



Novel insights into the associations between immune cell population distribution in mammary glands and milk minerals in Holstein cows

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ABSTRACT

Udder health has a crucial role in sustainable milk production, and various reports have pointed out that changes in udder condition seem to affect milk mineral content. The somatic cell count (SCC) is the most recognized indicator for the determination of udder health status. Recently, a new parameter, the differential somatic cell count (DSCC), has been proposed for a more detailed evaluation of intramammary infection patterns. Specifically, the DSCC is the combined proportions of polymorphonuclear neutrophils and lymphocytes (PMN-LYM) on the total SCC, with macrophages (MAC) representing the remainder proportion. In this study, we evaluated the association between DSCC in combination with SCC on a detailed milk mineral profile in 1,013 Holstein-Friesian cows reared in 5 herds. An inductively coupled plasma–optical emission spectrometry was used to quantify 32 milk mineral elements. Two different linear mixed models were fitted to explore the associations between the milk mineral elements and first, the DSCC combined with SCC, and second, DSCC expressed as the PMN-LYM and MAC counts, obtained by multiplying the proportion of PMN-LYM and MAC by SCC. We observed a significant positive association between SCC and milk Na, S, and Fe levels. Differential somatic cell count showed an opposite behavior to the one displayed by SCC, with a negative association with Na and positive association with K milk concentrations. When considering DSCC as count, Na and K showed contrasting behavior when associated with PMN-LYM or MAC counts, with decreasing of Na content and increasing K when associated with increasing PMN-LYM counts, and increasing Na and decreasing K when associated with increasing MAC count. These findings confirmed that an increase in SCC is associated with altered milk Na and K amounts. Moreover, MAC count seemed to mirror SCC patterns, with the worsening of

inflammation. Differently, PMN-LYM count exhibited patterns of associations with milk Na and K contents attributable more to LYM than PMN, given the non-pathological condition of the majority of the investigated population. An interesting association was observed for milk S content, which increased with increasing of inflammatory conditions (i.e., increased SCC and MAC count) probably attributable to its relationship with milk proteins, especially whey proteins. Moreover, milk Fe content showed positive associations with the PMN-LYM population, highlighting its role in immune regulation during inflammation. Further studies including individuals with clinical condition are needed to achieve a comprehensive view of milk mineral behavior during udder health impairment.

Key words: dairy cattle, differential somatic cell count, milk minerals, udder health

INTRODUCTION

Milk is an excellent nutritional product and a valuable source of nutrients, including vitamins and minerals (Cashman, 2002, 2006). Minerals constitute a minor fraction of milk components (approximately 0.7%; Kaufmann and Hagemeyer, 1987). Despite that, milk contains the complete variety of mineral elements in different chemical forms, such as inorganic ions and salts, or associated with proteins, nucleic acids, fats, and carbohydrates that are essential for human health (Gaucheron, 2005; Cashman, 2006).

As highlighted by previous reports, milk mineral content is first dependent on feeding practices and environmental features, although for some macrominerals (i.e., Ca, K, Mg) genetic and individual factors have proved to have a much more important role (Stocco et al., 2019). Various reports pointed out that udder health conditions also seem to affect milk mineral content, showing that decreased levels of Ca, Mg, and K with increased levels of Na in milk can be considered as indicators of mastitis (Summer et al., 2009; Gaucheron, 2013; Nogalska et al., 2020). In this framework, in-line sensors able to measure the electrical conductiv-

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ity are commonly used in automatic milking systems to detect mastitis (Steenefeld et al., 2010; Khatun et al., 2018). In fact, a higher electrical conductivity has been associated with the regulation of osmotic pressure induced by higher ion concentrations (e.g., Na, Cl) during mastitis-related damage of the blood-milk barrier (Khatun et al., 2019). In addition, some trace minerals such as Zn, Cu, and Mn are closely intertwined with immune function and udder health (O'Rourke, 2009), but there is limited information regarding alterations in trace mineral content in milk and udder health.

Udder health has a pivotal role in sustainable milk production: the detection of mastitis at early stage, especially in its subclinical form, is fundamental as this disease affects not only on individual cow's health but also on milk yield and quality, managerial costs, and antimicrobial handling (Neculai-Valeanu and Ariton, 2022). The most widely recognized and practical method that provides prompt information on udder health is the SCC (Bobbo et al., 2016). Somatic cell count represents the total count of immune cells in the milk and indicates the inflammatory response in the mammary gland, being a proxy for measuring intramammary infection and milk quality at quarter, cow, herd, and population level (Schukken et al., 2003). There is a general agreement on the association between an increase in milk SCC and changes in inflammatory cell population distribution in the milk, that is mainly due to the migration of polymorphonuclear leukocytes (PMN) from blood circulation to milk (Dal Prà et al., 2022). For this reason, several studies have suggested the use of differential SCC (DSCC), which distinguishes among the different immune cells in the milk allowing a more detailed evaluation of the udder health status (Schwarz et al., 2011; Damm et al., 2017). The recent possibility of using a milk analyzer (Fosomatic7DC, Foss A/S, Hillerød, Denmark) that enables performance of a partial DSCC combining the proportion of PMN and lymphocytes (LYM), may open new opportunities to fast evaluate DSCC in combination with SCC. Indeed, diverse studies have shown that this combined use can add significant information in describing intramammary infection status (Schwarz et al., 2019; Kirkeby et al., 2020, 2021). In the same vein, recent studies have been conducted on the association between DSCC (expressed either individually or in combination with SCC) and milk coagulation properties (Pegolo et al., 2021a, 2022a) and detailed protein profile (Bisutti et al., 2022). However, to date, there are no studies in relatively large population of dairy cattle on the associations between DSCC and a detailed set of milk mineral elements quantitatively determined with an analytical methodology (i.e., inductively coupled plasma-optical emission spectrometry; ICP-OES).

Hence, this study aimed at evaluating the association between DSCC in combination with SCC on a detailed milk mineral profile in 1,013 Holstein-Friesian cows reared in 5 herds. The effects were estimated using the DSCC expressed as a proportion and count.

MATERIALS AND METHODS

The required sample size for this study was calculated on the differences between groups of animals with different SCS and DSCC values for Na, which was chosen as a representative milk mineral element of mammary gland inflammation (Gaucheron, 2013). The minimum sample size was estimated based on the expected difference between the mean values of the extreme classes of SCS and DSCC (i.e., 0.92 vs. 6.33 and 46.7% vs. 83.9%, respectively), which was equal to 88 and 77 mg/kg milk, corresponding to roughly 90% and 80% of the standard deviation of the trait (SCS and DSCC, respectively). A 2-tailed test was performed with α of 0.05 and power $(1 - \beta)$ of 0.90. The minimum number of samples required was 320 and 470 (for SCS and DSCC, respectively). Nevertheless, because the study is part of broader research projects that involved not only phenotypic, but also genetic and genomic analyses, a larger sample size was available ($n = 1,013$ cows).

