J. Dairy Sci. 106:6577–6591
https://doi.org/10.3168/jds.2022-23161

Associations between the detailed milk mineral profile, milk composition, and metabolic status in Holstein cows

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ABSTRACT

The causes of variation in the milk mineral profile of dairy cattle during the first phase of lactation were studied under the hypothesis that the milk mineral profile partially reflects the animals' metabolic status. Correlations between the minerals and the main milk constituents (i.e., protein, fat, and lactose percentages), and their associations with the cows' metabolic status indicators were explored. The metabolic status indicators (MET) that we used were blood energy-protein metabolites [nonesterified fatty acids, β-hydroxybutyrate (BHB), glucose, cholesterol, creatinine, and urea], and liver ultrasound measurements (predicted triacylglycerol liver content, portal vein area, portal vein diameter and liver depth). Milk and blood samples, and ultrasound measurements were taken from 295 Holstein cows belonging to 2 herds and in the first 120 d in milk (DIM). Milk mineral contents were determined by ICP-OES; these were considered the response variable and analyzed through a mixed model which included DIM, parity, milk yield, and MET as fixed effects, and the herd/date as a random effect. The MET traits were divided in tertiles. The results showed that milk protein was positively associated with body condition score (BCS) and glucose, and negatively associated with BHB blood content; milk fat was positively associated with BHB content; milk lactose was positively associated with BCS; and Ca, P, K and S were the minerals with the greatest number of associations with the cows' energy indicators, particularly BCS, predicted triacylglycerol liver content, glucose, BHB and urea. We conclude that the protein, fat, lactose, and mineral contents of milk partially reflect the metabolic adaptation of cows during lactation and within 120 DIM. Variations in the milk mineral profile were consistent with changes in the major milk constituents and the metabolic status of cows.

Received December 16, 2022.
Accepted March 7, 2023.
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Key words: milk composition, milk mineral profile, negative energy balance, blood metabolites, liver ultrasound

INTRODUCTION

Studies of milk minerals have been conducted using a factorial design to estimate the mineral requirements of dairy cows (NASEM, 2021), whereas others have assessed the effects of minerals in milk on human health (Knowles et al., 2006), and evaluated the cheese-making properties of milk (Stocco et al., 2019). The nature and the distribution of macro and trace minerals in aqueous and solid milk fractions, particularly casein micelles, have been variously reviewed (Gaucheron, 2005; Šimun et al., 2012). Specifically, it has been shown that the health status and productivity of cows are improved by adjusting the dietary mineral content to maintain the cows' acid-base balance and antioxidant status during dry periods and lactation (Goff, 2018). Furthermore, milk mineral content is heritable (Buitenhuis et al., 2015), so the potentiality of varying mineral content through feeding or breeding become crucial.

Anyhow, the mineral content of milk is determined not only by genetic, nutritional, and environmental factors but also by the physiological and health status of the cow (Nogalska et al., 2020; Manuelian et al., 2022). Stocco et al. (2019) reported the effects of herd productivity, the parity and stage of lactation of cows on the mineral profile of milk. Although the mineral profile of milk has already been associated with udder health status (Summer et al., 2009; Nogalska et al., 2020), there is a lack of studies that evaluated the potential associations between milk minerals and energy metabolism.

During early lactation, the cow experiences a very high metabolic load with a rapid and massive increase in nutrient requirements that is not covered by feed intake. As a consequence, the cow enters a state of negative energy balance (**NEB**). This is manifested in a reduction in BCS and blood glucose content, an increase in circulating nonesterified fatty acids (**NEFA**)

and ketones, such as BHB, alteration to the milk fat-to-protein (**F:P**) ratio, and an increase in liver triacylglycerol content and liver dimensions (Bobe et al., 2004; Mulligan and Doherty, 2008). Individually or in combination, minerals are known to perform important biological functions, such as membrane transport and regulation of osmotic balance, and are components of energy molecules or cofactors of enzymes (NRC, 2001; Šimun et al., 2012). We therefore hypothesize that changes in the milk mineral profile and in the major milk components during the first phase of lactation reflect the metabolic status of the cow. The associations between the milk mineral profile and indicators of the metabolic status of cows have never been explored, and, in the view of enhancing mineral content in milk for both human and animal health (Denholm et al., 2019), intrinsic relationships with metabolic changes need to be established.

Finally, mineral chemical analyses are costly and time consuming (Soyeurt et al., 2009; Bonfatti et al., 2016), thus preventing the use of milk minerals for herd-scale investigations. Recent research is studying the potentiality of the infrared spectroscopy to predict milk minerals with reliable accuracy. Hence, the predictability of milk minerals combined with their potential relationship with energy metabolic status might allow their use as indicator of energy metabolism in dairy cows.

The aims of this study were (1) to evaluate the macro- and micromineral profile of the milk of 295 Holstein cows in the first 120 d of lactation; (2) to describe the correlations among minerals and the major milk constituents (i.e., protein, fat, and lactose); and (3) to explore the associations between milk minerals and the major milk components and indicators of the cows' metabolic status (i.e., BCS, F:P ratio, energy- and protein-related metabolites, and liver ultrasound measurements).

MATERIALS AND METHODS

Animal Data

This work is part of a larger project (BENELAT) aimed at developing short- and long-term interventions to improve welfare and production quality in the dairy cattle sector, as explained in detail in previous works (Pegolo et al., 2021). The research received prior approval from the Ethics Committee of the Università Cattolica del Sacro Cuore (Organismo Preposto al Benessere degli Animali) and the Italian Ministry of Health (Rome, Italy; protocol number 510/2019-PR of 19/07/2019). Each procedure was conducted in compliance with the appropriate guidelines and regulations.

For the present research we used the same cow population as that previously used by Piazza et al. (2022). Briefly, 295 lactating cows were selected on the basis of DIM (5–120 d) from 2 farms in the province of Piacenza (northwestern Italy). The clinical status of the cows was assessed by an experienced veterinarian, and individuals with overt disease (e.g., abscesses, laminitis, mastitis) were excluded. Of the selected cows, 184 were primiparous, average milk yield 34.2 kg/d, while the remaining 111 were multiparous, average milk yield 43.9 kg/d.

The cows were housed in freestalls, fed TMR, and milked twice daily. Drinking water was available in automatic water bowls. The ingredients and chemical compositions of the diets are reported in Giannuzzi et al. (2022).

Milk and Blood Collection

Two 50-mL aliquots of milk were taken once during evening milking operations. One of these was delivered to the Veneto Region Breeders' Association (ARAV) to determine milk composition, while the other was taken to the laboratories of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padua (Legnaro, Padua, Italy) to determine the milk mineral profile.

Concurrent or close in time to milk sampling and before meal distribution, blood samples (5 mL, 2 or more samplings per farm, depending on size; 15 herd/date) were collected from the jugular vein in vacuum tubes containing 150 units of lithium heparin UPS (Vacumed; FL Medical, Torreglia, Padua, Italy).

Metabolic Indicators

Regarding determination of the metabolic indicators, the acquisition of ultrasound liver measurements is described in detail in Giannuzzi et al. (2021), and the analysis of blood metabolites in Mezzetti et al. (2019). The procedures that were followed are briefly outlined below.

