J. Dairy Sci. 107:1413–1426
https://doi.org/10.3168/jds.2023-23818

Effect of intramammary infection and inflammation on milk protein profile assessed at the quarter level in Holstein cows

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ABSTRACT

In this study we wanted to investigate the associations between naturally occurring subclinical intramammary infection (IMI) caused by different etiological agents (i.e., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Prototheca* spp.), in combination with somatic cell count (SCC), on the detailed milk protein profile measured at the individual mammary gland quarter. An initial bacteriological screening (time 0; T0) conducted on individual composite milk from 450 Holstein cows reared in 3 herds, was performed to identify cows with subclinical IMI. We identified 78 infected animals which were followed up at the quarter level at 2 different sampling times: T1 and T2, 2 and 6 wk after T0, respectively. A total of 529 quarter samples belonging to the previously selected animals were collected at the 2 sampling points and analyzed with a reversed phase HPLC (RP-HPLC) validated method. Specifically, we identified and quantified 4 caseins (CN), namely α_{S1}-CN, α_{S2}-CN, κ-CN, and β-CN, and 3 whey protein fractions, namely β-lactoglobulin, α-lactalbumin, and lactoferrin (LF), which were later expressed both quantitatively (g/L) and qualitatively (as a percentage of the total milk nitrogen content, % N). Data were analyzed with a hierarchical linear mixed model with the following fixed effects: days in milk (DIM), parity, herd, SCC, bacteriological status (BACT), and the SCC × BACT interaction. The random effect of individual cow, nested within herd, DIM and parity was used as the error term for the latter effects. Both IMI (i.e., BACT) and SCC significantly reduced the proportion of β-CN and α_{S1}-CN, ascribed to the increased activity of both milk endogenous and microbial proteases. Less evident alterations were found for whey proteins, except for LF, which being a glycoprotein with direct and undirect antimicrobial activity, increased both with IMI and SCC, suggesting its involvement in the modulation of both the innate and adaptive immune response. Finally, increasing SCC in the positive samples was associated with a more marked reduction of total caseins at T1, and α_{S1}-CN at T2, suggesting a synergic effect of infection and inflammation, more evident at high SCC. In conclusion, our work helps clarify the behavior of protein fractions at quarter level in animals having subclinical IMI. The inflammation status driven by the increase in SCC, rather the infection, was associated with the most significant changes, suggesting that the activity of endogenous proteolytic enzymes related to the onset of inflammation might have a pivotal role in directing the alteration of the milk protein profile.

Key words: subclinical mastitis, somatic cell count, udder health, dairy cows

INTRODUCTION

Mastitis is a mammary gland inflammatory condition, which typically develops because of the penetration of a wide range of microorganisms (mostly bacteria) in the udder. In the context of dairy production, despite the ongoing effort toward the improvement of farm management strategies, bovine mastitis remains, especially in the subclinical form, one of the most common and impactful diseases, leading to the impairment of animal welfare and huge economic losses, mostly related to the decreased milk productivity and quality, veterinary costs, and premature culling (Antanaitis et al., 2021; Nayan et al., 2022).

To date, the gold standard for the detection of IMI and the identification of the respective causative pathogens remains the bacteriological examination (Nagasawa et al., 2020), even if its systemic application at a farm level is not always possible as it is a time consuming and expensive technique. Milk SCC gives instead information on the inflammatory status of the mammary gland, and at present is the most widespread tool for screening rapidly and on-site the udder condition (Ruegg, 2017). In this context, in dairy farm practice a threshold of 200,000 somatic cells/mL is generally

Received May 30, 2023.
Accepted September 21, 2023.
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considered appropriate for identifying animals having subclinical mastitis (NMC, 2017; Dal Prà et al., 2022). However, there have been recent studies that demonstrated that SCC may not always provide a complete picture of the animals' health condition (Bobbo et al., 2017). Cases of animals positive at the microbiological examination with low somatic cells have been reported as well as culture-negative samples with high SCC (Bobbo et al., 2017; Pegolo et al., 2022).

It has been demonstrated that milk having high SCC undergoes compositional changes that negatively affect its composition and quality (Bobbo et al., 2016; Pegolo et al., 2020) and, therefore, its economic value. In this context, it has been observed that the increased proteolytic activity associated with high SCC seems to have a particularly strong effect on milk protein fractions (Zhang et al., 2015) and especially on caseins (Caggiano et al., 2019), which are important traits influencing milk quality and technological characteristics.

In the context of the Italian dairy sector, where most of the produced milk is processed into high quality cheese, milk proteins, and especially caseins, play a pivotal role as their quantity and proportion can directly affect the milk coagulation and cheesemaking ability (Cipolat-Gotet et al., 2018; Amalfitano et al., 2019). Several studies have investigated the effects of clinical and subclinical mastitis on milk composition and quality (Paixão et al., 2017; Stocco et al., 2020) and technological traits (Pegolo et al., 2021). However, to the best of our knowledge, no extensive research focused on the specific interaction between subclinical IMI and SCC (i.e., the inflammatory status) and the detailed milk protein profile, when measured at the mammary gland quarter level.

Therefore, the aim of this study was to investigate the association between naturally occurring subclinical IMI caused by 4 different pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Prototheca* spp.) in combination with SCC on the detailed milk protein profile assessed at the quarter level in Holstein cows.

MATERIALS AND METHODS

Study Design, Animals, and Sampling

This study was conducted within the LATSAN project, which aimed at developing innovative tools for improving mammary gland health and milk quality in dairy cattle. The research was approved by the Ethical Animal Care and Use Committee (OPBA – Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019).

