Application of generalized additive models to explore minerals in sheep milk

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ABSTRACT

This study explores the complex relationships among ovine milk minerals (Ca, P, Na, K, Mg, K, Cl, respectively) and different factors employing generalized additive mixed models (GAMM). The GAMM included milk yield (MY), parity, and breed as parametric terms, and casein, fat, lactose, pH, SCS, DIM, and sampling day as smooth functions. The objectives were to investigate how these factors could affect minerals in sheep milk and to assess whether their patterns change over time and across different concentrations of major milk components. The GAMM identified distinct patterns in the mineral concentrations between Comisana and Massese breeds, with the Massese ewes having less P, Mg, K, and Cl compared with the Comisana. Moreover, these minerals were also affected by DIM; Mg, S, and Cl were influenced by parity; and P, K, and Na changed across MY levels. Regarding milk components, all the minerals were affected by casein, fat, and lactose concentrations (excluding P for fat). Milk pH was important for Ca, K, Na, and Cl, whereas SCS affected the variability of all minerals except Ca. This study provided valuable insights on the variability of macrominerals in sheep milk, by using GAMM and examining the trajectory of each element across factors as breed, MY, parity, and DIM, as well as across various concentrations of major milk components and their interactions. The dynamic nature of milk mineral content was evident through temporal variability, likely driven by dietary changes, environmental fluctuations, and physiological adaptations, as well as synergistic and antagonistic interactions between milk components and fixed factors. These findings enhance understanding of mineral composition in sheep milk, providing a comprehensive framework for future research on milk quality, animal health, and cheesemaking properties.

Key words: mineral variation, ovine milk, smooth functions, Comisana, Massese

INTRODUCTION

The investigation of bioactive milk components has been an ongoing process characterized by pioneering and innovative findings, scientific investigations, and the identification of novel opportunities and challenges that characterize the dairy research (Punia et al., 2020; Agregán et al., 2021). Minerals are among the many components that make sheep milk attractive and valuable, owing to their pivotal role in nutraceutical properties, as well as milk structural integrity (Goyal et al., 2022).

In Europe, dairy sheep farming systems are prominently established in Mediterranean regions, where they exert significant influence within the wider dairy market (Koluman and Paksoy, 2024). Italy, in particular, stands out as a leading producer of high-quality dairy products made from sheep milk, including renowned Protected Designation of Origin cheeses such as Pecorino Romano and Pecorino Toscano. These cheeses are predominantly made from milk obtained from cosmopolitan dairy breeds like East Friesian and Lacaune, which are highly productive and have been used in crossbreeding programs to enhance milk production in local breeds. However, local breeds remain crucial for maintaining the regional dairy industry due to their ability to produce milk with favorable nutraceutical and technological properties, including high concentrations of essential minerals and distinctive fatty acid profiles. These factors contribute to the unique flavors and exceptional quality of typical regional dairy products (Watkins et al., 2021).

The sheep industry requires innovative tools and practices to compete with dairy cattle products in the market. Despite recent advancements, the specific points of intervention in the ovine dairy industry remain incompletely

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

understood. One critical area requiring further research is the mineral composition of sheep milk (Nguyen, 2022), the variability of which is influenced by various factors, including breed, diet, and seasonal changes (Li et al., 2022). Research has shown that dietary mineral and vitamin supplementation, along with specific farming practices, can temporarily improve the mineral profile of sheep milk (Arshad et al., 2021; Adinepour et al., 2022). However, studies evaluating strategies for the permanent enhancement of sheep milk mineral content and overall composition are still limited, with findings often yielding variable results (Jones and Wilson, 2022; Marshall et al., 2024). To address these challenges, more comprehensive studies on the detailed aspects of dairy farming, milk quality, and cheese characteristics are essential. Specifically, a deeper understanding of how mineral concentrations fluctuate throughout the lactation period and under varying environmental and management conditions is crucial for improving both milk and cheese quality. In this context, this study seeks to fill the knowledge gap in dairy science regarding the variability of minerals in sheep milk. By utilizing generalized additive mixed models (**GAMM**), a statistical approach widely used in ecology, environmental science, and epidemiology (Keyghobadi et al., 2020; Ugwu and Zewotir, 2020), we aimed to capture the complex relationships and nonlinear patterns inherent in sheep milk composition, such as minerals. Specifically, GAMM can represent nonlinear trends and observe, for instance, changes in mineral concentrations throughout the lactation period, in response to varying concentrations of major milk components, or under different levels of milk acidity. Understanding these dynamics is crucial to plan interventions and management techniques aimed at optimizing the mineral content of sheep milk, improving the overall quality of dairy products and promoting the competitiveness of the sector as a whole. Therefore, the objectives of this study were to (1) investigate the factors affecting macrominerals in sheep milk (i.e., daily milk production, parity, days in milk, breed), and (2) to assess whether their trajectory changes over time (i.e., lactation period) and over additional features (i.e., different concentrations of major milk components).

MATERIALS AND METHODS

Sheep Breed and Milk Sampling

The present study is embedded within the project "Additional resources to study multi-functional traits in milk from indigenous sheep breeds" through the action "Bando di Ateneo 2023 per la ricerca" and was carried out as a part of the Sheep4Cheese project, which aimed at studying the milk protein profile for improving the cheesemaking efficiency of Comisana and Massese sheep breeds. A total of 740 ewes were milk-sampled during the morning milking, and daily milk production (kg/d) was measured. Eleven sampling sessions (~70 ewes per sampling) were carried out from November 2021 to March 2023. In each sampling session, ~500 mL of milk per ewe was collected, immediately refrigerated at 4°C, and analyzed within 24 h of sampling. All the dairy ewes involved in this study were reared at the National Association of Sheep Breeders nucleus farm (Asciano, Tuscany region, Italy; https://www.assonapa.it/centro-genetico), which manages the breeding and provides semen and rams for genetic improvement of these 2 breeds in commercial flocks. The farming system of the nucleus is semi-intensive. The sheep are housed in the barn throughout October to March. Generally, they are allowed access to pasture in the spring once they reach ~150 d of lactation. During this initial phase, their diet primarily consists of TMR. In the final phase of lactation (April to May), the sheep are moved to pasture each morning for ~3 h. Their nutritional needs are supplemented using TMR. During the summer months, the sheep are allowed to graze on pasture. The Comisana is an endangered Italian dairy sheep breed that is well adapted to both hills and plains. The population counts ~2,570 individuals, mainly distributed in the inland areas of central and southern Italy (FAO, 2023). The Comisana is a composite of Maltese and Sicilian sheep breeds. Despite being raised in typically extensive or semi-extensive systems, the Comisana displays adaptability to more intensive farming systems, including partially free housing, mechanical milking, and feeding with the use of concentrates. Its fertility rate stands at 95%, with a prolificity of 180%, and the average age of first parity is 16 mo (AIA, 2024). In the present study, ewes of this breed (n = 429) ranged at sampling from 8 to 148 DIM, and milk production was 0.72 kg/d for primiparous ewes, 0.83 kg/d for secondiparous, 0.95 kg/d for tertiparous, 0.85 g/d for quartiparous, and 0.83 kg/d for ewes with 5 or more parities. The Massese is a vulnerable Italian dairy sheep breed that is adapted to the hills and mountains environment. The population counts ~6,833 individuals, mainly distributed in central Italy (FAO, 2023). The Massese is a composite originating from various breeds in the Apennine mountains. This breed is typically raised in natural grazing supplemented with hay and, to a lesser extent, concentrates. Its fertility rate stands at 95%, with a prolificity of 135% and the average age of first parity is 16 mo (AIA, 2024). In the present study, ewes of this breed (n = 311) ranged at sampling from 8 to 170 DIM, and milk production was on average 0.71 kg/d in the first parity, 0.95 kg/d in the second, 0.87 kg/d in the third, and 1.00 kg/d in the fourth or greater parity.

Analysis of Milk Composition

All milk samples were analyzed for fat, casein, and lactose with a MilkoScan FT3 infrared analyzer (Foss Electric A/S, Hillerød, Denmark) calibrated according to the reference methods: ISO 1211/IDF for fat (ISO-IDF, 2010a), ISO 8968-2/IDF 20-2 for casein (ISO-IDF, 2014), and ISO 26462/IDF 214 (ISO-IDF, 2010b) for lactose. Milk pH was measured with a portable pH meter (Crison Basic 25 portable pH meter; Crison Instruments SA, Barcelona, Spain). Somatic cell count was determined using a Fossomatic DC7 somatic cell counter (Foss Electric A/S, Hillerød, Denmark) and transformed into the logarithmic SCS [$\log_2(\text{SCC} \times 10^{-5}) + 3$].