Ultrasound Measurements. Ultrasound scans were performed by a single experienced veterinarian using a Mylab OneVET portable ultrasound scanner (Esaote SpA,) connected to a linear probe (Animal Science Probe, SV3L11; Esaote SpA). Images were taken from the right side of the animal in a standing position at the 10th intercostal space, after the skin had been degreased with 70% alcohol, washed with water and coated with ultrasound gel. Cows with hepatic alterations that were not attributable to metabolic alterations (i.e., abscesses and neoplastic masses) were excluded. Multiple images were taken and stored, and the best one was selected

by a single experienced operator. From these final images the following measurements were taken using the MyLab Desk software (Esaote SpA): liver depth (**LD**, mm), portal vein area (**PVA**, mm^2), and portal vein depth (**PVD**, mm). The texture analysis software MaZda v.4.6 (Technical University of Lodz, Institute of Electronics, Poland) was used to estimate the liver triacylglycerol content (**pTAG**, mg/g), as described by Banzato et al. (2016).

Blood Metabolites. Blood samples were kept on ice until centrifugation (3,500 × *g*, 16 min, 6°C) within 2 h of collection. The ILAB-650 clinical auto analyzer (Instrumentation Laboratory) was used to determine the glucose, cholesterol, urea, creatinine, NEFA, and BHB contents. In addition, a portion of each sample was used for hematocrit determination with an ALC Centrifugette 4203 (15,300 × *g*, 12 min).

Body Condition Score. A single expert operator assigned all the cows a BCS on a 5-point scale from very thin (1) to very fat (5), as described in Edmonson et al. (1989).

Milk Mineral Contents

The procedure is described in detail in Stocco et al. (2019), and the 2 steps are briefly outlined below.

Treatment of Samples. Milk samples were mineralized using a Milestone Start D microwave digestion system (Milestone S.r.l.), microwave power 1,200 W, equipped with an SK10 high-pressure rotor (100 bar). Subsamples of 2 to 2.5 g were taken from each milk sample and placed in modified polytetrafluoroethylene containers, to which were added 7 mL of 67% nitric acid and 2 mL of 30% hydrogen peroxide, both Suprapur quality (Merck Chemicals GmbH). The subsamples were heated from 25 to 200°C in 18 min, maintained at 200°C for 15 min, then cooled to 35°C. The resulting mineralized material was made up to volume (25 mL) with ultrapure water, and the mineral content determined by ICP-OES.

Mineral Determination. A Spectro Arcos EOP ICP-OES (SPECTRO Analytical Instruments GmbH) was used to determine 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). Those elements present in quantities below the instrument's detection limit (Ag, As, Be, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sb, Se, Si, Sn, Ti, Tl, V) were excluded. Multi-element and single-element standard solutions (Inorganic Ventures Inc.) in 10% Suprapur nitric acid (Merck Chemicals GmbH) were used to calibrate the instrument. Using the same method as described above, certified reference material BCR-063 "skim milk powder" (Institute for Reference Materials and Measurements) was prepared to determine 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 in more than 75% of the samples were excluded from the analysis. Those minerals with values below the LOD in less than 25% of the samples, to the values below the LOD half the LOD were assigned.

Iodine Determination. Iodine content was determined with an ICP-MS analyzer (Spectro MS, Spectro Analytical Instruments GmbH) by Eurolab s.r.l (Vicenza, Italy). The samples were freeze-dried before extraction. The preparation used was a variation of that proposed by Landon et al. (2017). To 100 mg of the lyophilizate was added 1 mL of H_2O at 37°C and 24 mL of 2% concentrated NH_4OH. The resulting solution was placed in a thermostatic bath at 90°C for 1 h, after which it was passed through 0.45-μm filters (GVS North America). Lastly, 5 mL of filtrate and 5 mL of 2% NH_4OH were aliquoted into a 15-mL tube.

Statistical Analysis

Data Editing. Samples with SCC ≥500,000 were excluded from the statistical analysis. Minerals in concentrations outside ± 3 standard deviations of the mean of the residual values calculated with the linear mixed model described below were deemed outliers and excluded from the final data set. Descriptive statistics were run to characterize the milk samples in terms of means and variability.

Pearson Correlations Among Major Milk Components and Milk Minerals. Pearson correlations were calculated between the minerals and the major constituents of milk in the R environment (https://www.R-project.org/) with the level of significance set at $P < 0.05$.

Mixed Model Analysis. We used the PROC MIXED procedure of SAS (SAS Institute Inc.) to investigate the associations between the major components (i.e., protein, fat, and lactose) and minerals of the milk and the cows' indicators of metabolic status (**MET**), except for milk protein and fat with F:P ratio. To avoid multicollinearity among the explanatory variables (i.e., MET), these were included in the statistical model one at the time. In addition, all predictors were considered class variables using the classification specified in the following paragraph.

The operational model was as follows:

$$y_{ijklmn} = \mu + DIM_i + parity_j + milkyield_k + MET_l + herd/date_m + e_{ijklmn},$$

where y_{ijklmn} is the investigated trait (milk constituent or mineral); μ is the overall mean; DIM_i is the fixed effect of the ith class of days in milk ($i = 4$ classes; class $1 \leq 30$, n = 44; $30 <$ class $2 \leq 60$, n = 83; $60 <$ class $3 \leq 90$, n = 76; class $4 > 90$, n = 92); $parity_j$ is the fixed effect of the jth parity ($j = 2$ classes; primiparous, n = 184; multiparous, n = 111); $milkyield_k$ is the fixed effect of the kth class of milk yield ($k = 4$ classes according to the 25th, 50th, and 75th percentiles); MET_l is the fixed effect of the lth class of the metabolic indicators discretized on the basis of tertiles (or classes for BCS); $herd/date_m$ is the random effect of the mth herd/date ($m = 1$ to 15); e_{ijklmn} is the random residual. Both random effect and residual were assumed to be normally distributed.

The effects were considered significant at $P < 0.05$. Linear and quadratic contrasts ($P < 0.05$) were run to evaluate the pattern of variation of the milk constituents and minerals against that of the MET indicators.

RESULTS

Major Milk Components and Milk Mineral Profile

The milk yield of the cows used in the present study was in the order of 37 kg/d, and the milk contained an average 32.2 g/kg of protein, 35.6 g/kg of fat, and 49.7 g/kg of lactose. As expected, lactose content had a much lower standard deviation than the protein and fat contents (Table 1).

For each milk sample, the concentrations of 32 minerals were analyzed simultaneously. Fifteen minerals (Ca, P, Mg, K, Na, S, Cu, Fe, I, Mn, Zn, Al, B, Ba, and Sr) were present in quantities greater than the LOD in all or most of the milk samples. The other 18 minerals (Ag, As, Be, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sb, Se, Si, Sn, Ti, Tl, V) were detected in amounts lower than the LOD in most of the milk samples, and were therefore excluded from the analysis. These elements were either macrominerals (Ca, K, Mg, Na, P, S), microminerals, or trace elements (Cu, Fe, I, Mn, Zn) and, contaminants or environmental elements (Al, B, Ba, Sr). The coefficient of variation (**CV**) of the macro minerals ranged from 0.09 to 0.15, considerably less than the CV of the trace minerals, 0.18 to 0.69, and the contaminants, 0.22 to 0.73.