This research was a longitudinal observational study aimed at inferring the associations between milk protein profile, determined at the quarter level, and subclinical IMI. The null hypothesis was that no association existed between them. The experimental unit was the mammary quarter within cow, therefore all comparisons were made between positive and negative samples as well as between different classes of SCC, within cow. The required sample size for this study was calculated assuming that β-CN, being together with α_{S1}-CN the predominant milk casein fraction, could be the most representative milk protein fractions affected by udder health (Forsbäck et al., 2010). Therefore, the minimum sample size was calculated to achieve a 95% confidence and 80% power to detect differences between negative and positive milk samples at the bacteriological screening of 1.6-point percentage of total milk N, corresponding to roughly 38% of the standard deviation of the trait. Considering an expected prevalence of animals affected by subclinical IMI of 20% and the variability of milk protein profile, we considered 280 quarters enough to detect desired differences. Milk samples were collected during the evening milking from 450 lactating Holstein cows belonging to 3 different commercial farms in Veneto region (N = 144, 71, and 235 for herd A, B, and C, respectively). Herd selection was based on a prevalence study conducted by the Istituto Zooprofilattico Sperimentale delle Venezie (**IZSVe**) that investigated some of the most common causative agents of mastitis in the Veneto region (*Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis*, and *Prototheca* spp.). The detailed description of the structure of the experimental design, as well as the management and farming conditions, are reported in the work of Pegolo et al. (2022) and Pegolo et al. (2023). In brief, the first sampling (time 0, T0) consisted in performing a bacteriological screening on all lactating cows within each herd, by collecting aseptically 50 mL of composite milk, to identify healthy subjects (Neg) from the ones having subclinical IMI. Among the ones positive to the bacteriological screening, we selected those with no clinical signs of mastitis during that lactation period, and no previous history of antibiotic and other drug treatments. In total 78 animals were selected. Two subsequent milk sampling were performed after 2 (T1) and 6 (T2) weeks, from T0, respectively, to monitor the progress of the disease. On such occasions, we collected 2 milk aliquots of 50 mL from each mammary quarter. One aliquot was sent to the regional breeders association (Associazione Regionale degli Allevatori del Veneto) for milk composition analysis and SCC determination. The second aliquot was instead transferred to the Milk Laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of

the University of Padova for the evaluation of the detailed milk protein profile through HPLC. A schematic summary of the structure of the experimental design is also reported in Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.24961494).

All milk samplings were performed in accordance with the guidelines published by the National Mastitis Council (NMC, 2017). Briefly, before the aseptic milk collection, teats were cleaned by the veterinarian with a commercial pre-milking disinfectant, dried with individual towels then cleaned again with sterile gauzes and alcohol. Cows were fed TMR mostly based on corn silage, sorghum silage, and concentrate, and drinking water was available in automatic water bowls. A detailed description of the feeding regimen adopted in the 3 herds is available in the study of Pegolo et al. (2023b) and also reported in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.24961494).

Microbiological Analysis

Milk samples microbiological examination was carried out by the IZSVe laboratory (Legnaro, PD, Italy). Samples, which were delivered within 4 h from collection, were frozen and analyzed within 3 d. Pegolo et al. (2022) reported all the detailed procedures of the microbiological analyses. Briefly, 10 μL of milk from the composite samples was inoculated on each of the following selective media: Baird-Parker agar with rabbit plasma fibrinogen (BP-RPF, Biokar Diagnostic, Beauvais, France; identification of *S. aureus*), thallium sulfate-crystal violet-B toxin blood agar (TKT, IZSVe internal production; identification of *Strep. agalactiae*), and *Prototheca* isolation medium (**PIM**, IZSVe internal production). BP-RPF plates were prepared following the ISO 6888-2 (ISO, 2021) standards, TKT according to the method described by Hauge and Ellingsen (1953), and PIM in accordance with the NMC guidelines (NMC, 2017). All inoculated media were incubated at 37°C. For BP-RPF plates colonies growth was observed at 24 and 48 h from the incubation, and suspected *S. aureus* colonies were confirmed through the tube coagulase test (NMC, 2017). Concerning the TKT plates, growth was observed at 24 h, and suspected positive colonies for *Strep. agalactiae* were confirmed by CAMP test (NMC, 2017). *Prototheca* isolation medium plates were observed at 24, 48, and 72 h, and suspected colonies were confirmed by wet mount method (NMC, 2017).

Quarter milk samples were cultured following the NMC guidelines (NMC, 2017). Briefly, 10 μL of milk was streaked on blood esculin agar, incubated at 37°C and, after 16 to 18 h, single isolated colonies were picked, subcultured, and identified through both morphological examination and additional NMC tests (NMC, 2017). For mixed species colonies, it was possible to isolate and identify up to 5 different colonies, if more were present the sample was labeled as "contaminated."

Bacterial colonies with questionable test results were identified by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) performed by Microflex Biotyper LT (Bruker Daltonics GmbH, Bremen, Germany). A log (score) ≥2 represented the threshold for species level identification.

Analysis of Milk Composition and SCC

Milk composition analysis (fat, protein, casein, lactose, and urea contents) was carried out using the Milkoscan FT6000 infrared analyzer (Foss A/S, Hillerød, Denmark) while SCC were determined using the Fossomatic 7 DC analyzer (Foss A/S).

Milk Protein Profiling

The validated reversed-phase (**RP**) HPLC technique (Maurmayr et al., 2013) was applied to identify and quantify the following protein fractions: κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, β-LG, α-LA, and LF (lactoferrin). With this method we were also able to identify the portion of glycosylated and nonglycosylated (carbohydrate-free) κ-CN by separating the 2 chromatographic peaks. Total κ-CN was expressed as the sum of these 2 portions. The instrument used consisted in an Agilent 1260 Series chromatograph (Agilent Technologies) coupled to a quaternary pump (Agilent 1260 Series, G1311B). A C:8 reverse-phase analytical column (Aeris WIDEPORE XB-C8, Phenomenex) with large pore core-shell packaging (3.6 μm, 200 Å, 250 × 2.1 mm i.d.) was used to separate the protein fractions. The injection volume was set at 2 μL and the flow rate was 0.5 mL/min. The chromatographic run was carried out in a gradient elution, whose specifics are reported in detail by Maurmayr et al. (2013), with the combination of 2 solutions: solvent A was 94.9% water, 5.0% acetonitrile, and 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. All protein fractions were later expressed both quantitatively (g/L) and qualitatively (% N) as previously reported by Amalfitano et al. (2020).

