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Associations 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 associations between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition, and cheese-making traits. Forty-one multibreed 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 min 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 percentages 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, medium, or high SCC; and culture-positive samples divided into contagious, environmental, and opportunistic intramammary infection (IMI). Data were analyzed using a linear mixed model. Significant variations in the casein to protein ratio and lactose content were observed in all culture-positive samples and in culture-negative samples with medium to high SCC compared to 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 2 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 cheeses labeled as Protected Designation of Origin 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). Bovine mastitis, an inflammatory response of the mammary gland to infection, is well known to decrease milk yield and quality, with considerable adverse economic effects (Seegers et al., 2003; Halasa et al., 2007). Mastitis, in both its clini-

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cal and subclinical (no visible clinical symptoms are present) states, 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 high SCC on the quantity and quality of milk and dairy products (regardless of bacterial etiology) has been widely reviewed (Kitchen, 1981; Auldist and Hubble, 1998; Sharif and Muhammad, 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; Barłowska et al., 2009), and greater pH (Batavani et al., 2007; Vianna et al., 2008). The detrimental effect of high SCC on milk composition has additional effects on the cheese-making process, with several studies reporting slower milk coagulation, weak curd consistency, and lower cheese yields after processing high SCC milk (Grandison and Ford, 1986; Politis and Ng-Kwai-Hang, 1988; Summer et al., 2015).

However, different pathogens elicit different immune responses in the mammary gland (Bannerman et al., 2004). Depending on etiology, differences have been observed in SCC trends (de Haas et al., 2002) and in milk quality (Leitner et al., 2006). Therefore, identification of pathogens is crucial to fully understanding changes in milk. The effect of different 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 associations between SCC and milk composition traits, few studies have dealt with the relationships between changes in milk in cases of clinical and subclinical mastitis and specific etiologies. In particular, although 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 pathogens have only been reported 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. Further investigations are required to gain a better understanding of specific changes in milk (in terms of both composition and technological properties) during pathogen-specific cases of clinical and subclinical mastitis.

Direct measurements of 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 data set of different measures of individual cheese yields (**%CY**) and milk nutrient and energy recoveries in cheese (**REC**) taken at the laboratory level using a model cheese-making procedure (Cipolat-Gotet et al., 2013) recently became available. In a previous study (Bobbo et al., 2016), 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 this study was to investigate associations between pathogen-specific cases of subclinical mastitis and milk yield, composition, protein composition, coagulation properties, and the aforementioned new technological traits in milk obtained from dairy specialized and dual-purpose cows living in multibreed herds. Rather than simply focus on microbiologically positive infections, we investigated both the effect of the IMI and the effect of the resulting inflammatory response.

MATERIALS AND METHODS

Milk Sample Collection

This study is part of the Cowplus Project described in Stocco et al. (2017). Briefly, 41 multibreed herds (with at least 2 breeds/herd) raised under the different dairy farming systems of Trentino province (northeastern Italy) were selected from a sample of 610 dairy farms previously investigated (detailed environmental conditions are reported in Sturaro et al., 2013). The 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. Three were specialized dairy breeds, Holstein Friesian (**HF**, n = 471), Brown Swiss (**BS**, n = 663), and Jersey (**JER**, n = 40); and the other 3 were 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 aseptically collected from each cow, according to National Mastitis Council guidelines (NMC, 1999), for bacteriological analyses. Briefly, teat ends were cleaned externally with commercial premilking 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 24 h of collection at the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy). After the collection of the milk sample for bacteriological analysis, approximately 2,500 mL of milk was collected from each cow by trained technicians in a single container, subsequently divided into 2 subsamples, maintained at a temperature of 4°C (without preservative), and processed within 24 h 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, 2 aliquots containing 1 mL of milk (with Bronopol, 2-bromo-2nitropropan-1,3-diol, Sigma-Aldrich, St. Louis, MO) 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/100 g) 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 [SCS = $\log_2$ (SCC/100,000) + 3], according to 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 (**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) 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 RP analytical column C8 (Aeris Widepore XB-C8, Phenomenex, Torrance, CA) with a large pore core-shell packing (3.6 μm, 300Å, 250 × 2.1 mm internal diameter). Sample vials, maintained at a low constant temperature (4°C), were injected via an autosampler (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 min from rennet addition ($\mathbf{a_{30}}$, $\mathbf{a_{45}}$, and $\mathbf{a_{60}}$, mm). Experimental conditions were as reported in Stocco et al. (2017).