Table 1. Descriptive statistics of milk major constituents and mineral contents

Trait	n	Mean	SD	CV
Milk yield, kg/d	289	37.6	8.26	0.22
Protein, g/kg	289	32.2	4.11	0.13
Fat, g/kg	289	35.6	7.18	0.20
Lactose, g/kg	284	49.7	1.85	0.04
Macromineral, mg/kg				
Ca	261	1,143	142	0.12
P	261	921	117	0.13
S	262	285	34.3	0.12
Mg	262	91.0	11.6	0.13
K	261	1,463	137	0.09
Na	259	299	46.2	0.15
Trace mineral, mg/kg				
Zn	261	3.960	0.694	0.18
Fe	258	0.280	0.135	0.48
Cu	221	0.084	0.058	0.69
Mn	260	0.018	0.009	0.50
I	245	0.249	0.106	0.43
Contaminant, mg/kg				
Al	256	0.086	0.063	0.73
Sr	262	0.460	0.101	0.22
B	259	0.273	0.090	0.33
Ba	260	0.045	0.027	0.60

Indicators of Cows' Energy-Protein Balance

Descriptive statistics for the metabolic status indicators are given in Table 2. The BCS of the cows in our population ranged from 2.84 to 3.38, and the milk F:P ratio from 0.88 to 1.34. The average pTAG content was 58.2 mg/g in the first tertile, 69.0 in the second, and 81.1 in the third, with an overall mean of 69.3 mg/g, and a CV of 0.15. PVD averaged 130 mm, LD 149 mm with a CV in the order of 0.09 to 0.1, whereas PVA averaged 1,106 mm^2 with a CV in the order of 0.25.

Glucose in the blood averaged 4.35 mmol/L, ranging from 3.93 to 4.74 mmol/L going from the first to the third tertile, with a CV of 0.09. Cholesterol varied from 4.56 to 5.90 mmol/L, with a CV of 0.25, whereas the blood contents of NEFA and BHB were much lower, averaging 0.179 and 0.554 mmol/L respectively, with higher CV than glucose and cholesterol (1.21 and 0.47 respectively). Approximately 2% of the animals (n = 5) had NEFA concentrations greater than 0.70 mmol/L, while 4% (n = 11) had BHB concentrations greater than 1 mmol/L. The BHB of 39% (n = 115) of the animals was in the range of 0.5 to 1 mmol/L. Only 2 animals had high levels of both NEFA and BHB. Creatinine content ranging from 76.1 to 87.9 μmol/L with a CV of 0.07. Average blood and urea content was 6.28 mmol/L, with a CV in the order of 0.17.

Pearson Correlations Between Major Milk Components and Mineral Profile

The correlation map is depicted in Figure 1. Milk protein was strongly and positively correlated with the milk contents of S, P, Ca, Mg, and Zn ($P < 0.001$), Cu and Fe ($P < 0.010$), and I ($P < 0.05$). Milk fat was positively correlated with milk protein ($P < 0.001$), Ca, P, Mg, Zn ($P < 0.001$), S, and Sr ($P < 0.010$). In contrast, milk lactose was correlated negatively with

Table 2. Descriptive statistics of explanatory variables indicators of energy status

Trait[1]	n	Means				SD	CV
		Overall	1st tertile	2nd tertile	3rd tertile		
F:P ratio	284	1.11	0.882	1.10	1.34	0.224	0.20
BCS, classes	280	3.10	2.84	3.13	3.38	0.223	0.07
Energy/protein-related metabolite							
Glucose, mmol/L	257	4.35	3.93	4.39	4.74	0.38	0.09
Cholesterol, mmol/L	257	4.56	3.25	4.55	5.90	1.21	0.27
NEFA, mmol/L	257	0.179	0.066	0.115	0.353	0.217	1.21
BHB, mmol/L	257	0.554	0.366	0.503	0.793	0.259	0.47
Creatinine, μmol/L	257	81.7	76.1	80.9	87.9	6.25	0.07
Urea, mmol/L	257	6.28	5.14	6.28	7.42	1.05	0.17
Ultrasound liver trait							
pTAG, mg/g	289	69.3	58.2	69.0	81.1	10.6	0.15
PVA, mm^2	285	1,106	807	1,098	1,419	282	0.25
PVD, mm	289	130	116	129	144	13.1	0.10
LD, mm	279	149	134	149	162	13.2	0.09

[1]F:P ratio = milk fat-to-protein ratio; BCS is scored on a 5-point scale; NEFA = nonesterified fatty acids; pTAG = predicted liver triacylglycerol; PVA = portal vein area; PVD = portal vein depth; LD = liver depth.

Na ($P < 0.001$), I ($P < 0.010$), and K ($P < 0.050$), and positively with P, S, and Mn ($P < 0.001$).

Many minerals were found to be correlated with each other. For example, Ca was significantly and positively correlated with P, S, Mg, Zn, Sr, and K ($P < 0.001$), and Mg was correlated with S, Zn, P, Sr, Na, Ca, Ba, and B ($P < 0.001$). Iodine, Al, and Cr had few correlations with the other minerals.

Associations Between the Major Milk Constituents and Minerals in Milk and Cows' Metabolic Status Indicators

The associations between the minerals and the MET indicators are reported in Figure 2 and Table 3. Milk protein was associated positively and linearly with BCS ($P < 0.001$, Figure 2a) and blood glucose ($P = 0.009$, Figure 2a), and negatively with BHB ($P = 0.014$, Figure 2a). Milk fat was positively associated with BHB ($P = 0.022$, Figure 2b). Milk lactose was associated negatively with F:P ratio ($P = 0.023$, Figure 2c), positively with BCS ($P = 0.014$, Figure 2c) and showed a quadratic trend with NEFA ($P = 0.023$, Supplemental Figure S1b; https://doi.org/10.6084/m9.figshare.22323694.v1; Giannuzzi, 2023). None of the major milk constituents was associated with the ultrasound liver measurements except for the quadratic association between milk protein and PVD ($P = 0.043$, Supplemental Figure S1a).

The associations between the minerals and the MET indicators are reported in Figures 3 and 4 and Table 4, 5, and 6. Calcium was associated positively with BCS ($P = 0.013$, Figure 3a), blood urea ($P = 0.009$, Figure 3a), and the F:P ratio ($P = 0.007$, Figure 3a), and negatively with pTAG ($P = 0.049$, Figure 3a). Phosphorus was associated positively with BCS ($P < 0.001$, Figure 3b), glucose ($P = 0.046$, Figure 3b), and urea ($P = 0.022$, Figure 3b), and negatively with BHB ($P = 0.048$, Figure 3b). Sulfur was associated positively with BCS ($P = 0.003$, Figure 3c), and negatively with pTAG ($P = 0.043$, Figure 3c) and BHB ($P = 0.004$, Figure 3c).

Potassium and I were negatively associated with BHB ($P = 0.048$ and $P = 0.033$, Figure 4a and Figure 4c, respectively), and K was also positively associated with the blood urea content ($P = 0.009$, Figure 4a). Both Na and Mg were positively associated with the milk F:P ratio ($P = 0.043$ and $P = 0.048$, Figure 4b and Figure 4d, respectively). Iron was positively associated with creatinine content ($P = 0.039$, Figure 4e).

Several quadratic associations between the minerals and the various indicators of the cows' metabolic status were found, and the least squares means of these associations are shown in Supplemental Figure S1. Overall, the target mineral had the maximum concentration at the intermediate value of the various indicators considered, except for the associations of Mn with LD, and Zn with cholesterol.

DISCUSSION

Detailed Milk Mineral Profile

The milk mineral profile is frequently assumed to be relatively constant across animals (Gaucheron, 2005), yet in the present study we found a notable variation in the concentrations of the various minerals. This agrees with van Hulzen et al. (2009), who reported similar coefficients of variation.

