between least squares means of traits for all effects included in the model. To compare breeds, the following contrasts were used: a) specialized (HF, BS and JER) vs. dual-purpose breeds (SI, REN and GA); within specialized, b) HF + BS vs. JER and c) HF vs. BS; within dual-purpose, d) SI vs. REN + GA and e) REN vs. GA. Orthogonal contrasts were estimated also for the parity effect, as follow: a) parity 1 vs. (parities 2 + 3 + ≥ 4); b) parity 2 vs. (parities 3 + ≥ 4) and c) parity 3 vs. parity ≥ 4. For the effects of DIM and SCS first order comparisons measured linear relationships, while second and third order comparisons measured quadratic and cubic relationships, respectively. According to the contrasts results, linear, quadratic or cubic trendlines were then reported in the figures, together with equation and coefficient of determination ($R^2$) of the regression and *P*-value of the polynomial contrast.

Pearson product-moment correlations between blood serum proteins and milk yield and composition (i.e., fat, protein, casein, casein number, lactose, urea, pH and SCS) were estimated using the CORR procedure of SAS (SAS Institute Inc., Cary, NC). The analysis was carried out using residuals extracted from model 1, removing SCS as explanatory variable.

## RESULTS

### *Descriptive Statistics of Herd Productivity, Breeds and Blood Serum Proteins*

Table 1 reports a profile of low and high producing herds, with descriptive statistics regarding milk production and composition. Low producing herds had on average 28 cows/herd and a "Traditional" dairy system. High producing herds were mostly "Modern" farms with on average 46 cows/herd A detailed description of the different dairy farming systems has been reported in Sturaro et al. (2013). Holstein Friesian, BS and SI were present in farms with both high and low production, while JER was found only in high producing herds and REN and GA were found only in the herds classified as having low production. A preliminary study of the effect of herd productivity and breed on milk production and composition has been carried out by Stocco et al. (2016b) using the same dataset. Briefly, as expected, based on the herd classification system, milk yield of cows in high producing herds, was almost 10 kg greater than milk yield of cows in low producing herds ($P < 0.05$). Herds with high production exhibited also greater fat, protein and casein percentages and lower urea content in milk in comparison to the herds of the other group (Stocco et al., 2016b).

The average milk yield of HF cows was more than double that of the GA cows (Table 2). This difference is due also to the effect of the herd productivity and of individual herds. The analysis carried out by Stocco et al. (2016b) have shown that "within herd" the difference between these two breeds, still highly significant ($P < 0.05$), is reduced to 7.2 kg/d. Milk from

JER cows was characterized by the greatest concentration of fat, protein and casein, but had the least lactose percentages and pH values. Conversely, REN produced the milk with the lowest percentages in fat, protein and casein, but the greatest lactose content and pH. Milk urea ranged from 21.5 mg/100g (HF) to 37.5 mg/100g (JER).

The Figure 1 gives the mean, standard deviation, and corresponding distributions of raw data and kurtosis and skewness estimated using model residuals. Serum total protein content averaged 74.12 g/L, which corresponds approximately to the sum of albumin and globulin contents (30.81 and 43.23 g/L, respectively). Albumin-to-globulin ratio ranged from 0.39 to 1.03, with a mean value of 0.72. Coefficient of variation of all serum traits ranged from 6 to 15%. All the traits were almost normally distributed (Figure 1), with kurtosis and skewness values (estimated using model residuals) close to zero.

***Sources of Variation of the Blood Serum Proteins***

The proportion of variance explained by herd-date was approximately 20-25% for all the blood protein profile traits (Table 3). With the exception of herd productivity (only associated with albumin concentration), associations between blood serum proteins and all explanatory variables were observed.

After adjusting for herd-date, breed, parity, DIM and milk SCS, serum albumin concentration was greater in cows from herds classified as high than in cows from low producing herds (Figure 2).

Breed was an important source of variation for the blood serum proteins (Table 3). As compared to dual-purpose breeds (SI, REN and GA), on average cows of specialized dairy breeds (HF, BS and JER) had greater concentrations of total protein and globulin, and a lower A:G (Table 3 and Figure 3). Within the two groups, the differences among individual breeds were often greater than between groups. Among specialized dairy breeds, serum protein of

JER contained less globulin and very high albumin, resulting in a greater A:G (Table 3 and Figure 3) compared with the two larger sized dairy breeds. Between this two breeds, serum protein and globulin concentrations were greatest for HF, resulting in the lowest A:G. Among dual-purpose breeds, serum protein and globulin concentrations were greater for SI (resulting in a lower A:G) as compared to the other two local breeds (REN and GA), that did not present any appreciable difference in protein pattern (Table 3 and Figure 3).

Total serum protein increased for the first one-third of lactation and then decreased (Figure 4a) for the remainder of lactation. Albumin levels in blood increased following a quadratic pattern with stage of lactation, while globulin content decreased linearly (-2%) (Table 3 and Figure 4b,c). As a consequence, A:G increased linearly (+5%) (Table 3 and Figure 4d).

Parity was positively associated with all traits (Table 3). Whereas albumin concentration slightly increased from primiparous to multiparous cows, serum globulin increased markedly (+7%) at the increasing of parity number of cows (Figure 5), causing a parallel increase (+4%) of total protein and a decrease (-6%) of A:G ($P = 0.01$).

Serum albumin decreased slightly (-2%) with SCS (Table 3 and Figure 6) whereas globulin (and total protein) increased approximately 8% (and 4%). The decrease in albumin levels, together with the rise in globulin content, resulted in a strong decline (-9%) in A:G (Figure 6).

***Relationships between Blood Serum Proteins, Milk Yield and Composition***

The Pearson product-moment correlation coefficients, based on model residuals (according to the model which included the days in milk, parity, breed, herd productivity, and the herd-date within herd productivity) between blood serum proteins, milk yield and composition are presented in Table 4. Results showed that, after correction for the most

important sources of variations, coefficients of correlations were almost close to zero for all variables, except for those related to blood serum proteins and SCS ($P < 0.05$).

## DISCUSSION

The analysis of the concentration of proteins in serum represents a developing area of clinical biochemistry for disease diagnosis (Eckersall, 2008), as it provides valuable diagnostic information for assessing infection, inflammation and trauma in animals (Murata et al., 2004). For instance, haptoglobin is recognized as a marker for presence, severity and recovery of several diseases in cattle, including mastitis, endometritis, enteritis, peritonitis, fatty liver, pneumonia and endocarditis (Petersen et al., 2004). Both haptoglobin and serum amyloid A are useful diagnostic tool for discriminate between chronic and acute inflammation (Horadagoda et al., 1999). Furthermore, it has been reported that total protein and albumin changes in blood of cows affected by paratuberculosis are characteristic of the clinical stage of the disease (Whitlock and Buergelt, 1996).

The present study demonstrated that several factors not associated with disease (not only individual cow factors, but also breed and herd-related factors) can affect the variation of blood serum proteins and must be accounted for in order to appropriately interpret blood serum proteins as a tool to assess the health status of dairy cows. As previously reported in literature (Kitchenham and Rowlands, 1976; Eckersall, 2008; Cozzi et al., 2011), globulin fraction was obtained subtracting albumin from total protein concentration and A:G was then calculated, in order to assess the relative contribution of each fraction to total protein. Detailed analysis of protein fractions was not performed in the present study and we are aware that this represents a weak point of our study.

***Association between Herd Productivity and Blood Serum Proteins***

After adjusting for breed and cow factors, we observed greater albumin concentrations in serum of cows in higher producing herds (Figure 2). Classification of herds as low or high producers was based on the average daily milk energy yield. However, herd classification also includes many potential confounding factors. High producing herds are larger farms characterized more frequently by a "Modern" dairy system, where animals could move freely in newer buildings, were milked in a parlor and were fed using a silage based TMR. In contrast, low producing herds are primarily based on more "Traditional" dairy management, with tied animals housed in old buildings, fed using local forages and eventually moved to highland pastures during summer. Nevertheless, comparisons between herd productivity levels were performed after adjusting for the major confounding effect (breed of cows). Moreover, as the sample size was not adequate to compare the different dairy systems and, within each dairy system, differences in management and productivity were observed, we decided to classify the farms on the basis of daily milk energy yield. Preliminary analysis showed that structural and management factors were not able to singularly explain relevant production differences, after adjusting for breed effect. Thus, herd classification was based on the output (milk energy production of cows) rather than on input factors (e.g., management, diet). It is however known that differences in diet explain many differences in herd production level (Walsh et al., 2008) and, in our study, herds classified as high producers were fed almost twice the amount of concentrates as compared to low producing herds, independently of the dairy system. The classification of farms into two categories of productivity allowed the separation of a portion of variation due to the production efficiency of herds, thus reducing the confounding effect of herd-date.

Concentrations of serum albumin similar to our results have been reported by Rowlands et al. (1977). In that study, cows with higher milk production (>30 kg/d) had

greater albumin concentration in comparison to cows with a daily milk yield <15 kg/d (32.2 g/L vs. 29 g/L, respectively). However, as individual intake data were not available, it was not possible for the authors to attribute the observed results to insufficient dietary protein intake. The effect of dietary crude protein intake on albumin concentration in blood has been reported by previous authors (Lee et al., 1978). Hoffman et al. (2001) reported that Holstein heifers fed a diet in which the proportion of crude protein was increased from 8 to 15% had higher serum protein and albumin levels, while A:G was not affected by nutrition. Raggio et al. (2007) observed a 4% increase in serum albumin concentration comparing Holstein cows fed with a high metabolizable protein diet to cows fed with a low metabolizable protein diet. In a recent study (Law et al., 2009), increasing dietary crude protein concentration from 144 to 173 g/kg of DM significantly increased plasma albumin and total protein levels in Holstein cows. In this perspective, we can hypothesize that the diets administered in modern high producing herds could influence the level of albumin in blood.

***Association between Breed and Blood Serum Proteins***

All cows were housed in multi-breed farms. This allowed the comparison of different breeds reared in the same conditions for most environmental treatments, such as herd management, feeding practices, health management. As environmental and individual cow effects were included in the statistical model, we hypothesized that breed differences (Figure 3) could be associated with individual genetic variation and also be explained by the different selective breeding programs to which breeds have been subjected. Selection of bulls for increased milk production has been a key breeding goal for HF cows (Miglior et al., 2005); dual-purpose breeds are instead characterized by lower selective pressures for milk yield and are considered to have greater robustness, longevity, fertility and adaptability to the mountain environment, as compared to dairy breeds.

Most previous studies about blood serum proteins have been performed using HF cattle (Chorfi et al., 2004; Cozzi et al. 2011; Piccione et al., 2011). Therefore, literature investigations about breed-induced variation in blood traits are scarce, not recently published and thus related to different environmental and genetic backgrounds. The effect of breed on blood traits of SI, BS, Holstein and SI × Holstein crossbred cows was not detected by Blum et al. (1983), but the authors attributed the failure to insufficient statistical power. Kitchenham and Rowlands (1976) reported differences in total serum protein and albumin concentration among Friesian, Ayrshire and Friesian × Ayrshire crossbred cows in a single herd. Serum albumin concentration was least for crossbreed cows, while serum total protein level was greatest in Friesians. Shaffer et al. (1981) reported that, as compared to Guernsey, Jersey and Brown Swiss cows reared in the same single herd, Holstein cows had greatest concentrations of serum total protein and globulin, and lowest albumin and A:G. Jersey was the breed with highest albumin and A:G; Brown Swiss the one with lowest total protein and globulin values. Different globulin concentrations were found by Gibson et al. (1987), comparing British Friesian and JE cows and those differences were attributed by the authors to differences in immunoglobulin G2 levels, previously reported for a subset of the same animals. Aeberhard et al. (2001) found higher plasma albumin level in the Simmental × Red Holstein crossbred than in BS and HF cows. However, the comparisons of the study of Aeberhard et al. (2001) were confounded by potential herd effects as different breeds were housed in different farms.

***Association between DIM and Blood Serum Proteins***

Changes in blood serum proteins are expected during pregnancy, parturition and lactation (Eckersall, 2008). In dairy cows the period of transition from end of gestation to early lactation is characterized by physiological and metabolic changes, including mobilization of body fat and protein, and changes of several blood traits like glucose, β-

hydroxybutyric acide and nonesterified fatty acids (Van Dorland et al., 2009). During the periparturient period cows experience hormone changes, alterations in defense mechanisms, physical and metabolic stress (Mallard et al., 1998). For this reason, albumin, which is synthesized by the liver, was lower at the beginning of lactation due to negative energy balance and it increased with a quadratic trend as metabolic state improved after parturition and during the lactation period (Figure 4b). In particular, since albumin represents an important pool of amino acids, its concentration could decrease around parturition to supply protein precursors (Cornelius and Kaneko, 1963). Serum albumin concentration can also be affected by fatty liver, a frequent disorder in early lactation (Sevinc et al., 2001). However, in the present study, specific indicators of hepatic function were not analyzed. The tendency for cows to have lower serum albumin concentrations shortly after calving has been confirmed by other authors (Seifi et al., 2007). A rise in albumin concentration between 15 and 90 d after calving was previously reported also by Rowlands et al. (1975). Conversely, globulin is generally low in plasma at the end of pregnancy because γ-globulins are transferred from the blood to the mammary gland and are lost in the colostrum (Weaver et al., 2000). Previous authors (Larson and Kendall, 1957) observed a 10-30% decrease in serum protein levels of bovine plasma prior to parturition, due to the loss of $\beta_2$- and $\gamma_1$-globulins in colostrum. In the present study, at the beginning of lactation serum globulin was increased (possibly due to an increase of α-globulins), as a physiological response to parturition, which linearly decreased with the advance of DIM (Figure 4c).

Lower total protein values around parturition were reported by Blum et al. (1983); because albumin did not significantly change, the fall in total protein levels was due to a decrease in globulin, lost in the colostrum. At the onset of lactation, total protein increased rapidly, reaching the highest levels between 30 and 100 d and then decreasing slightly. Thus, as confirmed by our results, variation of total protein content during lactation follows a trend

comparable to that reported for the variation of milk yield, and significant positive correlations with milk yield ($r > 0.30$) were estimated (Blum et al., 1983). Similar variation in blood serum proteins concentration during lactation was described also by Aeberhard et al. (2001). Piccione et al. (2011) reported that stage of gestation and lactation affected serum total protein and globulins ($\alpha 1$, $\beta$ and $\gamma$) content and A:G of five HF cows, particularly during the transition from late gestation to early lactation, when cows must typically cope with a pronounced metabolic stress. Conversely, Cozzi et al. (2011) did not find any effect of stage of lactation when comparing total protein, albumin and globulin content of plasma from HF cows in early and mid-lactation.

***Association between Parity and Blood Serum Proteins***

Total protein concentration increased at the increasing of parity, mainly because of an increase in globulin content (Figure 5a,c). This is expected, as the immune system of older cows has been in contact with more pathogens and their antibodies are elevated (Larson and Touchberry, 1959; Eckersall, 2008). Greater globulin levels might be an indicator of good immunization of the animal. However, globulin comprises not only immunoglobulins, but also acute phase proteins, which might increase during inflammation (Eckersall and Bell, 2010). Nevertheless, in the present study, a detailed analysis of globulin fractions was not performed. Conversely, albumin level was only slightly affected by parity and seems to be more related to diet and health status of the animal rather than age.

The pattern of change of blood serum proteins with parity observed in this study is consistent with results reported by Kitchenham and Rowlands (1976) and by Shaffer et al. (1981). Conversely, Alberghina et al. (2011) did not find relationships between the age of Modicana cows and their total protein and albumin serum concentrations, and A:G, whereas the same authors observed greater content of plasma globulin in older cows (significant effect

on α and β fractions, but not on the γ). Alberghina et al. (2010) reported age-related differences also on serum protein fractions of goat, with older animals (5-12 years) showing greater total protein and α-globulins and lower albumin content and A:G. Effect of parity on blood serum proteins has been evidenced also by Cozzi et al. (2011), who reported a trend of plasma total proteins and globulins with age comparable with that observed in the present study.

***Association between SCC and Blood Serum Proteins***

In the present study we observed a linear relationship between SCS, standard indicator of mammary gland inflammation, and blood serum proteins (Figure 6). Namely, both total protein and globulin plasma contents increased with increasing SCS (Table 4). An increase of globulin with increasing SCS may be expected, as several immunoglobulin isotypes are involved in the immune response, both enhancing phagocytosis by macrophages and neutrophils (IgG1, IgG2 and IgM), or preventing the spread of bacteria in the mammary gland (IgA) (Korhonen et al., 2000). However, the globulin fraction contains many different proteins. In the present study, it is not clear whether the increase in globulin is related to an increase in γ-globulins or to an increase in α- and β-globulins. Milk SCS exhibited an inverse relationship with serum albumin content (Figure 6b). High albumin content is associated with low inflammation rate, and in case of infection albumin synthesis in the liver is expected to decrease in order to favour globulin production (Bertoni et al., 2008). The decrease of albumin in blood during mammary infections is due not only to a lower biosynthesis, but also to the damage of the blood- milk barrier and the leakage from the blood to the milk (Kitchen, 1981). The rise in globulin content, together with the decrease in albumin levels, resulted in a decline in A:G with the increase of SCC in milk (Figure 6). A lower A:G was observed by Gain et al. (2015) comparing 10 cows with subclinical mastitis, characterized by a statistically

higher milk SCC, and 10 healthy cows (0.40 vs 1.39, respectively). The decrease in A:G, as the increment in globulin content, is particularly visible from class 5 of SCS (which corresponds to SCC between 123,000 and 237,000 cells/mL); we can speculate that the first 4 classes are not different due to overlapping of standard error bars (Figure 6c). To notice, cows with composite milk SCC >200.000 cells/mL are considered to have subclinical mastitis (Ruegg and Pantoja, 2013). Thus, elevated SCC and low A:G could indicate an inflammatory status.

## CONCLUSIONS

In conclusion, several factors must be considered to appropriately interpret blood serum proteins as animal welfare indicators. Cows in high producing herds, characterized by presence of high yielding cows and by larger use of dietary concentrates, had greater serum albumin concentrations. Breed differences were observed for all traits, highlighting a possible genetic mechanism. The specialized dairy breed JE and the two dual-purpose local breeds (GA and REN) had the lowest globulin content and greatest A:G. Changes in blood serum proteins were observed throughout the entire lactation. Older cows had greater total protein and globulin contents, while albumin was only slightly affected by age. Linear relationships between blood serum proteins and SCS were reported and supported also by correlation estimates, confirming the important role of SCC as an indicator of mammary gland inflammation and highlighting the potential use of blood serum proteins as indicators of immune response of the mammary gland to infections. High milk SCS was associated with greater total protein and globulin contents in blood. The rise in globulin content, together with a decrease in albumin levels, resulted in a decline in A:G as SCC of milk increased. Nevertheless, repeated measures would be more informative about the inflammatory status and might be helpful in clarifying the relationships between blood serum proteins and SCC

and milk traits that are known to be affected by high SCC (e.g., milk yield, casein number and lactose).

Besides clinical relevance, the evaluation of non-genetic sources of variation of blood serum proteins in dairy cattle represents an important first-step for future analysis (especially integration of metabolomics, proteomic and genomic information for improving the robustness of dairy cows). Therefore, further studies to elucidate the role of the genetic background in explaining variation in blood serum proteins will be carried out.

## ACKNOWLEDGMENTS

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## TABLES AND FIGURES

**Table 1.** Characteristics of multi-breed herds in north-eastern Italy based on classification as low or high productivity[1]

| Item | Low productivity | High productivity |
|---|---|---|
| Number of herds | 21 | 20 |
| Number of cows | 588 | 920 |
| Average number of cows/herd | 28 | 46 |
| Dairy system | | |
| Traditional with summer pastures | 9 | 0 |
| Traditional without summer pastures | 6 | 5 |
| Traditional with silages | 0 | 2 |
| Modern | 6 | 13 |
| Utilized agricultural area, ha[2] | 24.0±13.2 | 38.2±26.3 |
| Concentrates, kg/d[2] | 6.7±2.8 | 19.9±4.9 |
| Breeds[3] | HF, BS, SI, REN, GA | HF, BS, JER, SI |
| Milk yield, kg/d[2] | 18.5±6.9 | 28.0±8.3 |
| Milk composition[2] | | |
| Fat, % | 3.75±0.80 | 4.19±1.00 |
| Protein, % | 3.48±0.50 | 3.73±0.48 |
| Casein, % | 2.73±0.36 | 2.92±0.37 |
| Casein number[4] | 0.79±0.01 | 0.78±0.01 |
| Lactose, % | 4.85±0.24 | 4.84±0.23 |
| Urea, mg/100g | 29.2±9.9 | 21.7±7.9 |
| pH | 6.51±0.10 | 6.51±0.11 |
| SCS[5], units | 2.79±1.94 | 2.89±1.81 |
| Milk energy production, MJ/d | 57.33 | 90.86 |

[1]according to average daily milk energy yield of the cows (Tyrrell and Reid, 1965) corrected for breed, DIM and parity.

