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INTERPRETIVE SUMMARY

The mineral profile affects the coagulation pattern and cheese-making efficiency of bovine milk. *By Stocco et al., page 000*. This study investigated the effects of the native contents of Ca, P, Na, K, and Mg on the coagulation process and cheese-making traits of individual bovine milk samples. Simultaneous inclusion in the statistical model of the fat, casein, and mineral contents allowed us to assess the specific effects of each mineral. Calcium and P positively affected the coagulation pattern and cheese-making traits, although high P concentrations led to lower fat recovery in the curd. High Na content only mildly affected coagulation, but reduced protein recovery in the curd. High Mg content slowed the coagulation process and lowered cheese yield measures.

The mineral profile affects the coagulation pattern and cheese-making efficiency of bovine milk

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ABSTRACT

Natural variations in milk minerals, their relationships, and their associations with the coagulation process and cheese-making traits present an opportunity for the differentiation of milk destined to high quality natural products, such as traditional specialties or Protected Designation of Origin (PDO) cheeses. The aim of this study was to quantify the effects of the native contents of Ca, P, Na, K, and Mg on 18 traits describing traditional milk coagulation properties (MCP), curd firming over time (CF_t) equation parameters, cheese yield (CY) measures, and nutrient recoveries in the curd (REC) using models that either included or omitted the simultaneous effects of milk fat and casein contents. The results showed that, by including milk fat and casein and the minerals in the statistical model, we were able to determine the specific effects of each mineral on coagulation and cheese-making efficiency. In general, about two thirds of the apparent effects of the minerals on MCP and the CF_t equation parameters are actually mediated by their association with milk composition, especially casein content, while only one third of the effects are direct and independent of milk composition. In the case of cheese-making traits, the effects of the minerals were mediated only negligibly by their association with milk composition. High Ca content had a positive effect on the coagulation pattern and cheese-making traits, favoring water retention in the curd in particular. Phosphorus positively affected the cheese-making traits, in that it was associated with an increase in CY in terms of curd solids, and in all the nutrient recovery traits. However, a very high P content in milk was associated with lower fat recovery in the curd. The variation in the Na content in milk only mildly affected coagulation, while with regard to cheese-making, protein recovery was negatively associated with high concentrations of this mineral. Potassium seemed not to be actively involved in coagulation and the cheese-making process. Magnesium content tended to slow coagulation and reduce CY measures. Further studies on the relationships of minerals with casein and protein fractions could deepen our knowledge of the role of all minerals in coagulation and the cheese-making process.

Key words: minerals, coagulation, cheese yield, protein recovery, fat recovery.

INTRODUCTION

One of the main factors influencing the processing characteristics of milk is its composition (Troch et al., 2017). As fat and protein are the most important milk components for the dairy industry, they are widely included in milk quality payment systems of sheep and goat (Pirisi et al., 2007) and cattle milk (Sneddon et al., 2013), and also in the selection indices of several cattle breeds reared for dairy purposes (Ghiroldi et al., 2005; Miglior et al., 2005; Pryce et al., 2009). The importance of protein, especially casein, lies in its active influence on the coagulation pattern (i.e., it increases the speed of curd-firming and curd firmness) and on cheese-making ability [i.e., high cheese yield (**CY**)] of the processed milk (Verdier-Metz et al., 2001; Wedholm et al., 2006). In contrast, fat plays a passive role during the coagulation process, as fat globules are entrapped in the para-casein matrix (Fox et al., 2017a), and thus positively affects CY and the recovery of total solids and energy in the curd (Pazzola et al., 2019). Besides fat and protein, other milk components influence milk processing characteristics, such as lactose, and bacterial and somatic cell counts (Leitner et al., 2016; Bobbo et al., 2017). Minerals, despite representing a small proportion of milk composition (about 0.7%; Kaufmann and Hagemeister, 1987), also have a powerful influence in defining the structural characteristics and functional properties of casein micelles, and during milk coagulation and the other phases of the cheese-making process (Lucey and Fox, 1993; Amenu and Deeth, 2007). Depending on their nature (e.g., nanoclusters or crystalline) and distribution (e.g., soluble or micellar forms), they are differently involved in the processing of the milk. Several studies have dealt with artificial modification of the mineral balance of usually reconstituted whole or skimmed milk from bovine species, mainly by adding Ca or chelating agents, in order to improve its rheological properties (Cooke and McSweeney, 2014; Bauland et al., 2020). When a mineral is added, the overall salt equilibrium in the milk changes, so the specific effects of the individual minerals on the coagulation pattern cannot be quantified. In contrast, very few studies have investigated the influence of the native mineral profile of raw milk on processing characteristics

(Malacarne et al., 2014), and these deal mainly with the effects of Ca (Tsioulpas et al., 2007; Gustavsson et al., 2014; Akkerman et al., 2019). The main issue of those studies is related to the fact that the native content of a given mineral in milk is not independent from the other minerals and milk components, and that a specific coagulation or cheese-making property is often the result of a sum of actions and interactions of different milk minerals and nutrients. Natural variations in milk minerals, and their relationships with each other and with coagulation and the cheese-making process present an opportunity for the differentiation of milk for the production of high quality natural products, such as Protected Designation of Origin (PDO) cheeses, where production specifications and restrictions prohibit milk treatments and the addition of minerals before and during cheese making. In this scenario it is important to characterize the milk supply for the native mineral profile, also considering that such studies at individual animal level are beneficial to possible genetic improvement of dairy populations for milk quality.