Analysis of Milk Minerals

Minerals (Ca, P, Na, K, Mg, K, and Cl) were measured via wavelength dispersive X-ray fluorescence (S6 Jaguar, Bruker Corporation, Billerica, MA) spectroscopy. Briefly, this is an analytical technique which allows assessment of the mineral composition of a sample by utilizing the fluorescence radiation emitted in response to X-rays. This method relies on the photoelectric effect, where incident X-ray radiation interacts with the atoms of the sample, leading to the excitation of electrons and subsequent emission of fluorescence radiation at longer wavelengths. This unique fluorescence radiation emitted by each chemical element enables the identification and quantification of constituent atoms within the sample. In the case of liquid samples like milk, no sample preparation is required. Approximately 3 mL of milk per ewe is introduced into a disposable cup, which is then positioned within the instrument for analysis. During analysis, the reading chamber is saturated with helium to optimize measurement accuracy, and milk samples are scanned at 30 kV for P, Mg, S, Na, and Cl, and at 50 kV for Ca and K, with a total scanning time of 3 min. Calibrations were developed using Spectra.Elements Advance v.3 software provided by Bruker.

Statistical Analysis

A GAMM was used to model the trajectory of the 7 minerals in milk from Comisana and Massese breeds throughout the observed period. A GAMM represents an extension of a general linear model, wherein the relationship of the dependent variable is not strictly linear, but can involve unknown smoothing functions, in conjunction with conventional regression coefficients and random effects. These smoothing functions are adaptable to the data and can take various patterns, such as linear, quadratic, cubic, spline, or a combination thereof. Importantly, the specific functional form does not need to be predetermined, enabling a highly flexible estimation of the connection between independent and dependent variables (Wood, 2017b). Indeed, GAMM are particularly valuable when dealing with longitudinal data, as they can be effective in modeling both the cyclical patterns over seasons and the extended-term trends in time-series data (Mundo et al., 2022). Furthermore, random effects can be incorporated into GAMM to cope with covariance of observations within individuals and across time.

In its general form, a GAMM can be written as:

$$y_i = \beta_0 + \sum_{j=1}^{p} f_j\left(x_{ij}\right) + \varepsilon_i = \beta_0 + f_1\left(x_{i1}\right) + f_2\left(x_{i2}\right) + \ldots + f_p\left(x_{ip}\right) + \varepsilon_i,$$

where y_i represents the response variable for the ith observation, β_0 is the intercept, and $f_j(x_{ij})$ are "smooth" or flexible nonlinear functions, which relate each p predictor variable (x_{ip}) to the observed response y_i. Finally, ε_i represents the error term for the ith observation. The term "additive" is used because a separate f_j is computed for each x_i, which are eventually added together (James et al., 2023). Additionally, a GAMM can include a parametric term (e.g., a categorical predictor) which captures overall differences in the height of the trajectories as a function of the levels of the predictor. This can result in 2 possible outcomes: (1) fitting a single smooth at the reference value of the categorical predictor, and (2) fitting an additional so-called "difference smooth" that captures the differences between the trajectories for the levels of the categorical predictor.

In the present study, the following model was used for all 7 observed minerals:

$$Y = intercept + breed\ intercept + milk\ production\ class$$
$$+ parity\ intercept + f_{dim}(DIM) + f_{dim}(DIM) \times breed$$
$$+ f_{SCS}(SCS) + f_{SCS}(SCS) \times breed + f_{casein}(casein)$$
$$+ f_{casein}(casein) \times breed + f_{lactose}(lactose) + f_{lactose}(lactose)$$
$$\times breed + f_{pH}(pH) + f_{pH}(pH) \times breed + f_{fat}(fat) + f_{fat}(fat)$$
$$\times breed + f_{sampling\ day}(sampling\ day) + \varepsilon,$$

where Y is recorded values for Ca, P, Mg, S, K, Na, and Cl at each sampling session. Intercept terms are the parametric model terms, representing categorical predictors, namely breed (n = 2; Comisana and Massese), class of milk production (n = 4; class 1 = 0.46 kg/d, class 2 = 0.65 kg/d, class 3 = 0.85 kg/d, class 4 = 1.27 kg/d) and parity (n = 5; parity 1, 2, 3, 4, and ≥5). The reference levels for the categorical predictors were Comisana, first class, and first parity for breed, class of milk produc-

tion, and parity, respectively. The $f_{casein}(casein)$, $f_{fat}(fat)$, $f_{lactose}(lactose)$, $f_{SCS}(SCS)$, and $f_{dim}(DIM)$ are the reference smooth terms for casein, fat, lactose, pH, SCS, DIM, respectively. $f_{dim}(DIM) \times breed$, $f_{SCS}(SCS) \times breed$, $f_{casein}(casein) \times breed$, $f_{lactose}(lactose) \times breed$, $f_{pH}(pH) \times breed$, and $f_{fat}(fat) \times breed$ represent the smooth for the difference between levels of the categorial breed effect. Finally, $f_{sampling\ day}(sampling\ day)$ is the random smooth term that models the sampling day variability throughout the observed period.

To model the effect of the categorical term and to separate the intercept difference and the nonlinear difference between Comisana and Massese breeds, the ordered factor difference smooths method described by Sóskuthy (M. Sóskuthy, University of York, York, England; unpublished data) and Wieling (2018) was used. The Comisana breed was used as reference level and results for the Massese breed are then expressed as the difference from the reference level.

Results from GAMM are summarized in tabular form, comprising 2 distinct sections: the parametric coefficients section and the smooth terms section. In the former there are the results for group means factors (breed, milk production and parity, respectively); in the latter there are the results for the linear or nonlinear association between macrominerals and casein, fat, lactose, pH, SCS, DIM, and sampling day. Linear or nonlinear associations were evaluated and identified by the effective degrees of freedom (**EDF**), which are proxies for the degree of nonlinearity between the predictor and the response: specifically, (1) an EDF of 1 is equivalent to a linear relationship, (2) an EDF >1 and ≤2 is a weakly nonlinear relationship, and (3) an EDF >2 indicates a highly nonlinear relationship (Zuur et al., 2009).

Model validation was based on 2 main steps: (1) optimization of the trade-off between smoothness (λ) and wiggliness (**k**); (2) distribution of the model residuals.

Each smooth in a GAM is essentially the weighted sum of many smaller functions, called basis functions. Both λ and k control the degree of smoothing of these basic functions and finding the right trade-off is fundamental to avoid an oversmoothed or an overfitted curve. In the first situation the curve will loosely fit the data and will therefore predict the missing point very poorly. In the second case the curve will fit the data very closely, following the signal as well as the noise surrounding it. The trade-off between λ and k was checked using the *k.check* function. When EDF were very close to k, the model was refit with a larger k. After fitting a different k, distribution of model residuals, homoscedasticity, and autocorrelation were checked using the *gam.check* function and eventually finding the optimized trade-off. The final results are shown in Supplemental File S1 (see Notes). Finding the optimal trade-off between λ and k allows

GAMM to cope efficiently with possible outliers, shrinking their influence and eventually avoiding overfitting.

Data preparation and all statistical analyses were carried out in the R environment version 4.4.1 (R Core Team, 2022) using a range of packages, including *tidyverse* (Wickham et al., 2019), *mgcv* (Wood, 2017a), *itsadug* (van Rij et al., 2022), and *broom* (Robinson et al., 2023). These were used, respectively, for data preparation and editing, GAMM model fitting, visualization of results, and outputs formatting.

RESULTS AND DISCUSSION

In the Mediterranean region, dairy sheep farming practices range from extensive to intensive systems and offer flexibility in managing mating and lambing activities, despite seasonal milk production. Within these systems, the progression of lactation stages correlates with seasonal fluctuations, thereby exerting a large influence on the composition of milk. In the present study, the variability of minerals in sheep milk using a GAMM approach was investigated. As mentioned previously, GAMM offer a versatile approach in such contexts by enabling the simultaneous capture of both linear and nonlinear relationships, and potential variations in the height and shape of trajectories attributable to specific factors (e.g., breed). Furthermore, using visual methods is a highly effective strategy for assessing results from GAMM. Indeed, this approach enables the observation of both the trajectory's shape and potential differences in this trajectory among various effects levels. A first comment regards the sampling day effect. Apart from Cl, the day of sampling was always significant, and EDF values ranged from a minimum of 4.15 (Ca) to a maximum of 7.80 (Na). These values suggest a strong, nonlinear relationship between the day of sampling and minerals. Sampling day effect was included in the GAMM as random effect and considers not only the variability due to that day (e.g., environmental conditions) but also the correlation among measurements taken at the same time. In the following sections, each macromineral is addressed individually.

