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Impact of somatic cell count combined with differential somatic cell count on milk protein fractions in Holstein cattle

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ABSTRACT

Udder health in dairy herds is a very important issue given its implications for animal welfare and the production of high-quality milk. Somatic cell count (SCC) is the most widely used means of assessing udder health status. However, differential somatic cell count (DSCC) has recently been proposed as a new and more effective means of evaluating intramammary infection dynamics. Differential SCC represents the combined percentage of polymorphonuclear neutrophils and lymphocytes (PMN-LYM) in the total SCC, with macrophages (MAC) accounting for the remaining proportion. The aim of this study was to evaluate the association between SCC and DSCC and the detailed milk protein profile in a population of 1,482 Holstein cows. A validated reversed-phase HPLC method was used to quantify 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, which were expressed both quantitatively (g/L) and qualitatively (as a percentage of the total milk nitrogen content, %N). A linear mixed model was fitted to explore the associations between somatic cell score (SCS) combined with DSCC and the protein fractions expressed quantitatively and qualitatively. We ran an additional model that included DSCC expressed as PMN-LYM and MAC counts, obtained by multiplying the percentages of PMN-LYM and MAC by SCC for each cow in the data set. When the protein fractions were expressed as grams per liter, SCS was significantly negatively associated with almost all the casein fractions and positively associated with the whey protein α-lactalbumin, while DSCC was significantly associated with α_{S1}-CN, β-CN, and α-lactalbumin, but in the opposite direction to SCS. We observed the same pattern with the qualitative data (i.e., %N), confirming opposite effects of SCS and DSCC on milk protein fractions. The PMN-LYM count was only slightly associated with the traits of concern, although the pattern observed was the same as when both SCS and DSCC were included in the model. The MAC count, however, generally had a greater impact on many casein fractions, in particular decreasing both β-CN content (g/L) and proportion (%N), and exhibited the opposite pattern to the PMN-LYM count. Our results show that information obtained from both SCS and DSCC may be useful in assessing milk quality and protein fractions. They also demonstrate the potential of MAC count as a novel udder health trait.

Key words: differential somatic cell count, macrophages, milk quality, protein fractions, HPLC, dairy cattle

INTRODUCTION

The Italian dairy sector has a substantial impact on the country's economy as most of the milk produced is processed into high-quality cheese, including Protected Designation of Origin (**PDO**) products (Bertoni et al., 2001; Bittante et al., 2012). The quantities and proportions of milk protein fractions are widely acknowledged to be among the most important factors involved in the coagulation and cheese-making process (Guinee, 2003; Caroli et al., 2009; Bittante et al., 2012). In particular, caseins, which account for almost 80% of the total milk proteins, are directly involved in curd formation and cheese yield (Wedholm et al., 2006). For example, β-CN content is positively associated with cheese yield, while κ-CN is responsible for the stability of casein micelles and influences DM yield (Cipolat-Gotet et al., 2018). In contrast, whey proteins, despite their high nutritional value, have less economic importance because they are not directly involved in the milk coagulation process, although they influence nutrient recovery in the curd. For example, Cipolat-Gotet et al. (2018) observed that

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higher milk β-LG contents were associated with lower fat and protein recoveries.

In the context of efficient dairy production, udder health is an important factor having a decisive influence on milk production, quality, and thus cheese production (Urech et al., 1999). This factor is the subject of continuously improving farm management strategies (Nørstebø et al., 2019). At present, the most widely used indicator for rapidly obtaining information on both udder health and milk quality is SCC (Guzzo et al., 2018). Somatic cells comprise a small, but constant proportion of mammary epithelial cells, as well as various immune cell populations, mainly lymphocytes (**LYM**), PMN, and macrophages (**MAC**). Each type of immune cells makes different contributions to the inflammatory response, and these actions can vary according to the infection stage. Lymphocytes, for example, are responsible for the regulation and suppression of immune responses (Nickerson, 1989), while PMN defend against invading bacteria during the early stage of an acute inflammatory process (Oviedo-Boyso et al., 2007). Finally, MAC, which have phagocytic and pinocytotic activity, are involved in nonspecific cell-induced immunity (Sordillo et al., 1997).

Somatic cell count is a robust indicator of the intensity of the mammary gland immune response. However, it does not provide information on the proportions of the individual cell populations and therefore does not indicate the stage of the inflammatory response or the progression of the disease. A step forward was recently made with the development of a new parameter to be coupled with SCC: the differential somatic cell count (**DSCC**). This trait represents the combined proportions of PMN and LYM in the milk expressed as a percentage, from which the MAC proportion can be calculated by subtracting the DSCC from 100% (Damm et al., 2017).

Milk with a high SCC undergoes compositional changes due to increased proteolytic activity (Wickström et al., 2009), which has detrimental effects on its technological properties, such as the clotting ability and cheese yield (Bobbo et al., 2016; Pegolo et al., 2021). Proteolytic activity seems to particularly affect the casein fraction in contrast to the whey proteins, and it has commonly been attributed to the PMN (Le Roux et al., 2003).

Recent studies have conducted phenotypic investigations of DSCC and how its variations are associated with total SCC (Damm et al., 2017), udder health traits (Zecconi et al., 2019), milk yield and composition (Zecconi et al., 2020; Stocco et al., 2020), and technological properties (Pegolo et al., 2021a,b).

To the best of our knowledge, no studies have as yet investigated the possible associations between DSCC and the fine composition of milk, specifically its detailed protein profile. Exploring these associations could shed light on some of the effects already found with respect to coagulation properties (Pegolo et al., 2021), which are strictly connected with milk protein fractions (especially caseins). In addition, because the different leukocyte populations exhibit different levels of proteolytic activity, the results might be useful for identifying potential novel indicator traits of mammary gland inflammation. Therefore, the aim of this work was to evaluate the association between DSCC, either combined with SCS or expressed as PMN-LYM and MAC counts, and the detailed milk protein profile, expressed both quantitatively (g/L) and qualitatively (% N), in 1,482 Holstein cows.

MATERIALS AND METHODS

Field Data and Sample Collection

The present study was carried out as part of the LATSAN and BENELAT projects, the aim of which is to develop short- and long-term interventions for improving animal welfare and efficiency, and the quality of dairy cattle production. 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).