Moreover, the content of some minerals in milk is highly correlated with other milk components, particularly casein (Lucey and Horne, 2009). This means that if these minerals are not included in the statistical models some of their effects on coagulation and cheese-making traits as reported in the literature will be confounded with the effects of milk composition, particularly the casein content, and vice versa. However, as far as we know, none of the studies published so far on this topic has considered the simultaneous effects of milk composition and mineral contents on cheese-making efficiency. The inclusion of casein in the statistical model is particularly important for minerals such as Ca and P, as they vary in proportion to the casein content of milk. As a result, the true effects of minerals on traditional coagulation properties (**MCP**) are still unclear, and their effects on curd firmness over time (**CF$_t$**) equation parameters (obtained from modeling individual curd firmness values recorded with a lactodynamograph; Bittante et al., 2013), and on cheese-making traits aside from traditional CY, such as

CY expressed as the cheese solids and water retained in the curd, and milk nutrient recoveries in the curd (**REC**; Cipolat-Gotet et al., 2018), are completely unknown.

The aim of this study, therefore, was to quantify the effects of Ca, P, Na, K, and Mg on 18 traits describing traditional MCP, CF_t equation parameters, different CY measures and REC traits using models either including or omitting the simultaneous effects of the main milk components (fat and casein).

MATERIALS AND METHODS

Experimental Design: Selection of Herds and Cows

This study is part of a research project (Cowplus project) aimed at quantifying the effects of different dairy breeds and farming systems, while avoiding confounding them, on milk coagulation properties and cheese-making efficiency (Stocco et al., 2017 and 2018). For the present study, we selected 27 multi-breed farms representing the different farming systems in the Trentino-Alto Adige region (north-eastern Italian Alps). Milk samples from 240 cows (at different parities and lactation stages) were analyzed for their mineral profiles. The cows belonged to six breeds, 3 specialized dairy: Holstein-Friesian (50 cows from 15 herds), Brown Swiss (50 cows from 16 herds), and Jersey (35 cows from 7 herds), and 3 dual-purpose: Simmental (35 cows from 11 herds), Rendena (34 cows from 8 herds) and Alpine Grey (34 cows from 9 herds). The herds were categorized as traditional farming system using summer pastures (n = 9), traditional without summer pastures (n = 11), traditional with silages (n = 2), and modern farming system using total mixed rations (n = 5). A detailed description of the types of farming system in the study area can be found in Berton et al. (2020).

Milk Sampling and Analysis of Milk Composition and Mineral Profiles

Samples were taken from the cows once during the evening milking (2 L of milk/cow) to carry out analyses of the milk chemical components, mineral profiles, and processing characteristics (coagulation properties and cheese-making traits). Immediately after collection, the samples were stored at 4 °C and were analyzed within 24 h of the time of sampling. The fat, protein, casein, lactose and total solids contents of each milk sample were measured with a MilkoScan FT2 infrared analyzer (Foss Electric A/S), calibrated according to reference methods [ISO 1211/IDF for fat (ISO-IDF, 2010); ISO 8968-2/IDF 20-2 for protein (ISO-IDF, 2014); ISO 17997-1/IDF 29-1 for casein (ISO-IDF, 2004); ISO 26462/IDF 214 for lactose (ISO-IDF, 2010b); ISO 6731/IDF 21 for total solids (ISO-IDF, 2010a)].

Mineral contents (Ca, P, Na, K and Mg) were determined using a Spectro Arcos EOP ICP-OES (Spectro A.I. GmbH, Kleve, Germany). All instrument operating parameters were optimized for a 10% nitric acid solution as follows: axial plasma observation, Crossflow nebulizer, Scott Double Pass spray chamber, 3.0 mm diameter quartz injector torch, plasma power 1400 W, coolant gas 12.0 L/min, auxiliary gas 0.6 L/min, nebulizer gas 0.85 L/min, additional gas 0.20 L/min, sample uptake rate 2.0 mL/min, replicate read time 28 s, 3 replicates, pre-flush time 60 s. The milk samples were analyzed after microwave closed vessel digestion (Ethos 1600; Milestone S.r.l., Sorisole, BG, Italy). Subsamples of between 1.950 and 2.050 g of each milk sample were placed in a vessel with 2 mL of 30% hydrogen peroxide and 7 mL of concentrated (65%) nitric acid, both Suprapur quality (Merck Chemicals GmbH, Darmstadt, Germany). These sub-samples were subjected to microwave digestion as follows: Step 1, 25-200 °C in 18 min at 1500 W with P max 45 bar; Step 2, 200 °C for 15 min at 1500 W with P max 45 bar; Step 3, 200-110 °C in 15 min. After cooling to room temperature, the dissolved sample was diluted with ultrapure water (resistivity 18.2 M Ω cm at 25°C) to a final volume of 20 mL. Calibration standards were prepared using multi-element and single-element standard solutions (Inorganic Ventures Inc., Christiansburg, VA, USA) in 10% Suprapur nitric acid (Merck Chemicals GmbH, Darmstadt, Germany) to obtain matrices similar to the samples. Calibration solutions of the analytes were prepared at common concentrations of 0, 0.002, 0.005, 0.02, 0.05, 0.2, 0.5 and 2 mg/L, as well as further concentrations of 5, 20, 50 and 200 mg/L, respectively, of calcium, potassium, magnesium, sodium, and phosphorous. The accuracy and precision of these calibration solutions were tested by analyzing a blank solution, a low-level control solution (recovery limits ± 30%), a medium-level control solution (recovery limits ± 10%), and the international standard reference material BCR - 063R "Skim milk powder" [Institute for Reference Materials and Measurements (IRMM), Geel, Belgium], prepared as described above. The measured values and the certified values were in excellent agreement for all five minerals. Detailed macro- and micro-mineral profiles of these milk samples and the effects of dairy system, breed, parity, and lactation stage of the cows were reported in a previous study (Stocco et al., 2019a).