Curd Firming Traits. Two hundred forty CF values were recorded for each replicate (60 min test, one datum every 15 s). 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. (2017):

$$CF_t = CF_P \times \left(1 - e^{-k_{CF} \times (t - RCT_{eq})}\right) \times e^{-k_{SR} \times (t - RCT_{eq})},$$

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 ($\mathbf{CF_{max}}$, mm) and the time at CF_{max} ($\mathbf{t_{max}}$, 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. (2017) was carried out using a small amount of milk (1,500 mL) to produce cheeses from individual cows. Three %CY traits, expressing the weights of total fresh curd ($\mathbf{\%CY_{CURD}}$) and dry matter ($\mathbf{\%CY_{SOLIDS}}$) and water ($\mathbf{\%CY_{WATER}}$) in the curd as percentages of the weight of the processed milk, and 4 REC traits ($\mathbf{REC_{PROTEIN}}$, $\mathbf{REC_{FAT}}$, $\mathbf{REC_{SOLIDS}}$, and $\mathbf{REC_{ENERGY}}$) 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 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 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.). Bacterial genus and species identification was confirmed definitively by multiplex-PCR assays, as previously described with minor changes (Shome et al., 2011). 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 pathogens, for which identification was performed even when 1 colony (≥100 cfu/mL) was isolated.

Statistical Analysis

Herds were classified as high or low 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., 2017). Briefly, individual milk energy values (kcal/kg) were converted to kilojoules per kilogram and multiplied by individual daily milk production (kg/d) to obtain the daily milk energy production of each cow (KJ/d). To estimate least squares means (**LSM**) of 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 LSM of their average daily milk energy yield, and classified into 2 categories (high or low 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 subgroups on the basis of the SCS 25th and 75th 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.) with the following linear mixed model:

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

where $y_{ijklmno}$ is the investigated milk trait; μ is the overall mean; DIM_i is the fixed effect of the ith class of days in milk ($i = 6$ classes of 60-d intervals, from 5 ≤ class 1 ≤ 65 d to class 6 >305 d); $Parity_j$ is the fixed effect of the jth parity ($j = 1$ to ≥4); $Breed_k$ is the fixed effect of the kth breed (k = HF, BS, JER, SI, REN, and GA); UH $status_l$ is the fixed effect of the lth group of UH status (l = Healthy, No Growth_L, No Growth_M, No Growth_H, Contagious, Environmental, Opportunistic); HP_m is the fixed effect of the mth herd productivity (m = high or low); $HTD_n(HP)_m$ is the random effect of the nth herd-date ($n = 1$ to 41) within the mth herd productivity; $e_{ijklmno}$ is the random residual. Given that herd effect is combined with date of sampling and season, a herd-test day (**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 the significance of the effects of DIM, parity, breed, and UH status was tested 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, and vat (20 levels) and water bath (2 levels) for %CY and REC traits. In addition, because 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 σ_h^2 and σ_e^2, respectively. The proportion of variance explained by herd-date (HTD, %) was calculated for each trait by dividing the corresponding variance component $\left(\sigma_h^2\right)$ by the total variance $\left(\sigma_h^2 + \sigma_e^2\right)$. 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 colonies (1,000 cfu/mL). Due to the low frequency of recovery of some pathogens, we classified them as contagious, environmental, and opportunistic, a commonly used classification scheme that 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*, *Strep. 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 subgroups on the basis of the SCS 25th and 75th 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

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

Udder health (UH) status group	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		
Staph. aureus + *Streptococcus dysgalactiae*	5	0.3	0.8		
Staph. aureus + *Streptococcus agalactiae*	2	0.1	0.3		
Staph. aureus + *Enterococcus* spp.	1	0.1	0.2		
Staph. aureus + *Streptococcus uberis*	1	0.1	0.2		
Staph. 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
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.

normal distributions, so only the 1st and 99th percentiles are reported (Tables 2 and 3). Milk production of cows on multibreed farms averaged 24.4 kg/d with large variability [coefficient of variation (**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 (16–26%), 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 CV of CY traits was 17 to 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. (2017). The proportion of variance explained by herd-test 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, and α_{S1}-, α_{S2}-, and β-casein (Table 4).

After adjustment 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 5). 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, α_{S1}-, α_{S2}-, and β-casein. Lower contents of casein and α_{S2}- and β-casein frac-

Table 2. Descriptive statistics of single test-day milk yield, composition and protein composition (n = 1,447)[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/100 g	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,[3] g/L				
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
α_{S1}-Casein	12.10	17.0	7.33	17.48
α_{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.

tions were also observed in No Growth_M samples, and infection with environmental pathogens was associated with lower β-casein content (Table 5). 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 5).

Sources of Variation Among Cheese-Making Traits

The proportion of variance explained by herd-test date was lower (<15%) for coagulation properties (both traditional MCP and curd firming) than for milk yield and composition traits (Table 6). Herd-test 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 6). 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 6).