Statistical Analysis

The association between quarter-level protein fractions and subclinical mastitis was explored using a hierarchical linear mixed model and the PROC MIXED procedure (SAS Institute Inc., Cary, NC). Two separate analyses were conducted for T1 and T2 due to hetero-

geneity of the variances and to the possible change in bacteriological status of the quarter between T1 and T2. The following model was run for T1 and T2, where the experimental unit was the individual mammary gland quarter:

$$y_{ijklm} = \mu + \text{DIM}_i + \text{Parity}_j + \text{Herd}_k + \text{SCC}_l + \text{BACT}_m + (\text{SCC} \times \text{BACT})_{lm} + Cow_{n:ijk} + e_{ijklmn},$$

where y_{ijklmn} is the investigated trait (milk protein fractions, expressed both quantitatively (g/L) and qualitatively as a percentage of the total milk nitrogen content, % N); μ is the overall mean; DIM_i is the fixed effect of the ith class of DIM ($i = 3$ classes; class 1 $\leq$ 120; 120 $<$ class 2 $\leq$ 240; class 3 $>$ 240); Parity_j is the fixed effect of the jth parity (j = primiparous or multiparous); Herd_k is the fixed effect of the kth herd/date ($k = 3$ herds); SCC_l is the fixed effect of the lth class of SCC ($l = 4$ classes; class 1 $<$ 50,000 cells/mL; 50,000 cells/mL $\leq$ class 2 $<$ 200,000 cells/mL; 200,000 cells/mL $\leq$ class 3 $<$ 400,000 cells/mL; class 4 $\geq$ 400,000 cells/mL); BACT_m is the fixed effect of the mth class of bacteriological status (m = positive or negative); $(\text{SCC} \times \text{BACT})_{lm}$ is the 2-way interaction between SCC_l and BACT_m; $Cow_{n:ijk}$ is the random effect of the nth animal nested within DIM_i, parity_j, and herd_k; and e_{ijklmn} is the random residual. Cow and the residuals were assumed to be normally distributed with a mean of zero and variances of σ^2_{Cow} and σ^2_e, respectively. With this nested design, the effects of DIM_i, parity_j, and herd_k were tested on the error line of the nth animal nested within DIM_i, parity_j, and herd_k, while the SCC_l, BACT_m $(\text{SCC} \times \text{BACT})_{lm}$, and animal effects were tested on the residual term.

We also tested the position and number of affected quarters, but because no significant outcome was obtained, we excluded them for the final analyses.

For SCC classes the orthogonal contrasts were set as follows:

- class 1 + class 2 versus class 3 + class 4 (low SCC vs. high SCC)
- class 1 versus class 2 (within low SCC)
- class 3 versus class 4 (within high SCC)

Although for BACT × SCC interaction, orthogonal contrasts were built as follows:

- negative (SCC class 1 + class 2 + class 3 + class 4) versus positive (SCC class 1 + class 2 + class 3 + class 4)
- negative (low SCC) versus positive (low SCC)
- negative (high SCC) versus positive (high SCC)
- negative versus positive (SCC class 1)
- negative versus positive (SCC class 2)
- negative versus positive (SCC class 3)
- negative versus positive (SCC class 4)

The significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

This longitudinal study evaluated for the first time the effect of naturally occurring subclinical IMI, inflammation status determined by the SCC level, and their combination, on milk protein fractions in Holstein cows at the quarter level.

The detailed results of the herd prevalence and the pathogens distribution for each farm have been described in the study of Pegolo et al. (2022). Briefly, among the positive animals identified during the first bacteriological screening (T0), *Strep. agalactiae* was the most prevalent microorganism found (n = 51, 2 herds), followed by *Prototheca* spp. (n = 19, 2 herds), *S. aureus* (n = 6, 2 herds), and finally *S. uberis* (n = 5, 1 herd). Three animals from one farm were co-infected by both *Strep. agalactiae* and *Prototheca*, and therefore excluded from the trial.

As reported by Pegolo et al. (2022), no significant differences were found in terms of milk production and composition between negative and positive animals to the bacteriological examination, underlying that subclinical infection may lead to no detectable alterations. In contrast, as expected, SCC significantly differed between negative (199,700 cells/mL) and positive animals (688,600 cells/mL) ($P < 0.001$).

Milk protein fractions were identified and quantified on 529 quarter milk samples from the 78 subclinically infected animals identified at T0, divided according to the sampling time as follows: 253 for T1 and 276 for T2, respectively. Nineteen samples (8 and 11 at T1 and T2, respectively) were found contaminated, thus they were excluded from the statistical analysis. In total, 63 quarter samples tested positive for the presence of microorganisms at T1 while the number of positive samples was reduced to 47 at T2. As previously stated, *Strep. agalactiae* was the prevalent pathogen, with 41 positive samples at T1 and 34 positive samples at T2 (Supplemental Table S2, https://doi.org/10.6084/m9.figshare.24961494). *Streptococcus agalactiae* is a reemerging pathogen in European countries, and is becoming one of the most important causative agents of subclinical mastitis, despite the implemented eradication programs (Barsi et al., 2022). In Italy, no systematic studies have been carried out to evaluate the prevalence of this microorganism. Only 2 regions, Emilia Romagna and Lombardia, have investigated

Table 1. Descriptive statistics of the milk protein fractions expressed both quantitatively (g/L) and qualitatively (% N)

Item[1]	N	Mean	SD	P1[2]	P99[2]
Protein composition (g/L of milk)					
True protein	528	33.60	4.04	24.43	43.09
Caseins	528	27.92	3.28	19.98	35.07
κ-CN	528	5.49	1.40	3.27	9.58
Glycosylated κ-CN	528	2.13	0.86	1.03	5.33
Nonglycosylated κ-CN	528	3.36	1.09	1.56	6.87
α_{S2}-CN	528	3.36	0.67	1.98	5.07
α_{S1}-CN	528	9.26	1.42	6.31	13.00
β-CN	527	9.83	2.19	5.30	14.50
Whey proteins	528	5.68	1.33	3.41	10.20
β-LG	528	4.47	1.19	2.32	8.30
α-LA	528	1.04	0.25	0.61	2.08
LF	526	0.16	0.19	0.03	0.97
NPN compounds					
Urea	524	0.22	0.05	0.12	0.36
Protein composition (% of total milk N)					
True protein	503	93.20	2.65	87.51	99.17
Caseins	528	77.81	1.13	74.05	79.73
κ-CN	528	15.24	3.07	10.18	24.78
Glycosylated κ-CN	528	5.87	1.98	2.95	12.65
Nonglycosylated κ-CN	528	9.37	2.73	4.23	17.82
α_{S2}-CN	528	9.36	1.53	5.63	13.50
α_{S1}-CN	528	25.89	3.27	18.92	32.93
β-CN	527	27.35	5.17	16.51	36.91
Whey proteins	528	15.84	3.34	10.40	27.61
β-LG	527	12.47	3.06	7.01	21.18
α-LA	528	2.91	0.67	1.74	5.04
LF	526	0.44	0.49	0.10	2.52
NPN compounds					
Urea	483	1.55	0.63	0.47	3.12

[1]κ-CN = sum of glycosylated κ-CN and carbohydrate-free κ-CN; caseins = sum of κ-CN + α_{S2}-CN + α_{S1}-CN + β-CN; whey proteins = sum of β-LG + α-LA + LF; LF = lactoferrin; NPN compounds = sum of urea and minor NPN compounds.