Traditional Milk Coagulation Properties

Milk coagulation properties were measured using a mechanical lactodynamograph (Formagraph, Foss Electric A/S, Hillerød, Denmark), with pendula calibration carried out before each session of the trial. Each sample (10 mL of milk) was heated to 35 °C, then mixed with 200 μL of rennet solution (Hansen Standard 215 with 80 ± 5% chymosin and 20 ± 5% pepsin; 215 international milk clotting units (IMCU)/mL; Pacovis Amrein AG, Bern, Switzerland) freshly diluted to 1.2% (wt/vol) in distilled water. Coagulation temperature was maintained at 35 °C and the duration of the analysis was 60 min. Traditional single-point measurements of each milk sample [rennet coagulation time (**RCT**; min), time interval between gelation and attainment of curd firmness of 20 mm ($\mathbf{k_{20}}$; min), and curd firmness at 30, 45 and 60 min after rennet addition ($\mathbf{a_{30}}$, $\mathbf{a_{45}}$, and $\mathbf{a_{60}}$, respectively, mm)] were obtained directly from the instrument.

Modeling the Coagulation Pattern

The Formagraph recorded the width (in mm) of the oscillatory graph of the pendulum submerged in the milk-filled wells every 15 s. Thus, 240 curd firmness (**CF**) values were recorded for each milk sample. A 4-parameter model was used to fit the CF over time values of each sample. This model, which uses all the information available for estimating the 4 coagulation parameters (Bittante et al., 2013), was as follows:

$$CF_t = CF_P \times [1 - e^{-k_{CF} \times (t - RCTeq)}] \times e^{-k_{SR} \times (t - RCTeq)}, \qquad [1]$$

where CF_t is curd firmness at time t (mm); **CF_P** is the asymptotic potential value of CF at an infinite time (mm); k_{CF} is the curd-firming instant rate constant (%/min); k_{SR} is the syneresis instant rate constant (%/min); and **RCT_{eq}** is RCT estimated by the CF_t equation on the basis of all data points (min). These parameters provide additional information to the traditional MCP, because i) CF_P is conceptually independent of test duration and does not depend on RCT (as a_{30} does); ii) **k_{CF}** describes the increase in curd firmness after RCT toward CF_P; iii) **k_{SR}** represents the expulsion of whey from the coagulum and describes the apparent decrease in curd firmness after RCT; soon after RCT the effect of k_{CF} prevails over the effect of k_{SR}, and the CF_t curve increases till its maximum firmness value (CF_{max}) is reached at a point in time (**t_{max}**) when the two effects are equal; after t_{max}, the effect of k_{SR} prevails over the effect of k_{CF}, and the CF_t curve declines asymptotically toward zero; iv) the RCT_{eq} has the same meaning as the traditional RCT, but is now estimated using all available data. To avoid convergence and estimation problems, the procedure described by Bittante et al. (2013) was modified to include curd firmness measurements up to 45 min from the addition of rennet, while CF_P was calculated by multiplying CF_{max} by 1.34, which is the coefficient resulting from the linear regression between CF_P and CF_{max} values obtained in a preliminary analysis. The other three CF_t model parameters (RCT_{eq}, k_{CF}, and k_{SR}) were estimated by curvilinear regression using the nonlinear procedure (PROC NLIN) in the SAS software (SAS Institute Inc., Cary, NC). The parameters of each individual equation were estimated using the

Marquardt iterative method (350 iterations and a 10^{-5} level of convergence), according to Bittante et al. (2013).

Model Cheese-Making and Related Traits

We used the individual cheese-making procedure described by Stocco et al. (2018) to measure CY and REC traits. Briefly, milk samples (1.5 L of milk/cow) were heated to 35 °C (30 min), then mixed with 8 mL of rennet solution [Hansen Standard 215 with 80 ± 5% chymosin and 20 ± 5% pepsin; 215 international milk clotting units (IMCU)/mL; Pacovis Amrein AG, Bern, Switzerland]. Gelation time was determined by visual observation of gelation of the milk with the aid of a spoon. Curd firming occurred at 50 °C (cooking phase, 20 min). The curd was cross-cut 10 min after gelation had occurred, then 10 min after cross-cutting the curd was separated from the whey (draining phase, 30 min). During the draining phase, the curd was gently pressed and turned over to facilitate whey expulsion. In the last 10 min of this phase, the curd was shaped into wheels in small cylindrical molds and was left in the whey. The model cheeses thus formed were pressed for 30 min, turning over every 10 min, and were then immersed in liquid brine for 30 min. The whey was analyzed for chemical composition (fat, protein, lactose, and total solids) with a MilkoScan FT2 infrared analyzer.

Cheese-making traits were calculated from the weights of the milk and whey (g) and their chemical compositions, as described by Cipolat-Gotet et al. (2018). Briefly, the traits measured were: $\mathbf{CY_{CURD}}$, $\mathbf{CY_{SOLIDS}}$, and $\mathbf{CY_{WATER}}$, calculated as the ratio of the weight (g) of fresh curd, curd dry matter, and curd water, respectively, to the weight of the milk processed (g); $\mathbf{REC_{PROTEIN}}$, $\mathbf{REC_{FAT}}$, and $\mathbf{REC_{SOLIDS}}$, calculated as the ratio of the weight (g) of the component (protein, fat, and dry matter, respectively) in the curd to the weight of the corresponding component in the milk (g). Recovery of energy in the curd ($\mathbf{REC_{ENERGY}}$) was determined by estimating the energy in the milk and in the curd using the equation proposed by NRC (2001) and converted into MJ/kg.