Individual milk samples were collected from 1,482 Holstein-Friesian cows belonging to 6 different herds (herds size ranging from 27 to 951 lactating cows) located in northern Italy. Three of the herds were located in the production area of the Grana Padano PDO cheese. All animals were housed in free stalls and fed TMR formulated in accordance with the nutritional requirements set by the NRC (2001) and based on corn silage, sorghum silage, and concentrates. Drinking water was distributed through automatic water bowls, and the animals were milked twice per day. Cows that had any clinical signs of disease or were under medical treatment were excluded from the sampling. Milk samples were collected once during the evening milking from March 2019 to February 2020 (1 or more samplings per herd, depending on the size of the herd, 21 herd-date combinations in total).

After collection, each sample was divided into 2 aliquots. Bronopol preservative was added to one, which was then transferred to the laboratory of the Breeders Association of the Veneto where it was stored at 4°C until analysis of milk composition and udder health traits (within 48 h of collection). The other aliquot was taken to the laboratory of the Department of Agronomy,

Food, Natural Resources, Animals and Environment of the University of Padova, Italy, within 3 h of sampling, where it was stored at −80°C until detailed analysis of the protein profile (within 4 mo of collection).

Analysis of Milk Composition and Udder Health Traits

Samples were analyzed for milk composition traits (fat, protein, casein, lactose, and urea contents) with an FT6000 MilkoScan infrared analyzer (Foss A/S), while SCC (cells/mL) and DSCC (%) were determined with a Fossomatic TM 7DC analyzer (Foss A/S). To obtain a normal distribution, the SCC were transformed to SCS according to the equation proposed by Ali and Shook (1980).

In accordance with Pegolo et al. (2021), the proportions of DSCC were converted to cell counts as follows:

$$\text{PMN-LYM count, } 10^3/\text{mL} = \text{DSCC} \times \text{SCC } (10^3/\text{mL});$$

$$\text{MAC count, } 10^3/\text{mL} = (1 - \text{DSCC}) \times \text{SCC } (10^3/\text{mL}).$$

In addition, the PMN-LYM and MAC counts were log-transformed as log(PMN-LYM or MAC count/100,000) + 3, in the same way as SCS, to obtain a normal distribution of the data.

Milk Protein Profiling

The validated reversed-phase HPLC method proposed by Maurmayr et al. (2013) and recently applied by Bisutti et al. (2022) was used to quantify the contents of κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, β-LG, α-LA, and lactoferrin in 1,264 individual milk samples. We were also able to discriminate between the proportions of glycosylated and nonglycosylated κ-CN, thus the total κ-CN content was the sum of the 2 peaks. Briefly, an Agilent 1260 Series chromatograph (Agilent Technologies) coupled to a quaternary pump (Agilent 1260 Series, G1311B) was used to separate the protein fractions using 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.). A gradient elution was carried out with a combination of 2 solvents as reported in Maurmayr et al. (2013): solvent A was 94.9% water, 5.0% acetonitrile, and 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The flow rate was 0.5 mL/min, and the injection volume was 2 μL.

All protein fractions were expressed both quantitatively (g/L) and qualitatively (%N), as reported in detail in Amalfitano et al. (2020). The quantitative measures were calculated by multiplying the proportion of each protein fraction determined by HPLC by the proportion of casein determined by the FT6000 infrared analyzer, while the qualitative measures were calculated as percentages of the total nitrogen content.

Statistical Analysis

The data set underwent preliminary editing for all the investigated traits to exclude records falling outside the interval defined by ±3 standard deviations of the mean. Only milk samples with >50,000 SCC/mL and <1,500,000 SCC/mL were used in the analysis, as the Fossomatic 7DC analyzer has a reportedly poor level of accuracy for extremely low or high SCC values (Damm et al., 2017). Following Pegolo et al. (2021), we did not assume any linear relationship between predictors and response variables. Therefore, we discretized the explanatory variables (i.e., SCC, DSCC, PMN-LYM count, and MAC count) and created classes based on the 25th, 50th, and 75th percentiles. This approach was taken to ensure better assessment of the pattern of DSCC and SCS effects and to ensure the classes were balanced. Moreover, SCC and DSCC were included in the same model to account for different degrees of mammary gland health as Damm et al. (2017) and Zecconi et al. (2020) reported that DSCC values may differ in animals with low SCC (healthy or at an early stage of the disease) and medium-high SCC (acute or chronic stage of the disease).

The associations between SCS and DSCC on the one hand and milk yield, composition, and protein fractions, expressed both as grams per liter and %N, on the other, were estimated using the following linear mixed model (Model 1) implemented in the *lme4* package of R software:

$$y_{ijklmn} = \text{DIM}_i + \text{Parity}_j + \text{SCS}_k + \text{DSCC}_l + \text{Herd-date}_m + e_{ijklmn}, \quad [1]$$

where y_{ijklmn} is the measure of the trait, DIM_i is the fixed effect of ith class of DIM (6 levels; i is <60, 60–120, 121–180, 181–240, 241–300, or >300), Parity_j is the fixed effect of the jth parity (4 levels; j is 1, 2, 3, or ≥4), SCS_k is the fixed effect of the kth class of SCS defined by quartiles (4 levels; k is <2.63, 2.63–3.33, 3.34–4.29, or >4.29), DSCC_l is the fixed effect of the lth class of DSCC defined by quartiles (4 levels; l is <59.9, 59.9–71.2, 72.3–79.7, or >79.7%), Herd-date_m is the random effect of the mth herd-date (21 levels), and e_{ijklmn} is the random residual assumed to have a normal

distribution with $e_{ijklmn} \sim N\left(0, \sigma_e^2\right)$, where σ_e^2 is the residual variance.

A second linear mixed model (Model 2) was built to evaluate the specific effects of PMN-LYM and MAC counts in explaining the total variance for the investigated traits:

$$y_{ijklmn} = \text{DIM}_i + \text{Parity}_j + \text{PMN\_LYM}_k + \text{MAC}_l + \text{Herd-date}_m + e_{ijklmn}. \quad [2]$$

In Model 2, SCS and DSCC were excluded from the vector of fixed effects, while the model included PMN-LYM_k as the fixed effect of PMN-LYM count class defined by quartiles (4 levels: k is <2.01, 2.01–2.68, 2.69–3.78, or >3.78), and MAC_l as the fixed effect of MAC count class defined by quartiles (4 levels: l is <1.03, 1.03–1.61, 1.62–2.36, or >2.36). All other terms were the same as in Model 1. Polynomial contrasts ($P < 0.05$) were estimated to describe the pattern of the effects of SCS, DSCC (%), and PMN-LYM and MAC counts. First-order comparisons measured the linear relationships; second-order comparisons, the quadratic relationships; and third-order comparisons, the cubic relationships. A model including the SCS × DSCC interaction was also tested, but it was significant for only a few traits and exhibited erratic patterns, so the results are not reported here.