[2]P1 = first percentile; P99 = 99th percentile.

this, with an estimated herd prevalence of 7% to 10% (Sora et al., 2022).

The descriptive statistics of the traits of concerns are reported in Table 1. As expected, the predominant casein fractions were β-CN and α_{S1}-CN, which averaged 9.83 g/L (27.35% N) and 9.26 g/L (25.89% N), respectively. Among the whey proteins, β-LG was the most abundant (4.47 g/L, 12.47% N), followed by α-LA (1.04 g/L, 2.91% N) and LF (0.16 g/L, 0.44% N). These values were overall in accordance with previous works carried out using the same RP-HPLC method on composite milk samples (Amalfitano et al., 2020; Bisutti et al., 2022a).

The pattern of the protein fractions at the 2 sampling points was overall similar. Moreover, we did not find differences when comparing the effects of IMI and inflammation status between the 2 ways of expressing the protein fractions (quantitatively, g/L and qualitatively, % N). Therefore, to avoid redundancy we focused on the results expressed as percentage of the total milk nitrogen. The plot displaying the results of the protein fractions expressed as g/L are reported in the supplemental materials (Supplemental Figures S2, S3, S4, and S5; https://doi.org/10.6084/m9.figshare.24961494). Moreover, as the behavior of the glycosylated and carbohydrate-free κ-CN had the same pattern as the total κ-CN (calculated as the sum of these 2 fractions; data not shown), we focused only on the effects toward the latter.

Associations Between Subclinical IMI and Milk Protein Fractions

The results of the ANOVA for the IMI effect on the investigated traits are reported in Table 2. The evaluation of pathogen-specific effects toward the investigated traits was not possible due to an excessive fragmentation of the data set which would have led to a significant reduction in the test power.

At T1, subclinical IMI was associated with most of the casein fractions. In particular, we observed that mammary quarters positive at the bacteriological examination had higher proportions of κ-CN ($P < 0.05$; +5.8%) and α_{S2}-CN ($P < 0.01$; +5.7%) but signifi-

Table 2. *F*-values and significances of the linear mixed model tested for quarter-level protein fractions at sampling time 1 (T1) and time 2 (T2)

	T1			T2		
Item[1]	BACT[2]	SCC[3]	SCC × BACT[4]	BACT[2]	SCC[3]	SCC × BACT[4]
Protein composition (g/L of milk)						
True protein	0.66	0.73	0.33	1.39	0.48	0.24
Caseins	0.00	1.91	2.16	0.61	0.97	0.67
κ-CN	5.94*	18.67***	1.00	0.19	8.86***	0.27
Glycosylated κ-CN	2.24	3.52*	1.51	0.01	9.99***	0.33
Carbohydrate-free κ-CN	3.32	15.56***	2.85*	0.97	5.06**	0.23
α_{S2}-CN	9.15**	3.96**	0.48	1.20	6.41***	1.19
α_{S1}-CN	0.77	5.35**	0.22	0.55	3.13*	3.53*
β-CN	6.97**	4.05**	1.33	0.34	3.53*	0.31
Whey proteins	3.58	1.94	0.73	0.03	0.12	0.63
β-LG	2.92	2.69*	1.03	0.44	0.20	1.03
α-LA	1.49	1.97	0.30	0.35	0.51	2.29
LF	1.72	19.24***	2.11	3.92*	5.77**	1.58
NPN compounds						
Urea	0.96	10.98***	0.35	3.22	8.26***	0.44
Protein composition (% of total milk N)						
True protein	1.54	1.86	0.93	0.27	5.24**	0.95
Caseins	4.81*	27.64***	4.58**	0.13	27.57***	0.72
κ-CN	5.95*	12.75***	0.87	0.03	8.68***	0.79
Glycosylated κ-CN	1.68	2.83*	1.84	0.03	9.13***	0.53
Carbohydrate-free κ-CN	3.59	11.04***	2.20	0.67	4.25**	0.62
α_{S2}-CN	9.47**	2.35	0.43	0.72	2.78*	0.79
α_{S1}-CN	0.11	9.76***	0.12	1.93	15.28***	3.74*
β-CN	10.36**	5.19**	1.26	0.02	9.51***	0.24
Whey proteins	1.54	1.87	1.22	0.18	0.49	0.59
β-LG	1.75	3.15*	1.64	0.70	0.67	0.81
α-LA	0.61	1.49	0.33	0.49	0.51	2.37
LF	0.55	16.90***	1.19	4.38*	5.91***	1.83
NPN compounds						
Urea	1.18	18.21***	1.10	1.40	5.98***	0.47

[1]κ-CN = sum of glycosylated κ-CN and carbohydrate-free κ-CN; caseins = sum of κ-CN + α_{S2}-CN + α_{S1}-CN + β-CN; whey proteins = sum of β-LG + α-LA + LF; LF = lactoferrin; NPN compounds = sum of urea and minor NPN compounds.

[2]BACT: bacteriological status (negative, positive).

[3]Somatic cell count expressed in classes (4 classes: class 1: SCC <50,000 cells/mL, 50,000 cells/mL ≤ class 2 < 200,000 cells/mL; 200,000 cells/mL ≤ class 3 < 400,000 cells/mL; class 4 ≥ 400,000 cells/mL).

[4]BACT × SCC: interaction between the bacteriological status and the SCC effects.

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

cantly lower β-CN ($P < 0.01$; −7.5%) (Figure 1). The milk compositional changes in the infected mammary gland are related to the activity of immune cells such as polymorphonuclear (PMN) cells and macrophages that release a great amount of proteases and other cellular components in the milk (Kelly et al., 2006; Pisanu et al., 2015) while fighting invading pathogens. Moreover, infecting bacteria can release into the milk exogenous enzymes, such as elastases (Guerrero et al., 2015), contributing to proteolysis. Furthermore, some bacteria can secrete activators of protease zymogens, which, synergically with native bovine enzymes lead to an increase in proteolytic potential of milk (Larsen et al., 2006). Enzymatic degradation seems to affect protein fractions in a different way. For example, Considine et al. (1999) observed a cleavage specificity of elastase on α_{S1}-CN and β-CN, suggesting that among the casein fractions those 2 might be the most suitable substrates for the proteolysis. Moreover, Hinz et al. (2012) assessed with 2-dimensional gel electrophoresis the peptides produced by proteolysis in milk samples from cows infused with *Escherichia coli* LPS and observed that almost all the obtained peptides derived from α_{S1}-CN and β-CN, in accordance with what observed in our study.