Statistical Analysis

The values of the 25 traits examined here (composition, mineral profile, coagulation, and cheese-making traits) outside the interval of the mean ±3 standard deviations (**SD**) were designated outliers and excluded. All traits were analyzed using two mixed linear models (MIXED procedure; SAS Institute Inc., Cary, NC). The first comprehensive linear mixed model (**M1**) was:

$$Y_{\mathrm{fghijklmnopqr}} = \mu + \mathrm{Herd}_f + \mathrm{Breed}_g + \mathrm{Parity}_h + \mathrm{DIM}_i + \mathrm{Ca}_j + \mathrm{P}_k + \mathrm{Na}_l + \mathrm{K}_m + \mathrm{Mg}_o + \mathrm{fat}_p + \mathrm{casein}_q + e_{fghijklmnopqr} \quad [2]$$

where $y_{\mathrm{fghijklmnopqr}}$ is the observed trait (fat, casein, Ca, P, Na, K, Mg, RCT, k_{20}, a_{30}, a_{45}, a_{60}, $\mathrm{RCT_{eq}}$, k_{CF}, k_{SR}, $\mathrm{CF_{max}}$, t_{max}, $\mathrm{CF_P}$, $\mathrm{CY_{CURD}}$, $\mathrm{CY_{SOLIDS}}$, $\mathrm{CY_{WATER}}$, $\mathrm{REC_{FAT}}$, $\mathrm{REC_{PROTEIN}}$, $\mathrm{REC_{SOLIDS}}$, $\mathrm{REC_{ENERGY}}$); μ is the overall intercept of the model; Herd_f is the random effect of the f^{th} herd (f = 1 to 27); Breed_g is the random effect of the g^{th} breed (g = Holstein-Friesian, Brown Swiss, Jersey, Simmental, Rendena and Alpine Grey); Parity_h is the fixed effect of the h^{th} parity (h = 1 to ≥3; 1st parity = 80 cows; 2nd parity = 59 cows; ≥3rd parity = 99 cows); DIM_i is the fixed effect of the i^{th} class of days in milk (i = 1 to 7; class 1 = 8-49 days, 25 cows; class 2 = 50-91 d, 27 cows; class 3 = 92-133 d, 39 cows; class 4 = 134-175 d, 42 cows; class 5 = 176-217 d, 43 cows; class 6 = 218-259 d, 32 cows; class 7 = >259 d, 30 cows); each mineral was included in classes according to quintiles based on its contents in the milk. Ranges of minerals per each quintile and the number of cows per each quintile is reported in Supplemental Table S1. Ca_j is the fixed effect of the j^{th} quintile of Ca (j = 1 to 5); P_k is the fixed effect of the k^{th} quintile of P (k = 1 to 5); Na_l is the fixed effect of the l^{th} quintile of Na (l = 1 to 5); K_m is the fixed effect of the m^{th} quintile of K (m = 1 to 5) Mg_o is the fixed effect of the o^{th} quintile of Mg (o = 1 to 5); fat_p is the fixed effect included in the model as a linear covariate; casein_q is the fixed effect included in the model as a linear covariate; $e_{fghijklmnopqr}$ is the random residual ~ N (0, σ_e^2). When fat, casein or one of the minerals in milk was considered a dependent variable, it was, of course, excluded from the model's independent variables.

A reduced version of model M1, named model **M2**, was obtained by excluding the fat and casein covariates. This model was used to carry out an auxiliary analysis to quantify the effects of the five minerals not corrected for fat and casein contents (i.e., the confounding effect of milk composition and mineral profile). The results obtained from the M2 model are not described and discussed analytically in this paper, but they are reported as Supplemental Table S2. Pearson's product-moment correlations were estimated among fat, casein, and the minerals, and are presented as supplemental material (Supplemental Figure S1).

RESULTS AND DISCUSSION

The rationale of this study relies on many aspects, among which the most important are: i) data are based on the comparison of individual milk samples of different characteristics in terms of composition, origin, farming system, breed and animals; ii) the results obtained are representative of many conditions, given that the experimental design and the statistical models adopted are able to avoid overlapping effects and multicollinearity; iii) the coagulation and cheese-making ability of milk in relation to the mineral content has never been studied before in terms of CF_t parameters (i.e., RCT_{eq}, k_{CF}, CF_{max}, t_{max}, k_{SR}, CF_P), different measures of CY (i.e., CY_{CURD}, CY_{SOLIDS}, CY_{WATER}), and REC traits (i.e., REC_{FAT}, $REC_{PROTEIN}$, REC_{SOLIDS}, REC_{ENERGY}). Beyond scientific relevance, we believe that the present study is important for the dairy industry, because the highest priced cheeses are often those protected by designations (like PDO by EU, or organic products) that forbidden any addition of chemicals during cheese-making.

Major Sources of Variation in Milk Fat, Casein and Mineral Contents

Table 1 reports the descriptive statistics and results of the analysis of variance of fat, casein, and minerals using the comprehensive model (M1). The effects of the six breeds, herds, parity, and DIM on the minerals were previously investigated and reported by Stocco et al. (2019a) using the same data, so they will not be discussed here. These factors, together with fat and casein, were of course included in the models to correctly quantify the effect of the minerals on the dependent variables. It is just worth noting that the importance of the effects of herd and breed of cow varied greatly according to the different traits: together they represented about half the total variance in the P content of milk, and only 13% in Ca and K (Table 1). Stage of lactation was very important for casein and Na contents, less important for Ca and P, and not significant for fat, K, and Mg contents, whereas the cow's parity affected only P, Na, and Mg.

As expected, fat and casein were associated with the mineral profile of milk. Milk fat affected the contents of Ca, Na, and Mg, but it was not in turn affected by any of the minerals (Table 1). Casein was much more interrelated with macro-minerals: it influenced all the minerals, except Na, and was in turn affected by all the minerals, except Na and Mg (Table 1).