Calcium

In the present study, the average milk Ca (± SD) was 2,174 mg/kg (± 260; Supplemental Table S1, see Notes). Specifically, the Ca levels in the Comisana and Massese breeds were 2,154 mg/kg (± 256) and 2,202 mg/kg (± 263), respectively (data not shown). These values are higher compared with those found for bovine (Malacarne et al., 2018; Zwierzchowski and Ametaj, 2019) and caprine milk (Park et al., 2007). Usually, this mineral in bovine milk is ~34% soluble, most of which occurs as

unionized salts of citrate, and ~30% exists as Ca^{2+} (Fox et al., 2015). However, a lower soluble Ca portion is reported in sheep milk compared with cow milk (~26%; Yabrir et al., 2014). Regarding the Ca^{2+} form, this is of major significance in various aspects of enzymatic milk coagulation (Fox et al., 2017). The insoluble Ca (~66% and 74%, respectively for cow and sheep milk) is mainly associated with casein micelles, either as colloidal calcium phosphate (**CCP**) or casein (micellar) Ca (Yabrir et al., 2014; Fox et al., 2015). Our findings showed that Ca was not affected by breed, production level, or parity. Indeed, the parametric section of Table 1 revealed no statistically significant differences in mean values among the various effect levels. This finding has practical implications for dairy producers, as maintaining consistent Ca levels may be more achievable through optimized feeding regimens and farm management rather than relying on breed selection alone. Interestingly, our study showed a nonlinear increase in Ca (EDF = 2.98) as casein in milk moved from 3.0% to 5.5% (Figure 1a). This suggests that as milk casein concentration increases, Ca levels also increase, which is important for dairy processors, especially in cheesemaking. Milk casein is fundamental in curd formation, and higher Ca concentrations can improve curd strength and cheese yield (Stocco et al., 2021). Additionally, a positive and essentially linear association (EDF = 1.00) was observed with other key milk components, such as fat and lactose (Figure 1b and c), providing further insight into the complex relationships between this mineral and milk composition. We also observed a nonlinear association (EDF = 1.97) between Ca and pH. Specifically, at pH levels of 6.6 or higher, Ca concentration seemed to reach a plateau (Figure 1d), further highlighting the pivotal role of milk pH in the coagulation kinetics. In the present study, changes in milk pH occurred throughout lactation, with values potentially exceeding 7.0 at very late DIM. These observations have practical applications in cheesemaking, where pH is a crucial factor in coagulation. By adjusting pH, dairy producers can influence the solubility of Ca and manage curd formation, achieving optimal texture and yield. Also, because milk pH changes throughout lactation, understanding how pH affects Ca levels allows dairy producers to better predict the behavior of milk at different stages of lactation, and whether it is more suitable for certain types of dairy products. In the literature, a previous study reported a physiological increase of milk pH in sheep from 6.60 to 6.89 at the beginning and at end of lactation, respectively (Pavić et al., 2002). Milk pH can rise also during mastitic infections because of the increased permeability of the mammary gland membranes, allowing more basic ions (OH) to enter the milk. Several factors contribute to the pH difference between blood and milk, including the active transport of different ions into milk and the precipitation of CCP, which causes the release of H^+ during casein micelle synthesis, higher concentrations of acidic groups in milk, and a relatively low buffering capacity of milk between pH 6.0 and 8.0 (Fox et al., 2017).

Phosphorus

Phosphorus in milk averaged $1{,}747 \pm 213$ mg/kg (Supplemental Table S1), showing values of $1{,}780 \pm 206$ mg/kg and $1{,}700 \pm 216$ mg/kg for Comisana and Massese, respectively (data not shown). In bovine milk, 43% of this mineral is soluble, with the majority occurring as $H_2PO_4^-$ (51%), followed by HPO_4^{2-} (39%), and a minor portion bound to Ca and Mg (10%). On the contrary, the insoluble P (~57%) occurs mainly associated with casein micelles as CCP (Fox et al., 2015). In sheep milk, Yabrir et al. (2014) reported soluble percentage of P similar to that of bovine milk (~41%). We observed a significant effect of both daily milk production and breed on this mineral. Indeed, sheep producing 0.850 kg/d showed a difference of 58.06 mg/kg than those producing less than 0.450 kg/d, and the Massese breed produced less P than Comisana (-83.01 mg/kg; $P < 0.001$; Table 1). This finding suggests that producers may need to tailor feeding strategies depending on the breed and milk output to ensure balanced P intake, which can affect both milk production and composition. For example, higher P levels in high-producing sheep could be beneficial for ensuring optimal milk composition, particularly in breeds such as Comisana, which showed higher P concentrations. Similar to Ca, milk P concentration showed a positive and nonlinear association with casein (EDF = 2.78), reflecting the linkage of these essential components (Figure 2a). This relationship between P and casein is of particular importance for cheese production. Dairy processors can optimize their milk sourcing by selecting milk with higher casein and P content to enhance curd formation and cheese yield. Understanding this relationship helps producers ensure the production of high-quality cheese, as casein is the primary protein involved in coagulation, and phosphorus contributes to this process.

Furthermore, lactose content exerted influence on P levels, and GAMM identified distinct patterns between Comisana and Massese breeds (Figure 2b). Notably, milk P content in Comisana decreased by 300 mg/kg when lactose content dropped below 4%, respect to a 100 mg/kg decrease in the Massese breed at the same content of lactose. This interesting correlation hints at the osmoregulatory role of lactose in milk production and is linked to the relationship previously found for P and MY (Table 1). Additionally, the nearly linear association between P concentrations and SCS (EDF = 1.34, Figure 2c) supported the complex relationship of mineral composition, milk

Table 1. Summary of fitted generalized additive mixed models (GAMM) tested on mineral of ovine milk

Term	Ca	P	Mg	S	K	Na	Cl
Parametric,[1] mg/kg							
Intercept	2,152	1,730	219	546	1,234	766	948
MY 2	20.22	39.45	4.56	2.33	9.22	−14.23	5.49
MY 3	46.67	58.06**	3.10	1.78	15.63	−2.193	8.58
MY 4	6.24	32.42	5.42	3.49	54.60***	−28.94*	3.54
Parity 2	−1.11	35.83	7.22*	11.23*	1.11	1.47	15.44
Parity 3	13.70	42.94	14.61***	15.60**	2.26	12.77	34.81**
Parity 4	−28.66	11.59	6.74	9.27	−3.82	−5.45	25.71*
Parity ≥5	−8.28	18.89	9.65**	10.19*	−10.14	−18.26	25.42*
Breed Massese	20.94	−83.01***	−8.01***	−6.49	−76.70***	−14.36	−46.87***
Smooth, EDF[2]							
Casein	2.98***	2.78***	2.28**	1.00***	1.00***	1.00*	2.12***
Casein × breed	2.13	1.00	1.00**	1.00	1.00*	1.00	1.00*
Fat	1.90***	1.00	1.37***	1.32*	1.00**	2.33	2.11***
Fat × breed	1.26	2.25	1.65	1.00	1.13	1.00	1.00
Lactose	1.00**	1.00*	2.18***	1.00	1.90***	2.94***	2.34***
Lactose × breed	1.00	1.00**	1.00	1.00	1.00	1.00	1.00
pH	1.97*	1.00	2.67	1.00	3.22**	1.04**	1.81**
pH × breed	1.00	1.87	2.77	1.96	2.19	1.00	1.00
SCS	1.20	1.34*	1.00*	2.57**	2.25***	3.25***	3.51***
SCS × breed	1.00	1.82	1.00	1.45	1.00	1.00	1.00
DIM	1.61	1.86**	1.00***	1.00	2.27**	1.00**	1.00***
DIM × breed	1.00	1.00	1.00***	1.25	1.00**	1.00	1.00
Sampling day	4.15**	5.41***	7.63***	6.60***	4.82**	7.80***	2.18
Adjusted R[2]	0.43	0.34	0.65	0.73	0.48	0.76	0.47

[1]Parametric term, which captures overall differences in the height of the trajectories as a function of the observed effect: classes of daily milk yield (MY), parity, and breed. All estimates are expressed as differences from a reference level. The reference levels were the lowest class of daily milk production (MY = 1), primiparous (parity = 1), and Comisana breed.