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}; Figure 1). 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.

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
CF_{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 the time to a curd firmness of 20 mm; $a_{30\ (45,\ 60)}$ = curd firmness at 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.

DISCUSSION

Prevalence of Pathogens and Classification of UH Status

This study was carried out with data collected from dairy specialized and dual-purpose cattle living in multibreed herds in northeastern Italy. Although it is ideal to have multiple microbiological examinations of milk samples, collection of single milk samples is the most practical and cost-effective sampling methodology in large field studies (Torres et al., 2009). A recent study (Reyher and Dohoo, 2011) 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 effect 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 as a criterion to perform subsequent 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 also suggested 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 when 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 the 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, because 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 of affected cows compared to healthy animals (Table 5). 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 in cows with subclinical mastitis due

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[3]
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,[4] %	0.1	17.3	4.4***	6.6***	18.8***	12.8***
Urea, mg/100 g	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,[5] g/L						
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***
α_{S1}-Casein	12.5**	13.8	44.3***	33.9***	26.4***	5.5***
α_{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]HTD = 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]UH = udder health.

[4]Casein number = (casein/protein) × 100.

[5]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. Least squares means (LSM) and standard errors (SE) of milk yield, composition, and protein composition by udder health (UH) status[1]

	Healthy		No Growth_L		No Growth_M		No Growth_H		Contagious		Environmental		Opportunistic	
Trait	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/100 g	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,[3] g/L														
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
α_{S1}-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
α_{S2}-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

[a–c]LSM with different letters are statistically different (Tukey adjusted $P < 0.05$).

[1]No-growth samples were divided into 3 classes based on the SCS 25th and 75th percentiles: 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.

Table 6. Results of ANOVA (*F*-value and significance) for traditional milk coagulation properties (MCP), curd firming, cheese yields (%CY), and curd nutrient recoveries (REC)

Trait[1]	HP[2]	HTD, %[3]	Breed	DIM	Parity	UH status[4]
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
CF_{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]RCT = rennet coagulation time; k_{20} = curd firming rate as the time to a curd firmness of 20 mm; $a_{30\ (45,\ 60)}$ = curd firmness at 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.

[2]HP = herd productivity.

[3]HTD = herd-test day effect expressed as proportion of variance explained by herd-date calculated by dividing the corresponding variance component by the total variance.

[4]UH = udder health.

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

to environmental and opportunistic pathogens, which did not impair milk production (Table 5). 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 any differences in fat and protein percentages in milk observed 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

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

	Healthy		No Growth_L		No Growth_M		No Growth_H		Contagious		Environmental		Opportunistic	
Trait[2]	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}, % $\times$ 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}, % $\times$ 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
CF_{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

[a,b]LSM with different letters are statistically different (Tukey adjusted $P < 0.05$).

[1]No-growth samples were divided into 3 classes based on the SCS 25th and 75th percentiles: low (L; 100–137 cells $\times$ 10^3/mL), medium (M; 137–425 cells $\times$ 10^3/mL), and high (H; >425 cells $\times$ 10^3/mL) SCC.

[2]RCT = rennet coagulation time; k_{20} = curd firming rate as the time to a curd firmness of 20 mm; $a_{30\,(45,\,60)}$ = curd firmness at 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.

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 5). However, no differences were observed among contagious, environmental, and opportunistic IMI pathogens. A lower casein to 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, compared to uninfected milk, lower lactose concentrations 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), significant changes in lactose content were observed in milk infected by a single specific pathogen compared with normal milk; 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 those from healthy animals (Table 5). 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 other UH status groups. The process of infection triggers an inflammatory response that should reduce 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), but the process itself has potentially affected milk composition. Thus, the classification system used in this study allowed us to evaluate the effect of inflammation even when microbiological analysis yields no microbial growth. Interestingly, culture-negative samples with high SCC were collected from herds in which at least one case