RESULTS AND DISCUSSION

Previous studies investigated the phenotypic associations between DSCC traits and milk composition (Stocco et al., 2020; Zecconi et al., 2020) or technological properties (Pegolo et al., 2021a). Furthermore, correlations previously obtained between SCS and DSCC (r = 0.44) and between PMN-LYM count and MAC (r = 0.65) count appeared to suggest that they capture different biological features (Pegolo et al., 2021). Given the essential role of protein fractions in determining the cheese-making ability of milk, and the effects of some leucocyte populations on the integrity and functionality of milk proteins, this study focused, for the first time, on evaluating the relationship between DSCC and the detailed milk protein profile.

Descriptive Statistics of Udder Health Traits and Protein Fractions

Descriptive statistics for the investigated traits are reported in Table 1. The average values of the main milk constituents are consistent with previous studies on Holstein cows (Maurmayr et al., 2018; Saha et al., 2020). Among the udder health traits, average SCS was 2.65 (±1.94), in agreement with other studies on Holstein cattle (Malchiodi et al., 2014), showing that the farming systems and the overall hygiene conditions in which the sampled herds were kept were good. Mean DSCC was 69.09% (±13.51%), while the mean MAC percentage, calculated as 100% minus DSCC, was 30.97% (±13.62%). When DSCC was expressed quantitatively as logPMN-LYM and logMAC counts, the average values were 3.09 ± 1.35 and 1.82 ± 1.04, respectively. In the case of DSCC, the mean value was also found to be consistent with previous studies (Stocco et al., 2020).

Regarding the protein profile expressed both quantitatively and qualitatively, the predominant casein fractions were α_{S1}-CN and β-CN, with mean values of 9.06 g/L (26.47%N) and 8.97 g/L (26.19%N), respectively. The fractions α_{S2}-CN and κ-CN (calculated as the sum of the glycosylated and carbohydrate-free forms) were less represented and averaged 3.48 g/L (10.13%N) and 5.25 g/L (15.29%N), respectively. The most abundant of the whey protein fractions was β-LG, with a mean value of 3.44 g/L (10.02%N), followed by α-LA (0.99 g/L, 2.88%N) and lactoferrin (0.16 g/L, 0.47%N). Overall, the mean values for both the quantitative (g/L) and qualitative (%N) measures were similar to those found by Amalfitano et al. (2020) and Maurmayr et al. (2018), with the exception of total caseins and β-CN, which were lower than those reported by Amalfitano et al. (2020) (28.36 g/L and 78.49%N, 10.69 g/L and 29.69%N, respectively), and α_{S2}-CN, which was instead slightly higher (2.78 g/L and 7.2%N). These differences could be due to several factors, such as differences in population size and breed and in the analytical method used (Bonfatti et al., 2011; Gebreyesus et al., 2016).

Effects of Herd-Date

We observed that herd-date explained a relatively low proportion of the total variance (<25%) for all the investigated traits (Table 2), with the exception of urea (56%), which is well known to be influenced by diet (Roseler et al., 1993; Schiavon et al., 2015), as well as environmental and management conditions (Maurmayr et al., 2018). This result accords with previous studies (Schopen et al., 2009; Bonfatti et al., 2011), confirming that variation in the milk protein composition is mainly related to genetic factors.

Association of SCS and DSCC with Milk Yield and Composition

The results of the ANOVA between SCS combined with DSCC and milk composition and the protein profile

Table 1. Descriptive statistics of single test-day milk yield, composition, udder health, and protein fractions expressed both as quantitative (g/L) and qualitative measures (%N)

Trait[1]	N	Mean	SD	P1[2]	P99[2]
Milk yield, kg/d	1,481	33.60	9.41	11.78	57.30
Milk composition					
Fat, %	1,472	3.66	0.83	1.47	5.71
Protein, %	1,479	3.43	0.34	2.72	4.32
Casein, %	1,480	2.68	0.28	2.09	3.43
Lactose, %	1,470	4.86	0.23	4.21	5.29
Urea, mg/100 g	1,480	26.87	5.90	13.80	40.60
Udder health					
SCC, 10^3/mL	1,482	272.30	1,192.41	7.00	2,972.03
SCS, units	1,482	2.65	1.94	−0.84	7.89
DSCC, %	1,260	69.09	13.51	30.97	91.90
MAC, %	1,260	30.97	13.62	8.10	69.48
Log PMN-LYM count	1,260	3.09	1.35	0.81	6.43
Log MAC count	1,260	1.82	1.04	−0.14	4.68
Protein composition, g/L of milk					
True protein	1,264	31.38	3.30	24.39	39.96
Caseins	1,262	26.75	2.77	21.00	33.78
α_{S1}-CN	1,260	9.06	1.08	6.65	11.88
α_{S2}-CN	1,263	3.48	0.68	2.07	5.20
β-CN	1,264	8.97	1.34	5.80	12.24
κ-CN	1,262	5.25	1.05	3.16	8.14
Glycosylated κ-CN	1,243	1.60	0.58	0.66	3.33
Whey proteins	1,259	4.58	0.92	2.77	7.19
β-LG	1,259	3.44	0.84	1.76	5.94
α-LA	1,258	0.99	0.14	0.68	1.36
LF	1,256	0.16	0.06	0.05	0.33
NPN compounds	1,252	2.93	0.91	0.70	5.30
Urea	1,263	0.28	0.06	0.15	0.41
Minor N compounds	1,234	2.13	0.84	0.40	4.35
Protein composition, % of total milk N					
True protein	1,253	91.41	2.62	85.31	98.19
Caseins	1,259	78.07	1.20	75.08	80.63
α_{S1}-CN	1,261	26.47	2.05	22.07	31.83
α_{S2}-CN	1,262	10.13	1.64	6.25	14.13
β-CN	1,257	26.19	2.60	19.45	31.57
κ-CN	1,260	15.29	2.60	10.15	22.63
Glycosylated κ-CN	1,245	4.67	1.62	1.98	9.34
Whey proteins	1,257	13.38	2.35	8.30	19.88
β-LG	1,258	10.02	2.21	5.31	16.09
α-LA	1,258	2.88	0.39	2.03	3.99
LF	1,257	0.47	0.16	0.16	0.91
NPN compounds	1,252	8.59	2.62	1.81	14.69
Urea	1,236	2.45	0.54	1.31	3.78
Minor N compounds	1,235	6.23	2.39	1.11	12.17

[1]SCS = log2 (SCC/100,000) + 3; DSCC = differential SCC; log PMN-LYM count = polymorphonuclear neutrophils-lymphocytes count expressed as log2 (PMN-LYM count/100,000) + 3; log MAC count = macrophages count expressed as log2 (MAC count/100,000) + 3; caseins = sum of α_{S1}-CN + α_{S2}-CN + β-CN + κ-CN; Whey proteins = sum of β-LG + α-LA + LF; LF = lactoferrin; NPN compounds = nonprotein nitrogen compounds, sum of urea and minor nonprotein N compounds (e.g., small peptides, ammonia, creatine, creatinine).