[2] Mean±SD

[3]Holstein Friesian (HF), Brown Swiss (BS), Jersey (JER), Simmental (SI), Rendena (REN) and Grey Alpine (GA).

[4]Casein number = casein/protein.

[5]SCS = $\log_2$ (SCC/100,000) + 3

**Table 2.** Descriptive statistics of milk yield and composition by breed of cows on 41 mixed breed farms in north-eastern Italy[1]

| | HF | BS | JER | SI | REN | GA |
|---|---|---|---|---|---|---|
| Number of cows | 471 | 663 | 40 | 158 | 103 | 73 |
| Milk yield, kg/d[2] | 28.4±9.8 | 25.0±7.7 | 21.1±4.8 | 20.2±6.8 | 16.9±5.7 | 13.1±4.8 |
| Milk composition[2] | | | | | | |
| Fat, % | 4.02±1.00 | 4.12±0.84 | 6.04±0.65 | 4.17±1.12 | 3.28±0.64 | 3.57±0.76 |
| Protein, % | 3.51±0.56 | 3.80±0.44 | 4.09±0.74 | 3.49±0.43 | 3.26±0.36 | 3.49±0.38 |
| Casein, % | 2.75±0.42 | 2.98±0.33 | 3.23±0.54 | 2.74±0.32 | 2.57±0.28 | 2.75±0.28 |
| Casein number[3] | 0.79±0.01 | 0.78±0.01 | 0.79±0.02 | 0.79±0.01 | 0.79±0.01 | 0.79±0.02 |
| Lactose, % | 4.83±0.26 | 4.84±0.22 | 4.77±0.19 | 4.83±0.21 | 4.91±0.28 | 4.89±0.22 |
| Urea, mg/100g | 21.5±7.9 | 25.8±9.8 | 37.5±11.0 | 27.7±10.9 | 25.2±6.8 | 30.5±9.7 |
| pH | 6.51±0.09 | 6.51±0.10 | 6.48±0.09 | 6.49±0.13 | 6.53±0.09 | 6.52±0.10 |
| SCS[4], units | 3.05±1.94 | 2.89±1.80 | 2.70±1.73 | 2.37±1.78 | 2.61±1.75 | 2.60±2.12 |

[1]Holstein Friesian (HF), Brown Swiss (BS), Jersey (JER), Simmental (SI), Rendena (REN) and Grey Alpine (GA).

[2]Mean±SD.

[3]Casein number = casein/protein.

[4]SCS = $\log_2$ (SCC/100,000) + 3

**Table 3.** Results from ANOVA (*F*-value and significance) for blood serum proteins

| Effect | Total protein, g/L | Albumin, g/L | Globulin, g/L | Albumin:Globulin |
|---|---|---|---|---|
| Production Level[1] | 3.06 | 12.82*** | 0.14 | 1.73 |
| HTD, %[2] | 21.6 | 24.5 | 19.9 | 20.9 |
| Breed | 7.24*** | 5.45*** | 10.25*** | 12.88*** |
| *Contrasts*[3] | | | | |
| *HF+BS+JER vs. SI+REN+GA* | 6.11* | 0.32 | 6.31* | 7.09** |
| *HF+BS vs. JER* | 0.15 | 14.40*** | 4.90* | 13.52*** |
| *HF vs. BS* | 14.87*** | 0.06 | 16.84*** | 11.94*** |
| *SI vs. REN+GA* | 17.61*** | 0.10 | 18.38*** | 15.59*** |
| *REN vs. GA* | 0.48 | 0.18 | 1.08 | 0.96 |
| DIM | 2.66* | 9.61*** | 2.64* | 5.15*** |
| *Contrasts* | | | | |
| *Linear* | 1.89 | 34.96*** | 10.02** | 25.70*** |
| *Quadratic* | 5.94* | 5.15* | 2.33 | 0.07 |
| *Cubic* | 4.88* | 3.12 | 1.78 | 0.30 |
| Parity | 30.11*** | 2.73* | 22.34*** | 14.71*** |
| *Contrasts* | | | | |
| *1 vs. (2+3+≥ 4)* | 71.09*** | 6.34* | 47.47*** | 24.65*** |
| *2 vs. (3+≥ 4)* | 17.57*** | 0.22 | 18.22*** | 15.52*** |
| *3 vs. ≥ 4* | 4.78* | 1.50 | 4.15* | 5.21* |
| SCS[4] | 7.36*** | 3.19** | 9.84*** | 10.15*** |
| *Contrasts* | | | | |
| *Linear* | 31.93*** | 14.85*** | 47.84*** | 50.86*** |
| *Quadratic* | 2.79 | 1.52 | 3.73 | 3.75 |
| *Cubic* | 0.04 | 0.00 | 0.06 | 0.55 |
| RMSE[5] | 4.54 | 1.67 | 4.68 | 0.09 |

[1]Herd's Production Level (low or high).

[2]Herd-date effect expressed as proportion of variance explained by herd-test date calculated by dividing the corresponding variance component by the total variance.

[3] Holstein Friesian (HF), Brown Swiss (BS), Jersey (JER), Simmental (SI), Rendena (REN) and Grey Alpine (GA).

[4]SCS = $\log_2$ (SCC/100,000) + 3.

[5]RMSE = root mean square error.

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

**Table 4.** Pearson product-moment correlations between blood serum proteins, milk yield and composition[1]

| Trait | Total protein, g/L | Albumin, g/L | Globulin, g/L | Albumin:Globulin |
|---|---|---|---|---|
| Milk yield, kg/d | -0.01$^{ns}$ | 0.11$^{***}$ | -0.04$^{ns}$ | 0.08$^{**}$ |
| Milk composition | | | | |
| Fat, % | 0.00$^{ns}$ | 0.00$^{ns}$ | 0.01$^{ns}$ | -0.02$^{ns}$ |
| Protein, % | -0.02$^{ns}$ | 0.04$^{ns}$ | -0.04$^{ns}$ | 0.05$^{ns}$ |
| Casein, % | -0.03$^{ns}$ | 0.05$^{ns}$ | -0.05$^{ns}$ | 0.06$^{*}$ |
| Casein number[2] | -0.06$^{ns}$ | 0.03$^{ns}$ | -0.07$^{*}$ | 0.07$^{*}$ |
| Lactose, % | 0.01$^{ns}$ | 0.02$^{ns}$ | -0.01$^{ns}$ | 0.02$^{ns}$ |
| Urea, mg/100g | 0.05$^{ns}$ | 0.05$^{ns}$ | 0.03$^{ns}$ | 0.00$^{ns}$ |
| pH | 0.04$^{ns}$ | 0.02$^{ns}$ | 0.04$^{ns}$ | -0.02$^{ns}$ |
| SCS[3], units | 0.15$^{***}$ | -0.10$^{***}$ | 0.18$^{***}$ | -0.19$^{***}$ |

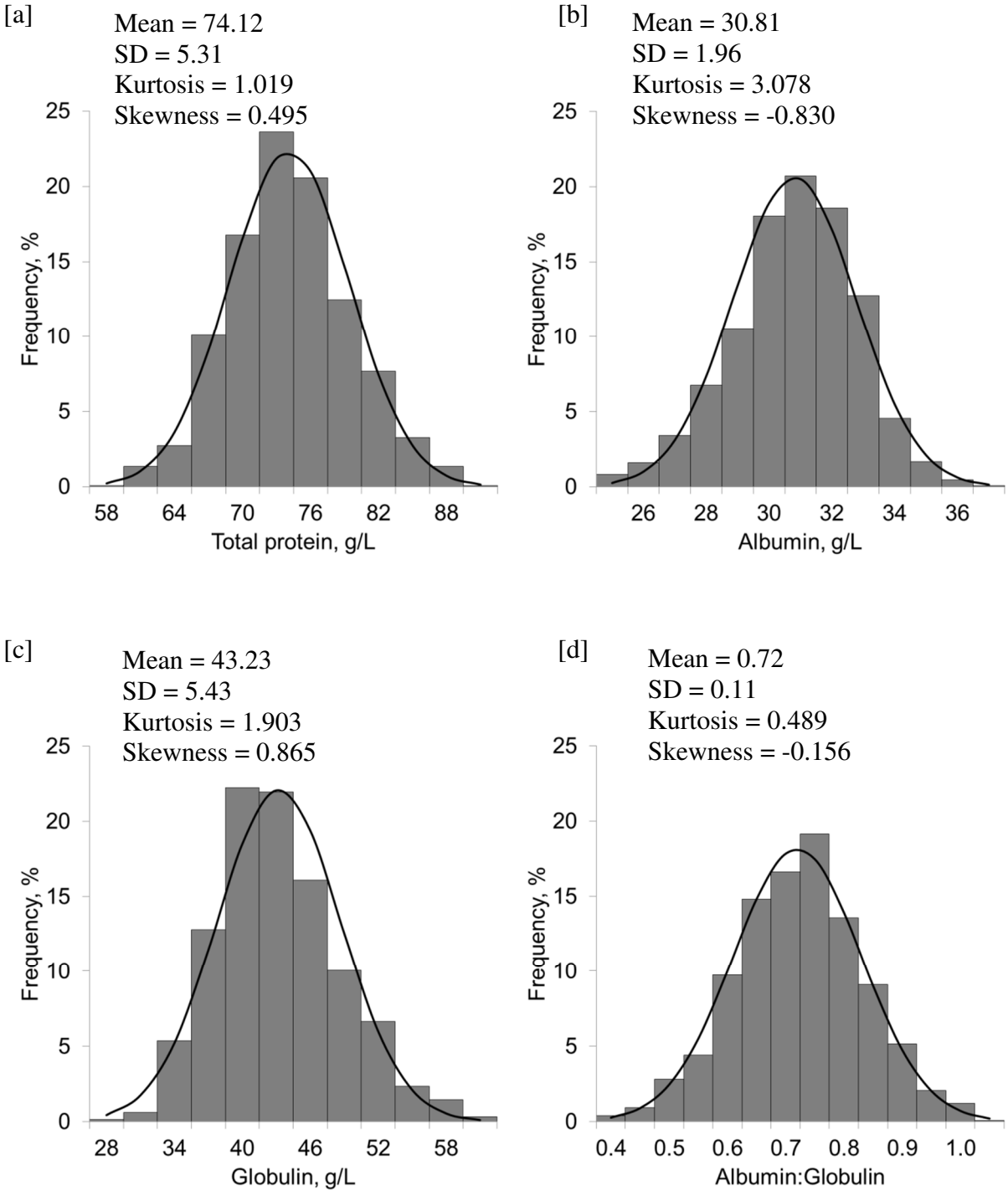
[1]correlations coefficients reported in the table were computed between model residuals (according to the model which included the days in milk, parity, breed, herd productivity, and the herd-date within herd productivity)

[2]Casein number = casein/protein

[3]SCS = $\log_2$ (SCC/100,000) + 3

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

**Figure 1.** Distribution, mean and standard deviation of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio[1]




[1]Skewness and kurtosis were estimated using model residuals.

**Figure 2.** Least square means and standard errors of albumin for herd productivity

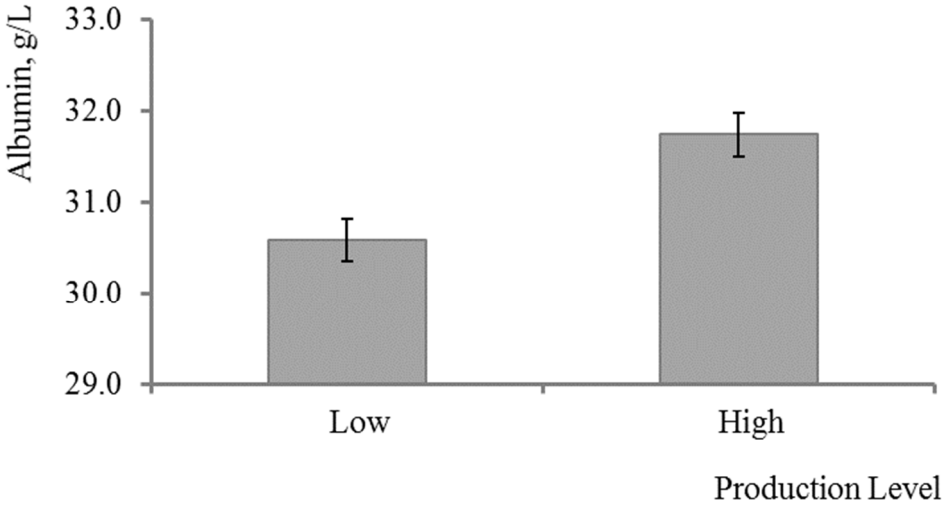
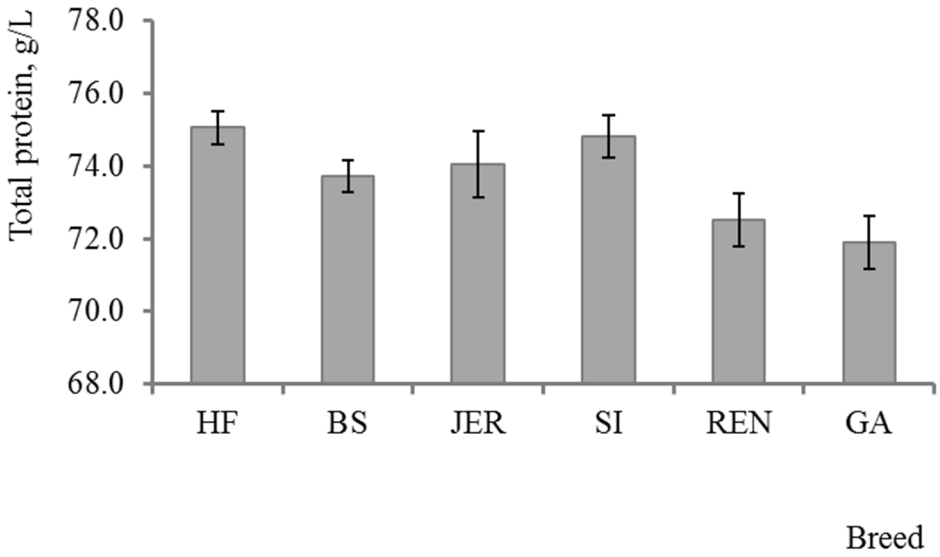
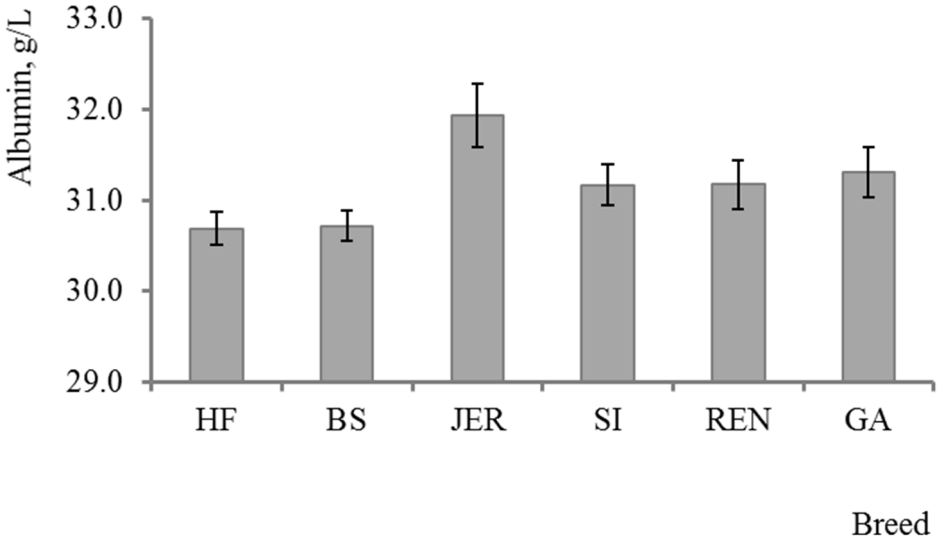


**Figure 3.** Least square means and standard errors of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio across breeds[1]

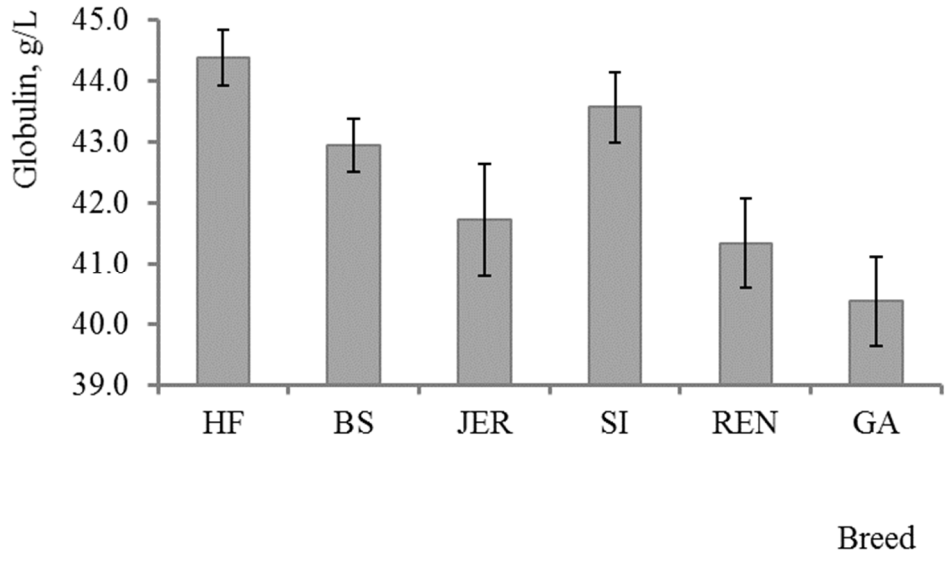
[a]



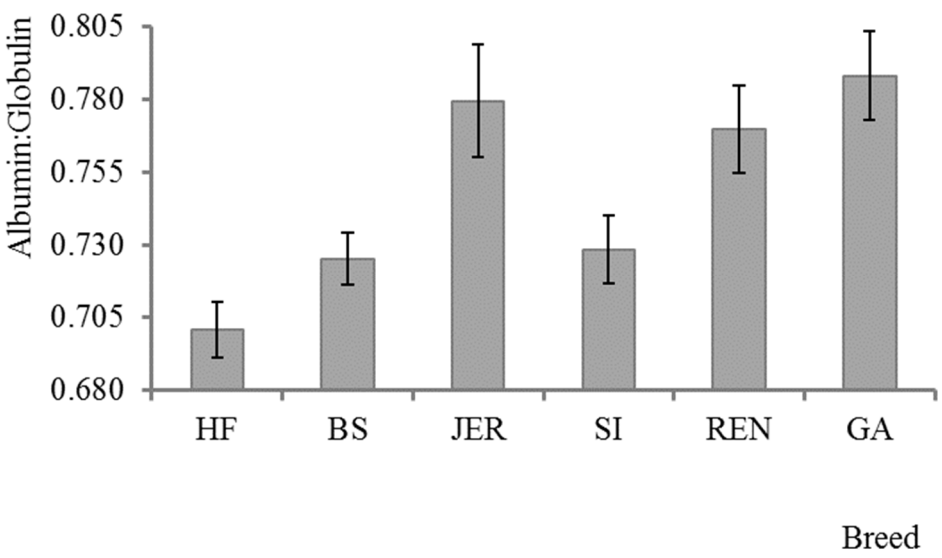

[b]




[c]



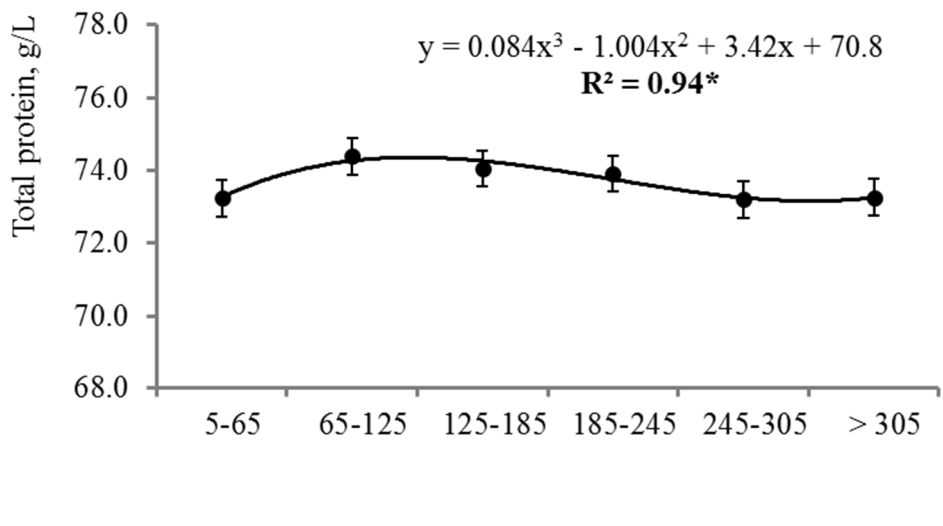
[d]



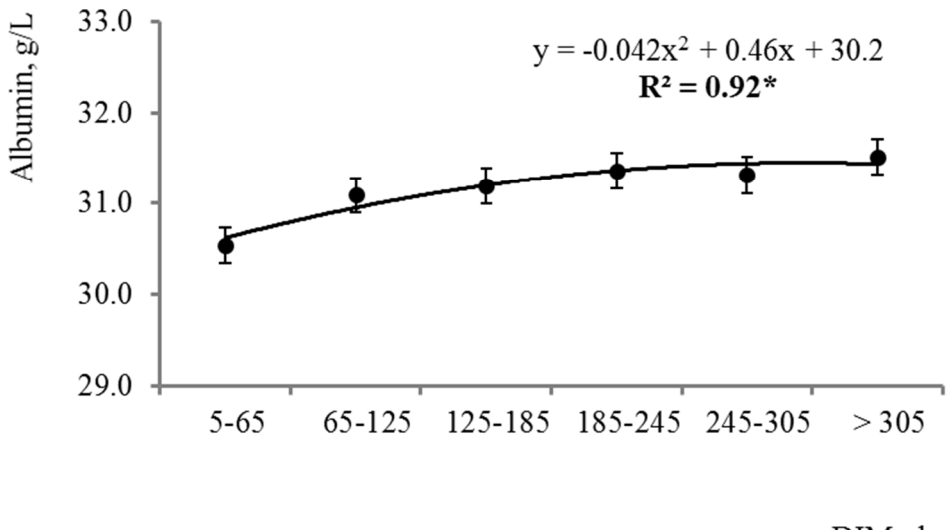

[1]Holstein Friesian (HF), Brown Swiss (BS), Jersey (JER), Simmental (SI), Rendena (REN) and Grey Alpine (GA).