[2]Smooth term which allows the model to capture the nonlinear pattern in the trajectories using a smooth function; EDF = effective degrees of freedom.
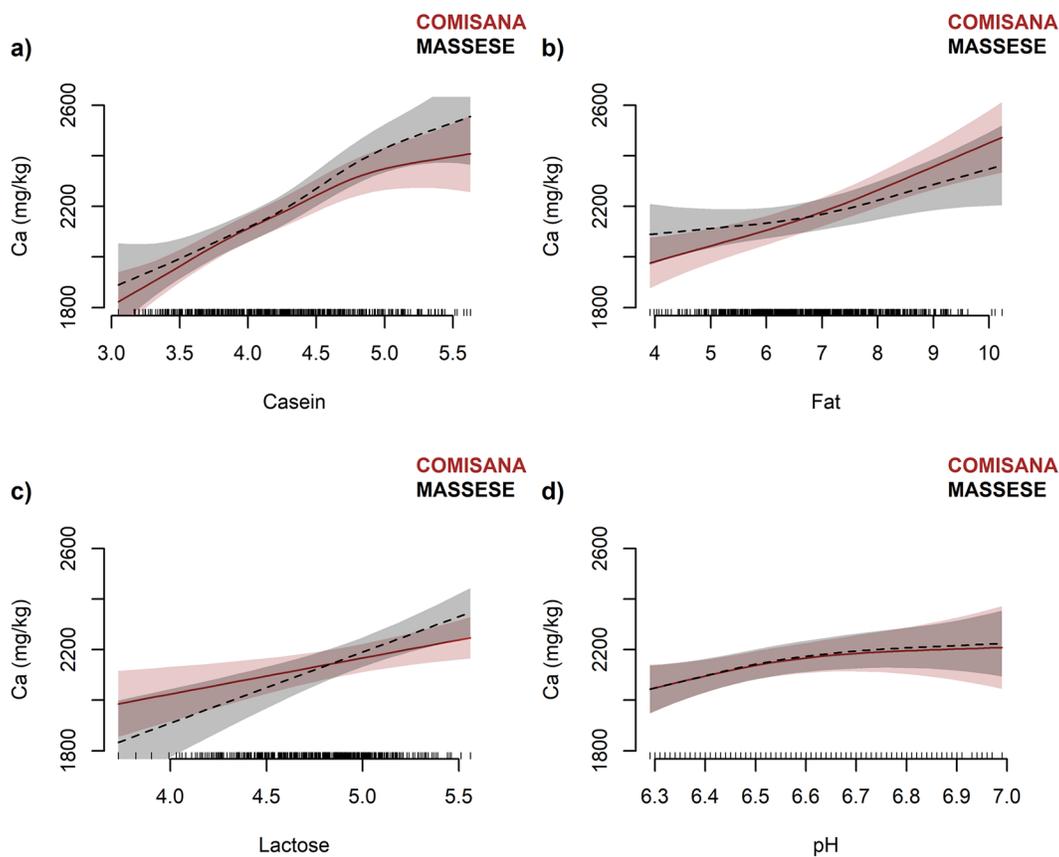*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

quality, and udder health. The positive association with SCS may be attributed to the indirect effect of reduced MY that is also usually observed at high SCS levels in dairy sheep (Tančin et al., 2017). Moving to the DIM effect, P levels exhibited a decreasing trend throughout the lactation period (Figure 2d), reflecting the concentration effect of this essential mineral in milk over time. The GAMM smooth function was also helpful for managing temporal correlation of this mineral with DIM.

Magnesium

The Mg content in milk samples averaged 227 mg/kg (±40; Supplemental Table S1), with values of 228 ± 39 mg/kg for Comisana and 226 ± 42 mg/kg for Massese breeds, respectively (data not shown). The observed concentration of Mg was notably higher than those reported in the scientific literature for sheep and other major dairy species (Zamberlin et al., 2012). Approximately 67% of this mineral is soluble in bovine milk, whereas the colloidal fraction is associated with CCP, and roughly half of it bound to casein phosphoserine residue (Cashman, 2002). In sheep milk, ~59% of this mineral is in soluble form (Yabrir et al., 2014). Despite Ca traditionally overshadowed the significance of Mg in milk due to its role in heat stability and structural integrity, recent studies

emphasize its vital bioactive role of in the dairy industry. However, a limited number of papers have addressed the variability of native Mg in milk (Fox et al., 2017; Oh and Deeth, 2017; Stocco et al., 2019a). Ovine milk has received even less attention in this regard, as discussed by de la Fuente et al. (1997) and Chia et al. (2017). Stocco et al. (2021) specifically highlighted significant associations between Mg in bovine milk and coagulation, as well as cheesemaking traits, thereby emphasizing the bioactive role of Mg in dairy processing. Recognizing unique binding sites on casein for Ca and Mg, Cuomo et al. (2011) underscored the cooperation between these minerals. The affinity of Ca for phosphoserine residues on casein aids the making of additional binding sites for Mg, which, in turn, bonds with glutamic and aspartic acids. This synergistic action may elucidate for the observed positive correlation between Mg and casein content, as depicted in Figure 3a.

Breed effect was statistically significant in terms of both average height (parametric term, Table 1) and the relationship between Mg and casein (smooth term, Table 1). On average, Massese breed produced 8.01 mg/kg less Mg than Comisana. This breed-specific variation in Mg levels can be used by dairy producers to select milk from specific breeds for processing, ensuring optimal mineral content for different dairy products. Moreover,
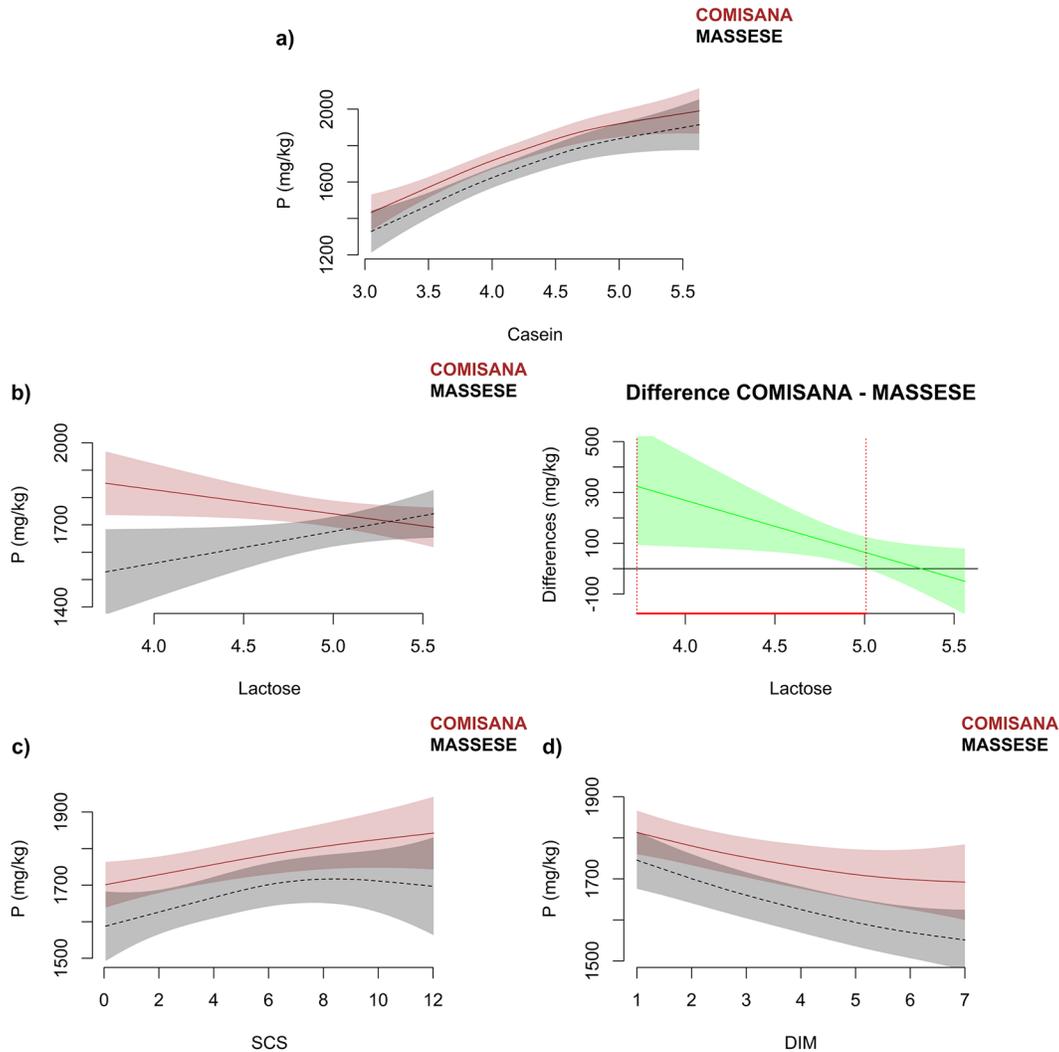
Figure 1. Calcium trajectory across percent of casein (a), fat (b), lactose (c), and pH (d) in milk from Massese (dotted black line) and Comisana (solid red line) lactating ewes. Shaded regions identify the 95% CI for smooth terms for each breed.