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

are reported in Table 2. In agreement with Amalfitano et al. (2020), DIM and parity were notable sources of variation for almost all the traits considered, confirming the importance of including these factors in the statistical models used to analyze these kind of traits.

The effects of SCS and DSCC on milk yield and composition have been extensively investigated by Stocco et al. (2020) and Pegolo et al. (2021). A significant decrease in milk yield and lactose was observed as SCS increased (data not shown), while increased DSCC was associated with increased milk yield and lactose (data not shown). Our results generally agree with previous studies, with the only exception of the fat proportion, which was not significantly affected by either SCS or DSCC in our study, similar to findings reported by Bansal et al. (2005).

Association of SCS and DSCC with Milk Detailed Protein Profile

Regarding protein composition measured quantitatively (g/L), Table 2 shows that SCS had a significant

Table 2. Results from ANOVA (*F*-value and significance) for single test-day milk yield, composition traits, and protein fractions, expressed both as quantitative (g/L) and qualitative measures (%N)

Trait[1]	Parity	DIM	SCS[2]	DSCC[3]	Herd-date
Milk yield, kg/d	9.52***	38.90***	5.10**	8.52***	10
Milk composition					
Fat, %	3.07*	2.73*	2.49	1.62	25
Protein, %	7.78***	30.16***	3.02*	1.05	11
Casein, %	9.65***	31.78***	1.83	0.91	9
Lactose, %	18.61***	4.96***	17.99***	14.74***	7
Urea, mg/100 g	0.02	2.26*	0.86	0.50	56
Protein composition, g/L of milk					
True protein	7.13***	33.20***	3.43*	2.12	7
Caseins	10.27***	31.88***	1.42	0.82	8
α_{S1}-CN	2.17	12.39***	2.68*	2.10	20
α_{S2}-CN	3.27*	6.76***	0.74	1.94	25
β-CN	16.52***	15.09***	1.48	3.56*	17
κ-CN	1.05	14.80***	4.57**	0.91	5
Glycosylated κ-CN	1.33	25.30***	2.47	1.70	10
Whey proteins	1.15	8.22***	3.88**	4.27**	5
β-LG	1.82	6.34***	4.79**	4.73**	3
α-LA	2.08	1.63	5.11**	6.87***	16
LF	1.22	7.84***	0.08	0.39	16
N compound	0.03	0.56	0.13	1.60	14
Urea	0.03	2.40*	0.98	0.43	56
Minor N compound	0.09	0.67	0.11	1.50	13
Protein composition, % of total milk N					
True protein	0.26	3.08**	0.45	2.60	11
Caseins	14.85***	10.55***	7.55***	9.32***	24
α_{S1}-CN	1.23	2.43*	0.59	0.55	23
α_{S2}-CN	1.36	1.53	0.47	1.46	33
β-CN	13.33***	2.88*	11.50***	11.86***	18
κ-CN	1.72	2.70*	2.20	0.74	8
Glycosylated κ-CN	0.90	14.77***	1.82	1.34	11
Whey proteins	2.11	0.76	2.70*	4.85**	8
β-LG	2.71*	1.40	2.73*	3.85**	6
α-LA	3.37*	4.77***	2.21	3.87**	18
LF	0.36	3.14**	0.20	0.44	16
N compound	0.26	3.08**	0.45	2.60	11
Urea	1.61	10.66	2.96	1.45	50
Minor N compound	0.26	1.77	0.09	2.11	10

[1]Caseins = sum of α_{S1}-CN + α_{S2}-CN + β-CN + κ-CN; Whey proteins = sum of β-LG + α-LA + LF; LF = lactoferrin; NPN compounds = nonprotein nitrogen compounds, sum of urea and minor nonprotein N compounds (e.g., small peptides, ammonia, creatine, creatinine).

[2]SCS = log2 (SCC/100,000) + 3.

[3]DSCC = differential SCC.

[4]L = linear; Q = quadratic, C = cubic.

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

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

effect on true protein ($P < 0.05$). Among the casein fractions, SCS appeared to be significantly associated with κ-CN ($P < 0.01$) and α_{S1}-CN ($P < 0.05$), which surprisingly, increased linearly with increasing SCS (Figure 1). With regard to the whey proteins, SCS had a significant positive effect not only on total whey protein content ($P < 0.01$), but also on β-LG and α-LA ($P < 0.01$). All whey proteins increased linearly with increasing SCS (Figure 1). In addition, DSCC showed a significant ($P < 0.05$) and positive effect on β-CN, but a significant negative effect on the total whey protein content ($P < 0.01$), β-LG ($P < 0.01$), and α-LA ($P < 0.001$), an opposite pattern to SCS (Table 2 and Figure 1).

When the protein fractions were expressed as percentages of total milk nitrogen (%N), SCS negatively affected the total casein proportion ($P < 0.001$) and β-CN ($P < 0.001$). Meanwhile, DSCC showed the opposite pattern, and its increase was associated with a linear increase of both total casein and β-CN ($P < 0.001$, Figure 2). Both SCS and DSCC had significant effects on the total proportion of whey protein ($P < 0.05$ and $P < 0.01$, respectively) and β-LG ($P < 0.05$ and $P < 0.01$, respectively), confirming once again an opposite pattern between them: a higher SCS was associated with higher proportions of total whey protein and β-LG, while higher DSCC was associated with