**Figure 4.** Least square means and standard errors of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio across classes of DIM[1]

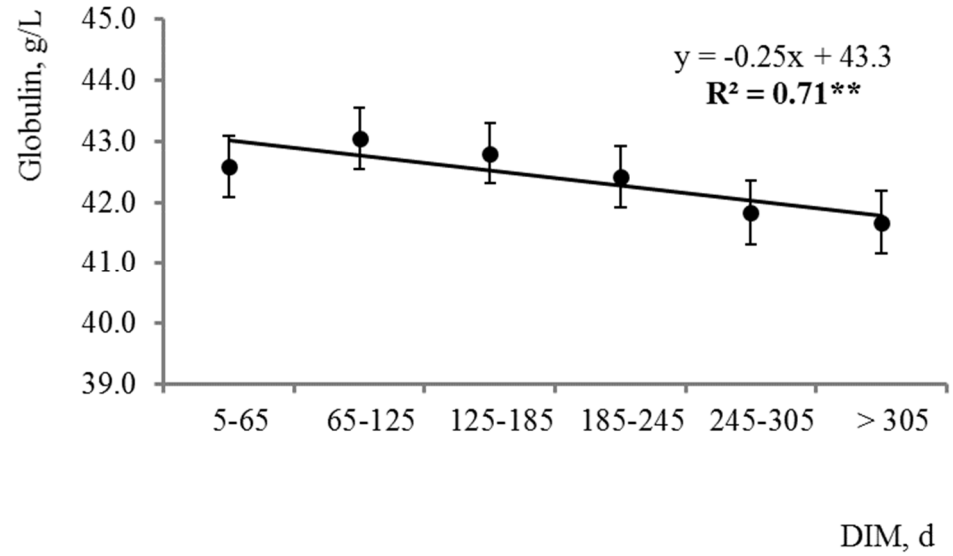
[a]



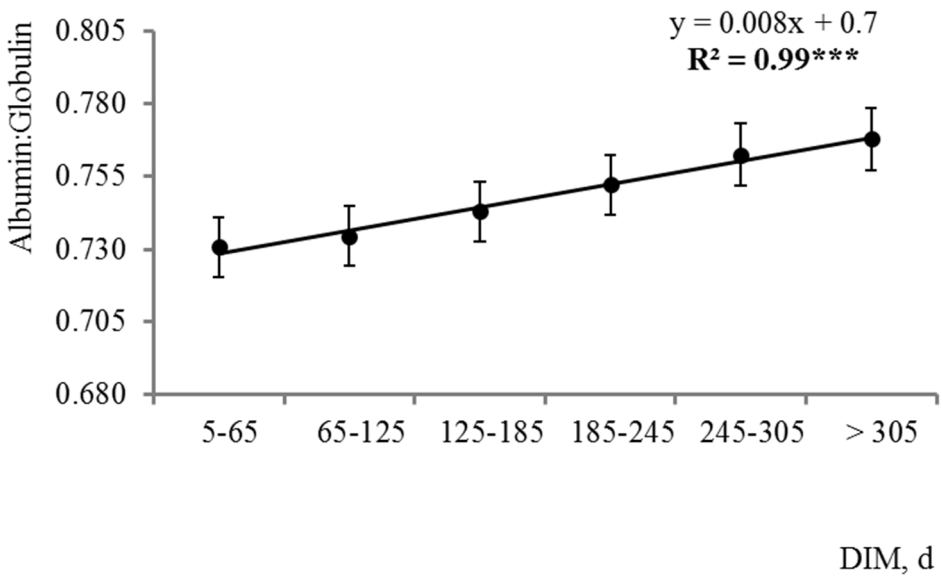

[b]




[c]



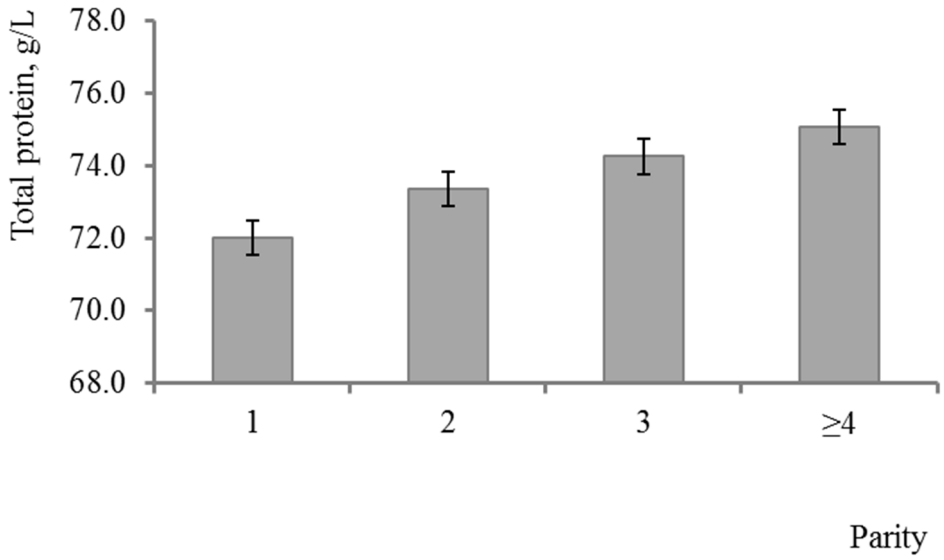
[d]



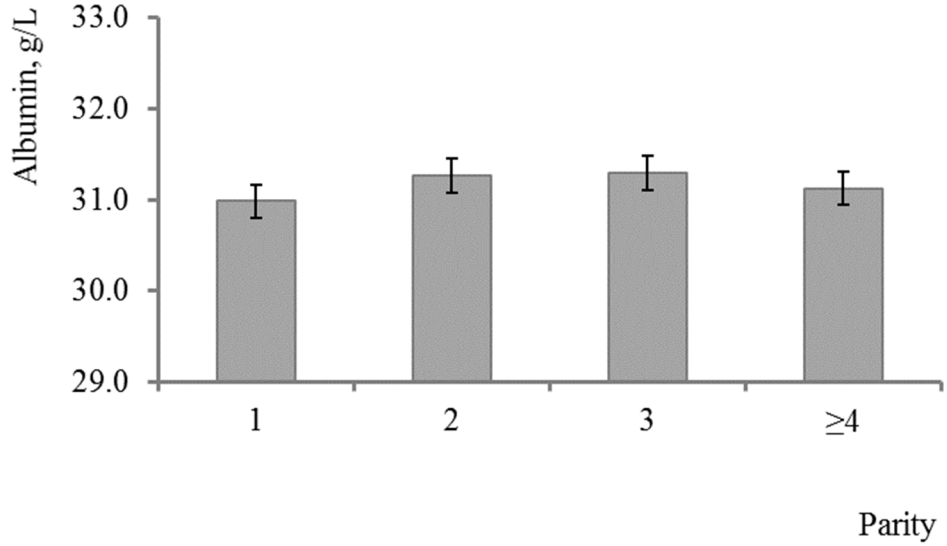

[1]Trendlines of data (linear, quadratic or cubic, according to the results of the polynomial contrasts reported in Table 3), equations and coefficients of determination ($R^2$) of the regression, and *P*-values of the polynomial contrasts reported in Table 3 (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) are shown.

**Figure 5.** Least square means and standard errors of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio across parities

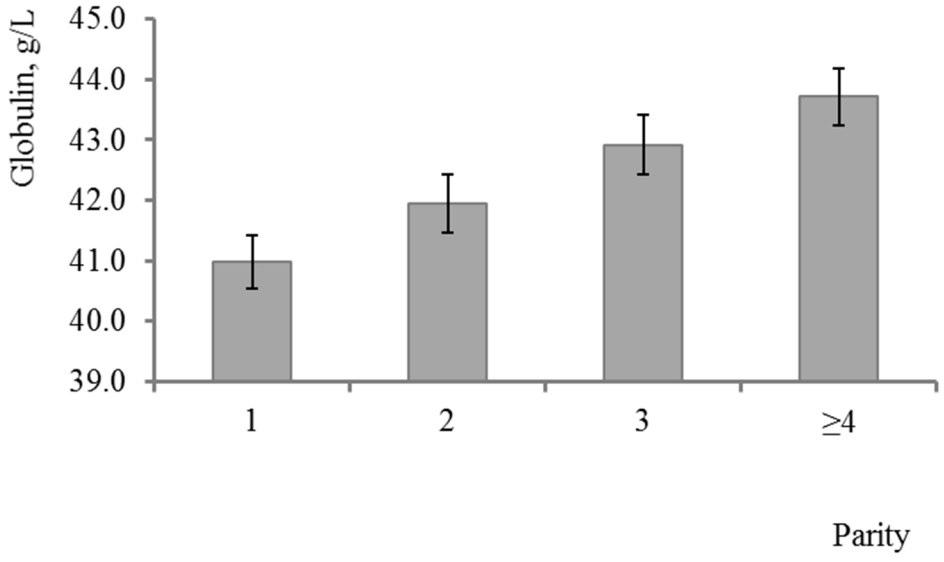
[a]




[b]




[c]



[d]

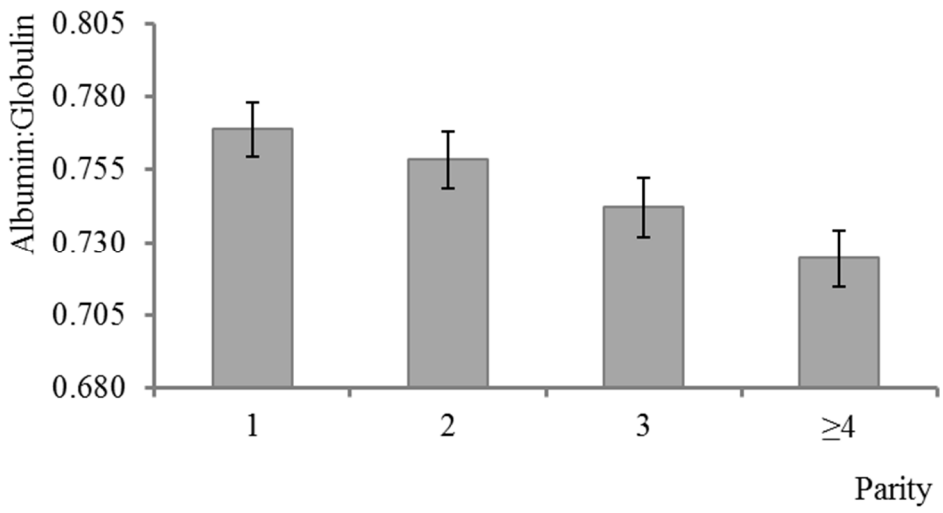
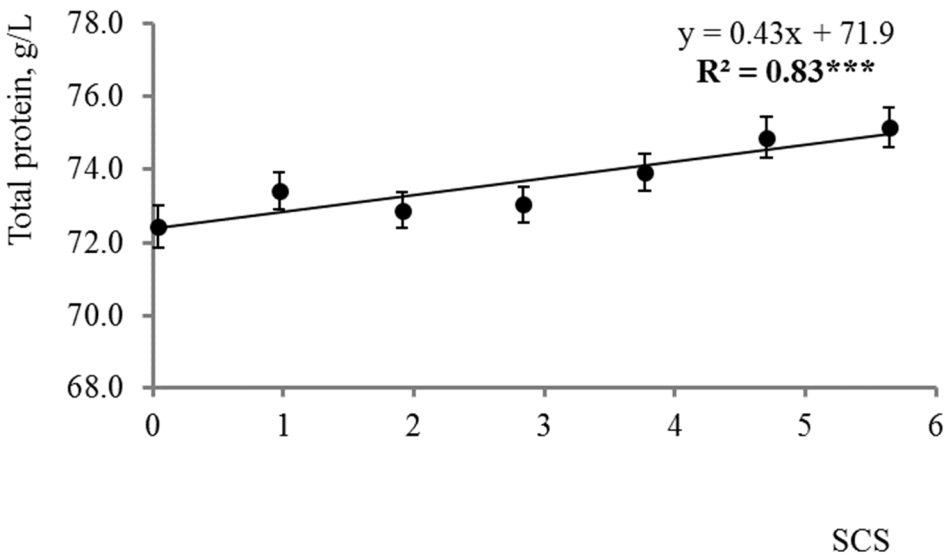
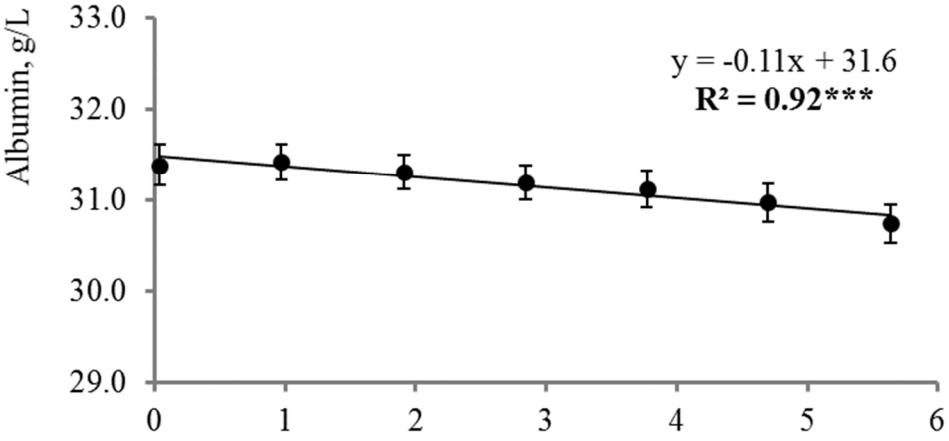


**Figure 6.** Least square means and standard errors of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio across classes of SCS[1]

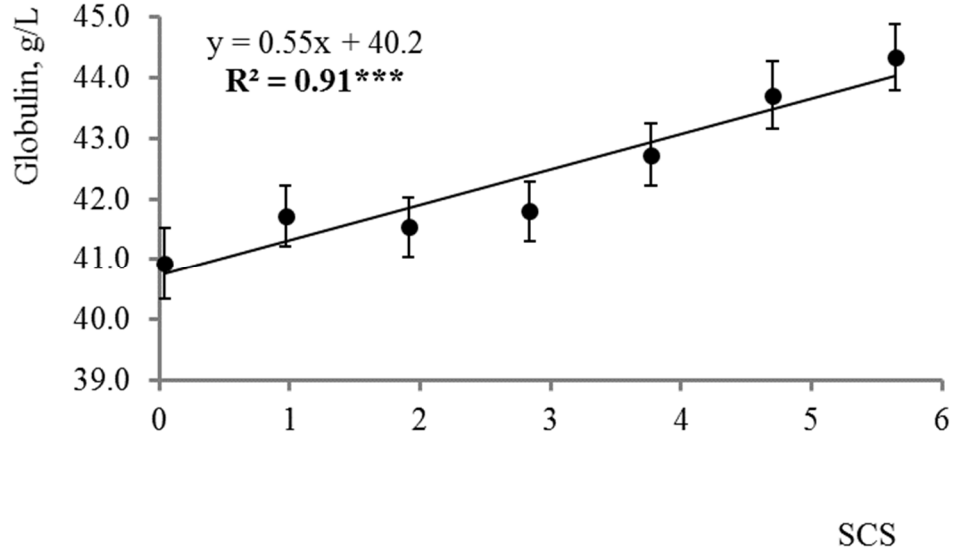
[a]



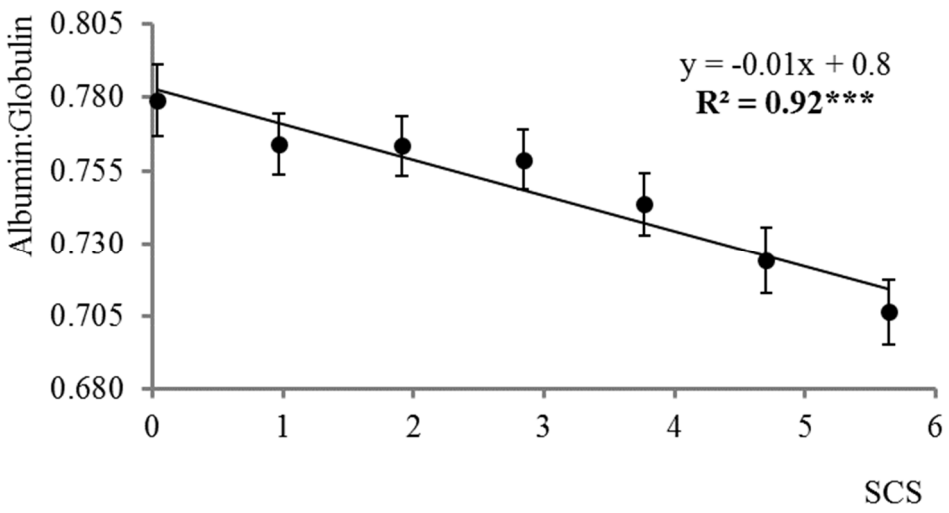

[b]




[c]



[d]




[1]Linear trendlines of data (according to the results of the polynomial contrasts reported in Table 3), equations and coefficients of determination ($R^2$) of the regression, and *P*-values of the polynomial contrasts reported in Table 3 (*** $P < 0.001$) are shown.