At T2, subclinical IMI was associated with higher LF ($P < 0.05$; +51%; Figure 1). Lactoferrin is an iron-binding glycoprotein synthesized by specific granules in PMN and glandular epithelial cells (Cheng et al., 2008), that is characterized by antimicrobial properties. The increased concentration of LF found in our study was in accordance to Chaneton et al. (2008) who also found a greater content of this protein in infected quarters of animal having clinical mastitis.

Apart from LF, we did not observe further significant effects of the bacteriological status toward the whey proteins. Different studies on the milk peptidome in cows with mastitis highlighted that peptides derived from the degradation of whey proteins were few or

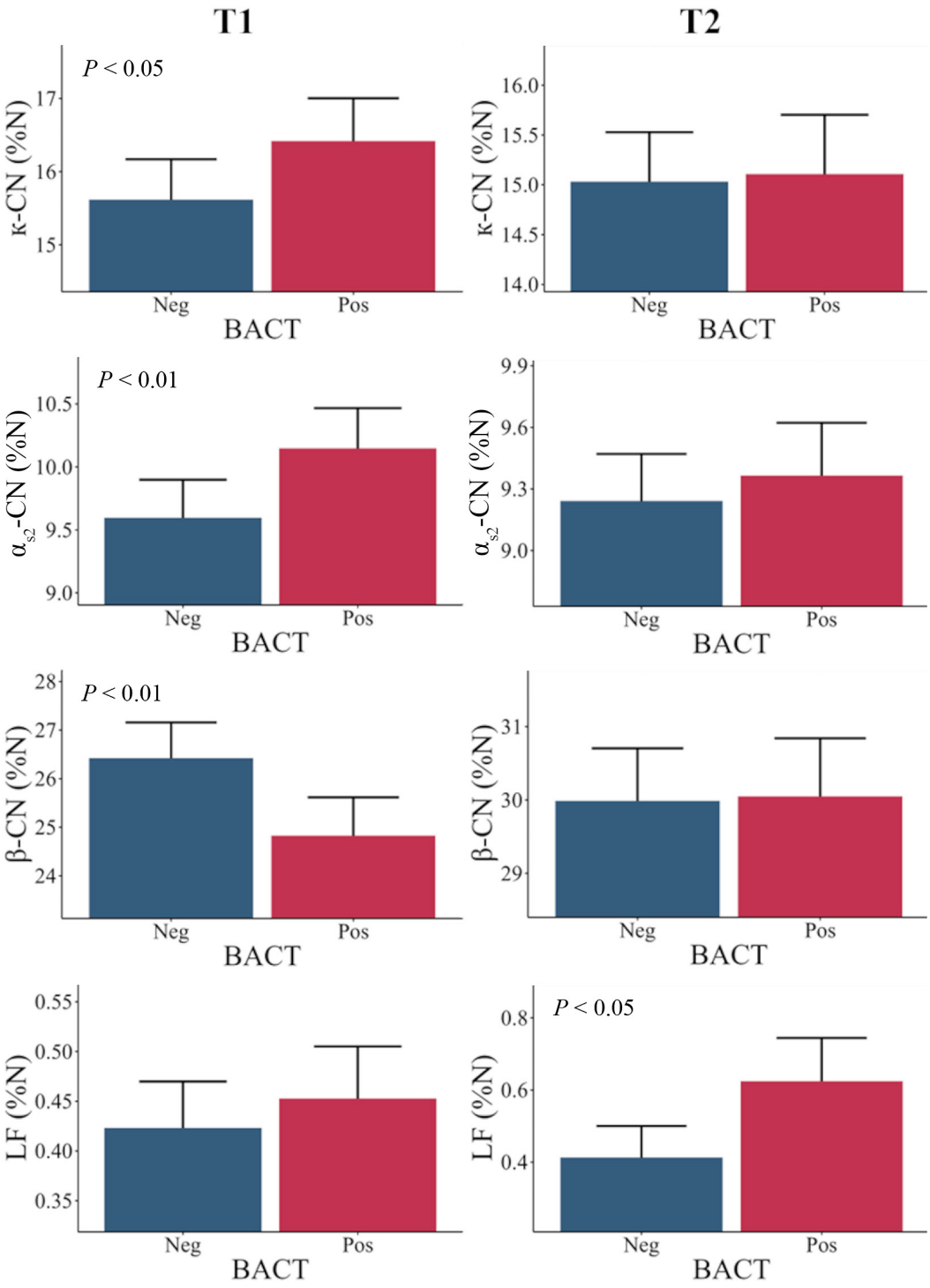

Figure 1. Least squares means and SE of the bacteriological status on the protein fractions expressed qualitatively (% N) and measured at the quarter level, at time 1 (T1) and time 2 (T2), respectively. Bacteriological status (BACT) classes were defined as negative at bacteriological examination (Neg) and positive at bacteriological examination (Pos). Only significant ($P < 0.05$) traits for at least one sampling time are displayed. LF = lactoferrin.

not even detected (Baum et al., 2013; Mansor et al., 2013). Hence, previous studies that observed a reduction of β-LG and α-LA in animals affected from clinical mastitis (Hogarth et al., 2004; Guerrero et al., 2015) attributed this alteration as a result of either the physical damage of the mammary epithelial cells from the pathogen invasion or the reduced synthesis and secretion of those proteins (McFadden et al., 1988), rather than from enzymatic degradation. Conversely, evaluation of whey from cows with subclinical mastitis by 2-dimensional gel electrophoresis did not show a reduction in either one of those proteins (Baeker et al., 2002), in accordance with what we observed in our study.

We might speculate that this result might partly be related to the chronic nature of infection, and partially to the fact that animals involved in our study, even if all displayed a subclinical condition, were not in the same stage of infection, and therefore the changes in the whey population might have been less evident than the ones found for caseins.