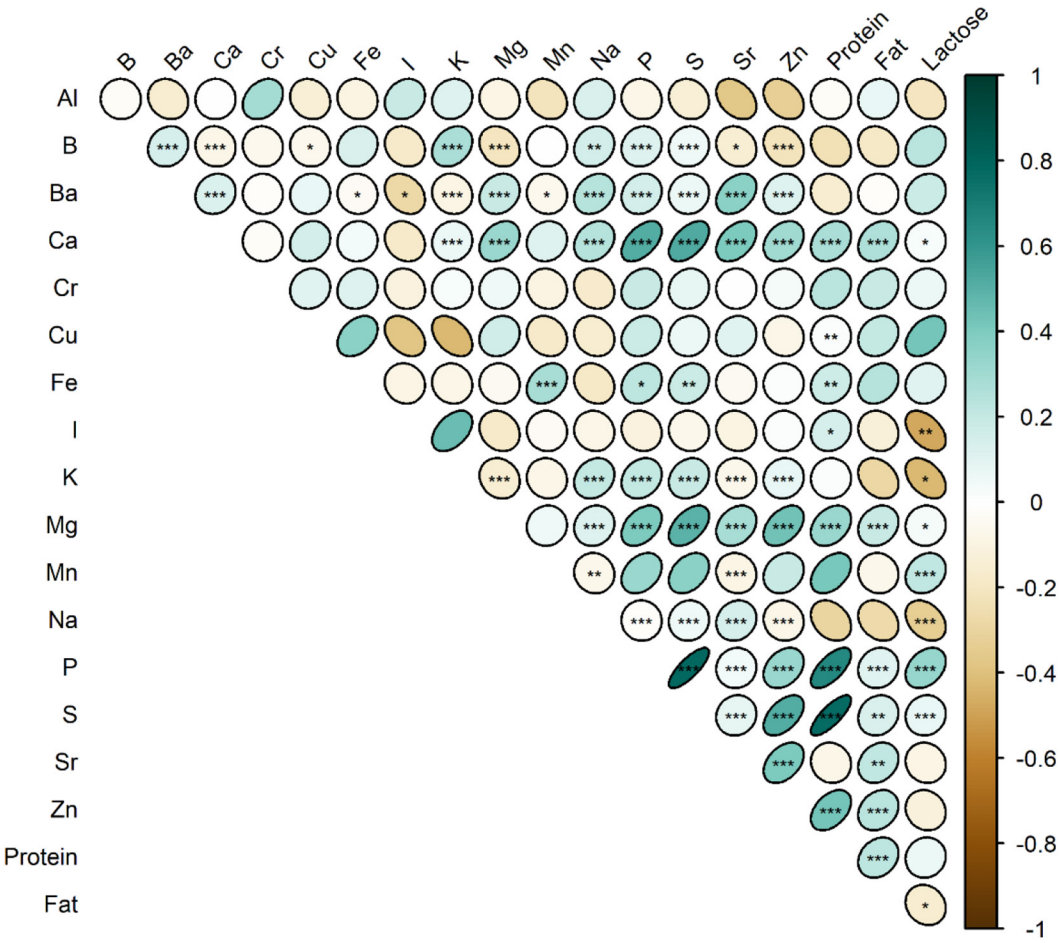

Figure 1. Map of Pearson correlations between milk minerals and milk major constituents. Ellipse colors represent the strength and the direction of the correlation, −1 to 0 to 1 (brown to white to petrol blue). $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

The average milk mineral contents found in this study agree with other values reported in the literature (Stocco et al., 2019; NASEM, 2021; Saha et al., 2021), with some exceptions. Previous NRC (2001) reports set the Na concentration in milk at 630 mg/kg, whereas the most recent NASEM (2021) sets it at 410 mg/kg, yet in our research it averaged 299 mg/kg. In the large survey carried out by Stocco et al. (2019) under similar productivity conditions to those of the present study, the Na content in milk ranged from 252 to 646 mg/kg, with an average value of 334 mg/kg. Harmon (1994) found milk Na to be positively related to the incidence of mastitis, increasing as SCC increased. In fact, the Na content of milk has decreased over the past 50 yr as a result of effective mastitis prevention strategies (NASEM, 2021).

The milk Fe content in the present study was markedly lower (0.280 mg/kg) than that proposed by NASEM (2021), which is 1 mg/kg, although the current literature reports it as being very variable and often lower than this value. For instance, Stocco et al. (2019) reported Fe contents ranging from 0.131 to 0.645 mg/kg, with a mean of 0.261 mg/kg, whereas Perez et al. (2002) reported a range of 0.084 to 0.345 mg/kg, with a mean of 0.201 mg/kg. Similarly, Knowles et al. (2006) report a mean of 0.270 mg/kg for grazing cattle, with a minimum of about 0.100 and a maximum of 0.400 mg/kg. In addition, Saha et al. (2021) found an average Fe content of milk from purebred Holstein and crossbred cows equal to 0.285 mg/kg.

Pearson Correlations Among Major Milk Components and Minerals

Given that direct correlation between the mineral contents of milk and blood minerals is scarce due to the different ionization state in which they are found in the 2 matrices (Buitenhuis et al., 2015), we studied the correlations between minerals in milk and with the major components of milk to better understand the