# CHAPTER 3.

## Association between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition and cheese-making traits in dairy cows

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**ABSTRACT**

The aim of this study was to investigate the association between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition and cheese-making traits. Forty-one multi-breed herds were selected for the study and composite milk samples were collected from 1,508 cows belonging to 3 specialized dairy breeds (Holstein Friesian, Brown Swiss, and Jersey) and 3 dual-purpose breeds of Alpine origin (Simmental, Rendena, and Grey Alpine). Milk composition [i.e., fat, protein, casein, lactose, pH, urea and somatic cell count (SCC)] was analyzed, and separation of protein fractions was performed by reversed-phase High Performance Liquid Chromatography. Eleven coagulation traits were measured: 5 traditional milk coagulation properties [time from rennet addition to milk gelation (RCT, min), curd-firming rate as the time to a curd firmness (CF) of 20 mm ($k_{20}$, min), and CF at 30, 45 and 60 minutes from rennet addition ($a_{30}$, $a_{45}$ and $a_{60}$, mm)], and 6 new curd firming and syneresis traits [potential asymptotical CF at an infinite time ($CF_P$, mm), curd-firming instant rate constant ($k_{CF}$, %×min$^{-1}$), curd syneresis instant rate constant ($k_{SR}$, %×min$^{-1}$), modeled RCT ($RCT_{eq}$, min), maximum CF value ($CF_{max}$, mm), and time at $CF_{max}$ ($t_{max}$, min)]. We also measured 3 cheese yield traits, expressing the weights of total fresh curd (%$CY_{CURD}$), dry matter (%$CY_{SOLIDS}$) and water (%$CY_{WATER}$) in the curd as percentage of the weight of the processed milk, and 4 nutrient recovery traits ($REC_{PROTEIN}$, $REC_{FAT}$, $REC_{SOLIDS}$ and $REC_{ENERGY}$), representing the percentage ratio between each nutrient in the curd and milk. Milk samples with SCC > 100,000 cells/mL were subjected to bacteriological examination. All samples were divided into 7 clusters of udder health (UH) status: Healthy (cows with milk SCC < 100,000 cells/mL and uncultured); culture-negative samples with low (No Growth_L), medium (No Growth_M) or high (No Growth_H) SCC; and culture-positive samples divided into Contagious, Environmental and Opportunistic IMI. Data were analyzed using a linear mixed model. Significant variations in the casein/protein ratio and lactose content were

observed in all culture-positive samples and in culture-negative samples with medium to high SCC compared with normal milk. No differences were observed among contagious, environmental and opportunistic pathogens, suggesting an effect of inflammation rather than infection. The greatest impairment in milk quantity and composition, clotting ability and cheese production was observed in the two UH status groups with the highest milk SCC (i.e., contagious IMI, and culture-negative samples with high SCC), revealing a discrepancy between the bacteriological results and inflammatory status, and thus confirming the importance of SCC as an indicator of udder health and milk quality.

**Key words**: subclinical mastitis, intramammary infection, milk composition, coagulation properties, cheese yield

## INTRODUCTION

Production of high-quality dairy products, especially of those cheeses labeled as Protected Designation of Origin (**PDO**) by the European Union, relies on the quality of the raw milk, which in turn is influenced by several environmental and individual cow factors, including health status (Laben, 1963). It is well known that bovine mastitis, an inflammatory response of the mammary gland to infection, decreases milk yield and quality, with considerable adverse economic effects on dairy farms (Seegers et al., 2003; Halasa et al., 2007). Mastitis, both in its clinical and subclinical (no visible clinical symptoms are present) forms, is characterized by an increase in milk SCC, which is recognized as the international standard measurement of udder health (**UH**) and milk quality (Harmon, 2001). The negative effect of a high SCC on the quantity and quality of milk and dairy products, regardless of the bacterial species causing mastitis, has already been widely reviewed in the literature (Kitchen et al., 1981; Auldist and Hubble, 1998; Sharif et al., 2008). A higher milk SCC is associated with lower milk production (Hortet and Seegers, 1998; Koldeweij et al., 1999; de los Campos et al., 2006), lower contents of casein (Haenlein et al., 1973; Urech et al., 1999; Mazal et al., 2007) and lactose (Auldist et al. 1995; Klei et al., 1998; Barlowska et al., 2009), and greater pH (Batavani et al., 2007; Vianna et al., 2008). The detrimental effect of a high SCC on milk composition has knock-on effects on the cheese-making process, with several studies reporting slower milk coagulation, weak curd consistency and lower cheese yields after processing milk with high SCC (Grandison and Ford, 1986; Politis and Ng-Kwai-Hang, 1988; Summer et al., 2015).

However, different IMI pathogens elicit different immune responses in the mammary gland (Bannerman et al., 2004). Differences have been observed in SCC variations (de Haas et al., 2002) and in milk quality (Leitner et al., 2006) according to the agent of infection. Therefore, identification of IMI pathogens is crucial to fully understanding the changes in

milk. The effect of different IMI bacteria (mostly recovered from clinical cases of mastitis) on milk production has been investigated in previous studies (Coulon et al., 2002; Gröhn et al., 2004; Schukken et al. 2009a). Despite the abundance of literature on the association between SCC and milk composition traits, few studies have dealt with the relationships between the changes in milk in cases of clinical and subclinical mastitis and specific causative agents. In particular, while some authors have observed pathogen-specific changes in milk composition (Coulon et al., 2002; Leitner et al. 2006; Chaneton et al., 2008), variations in milk clotting ability due to specific IMI pathogens have been reported only in a few studies (Leitner et al., 2006; Merin et al., 2008; Fleminger et al., 2011). However, current scientific knowledge is based mostly on quarter-level analysis performed on relatively small sample sizes for a small number of traits related to the technological properties of milk. Therefore, further investigations are required to gain a better understanding of the specific changes in milk (in terms of both composition and technological properties) during pathogen-specific cases of clinical and subclinical mastitis.

Direct measurements of the phenotypes related to the cheese-making process using a large sample size and at the individual cow level are expensive and time-consuming. However, a large dataset of different measures of individual cheese yields (**%CY**) and milk nutrient and energy recoveries in cheese (**REC**) taken at the lab level using a model cheese-making procedure (Cipolat-Gotet et al., 2013) recently became available. In a previous study (Bobbo et al., 2016), based on the aforementioned database, we reported linear and nonlinear relationships between SCS and milk yield and composition, traditional milk coagulation properties (**MCP**; developed by Annibaldi et al., 1977), and new technological traits related to cheese processing (i.e., curd firming and syneresis traits, %CY and REC).

The objective of the present study was to investigate the association between pathogen-specific cases of subclinical mastitis and milk yield, composition, protein

composition, coagulation properties and the aforementioned new technological traits in specialized dairy and dual-purpose cows housed in multi-breed herds. Rather than simply focus on microbiologically positive infections, we investigated both the impact of the intramammary infection and the impact of the resulting inflammatory response.

## MATERIALS AND METHODS

### *Milk Sample Collection*

This study is part of the Cowplus Project described in Chapter 2. Briefly, 41 multi-breed herds (with at least 2 breeds/herd) raised under the different dairy farming systems of Trentino province (north-eastern Italy) were selected from a sample of 610 dairy farms previously investigated (detailed environmental conditions are reported in Sturaro et al., 2013). Average herd size was 31 cows, ranging from 12 to 80 cows/herd. One farm per day was visited once during the study period (March-December 2013). Only clinically healthy animals at the time of the visit were selected. Health status was determined on the basis of rectal temperature, heart rate, respiratory profile, appetite and fecal consistency. Animals with obvious clinical symptoms of diseases (e.g., retained placenta, metritis, clinical mastitis, abomasal displacement, uterine prolapse, milk fever, clinical ketosis) were excluded from the trial. Milk samples were collected from 1,508 cows of 6 different breeds, 3 of which are specialized dairy breeds: Holstein Friesian (**HF**, n = 471), Brown Swiss (**BS**, n = 663), and Jersey (**JER**, n = 40), while the other 3 are dual-purpose breeds of Alpine origin: Simmental (**SI**, n = 158), Rendena (**REN**, n = 103), and Grey Alpine (**GA**, n = 73). Rendena and GA are local dual-purpose breeds with medium milk production, good functional traits, and greater adaptability to the mountain environment compared with the major breeds. During the evening milking, a milk sample (40 mL) was collected aseptically from each cow, according to National Mastitis Council guidelines (NMC, 1999), for bacteriological analyses. Briefly,

teat ends were cleaned externally with commercial pre-milking disinfectants by the veterinarian, dried with individual towels and then cleaned again with alcohol. After discarding the first streams of foremilk, approximately 10 mL of milk from each quarter was collected in sterile tubes, pooled, stored at 4°C and cultured within 24h of collection at the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy). A second sample, subsequently divided into two subsamples, was collected by trained technicians, maintained at a temperature of 4°C (without preservative) and processed within 24h of collection. One subsample (50 mL) was transferred to the Milk Quality Laboratory of the Provincial Federation of Breeders (Trento, Italy) for quality analysis. The other (2,000 mL) was taken to the Milk Laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padova (Italy) for analysis of cheese-making traits. In addition, two aliquots containing 1 mL of milk (with Bronopol, 2-bromo-2nitropropan-1,3-diol) taken from each sample were frozen at -20°C at the time of milk collection, transferred at -80°C to the DAFNAE Laboratory and kept there until protein composition analysis. Information on the herds and cows was obtained from the Provincial Federation of Breeders (Trento, Italy).

***Analysis of Composition Traits, Protein Composition, and Cheese-Making Traits***

***Milk Composition.*** Milk was analyzed within 24 h of collection for fat, protein, casein, lactose (%) and urea (mg/100g) using a Milkoscan FT6000 (Foss Electric A/S, Hillerød, Denmark). Details of instrument calibration and reference methods are reported in Bobbo et al. (2016). Somatic cell count was obtained with a Fossomatic Minor (Foss Electric A/S) and log-transformed to SCS (Ali and Shook, 1980). Milk pH was measured after sample temperature adjustment using a Crison Basic 25 electrode (Crison Instruments SA, Barcelona, Spain).

***Milk Protein Composition.*** Separation of milk protein fractions was performed by reversed-phase High Performance Liquid Chromatography (**RP-HPLC**) using the method described by Maurmayr et al. (2013). Milk samples were prepared following the method suggested by Bobe et al. (1998). Analysis was carried out using an Agilent 1260 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump (Agilent 1260 Series, G1311B), and a Diode Array Detector (Agilent 1260 Series, DAD VL+, G1315C). Protein separation was performed using a reversed-phase analytical column C8 (Aeris WIDEPORE XB-C8, Phenomenex) with a large pore core-shell packing (3,6µm, 300Å, 250 x 2,1 I.D.). Sample vials, maintained at a low constant temperature (4°C), were injected via an auto-sampler (Agilent 1100 Series, G1313A).

***Traditional MCP.*** Traditional parameters of milk clotting ability were determined in duplicate using a mechanical lactodynamograph (Formagraph, Foss Electric A/S). These parameters were: time from rennet addition to milk gelation (**RCT**, min), curd-firming rate as time to a curd firmness (**CF**) of 20 mm ($\mathbf{k_{20}}$, min), and CF at 30, 45 and 60 minutes from rennet addition ($\mathbf{a_{30}}$, $\mathbf{a_{45}}$ and $\mathbf{a_{60}}$, mm). Experimental conditions were as reported in Stocco et al. (2016b).

***Curd Firming Traits.*** 240 CF values were recorded for each replicate (60 min test, one datum every 15 sec). Curd firming and syneresis traits were estimated for each individual milk sample using the equation proposed by Bittante et al. (2013) and modified by Stocco et al. (2016b):

$$CF_t = CF_P \times (1 - e^{-k_{CF} \times (t - RCTeq)}) \times e^{-k_{SR} \times (t - RCTeq)}$$

where $\mathbf{CF_t}$ (mm) is the CF modeled as a function of time t, $\mathbf{CF_P}$ (mm) is the potential asymptotical CF at an infinite time, $\mathbf{k_{CF}}$ (%×min$^{-1}$) is the curd-firming instant rate constant, $\mathbf{k_{SR}}$ (%×min$^{-1}$) is the curd syneresis instant rate constant, and $\mathbf{RCT_{eq}}$ (min) is the rennet

coagulation time. Two other traits related to maximum CF were also measured: the maximum $CF_t$ value (**$CF_{max}$**, mm) and the time at $CF_{max}$ (**$t_{max}$**, min).

***Individual Cheese Yield and Curd Nutrient Recovery.*** The model cheese-making procedure developed by Cipolat-Gotet et al. (2013) and modified by Stocco et al. (2016a) was carried out using a small amount of milk (1,500 mL) in order to produce cheeses from individual cows. Three %CY traits, expressing the weights of total fresh curd (**%CY$_{CURD}$**), and dry matter (**%CY$_{SOLIDS}$**) and water (**%CY$_{WATER}$**) in the curd as percentages of the weight of the processed milk, and 4 REC traits (**REC$_{PROTEIN}$**, **REC$_{FAT}$**, **REC$_{SOLIDS}$** and **REC$_{ENERGY}$**) were measured. Recovery traits represent the proportion of a given milk component and energy retained in the curd (calculated as the difference between the nutrient or energy in the milk and in the whey).

***Bacteriological Analysis***

Cows were considered potentially healthy if their milk SCC was < 100,000 cells/mL. These milk samples were not cultured. Composite milk samples with SCC above the selected threshold were subjected to bacteriological examination. Ten microliters from each milk sample were plated onto blood agar containing 5% defibrinated sheep blood (Oxoid Ltd., Basingstoke, UK). The plates were incubated aerobically at 37 +/-1°C and examined after 24 and 48 h. Bacteria were identified according to National Mastitis Council guidelines (NMC, 1999), which include morphology, Gram staining, catalase and coagulase reactions, oxidase reaction, biochemical properties and hemolysis pattern. Gram-positive microorganisms were differentiated as staphylococci and streptococci by the catalase reaction. The coagulase tube test in rabbit plasma (bioMérieux Italia S.p.A., Grassina. Italy) was used to differentiate *Staphylococcus aureus* from coagulase-negative *Staphylococcus* spp. (**CNS**). Gram-negative bacteria were identified by oxidase test as well as by growth features on MacConkey agar and

Eosin Methylene Blue agar (Oxoid Ltd., Basingstoke, UK). Bacterial genus and species identification was confirmed definitively by multiplex-PCR assays, as previously described by Shome et al. (2011), with minor changes related to Taq DNA polymerase (KAPA2G Fast Multiplex PCR Kit, Kapabiosystems, Massachusetts, USA) and cycling protocol. A sample was considered contaminated when 3 or more dissimilar colony types were observed with no single colony type predominating (NMC, 1999). Milk samples were considered culture-negative when no pathogens were isolated or no significant growth (< 1,000 cfu/mL) was observed within 48 h of incubation, with the exception of suspected cases of contagious IMI pathogens, for which identification was performed even when 1 colony (≥ 100 cfu/mL) was isolated.

***Statistical Analysis***

Herds were classified as low or high production according to the cows' average daily milk energy yields (Tyrrell and Reid, 1965), adjusted for stage of lactation, parity and breed (Stocco et al., 2016b). Briefly, individual milk energy values (kcal/kg) were converted to KJ/kg and multiplied by individual daily milk production (kg/d) to obtain the daily milk energy production of each cow (KJ/d). To estimate the least square means of the average daily milk energy production of each farm, data were analyzed using the SAS GLM procedure (SAS Institute Inc., Cary, NC) and including herd, breed, parity and DIM as fixed effects. Herds were ranked according to the estimated least square means of their average daily milk energy yield, and classified into two categories (low or high production) based on the median.

All milk samples were initially grouped into 5 clusters of UH status for statistical analysis: Healthy (cows with milk SCC < 100,000 cells/mL and not cultured for the presence of pathogens), No Growth, and Contagious, Environmental and Opportunistic pathogens. To ensure better analysis of culture-negative samples and possibly identify false negative results,

the "No Growth" group was divided into 3 sub-groups on the basis of the SCS 25$^{th}$ and 75$^{th}$ percentiles: culture-negative samples with low (**No Growth_L**), medium (**No Growth_M**) and high (**No Growth_H**) SCS. Contaminated samples were excluded from the analysis.

To investigate the associations between pathogen-specific cases of subclinical mastitis and milk traits, data (milk yield, composition, protein composition, and cheese-making traits) were analyzed using the SAS MIXED procedure (SAS Institute Inc., Cary, NC) with the following linear mixed model:

$$y_{ijklmno} = \mu + DIM_i + Parity_j + UH\ status_k + Breed_l + HP_m + HTD_n(HP)_m + e_{ijklmno}, \quad [1]$$

where $y_{ijklmno}$ is the investigated milk trait; $\mu$ is the overall mean; $DIM_i$ is the fixed effect of the $i$th class of days in milk ($i$ = 6 classes of 60-d intervals, from 5 ≤ class 1 ≤ 65d to class 6 > 305d); $Parity_j$ is the fixed effect of the $j$th parity ($j$ = 1 to ≥ 4); $UH\ status_k$ is the fixed effect of the $k$th group of udder health status ($k$ = Healthy, No Growth_L, No Growth_M, No Growth_H, Contagious, Environmental, Opportunistic); $Breed_l$ is the fixed effect of the $l$th breed ($l$ = HF, BS, JER, SI, REN and GA); $HP_m$ is the fixed effect of the $m$th herd productivity ($m$ = low or high); $HTD_n(HP)_m$ is the random effect of the $n$th herd-date ($n$ = 1 to 41) within the $m$th herd productivity; $e_{ijklmno}$ is the random residual. Given that herd effect is combined with date of sampling and season, a herd-date (**HTD**) effect was included in a 2-level nested model. The significance of the HP effect was tested on the error line of herd-date within herd productivity, and of the effects of DIM, parity, breed and UH status on the error line of the residual variance. For cheese-making traits, fixed effects related to the analytical devices were added to model 1: pendulum (20 levels) for traditional MCP and curd firming traits; vat (20 levels) and water bath (2 levels) for %CY and REC traits. In addition, as all coagulation traits were measured in duplicate, the fixed effect of repeated measures and the

random effect of animal were also taken into account. Herd-date and residuals were assumed to have a normal distribution with a mean of zero and variances of $\sigma_h^2$ and $\sigma_e^2$, respectively. The proportion of variance explained by herd-date (HTD, %) was calculated for each trait by dividing the corresponding variance component ($\sigma_h^2$) by the total variance ($\sigma_h^2 + \sigma_e^2$). Pairwise comparisons between infection groups were made using the Tukey correction ($P < 0.05$).

## RESULTS

### *Bacterial Findings and Classification of UH Status*

About 58% of the cows had milk SCC < 100,000 cells/mL (mean SCS = 1.48, SD = 0.97) and were defined as "Healthy". An IMI was determined when composite milk samples had milk SCC > 100,000 cells/mL and were microbiologically positive with at least 10 grown colonies/type (1,000 cfu/mL). Due to the low frequency of recovery of some pathogens, we classified them as contagious, environmental and opportunistic, a classification commonly used which is based on reservoir and mode of transmission. Contagious pathogens were considered to cause an IMI if at least 1 colony (≥ 100 cfu/mL) was isolated. Contagious bacteria were the most numerous (11% of the total population, and 27% of the cultured samples), and *Staph. aureus* was the most frequently isolated pathogen (Table 1). Environmental pathogens (about 7% of our population and 16% of the tested samples) included *Enterococcus* spp, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Proteus* spp, *Aerococcus viridans*, *Escherichia coli*, *Klebsiella* spp, *Bacillus* spp, *Enterobacter* spp, *Lactococcus lactis* and other Streptococci. Coagulase-negative Staphylococci, isolated in approximately 9% of the cultured samples, were classified as opportunistic pathogens. Of the 639 composite milk samples tested, 245 were culture-negative (No Growth) and 61 were contaminated (Table 1). Culture-negative samples (mean SCS = 4.38, SD = 1.19) were then divided into 3 sub-groups on the basis of the SCS 25$^{th}$ and 75$^{th}$ percentiles: culture-negative

samples with low SCS (No Growth_L; 61 samples with mean SCS = 3.21 and SD = 0.14), medium SCS (No Growth_M; 122 samples with mean SCS = 4.07 and SD = 0.44) and high SCS (No Growth_H; 62 samples with mean SCS = 6.13 and SD = 0.79).

***Descriptive Statistics***

All investigated traits (i.e., milk yield, composition, protein composition, and cheese-making traits) had normal distributions, so only the 1st and 99th percentiles were reported (Tables 2 and 3). Milk production of cows on multi-breed farms averaged 24.4 kg/d with large variability (CV = 36.8%) (Table 2). Of the quality traits, casein number (ratio between casein and total protein) was the least variable trait, with CV = 1.6%. The variabilities of the other quality traits ranged from approximately 6 to 38% (Table 2). Values of the CV of the detailed milk protein composition, determined by RP-HPLC analysis, were intermediate (16 to 26%), with the exception of lactoferrin (CV = 53%) (Table 2). Coagulation of milk samples started on average 19 min after rennet addition (both traditional and estimated RCT), and a curd firmness of 20 mm ($k_{20}$) was attained after about 4 min (Table 3). The variabilities of curd firming traits ranged from 17% (time of achievement of maximum curd firmness) to 41% (syneresis instant rate constant). The mean $\%CY_{CURD}$ was 15.7%, which corresponded to the sum of $\%CY_{SOLIDS}$ (7.2%) and $\%CY_{WATER}$ (8.5%) (Table 3). The coefficient of variation of CY traits was 17-19%. Recoveries of protein, fat, solids and energy in the curd ranged from an average of 53.3% ($\%CY_{SOLIDS}$) to 84.5% ($\%CY_{FAT}$).