Animal Data

Data for this study result from previous research projects (LATSAN and BENELAT) and were generated using the same methodology and in the same laboratory of our research group. The study was performed on 1,013 Holstein-Friesian lactating cows pertaining to 5 herds located in the Veneto (3 herds, $n = 103$ cows) and Emilia Romagna (2 herds, $n = 910$ cows) regions (northern Italy). The sampled cows were of different parities (1 to 6) and stages of lactation (5 to 400 DIM) with a well-balanced distribution of parities and stages of lactation. The cows were housed in sand-bedded free stalls and fed twice daily on TMR based on corn and sorghum silage supplemented with concentrates. Detailed diets of the farms are reported in Giannuzzi et al. (2022) and Pegolo et al. (2023). The cows were sampled once during the evening milking after medical checks from May 2019 to January 2021. Any animals with clinical disease or under pharmaceutical treatments were excluded to avoid introducing bias into the analysis. The research was approved by the Committee for the Protection and Welfare of Experimental Animals (Organismo Preposto al Benessere degli Animali) of the Catholic University of the Sacred Heart and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019).

Milk Sampling and Analysis

Milk samples were collected in 18 batches (i.e., herd/date combinations): 12 in 2019 ($n = 889$ cows), 5 in 2020 ($n = 114$ cows), and 1 in 2021 ($n = 10$ cows). Herds were sampled one per day. Large herds were sampled on more than one day as the laboratory for assessing cheese-making traits (not reported in this study) could only process around 65 milk samples per day (Pegolo et al. 2021a). The individual composite milk samples (100 mL), taken following the standard procedures adopted by Breeder Associations within the milk recording schemes (ICAR, 2020) were mixed by gentle inversion and divided into 2 aliquots (50 mL each). One aliquot was added with bronopol preservative and transferred to the laboratory of the Breeders Association of Veneto region where it was stored at 4°C until analysis of udder health traits (i.e., SCC, DSCC) and milk composition (i.e., fat, protein, casein, and lactose percentages) within 48 h from 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 mineral profile (within 4 mo from collection).

Analysis of Udder Health Traits

Samples were analyzed for SCC (cells/mL) and DSCC (%) with a Fossomatic 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. (2021a,b) and Bisutti et al. (2022), the proportions of DSCC were converted to cell counts as follows:

$$\text{PMN_LYM count, } \frac{10^3}{\text{mL}} = \text{DSCC} \times \text{SCC};$$

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

In addition, the PMN-LYM and macrophage (MAC) counts were transformed as $\log(\text{PMN-LYM or MAC count}/100,000) + 3$, similarly to SCS, to obtain a normal distribution of the data.

Milk Mineral Profiling

Detailed procedure of the analysis is described in Toscano et al. (2023). Briefly, the analysis includes 2 steps: first, milk samples were mineralized using the Milestone Start D instrument (Milestone S.r.l., Bergamo, Italy)

power 1,200 W, equipped with a SK10 high-pressure rotor (100 bar). From each sample, 2 to 2.5 g of milk was taken and placed in modified polytetrafluoroethylene containers, to which 7 mL of 67% nitric acid and 2 mL of 30% hydrogen peroxide, both Suprapur quality (Merck Chemicals GmbH, Darmstadt, Germany), were added. Mineralization occurred in 3 heating steps: (1) from 25°C to 200°C in 18 min; (2) 15 min at 200°C; (3) cooling to 35°C, which took approximately 15 min. The resulting mineralized material was made up to volume (25 mL) in ultrapure water for determination of mineral content in ICP-OES. Second, a Spectro Arcos EOP ICP-OES (SPECTRO Analytical Instruments GmbH, Kleve, Germany) was used for the determination of 32 mineral elements (Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sn, Sr, Ti, Tl, V, Zn). Mineral elements present below the detection limit (Ag, As, Be, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sb, Se, Si, Sn, Ti, Tl, V) of the instrument were not considered. Multi-element and single-element standard solutions (Inorganic Ventures Inc., Christiansburg, VA) in 10% Suprapur nitric acid (Merck Chemicals GmbH) were used to calibrate the instrument. Using the same method described above, certified reference material BCR-063 skim milk powder (Institute for Reference Materials and Measurements, Geel, Belgium) was prepared for the determination of the accuracy and precision of the analysis. The measured values and the certified values were in excellent agreement for all the elements. Minerals with values below the limit of detection (LOD) of the instrument for more than 75% of the samples were excluded from the analysis. For those minerals where the value was below LOD for less than 25% of the samples, that value was replaced with half of the LOD.

Statistical Analysis

Given that the accuracy of the Fossomatic 7DC analyzer has been reported low for extremely low or high values of SCC (Damm et al., 2017), only milk samples with $\text{DSCC} > 50,000$ SCC/mL and $< 1,500,000$ SCC/mL were considered. In addition, using the rationale of Pegolo et al. (2021a) and Bisutti et al. (2022), 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 adopted to better assess 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. Before fitting the operational model for

estimating the effects of DSCC and SCC, we carried out a preliminary analysis to quantify the relative importance of individual sources of variation due to DIM, parity, and the herd-date effect on the milk mineral profile. To do this, the latter factors have been included as random class effects and the variances of these 3 sources of variation were expressed as percentages of their sum (total variance). The linear mixed model implemented in R software (version 4.3.0; R Foundation for Statistical Computing), using the package lme4, was as follows:

$$y_{ijklmn} = \text{DIM}_i + \text{parity}_j + \text{herd}_m + e_{ijklmn}, \quad [1]$$

where y_{ijklmn} is the phenotype (milk mineral), DIM_i is the random effect of DIM class i (12 levels: <30; 31–60; 61–90; 91–120; 121–150; 151–180; 181–210; 210–240; 241–270; 271–300; 301–330; >330), parity_j is the random effect of parity j of the cow (4 levels: 1, 2, 3, >3), herd_m is the random effect of the m herd (5 levels), and e_{ijklmn} is the random residual. Days in milk, parity, and the herd effects, as well as residuals, were assumed to be independently and normally distributed with a mean of zero and variance σ_{DIM}^2 , σ_{parity}^2 , $\sigma_{\text{herd-date}}^2$, σ_e^2 respectively; restricted maximum likelihood was used as the method of estimation of variance components.

The associations among SCS and DSCC and milk minerals were estimated using the linear mixed model implemented in R using the package lme4 as follows:

$$y_{ijklmn} = \text{DIM}_i + \text{parity}_j + \text{SCS}_k + \text{DSCC}_l + \text{herd/date}_m + e_{ijklmn}, \quad [2]$$

where y_{ijklmn} is the phenotype (milk mineral), SCS_k is the fixed effect of SCS k class, categorized into quartiles (4 levels: <1.20 [n = 253]; 1.20–2.42 [n = 252]; 2.42–3.65 [n = 254]; >3.65 [n = 253]), DSCC_l is the fixed effect of DSCC l class categorized into quartiles (4 levels: <59.9 [n = 134]; 59.9–70.9 [n = 136]; 70.9–79 [n = 133]; >79 [n = 133]), herd/date is the random effect of the m herd/date (18 levels), and other factors as previously defined in model 1.