GAMM effectively captured the distinct and significant ($P < 0.01$) increase in Mg levels observed in the milk of Comisana and Massese ewes within the specific range of 3.0% to 4.4% of casein. Notably, even at the lowest casein concentrations, Comisana exhibited a higher Mg content in milk compared with the Massese breed. The GAMM proved particularly effective in elucidating this interaction term, allowing to assess the relationship between Mg and casein across different breeds. Furthermore, Mg showed a linear and positive association with fat (Figure 3b), as observed in bovine milk studies by Stocco et al. (2021). Conversely, its relationship with lactose (Figure 3c) exhibited a negative pattern, whereas that with SCS was positive (Figure 3d). This combined information provided by GAMM agrees with previous studies focused on the associations between minerals in bovine milk and subclinical mastitis. Indeed, alkaline phosphatase, a reliable trait for early subclinical mastitis diagnosis, has been well-established (Guha et al., 2012). El Zubeir et al. (2005) found a positive correlation between Mg and the alkaline phosphatase (r = 0.53, $P < 0.05$) in milk samples from dairy cows with subclinical mastitis, further affirming the fundamental role of Mg in the innate immune

response in presence of inflammation (Libera et al., 2021). Ataollahi et al. (2018) highlighted the role of Mg in energy regulation through controlling ATP production and utilization in mitochondria. Additionally, Mg serves as a cofactor for 2 catabolic regulatory enzymes, fructose bisphosphatase and pyruvate carboxylase. Nevertheless, the precise role of Mg in responding to udder inflammation in dairy species remains a subject requiring further exploration, but these findings highlight its importance as a potential marker for udder health, helping producers take preventative measures to maintain milk quality.

A significant ($P < 0.001$) effect of parity was observed, with ewes at their third and fifth parity exhibiting higher Mg levels in milk than others (Table 1). This observation may be influenced by factors related to the age and reproductive history of the ewes, as well as by breed imbalances within parity classes, as Comisana ewes outnumbered Massese ewes at both the third and fifth parities. Furthermore, the impact of DIM on Mg content was significant ($P < 0.001$), revealing differences between the 2 breeds throughout lactation. Specifically, Mg content of milk increased during lactation in Comisana, whereas it tended to decrease in Massese
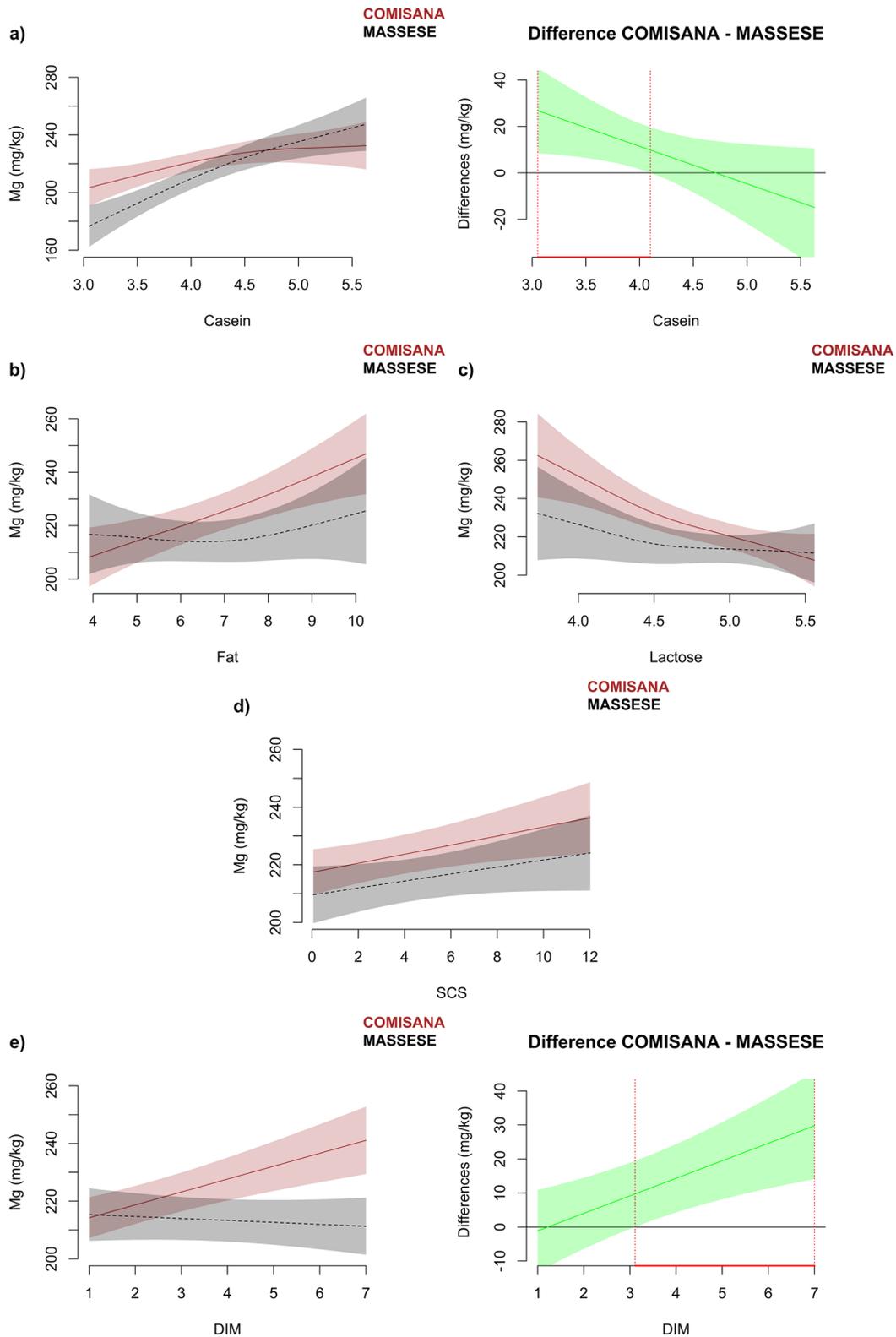
Figure 2. Phosphorus trajectory across percent of casein (a), lactose (b), SCS (c), and DIM (d) in Massese (dotted red line) and Comisana (solid black line) lactating ewes, and estimated differences (green solid line and related shaded area) for lactose effect. Shaded regions identify the 95% CI for smooth terms for each breed. The vertical red dotted lines mark the interval where the differences are significantly different from zero.