Relationships among minerals were also observed: 8 out of 20 possible mineral-on-mineral effects were significant (Table 1). When we compared these results with those obtained from the model that did not include fat and casein (M2), summarized in Supplemental Table S2, we found differences in the relationships among the minerals, as also evidenced by the different number of significant mineral-on-mineral effects (13 out of 20), as summarized in Figure 1. This means that some of the mineral-on-mineral effects are most likely due to an indirect effect of milk gross composition, especially the casein content. In particular, the effects of Ca on K, Ca on Mg, P on Na, P on Mg, and Mg on P were significant in the model that did not correct for milk composition, but were no longer significant when milk fat and casein were taken into account. The other 8 mineral-on-mineral effects reported in Table 1 were still significant, although their effect tended to lessen after fat and casein correction.

Major Sources of Variation in Coagulation and Cheese-Making Traits

The statistical analyses of the traditional MCP and CF_t equation parameters are summarized in Table 2. After including in the model the breed, parity, and lactation stage of the cows, and the composition and mineral profile of the milk, the effect of herd on coagulation and curd firming traits was moderate (6.3 to 18.0% of total variance) for all traits, except for the curd firming (k_{CF}) and curd syneresis (k_{SR}) instant rate constants, which were almost unaffected by herd.

The effect of breed was even lower than that of herd (0.2 to 9.1%, Table 2), due to the inclusion of milk composition and mineral profile in the model, which explained a large part of the differences among breeds observed for these traits in a previous study (Stocco et al., 2017). The effects of parity and lactation stage after including milk composition and mineral profile were also smaller here compared to those reported by Stocco et al. (2017).

Milk fat content did not have a direct effect on milk coagulation, curd firming and syneresis, while casein, as expected, exerted quite a large influence on these traits. Casein favorably affected all traditional MCP, except RCT, as well as the CF_P (and CF_{max}) of the CF_t equation parameters (Table 2). As the casein content is interrelated with the milk mineral profile, as can be seen in Table 1, including it in the statistical model together with the minerals made it possible to interpret the results more accurately. It is worth noting that the minerals were significantly involved in 9 of the 25 possible effects on traditional MCP, and 10 of the 30 possible effects on the CF_t equation parameters when the experimental dataset was analyzed using the model that did not include the milk fat and casein covariates (Figure 1). However, the effect of some minerals remained significant after including milk composition: Ca content on RCT, a_{30}, RCT_{eq}, k_{CF}, and t_{max}, Na content on t_{max}, and Mg content on k_{20} (Table 2). This confirms that about two thirds of the apparent effects of minerals on MCP and the CF_t equation parameters are in fact mediated by their association with milk composition, especially casein content; only one third of the effects on these traits can be directly attributed to the minerals (particularly Ca) independently of milk composition.

Moving to cheese-making traits (Table 3), the herd effect was moderate (15.6 to 19.5% of total variance) for the three CYs and for REC_{SOLIDS}, and much smaller (3.1 to 6.6%) for the other recovery traits. Again, the effect of breed was much smaller (<7.0%), with the only exception of REC_{FAT} (10.8%). The effects of parity and lactation stage on cheese-making traits were never significant (Table 3), unlike in other studies where milk composition and mineral profile were not included in the statistical model (Cipolat-Gotet et al., 2013; Stocco et al., 2018).

As expected, milk fat and casein contents played an essential role in explaining the variability in the three CY measures and in the recovery traits, with the only exception of REC_{FAT} (Table 3). Nevertheless, the numbers of significant effects of milk mineral content on these traits changed little whether or not the milk fat and casein covariates were included in the statistical model (Figure 1). Although insoluble minerals (Ca and phosphate) associated with the para-casein matrix are known to influence %CY (Fox et al., 2017b), our results suggest that the effects of minerals on cheese-making traits are barely mediated by their association with the milk composition.

Calcium

Calcium is one of the most important minerals in milk. In the aqueous phase, Ca is present in ionic form, and is associated to citrate and inorganic phosphate to form calcium citrate and calcium phosphate, respectively. In the micellar phase, Ca is bound to phosphoseryl residues of casein molecules and inorganic phosphate (i.e., colloidal calcium phosphate, **CCP**). The presence of calcium phosphate clusters in the micelles is essential to the structure of the protein particles and to their technological functionality (Dalgleish and Corredig, 2012). In this study, Ca appeared to be the mineral with the greatest impact on milk quality and technological properties: 19 of the 23 traits studied were significantly affected by milk Ca content. This number decreased after milk fat and casein were also included in the model (Figure 2), but nonetheless remained substantial (11 out of 23 traits). Milk Ca was associated with fat and casein, and was also related to the contents of P and Mg (Supplemental Figure S1).

Figure 3 depicts the pattern of the CF_t equation parameters across different concentrations of Ca in milk. Clearly, the overall coagulation process improved at increasing levels of Ca in milk. In particular, coagulation time traits (RCT and RCT_{eq}) were shortened by about 4 min moving from the lowest (1,059 mg/L) to the highest (1,445 mg/L) average Ca concentration quintile. Curd firming was faster (about + 2%/min of k_{CF}), so that at 30 min the curd was also firmer (about +10 mm of a_{30}), and CF_{max} was reached faster (about -4 min of t_{max}) in milk samples with high compared with low Ca concentrations.