In addition to model 2, another linear mixed model (model 3) was used to evaluate the specific effect of PMN-LYM and MAC counts in explaining the total variance for the investigated traits:

$$y_{ijklmn} = \text{DIM}_i + \text{parity}_j + \text{PMN} - \text{LYM}_k + \text{MAC}_l + \text{herd/date}_m + e_{ijklmn}. \quad [3]$$

In model 3, SCS and DSCC were excluded from the vector of fixed effects, while the model included PMN-

LYM $_k$, the fixed effect of PMN-LYM count class k categorized into quartiles (4 levels: <2.02 [n = 134]; 2.02–2.67 [n = 134]; 2.67–3.75 [n = 133]; >3.75 [n = 135]) and MAC $_l$, the fixed effect of MAC count class l categorized into quartiles (4 levels: <1.00 [n = 134]; 1.00–1.62 [n = 134]; 1.62–2.37 [n = 134]; >2.37 [n = 134]). All the other terms were equal to model 2. A model including DIM \times parity interaction was tested, but it was not significant for all the traits; therefore, this additional source of variation was excluded from the final model. In the same line, a model including SCS \times DSCC interaction was also tested. Because it was slightly significant only for few traits and it exhibited erratic patterns, the results were omitted. The differences in means were tested using the Tukey-Kramer multiple means comparison test ($P < 0.05$). Only significant results ($P < 0.05$) were displayed in the figures.

RESULTS AND DISCUSSION

The phenotypic associations existing between DSCC and milk composition (Zecconi et al., 2020), milk protein profile (Bisutti et al., 2022), and technological characteristics (Pegolo et al., 2021a) have been previously explored. Notably, these recent studies have highlighted that the correlation between SCS and DSCC and between PMN-LYM count and MAC count was not 1, emphasizing that they can capture diverse biological features, which justifies the inclusion of both traits in the respective model (Pegolo et al., 2021a). Herein, we observed similar relationships, with correlations between SCS and DSCC ($r = 0.36$), and between PMN-LYM and MAC counts ($r = 0.57$). Given the well-assessed link between milk minerals and udder health traits, we evaluated the associations existing among the DSCC, considered as a proportion and as a count, and the detailed milk mineral profile.

Descriptive Statistics of Udder Health Traits and Mineral Elements

A summary of descriptive statistics for the studied traits is reported in Table 1. Milk yield was on average 32.85 ± 9.38 kg/d, and major milk components were in line with previous reports on Holstein-Friesian cows (Premi et al., 2021). With regard to udder health traits, SCC has a median of 66,000 cells/mL. As reported in Table 1, the majority of samples (>86%) showed concentrations above the LOD threshold for mineral elements, with the exception of Cu, Ba, Cr, Ti. In the case of contaminant mineral elements (i.e., Ba, Cr, Ti) the absence or presence under the LOD in certain samples is expected, being their presence mainly related to en-

Table 1. Descriptive statistics of single test-day milk yield, composition, udder health, and milk mineral profile expressed as mg/kg milk (n = 1,013)

Item	Mean	SD	P1 ¹	P99 ¹	% >LOD ¹
Milk yield, kg/d	32.8	9.38	5.55	56.2	
Milk composition, %					
Fat	3.77	0.77	1.74	5.69	
Protein	3.42	0.37	2.71	4.29	
Casein	2.68	0.28	2.09	3.43	
Lactose	4.87	0.24	4.06	5.29	
Udder health ²					
SCC, 10 ³ mL ⁻¹	206	578	8	2,426	
SCS, score	2.57	1.82	-0.64	7.60	
DSCC, %	68.4	14.3	28.8	92.0	
MAC, %	31.6	14.3	7.97	71.1	
logPMN-LYM count	2.97	1.31	0.85	6.50	
logMAC count	1.74	1.04	-0.13	4.38	
Macrominerals, mg/kg milk					
Ca	1,159	179	771	1,670	99
Mg	98.3	16.0	66.6	137	100
P	939	169	615	1,463	98
K	1,430	206	1,032	2,079	98
Na	344	98.5	215	676	98
S	304	49.1	208	450	99
Trace minerals, mg/kg milk					
Cu	0.05	0.02	0.02	0.14	65
Fe	0.31	0.19	0.12	1.23	99
Mn	0.02	0.01	0.01	0.04	87
Zn	4.10	0.86	2.21	6.25	99
Contaminants, mg/kg milk					
Al	0.12	0.08	0.01	0.34	86
B	0.23	0.07	0.06	0.43	98
Ba	0.05	0.02	0.01	0.14	70
Cr	0.03	0.01	0.01	0.07	21
Sr	0.42	0.10	0.22	0.71	99
Ti	0.02	0.01	0.01	0.06	48

¹% >LOD = proportion of records above the limit of detection (LOD). The LOD for macrominerals >1 mg/kg. Other minerals (expressed as mg/kg): Cu >0.1, Fe >0.2, Mn >0.1, Zn >0.1, Al >0.2, B >0.2, Ba >0.2, Cr >0.1, Sr >0.1, Ti >0.1; P1 = first percentile; P99 = 99th percentile.

²SCS = $\log_2(\text{SCC}/100,000) + 3$; logPMN-LYM count = polymorphonuclear neutrophils-lymphocytes count expressed as $\log_2\{(\text{DSCC} \times \text{SCC})/100,000\} + 3$; logMAC count = macrophages count expressed as $\log_2\{(100 - \text{DSCC}) \times \text{SCC}/100,000\} + 3$.

environmental factors; with regard to Cu, it is known to be relatively high in early lactation, and then concentrations drop down for the rest of lactation (King and Dunkley, 1959), during which the investigated population have been sampled (median 177 DIM).

Milk macrominerals are generally recognized as relatively constant, whereas trace minerals and contaminants are much more variable (Zamberlin et al., 2012). Indeed, we observed that macrominerals have lower coefficients of variation (range 0.14 to 0.28), compared with trace minerals and contaminants, that have higher coefficients of variation (range 0.20 to 0.68). Overall, the observed milk mineral content is consistent with the ranges proposed by NASEM (2021) and also with previous literature using comparable analytical methods, despite a few exceptions. Indeed, we observed lower contents of Mg (98.38 mg/kg) compared with those reported by Visentin et al. (2016; 128.30 mg/kg) and by Buitenhuis et al. (2015; 108 mg/kg) in a Danish