***Sources of Variation Among Milk Yield, Composition and Protein Composition***

All the effects included in the model played important roles in explaining the variation of single test-day milk yield, composition and protein composition (Table 4). With the exception of UH status, the effects of the other sources of variation have already been

discussed in Stocco et al. (2016b). The proportion of variance explained by herd-date was highest for urea (77%) and pH (52%), about 30% for milk production, and less than 20% for all the other traits. Herd productivity influenced milk yield, protein, casein, urea, and almost all milk protein fractions, except for β-casein and lactoferrin. As expected, breed, DIM and parity were important sources of variation for all traits, with the exception of fat, which was not affected by age. Udder health status was associated with milk yield, casein number, lactose, pH, and, of the protein composition traits, total protein, whey protein, casein, $\alpha s_1$-, $\alpha s_2$- and β-casein (Table 4).

After adjusting for herd productivity, herd-date, breed, stage of lactation and parity, daily milk production of cows subclinically infected with contagious pathogens was lower than that of healthy animals (-1.6 kg/d) (Table 6). All milk samples in which, independently from the group, a pathogen was recovered, and culture-negative samples with medium to high SCC (No Growth_M and No Growth_H) had a lower casein number and lactose content than samples with SCC < 100,000 cells/mL. No Growth_H samples also had greater pH and, of the protein composition traits, lower total protein, casein, $\alpha s_1$-, $\alpha s_2$- and β-casein. Lower contents of casein, $\alpha s_2$- and β-casein fractions were also observed in No Growth_M samples, and infection with environmental pathogens was associated with lower β-casein content (Table 6). Culture-negative samples with low SCC (No Growth_L; average SCC = 116,000 cells/mL) did not differ statistically from healthy samples in milk composition (Table 6).

***Sources of Variation Among Cheese-making Traits***

The proportion of variance explained by herd-date was lower (< 15%) for coagulation properties (both traditional MCP and curd firming) than for milk yield and composition traits (Table 5). Herd-date explained 18 to 28% of the variation in cheese yield traits, while values ranged from 4 to 19% for milk nutrient recoveries in the curd. Herd productivity was

associated with some MCP (RCT and $a_{60}$), all curd firming traits, and $\%CY_{SOLIDS}$. Breed strongly affected all technological traits ($P < 0.001$), and DIM influenced almost all traits, with a few exceptions ($k_{SR}$, $REC_{PROTEIN}$ and $REC_{FAT}$). Parity was significant in explaining the variation of RCT and $a_{60}$ among the MCP, of $RCT_{eq}$ and 2 CF traits ($CF_p$ and $C_{max}$) among the curd firming traits, of all cheese yield traits, and of $REC_{PROTEIN}$ (Table 5). The effects of pendulum and of repeated measures, included in model 1 only for coagulation traits, were important in explaining the variation of all these traits, except for $a_{45}$, which was not affected by repeated measures (data not shown). Conversely, the effects of instrument (vat and water bath), included in model 1 for cheese yield and nutrient recovery traits, only influenced $\%CY_{WATER}$ (data not shown). Udder health status was associated with most of the coagulation traits (except $k_{20}$, $k_{SR}$ and $t_{max}$) and with protein and fat recoveries in the curd ($REC_{PROTEIN}$ and $REC_{FAT}$) (Table 5).

Compared with healthy samples, coagulation was slower (greater RCT and $RCT_{eq}$) in milk samples subclinically infected with contagious pathogens and culture-negative samples with high SCC (No Growth_H). These 2 groups also displayed weaker curd firmness at 30 min ($a_{30}$) after rennet addition, while No Growth_H also displayed weaker curd firmness at 45 and 60 min ($a_{45}$ and $a_{60}$) (Table 7). Culture-negative samples with high SCC also had the lowest asymptotic potential CF ($CF_p$) and the lowest maximum CF attained after 45 min ($C_{max}$). No association was found between cheese yield traits and UH status. Protein recovery in the curd was approximately 1% lower in milk samples infected by contagious or opportunistic pathogens (Table 7) than in healthy samples. The lowest recovery of fat in the curd was observed in the milk of cows with IMI contagious pathogens.

## DISCUSSION

### *Prevalence of Pathogens and Classification of UH Status*

This study was carried out with data collected from specialized and dual-purpose dairy cattle housed in multi-breed herds in north-eastern Italy. While it is ideal to have multiple microbiological examinations of milk samples, in large field studies collection of single milk samples is the most practical and cost-effective sampling methodology (Torres et al., 2009). A recent study (Reyher and Dohoo, 2011) has indicated that the use of composite milk samples results in lower sensitivity but acceptable specificity and thus the compromise of using single composite milk samples can be acceptable for the purpose of evaluating the impact of IMI on composition.

An SCC threshold of 100,000 cells/mL was established to differentiate between composite milk samples collected from potentially healthy cows and those from animals potentially affected by naturally occurring subclinical mastitis and thus subjected to bacteriological analysis. Somatic cell count > 100,000 cells/mL is commonly associated with inflammatory response of the mammary gland (Schwarz et al., 2010), and this threshold has been previously used to differentiate cows with IMI from those without, at both the quarter (Hamann, 2003; Hiss et al., 2007) and cow composite levels (Eberhart et al., 1979; Krömker et al., 2001). Furthermore, Pyörälä (2003) reported that the SCC of composite milk should not exceed 100,000 cells/mL and a SCC threshold of 100,000 cells/mL was suggested also by Dohoo and Meek (1982) to identify uninfected and infected cows.

The higher prevalence of contagious pathogens, and in particular of *Staph. aureus*, than of the other IMI bacteria in our study population, is in agreement with results from previous studies conducted in northern Italy (Bertocchi et al., 2012; Bortolami et al., 2015). About 40% of the cultured samples were culture-negative (Table 1). However, the mean SCS of these samples was relatively high (4.38, corresponding to a SCC of 260,000 cells/mL). A

possible explanation is that some of the cows were in the healing process at the time of sampling and the infection was spontaneously eliminated (Smith et al., 1985). In such a case, even where the inflammatory response is still active, the pathogens have been cleared and cannot be recovered. In the culture-negative samples with the highest milk SCC values, it is possible that inflammatory status was at the maximum level and pathogens were engulfed by phagocytes and could therefore not be isolated (Newbould and Neave, 1965; Hill et al., 1978). Moreover, since composite milk samples were analyzed, a certain percentage of false negative results could be due to a dilution effect of healthy quarters, so that the few colonies of the infected quarter could not be detected by culture analysis (Dohoo and Meek, 1982). Given the relatively high SCS observed in the culture-negative samples, we decided to divide them into 3 sub-groups.

***Association between UH Status and Milk Yield, Composition and Protein Composition***

Subclinical infections with contagious pathogens were found to reduce daily milk production compared with healthy animals (Table 6). Given the pathogenesis, chronicity and lower cure rate of *Staph. aureus* infections, greater milk yield loss was expected in cows affected by subclinical mastitis due to contagious pathogens than due to environmental and opportunistic pathogens, which did not impair milk production (Table 6). Associations between lower daily milk production and subclinical IMI caused by contagious pathogens (Reksen et al., 2007; Schukken et al., 2009b) and by streptococcal species (Schukken et al., 2009b; Pearson et al., 2013) have previously been observed. Pathogen-specific patterns of milk production losses due to clinical mastitis have been reported in the literature (Coulon et al., 2002; Gröhn et al., 2004; Hertl et al., 2014). In those studies, a large reduction in milk yield was found to have been caused by *E. coli*. However, *Staph. aureus* and *Klebsiella* spp. also negatively affect milk production, in both primiparous and multiparous cows (Gröhn et

al., 2004; Hertl et al., 2014). In agreement with previous studies (Paradis et al., 2010; Pearson et al., 2013; Tomazi et al., 2015), CNS infections had no detrimental effect on milk production. Generally, minor pathogens cause less damage to the udder than major pathogens, such as *Staph. aureus*, *E. coli*, *Streptococcus* spp. and *Klebsiella* spp (Reyher et al., 2012). Moreover, because CNS are commonly found on the teat skin and canal, some of the culture-positive samples could have resulted from udder skin contamination during composite milk samples collection, rather than from real infection of the gland (Thorberg et al., 2009).

As previously reported by other authors (Leitner et al., 2006; Silanikove et al., 2014; Gonçalves et al., 2016), fat, protein and casein contents were not affected by naturally-occurring pathogen-specific subclinical IMI. Nor were there any differences in fat and protein percentages in milk following experimentally-induced *Strep. uberis* IMI, compared with milk from uninfected animals (Kester et al., 2015). Nevertheless, comparison of infected and uninfected quarters in Gyr cows in the tropics showed that IMI caused by different bacteria (*Staph. aureus*, CNS, *Streptococcus* spp. and *Corynebacterium* spp.) altered total solids, nonfat solids, protein and fat percentages (Malek dos Reis et al., 2013). Moreover, greater protein concentrations were reported in cases of clinical mastitis caused by *Staph. aureus*, *Strep. uberis* and *E. coli* (Coulon et al., 2002) as a result of the influx of soluble proteins from the bloodstream. Confirming findings previously reported by Bobbo et al. (2016) concerning the influence of SCC on milk composition, the milk produced by infected cows, characterized by an average SCC above 300,000 cells/mL, had a lower casein number and lactose content than normal milk (Table 6). However, no differences were observed among contagious, environmental and opportunistic IMI pathogens. A lower casein/protein ratio has previously been observed in quarters affected by subclinical IMI caused by *Staph. aureus* or by infections associated with clinical signs than in healthy quarters (Coulon et al., 2002). The negative effect of IMI caused by different bacteria on lactose content is well established in the

literature. In particular, lower lactose concentrations than in uninfected milk have been measured in milk infected by *Staph. aureus* (Coulon et al., 2002), *Strep. dysgalactiae* (Leitner et al., 2006; Merin et al., 2008; Fleminger et al., 2011), *Strep. uberis* (Coulon et al., 2002; Kester et al., 2015), *E. coli* (Coulon et al., 2002; Leitner et al., 2006; Fleminger et al., 2011), *Corynebacterium* spp. (Malek dos Reis et al., 2013; Gonçalves et al., 2016) and CNS (Coulon et al., 2002). However, when the effects of different subclinical IMI bacteria were analyzed (Coulon et al., 2002; Leitner et al., 2006; Fleminger et al., 2011), compared with normal milk significant changes in lactose content were observed in milk infected by a single specific pathogen; as in our study, no significant variation was observed among the IMI pathogens. Moreover, culture-negative samples with medium to high SCC (No Growth_M and No Growth_H) had a lower casein number and lactose percentages compared to healthy animals (Table 6). The greatest composition changes were observed in culture-negative samples with high SCC (on average 880,000 cells/mL), which differed in lactose content and pH from all the other UH status groups. The process of infection triggers an inflammatory response that has a goal of reducing the number of viable pathogens. In many instances this process is effective, and viable bacteria cannot be recovered (in sufficient quantities for detection using routine methods) even though this process has potentially affected milk composition. Thus the classification system used in this study allowed us to evaluate the impact of inflammation even when microbiological analysis results in no microbial growth. Interestingly, culture-negative samples with high SCC were collected from herds where at least one case of contagious IMI was found. Hence, we can speculate that those samples classified as 'no growth' were most likely infected by contagious pathogens that could not be recovered by bacteriological analysis because they were engulfed by neutrophils or due to the intermittent shedding of *Staph. aureus* IMI. Therefore, independently of the recovery of a pathogen, milk samples with high SCC were of lower quality, indicating an effect of inflammation rather than

infection. The association between high SCC and poor milk quality as a result of increased proteinase activity, lower biosynthesis and damage of the blood-milk barrier during the inflammatory response is well established in the literature (Auldist and Hubble, 1998; Le Maréchal et al., 2011).

Detailed exploration of milk protein composition by RP-HPLC analysis of skimmed milk confirmed important changes in culture-negative samples with medium to high SCC (Table 6). In particular, lower true protein and casein contents were found in milk samples where the level of inflammation was most likely so high that the pathogens may have been internalized by neutrophils and could not be recovered by the bacteriological test (No Growth_H), than in milk collected from healthy cows and animals infected by a specific bacterium. Whey proteins were not influenced by UH status, while $\alpha s_1$-, $\alpha s_2$- and β-caseins were highly affected (Table 6). Milk with a high SCC is characterized by greater proteolytic activity (Le Roux et al., 1995). Activation of the plasmin-plasminogen system during the innate immune response of the mammary gland to infection is responsible for degradation of β-casein into γ-casein and proteose peptones (Politis and Ng-Kwai-Hang, 1988). Therefore, in our study, the more active inflammatory status of the No Growth_H samples, explained by high milk SCC, may be associated with greater enzymic breakdown of caseins, explaining the estimated 1% decrease in casein number compared with normal milk (Table 6). A significantly lower β-casein fraction was also observed in milk samples infected with environmental pathogens (Table 6). Compared with healthy quarters, milk collected from quarters with subclinical IMI caused by streptococci other than *Strep. agalactiae* (classified as environmental pathogens in our study) contained a higher proportion of β-casein hydrolysis products (Urech et al., 1999). Increased hydrolysis of casein by plasmin activity following *E. coli* (Moussaoui et al., 2004) and *Strep. uberis* (Larsen et al., 2004) infection has also been reported in the literature. Moreover, it has been shown that specific bacterial termolysin- and

elastin-like proteases, which are responsible for β-casein fragments formation, are synthetized during *Strep. dysgalactiae* IMI (Fleminger et al., 2011).

***Association between UH Status and Cheese-making Traits***

Variations in milk composition due to mastitis may impair the transformation process and the quality of dairy products (Le Maréchal et al., 2011). Studies have previously been carried out on the impact of milk SCC on coagulation properties and the cheese-making process (Grandison and Ford, 1986; Politis and Ng-Kwai-Hang, 1988; Bobbo et al., 2016), although there is little information concerning the influence of pathogen-specific IMI on milk technological traits. In the present study, milk samples subclinically infected with contagious pathogens exhibited longer coagulation time (RCT) and weaker CF ($a_{30}$) than milk samples collected from healthy animals (Table 7). An even greater deterioration in milk clotting ability was observed in culture-negative samples with high SCC (No Growth_H), reflecting the poor milk composition previously described for these samples. High SCC can delay gelation time, increasing the incidence of samples that do not coagulate within the 30 min test period [noncoagulating milk, as reported by Bittante et al. (2012)], meaning that traditional MCPs are not fully representative of the effect of mastitis on milk clotting ability. Prolongation of the observation time from 30 to 60 min considerably reduced the number of noncoagulating samples, and modeling of all the available information (240 data points for each sample) allowed the new curd firming and syneresis traits to be estimated (Bittante et al., 2013; Stocco et al., 2016b). The modeled traits confirmed the poor coagulation of samples infected by contagious bacteria (slower $RCT_{eq}$), and in particular of culture-negative samples with high SCC, which had slower coagulation time ($RCT_{eq}$) and weaker CF values: both the asymptotic potential ($CF_p$) and maximum CF achieved within 45 min ($C_{max}$) (Table 7 and Figure 1). Worsening of coagulation properties is a consequence of higher milk pH, lower lactose

content, and the degradation of casein fractions (Table 6). Higher milk pH causes a decrease in the enzymatic activity involved in milk clotting (Swaisgood, 1982), which negatively affects both traditional and modeled coagulation properties (Stocco et al., 2015; Bobbo et al., 2016). In addition, it has been shown that greater casein breakdown (Auldist et al., 1996) and lower lactose (Leitner et al., 2011) are associated with lower clotting ability and curd firmness. Impairment of rennet clotting time and CF was reported by Leitner et al. (2006), who compared milk from glands infected by different subclinical IMI bacteria with normal milk. The greatest effect was observed in milk infected by *Strep. dysgalactiae*, although it is worth noting that they used different instruments to those used in our study (Optigraph versus Formagraph), the sample size was smaller, and the standard errors of means were larger. The detrimental effect of *Strep. dysgalactiae* IMI on milk clotting was confirmed by Fleminger et al. (2011), while Merin et al. (2008) demonstrated that such deterioration is also found in yogurt and cheese made from milk of *Strep. dysgalactiae*-infected glands. In particular, yogurt made from infected milk was softer, the cheese curd had a fragile texture (resulting in greater curd losses), and the cheese yield was lower at the end of maturation as a result of pathogen-specific proteolytic activity and release of proteose-peptones. In the present study, estimates of cheese yield and recovery were lower for culture-negative samples with high SCC, although not statistically different from the other UH status groups (Table 7). Given that cheese-related traits were analyzed only on a subset of data, standard errors of means of some UH status groups could be inflated by the smaller sample size, and it is possible that a significant variation in No Growth_H samples may not have been detected. The lower casein number may justify the lower recoveries of protein and fat in cheese observed in milk samples infected by contagious pathogens. In fact, aggregation of casein during the cheese-making process also incorporates the fat into the curd (Dalgleish, 1993). Therefore, variations in casein as a percentage of total protein may result in a greater loss of fat in the whey.

## CONCLUSIONS

In the present study we report associations between pathogen-specific cases of subclinical mastitis and several milk composition and cheese-making traits in specialized and dual-purpose dairy cows housed in multi-breed herds. Significant variations in the casein/protein ratio and the lactose content were observed in all culture-positive samples and culture-negative samples with medium to high SCC compared with normal milk. No differences were observed among contagious, environmental and opportunistic pathogens, suggesting an effect of inflammation rather than infection. Given that environmental pathogens are also responsible for a worsening of milk composition, great importance should be given to herd health management. The greatest impairment in milk quantity and quality, clotting ability and cheese production was observed in the two UH status groups with highest milk SCC, i.e., milk samples subclinically infected with contagious pathogens and culture-negative samples with high SCC, pointing to a discrepancy between bacteriological results and inflammatory status, and thus confirming the importance of SCC as an indicator of udder health and milk quality. Culture-negative samples with high SCC may be infected by contagious bacteria that it was not possible to recover by bacteriological analysis due to their being engulfed by neutrophils. Molecular analysis would be required to detect bacterial DNA in culture-negative samples to support this hypothesis. In addition, further studies will be required to confirm the present findings, and also to evaluate the effect of cases of subclinical mastitis at the quarter level (to avoid possible contamination and dilution effects) and at the individual pathogen level. Repeated sampling, in order to establish the exact infection stage, should also be considered.

## ACKNOWLEDGMENTS

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## TABLES AND FIGURES

**Table 1.** Bacterial findings and classification in our study population (N = 1,508)

| UH status groups | N | % tot[1] | % test[2] | Mean SCS[3] | SD |
|---|---|---|---|---|---|
| Healthy | 869 | 57.6 | - | 1.48 | 0.97 |
| Contagious | 172 | 11.4 | 26.9 | 4.81 | 1.14 |
| *Staphylococcus aureus* | 151 | 10.0 | 23.6 | | |
| *Streptococcus agalactiae* | 11 | 0.7 | 1.7 | | |
| *Staphylococcus aureus* + *Streptococcus dysgalactiae* | 5 | 0.3 | 0.8 | | |
| *Staphylococcus aureus* + *Streptococcus agalactiae* | 2 | 0.1 | 0.3 | | |
| *Staphylococcus aureus* + *Enterococcus spp* | 1 | 0.1 | 0.2 | | |
| *Staphylococcus aureus* + *Streptococcus uberis* | 1 | 0.1 | 0.2 | | |
| *Staphylococcus aureus* + other Streptococci | 1 | 0.1 | 0.2 | | |
| Environmental | 102 | 6.8 | 16.0 | 4.70 | 1.28 |
| other Streptococci | 26 | 1.7 | 4.1 | | |
| *Enterococcus spp* | 21 | 1.4 | 3.3 | | |
| *Streptococcus dysgalactiae* | 16 | 1.1 | 2.5 | | |
| *Streptococcus uberis* | 14 | 0.9 | 2.2 | | |
| *Proteus spp* | 9 | 0.6 | 1.4 | | |
| *Aerococcus viridans* | 5 | 0.3 | 0.8 | | |
| *Escherichia coli* | 5 | 0.3 | 0.8 | | |
| *Klebsiella spp* | 2 | 0.1 | 0.3 | | |
| *Bacillus spp* | 1 | 0.1 | 0.2 | | |
| *Enterobacter spp* | 1 | 0.1 | 0.2 | | |
| *Lactococcus lactis* | 1 | 0.1 | 0.2 | | |
| *Aerococcus viridans* + CNS | 1 | 0.1 | 0.2 | | |
| Opportunistic | 59 | 3.9 | 9.2 | 4.60 | 1.26 |
| Coagulase Negative Staphylococci (CNS) | 59 | 3.9 | 9.2 | | |
| No Growth | 245 | 16.2 | 38.3 | 4.38 | 1.19 |
| Contaminated | 61 | 4.0 | 9.5 | 4.69 | 1.26 |

[1]Percentage calculated on the total number of collected samples (N = 1,508)

[2]Percentage calculated on the number of cultured samples (N = 639)

[3]SCS = $\log_2$ (SCC/100,000) + 3

**Table 2.** Descriptive statistics of single test-day milk yield, composition and protein composition (n = 1447)[1]

| Trait | Mean | CV, % | P1 | P99 |
|---|---|---|---|---|
| Milk yield, kg/d | 24.4 | 36.8 | 7.0 | 49.0 |
| Milk composition | | | | |
| Fat, % | 4.21 | 21.9 | 1.87 | 7.06 |
| Protein, % | 3.62 | 13.9 | 2.66 | 4.85 |
| Fat:Protein | 1.15 | 19.8 | 0.52 | 1.84 |
| Casein, % | 2.84 | 13.3 | 2.10 | 3.80 |
| Casein number, %[2] | 78.5 | 1.6 | 75.1 | 81.3 |
| Urea, mg/100g | 24.98 | 38.1 | 7.39 | 49.04 |
| Lactose, % | 4.98 | 5.9 | 4.09 | 5.52 |
| pH | 6.51 | 1.6 | 6.27 | 6.74 |
| Protein composition, g/L[3] | | | | |
| Total protein | 43.22 | 15.8 | 29.52 | 61.58 |
| Whey protein | 7.02 | 21.7 | 3.49 | 10.66 |
| Casein | 36.20 | 16.6 | 24.18 | 51.51 |
| $\alpha_{s1}$-casein | 12.10 | 17.0 | 7.33 | 17.48 |
| $\alpha_{s2}$-casein | 3.60 | 26.2 | 1.66 | 5.97 |
| β-casein | 13.51 | 16.8 | 8.38 | 19.07 |
| κ-casein | 4.31 | 26.2 | 2.01 | 6.93 |
| Lactalbumin | 0.97 | 18.2 | 0.59 | 1.45 |
| Lactoglobulin | 5.95 | 25.1 | 2.59 | 9.44 |
| Lactoferrin | 0.10 | 53.5 | 0.03 | 0.24 |

[1]P1 = 1st percentile; P99 = 99th percentile

[2]Casein number = (casein / protein) *100

[3]Contents of all protein fractions were measured by reversed-phase HPLC on skim milk. Total protein = Whey protein + Casein; Whey protein = sum of total whey fractions; Casein = sum of total casein fractions.