Associations Between Milk SCC and Protein Fractions

The results of the ANOVA for the SCC effect on the investigated traits are reported in Table 2. At T1, SCC was associated with all the casein fractions. Increases in SCC led to a reduction α_{S1}-CN (−8%; $P < 0.001$) and β-CN (−8%; $P < 0.01$). In contrast, κ-CN and α_{S2}-CN showed an opposite trend, as we observed for both these casein fractions a higher proportion at increasing SCC (+17% and 4.9% for κ-CN and α_{S2}-CN, respectively; Figure 2). Indeed, the reduction in α_{S1}-CN and β-CN, which are the 2 most abundant protein fractions, is probably to be ascribed to the increase of milk endogenous proteases, which include plasmin, enzymes produced by leucocytes and those derived from the transition through the altered blood-milk barrier (Le Roux et al., 2003; Forsbäck et al., 2010). Plasmin, the main milk native enzyme (Crudden et al., 2005), exists as a component of a complex system, which includes its zymogen, plasminogen and plasminogen activators (Kelly et al., 2006). Even though it is normally present in milk, both plasmin and plasminogen significantly increase with elevated SCC (Dallas et al., 2015), together with the urokinase type I plasminogen activator, which activates plasminogen into plasmin (Guerrero et al., 2015). Along with plasmin, other proteases such as cathepsin B and D, collagenases, and elastases, are released by lysosomes contained in macrophages and PMN cells with the onset of inflammation. Some of these enzymes (such as elastase and cathepsin B) have the ability to kill different pathogens that are causative agents of mastitis (Ezzat Alnakip et al., 2014; Abdelmegid et al., 2020), but at the same time they significantly concur in the degradation of milk protein fractions (Le Roux et al., 2003; Wedholm et al., 2008).

It was observed that, among all caseins, β-CN, followed by α_{S1}-CN were the fractions most subjected to the enzymatic degradation (Urech et al., 1999; Ramos et al., 2015). In addition, studies that evaluated changes in peptidome in animals affected by subclinical mastitis evidenced a more pronounced production of β-CN and α_{S1}-CN derived peptides (Guerrero et al., 2015; Addis et al., 2020), suggesting that κ-CN might be more resistant to the proteolysis by these enzymes than the other casein fractions. Therefore, we might speculate that the higher sensitivity to proteolysis of these 2 casein fractions might be attributable either to their aminoacidic structure or to their tertiary structure, which could make the proteases cleavage sites more accessible.

Among the whey proteins, SCC was associated with β-LG ($P < 0.05$) and LF ($P < 0.001$; +95%) (Figure 3). However, in our study the pattern of variation of β-LG across the SCC classes was erratic, suggesting further evaluations. Aside from protein fractions, we observed a significant reduction in the urea proportion at the increase of SCC ($P < 0.001$; −9.6%). Urea is the primary metabolite derived from dietary protein and tissue protein turnover and it is released from liver to the bloodstream and then to the milk in a stable form (Hayton et al., 2012). Urea is greatly influenced by management and feeding conditions, such as the lack of rumen degradable proteins, alteration in dry matter intake, or the energy in the feed ration (Rezamand et al., 2007). There have been studies which reported a reduction in milk urea proportion in cows with IMI and increased SCC (Nyman et al., 2014; Timonen et al., 2017). The molecular mechanisms behind this association are not fully understood and therefore require further evaluations, however we might speculate being partially linked to the reduction of feed intake that is observed in animals having masitits .

Overall, the patterns of milk protein fractions according to SCC classes agreed at the 2 sampling points. In fact, at T2 SCC was associated with almost all the protein fractions, except for β-LG and α-LA. We observed a 9% reduction in β-CN ($P < 0.001$). On the flipping side, we confirmed the same behavior as at T1 for κ-CN (+14%; $P < 0.001$) and α_{S2}-CN (+5%; $P < 0.05$), whose proportion increased with higher SCC. The increase in κ-CN was found in accordance to a previous study which evaluated the effect of SCC and differential SCC (**DSCC**) in clinically healthy animals (Bisutti et al., 2022b). We observed the same trend also for LF ($P < 0.001$; +121%). Aside from its antimicrobial effect, it has been reported that LF can exert