Holstein population. We observed also lower contents of K (1,403.6 mg/kg) compared with the reports of van Hulzen et al. (2009; 1,612 mg/kg), and Denholm et al. (2019; 1,774.39 mg/kg) in Holstein-Friesian cows, and Christophe et al. (2021; 1,513 mg/kg) which considered multiple breeds. Sodium (344 mg/kg) was in line with the findings of Buitenhuis et al. (2015; 349 mg/kg) and Christophe et al. (2021; 354 mg/kg) but has a slightly lower content compared with Saha et al. (2021; 401 mg/kg) and Visentin et al. (2016; 399.18 mg/kg): given the tight link between higher Na and increasing presence of mastitis at herd level (Harmon, 1994), this result highlighted the high welfare conditions of the investigated population. In regard to trace elements and contaminants, we observed some deviation with respect to previous studies, that could be ascribed to feeding, management and environmental differences between the populations analyzed in other studies. Specifically, Cu and Fe contents are much lower compared with the

study of Denholm et al. (2019; 0.059 mg/kg vs. 0.121 mg/kg and 0.319 mg/kg vs. 1.08 mg/kg, respectively). If Cu is known to be affected by individual animal factors and feeding, Fe is highly resistant to changes in its amount in cow's diet (Murthy et al., 1972), and therefore its variability might be related to individual animal factors. The B (0.233 mg/kg) has lower contents than the ones reported by Saha et al. (2021; 0.393 mg/kg) and Stocco et al. (2019; 0.301 mg/kg). A lower Sr content (0.377 mg/kg) was observed in Stocco et al. (2019; 0.428 mg/kg).

Effect of DIM, Parity, and Herd on Detailed Milk Mineral Profile

Previous studies have explored the main sources of variation of milk mineral profile (Stocco et al., 2019), and the reported least squares means patterns of DIM and parity, as well as the effect of herd-date were similar to those of the current study. In this study we wanted to assess the relevance of these factors on milk minerals in the investigated population to better appreciate the association to SCS and DSCC.

Herd effect, which includes several management factors (i.e., diet, water, soil, equipment), had a great influence on milk minerals content (up to 67%, expressed as incidence on the total phenotypic variation), whereas individual factors, namely parity and DIM, have a lower effect (<7% and <14%, respectively, using the same way to express the relevance; Figure 1). Specifically, the highest effect of herd is evidenced in contaminants, such as Al and B (67% and 55%, respectively). This finding is expected, given that milk is susceptible to environmental contaminants and feed consumption is the most common route of contamination (Calahorrano-Moreno et al., 2022). A significant effect of diet and management is remarkable for all the macrominerals (Stocco et al., 2019); anyhow, Mg, Na and Ca seemed to be mildly influenced by herd effect (31%, 38%, and 39%, respectively), whereas K, S and P showed to be strongly influenced by herd (48%, 49%, and 59%, respectively). This is consistent with previous reports, showing that Mg, Na and Ca are more dependent from individual and genetic factors and less influenced from dietary intake (Zamberlin et al., 2012; Stocco et al., 2019), whereas K, S and P are influenced by difference in housing, especially feedstuffs, which contain an high amount of these minerals (Meyer et al., 2014) and are also associated with milk protein content (Toscano et al., 2023). Magnesium, K, Na and S are mildly influenced by DIM, as already reported (Stocco et al., 2019). An unexpected result was observed for Mn, whose content normally strictly depends on soil and plant species used for feedstuffs (Hurley and Keen,

1987) and that in our analysis showed no variability related to herd effect. We can thus hypothesize that soil and plant species were similar for Mn content in the investigated herds.

Association of SCS and DSCC with Milk Mineral Profile

Somatic cell count and DSCC showed to have an influence on milk mineral content; the results of ANOVA (model 2) are displayed in Table 2. For both SCS and DSCC, we observed that the most significant associations were the ones with Na, K, and Fe (*F*-values ranging from 5 to 29, $P < 0.001$).

The increase in SCS was positively associated with the increase in milk Na levels ($P < 0.001$; Figure 2A). An increase in SCS is widely accepted as an early sign of inflammation in the mammary gland. This condition causes an increased permeability of the blood-milk barrier promoting blood transudation of molecules into milk including Na, that is osmotically complemented by a reduction of lactose and K (Wegner and Stull, 1978; Kandeel et al., 2019). Differential somatic cell count showed an opposite behavior to the one displayed by SCS, with increasing values of DSCC that were associated with a linear decrease in Na and a linear increase in K milk concentrations ($P < 0.001$; Figure 2B and 2C, respectively). This is surprising, given that higher DSCC is generally ascribed to a worsening of udder health condition, with increasing proportions of PMN while LYM remaining constant with low proportions across the entire SCC range (Schwarz et al., 2011; Pilla et al., 2013; Damm et al., 2017), so increasing concentration of milk Na and decreasing concentrations of milk K are expected with increasing DSCC. Given that the considered population was mostly clinically healthy, as also confirmed by SCS values, the LYM proportion in the DSCC is probably predominant with respect to PMN (Schwarz et al., 2011), so the high level of DSCC is not exactly mirroring an increased inflammation but rather a different cell type distribution. These findings support the importance of discriminating between leukocyte populations within DSCC as previously evidenced (Pegolo et al., 2021a, 2022a; Bisutti et al., 2022).

An increase in SCS was also associated with higher S content ($P < 0.01$; Figure 3A). Sulfur is an essential element in dairy cow's diet, having a central role in rumen microbial synthesis of specific amino acids, vitamins, and enzymes. Specifically, serum (whey) proteins are rich in S-containing amino acids (Jo et al., 2019). The rise in milk noncasein proteins concentration (especially α -LA and, with a minor extent, immunoglobulins) during inflammatory impairment of the blood-milk perme-

Table 2. Results from ANOVA (*F*-value and significance) for milk mineral profile expressed as mg/kg milk (model 2)

Trait, mg/kg milk	Parity ¹	DIM ¹	SCS ^{1,2}	DSCC ¹	Herd-date, ³ %
Macrominerals					
Ca	0.67	2.80**	2.34	0.54	31
Mg	0.57	16.68***	2.55	0.60	27
P	3.49*	2.25*	1.58	0.41	55
K	3.29*	8.311***	1.98	5.00***	46
Na	9.75***	8.03***	29.97***	17.03***	16
S	1.17	15.09***	5.23**	0.69	46
Trace minerals					
Cu	1.45	3.11***	0.72	0.20	10
Fe	0.01	1.28	6.76***	0.23	13
Mn	0.18	8.24***	0.77	1.94	4
Zn	5.00**	4.77***	0.22	0.54	23
Contaminants					
Al	1.38	0.91	1.13	1.97	28
B	1.36	2.95***	0.92	0.85	56
Ba	1.66	4.40***	0.82	0.47	6
Cr	0.71	1.47	0.86	0.67	26
Sr	0.26	4.48***	2.39	1.89	21
Ti	2.99*	2.19*	3.60*	1.19	11

¹Parity, DIM, SCS, and differential SCC (DSCC) are divided in classes.

²SCS = $\log_2(\text{SCC}/100,000) + 3$.

³Herd-date effect expressed as proportion of variance explained by herd/test date and calculated by dividing the corresponding variance component by the total variance.