Our results are in agreement with those reported by Tsioulpas et al. (2007) for the effects of the natural mineral contents of 235 milk samples on casein micelle stability and some technological traits (i.e., RCT and coagulum firmness, measured by rheometer), and by Akkerman et al. (2019), who investigated the natural variation in Ca and citrate contents in skim milk in relation to RCT and the curd firming rate (measured by rheometer). Ketto et al. (2017) analyzed the correlations between Ca, P and Mg contents and coagulation properties (measured by a mechanical instrument) in 99 milk samples and found that Ca was associated negatively with RCT ($r = -0.21$, $P < 0.01$) and k_{20} ($r = -0.23$, $P < 0.01$), and positively with a_{30} ($r = 0.27$, $P < 0.001$), although the coefficients were low. Those authors used Pearson's correlations to assess only the linear relationships between minerals and coagulation properties, without correcting for any other affecting factor (i.e., herd, animal, and milk components).

Other studies have found several differences in the mineral contents of milk between samples exhibiting good and poor coagulation. In an investigation of the causes of non-coagulating milk from Danish-Holstein cows (n = 20), Frederiksen et al. (2011) found no differences in total Ca, P and Mg contents between well and poorly coagulating milk samples. In contrast, Jensen et al. (2012), also looking at the underlying causes of poorly coagulating milk from Holstein-Friesian and Jersey cows (n = 102), found some differences in the total, soluble and micellar fractions between milk samples exhibiting good and poor coagulation. They found that total and micellar Ca, and soluble and micellar P were higher in well than in poorly coagulating Jersey milk samples, while both total and micellar Ca and P, and micellar Mg were higher in well than in poorly coagulating Holstein-Friesian milk samples. However, those authors did not study the direct effects of each mineral on the coagulation properties of their samples, and therefore did not quantify them.

Milk Ca content also strongly affected CY measures and REC traits (Table 3). Fresh cheese yield was higher in milk samples with elevated Ca concentrations than in milk samples with low Ca (about +1% on an average of 15.7%, i.e., a favorable effect of +6%) (Figure 4a). This effect seems due mainly to the increased retention of water in the curd (CY_{WATER}), although the trends were rather cubic. Regarding the REC traits, a higher Ca content in milk also resulted in higher REC_{FAT} (about +5%) and REC_{SOLIDS} (+2%; Figure 4b), leading to a 3% higher REC_{ENERGY} (data not shown). Although not related to the native mineral content of milk, previous studies evidenced that the positive effects of the addition of $CaCl_2$ on the recovery of fat and protein and cheese yield were probably due to the increased aggregation of caseins (Fox et al., 2017b).

It is important to remember that our results for Ca are adjusted for the effects of fat and casein, the main factors influencing MCP, the CF_t equation parameters, and cheese-making traits (Bland et al., 2015; Pazzola et al., 2019; Cipolat-Gotet et al., 2020). Possibly, further understanding could be achieved by analyzing the mineral profile of standardized milk samples (e.g. fat to protein ratio) and by quantifying the effect of each mineral in milk samples with the same composition (Auldist et al., 2004). Moreover, since Bauland et al. (2020) confirmed that the soluble and colloidal forms of Ca are important in explaining the changes in the coagulation properties of milk, it would be interesting to assess the effect of each mineral form on coagulation and cheese-making properties of milk.

Phosphorus

Phosphorus is present in milk as organic (i.e., bound to casein) and inorganic phosphates (i.e., ions). Inorganic phosphates are equally distributed between the aqueous and micellar phases (i.e., CCP) at a milk pH of 6.7. In this study, P appeared to have the second largest impact on milk quality and technological properties after Ca. Thirteen of the 23 traits studied here were significantly affected by milk P concentrations when analyzed with the M2 statistical model, and the number of traits decreased to 7 when milk fat and casein were also included in the model (Figure 2). Milk P content was not associated with fat, but it was the mineral with the strongest association with casein content (Table 1). It should also be pointed out that P, Ca and K contents are mutually influential (Table 1).

The quantity of CCP and the number of phosphate groups in the casein micelle seem to influence the rennet coagulation of milk (Malacarne et al., 2014), as well as the interaction of caseins with CCP enhances the aggregation of the para-casein micelles (Bauland et al., 2020). However, in the present study, the P content of milk had no effect on either MCP nor the CF_t equation parameters when fat and casein were included in the model, but when they were not included, P showed significant associations with coagulation, curd firming and syneresis traits (Supplemental Figure S2b). This means that the effects of P sometimes reported in the literature were probably mediated by its strong association with casein. The effects of P on coagulation traits were not linear, since milk samples with P concentrations between 983 and 1,047 mg/L showed shorter RCT, faster k_{CF}, the highest k_{SR} and higher CF compared with both low and high milk P concentrations. Ketto et al. (2017) reported low linear correlation coefficients between P and some coagulation traits: -0.22 for k_{20}, and 0.22 for and a_{30} and gel firming rate. Gustavsson et al. (2014) observed a significant effect of P on the gelation time of 98 individual milk samples from Swedish Red cows. The non-linearity of the relationship between P and coagulation found in our study could be due to the several interactions between P and the other milk components and minerals, especially casein (i.e., organic phosphate linked to phosphoserine residues) and Ca (i.e., CCP). Jensen et al. (2012) reported differences in the proportions of soluble and micellar fractions of P between well and poorly coagulating milk samples from Jersey and Holstein-Friesian cows. In particular, micellar P was higher than soluble P in well compared with poorly coagulating Jersey milk samples, but in Holstein-Friesian cows they found only higher micellar P - but not lower soluble P - in well compared with poorly coagulating milk samples. The differences can probably be attributed to the different total casein contents and casein profiles of the two breeds. In fact, the cation binding ability of the casein fractions for the organic form of P decreases moving from α_{s2}, α_{s1}, β, to κ-casein, corresponding with their decreasing phosphoserine residues (Lucey et al., 2017). In fact, caseins and whey proteins are the main mineral-binding components in milk. For example, α_{s2} and α_{s1} caseins bind Ca and Fe; β-casein, α-Lactalbumin and β-Lactoglobulin bind Ca, Zn, Mg, Mn and Cu; lactoferrin binds Fe and Zn (Vegarud et al., 2000). A recent study on the detailed protein fractions of milk from 1,504 cows of the same breeds as in this study reported large differences among breeds in their protein profiles (caseins, whey proteins, and minor NPN compounds; Amalfitano et al., 2020). Because of the mineral-binding ability of casein and whey proteins, it would be very interesting to combine these data with data on minerals to further elucidate the effects of each single component and their interactions on coagulation and cheese-making traits.