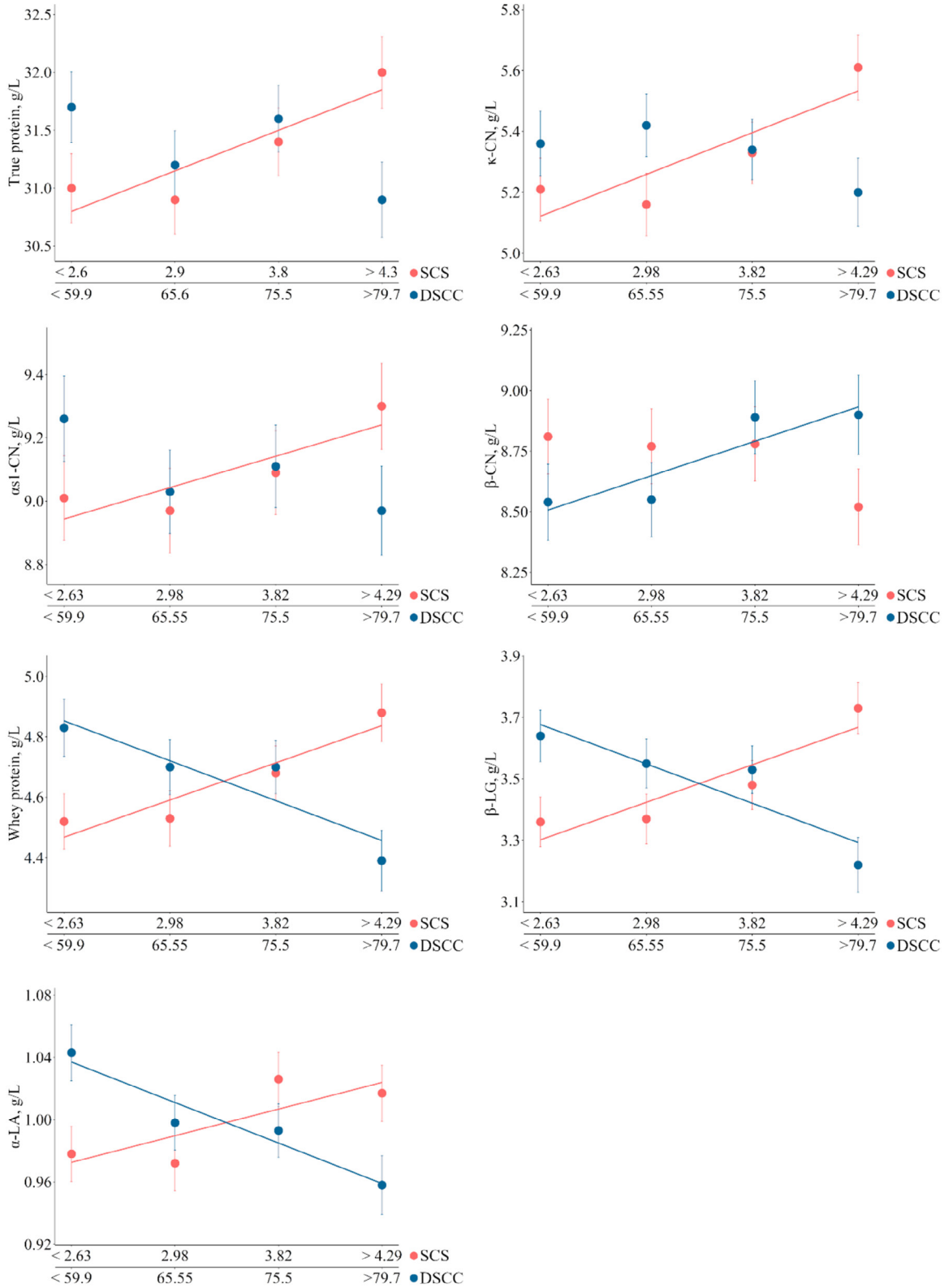

Figure 1. Least squares means and SE of milk protein fractions expressed as quantitative measures (g/L) across SCS and differential somatic cell count (DSCC) classes. Only significant results ($P < 0.05$) are displayed. When the trend line across SCS or DSCC LSM classes is presented, it means that the polynomial contrast was significant at $P < 0.05$. Only linear contrasts were significant. Values on the x-axis refer to the average value for each class of SCS [top row; expressed as log2 (SCC/100,000) + 3] and DSCC (bottom row; expressed as percentage).

lower proportions of those fractions. α-Lactalbumin was significantly affected only by DSCC ($P < 0.01$), and the association was negative, as with the other whey proteins. β-Lactoglobulin is the most abundant whey protein in milk, and it is synthetized in the epithelial cells of the mammary gland. The reduction of β-LG with the increase of DSCC needs to be further evaluated, as its biological role is currently unclear at the moment. However, we might speculate that β-LG, a member of the lipocalin family, which includes, among others, fatty acid-binding proteins and bacterial metalloprotease inhibitors (Broersen, 2020),