**Table 3.** Descriptive statistics of traditional milk coagulation properties (MCP; n = 2,894), curd firming (n = 2,894), cheese yields (%CY; n = 488) and curd nutrient recoveries (REC; n = 488)[1]

| Trait[2] | Mean | CV, % | P1 | P99 |
|---|---|---|---|---|
| Traditional MCP | | | | |
| RCT, min | 18.6 | 37.9 | 8.2 | 45.0 |
| $k_{20}$, min | 4.3 | 71.6 | 1.3 | 16.2 |
| $a_{30}$, mm | 39.7 | 48.6 | 0.0 | 73.6 |
| $a_{45}$, mm | 51.0 | 31.6 | 0.0 | 80.5 |
| $a_{60}$, mm | 53.6 | 27.8 | 1.3 | 81.6 |
| Curd firming | | | | |
| $RCT_{eq}$, min | 18.8 | 37.1 | 7.9 | 44.6 |
| $CF_p$, mm | 74.5 | 23.4 | 23.4 | 109.3 |
| $k_{CF}$, %×min$^{-1}$ | 8.1 | 30.6 | 4.7 | 17.9 |
| $k_{SR}$, %×min$^{-1}$ | 0.7 | 41.2 | 0.0 | 1.6 |
| $C_{max}$, mm | 55.6 | 23.4 | 17.4 | 81.6 |
| $t_{max}$, min | 51.8 | 17.3 | 27.8 | 60.0 |
| Cheese yields, % | | | | |
| $\%CY_{CURD}$ | 15.7 | 17.4 | 10.5 | 23.6 |
| $\%CY_{SOLIDS}$ | 7.2 | 17.5 | 4.8 | 11.4 |
| $\%CY_{WATER}$ | 8.5 | 19.2 | 5.3 | 13.0 |
| Recoveries, % | | | | |
| $REC_{PROTEIN}$ | 79.3 | 2.5 | 73.6 | 83.1 |
| $REC_{FAT}$ | 84.5 | 6.0 | 67.6 | 91.5 |
| $REC_{SOLIDS}$ | 53.3 | 8.8 | 43.1 | 64.8 |
| $REC_{ENERGY}$ | 68.8 | 5.7 | 58.2 | 77.8 |

[1]P1 = 1st percentile; P99 = 99th percentile

[2]RCT = rennet coagulation time; $k_{20}$ = curd firming rate as min to a curd firmness of 20 mm; $a_{30\ (45\text{-}60)}$ = curd firmness after 30 (45-60) min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using the equation; $CF_P$ = asymptotic potential curd firmness; $k_{CF}$ = curd firming instant rate constant; $k_{SR}$ = syneresis instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$; $\%CY_{CURD}$ = weight of fresh curd as percentage of weight of milk processed; $\%CY_{SOLIDS}$ = weight of curd solids as percentage of weight of milk processed; $\%CY_{WATER}$ = weight of water curd as percentage of weight of milk processed; $REC_{PROTEIN}$ = protein of the curd as percentage of the protein of the milk processed; $REC_{FAT}$ = fat of the curd as percentage of the fat of the milk processed; $REC_{SOLIDS}$ = solids of the curd as percentage of the solids of the milk processed; $REC_{ENERGY}$ = energy of the curd as percentage of energy of the milk processed.

**Table 4.** Results of ANOVA (*F*-value and significance) for single test-day milk yield, composition and protein composition

| Trait | HP[1] | HTD, %[2] | Breed | DIM | Parity | UH status |
|---|---|---|---|---|---|---|
| Milk yield, kg/d | 61.8*** | 32.0 | 35.6*** | 110.3*** | 53.7*** | 2.7* |
| Milk composition | | | | | | |
| Fat, % | 3.5 | 12.3 | 29.2*** | 33.5*** | 2.2 | 1.6 |
| Protein, % | 11.9** | 16.3 | 23.9*** | 92.1*** | 9.9*** | 1.4 |
| Fat:Protein | 0.0 | 11.6 | 10.7*** | 4.9*** | 3.1* | 1.4 |
| Casein, % | 14.0*** | 15.6 | 25.5*** | 93.5*** | 13.1*** | 0.6 |
| Casein number, %[3] | 0.1 | 17.3 | 4.4*** | 6.6*** | 18.8*** | 12.8*** |
| Urea, mg/100g | 10.1** | 77.0 | 7.9*** | 6.0*** | 5.5*** | 0.9 |
| Lactose, % | 0.0 | 9.6 | 3.9** | 14.9*** | 21.5*** | 26.7*** |
| pH | 0.0 | 51.6 | 2.3* | 13.3*** | 10.0*** | 2.8* |
| Protein composition, g/L[4] | | | | | | |
| Total protein | 14.8*** | 11.0 | 77.2*** | 53.6*** | 27.2*** | 7.1*** |
| Whey protein | 31.3*** | 6.8 | 22.1*** | 25.6*** | 3.8** | 2.2* |
| Casein | 7.6** | 13.3 | 78.0*** | 46.6*** | 30.4*** | 6.9*** |
| $\alpha_{s1}$-casein | 12.5** | 13.8 | 44.3*** | 33.9*** | 26.4*** | 5.5*** |
| $\alpha_{s2}$-casein | 6.0* | 7.8 | 62.0*** | 4.9*** | 27.0*** | 4.2*** |
| β-casein | 1.6 | 19.9 | 21.3*** | 36.5*** | 31.9*** | 10.2*** |
| κ-casein | 7.4** | 7.0 | 142.8*** | 8.1*** | 12.0*** | 1.2 |
| Lactalbumin | 14.8*** | 18.2 | 16.1*** | 2.4* | 16.8*** | 1.5 |
| Lactoglobulin | 25.0*** | 6.6 | 22.5*** | 26.9*** | 2.8* | 1.9 |
| Lactoferrin | 2.7 | 16.7 | 3.4** | 8.2*** | 4.0** | 1.0 |

[1]HP = Herd productivity

[2]Herd/Test Day effect expressed as proportion of variance explained by herd-date calculated by dividing the corresponding variance component by the total variance

[3]Casein number = (casein / protein) *100

[4]Contents of all protein fractions were measured by reversed-phase HPLC on skim milk. Total protein = Whey protein + Casein; Whey protein = sum of total whey fractions; Casein = sum of total casein fractions.

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

**Table 5.** Results of ANOVA (*F*-value and significance) for traditional milk coagulation properties (MCP), curd firming, cheese yields (%CY) and curd nutrient recoveries (REC)

| Trait[3] | HP[1] | HTD, %[2] | Breed | DIM | Parity | UH status |
|---|---|---|---|---|---|---|
| Traditional MCP | | | | | | |
| RCT, min | 9.8** | 8.2 | 11.0*** | 19.8*** | 2.9* | 4.7*** |
| $k_{20}$, min | 0.8 | 5.0 | 22.6*** | 3.7** | 1.4 | 1.3 |
| $a_{30}$, mm | 0.3 | 9.9 | 17.7*** | 9.1*** | 1.8 | 4.4*** |
| $a_{45}$, mm | 1.9 | 8.5 | 14.9*** | 4.9*** | 1.9 | 5.9*** |
| $a_{60}$, mm | 6.8* | 9.8 | 17.3*** | 4.0** | 2.8* | 8.5*** |
| Curd firming | | | | | | |
| $RCT_{eq}$, min | 8.8** | 9.1 | 10.9*** | 21.8*** | 2.9* | 5.5*** |
| $CF_p$, mm | 6.6* | 9.4 | 28.3*** | 5.1*** | 5.8*** | 4.1*** |
| $k_{CF}$, %×min$^{-1}$ | 5.2* | 10.6 | 36.6*** | 7.7*** | 0.8 | 2.2* |
| $k_{SR}$, %×min$^{-1}$ | 4.8* | 8.2 | 22.0*** | 1.8 | 0.9 | 1.2 |
| $C_{max}$, mm | 6.6* | 9.4 | 28.3*** | 5.1*** | 5.8*** | 4.1*** |
| $t_{max}$, min | 13.3*** | 14.5 | 20.6*** | 3.5** | 2.2 | 1.4 |
| Cheese yields, % | | | | | | |
| $\%CY_{CURD}$ | 4.0 | 28.1 | 29.0*** | 27.2*** | 4.9** | 0.5 |
| $\%CY_{SOLIDS}$ | 5.3* | 18.0 | 20.9*** | 23.2*** | 2.8* | 1.1 |
| $\%CY_{WATER}$ | 2.8 | 28.4 | 26.7*** | 20.1*** | 5.9*** | 0.1 |
| Recoveries, % | | | | | | |
| $REC_{PROTEIN}$ | 1.4 | 19.2 | 7.3*** | 2.2 | 6.4*** | 5.6*** |
| $REC_{FAT}$ | 0.2 | 3.7 | 8.4*** | 0.9 | 1.2 | 2.5* |
| $REC_{SOLIDS}$ | 2.8 | 17.6 | 16.4*** | 23.1*** | 1.3 | 0.9 |
| $REC_{ENERGY}$ | 4.1 | 10.7 | 13.8*** | 9.4*** | 2.1 | 1.7 |

[1]HP = Herd productivity

[2]Herd/Test Day effect expressed as proportion of variance explained by herd-date calculated by dividing the corresponding variance component by the total variance

[3]RCT = rennet coagulation time; $k_{20}$ = curd firming rate as min to a curd firmness of 20 mm; $a_{30\ (45\text{-}60)}$ = curd firmness after 30 (45-60) min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using the equation; $CF_P$ = asymptotic potential curd firmness; $k_{CF}$ = curd firming instant rate constant; $k_{SR}$ = syneresis instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$; $\%CY_{CURD}$ = weight of fresh curd as percentage of weight of milk processed; $\%CY_{SOLIDS}$ = weight of curd solids as percentage of weight of milk processed; $\%CY_{WATER}$ = weight of water curd as percentage of weight of milk processed; $REC_{PROTEIN}$ = protein of the curd as percentage of the protein of the milk processed; $REC_{FAT}$ = fat of the curd as percentage of the fat of the milk processed; $REC_{SOLIDS}$ = solids of the curd as percentage of the solids of the milk processed; $REC_{ENERGY}$ = energy of the curd as percentage of energy of the milk processed.

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

**Table 6.** Least square means (LSM) and standard errors (SE) of milk yield, composition and protein composition by UH status[1]

| Trait | Healthy | | No Growth_L | | No Growth_M | | No Growth_H | | Contagious | | Environmental | | Opportunistic | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE |
| Milk yield, kg/d | 21.9$^{a}$ | 0.6 | 21.8$^{ab}$ | 0.9 | 21.6$^{ab}$ | 0.8 | 20.5$^{ab}$ | 0.9 | 20.3$^{b}$ | 0.7 | 20.7$^{ab}$ | 0.8 | 22.1$^{ab}$ | 0.9 |
| Milk composition | | | | | | | | | | | | | | |
| Fat, % | 4.35 | 0.06 | 4.59 | 0.11 | 4.43 | 0.09 | 4.35 | 0.11 | 4.25 | 0.08 | 4.39 | 0.09 | 4.40 | 0.11 |
| Protein, % | 3.61 | 0.04 | 3.64 | 0.06 | 3.65 | 0.05 | 3.66 | 0.06 | 3.67 | 0.05 | 3.69 | 0.06 | 3.70 | 0.07 |
| Fat:Protein | 1.23 | 0.02 | 1.28 | 0.04 | 1.23 | 0.03 | 1.23 | 0.04 | 1.18 | 0.03 | 1.20 | 0.03 | 1.23 | 0.04 |
| Casein, % | 2.84 | 0.03 | 2.86 | 0.05 | 2.86 | 0.04 | 2.85 | 0.05 | 2.88 | 0.04 | 2.89 | 0.04 | 2.89 | 0.05 |
| Casein number, %[2] | 78.9$^{a}$ | 0.1 | 78.6$^{ab}$ | 0.2 | 78.5$^{b}$ | 0.2 | 77.9$^{c}$ | 0.2 | 78.4$^{bc}$ | 0.2 | 78.3$^{bc}$ | 0.2 | 78.4$^{bc}$ | 0.2 |
| Urea, mg/100g | 26.17 | 1.46 | 26.21 | 1.58 | 26.21 | 1.51 | 25.25 | 1.57 | 25.61 | 1.50 | 25.29 | 1.54 | 26.24 | 1.6 |
| Lactose, % | 5.02$^{a}$ | 0.02 | 4.95$^{ab}$ | 0.04 | 4.92$^{b}$ | 0.03 | 4.65$^{c}$ | 0.04 | 4.89$^{b}$ | 0.03 | 4.86$^{b}$ | 0.03 | 4.89$^{b}$ | 0.04 |
| pH | 6.50$^{b}$ | 0.01 | 6.52$^{ab}$ | 0.02 | 6.51$^{ab}$ | 0.01 | 6.53$^{a}$ | 0.02 | 6.51$^{ab}$ | 0.01 | 6.50$^{ab}$ | 0.01 | 6.51$^{ab}$ | 0.02 |
| Protein composition, g/L[3] | | | | | | | | | | | | | | |
| Total protein | 44.45$^{a}$ | 0.36 | 43.76$^{ab}$ | 0.73 | 42.67$^{abc}$ | 0.56 | 40.61$^{c}$ | 0.72 | 43.50$^{ab}$ | 0.51 | 43.26$^{ab}$ | 0.59 | 43.57$^{ab}$ | 0.73 |
| Whey protein | 7.00 | 0.08 | 6.93 | 0.19 | 6.70 | 0.14 | 6.49 | 0.18 | 6.90 | 0.13 | 6.78 | 0.15 | 6.97 | 0.19 |
| Casein | 37.46$^{a}$ | 0.34 | 36.81$^{ab}$ | 0.65 | 35.97$^{bc}$ | 0.50 | 34.12$^{c}$ | 0.64 | 36.58$^{ab}$ | 0.46 | 36.49$^{ab}$ | 0.53 | 36.62$^{ab}$ | 0.65 |
| $\alpha s_1$-casein | 12.45$^{a}$ | 0.13 | 12.20$^{abc}$ | 0.24 | 11.98$^{bc}$ | 0.18 | 11.36$^{c}$ | 0.24 | 12.19$^{ab}$ | 0.17 | 12.15$^{ab}$ | 0.19 | 12.18$^{abc}$ | 0.24 |
| $\alpha s_2$-casein | 3.73$^{a}$ | 0.05 | 3.71$^{a}$ | 0.11 | 3.64$^{a}$ | 0.08 | 3.25$^{b}$ | 0.11 | 3.62$^{a}$ | 0.07 | 3.67$^{a}$ | 0.09 | 3.54$^{ab}$ | 0.11 |
| β-casein | 14.11$^{a}$ | 0.16 | 13.77$^{ab}$ | 0.28 | 13.15$^{bc}$ | 0.23 | 12.71$^{c}$ | 0.28 | 13.65$^{ab}$ | 0.21 | 13.40$^{bc}$ | 0.24 | 13.59$^{abc}$ | 0.28 |
| κ-casein | 4.45 | 0.05 | 4.46 | 0.12 | 4.33 | 0.09 | 4.23 | 0.12 | 4.33 | 0.08 | 4.37 | 0.09 | 4.39 | 0.12 |
| Lactalbumin | 1.00 | 0.01 | 0.97 | 0.02 | 0.97 | 0.02 | 0.97 | 0.02 | 1.00 | 0.02 | 0.98 | 0.02 | 1.02 | 0.02 |
| Lactoglobulin | 5.90 | 0.08 | 5.87 | 0.18 | 5.63 | 0.14 | 5.43 | 0.18 | 5.81 | 0.12 | 5.71 | 0.15 | 5.85 | 0.18 |
| Lactoferrin | 0.099 | 0.004 | 0.089 | 0.007 | 0.102 | 0.006 | 0.092 | 0.007 | 0.092 | 0.005 | 0.096 | 0.006 | 0.099 | 0.007 |

[1]No Growth samples were divided into 3 classes on the basis of SCS 25$^{th}$ and 75$^{th}$ percentiles: with low (L; 100-137 cells*10$^{3}$/mL), medium (M; 137-425 cells*10$^{3}$/mL) and high (H; > 425 cells*10$^{3}$/mL) SCC

[2]Casein number = (casein / protein) *100

[3]Contents of all protein fractions were measured by reversed-phase HPLC on skim milk. Total protein = Whey protein + Casein; Whey protein = sum of total whey fractions; Casein = sum of total casein fractions.
LSM with different letters are statistically different (Tukey AdJ $P < 0.05$).