Although P did not seem to be strictly associated with milk coagulation, it exerted a large effect on the cheese-making traits (Table 3 and Figure 5). Unlike Ca, which increased CY_{CURD} mainly through increased water retention, milk samples with elevated P concentrations showed higher $CY_{SOLIDS,}$ and CY_{CURD} followed the same trend, but it was not significant (Figure 5a). This effect was not linear, but quadratic, with the highest values being for milk samples in the 4th quintile (1,048-1,100 mg/L). This pattern is a clear consequence of two different trends observed in milk fat and protein recovery in the curd. Milk samples with intermediate concentrations of P (2nd, 3rd and 4th quintiles) showed higher REC_{FAT} (Figure 5b); $REC_{PROTEIN}$, on the other hand, followed a linear pattern, with the highest values corresponding with the highest concentrations of P in the milk (Figure 5b). As expected, REC_{SOLIDS} and REC_{ENERGY} followed a similar trend to CY_{SOLIDS}, with the highest values corresponding with the 4th quintile, and a slight decrease in the last quintile of P (Figure 5c). A possible explanation for these non-linear associations could lie in the interaction of P with the other milk components and minerals, especially Ca (formation of CCP). Calcium and inorganic phosphates are in dynamic equilibrium between the aqueous and micellar phases, and this equilibrium is influenced by the physico-chemical conditions of milk (e.g., pH, temperature), while modifications occurring between the aqueous and micellar phases affect the structure and stability of casein micelles (Gaucheron, 2013). This certainly affects the cheese-making process. For example, the lower REC_{FAT} observed at the highest P concentrations could be explained by excessive mineralization of the casein micelle (high CCP content), which determines a reduction in the phosphate groups of caseins, and, as a consequence, a reduction in the interaction between these groups and soluble ionic Ca during the second phase of the coagulation process (Malacarne et al., 2014). Similarly, an excess of phosphates in soluble form could sequester soluble ionic Ca, leading to a weak coagulum that is no longer able to retain fat globules in the casein network.

Sodium

Sodium is present in milk mainly in the aqueous phase, where it is free or weakly associated with ions of the opposite charge. Together with K and Cl, Na contributes to the ionic strength of milk (Gaucheron, 2013). Since it is in osmolar equilibrium between milk and blood, a higher milk Na concentration than normal is often indicative of an inflammatory process affecting the mammary gland, and is associated with increased solubilization of casein and proteolytic activity in milk (El Zubeir et al., 2005; Batavani et al., 2007). Five of the 23 traits we studied were significantly affected by Na concentration, whether or not fat and casein were included in the statistical model. Milk Na was negatively associated with fat, but was not associated with casein content (Table 1). Moreover, Na content was influenced by Mg, and affected K and Mg contents (Table 1).

The CF_t curves across different concentrations of Na in milk are illustrated in Figure 6. Although Na was significant only on t_{max}, it is interesting that both the lowest and the highest levels of Na were associated with delayed RCT and RCT_{eq}, and with the lowest a_{30} values. Milk samples with intermediate Na contents showed the most favorable coagulation and curd-firming patterns (Figure 6). The effect of the natural content of Na in milk on coagulation and curd firming was therefore not linear, but instead curvilinear.

Most of the previous studies have focused on the effect of adding NaCl to milk on the dissociation between Ca and P in the casein micelles (Lucey and Fox, 1993), and the coagulation properties of reconstituted (Sbodio et al., 2006) or fresh pasteurized milk (Awad, 2007). Awad (2007) showed that RCT slowed and CF decreased with increasing NaCl concentrations in milk. In this study, however, the high natural content of milk Na had only a marginal effect on coagulation, which could be due to the fact that we examined the native content instead of its addition, and investigated only the mineral Na and not the compound NaCl. The contribution of this mineral to coagulation and the cheese-making traits did not change when fat and casein were included in the statistical model (Figure 2). This is probably due to the fact that we did not sample any clinically mastitic cows, and sampled only a few cows with high somatic cell counts. The range of variation in Na here (281-488 mg/L) was much narrower than when mastitic milk was also included (El Zubeir et al., 2005; Batavani et al., 2007). In goats' milk, a high native content of NaCl (i.e., >319 mg/dL) impaired coagulation (i.e., slowed k_{20}, decreased CF traits, and inhibited syneresis; Stocco et al., 2019b) and the overall cheese-making process (Stocco et al., 2019c).

Regarding bovine cheese-making traits, native milk Na affected $REC_{PROTEIN}$ and REC_{SOLIDS}: The former was about 2% lower in milk samples with a high Na content compared with samples with a low Na content, although the trend was not linear, but quadratic, while the trend for REC_{SOLIDS} was rather erratic (Figure 7).