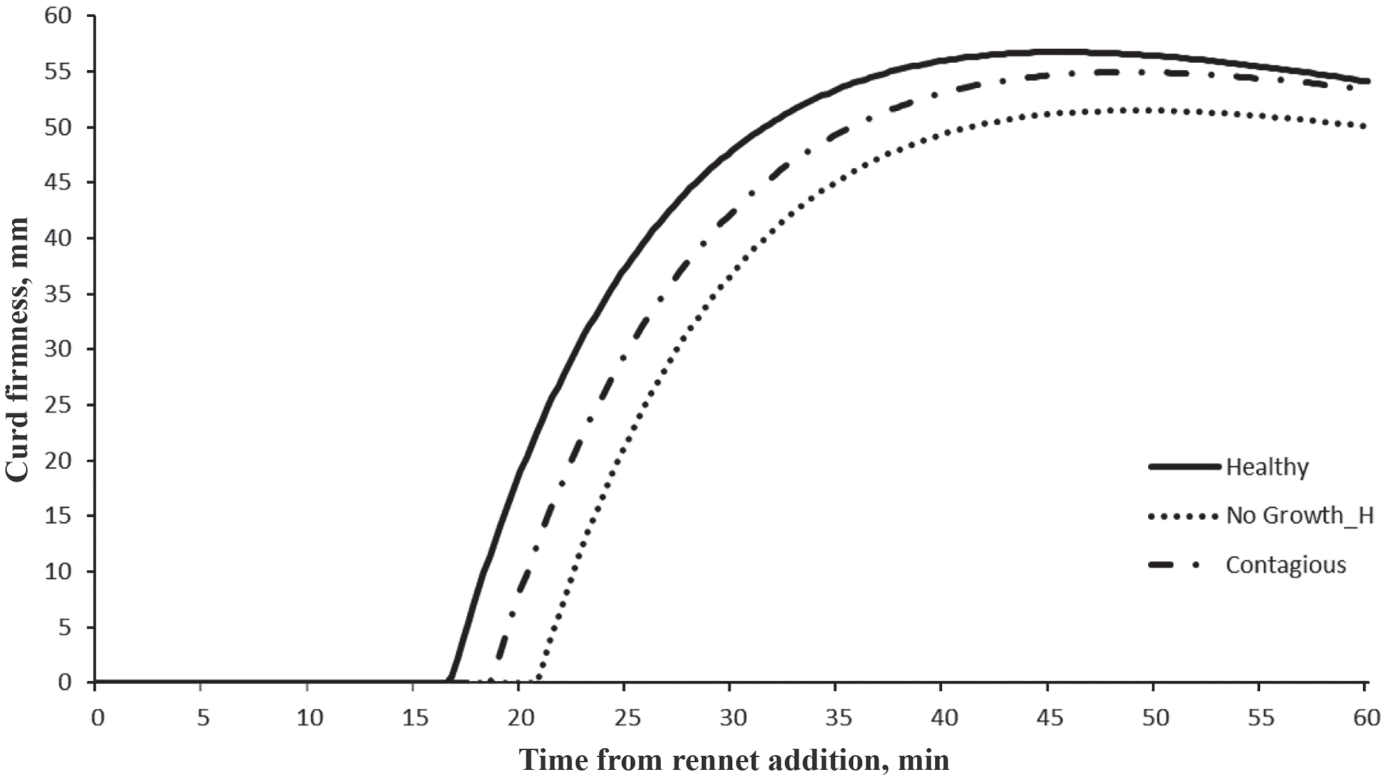

Figure 1. Curd firmness modeling for 3 udder health (UH) status groups: healthy, culture-negative with high SCC (No Growth_H), and contagious.

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 because of 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 skim milk confirmed important changes in culture-negative samples with medium to high SCC (Table 5). In particular, lower true protein and casein contents were found in milk samples for which 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) in comparison with milk collected from healthy cows and animals infected by a specific bacterium. Whey proteins were not influenced by UH status, but α_{S1}-, α_{S2}-, and β-caseins were highly affected (Table 5). 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 to normal milk (Table 5). A significantly lower β-casein fraction was also observed in milk samples infected with environmental pathogens (Table 5). Compared with milk from 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 formation of β-casein fragments, 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 effect 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), but little information exists 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 MCP 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., 2017). 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 5). 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, greater casein breakdown (Auldist et al., 1996) and lower lactose (Leitner et al., 2011) have been shown to be 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 to normal milk. The greatest effect was observed in milk infected by *Strep. dysgalactiae*, although it is worth noting that Leitner et al. (2006) 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), and Merin et al. (2008) previously 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 they were not statistically different from the other UH status groups (Table 7). Given that cheese-related traits were analyzed only on a subset of data, SEM 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 living in multibreed herds. Significant variations in the casein to protein ratio and the lactose content were observed in all culture-positive samples and culture-negative samples with medium to high SCC compared to normal milk. No differences were observed among samples with 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 2 UH status groups with the highest milk SCC (i.e., milk samples subclinically infected with contagious pathogens and culture-negative samples with high SCC), highlighting a discrepancy between bacteriological results and inflammatory status and thus confirming the importance of SCC as an indicator of UH and milk quality. Culture-negative samples with high SCC may have been infected by contagious bacteria that could not be recovered by bacteriological analysis due to 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 to establish the exact infection stage should also be considered.

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