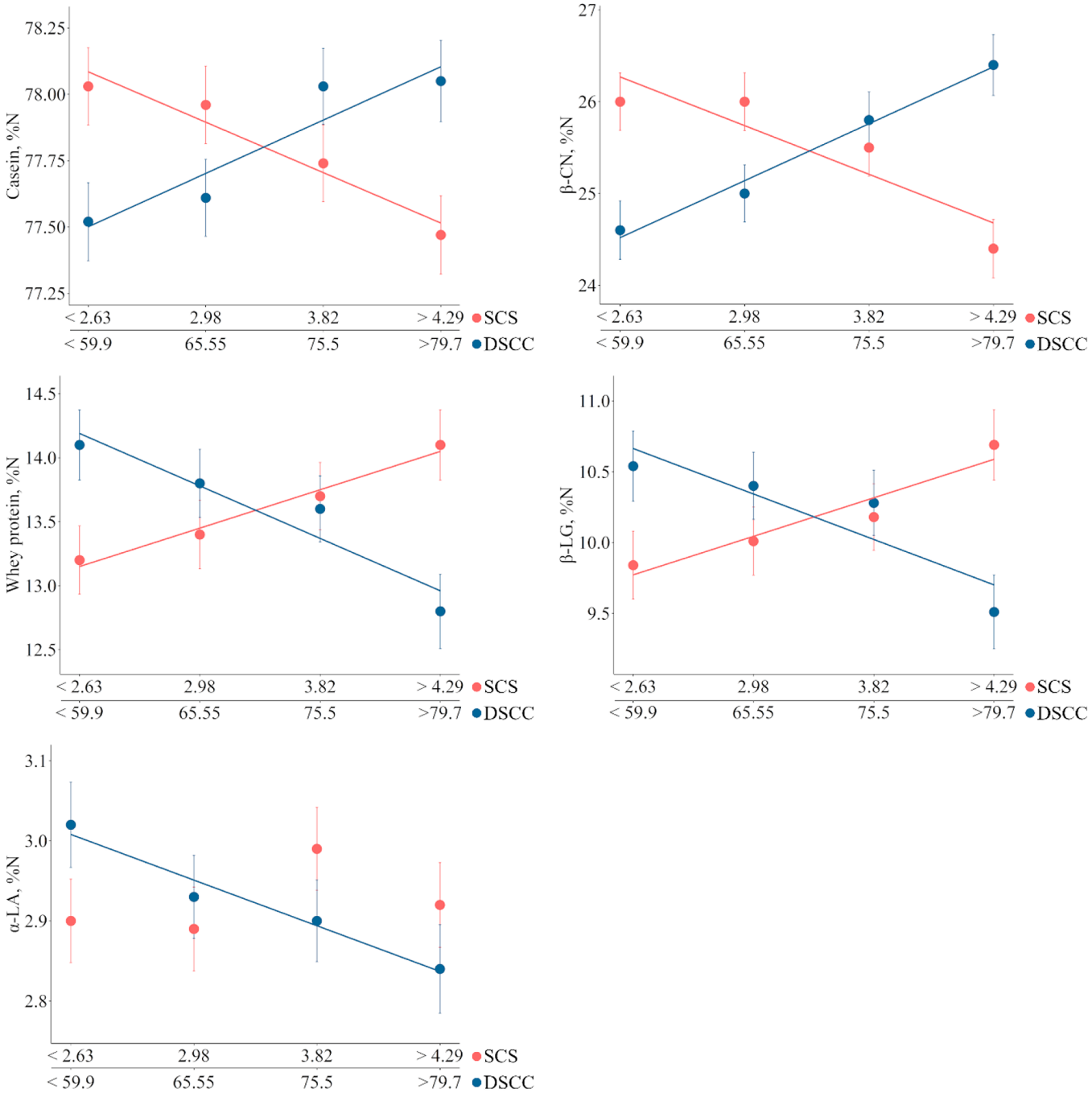

Figure 2. Least squares means and SE of milk protein fractions expressed as qualitative measures (%N) across SCS and differential somatic cell count (DSCC) classes. Only significant results ($P < 0.05$) are displayed. When the trend line across SCS or DSCC LSM classes is presented, it means that the polynomial contrast was significant at $P < 0.05$. Only linear contrasts were significant. Values on the x-axis refer to the average value for each class of SCS [top row; expressed as log2 (SCC/100,000) + 3] and DSCC (bottom row; expressed as percentage).

Table 3. Results from ANOVA (*F*-value and significance) for single test-day milk yield, composition traits, and protein fractions, expressed both as quantitative (g/L) and qualitative measures (%N) using log counts of PMN and lymphocytes (PMN-LYM) and macrophages (MAC)

Trait[1]	Parity	DIM	Log PMN-LYM[2]	Log MAC[3]	Herd-date,[4] %
Milk yield, kg/d	9.61***	39.39***	1.88	7.71***	10
Milk composition					
Fat, %	3.59*	2.77*	0.33	3.06*	25
Protein, %	8.16***	30.67***	1.78	1.25	11
Casein, %	10.01***	32.27***	2.39	1.49	9
Lactose, %	19.29***	5.35***	2.82*	20.52***	7
Urea, mg/100 g	0.04	2.27*	0.99	0.43	56
Protein composition, g/L of milk					
True protein	7.21***	33.90***	1.14	0.85	7
Caseins	10.69***	32.20***	2.09	1.73	8
α_{S1}-CN	2.42	12.68***	1.88	0.78	20
α_{S2}-CN	3.22*	6.99***	0.50	1.54	25
β-CN	17.54***	15.13***	2.97*	6.07***	16
κ-CN	1.14	14.81***	1.25	0.79	5
Glycosylated κ-CN	1.48	25.56***	0.43	1.71	10
Whey proteins	1.20	8.90***	0.87	4.44*	5
β-LG	1.84	7.11***	0.64	3.73*	4
α-LA	1.57	1.80	4.76**	8.50***	16
LF	1.26	8.38	0.09	0.32	16
N compound	0.05	0.50	1.47	0.71	14
Urea	0.04	2.41*	0.95	0.42	56
Minor N compound	0.11	0.62	1.46	0.67	13
Protein composition, % of total milk N					
True protein	0.20	3.30**	0.66	0.61	11
Caseins	15.08***	10.59***	2.37	13.85***	25
α_{S1}-CN	1.38	2.27*	1.35	0.10	23
α_{S2}-CN	1.31	1.78	2.40	1.67	33
β-CN	14.14***	3.05**	3.16*	13.16***	18
κ-CN	1.90	2.55*	0.81	0.77	8
Glycosylated κ-CN	0.98	14.99***	0.61	1.24	11
Whey proteins	2.35	1.09	2.51	5.40**	8
β-LG	2.87*	1.78	1.38	3.62*	6
α-LA	3.21*	4.53***	7.71***	8.15***	18
LF	0.37	3.69**	0.09	0.45	16
N compound	0.20	3.30**	0.66	0.61	11
Urea	1.60	10.87***	1.51	0.43	50
Minor N compound	0.20	1.90	1.04	0.58	10

[1]Caseins = sum of α_{S1}-CN + α_{S2}-CN + β-CN + κ-CN; whey proteins = sum of β-LG + α-LA + LF; LF = lactoferrin; NPN compounds = nonprotein nitrogen compounds, sum of urea and minor nonprotein N compounds (e.g., small peptides, ammonia, creatine, creatinine).

[2]Log PMN-LYM count = polymorphonuclear neutrophils-lymphocytes count expressed as log2 (PMN-LYM count/100,000) + 3.

[3]Log MAC count = macrophages count expressed as log2 (MAC count/100,000) + 3.

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

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

could have a role in the innate immune response of the mammary gland.

A significant reduction in β-LG and α-LA has been previously observed in mastitic animals with elevated SCC (Ishikawa et al., 1982; Hogarth et al., 2004; Ramos et al., 2015). As no cow enrolled in our study exhibited clinical signs of mastitis, we could assume that no inflammatory damage of mammary secretory tissue or increased leakage between tight junctions in the mammary gland could have affected the reduction of either β-LG and α-LA.

The reduction in the proportion of total casein, and more specifically the β-CN fraction, observed at the increasing of SCS could be ascribed either to reduced synthesis of caseins or to the proteolytic activity of plasmin, the principal indigenous proteinase in milk, which seems to break the β-casein protein chain, in particular (Le Roux et al., 2003; Crudden et al., 2005; Forsbäck et al., 2010). The opposite pattern shown by DSCC could, however, be ascribed to the fact that the present study was carried out on a population of clinically healthy animals, and it is therefore likely that the LYM predominated over the PMN and MAC, cells which are known for their proteolytic activity.