(Figure 3e). This result could potentially be attributed to the more pronounced reduction in daily MY from Comisana ewes toward the end of lactation compared with Massese. In the case of Mg, the GAMM confirmed the significance of factors such as age-related factors and breed-specific characteristics, but also provided a robust framework for visualizing and quantifying their impact on the variability in this mineral in milk throughout lactation. This comprehensive approach highlights the versatility of GAMM in uncovering nuanced patterns within complex data sets.
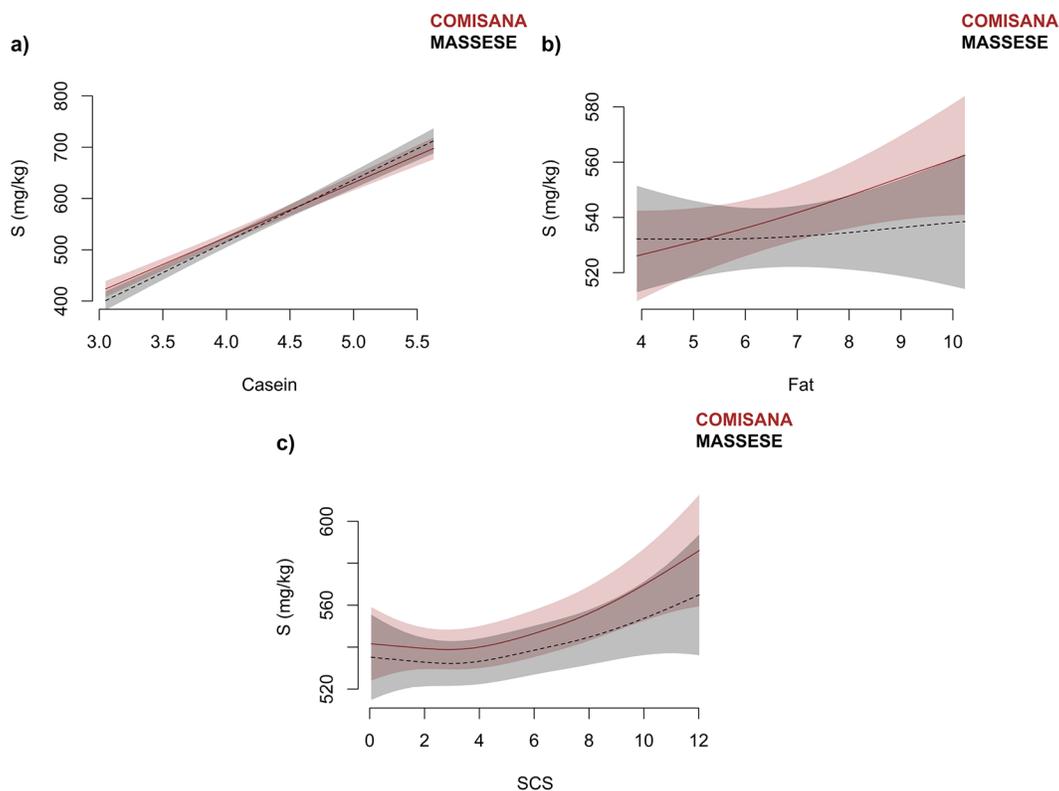
Sulfur

Sulfur in milk averaged 555 ± 70 mg/kg (Supplemental Table S1), showing values of 553 ± 68 mg/kg and 557 ± 73

mg/kg for Comisana and Massese, respectively (data not shown). It is challenging to compare our values with those of recent studies; however, past literature reports that this mineral is ~290 mg/kg in sheep milk (Gaucheron, 2013; Park et al., 2007). Despite the limited scientific exploration of S in milk, attributed to its underexplored nature and the scarcity of technological involvement during coagulation and cheesemaking, recognizing its importance can be crucial, especially given its pivotal role in various biological processes (Hewlings and Kalman, 2019). Sulfur compounds affect the organoleptic aspects of milk, contributing to distinctive flavors, including the characteristic egg flavor observed in certain dairy products (Jo et al., 2019). Understanding the variability of S not only deepens our comprehension of sheep milk composition, but also have implications for developing high-quality

Figure 3. Magnesium trajectory across percent of casein (a), fat (b), lactose (c), SCS (d), and DIM (e) in Massese (dotted red line) and Comisana (solid black line) lactating ewes, and estimated differences (green solid line and related shaded area) for casein and DIM effects. Shaded regions identify the 95% CI for smooth terms for each breed. The vertical red dotted lines mark the interval where the differences are significantly different from zero.

Figure 4. Sulfur trajectory across percent of casein (a), fat (b), and SCS (c) in milk from Massese (dotted black line) and Comisana (solid red line) lactating ewes, and estimated differences (green solid line and related shaded area) for lactose effect. Shaded regions identify the 95% CI for smooth terms for each breed.

dairy products with desirable sensory attributes (Jo et al., 2019). Notably, our investigation has revealed positive correlations between S content and both casein (Table 1, Figure 4a) and fat (Table 1, Figure 4b). This agrees with findings from Fantuz et al. (2020) about S distribution in donkey milk, which highlighted a significant correlation between S and both protein (r = 0.87) and casein (r = 0.57), aligning closely with our own results. Fantuz et al. (2020) further demonstrated that the majority of S in donkey milk (63.6%) was associated with whey proteins, indicating a higher content of sulfur-containing amino acids in whey proteins than in casein. This mirrors the observations in other species, such as cows, where whey proteins, particularly β-LG and α-LA, are rich in S. Moreover, in Figure 4c the GAMM was very useful in revealing a positive nonlinear association between S content and SCS, highlighting the potential important role of S in udder health and its involvement in the immune response. Specifically, the increase of S in milk started from 200,000 cells/mL (SCS = 4), and this could suggest a physiological range for S levels in relation to SCS, as well as that there might be a threshold level of SCS at which the impact on S levels in milk becomes

more pronounced. This insight highlights the bioactive importance of S in maintaining milk quality and health in dairy ewes. Understanding this relationship could allow dairy producers to monitor S levels as an indirect indicator of udder health, combined with SCS, enabling early detection of potential infections and improving overall herd management strategies in the ovine dairy industry.

Potassium

The content of K in milk averaged 1,223 ± 26 mg/kg (Supplemental Table S1), showing values of 1,261 ± 119 mg/kg and 1,168 ± 117 mg/kg for Comisana and Massese, respectively (data not shown). Our values are lower compared with those reported in the scientific literature (Gaucheron, 2013; Park et al., 2007), although a large variability exists among studies and sheep breeds (Gerchev and Mihaylova, 2012; Yabrir et al., 2014). This mineral predominantly exists in an ionized form in bovine milk (92%), with only a small fraction being colloidal (8%; Fox et al., 2017). Potassium as monovalent cation is subject to fluctuations in milk composition, particularly influenced by conditions that promote the open-

ing of tight junctions between epithelial cells. Indeed, a pivotal process altering the presence of monovalent cation content in milk is mastitis or localized inflammation of the mammary tissue (Zhao and Lacasse, 2008). Within the context of the present study, K levels were intricately influenced by milk components, demonstrating an overall K decrease with increasing levels of those included as effects in the model (Figure 5). A noteworthy finding provided by GAMM was the breed-specific influence on K levels, especially related to casein content. The Comisana breed, having a higher casein content than the Massese breed, showed a statistically significant ($P < 0.05$) drop in K concentrations at increasing levels of casein (Figure 5a), particularly in the interval between 3.0% and 4.8%. This pattern was also observed when K was related to other milk components such as fat (Figure 5b) and lactose (Figure 5c). Particularly interesting was the association of K with milk pH (Figure 5d), revealing more acidic milk at higher K levels and a shift to basic conditions as K levels reduced. This finding, coupled with the negative and nonlinear association with SCS (Figure 5e), reflects the intricate relationships among milk constituents during udder inflammation and the adaptation of the acid-base equilibrium and buffering capacity of milk. Throughout lactation, a significant ($P < 0.01$) difference in milk K variation was noted between the 2 breeds, with Comisana ewes consistently exhibiting higher K content in their milk (Figure 5f). Given that K primarily acts as an osmotic solute, regulating acid-base balance and osmotic pressure, its negative correlations with milk components are likely indirect and linked to milk production dynamics. Indeed, in this study, daily MY was associated with significant ($P < 0.001$) variations of K in milk, being K at highest concentrations (1,320 mg/kg) at the highest production level (MY = 1.27 kg/d; data not shown). These findings underline the importance of K as a bioactive compound not only in its quantity but also in its responsiveness to the complex phenomenon of lactation and milk production in sheep.
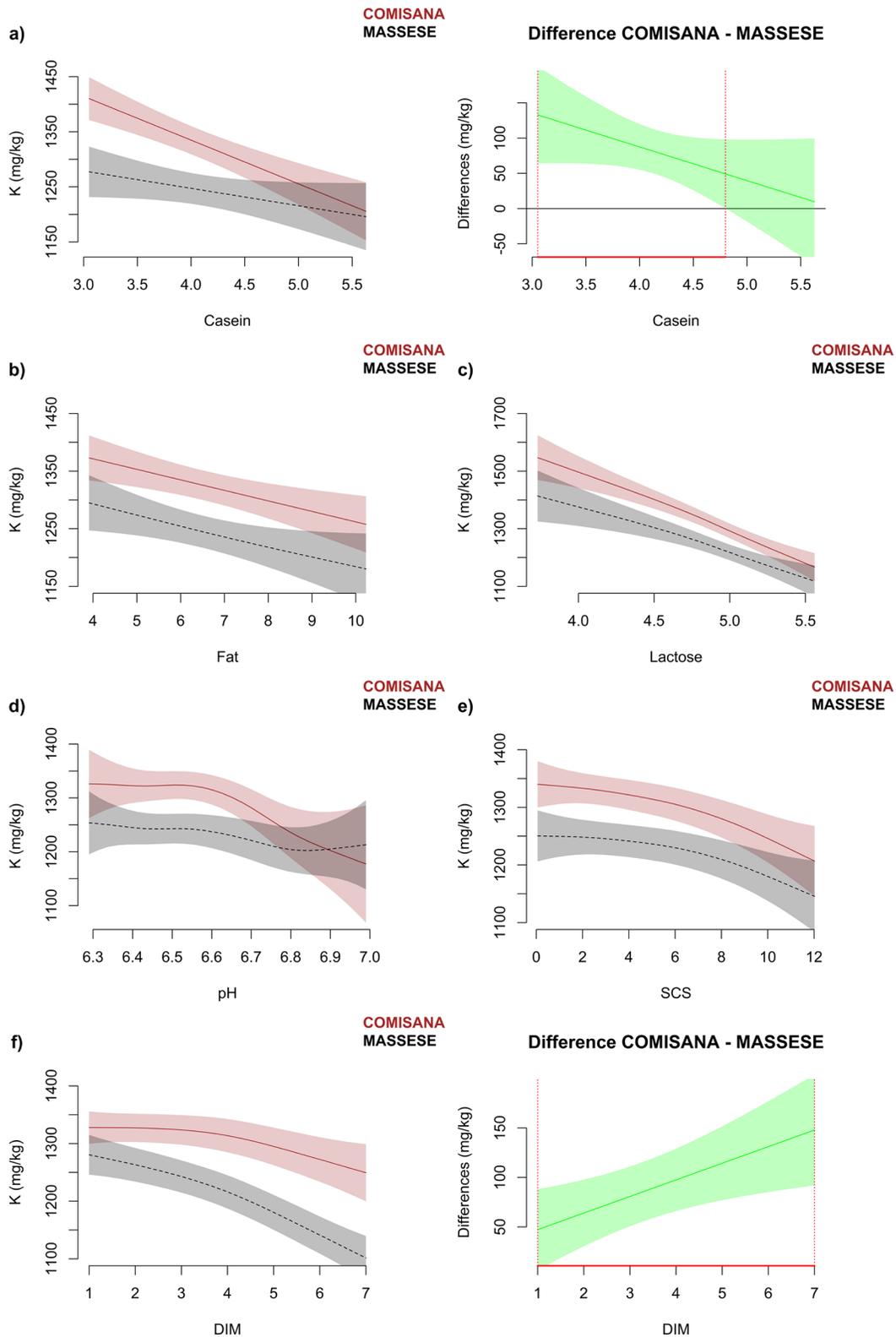
Sodium