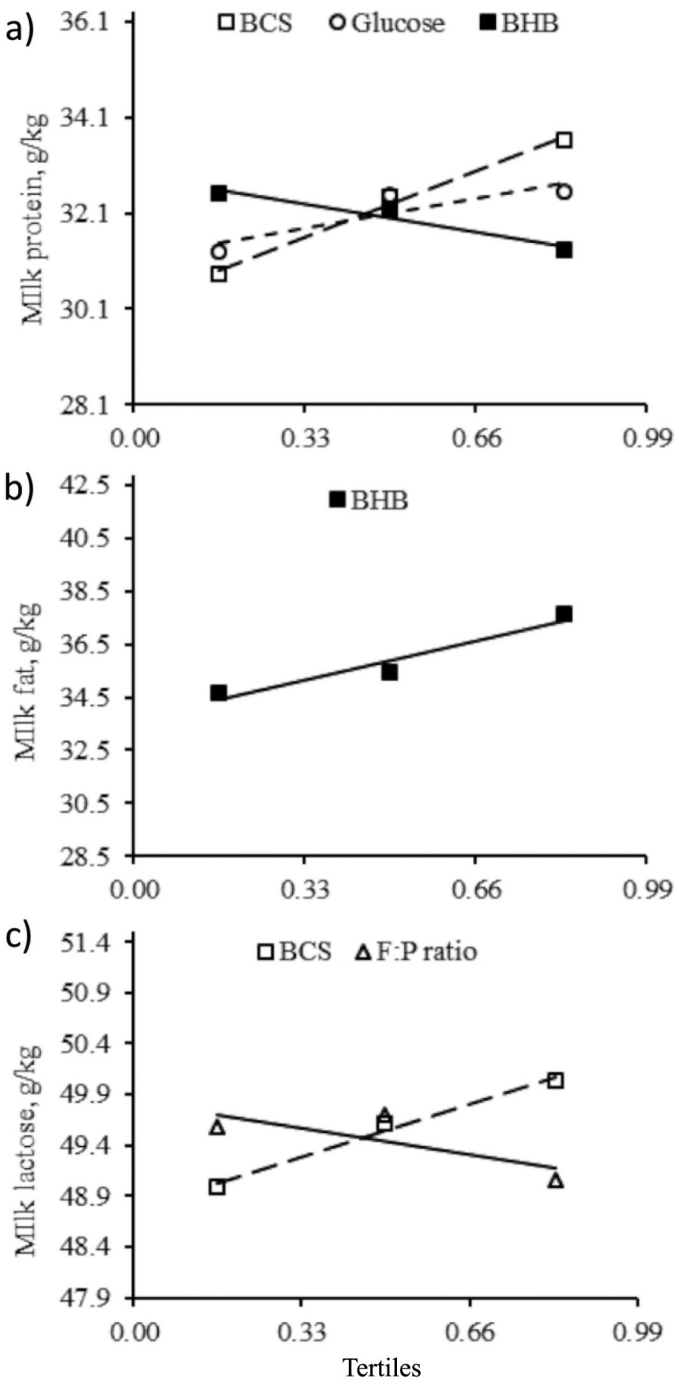

Figure 2. Least squares means of milk constituents over classes of milk fat-to-protein ratio (F:P ratio), BCS, glucose, and BHB. Explanatory variables were discretized according to tertiles, and only significant linear contrasts were given ($P < 0.05$).

mechanisms that link them. The 2 major forms of minerals in milk are the diffusible (soluble) fraction, and the nondiffusible fraction. Potassium, Na, and ions are present almost exclusively in soluble form (Gaucheron, 2005). Depending on the form in which we find these minerals, they may or may not be related to the major constituents of milk or other minerals.

Correlation Among Mineral and Major Milk Components. We found that Ca, Mg, S, Mn, and Zn, along with P, were positively correlated with the milk protein content. Sulfur was the mineral element most closely related to the milk protein content, which can be explained by the fact that S is a constitutive element of the sulfur-containing amino acids comprising milk protein (NASEM, 2021). Calcium, P, Mg, and milk protein are partially bound to casein micelles in proportions of approximately 69, 46, and 35%, respectively (Singh et al., 1989; Gaucheron, 2005; NASEM, 2021). Furthermore, as reported in previous studies (Buitenhuis et al., 2015; Stocco et al., 2021), a low content of these minerals in milk (particularly Ca) is associated with poor cheese-making attitudes. Manganese and Zn are also partially bound to the milk protein fraction, in proportions of about 67 and 50%, respectively (Singh et al., 1989; Stocco et al., 2019).

In this study, many minerals (Ca, Mg, P, S, Zn) were found to be positively correlated with milk fat. Gaucheron (2005) reported that no macromineral is bound to fat globules in significant amounts. Therefore, the correlations found in the current research can be indirectly attributed to the correlation between milk fat and milk protein.

We found K and Na to be negatively related to the lactose content and positively related to each other. This negative correlation may reflect osmotic regulation of the liquid milk phase, as described by others (Peaker, 1977; Šimun et al., 2012; Bijl et al., 2013). Moreover, previous studies have found that an elevated Na content with a low lactose content indicates inflammatory status of the mammary gland (NASEM, 2021). Iodine also had a negative correlation with lactose, in agreement with Niero et al. (2020) results, but was not correlated with K and Na, suggesting that the milk iodine concentration is regulated by other factors. Indeed, the concentration of I in milk may depend on various factors such as its presence in the diet (or conversely the presence of antagonists such as glucosinolate), farm management practices, or the presence of I in udder dipping (Denholm et al., 2019).

Correlations Between Milk Minerals. The results of this study showed numerous positive correlations between mineral elements as previously reported (Buitenhuis et al., 2015; Denholm et al., 2019). The strongest were found among Ca, Mg, P, S, and Zn. These correlations may be due to several factors, such as their binding to caseins (as described above) or absorption mechanisms (as between Ca and P, both controlled by parathyroid hormone). Weak negative correlations were found between K and Mg. This may be due to the

Table 3. Results from the mixed model [*F*-values, significance, and root mean square error (RMSE)] for milk macrominerals (mg/kg) as affected by classes of explanatory variables[1]

	Protein		Fat		Lactose	
Trait[2]	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE
F:P ratio	—	—	—	—	3.83*	1.57
BCS	13.4***	2.32	2.09	6.55	4.29*	1.55
Energy/protein-related metabolite						
Glucose, mmol/L	4.75**	2.37	0.51	6.13	1.61	1.56
Cholesterol, mmol/L	0.1	2.41	1.26	6.59	1.21	1.56
NEFA, mmol/L	2.17	2.39	2.06	6.56	3.82*	1.57
BHB, mmol/L	4.32*	2.37	3.87*	6.56	0.5	1.57
Creatinine, μmol/L	1.48	2.39	0.91	6.61	0.34	1.57
Urea, mmol/L	0.012	2.42	0.4	6.61	0.93	1.56
Ultrasound liver trait						
pTAG, mg/g	1.99	2.38	0.055	6.6	0.82	1.59
PVA, mm^2	0.032	2.41	0.63	6.61	0.15	1.6
PVD, mm	3.18*	2.39	0.29	6.62	1.03	1.59
LD, mm	0.81	2.43	0.42	6.58	0.79	1.58

[1]Model that considered DIM, parity, milk yield, and herd/date as sources of variation. DIM was constituted by 4 classes: class 1 ≤ 30; 30 < class 2 ≤ 60; 60 < class 3 ≤ 90; 90 < class 4 ≤ 120. Parity was constituted by 2 classes: primiparous and multiparous. Milk yield was constituted by 4 classes discretized according the 25th, 50th, and 75th percentiles.

[2]All explanatory variables were discretized according to tertiles, therefore 3 levels and 2 degrees of freedom are absorbed by each linear model; F:P ratio = milk fat-to-protein ratio; BCS scored on a 5-point scale; NEFA = nonesterified fatty acids; pTAG = predicted liver triacylglycerol; PVA = portal vein area; PVD = portal vein depth; LD = liver depth.

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

fact that K is able to disrupt the electrical potential required for Mg absorption (NASEM 2021). We observed from weak to modest negative correlations of B with Ca, Mg, Zn, of Ba with Fe, I, K, Mn, and of Sr with K and Mn. We can hypothesize that these correlations are due to physiological mechanisms, such as competition for the absorption site or enzymatic mechanisms. Anyhow, in some cases (e.g., Ca and Ba, or K and B) the correlation is positive and this should be considered when nutritional or managerial variations are made.

Indicators of Metabolic Status

Only a small proportion of the animals involved in this study had BHB and NEFA levels that could be attributed to subclinical ketosis (>1 mmol/L; Ospina et al., 2010). Bearing in mind that this is the most prevalent metabolic disease (Mezzetti et al., 2019), the small number of hyperketonaemic cows found in this study may be considered an indication of good farm management. With regard to improved farm management, a recent study (Lisuzzo et al., 2022) suggested lowering critical value for monitoring for hyperketonaemia, given that individuals with serum BHB levels ranging from 0.5 mmol/L to 1 mmol/L may be at potential risk of subclinical ketosis in high-welfare dairy farms. At that threshold, around 39% of animals in the investigated population would need to be monitored.