**Table 7.** Least square means (LSM) and standard errors (SE) of traditional milk coagulation properties (MCP), curd firming, cheese yields (%CY) and curd nutrient recoveries (REC) by UH status[1]

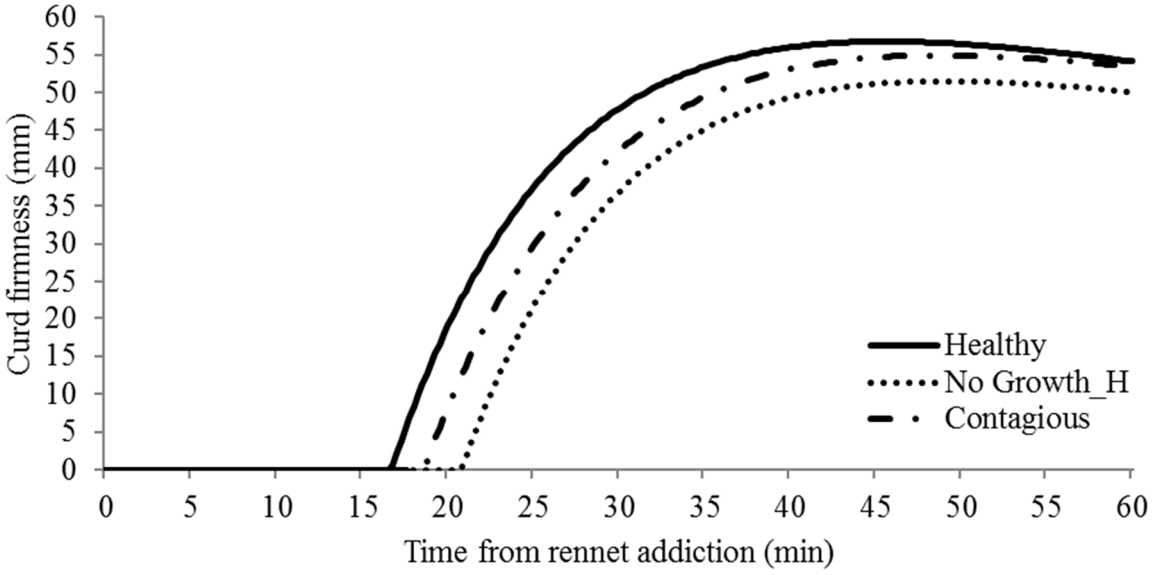
| Trait[2] | Healthy | | No Growth_L | | No Growth_M | | No Growth_H | | Contagious | | Environmental | | Opportunistic | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE | LSM | SE |
| Traditional MCP | | | | | | | | | | | | | | |
| RCT, min | $16.5^{b}$ | 0.4 | $18.0^{ab}$ | 0.9 | $17.7^{ab}$ | 0.7 | $20.4^{a}$ | 0.9 | $18.3^{a}$ | 0.6 | $17.4^{ab}$ | 0.7 | $17.6^{ab}$ | 0.9 |
| $k_{20}$, min | 3.9 | 0.2 | 4.0 | 0.4 | 4.1 | 0.3 | 4.6 | 0.4 | 4.4 | 0.3 | 4.0 | 0.3 | 4.3 | 0.4 |
| $a_{30}$, mm | $44.5^{a}$ | 1.2 | $40.4^{ab}$ | 2.5 | $41.5^{ab}$ | 1.9 | $34.5^{b}$ | 2.5 | $39.8^{b}$ | 1.8 | $42.1^{ab}$ | 2.0 | $41.3^{ab}$ | 2.5 |
| $a_{45}$, mm | $52.8^{a}$ | 1.0 | $51.0^{a}$ | 2.1 | $51.6^{a}$ | 1.6 | $41.5^{b}$ | 2.1 | $50.1^{a}$ | 1.4 | $51.8^{a}$ | 1.7 | $53.2^{a}$ | 2.1 |
| $a_{60}$, mm | $53.7^{a}$ | 0.9 | $52.3^{a}$ | 1.7 | $53.3^{a}$ | 1.3 | $42.4^{b}$ | 1.7 | $52.3^{a}$ | 1.2 | $54.2^{a}$ | 1.4 | $53.3^{a}$ | 1.7 |
| Curd firming | | | | | | | | | | | | | | |
| $RCT_{eq}$, min | $16.7^{b}$ | 0.4 | $18.2^{ab}$ | 0.9 | $18.0^{ab}$ | 0.7 | $20.8^{a}$ | 0.9 | $18.6^{a}$ | 0.6 | $17.7^{ab}$ | 0.7 | $18.0^{ab}$ | 0.9 |
| $CF_p$, mm | $75.5^{a}$ | 1.0 | $72.8^{ab}$ | 2.0 | $74.1^{a}$ | 1.6 | $66.7^{b}$ | 2.1 | $72.9^{ab}$ | 1.4 | $76.3^{a}$ | 1.7 | $75.6^{a}$ | 2.1 |
| $k_{CF}$, %×min$^{-1}$ | $9.0^{ab}$ | 0.1 | $8.7^{ab}$ | 0.3 | $8.8^{ab}$ | 0.2 | $9.6^{a}$ | 0.3 | $8.7^{b}$ | 0.2 | $9.0^{ab}$ | 0.2 | $9.0^{ab}$ | 0.3 |
| $k_{SR}$, %×min$^{-1}$ | 0.72 | 0.02 | 0.69 | 0.03 | 0.70 | 0.02 | 0.68 | 0.03 | 0.68 | 0.02 | 0.72 | 0.02 | 0.71 | 0.03 |
| $C_{max}$, mm | $56.4^{a}$ | 0.8 | $54.3^{ab}$ | 1.5 | $55.3^{a}$ | 1.2 | $49.8^{b}$ | 1.6 | $54.4^{ab}$ | 1.1 | $57.0^{a}$ | 1.2 | $56.4^{a}$ | 1.5 |
| $t_{max}$, min | 48.4 | 0.6 | 49.5 | 1.0 | 49.5 | 0.8 | 49.2 | 1.0 | 49.8 | 0.7 | 49.0 | 0.8 | 49.4 | 1.0 |
| Cheese yields, % | | | | | | | | | | | | | | |
| $\%CY_{CURD}$ | 16.3 | 0.2 | 16.3 | 0.5 | 16.0 | 0.4 | 15.6 | 0.6 | 16.2 | 0.3 | 16.4 | 0.4 | 16.5 | 0.4 |
| $\%CY_{SOLIDS}$ | 7.5 | 0.1 | 7.6 | 0.2 | 7.4 | 0.2 | 7.1 | 0.3 | 7.5 | 0.1 | 7.7 | 0.2 | 7.7 | 0.2 |
| $\%CY_{WATER}$ | 8.7 | 0.2 | 8.7 | 0.3 | 8.6 | 0.2 | 8.5 | 0.4 | 8.7 | 0.2 | 8.7 | 0.3 | 8.7 | 0.3 |
| Recoveries, % | | | | | | | | | | | | | | |
| $REC_{PROTEIN}$ | $79.7^{a}$ | 0.2 | $78.9^{ab}$ | 0.4 | $78.7^{ab}$ | 0.4 | $78.1^{ab}$ | 0.6 | $78.7^{b}$ | 0.3 | $79.0^{ab}$ | 0.4 | $78.5^{b}$ | 0.4 |
| $REC_{FAT}$ | $85.5^{a}$ | 0.4 | $85.8^{ab}$ | 1.2 | $84.6^{ab}$ | 0.9 | $83.1^{ab}$ | 1.5 | $83.3^{b}$ | 0.6 | $84.8^{ab}$ | 1.0 | $83.9^{ab}$ | 1.0 |
| $REC_{SOLIDS}$ | 54.2 | 0.4 | 54.6 | 0.9 | 53.8 | 0.7 | 53.0 | 1.1 | 53.9 | 0.5 | 55.3 | 0.8 | 54.7 | 0.8 |
| $REC_{ENERGY}$ | 69.7 | 0.3 | 70.1 | 0.8 | 69.1 | 0.7 | 68.1 | 1.0 | 68.6 | 0.5 | 69.7 | 0.7 | 69.3 | 0.7 |

[1]No Growth samples were divided into 3 classes on the basis of SCS 25$^{th}$ and 75$^{th}$ percentiles: with low (L; 100-137 cells*10$^3$/mL), medium (M; 137-425 cells*10$^3$/mL) and high (H; > 425 cells*10$^3$/mL) SCC

[2]RCT = rennet coagulation time; $k_{20}$ = curd firming rate as min to a curd firmness of 20 mm; $a_{30\ (45\text{-}60)}$ = curd firmness after 30 (45-60) min from rennet addition; $RCT_{eq}$ = rennet coagulation time estimated using the equation; $CF_P$ = asymptotic potential curd firmness; $k_{CF}$ = curd firming instant rate constant; $k_{SR}$ = syneresis instant rate constant; $CF_{max}$ = maximum curd firmness achieved within 45 min; $t_{max}$ = time at achievement of $CF_{max}$; $\%CY_{CURD}$ = weight of fresh curd as percentage of weight of milk processed; $\%CY_{SOLIDS}$ = weight of curd solids as percentage of weight of milk processed; $\%CY_{WATER}$ = weight of water curd as percentage of weight of milk processed; $REC_{PROTEIN}$ = protein of the curd as percentage of the protein of the milk processed; $REC_{FAT}$ = fat of the curd as percentage of the fat of the milk processed; $REC_{SOLIDS}$ = solids of the curd as percentage of the solids of the milk processed; $REC_{ENERGY}$ = energy of the curd as percentage of energy of the milk processed.

LSM with different letters are statistically different (Tukey AdJ $P < 0.05$).

**Figure 1.** Curd firmness modelling for 3 different UH status groups: Healthy, culture-negative with high SCC (No Growth_H) and Contagious



# CHAPTER 4.

## Association between pathogen-specific cases of subclinical mastitis and blood serum proteins in dairy cows

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**ABSTRACT**

The aim of this study was to assess the association between pathogen-specific cases of subclinical mastitis and blood serum proteins (i.e., total protein, albumin, globulin and the ratio of albumin-to-globulin) in dairy cows. Blood and milk samples were collected from 1,508 cows of 6 breeds housed in 41 multi-breed herds. Bacteriological analysis was performed on milk samples with somatic cell count (SCC) > 100,000 cells/mL and bacteria identification was confirmed by multiplex-PCR assays. Data of blood serum proteins were analyzed using a linear mixed model which included the fixed effects of stage of lactation, parity, breed, udder health (UH) status (healthy cows, culture-negative samples with low - medium and high SCC, and culture-positive samples with contagious, environmental and opportunistic IMI), herd productivity (low or high production, defined according to the average net energy of milk yielded daily by the cows), and the random effect of herd-date within herd productivity. Culture-negative samples with high milk SCC, which were possibly undergoing a strong inflammatory response and pathogens could not be isolated because engulfed by macrophages, and milk samples infected by contagious and environmental bacteria were associated with greater globulin content (and lower albumin-to-globulin ratio) in blood. Variation in blood serum proteins seemed to be associated with inflammation status rather than infection, as globulin significantly increased in UH status groups with highest milk SCC and no differences were observed among IMI pathogens. Blood serum proteins can be indicators of mammary gland inflammation, but cannot be used to differentiate pathogen-specific IMI.

**Key words**: subclinical mastitis, intramammary infection, blood serum proteins, dairy cattle

## INTRODUCTION

Udder health (**UH**) represents a critical issue for milk production and monitoring the health status of the animals is of great importance for dairy farm economics. Bovine mastitis, an inflammatory status of the mammary gland in response to an infection, is the most prevalent production disease and strongly affects dairy herds income by decreasing milk yield and quality, cow fertility and longevity (Seegers et al., 2003). Thus, a successful immune response of the animal to infection can improve milk production and reduce the treatment costs.

Blood serum proteins have been suggested as markers for assessing the immune status in dairy cows (Piccinini et al., 2004) and their analysis possibly represents an initial screening test to identify animals which need further clinical investigations. In Chapter 2 we characterized the variation in blood serum proteins [i.e., total protein, albumin, globulin and the ratio of albumin-to-globulin (**A:G**)] in dairy cattle housed in multi-breed herds and we concluded that environmental factors (e.g., herd productivity), breed and individual cow factors (stage of lactation and parity) must be considered to appropriately interpret blood serum proteins as a cow welfare indicators. After adjusting for those factors, linear relationships between blood serum proteins and milk SCS were identified and supported by correlation estimates, highlighting the possible use of blood serum proteins as UH indicators. Cows with high SCC in milk had greater total protein and globulin contents in blood, an expected result given that α- and γ-globulins are involved in the immune response of the mammary gland.

The inflammatory status of the mammary gland is commonly detected by an increased milk SCC; however, specific IMI pathogens can elicit different immune responses (Bannerman et al., 2004) and differences in SCC variation (de Haas et al., 2002). To our knowledge, variation in blood serum proteins during pathogen-specific cases of subclinical

mastitis has not been investigated yet. Therefore, the objective of the present study was to assess the association between UH status and blood serum proteins in dairy cows.

## MATERIALS AND METHODS

### *Samples Collection and Analysis*

This study is part of the Cowplus Project, described in details in Chapter 2. Briefly, blood and milk samples were collected from 1,508 cows belonging to 3 specialized dairy breeds [Holstein Friesian (**HF**), Brown Swiss (**BS**) and Jersey (**JER**)] and 3 dual-purpose breeds of Alpine origin [Simmental (**SI**), Rendena (**REN**) and Grey Alpine (**GA**)] housed in 41 multi-breed herds of Trentino region (north-eastern Italy). In a calendar year, one herd per day was visited once and only clinically healthy cows at the time of the visit were sampled during an evening milking. Details on procedures used to collect and analyze blood samples for serum proteins determination can be found in Chapter 2. From each selected cow, a composite milk sample (40 mL) for bacteriological analyses and a second sample (50 mL) for SCC determination were collected. Details on milk samples collection, storage and analyses have already been described in materials and methods of Chapter 3. Cows with milk SCC < 100,000 cells/mL were considered potentially healthy and were not subjected to microbiological examination. Bacteriological analysis was performed on milk samples with SCC > 100,000 cells/mL according to the guidelines of National Mastitis Council (NMC, 1999) and bacteria identification was confirmed by multiplex-PCR assays, as previously reported in Chapter 3. Contaminated plates presented 3 or more different colony types, with no prevalence of a single colony type (NMC, 1999). Samples were classified as culture-negative when no bacteria were isolated or no significant growth (<1,000 cfu/mL) was observed within 48 h of incubation, with the exception of potential contagious IMI cases, for which identification was performed even when 1 colony (≥ 100 cfu/mL) was isolated.

***Statistical Analysis***

For the statistical analysis, farms were classified as low or high production according to the cow's average daily milk energy yield (Tyrrell and Reid, 1965) corrected for breed, parity and stage of lactation (Stocco et al., 2016b). Seven clusters of UH status were identified: Healthy (cows with milk SCC < 100,000 cells/mL and not tested for presence of bacteria), culture-negative samples with low (**No Growth_L**), medium (**No Growth_M**) and high (**No Growth_H**) SCC (divided on the basis of SCS 25$^{th}$ and 75$^{th}$ percentiles, calculated considering all culture-negative samples), culture-positive samples with Contagious, Environmental and Opportunistic IMI. Data of blood serum proteins were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with the linear mixed model applied also in Chapter 3:

$$y_{ijklmno} = \mu + DIM_i + Parity_j + Breed_k + UH\ status_l + HP_m + HTD_n(HP)_m + e_{ijklmno},$$

where $y_{ijklmno}$ is the investigated trait (blood serum proteins); $\mu$ is the overall mean; $DIM_i$ is the fixed effect of the $i$th class of days in milk ($i$ = 6 classes of 60-d intervals); $Parity_j$ is the fixed effect of the $j$th parity ($j$ = 1 to ≥ 4); $Breed_k$ is the fixed effect of the $k$th breed ($k$ = HF, BS, JER, SI, REN and GA); $UH\ status_l$ is the fixed effect of the $l$th group of udder health status ($l$ = Healthy, No Growth_L, No Growth_M, No Growth_H, Contagious, Environmental, Opportunistic); $HP_m$ is the fixed effect of the $m$th herd productivity ($m$ = low or high production); $HTD_n(HP)_m$ is the random effect of the $n$th herd-date ($n$ = 41 levels) within the $m$th herd productivity; $e_{ijklmno}$ is the random residual. A normal distribution was assumed for herd-date and residuals, with a mean of zero and a variance of $\sigma_h^2$ and $\sigma_e^2$, respectively. Proportion of variance explained by herd-date (HTD, %) was determined by dividing the

corresponding variance component by the total variance. Pairwise comparisons between UH status groups were performed using the Tukey correction ($P < 0.05$).

## RESULTS AND DISCUSSION

### *Bacteriological Results and Classification of UH Status*

Results of the bacteriological analysis have been reported in Table 1. Healthy animals represented about 58% of the samples and had a mean milk SCS of 1.48. Contagious IMI (mean milk SCS = 4.81), identified when at least 1 colony (≥ 100 cfu/mL) of *Staphylococcus aureus* or *Streptococcus agalactiae* was isolated, were the most frequent, corresponding to about 27% of the cultured samples. Environmental and opportunistic pathogens were considered to cause an IMI if at least 10 grown colonies/type (1,000 cfu/mL) were isolated in the plate. Environmental IMI (16%, mean milk SCS = 4.70) were caused by *Enterococcus* spp, *Streptococcus dysgalactiae*, *Strepococcus uberis*, *Proteus* spp, *Aerococcus viridans*, *Escherichia coli*, *Klebsiella* spp, *Bacillus* spp, *Enterobacter* spp, *Lactococcus lactis* and other Streptococci. Opportunistic IMI (9%, mean milk SCS = 4.60) were related to the presence of Coagulase-negative Staphylococci (**CNS**) in milk. Sixty-one samples were contaminated and thus excluded from the analysis, whereas 245 were culture-negative (No Growth), even if the mean milk SCS was relatively high (4.38). Three hypothesis were considered to explain the high SCC in samples with no bacterial growth: (1) healing process and spontaneous elimination of the infection at the time of the sampling, (2) high level of inflammation and internalization of the pathogens by phagocytes and iii) presence of false negative results due to a dilution effect of composite samples. Culture-negative samples were thus divided in 3 sub-groups on the basis of SCS 25$^{th}$ and 75$^{th}$ percentiles: culture-negative samples with low SCS (No Growth_L; 61 samples with mean SCS = 3.21), medium SCS (No Growth_M; 122

samples with mean SCS = 4.07) and high SCS (No Growth_H; 62 samples with mean SCS = 6.13).

***Association between UH Status and Blood Serum Proteins***

Coefficient of variation of blood serum proteins ranged from 7 to 16% (Table 2). Serum total protein averaged 74.17 g/L, corresponding to the sum of albumin and globulin (30.77 and 43.40 g/L, respectively). Albumin-to-globulin ratio had a mean value of 0.72.

In accordance to our previous results reported in Chapter 2, the proportion of variance explained by herd-date was approximately 20%. All explanatory variables were important sources of variation of blood serum proteins, with the exception of herd productivity, which was only associated with albumin content in blood (Table 3). In particular, the association previously observed between blood serum proteins and mammary gland inflammation, determined by the measure of SCC in milk, is now supported by the evidence of an association also with UH status (Table 3).

In comparison to healthy cows, greater total protein concentrations were measured in blood of cows infected by contagious pathogens and in animals whose milk was characterized by medium to high SCC but no bacteria were isolated (No Growth_M and No Growth_H) (Figure 1a). As serum albumin concentration did not differ among UH status groups (Figure 1b), with the exception of a slightly decrease during environmental IMI in comparison to healthy animals, the increase in total protein can be explained by an increase in the globulin fraction. Culture-negative samples with high milk SCC (NoGrowth_H), which were possibly undergoing a strong inflammatory response and pathogens could not be isolated because engulfed by macrophages (as hypothesized in Chapter 3), and milk samples infected by contagious and environmental bacteria were associated with greater globulin content in blood (Figure 1c). As a consequence, lower A:G was reported for those three UH clusters (Figure

1d). Variation in blood serum proteins was not observed with opportunistic IMI. Because CNS are commonly found on udder skin, some of the culture-positive results can be due to teat skin contamination during the collection of composite samples, rather than represent a real mammary gland infection (Thorberg et al., 2009). Thus, as the increase in globulin content was observed in UH status groups with the highest milk SCS (6.13, 4.80 and 4.70, for No Growth_H, Contagious and Environmental IMI, respectively) and no differences were observed among IMI pathogens, variation in blood serum proteins seemed to be associated with inflammatory status rather than infection. These findings support the results previously reported in Chapter 3, in which the greatest impairment in milk composition and technological properties related to cheese production was observed in the two UH status groups with the highest milk SCC, i.e. contagious IMI and culture-negative samples with high SCC.

Somatic cells and blood serum proteins seems to be two key components of the immune response. Once inside the teat cistern, bacteria release toxins and the contact between pathogens and leukocytes and epithelial cells induces the activation of the innate immune system. After pathogen recognition, realized through the expression of specific receptors like the cytokines toll-like receptors (**TLR**) and CD14, bacterial growth is inhibited through the recruitment of circulating neutrophils from the bloodstream to the mammary tissue (Wellnitz and Bruckmaier, 2012). Leukocytes release several cytokines, including tumor necrosis factor-α (**TNF-α**) and interleukin 8 (**IL-8**), which induce the synthesis of positive acute phase proteins, like the α-globulins serum amyloid A (**SAA**) and haptoglobin (**Hp**) (Viguier et al., 2009). Alfa-globulins are produced in the liver and migrate to the site of infection to inhibit growth of microbes; thus, higher levels can be detected in blood during inflammatory status, partially explaining the increase in globulin content observed in serum of cows with high SCC

in milk (Figure 1c). Serum amyloid A and Hp have already been recognized as possible mastitis indicators, in association with the traditional SCC (Petersen et al., 2004).

Also the γ-globulin fraction is actively involved in the defense system. In fact, different immunoglobulin isotypes prevent colonization by bacteria (IgA) and support phagocytosis by neutrophils (IgG and IgM) (Korhonen et al., 2000). In particular, animals with a successful immune response to IMI pathogens should have a rapid and effective neutrophils recruitment ability and high levels in blood of the opsonizing antibodies IgG2, with subsequent leakage into milk (Burton and Erskine, 2003). Thus, when appropriate antibodies bind bacteria, pathogen recognition and destruction by neutrophils is facilitated. A positive correlation between IgG2 level in serum and clinical mastitis has been observed in Holstein Friesian cows (Mallard et al., 1983) and in Danish Red cattle a genetic deficiency in IgG2 synthesis was associated with a higher frequency of disease caused by pyogenic bacteria, including mastitis (Nansen, 1972). To synthesized pathogen-reactive IgG2 antibodies, B lymphocytes need interferon (**IFN- γ**) and other cytokines, secreted by T cells only after recognition of specific antigens during infection (Burton and Erskine, 2003). Thus, the increased synthesis of γ-globulins during mammary gland inflammation can further explain the greater globulin concentration in serum of cows with high milk SCC.