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

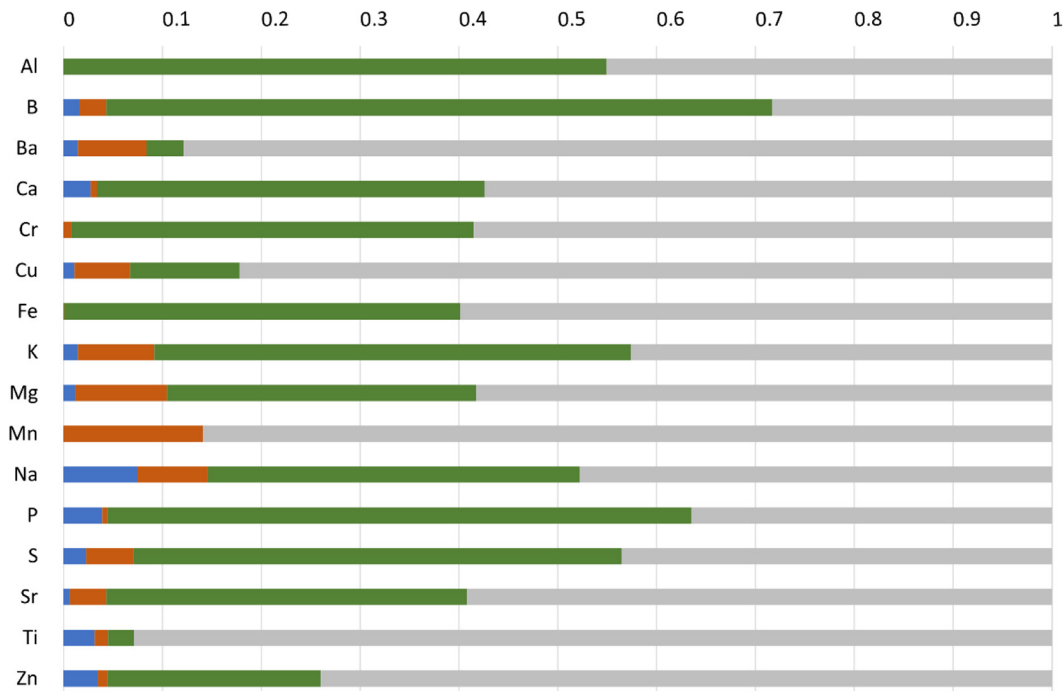


Figure 1. Individual sources of the variation (expressed as percentage of total variance) of the detailed milk mineral profile (mg/kg milk). The blue color refers to parity effect (expressed in classes), the orange to DIM effect (expressed in classes), the green to herd effect, and the gray to the residual.

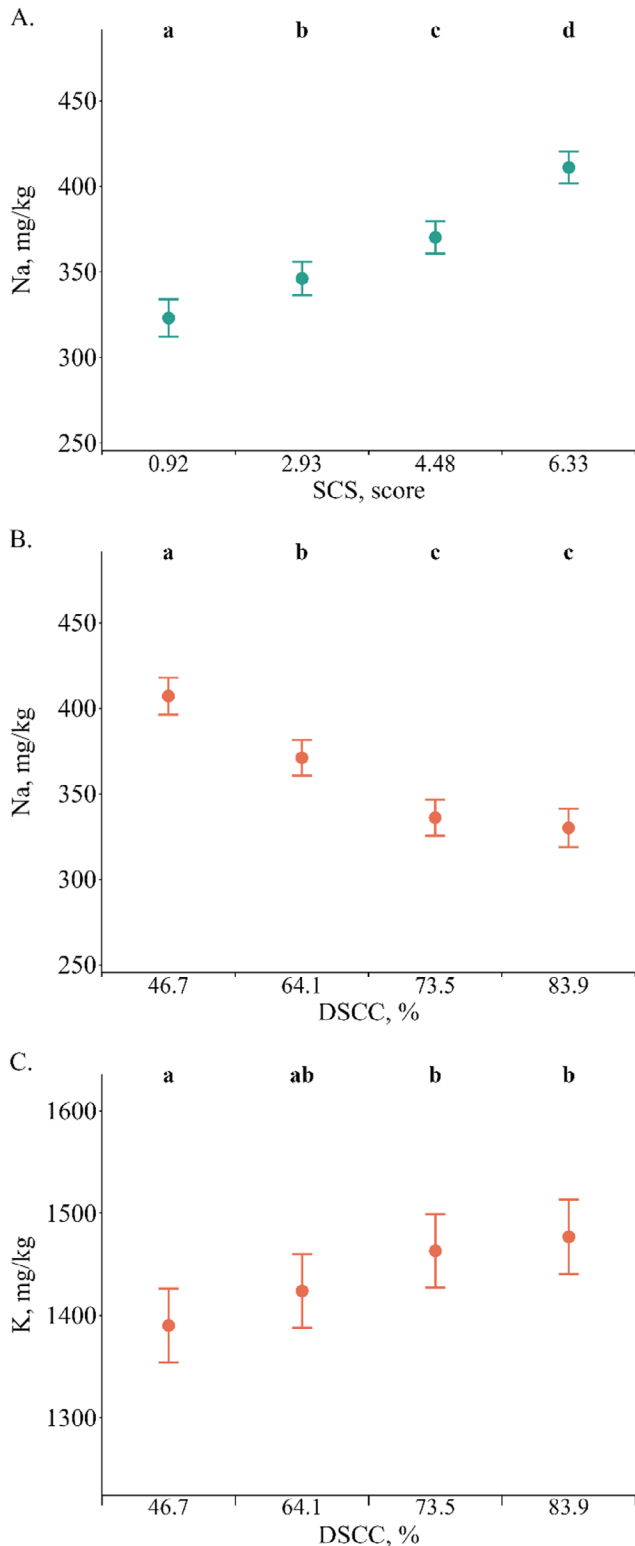


Figure 2. Least squares means and SE of Na (A, B) and K (C) milk content across SCS classes and differential SCC (DSCC) classes. Values on the x-axis refer to the average value for each class of SCS (expressed as $\log_2 [\text{SCC}/100,000] + 3$) and DSCC (expressed as percentage). Different lowercase letters (a-d) indicate significant differences among SCS or DSCC classes.

ability barrier and consequently with increasing levels of milk SCS has been repeatedly reported (Ishikawa et al., 1982; Urech et al., 1999; Bisutti et al., 2022). Hence, the positive association between SCS and S milk content may be related to the progressive increase in whey proteins with the onset of inflammatory condition, in particular of α -LA that contains a high amount of cysteine/cysteine and immunoglobulins, that have high content of S-containing amino acids per monomeric protein and intramolecular disulfide bonds (Jo et al., 2019).