With the onset of lactation, the mammary gland's need for glucose causes an increase in gluconeogenesis and glycogenolysis in the liver, lipolysis in adipose tissue with release of NEFA into the bloodstream, and mobilization of labile protein from the muscle tissue. The increased requirements cause profound changes in lipid, protein and mineral metabolism, with the liver and adipose tissues being the major organs involved. Lipid and protein mobilization causes the cow to lose weight, with a subsequent decrease in BCS (Komaragiri and Erdman, 1997). Liver uptake of NEFA from the bloodstream is commonly followed by esterification of these fatty acids to triacylglycerol (**TAG**). However, when the inflow of NEFA exceeds the liver's metabolic capacity, TAG starts to accumulate in the hepatocytes leading to hepatic steatosis (Bobe et al., 2004) and alterations to the dimensions of the liver and connected structures (i.e., the portal vein), as described by Piazza et al. (2022). Negative energy balance and the consequent reduction in blood glucose stimulates fat mobilization, NEFA formation, and liver ketogenesis, with formation of BHB, acetoacetate and acetone (Bobe et al., 2004). The incidence of ketosis is not only a matter of NEB, it also has to do with the individual's ability to cope with metabolic adaptation during lactation (Kessel et al., 2008). The Ca requirement also increases enormously with the onset of lactation because a large amount of this mineral is released into the milk. Hypocalcemia occurs when the homeostasis of blood Ca concentration fails to provide the required amount of Ca from intestinal and renal absorption and bone mobilization and in the presence of acute or chronic

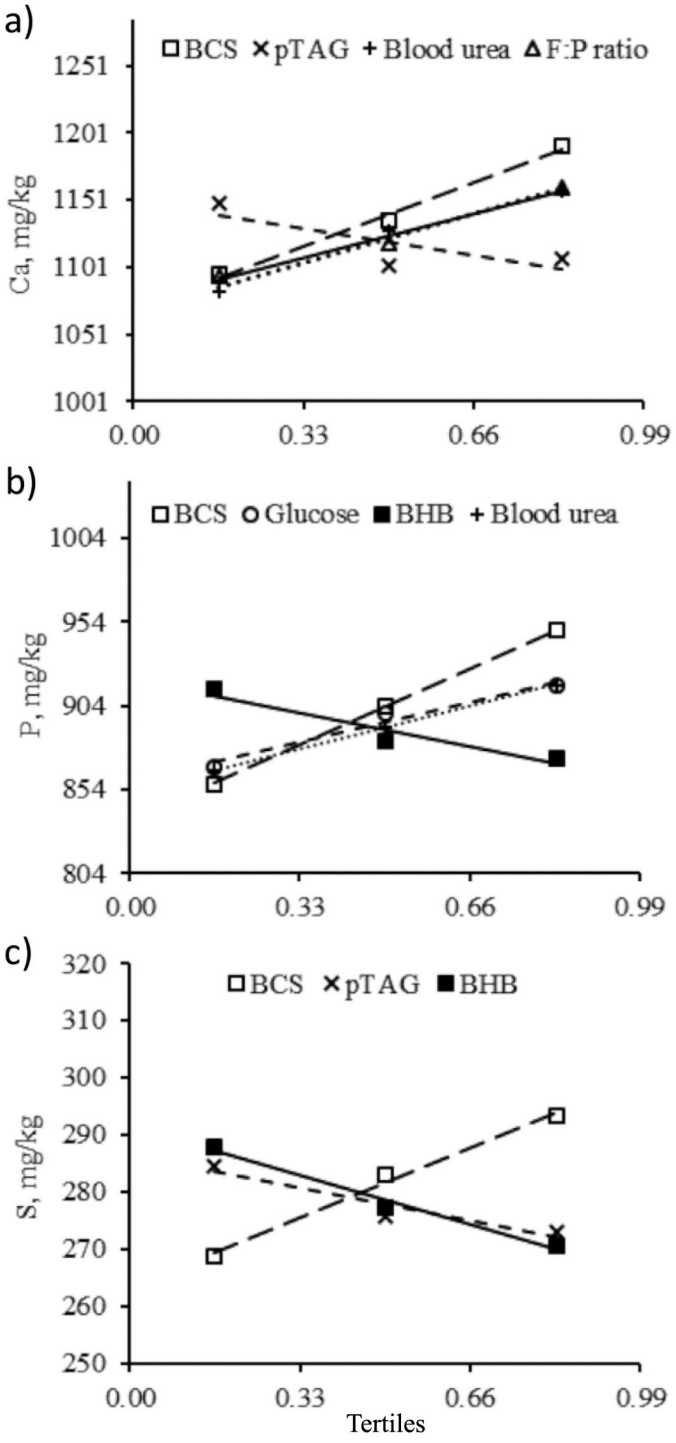

Figure 3. Least squares means of Ca, P, and S over classes of milk fat-to-protein ratio (F:P), BCS, glucose, BHB, blood urea, and predicted liver triacylglycerol (pTAG). Explanatory variables were discretized according to tertiles, and only significant linear contrasts were given ($P < 0.05$).

inflammatory processes (Trevisi and Minuti, 2018; NASEM, 2021), and is associated with reduced motility of the gastrointestinal tract, and decreased DMI, which aggravates NEB and intensifies the impact of resulting metabolic disorders (Steiner, 2003).

Consistent with these metabolic changes and with the current literature (Benedet et al., 2019), milk protein was found to be associated positively with BCS and blood glucose, and negatively with the BHB content. Milk lactose was positively associated with BCS, whereas milk fat was positively associated with the blood BHB content. This confirms that a cow in better body condition will produce milk with greater protein and lactose contents (Roche et al., 2009). Furthermore, milk lactose was negatively associated with the F:P ratio, as we expected due to the normal pattern of lactose to fat during lactation (Costa et al., 2019). The positive association between blood BHB and milk fat is consistent with BHB being a substrate for the formation of de novo fatty acids in milk (Urrutia and Harvatine, 2017).

Minerals and Indicators of Metabolic Status

There have been few or no systematic investigations testing the hypothesis that the milk mineral profile reflects changes in the indicators of a cow's metabolic status during the first phase of lactation. Stocco et al. (2019) suggested that variations in milk mineral contents should be evaluated by correcting the response variables for individual factors, such as milk yield, DIM, parity, and herd, as we have done in the present work. Given the minimal differences between the diets of the 2 herds, the observed variations are likely largely due to individual factors related to intake, absorption, metabolism, and the secretion of minerals into the milk.

Our results show that variations in the milk mineral profile are associated with metabolic status indicators. Calcium and S were positively associated with BCS, while P was associated positively with BCS and glucose, and negatively with BHB. Sulfur, K, and I were also negatively associated with BHB. These trends are consistent with expectation that the fatter the cow, the higher the glycemia, and the lower the serum BHB levels during the first phase of lactation, the better the metabolic status (Cardoso et al., 2020). These results suggest that the mineral and protein content of milk partially reflect the cows' metabolic adaptation in first 120 DIM. In this regard, we observed that when milk protein was included in the model almost all the significant associations disappeared. However, to avoid