## CONCLUSIONS

Results of this study suggest that blood serum proteins (i.e., total protein, albumin, globulin and A:G) can be indicators of mammary gland inflammation, but cannot be used to differentiate pathogen-specific IMI. However, as different etiological agents can induce different immune responses and stimulate the synthesis of different pro-inflammatory cytokines (Oviedo-Boyso et al., 2007), further studies will be required to deeply investigate variation in specific globulin fractions during pathogen-specific cases of subclinical mastitis.

## ACKNOWLEDGMENTS

The authors thank the Autonomous Province of Trento (Italy) for funding.

## TABLES AND FIGURES

**Table1.** Results of the bacteriological analysis performed on 639 composite milk samples with SCC > 100,000 cells/mL (modified from Chapter 3)

| Pathogen classification | N | % |
|---|---|---|
| Contagious | 172 | 26.9 |
| *Staphylococcus aureus* | 151 | 23.6 |
| *Streptococcus agalactiae* | 11 | 1.7 |
| *Staphylococcus aureus* + *Streptococcus dysgalactiae* | 5 | 0.8 |
| *Staphylococcus aureus* + *Streptococcus agalactiae* | 2 | 0.3 |
| *Staphylococcus aureus* + *Enterococcus spp* | 1 | 0.2 |
| *Staphylococcus aureus* + *Streptococcus uberis* | 1 | 0.2 |
| *Staphylococcus aureus* + other Streptococci | 1 | 0.2 |
| Environmental | 102 | 16.0 |
| other Streptococci | 26 | 4.1 |
| *Enterococcus spp* | 21 | 3.3 |
| *Streptococcus dysgalactiae* | 16 | 2.5 |
| *Streptococcus uberis* | 14 | 2.2 |
| *Proteus spp* | 9 | 1.4 |
| *Aerococcus viridans* | 5 | 0.8 |
| *Escherichia coli* | 5 | 0.8 |
| *Klebsiella spp* | 2 | 0.3 |
| *Bacillus spp* | 1 | 0.2 |
| *Enterobacter spp* | 1 | 0.2 |
| *Lactococcus lactis* | 1 | 0.2 |
| *Aerococcus viridans* + CNS | 1 | 0.2 |
| Opportunistic | 59 | 9.2 |
| Coagulase Negative Staphylococci (CNS) | 59 | 9.2 |
| No Growth | 245 | 38.3 |
| Contaminated | 61 | 9.5 |

[1]SCS = $\log_2$ (SCC/100,000) + 3

**Table 2.** Descriptive statistics of blood serum proteins (n = 1,447)[1]

| Trait | Mean | CV, % | P1 | P99 |
|---|---|---|---|---|
| Serum proteins | | | | |
| Total protein, g/L | 74.17 | 7.5 | 61.29 | 88.66 |
| Albumin, g/L | 30.77 | 6.9 | 24.84 | 35.22 |
| Globulin, g/L | 43.40 | 13.5 | 32.83 | 61.03 |
| Albumin:Globulin | 0.72 | 15.9 | 0.43 | 0.99 |

[1]P1 = 1st percentile; P99 = 99th percentile

**Table 3.** Results from ANOVA (F-value and significance) for blood serum proteins

| Trait | HP[1] | HTD, %[2] | Breed | DIM | Parity | UH status | RMSE[3] |
|---|---|---|---|---|---|---|---|
| Serum proteins | | | | | | | |
| Total protein, g/L | 2.4 | 22.0 | $7.2^{***}$ | $3.2^{**}$ | $28.7^{***}$ | $6.9^{***}$ | 4.75 |
| Albumin, g/L | $14.8^{***}$ | 22.5 | $7.1^{***}$ | $7.6^{***}$ | $2.8^{*}$ | $3.2^{**}$ | 1.80 |
| Globulin, g/L | 0.0 | 20.3 | $8.9^{***}$ | $3.3^{**}$ | $23.2^{***}$ | $8.4^{***}$ | 5.11 |
| Albumin:Globulin | 1.8 | 21.4 | $12.4^{***}$ | $4.7^{***}$ | $15.6^{***}$ | $8.2^{***}$ | 0.10 |

[1]HP = Herd productivity

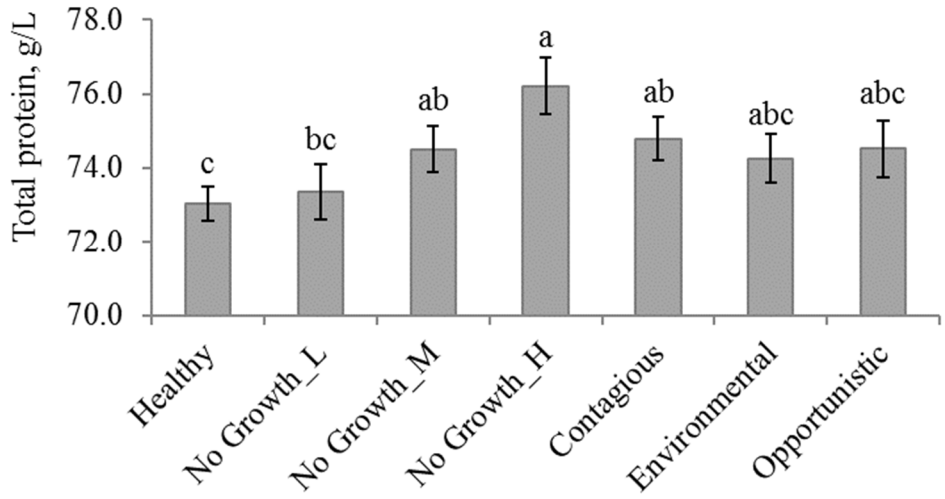
[2]Herd/Test Day effect expressed as proportion of variance explained by herd-date calculated by dividing the corresponding variance component by the total variance

[3]RMSE = root mean square error.

$^{*}$ $P < 0.05$; $^{**}$ $P < 0.01$; $^{***}$ $P < 0.001$

**Figure 1.** Least square means and standard errors of (a) total protein, (b) albumin, (c) globulin and (d) albumin-to-globulin ratio by UH status[1]

[a]

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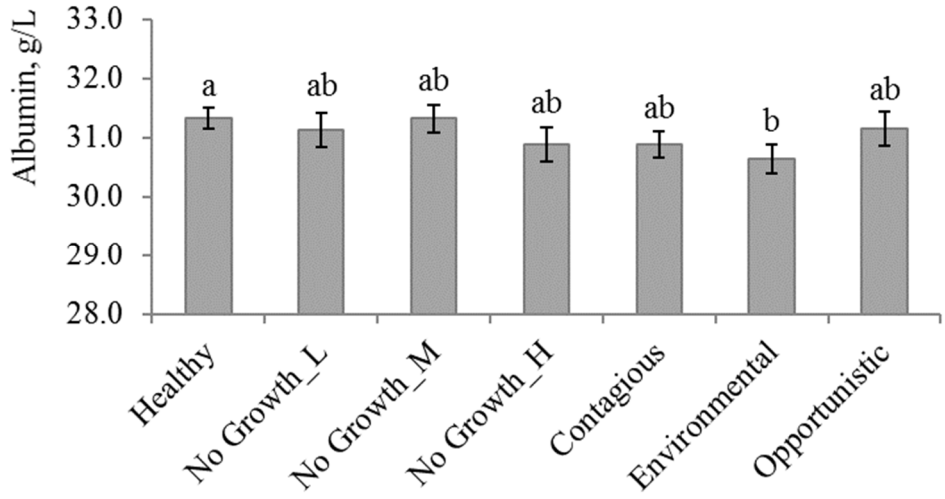
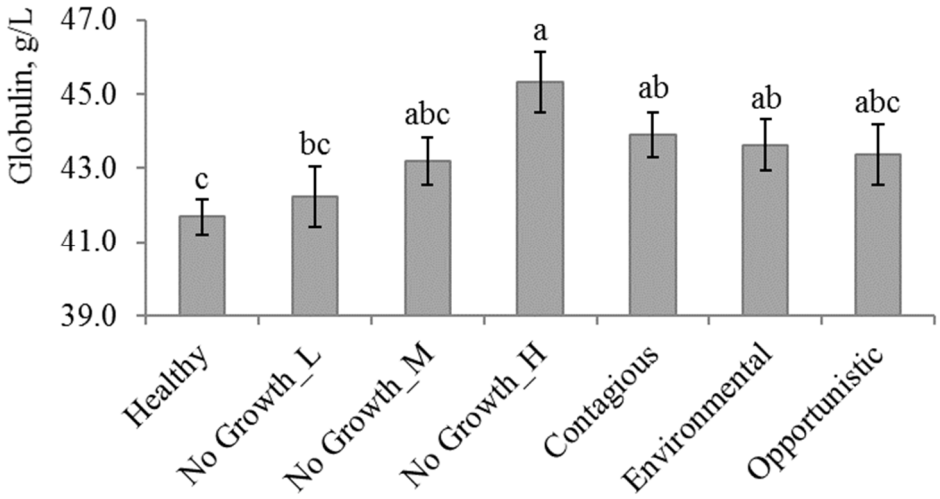
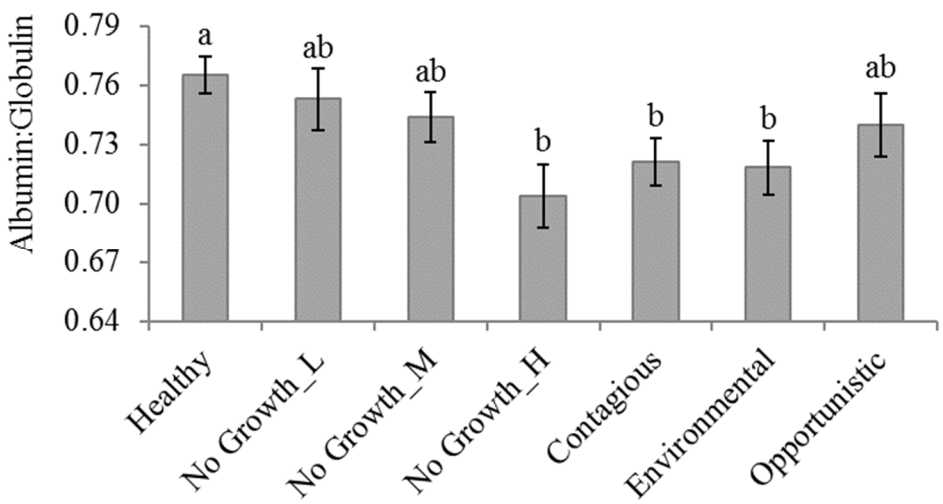



[c]



[d]




[1]No Growth samples were divided into 3 classes on the basis of SCS 25th and 75th percentiles: with low (L; 100-137 cells*$10^3$/mL), medium (M; 137-425 cells*$10^3$/mL) and high (H; > 425 cells*$10^3$/mL) SCC. LSM with different letters are statistically different (Tukey AdJ $P < 0.05$)

# GENERAL DISCUSSION AND CONCLUSIONS

Our results have provided with new insights on the relationships between udder health (with a focus on subclinical cases of mastitis identified by SCC and bacteriological analyses) and milk traits (i.e., composition, detailed protein profile and new milk technological traits related to the cheese-making process), and blood serum proteins, as possible indicators of immune response, in dairy cows.

Our findings confirmed the negative effect of high SCC on milk yield, composition, coagulation properties, as well as in a new set of cheese-making traits, measured at individual cow level, highlighting the poor technological properties of milk with very low SCC. As SCS increased, a linear loss in milk production was observed accompanied by changes in milk composition. This, in turn resulted in a decreased quality, clotting and cheese-making ability of the processed milk. Nonlinear trends were observed for some milk traits with respect to different classes of SCS (from very low to very high). Such nonlinear relationships should be considered in future studies.

Linear relationships between SCS and blood serum proteins were detected, confirming the important role of SCC as an indicator of mammary gland inflammation and highlighting the potential use of blood serum proteins as indicators of immune response of the mammary gland to infections. Nevertheless, several factors (e.g., stage of lactation, parity, breed and herd productivity) should be considered to appropriately interpret blood serum proteins as animal welfare indicators. The evaluation of such non-genetic sources of variation of blood serum proteins represent an important first-step for future genetics/genomics analysis (especially for studies integrating genomic, proteomic and metabolomic information for improving the robustness of dairy cows).

Pathogen-specific information revealed no differences among contagious, environmental and opportunistic pathogens, since the greatest impairment in milk and cheese

quantity and quality was observed for samples characterized by the highest level of somatic cells. Hence our results suggest an inflammation rather than an infection effect. Given that environmental pathogens are also responsible of a worsening of milk composition, special focus should be placed on the herd health management. Moreover, our findings detected a discrepancy between bacteriological results and inflammatory status. In fact, culture-negative samples with high SCC were characterized by the poorest milk quality and clotting ability, confirming the important role of SCC as indicator of udder health and milk quality.

The hypothesis of the detrimental effect of inflammatory status rather than infection of the mammary gland on milk traits was supported by the results obtained on blood serum proteins. In fact, an increase of globulin concentration in serum was observed in cows with the greatest SCC in milk, independently from the recovery of a pathogen or not. Thus, blood serum proteins can be indicators of mammary gland inflammation, but cannot be used to differentiate pathogen-specific IMI.

Although our findings provide new insights about the important role of mammary health in the bovine dairy sector, several additional aspects need to be explored in future studies. In particular, new studies are needed to investigate the negative effect of very low SCC on milk composition and cheese-related traits. Moreover, repeated sampling, in order to establish the inflammatory status of the mammary gland and the exact infection stage, might help in clarifying the relationship between SCC, milk traits and blood serum proteins. Further studies shall focus in evaluating the effect of subclinical cases of mastitis at a quarter level (to avoid possible contamination and dilution effects) and at a single pathogen level. In addition, molecular analysis could help to detect bacterial DNA in culture-negative samples with high SCC to support the hypothesis of the presence of bacteria engulfed by neutrophils.

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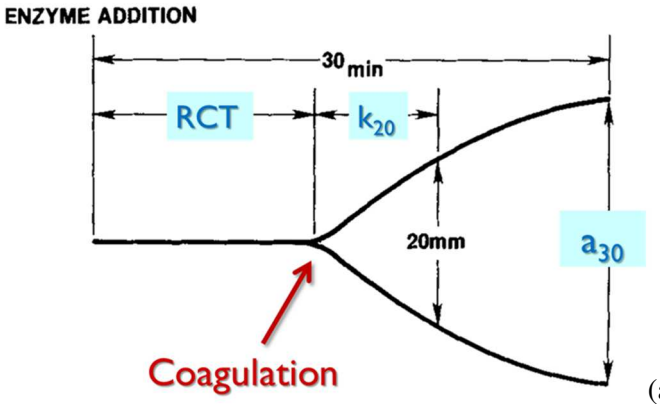
# APPENDIX I

***Traditional milk coagulation property (MCP)***

- RCT (rennet coagulation time, min) = time from addition of enzyme to the beginning of coagulation
- $k_{20}$ (curd-firming rate, min) = interval from RCT to the time at which a curd firmness (CF) of 20 mm is attained
- $a_{30(45–60)}$ (curd firmness, mm) = measure of the extent of CF 30 (45-60) min after coagulant addition




Ideally:

- ✓ Fast coagulation time (small RCT)
- ✓ Good consistency of the curd (large $a_{30}$)

(adapted from McMahon and Brown, 1982)

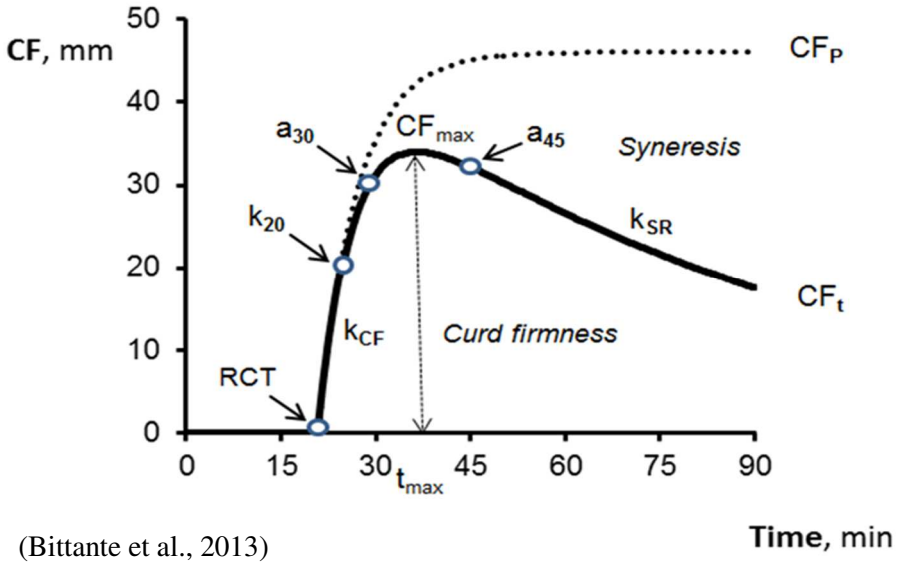
***New curd firming and syneresis traits***

$$CF_t = CF_P \times (1 - e^{-k_{CF} \times (t - RCTeq)}) \times e^{-k_{SR} \times (t - RCTeq)}$$

(Bittante et al., 2013)

- $CF_t$ (mm) = curd firmness (CF) at time t
- $CF_P$ (mm) = asymptotic potential CF at infinite time in absence of syneresis
- $RCT_{eq}$ (min) = RCT estimated by 4-parameters equation on the basis of all data points
- $k_{CF}$ (% × $min^{-1}$) = curd firming instant rate constant
- $k_{SR}$ (% × $min^{-1}$) = syneresis instant rate constant

- $CF_{max}$ (mm) = maximum CF achieved within 45 min
- $t_{max}$ (min) = time at achievement of $CF_{max}$




(Bittante et al., 2013)

***<u>Individual cheese yield (considering also the recovery of nutrients in curd)</u>***

***Percentage cheese yield (%CY)***

- $\%CY_{CURD} = \frac{\text{weight of wheel (g)}}{\text{weight of milk (g)}} \times 100$
- $\%CY_{SOLIDS} = \frac{\text{milk TS (g)} - \text{whey TS(g)}}{\text{weight of milk (g)}} \times 100$
- $\%CY_{WATER} = \frac{\text{milk water (g)} - \text{whey water (g)}}{\text{weight of milk (g)}} \times 100$

Where TS is the total solids and wheel represents the cheese.

***Recovery of nutrients in curd (REC)***

- $REC_{PROTEIN}(\%) = \frac{\text{milk protein (g)} - \text{whey protein (g)}}{\text{milk protein (g)}} \times 100$
- $REC_{FAT}(\%) = \frac{\text{milk fat (g)} - \text{whey fat (g)}}{\text{milk fat (g)}} \times 100$
- $REC_{SOLIDS}(\%) = \frac{\text{milk TS (g)} - \text{whey TS (g)}}{\text{milk TS (g)}} \times 100$
- $REC_{ENERGY}(\%) = \frac{\text{milk energy (kJ)} - \text{whey energy (kJ)}}{\text{milk energy (kJ)}} \times 100$

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*Tania Bobbo*

# CURRICULUM VITAE

Tania Bobbo was born on the 1st of January 1988 in Venice (Italy). After graduation of her Bachelor in Biology at the University of Padova (Italy) in 2009, she enrolled in the Master in Evolutionary Biology at the same University. The MSc thesis project, entitled "Linking mRNA expression and allelic variation in physiologically important genes of the three-spined stickleback", was developed in collaboration with EGRU (Ecological Genetics Research Unit) of the University of Helsinki (Finland). After graduation from the master program in 2011, she worked as a field assistant (in 2012) on a PhD project of the University of Zurich (Switzerland) on the evolution of family living and cooperative breeding in several species of birds. In 2013, she worked as a field assistant at the Environment Office of North Island (Seychelles) and she attended a post-graduate course in "New techniques in molecular medicine" at the University of Padova (Italy). In 2014, she was offered a PhD in Animal Science, aimed at studying the association between udder health (focusing on subclinical cases of bovine mastitis identified by somatic cell count and bacteriological analyses) and milk composition and detailed protein profile, milk technological traits related to the cheese-making process, and other immune response indicators, in dairy cows.

## LIST OF PUBLICATIONS

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