When considering trace minerals, increasing level of SCS (above 3.65) was associated with a raise in milk Fe and Ti concentrations, with Fe having an almost linear trend, whereas Ti having a curvilinear pattern, decreasing mildly until 3.65 (nonsignificant) and then increasing with the highest class of SCS ($P < 0.01$; Figures 3B and 3C, respectively). Controversial reports have been provided in evaluating milk Fe content in mastitic versus healthy cows: Hussain et al. (2013) observed a decrease in Fe content during mastitis, whereas Yildiz and Kaygusuzoglu (2005) observed an increase of Fe content in mastitic samples. In both studies California mastitis test was performed as diagnostic test, therefore considering the SCS as indication of disease, but different clinical types of mastitis have been considered. However, with the increase of SCS it may be hypothesized a transudation of trace elements (e.g., Fe, Ti) from bloodstream to milk, as a result of inflammation-induced increase in vascular permeability (Yildiz and Kaygusuzoglu, 2005).

Different Milk Leucocyte Populations Showed Opposite Associations with Milk Minerals

Considering that the diverse somatic cell types exert different biological activity (Halasa and Kirkeby, 2020) and the DSCC gives an information about their proportion, we built up this analysis to evaluate if also the amount of DSCC gives insights on biological population patterns. The associations between DSCC expressed as a count (i.e., PMN-LYM and MAC counts) and milk minerals (model 3) are shown in Table 3. The phenotypic correlation between them is less than 1 ($r = 0.57$) and this justifies the statistical analysis which aimed to appreciate the differential effects of PMN-LYM and MAC in a quantitative way.

For both PMN-LYM and MAC counts, we observed that the most significant associations were the ones with Na and K (F -value ranging from 5 to 32 and $P < 0.01$). Sodium and K showed contrasting behavior when associated with PMN-LYM or MAC counts. On one hand, the patterns evidenced by PMN-LYM count mirrored the trends of DSCC expressed as proportion

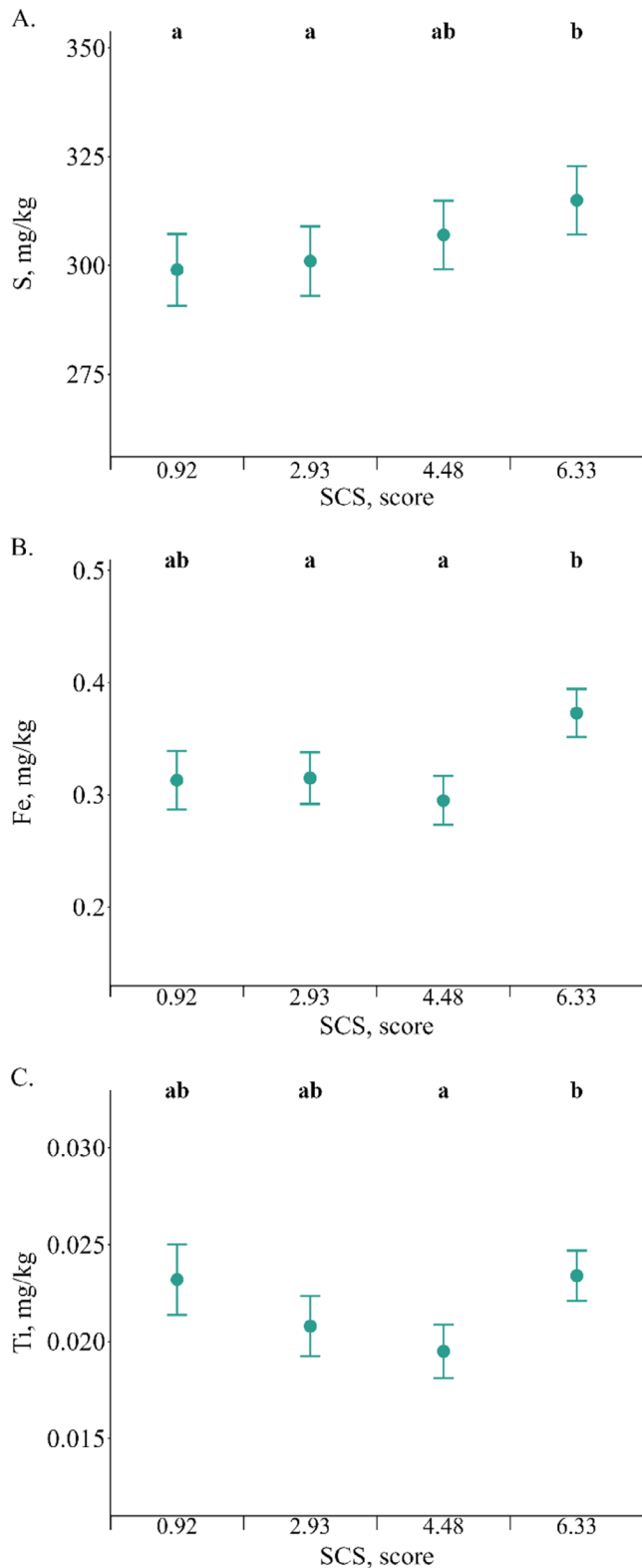


Figure 3. Least squares means and SE of S (A), Fe (B), and Ti (C) milk content across SCS classes. Values on the x-axis refer to the average value for each class of SCS (expressed as \log_2 [SCC/100,000] + 3). Different lowercase letters (a, b) indicate significant differences among SCS classes.

as the increase of PMN-LYM count (>3.75 , log count) is associated with a sharp decrease in milk Na content ($P < 0.001$; Figure 4A), and a linear increase in K milk concentrations ($P < 0.05$ Figure 4C). In contrast, the increase in MAC count is associated with a linear increase in Na milk concentrations ($P < 0.001$; Figure 4B), and a decrease of K milk content, significant in the last class (>2.37 , log count; $P < 0.01$; Figure 4D). To correctly interpret these results, it has to be taken into account that, when a healthy mammary gland is considered, the somatic cell population is mainly constituted by LYM and MAC, with only a small fraction of PMN (Schwarz et al., 2011). With the onset of inflammation in the udder, the distribution of milk leukocyte populations markedly fluctuates, even if their pattern has not been well defined yet. In fact, there have been studies showing a shift in MAC and PMN, and rather unchanged LYM (Damm et al., 2017; Stocco et al., 2020; Silva et al., 2021), whereas others highlighted a sharp alteration in LYM and PMN proportion, while MAC remained fairly constant (Schwarz et al., 2011; Pilla et al., 2013). Furthermore, the different phases of inflammation and the type of pathogen involved are critical components in the predominance of one over another immune cell type (Bruckmaier and Wellnitz, 2017; Pegolo et al., 2022b). Here we evidenced, as already reported in previous studies (Pegolo et al., 2021a, 2022a; Bisutti et al., 2022), that the trend of MAC expressed as count seemed to better mirror the inflammatory onset, also mimicking the patterns of SCS. Macrophages represent the sentinels of the mammary gland to invading pathogens (Sarıkaya et al., 2006). When MAC are exposed to inflammatory stimuli, they release cytokines such as tumor necrosis factor α , interleukin 1β , and 6 (Arango Duque and Descoteaux, 2014) which, in addition to inducing the rapid influx of PMN from bloodstream, contribute to the increase of the blood-milk barrier permeability, a crucial process to contrast invading pathogens (Paape et al., 2002; Wellnitz and Bruckmaier, 2021). Thus, a permeable blood-milk barrier triggers changes in milk composition including those of mineral elements, as described above, and could explain the positive association between MAC count and milk Na content and the inverse association with K milk concentrations that we observed. Conversely, PMN-LYM count patterns of association with Na and K milk content seemed to represent more the trend of LYM cells in milk, likely because the investigated population is constituted mainly by clinically healthy cows where in the SCC distribution LYM predominate over PMN (Schwarz et al., 2011).