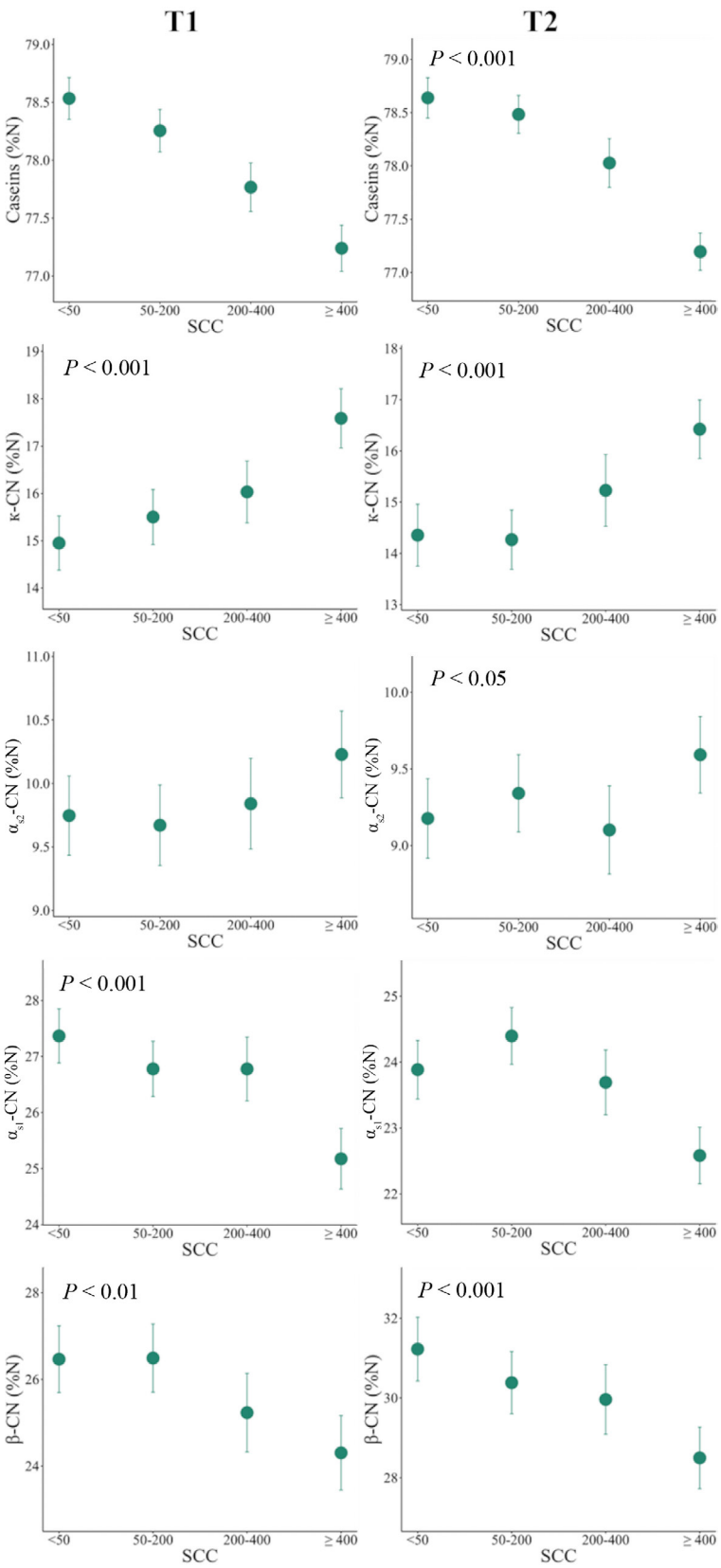

Figure 2. Least squares means and SE of the SCC classes (SCC × 1,000) on the casein fractions expressed qualitatively (% N) and measured at the quarter level, at time 1 (T1) and time 2 (T2), respectively. Only significant ($P < 0.05$) traits for at least one sampling time are displayed.

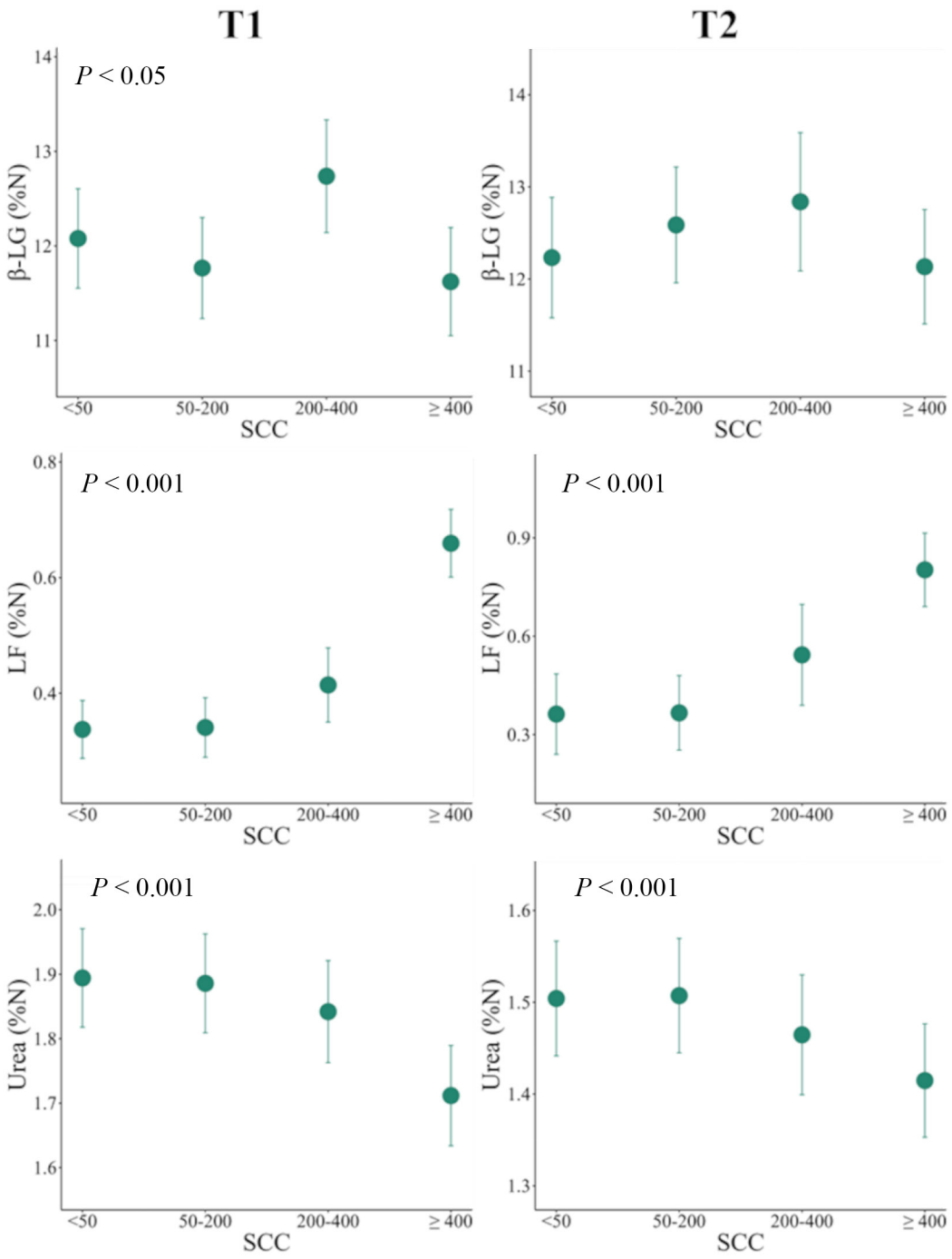

Figure 3. Least squares means and SE of the SCC classes (SCC × 1,000) on the whey protein fractions and milk urea expressed qualitatively (% N) and measured at the quarter level, at time 1 (T1) and time 2 (T2), respectively. Only significant ($P < 0.05$) traits for at least one sampling time are displayed. LF = lactoferrin.

a wide range of actions toward the immune system, from the inhibition of the inflammatory process to the modulation of both the innate and adaptive immune response (Drago-Serrano et al., 2017; Shimazaki and Kawai, 2017). In our study we observed an increase of LF from T1 to T2, and this result might be ascribed to the fact that, with the progression of inflammation, LF might have accumulated due to both its induced production and additional degranulation of PMN (de la Rosa et al., 2008).

Association of the Interaction Between BACT and SCC with Milk Protein Fractions

The results of the ANOVA for the IMI combined with SCC effect on the investigated traits are reported in Table 2. Evaluating the interaction between the infection status and inflammation process allowed us to focus directly on the effect of subclinical IMI within each SCC class to better understand which could be the major forces of alteration of milk protein fractions.