Different Leucocyte Populations Have Contrasting Behavior on Milk Protein Fractions

Given that DSCC is a parameter that provides information on the proportions of the different leucocyte

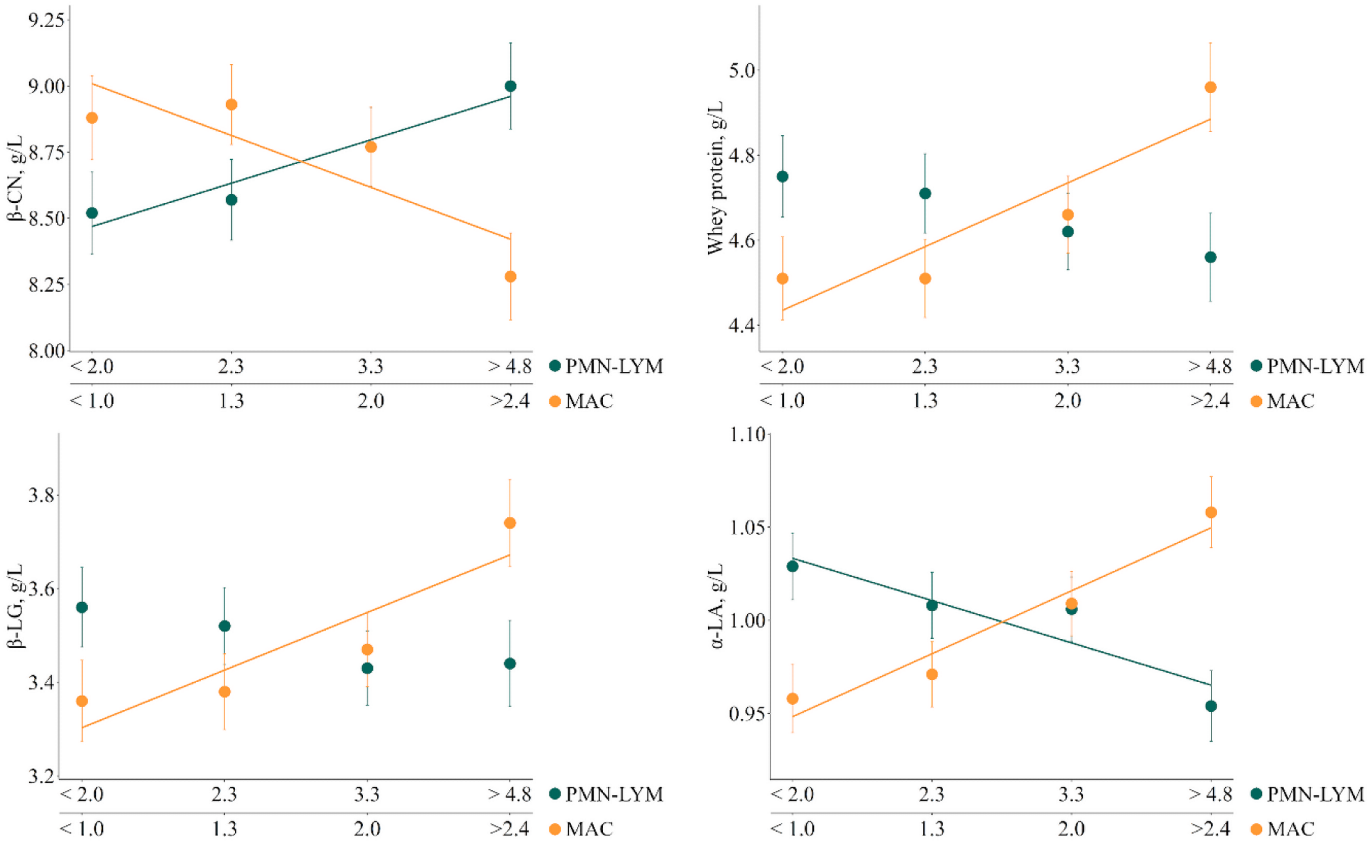

Figure 3. Least squares means and SE of milk protein fractions expressed as quantitative (g/L) measures across polymorphonuclear neutrophils and lymphocytes (PMN-LYM), and macrophages (MAC) classes. Only significant results ($P < 0.05$) are displayed. When the trend line across SCS or DSCC LSM classes is presented, it means that the polynomial contrast was significant at $P < 0.05$. Only linear contrasts were significant. Values on the x-axis refer to the average value for each class of PMN-LYM [top row; expressed as log2 (PMN-LYM count/100,000) + 3] and MAC [bottom row; expressed as log2 (MAC count/100,000) + 3].

populations in milk, it would be interesting to evaluate if the trends previously observed would either change or be confirmed when the quantitative variables (i.e., the PMN-LYM and MAC counts) are considered. Including both variables in the model did not induce any bias because they were only moderately correlated (Pegolo et al., 2021).

Table 3 reports the results of the ANOVA for the associations between PMN-LYM and MAC counts and milk composition and protein profile. Expressed quantitatively, β-CN was the only casein fraction significantly affected by both PMN-LYM ($P < 0.05$) and MAC ($P < 0.001$). In particular, an increase across PMN-LYM count classes was associated with an increase in β-CN contents (+5.63%), while an increase across MAC count classes was associated with lower β-CN contents (−6.75%; Figure 3).

Moreover, MAC count significantly affected total whey protein and β-LG ($P < 0.05$), as well as α-LA ($P < 0.001$), which was also significantly associated with PMN-LYM count ($P < 0.01$). The effect of PMN-LYM and MAC counts on the whey protein fractions was the opposite to what we observed for β-CN, with MAC count associated with an increase in total whey proteins, (+9.98%), β-LG (+ 11.31%), and α-LA (+10.44%), whereas PMN-LYM was negatively associated with α-LA (−7.29%).

Regarding the protein fractions expressed as percentages of total nitrogen (%N), we confirmed the previously observed trend, with MAC count having a significant negative association with total caseins and β-CN ($P < 0.001$) and a positive association with total whey proteins ($P < 0.01$), β-LG ($P < 0.05$), and α-LA ($P < 0.001$; Figure 4). Once again, PMN-LYM count showed the opposite trend to MAC count, being associated with an increase in the β-CN proportion ($P < 0.05$) and a decrease in the α-LA proportion ($P < 0.001$).