It is commonly reported that Na levels are lower in milk of ovine species compared with bovine (Chia et al., 2017). However, in the present study, Na content in milk samples ranged from 722 ± 186 mg/kg in Comisana to 776 ± 219 mg/kg in Massese breeds, respectively. These values are not only higher than what found in ovine milk (Gaucheron, 2013; Park et al., 2007) but also exceeds the most recent studies in bovine milk (Singh et al., 2019; Stocco et al., 2019a). As for K, this mineral is almost all ionized in milk (92%) and only a small fraction is colloidal (8%; Fox et al., 2015). High values of Na in milk maintain osmolar equilibrium between milk and blood
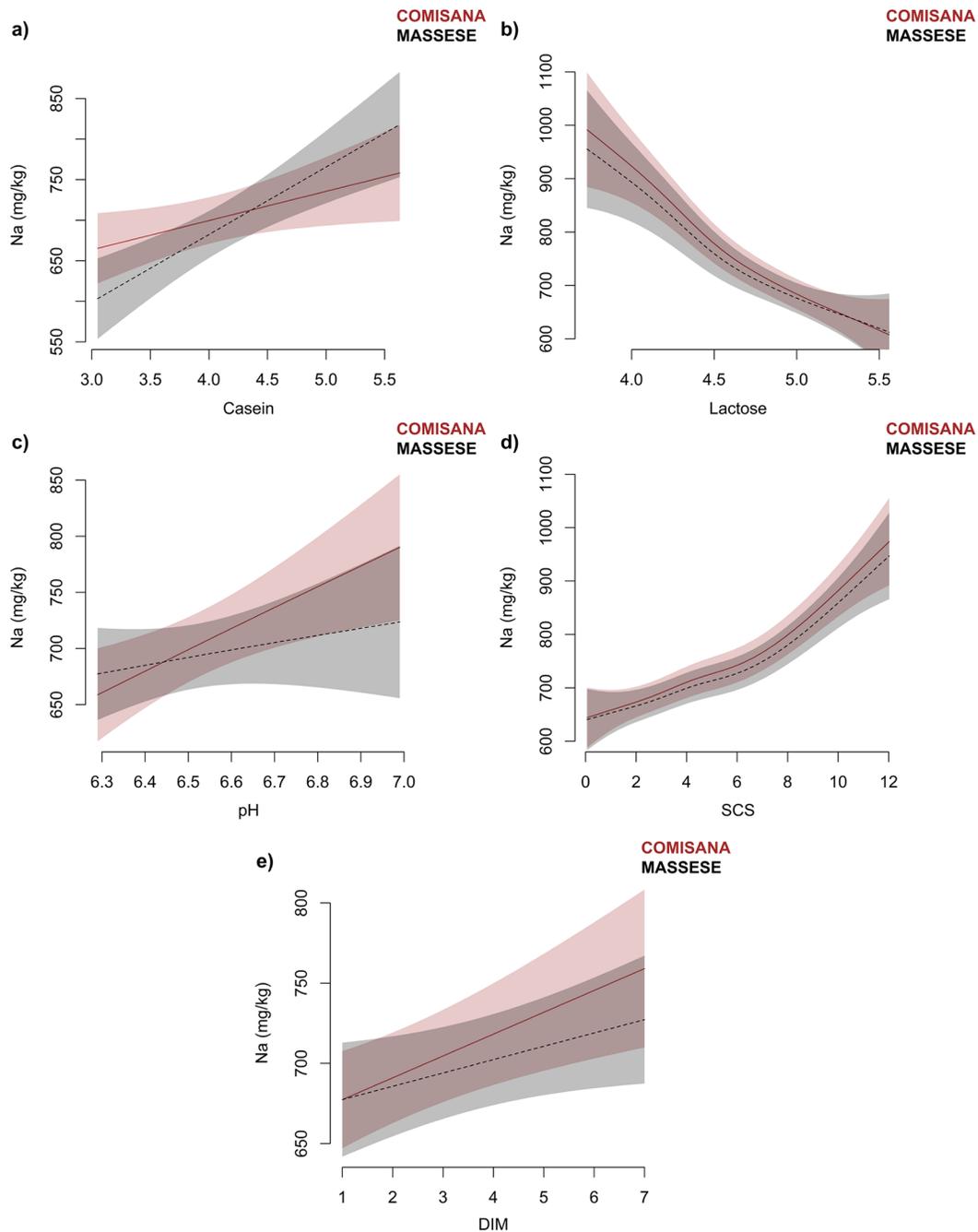
in the presence of an inflammatory process affecting the mammary gland. This condition is linked to an increased solubilization of casein and heightened proteolytic activity in milk (El Zubeir et al., 2005; Batavani et al., 2007). Notably, positive associations of Na with various milk components were identified in this study (Table 1 and Figure 6), except for lactose. In bovine milk, a positive association has been reported between natural Na content and fat, with no association with casein (Stocco et al., 2021). In caprine species, natural NaCl content showed a negative association with casein, protein, fat (Stocco et al., 2018), lactose, and pH, but a positive correlation with SCS and bacterial count (Stocco et al., 2019b). The increased osmotic pressure due to the high Na concentration in milk is counteracted by decreased lactose content (Figure 6b) and altered membrane permeability, potentially indicating an inflammatory status (e.g., high SCS; Figure 6d). These changes facilitate the flow of blood components into the milk, leading to an increase in milk pH (Figure 6c). These factors provide primary explanations for our findings, emphasizing the important dynamic role of Na in milk as a mineral element. Furthermore, the variation of Na throughout the lactation was significant ($P < 0.01$; Figure 6e), showing higher values when DIM increased. This pattern differs slightly from the observed cubic pattern in bovine species, characterized by an increase mainly during the middle phase of lactation (Stocco et al., 2019a). The disparities in milk Na variations between sheep and cattle may be attributed to various biological and physiological factors specific to each species, including milk composition and metabolic dynamics during lactation. Nevertheless, given that high Na concentrations in milk hamper the cheesemaking process, in particular reducing the recovery of protein in the curd (Stocco et al., 2021), understanding the factors that affect the variability of minerals can help to properly address the dairy sheep management.