At T1, the BACT × SCC interaction was associated with the total casein proportion ($P < 0.01$, Figure 3). Specifically, both positive and negative samples showed the highest proportion of casein with very low SCC (<50,000 cells/mL), and then decreased across higher SCC classes, the behavior of negative and positive samples differed for the SCC class ≥400,000, with these latter having the lowest casein content (Figure 4). At T2, we observed an association of the BACT × SCC with α_{S1}-CN. In positive samples we observed an increase in α_{S1}-CN from the first SCC class to the second one (≥50,000 and <200,000 cells/mL) but then a reduction, reaching the lowest value with SCC >400,000 cells/mL.

These results suggest that the effect of IMI on milk caseins seems to be more evident at very high SCC value, probably linked to a synergic effect of infection and inflammation. In fact, aside from the pathogen release of exogenous enzymes, some bacteria are able to induce the production of protease activators, which by enhancing the proteolysis in the mammary gland, release important substrates for the bacterial growth (Mehrzad et al., 2005; Kelly et al., 2006).

These results further support that the level of inflammation (i.e., SCC) strongly affects the milk protein profile, which is known to be a factor of interest for the dairy sector, given the crucial role that protein fractions play in determining the milk cheesemaking aptitude. It is however worth remembering that IMI,

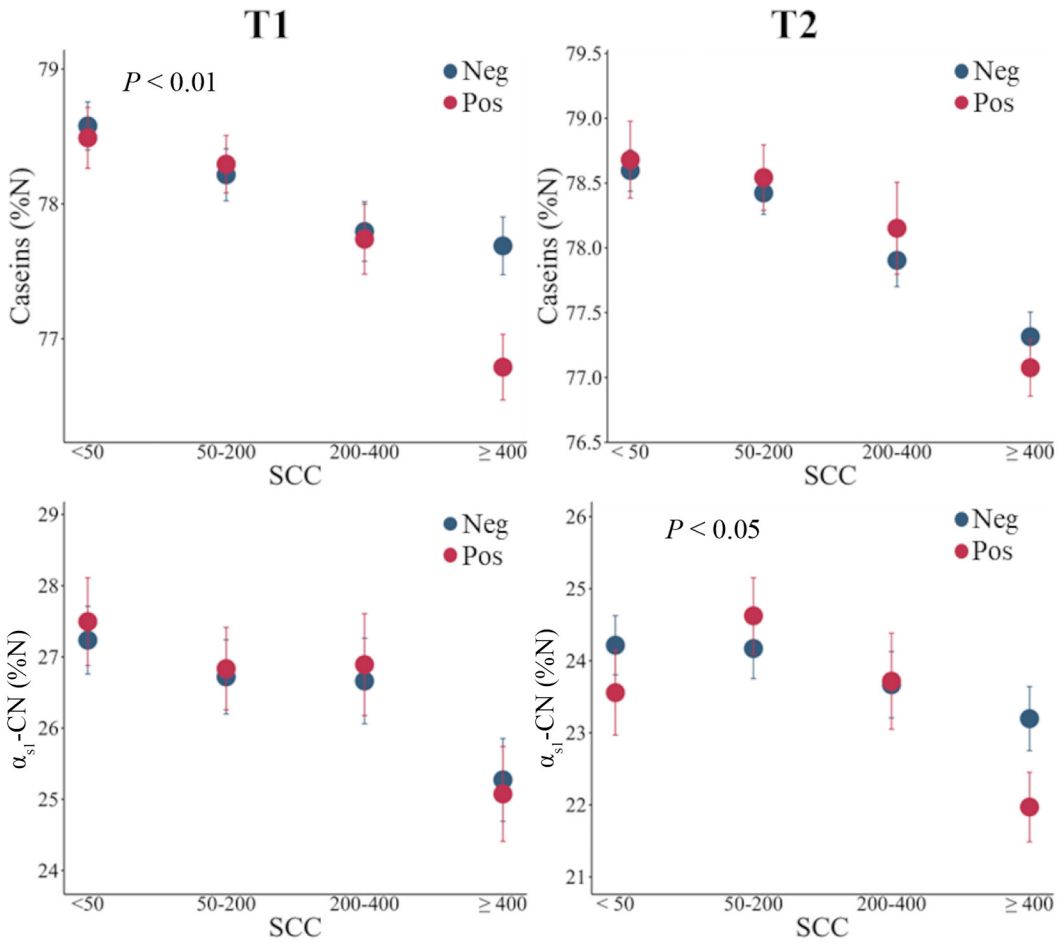

Figure 4. Least squares means and SE of the bacteriological status × SCC interaction (SCC × 1,000) on the protein fractions expressed qualitatively (% N) and measured at the quarter level, at time 1 (T1) and time 2 (T2), respectively. Only significant ($P < 0.05$) traits for at least one sampling time are displayed. Neg = negative at bacteriological examination; Pos = positive at bacteriological examination.

especially when subclinical, is not always associated with increased SCC. Indeed, cases of animals positive at the microbiological examination but with low SCC (<200,000 cells/mL) have been reported, as well as culture-negative samples with high SCC (Bobbo et al., 2017; Pegolo et al., 2022). This can be due to different reasons, including the stage of infection, the animals' immune response to infection, or the pathogens' infection patterns. For instance, *Staphylococcus aureus* can induce chronic infections with intermittent and cyclical shedding (Sears et al., 1990).

CONCLUSIONS

This study added new insights on the behavior of milk protein fractions in animals having subclinical IMI. Moreover, it explored for the first time and in an extensive way the pattern of alteration of the protein fractions at the quarter level. The IMI had a significant effect on mostly casein fractions, negatively affecting β-CN and α_{S1}-CN proportions. However, we have substantiated that the increase in SCC seemed to be the factor associated with the most significant changes in the protein fractions. This suggests that the increase in endogenous proteolytic enzymes related to the onset of inflammation, and not the infection (i.e., the bacteriological status), might be the pivotal aspect driving the alteration of the milk protein profile. Finally, our results further confirmed the importance of SCC as an indicator of both udder health and milk quality.

ACKNOWLEDGMENTS

This study was part of the LATSAN project, funded by the Ministero dell'agricoltura, della sovranità alimentare e delle foreste (MASAF, Rome, Italy). The authors thank Sofia Ton of the Breeders Association of the Veneto Region (Padova, Italy) for the analyses of milk composition. The PhD grant of Vittoria Bisutti is funded by the CARIPARO foundation (Padova, Italy). The authors have not stated any conflicts of interest.

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