To correctly interpret these results, it is important to remember that DSCC represents the combined proportions of PMN and LYM, which have very different

distributions in healthy and diseased udders. Notably, LYM and MAC are thought to predominate among the immune cells in a healthy mammary gland, while PMN constitute only a small proportion (Wickström et al., 2009; Schwarz et al., 2011) but predominate once inflammation occurs (Kehrli and Shuster, 1994). Some uncertainty remains concerning the distribution of immune cells during inflammation. Damm et al. (2017) and Stocco et al. (2020) observed that PMN and MAC seemed to change dramatically and in opposite directions across the whole SCC range, while LYM remained fairly constant and were able to sustain the immune response. In contrast, other studies reported that the most marked change related to inflammation process

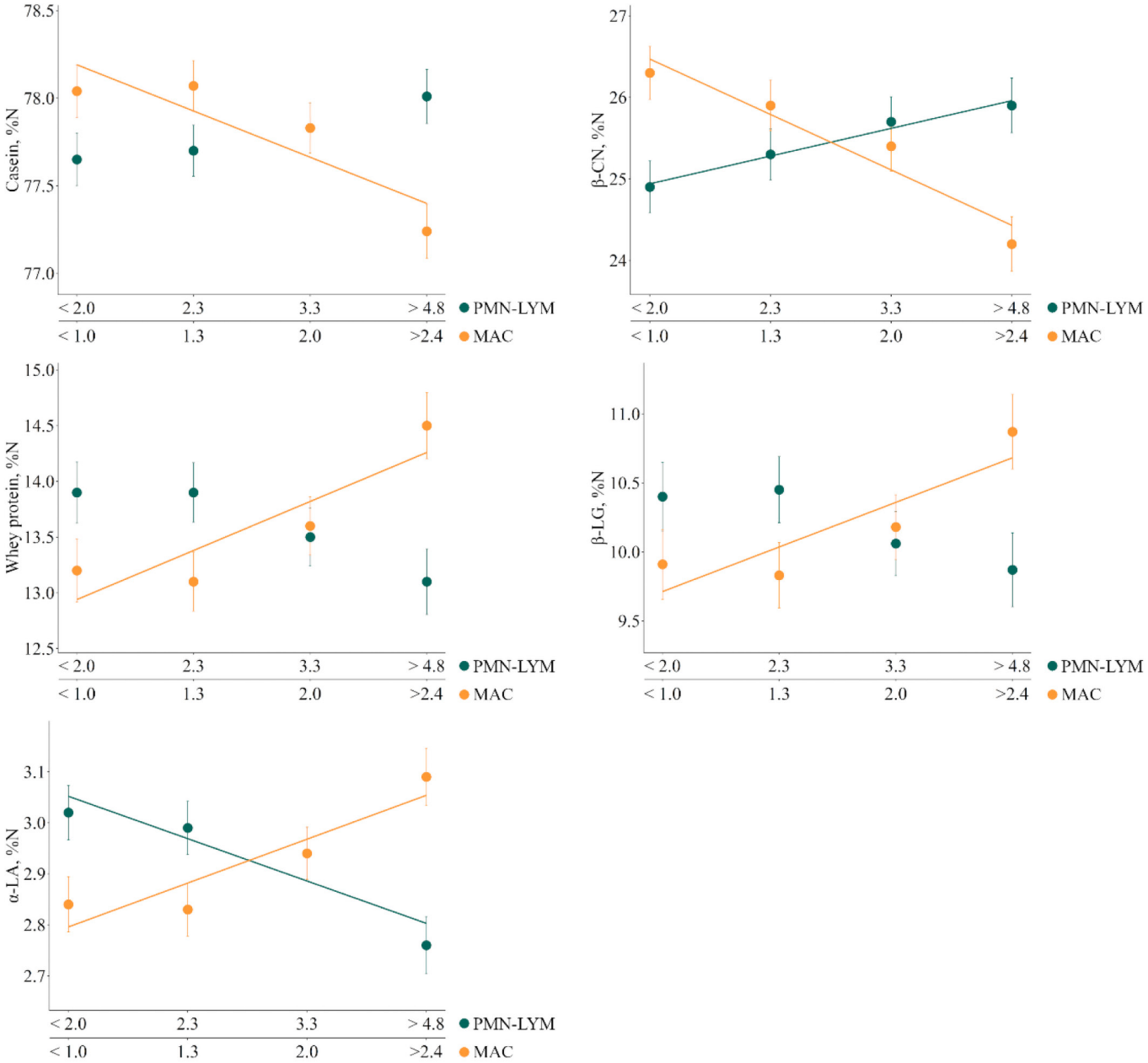

Figure 4. Least squares means and SE of milk protein fractions expressed as qualitative measures (%N) across polymorphonuclear neutrophils and lymphocytes (PMN-LYM), and macrophages (MAC) classes. Only significant results ($P < 0.05$) from model are displayed. When the trend line across SCS or differential somatic cell count (DSCC) LSM classes is presented, it means that the polynomial contrast was significant at $P < 0.05$. Only linear contrasts were significant. Values on the x-axis refer to the average value for each class of PMN-LYM [top row; expressed as log2 (PMN-LYM count/100,000) + 3] and MAC [bottom row; expressed as log2 (MAC count/100,000) + 3].

concerned the proportions of LYM and PMN, with MAC remaining relatively unchanged (Schwarz et al., 2011; Pilla et al., 2013).

The general low SCS values that we observed highlight the overall good condition of the cows, so it was most likely that the predominant leucocyte populations were LYM and MAC, rather than PMN. The main function of MAC, which contain lysosomes with proteolytic enzymes such as collagenase, elastase, and cathepsins, is to defend the udder from infection (Kelly and McSweeney, 2003). They have also been found to produce in vitro the urokinase-type plasminogen activator (Politis et al., 1991), which converts inactive plasminogen into plasmin, the main native proteolytic enzyme in milk. The contribution of MAC to proteolysis in milk has been investigated by Caroprese et al. (2007), who found appreciable casein hydrolysis in bulk ewe milk, particularly of α-CN, while β-CN did not undergo any appreciable degradation. This difference might be related to the slightly different structures and distributions of casein fractions in different animal species.

CONCLUSIONS

Our results confirm the unfavorable effect of SCC on milk caseins, and specifically on β-CN, which is probably related to the proteolytic activity of milk native enzymes, such as plasmin. These results shed new light on the association between DSCC and milk proteins. In particular, the unfavorable association we found between MAC count and casein fractions might be related to the proteolytic activity of MAC and the fact that they were present in larger amounts than PMN, given the nonpathological status of the animals. These results highlight the importance of this trait as a potential novel udder health indicator and could be of interest for the dairy industry, given the important role of caseins in milk coagulation properties and cheese production. To validate these findings and assess the association between DSCC and milk protein fractions in greater detail, it will be necessary to be able to accurately differentiate the PMN and LYM populations, which are combined in the DSCC parameter, and investigate their individual effects. Furthermore, possible extensions of this study include the study of genetic correlations between DSCC and protein fractions. These correlations could provide useful information from breeding perspectives.

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