Among the other major milk minerals, the increase in MAC count is associated with an increase in S ($P < 0.05$; Figure 5A), mimicking the trend of SCS. Again,

Table 3. Results from ANOVA (*F*-value and significance) for milk mineral profile expressed as mg/kg milk (model 3)

Trait, mg/kg milk	Parity ¹	DIM ¹	logPMN-LYM ^{1,2}	logMAC ^{1,3}	Herd-date, ⁴ %
Macrominerals					
Ca	0.61	1.28	0.80	0.93	30
Mg	0.33	7.63***	2.42	2.10	24
P	2.32	1.53	0.16	0.54	54
K	3.39*	5.99***	3.40*	5.02**	44
Na	6.25***	4.11***	10.99***	32.7***	11
S	1.01	6.74***	1.16	2.85*	44
Trace minerals					
Cu	0.96	1.80	1.32	1.43	11
Fe	0.70	1.45	3.34*	0.70	17
Mn	0.02	3.42***	0.95	1.21	1
Zn	3.70*	2.32**	0.27	0.59	21
Contaminants					
Al	1.43	0.60	0.41	0.33	33
B	1.00	1.73	1.06	0.84	55
Ba	1.57	3.20***	1.45	0.33	4
Cr	1.08	0.93	0.77	1.01	19
Sr	0.34	3.64***	2.69*	0.24	21
Ti	0.97	2.24*	0.37	1.69	6

¹Parity, DIM, logPMN-LYM, and logMAC are divided in classes.

²logPMN-LYM = polymorphonuclear neutrophils-lymphocytes count expressed as $\log_2 [(DSCC \times SCC)/100,000] + 3$.

³logMAC = macrophages count expressed as $\log_2 \{[(100-DSCC) \times SCC]/100,000\} + 3$.

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

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

the MAC increase in milk can be associated with an impaired permeability (Arango Duque and Descoteaux, 2014) with an increase of whey proteins with a high content of S-containing amino acids (Jo et al., 2019).

As regards trace minerals, the increase in PMN-LYM count is associated with higher Fe content ($P < 0.05$; Figure 5B). Iron has an essential role in immune processes, both innate and adaptive (Kuźmicka et al., 2022). Specifically, PMN are key players in the so-called nutritional immunity and compete intensively for Fe with bacterial pathogens during infections, preventing the release of Fe from their stores through the production of hepcidin (Peyssonnaud et al., 2006). In addition, PMN have a high Fe content compared with other immune cells (Niemiec et al., 2015). Finally, the peroxidase-catalyzed halogenation of myeloperoxidase, an enzyme present in azurophilic granules of PMN that produces hypohalous acids, is Fe dependent (Arnhold et al., 2003). As regards to adaptive immunity, Fe promotes activation and proliferation of LYM (Cronin et al., 2019). In this context, a positive association with milk Fe content was expected, regardless of the predominance of PMN or LYM in the PMN-LYM count.

Considering contaminants, MAC count was associated with an increase in Sr content in milk, highlighting a quadratic pattern ($P < 0.05$; Figure 5C). Information about Sr content in milk and its association with udder health is scarce, nevertheless it can be hypothesized

that as Sr is a contaminant MAC may have a role in its clearance. Further studies are needed to explore the relationship between Sr and MAC population in milk.

The Contribution of Milk Minerals for Udder Health Monitoring at the Field Level

In the view of early monitoring and early detection of udder impairments, combining the information given by SCS, DSCC, mineral element variations, and also other biomarkers (i.e., electrical conductivity, lactate dehydrogenase, acute phase proteins) is attractive. Kamphuis et al. (2008) explored this possibility in automatic milking systems, combining the potential of an in-line sensor able to estimate SCC with an electrical conductivity sensor, that indirectly evaluates ion variations in milk. Their results showed increasing performance in identifying clinical mastitis with the data integration of both inputs, underpinning the relevance of combining more indicators to early identify udder impairments. In the same line, recent studies highlighted that, using a combined SCC and lactate dehydrogenase activity, it is possible to differentiate mastitis from gram-positive and gram-negative bacteria (Hernández-Castellano et al., 2017; Khatun et al., 2019). Indeed, different pathogens might induce diverse leukocyte responses (Pegolo et al., 2022b). Our results suggested that milk minerals have a different behavior when associated with different