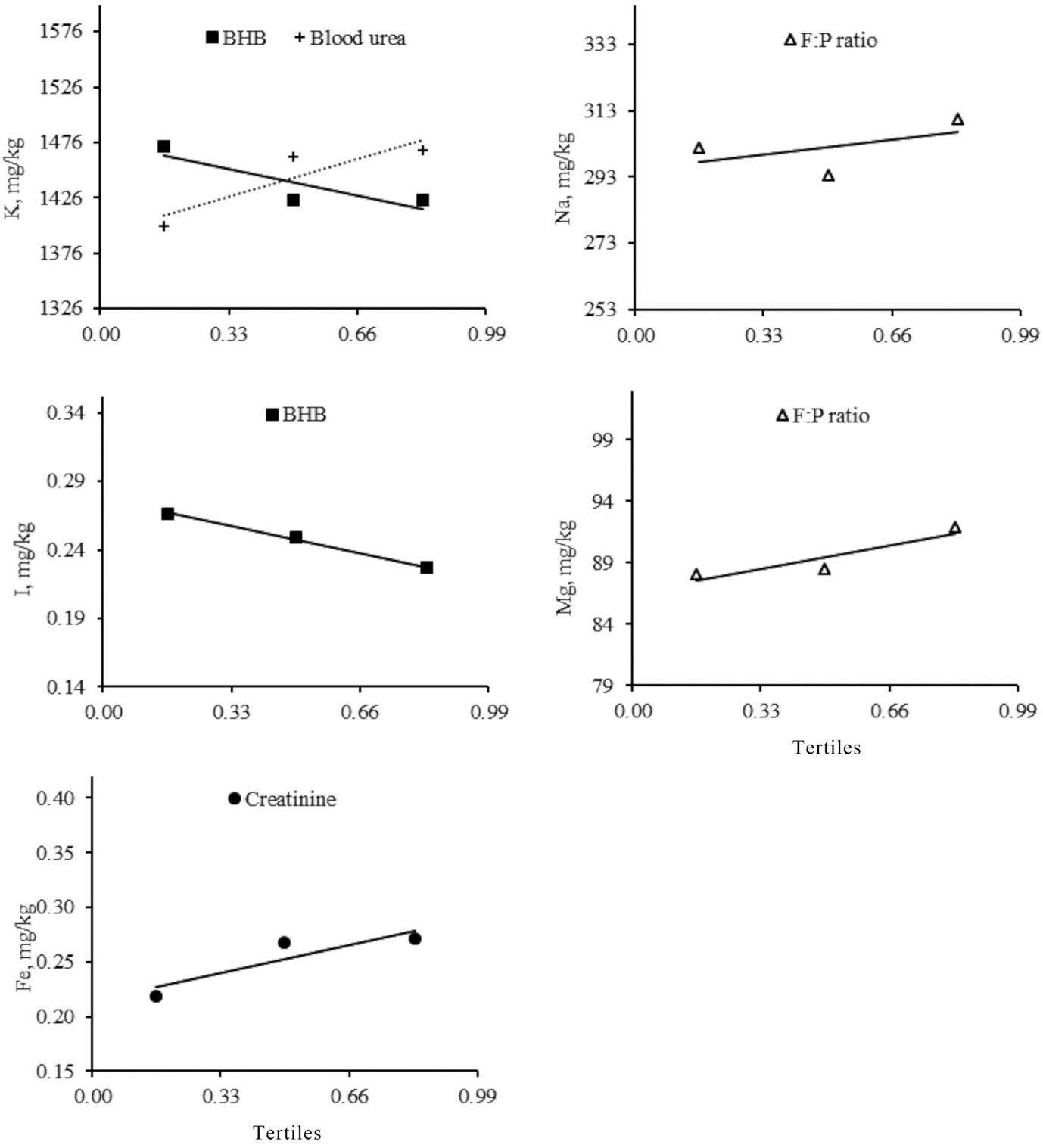

Figure 4. Least squares means of K, Na, I, Mg, and Fe over classes of BHB, blood urea, milk fat-to-protein ratio (F:P ratio), and creatinine. Explanatory variables were discretized according to tertiles, and only significant linear contrasts were given ($P < 0.05$).

Table 4. Results from the mixed model [*F*-values, significance, and root mean square error (RMSE)] for milk macrominerals (mg/kg) as affected by classes of explanatory variables[1]

	Ca		K		Mg		Na		P		S	
Trait[2]	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE
F:P ratio	5.07**	123	1.69	120	3.06*	10.3	3.19*	41.5	0.595	94.1	0.204	28.3
BCS	4.44*	124	2.23	120	0.78	10.3	0.61	40.9	7.15***	91.9	5.915**	27.6
Energy/protein-related metabolite												
Glucose, mmol/L	0.31	124	1.66	122	2.85	10.4	1.27	42.5	3.13*	94.2	2.652	28.3
Cholesterol, mmol/L	0.53	124	0.92	121	0.97	10.4	0.03	42.6	0.32	95.0	0.259	28.5
NEFA, mmol/L	0.98	123	3.08*	120	1.17	10.4	0.64	42.4	2.33	93.8	2.002	28.2
BHB, mmol/L	0.99	124	3.22*	120	1.23	10.4	1.84	42.1	3.07*	93.6	5.682**	27.7
Creatinine, μmol/L	0.77	124	0.06	122	0.44	10.4	0.57	42.5	1.02	94.6	1.78	28.2
Urea, mmol/L	4.79**	122	4.74**	120	2.81	10.4	0.56	42.4	3.85*	93.8	2.986	28.2
Ultrasound liver trait												
pTAG, mg/g	3.04*	120	1.77	119	1.38	10.3	0.44	42.0	2.66	91.7	3.172*	27.6
PVA, mm^2	0.92	124	0.22	121	0.03	10.3	1.97	41.6	0.41	94.0	0.482	28.5
PVD, mm	3.51*	125	4.77**	118	3.68*	10.1	1.15	41.6	6.28**	92.1	7.13***	27.6
LD, mm	0.18	127	0.46	120	0.36	10.4	1.88	41.4	0.05	95.0	0.549	28.7

[1]Model that considered DIM, parity, milk yield, and herd/date as sources of variation. DIM was constituted by 4 classes: class 1 $\leq$ 30; 30 < class 2 $\leq$ 60; 60 < class 3 $\leq$ 90; 90 < class 4 $\leq$ 120. Parity was constituted by 2 classes: primiparous and multiparous. Milk yield was constituted by 4 classes discretized according the 25th, 50th, and 75th percentiles.

[2]All explanatory variables were discretized according to tertiles; therefore 3 levels and 2 degrees of freedom are absorbed by each linear model. F:P ratio = milk fat-to-protein ratio; BCS scored on a 5-point scale; NEFA = nonesterified fatty acids; pTAG = predicted liver triacylglycerol; PVA = portal vein area; PVD = portal vein depth; LD = liver depth.