Chloride

The content of Cl in milk averaged 952 ± 111 mg/kg (Supplemental Table S1), showing values of 961 ± 108 mg/kg and 937 ± 113 mg/kg for Comisana and Massese, respectively (data not shown). This mineral is 100% ionized in milk (Fox et al., 2015). As for K and Na, high content of this mineral in milk is associated with an inflammatory status of the mammary gland in dairy cattle (Batavani et al., 2007). Similarly, its potential involvement in inflammatory processes in dairy goats has been suggested by Tonin and Nader Filho (2002). Chloride ions are indeed essential electrolytes that contribute to various physiological processes, including those related to the immune system, such as phagocytosis and generation of reactive oxygen species within immune cells
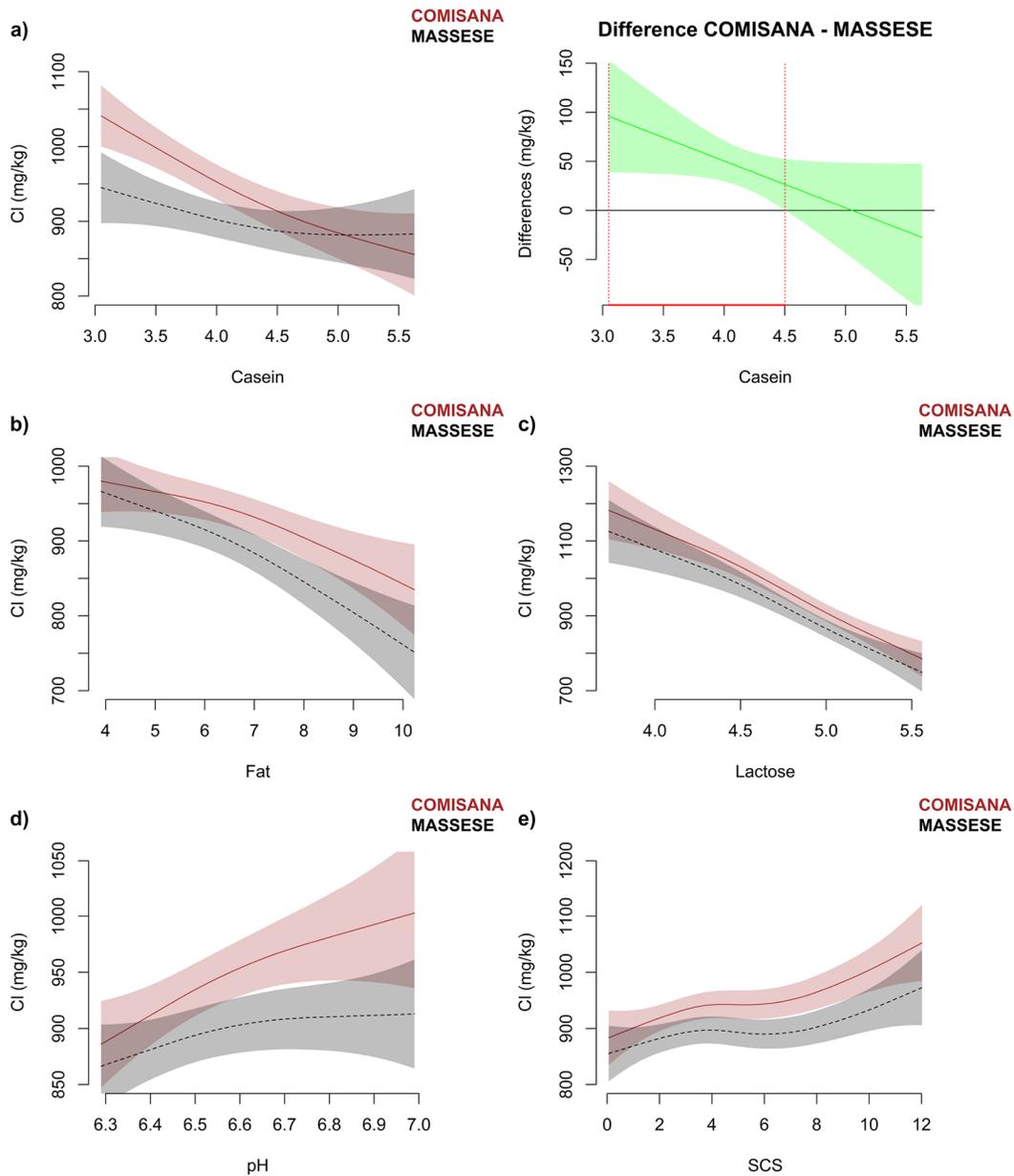
Figure 5. Potassium trajectory across percent of casein (a), fat (b), lactose (c), pH (d), SCS (e), and DIM (f) in Massese (dotted red line) and Comisana (solid black line) lactating ewes, and estimated differences (green solid line and related shaded area) for casein and DIM effects. Shaded regions identify the 95% CI for smooth terms for each breed. The vertical red dotted lines mark the interval where the differences are significantly different from zero.

Figure 6. Sodium trajectory across percent of casein (a), lactose (b), pH (c), SCS (d), and DIM (e) in Massese (dotted red line) and Comisana (solid black line) lactating ewes. Shaded regions identify the 95% CI for smooth terms for each breed.

(Al-Shehri, 2021). However, despite the importance of Cl in milk, there is a noticeable scarcity of studies focusing on this mineral in ovine species. The negative association of Cl with casein was observed in this study, and it was noteworthy that this relationship varied between breeds, as depicted in Figure 7a. In particular, the smooth function of GAMM detected a significant ($P < 0.05$) difference in Cl content in the milk of Comisana and Massese ewes, when casein ranged from 3.0% to 4.5%. A similar negative pattern was evident when milk Cl was associated with fat (Figure 7b) and lactose (Figure 7c) contents. Furthermore, we observed a positive

Figure 7. Chloride trajectory across percent of casein (a), fat (b), lactose (c), pH (d), and SCS (e) in Massese (dotted red line) and Comisana (solid black line) lactating ewes, and estimated differences (green solid line and related shaded area) for casein effect. Shaded regions identify the 95% CI for smooth terms for each breed. The vertical red dotted lines mark the interval where the differences are significantly different from zero.

association between Cl levels and pH (Figure 7d), as well as with SCS (Figure 7e). In small ruminants, the SCS is influenced by numerous noninfectious factors, including the stage of lactation (Puggioni et al., 2020). However, if the SCS increases because of an infection, the relative increase in polymorphonuclear against all other cell types leads to a corresponding increase in cathelicidins (Puggioni et al., 2020). In the latter case, probably due to the hydrolysis of Cl ions resulting from cellular damage,

which generates basic ions (e.g., hydroxide ions), the increase in Cl levels can shift the equilibrium toward the basic side, increasing the pH of the milk. The complex interactions between different milk components and Cl levels highlight the diverse functions of this bioactive substance in determining the characteristics of milk in ovine species. These findings emphasize the potential of Cl in milk as a marker for udder health, particularly in relation to SCS. Monitoring Cl content could help dairy

producers identify early signs of udder inflammation or infection, allowing for timely intervention. Additionally, understanding the relationships between Cl and milk components such as casein, fat, and lactose can guide producers in optimizing milk quality for cheese production or other dairy products, where mineral composition plays a significant role in texture and yield.

CONCLUSIONS

This study provided valuable insights on the variability of macrominerals in sheep milk by using GAMM and examining the trajectory of each element across factors such as breed, MY, parity, and DIM, as well as across various concentrations of major milk components and their interactions. The dynamic aspect of milk minerals content was highlighted by the temporal variability (e.g., dietary changes, environmental fluctuations, physiological changes) in sheep throughout time, as well as by the synergic or antagonistic effect of the interactions between milk components and parametric terms. Our findings clarified the complex relationships between these variables, contributing to a deeper understanding of the composition and variability of macrominerals in sheep milk. Additional research is needed to improve modeling techniques and accuracy including the aspects related to animal health, milk coagulation, and cheesemaking.

NOTES

Nonstandard abbreviations used: CCP = colloidal calcium phosphate; EDF = effective degrees of freedom; GAMM = generalized additive mixed models; MY = milk yield.

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