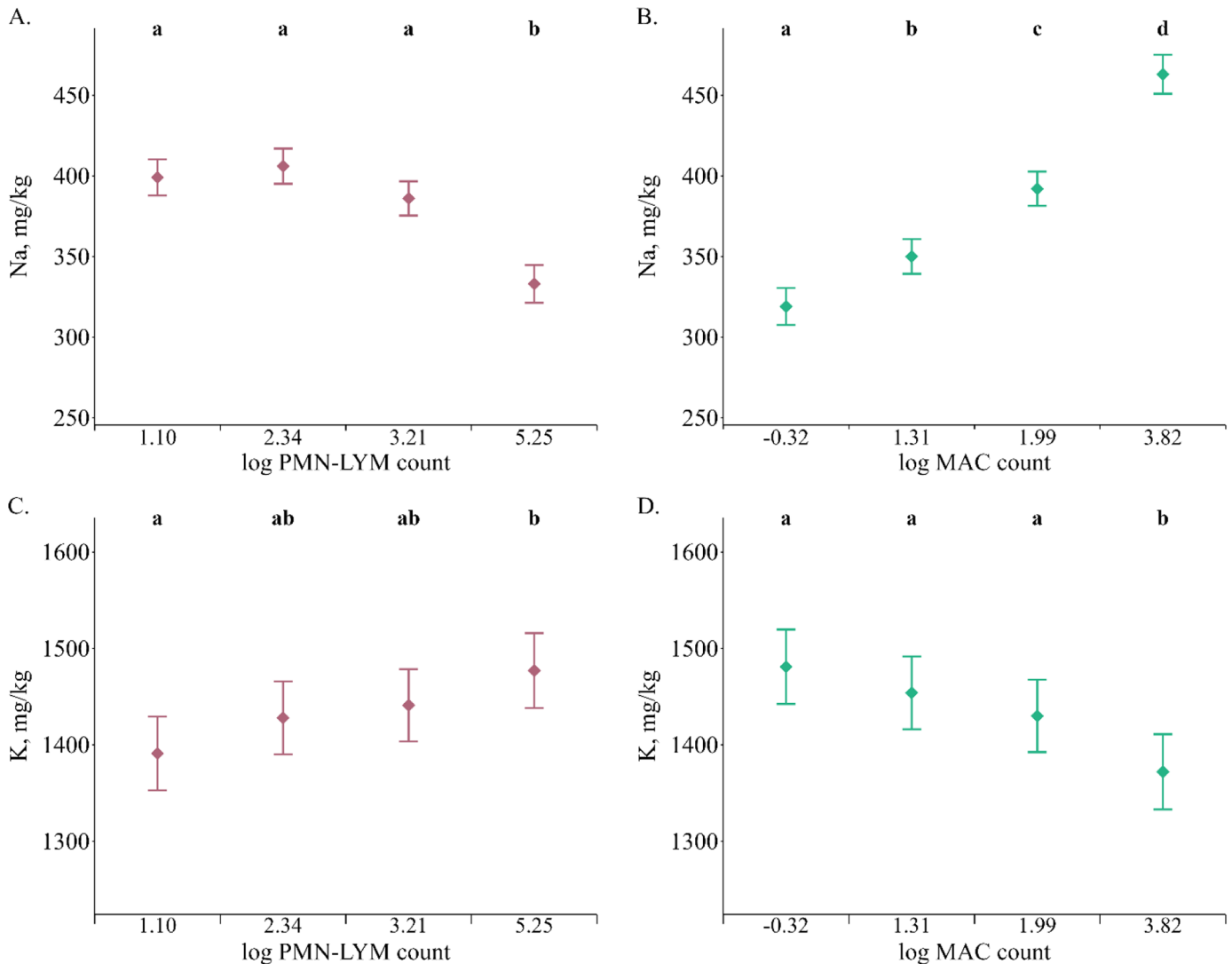


Figure 4. Least squares means and SE of Na (A, B) and K (C, D) across polymorphonuclear neutrophil and lymphocyte (PMN-LYM) and macrophage (MAC) classes. Values on the x-axis refer to the average value for each class of PMN-LYM (expressed as \log_2 [PMN-LYM count/100,000] + 3) and MAC (expressed as \log_2 [MAC count/100,000] + 3). Different lowercase letters (a–d) indicate significant differences among PMN-LYM or MAC classes.

immune cell populations, paving the way for their potential use as indicators of specific immune responses. Nevertheless, the use of milk minerals as markers for specific leukocyte populations can only be achieved through the application of noninvasive sensing equipment, such as infrared spectroscopy, which would accurately predict milk minerals at the herd level.

CONCLUSIONS

Our study highlighted that some milk mineral elements are associated with SCS and DSCC and behave differently in association with diverse immune cell populations. The MAC count seemed to mirror SCS, being

associated with an increase in Na and a decrease in K milk contents. Milk S content increased in association with the increase in SCS and MAC count, likely due to its relationship with milk whey proteins. Milk Fe content showed positive associations with the PMN-LYM population, emphasizing its role in immune regulation during inflammation. Finally, the PMN-LYM count exhibited associations with milk Na and K contents, attributable more to LYM than to PMN, given the non-pathological condition of the animals in this study. For a more accurate evaluation of the associations between milk mineral profile and DSCC, it would be necessary to examine PMN and LYM as separate populations and not combined, through methods such as flow cytometry.

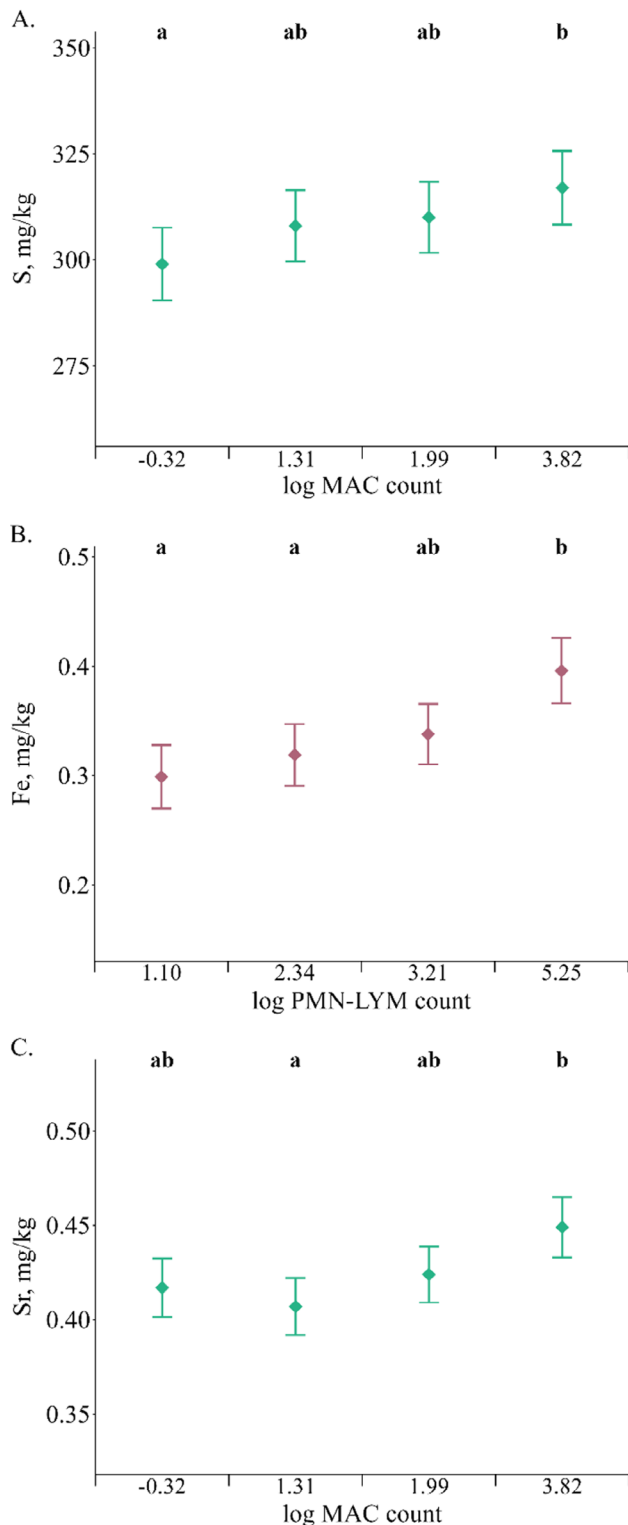


Figure 5. Least squares means and SE of S (A), Fe (B), and Sr (C) across polymorphonuclear neutrophil and lymphocyte (PMN-LYM) and macrophage (MAC) classes. Values on the x-axis refer to the average value for each class of PMN-LYM (expressed as \log_2 [PMN-LYM count/100,000] + 3) and MAC (expressed as \log_2 [MAC count/100,000] + 3). Different lowercase letters (a, b) indicate significant differences among PMN-LYM or MAC classes.

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




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