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

Table 5. Results from the mixed model [*F*-values, significance, and root mean square error (RMSE)] for milk microminerals (mg/kg) as affected by classes of explanatory variables[1]

	Cu		Fe		I		Mn		Zn	
Trait[2]	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE
F:P ratio	0.179	0.055	0.275	0.125	1.01	0.081	9.46***	0.007	0.501	0.605
BCS	0.12	0.056	0.77	0.126	1.81	0.081	2.52	0.007	0.22	0.608
Energy/protein-related metabolite										
Glucose, mmol/L	1.43	0.054	0.11	0.126	0.62	0.078	0.68	0.007	2.30	0.603
Cholesterol, mmol/L	0.13	0.055	0.53	0.126	0.59	0.078	1.25	0.007	3.72*	0.596
NEFA, mmol/L	1.56	0.054	0.61	0.126	0.95	0.078	0.15	0.007	4.52*	0.591
BHB, mmol/L	0.59	0.055	2.17	0.125	3.47*	0.077	0.09	0.007	1.00	0.603
Creatinine, μmol/L	0.99	0.055	3.30*	0.124	0.41	0.078	0.27	0.007	0.14	0.606
Urea, mmol/L	2.49	0.054	1.20	0.126	0.77	0.078	1.07	0.007	1.39	0.604
Ultrasound liver trait										
pTAG, mg/g	2.05	0.055	0.15	0.126	0.24	0.081	0.18	0.007	1.59	0.602
PVA, mm^2	0.18	0.056	2.83	0.121	1.37	0.081	0.78	0.007	0.14	0.610
PVD, mm	1.29	0.056	1.04	0.121	0.55	0.081	0.22	0.007	0.65	0.607
LD, mm	0.86	0.055	0.04	0.124	1.69	0.080	4.09*	0.007	0.04	0.606

[1]Model that considered DIM, parity, milk yield, and herd/date as sources of variation. DIM was constituted by 4 classes: class 1 ≤ 30; 30 < class 2 ≤ 60; 60 < class 3 ≤ 90; 90 < class 4 ≤ 120. Parity was constituted by 2 classes: primiparous and multiparous. Milk yield was constituted by 4 classes discretized according the 25th, 50th, and 75th percentiles.

[2]All explanatory variables were discretized according to tertiles; therefore 3 levels and 2 degrees of freedom are absorbed by each linear model. F:P ratio = milk fat-to-protein ratio; BCS scored on a 5-point scale; NEFA = nonesterified fatty acids; pTAG = predicted liver triacylglycerol; PVA = portal vein area; PVD = portal vein depth; LD = liver depth.

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

bias due to multicollinearity, we conducted the association analysis with the major milk constituents excluded from the model.

Among the microminerals, Fe was found to be positively associated with creatinine. Creatinine is an indicator of muscle metabolism in animals, as it is a waste product of nonenzymatic muscle breakdown (McCabe and Boerman, 2020). Causes for this association need to be investigated further, but we can hypothesize that

Table 6. Results from the mixed model [*F*-values, significance, and root mean square error (RMSE)] for milk contaminants (mg/kg) as affected by classes of explanatory variables[1]

	Al		B		Ba		Sr	
Trait[2]	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE
F:P ratio	0.188	0.063	4.15*	0.050	0.202	0.023	1.85	0.088
BCS	1.71	0.063	1.63	0.051	0.21	0.023	0.37	0.088
Energy/protein-related metabolite								
Glucose, mmol/L	0.50	0.062	1.28	0.052	0.87	0.022	0.67	0.085
Cholesterol, mmol/L	2.26	0.062	0.07	0.052	1.08	0.022	0.05	0.086
NEFA, mmol/L	1.88	0.062	0.14	0.052	0.13	0.022	1.28	0.085
BHB, mmol/L	1.13	0.062	1.91	0.052	0.28	0.022	0.31	0.086
Creatinine, μmol/L	0.64	0.062	0.02	0.052	0.36	0.022	1.74	0.085
Urea, mmol/L	0.79	0.062	2.37	0.052	2.19	0.022	3.37*	0.084
Ultrasound liver trait								
pTAG, mg/g	0.84	0.063	0.20	0.051	0.35	0.023	0.89	0.088
PVA, mm^2	1.17	0.063	0.64	0.052	0.18	0.023	1.44	0.087
PVD, mm	3.62*	0.062	2.23	0.051	2.78	0.022	0.36	0.088
LD, mm	1.65	0.064	1.56	0.051	0.12	0.023	0.12	0.086

[1]Model that considered DIM, parity, milk yield, and herd/date as sources of variation. DIM was constituted by 4 classes: class 1 ≤ 30; 30 < class 2 ≤ 60; 60 < class 3 ≤ 90; 90 < class 4 ≤ 120. Parity was constituted by 2 classes: primiparous and multiparous. Milk yield was constituted by 4 classes discretized according the 25th, 50th and 75th percentiles.

[2]All explanatory variables were discretized according to tertiles; therefore 3 levels and 2 degrees of freedom are absorbed by each linear model. F:P ratio = milk fat-to-protein ratio; BCS scored on a 5-point scale; NEFA = nonesterified fatty acids; pTAG = predicted liver triacylglycerol; PVA = portal vein area; PVD = portal vein depth; LD = liver depth.

*$P < 0.05$.

it is related to more or less pronounced loss of BCS and muscle tone in the early lactation period.

Quadratic associations were found for P with NEFA, and for several minerals with liver dimensions (i.e., LD and PVD). Alterations to the liver dimensions, particularly PVD, are commonly associated with NEB and various grades of liver lipidosis (Haudum et al., 2011; Piazza et al., 2022). As previously discussed by Piazza et al. (2022), the average PVD, LD, and PVA measurements in our population were indicative of physiological variations during the lactation period. Furthermore, the proportion of cows with a high pTAG content (>100 mg/g; Bobe et al., 2004) was negligible, whereas a large number of cows in the population had a moderate pTAG content (50 to 100 mg/g). This is consistent with previous studies which have shown that identifying cows with moderate hepatic lipidosis is challenging because DMI and milk yield are unaffected, so there is an increased risk of progression to a severe fatty liver condition (Bobe et al., 2004; Haudum et al., 2011).

We observed that Ca and S were negatively associated with pTAG, patterns that are consistent with those observed for BCS. Indeed, major drops in BCS during the first months of lactation are associated with a more severe NEB status and higher TAG content in the liver, due to a high energy demand that is not met by feed intake, and consequently higher lipomobilization (Fiore et al., 2018).

Among the contaminants, Al had a significant linear association with PVD, whereas Sr had a positive association with blood urea content. In humans, the toxic effect of Al is due to interference with mitochondrial function. The effect of Al on mitochondrial metabolism is very common in obese subjects or those suffering from hepatic steatosis (Mailloux et al., 2011). In normal conditions, the cows ingest Al when they lick the metal pen bars, behavior that is subject to notable individual variation (Redbo and Nordblad, 1997). The results of the research presented here suggest that an increase in milk Al content could indicate putative toxic effects on the liver, which is reflected in an increase in the portal vein thickness (Roberts et al., 2000).

Finally, mineral chemical analysis is expensive and time consuming, and cannot be applicable on a herd-scale investigation (Soyeurt et al., 2009). Ongoing research are testing the potential of using infrared spectroscopy on milk to predict milk minerals, achieving moderate to good accuracy (Soyeurt et al., 2009; Bonfatti et al., 2016). Prediction of milk minerals, in combination with further investigation on the suitability of mineral components as indicator of energy metabolism might allow the early detection of an altered metabolic status in cows.

CONCLUSIONS

This study analyses the variation in the mineral profile of cows in the first 120 d of lactation, and, for the first time, the relationships between the milk mineral profile and various MET indicators. As we found strong associations between the milk protein content and MET indicators, and between the mineral profile and MET indicators, we conclude that protein may be the major element in mediating this relationship. The most significant associations were observed between the macrominerals and BCS, pTAG, and BHB. Future investigations are needed to assess the suitability of using the mineral profile as an indicator of energy metabolism. Specifically, this will only be feasible along with the application of a noninvasive and easy-to-use tools, such as the infrared spectroscopy, able to predict milk mineral elements at the population level during milk recording schemes with reliable accuracy.

ACKNOWLEDGMENTS

This research was carried out as part of the BENELAT project, funded by the Regione Lombardia (Bando d.d.s. no. 4403, 28/03/2018). The authors have not stated any conflicts of interest.

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