Potassium

Potassium is a monovalent ion contributing a quarter of the osmolality of bovine milk together with Na and Cl (Atkinson et al., 1995). Potassium balance closely interacts with glucose and electrolyte metabolism (Berg et al., 2017), and its concentration in milk is regulated mostly by secretion mechanisms in the mammary cell. The dairy industry's use of K salts (e.g., KCl) is aimed at reducing the Na content of cheese (Grummer et al., 2013), but this practice is generally not favored because the salts tend to impart a bitter flavor to the cheese. Bauland et al. (2020) reported that the addition of KCl to milk did not affect mineral partitioning between colloidal and soluble phase, neither the aggregation of casein micelles and curd firming. However, no studies are available on the effect of native milk K on coagulation and cheese making. Potassium interacts with casein and with the minerals P and Na, as can be seen in Table 1 and in Supplemental Figure S1. However, the correlation coefficient between K and casein was low, and indeed K has a weak affinity with caseins, as does Na (Le Graet and Brulé, 1993). According to our results, this mineral seemed not to have a specific role of its own during coagulation and the cheese-making process when fat, casein and the other minerals were included in the model (Tables 2 and 3, Figure 2). In fact, when fat and casein were not included in the model, it was found to affect k_{20}, a_{30}, k_{CF}, CY_{CURD}, and CY_{WATER}: all these traits worsened at increasing levels of K in the milk (Supplemental Table S2 and Supplemental Figure 2c). Given the general unfavorable association of K with casein and the concentrations of the other minerals, and that it was found to have an effect only after removing fat and casein from the model, we can speculate that the apparent contribution of K to coagulation and the cheese-making traits is instead attributable to casein and to the changes in the equilibrium of the other milk constituents and the overall mineral profile.

Magnesium

The technological importance of Mg in milk has been largely eclipsed by Ca, which plays an essential role in the structure and stability of casein micelles via CCP (Oh and Deeth, 2017). However, these two minerals act cooperatively during coagulation, as they have different coupling sites on casein, and, in particular, Ca aids the binding of Mg by making more casein sites available (Cuomo et al., 2011). Bauland et al. (2020) evidenced that after addition of $MgCl_2$, Mg was mainly exchanged with casein micelles through the bound form, whereas 70% of added Ca precipitated as CCP. In our study, we were able to disentangle the contribution of each mineral to coagulation and the cheese-making traits from the other minerals included in the model and milk composition. Unlike the other minerals, the effect of Mg was more evident when fat and casein were included in the statistical model (Figure 2). It seems that fat and casein, which are associated with Mg content (Table 1), masked the effect of this mineral. Magnesium was also influenced by Na content, and it affected Ca and Na (Table 1). Tables 2 and 3 show clearly that Mg had an effect on k_{20} and the three CY measures. In particular, moving from low to high levels of Mg in milk, a slight linear increase in k_{20} values was observed (more than 1 min difference between low and high Mg content). The results on the effect of Mg on coagulation traits reported in the literature are limited to the association between this mineral and the overall good or poor coagulation ability of milk. Ketto et al. (2017) reported weak associations between Mg and the gel firming rate (r = 0.18, $P < 0.01$) and gel firmness at 30 min (r = 0.22, $P < 0.01$); Frederiksen et al. (2011) did not find any differences in Mg content between well and poorly coagulating milk samples, but Jensen et al. (2012) did find some differences in the milk of Holstein Friesian cows.

Regarding cheese-making traits, high levels of Mg were associated with reduced CY_{SOLIDS} (about -0.2%) and CY_{WATER} (the trend here was erratic), that consequently tended to reduce the total CY_{CURD} (about -0.5%) (data not shown). The correlation coefficient between Mg and casein found in our study (r = 0.62, $P < 0.001$; Supplemental Figure S1) was similar to that between Mg and protein reported by Bijl et al. (2013) (r = 0.64, $P < 0.01$). It is interesting that this linear relationship was not accompanied by the same trend when Mg was associated with cheese-making traits. Since no published studies provide this type of information, we can only speculate that these results are related to different interactions with the other minerals (i.e., inorganic phosphates, Ca) and milk components (i.e., citrate, nanoclusters of casein micelles), and some of the enzymatic reactions in which Mg is involved (i.e., β-galactosidase, alkaline phosphatase activities; Rankin et al., 2010; Banerjee et al., 2018).

CONCLUSIONS

The results presented here provide new knowledge about the relationships between the mineral contents, coagulation ability and cheese-making traits of bovine milk. This novel knowledge is possible thanks to the experimental design used, the statistical approach employed (avoidance of overlapping effects and multicollinearity) and the phenotypes investigated (CF_t parameters, %CY measures, %REC traits). Simultaneous inclusion in the statistical model of the fat, casein and mineral fractions in the milk allowed us to investigate the specific effects of each mineral on coagulation and cheese-making efficiency. We found, in particular, that a high Ca content had a positive effect on both the coagulation pattern and cheese-making traits, favoring water retention in the curd. Phosphorus positively affected the cheese-making traits, increasing CY in terms of curd solids, and all the nutrient recovery traits, although a very high P content in milk was associated with less fat recovered in the curd. The variation in the Na content of milk only mildly affected coagulation, while protein recovery was negatively associated with high concentrations of this mineral, probably reflecting the association with sub-clinical mastitis. The role of K during coagulation and the cheese-making process seemed to be more passive and linked to milk composition and the overall milk salt equilibrium, while high Mg content tended to slow coagulation and reduce CY traits. Greater understanding of the dynamics of coagulation and cheese making could be gained by analyzing the relationships of these minerals to casein and the protein fractions, and by investigating the recovery of each mineral in the cheese. However, these findings are important for the dairy industry, in particular for cheeses whose productions prohibit any addition of chemicals to the milk during cheese-making, and preconize the possible genetic improvement of dairy populations for the native mineral profile.

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Wedholm, A., L.B. Larsen, H. Lindmark-Månssom, A. H. Karlsson, and A. Andrén. 2006. Effect of protein composition on the cheese-making properties of milk from individual dairy cows. J. Dairy Sci. 89:3296-3305.

TABLES AND FIGURES

Table 1. Descriptive statistics (mean ± SD) and analysis of variance of milk components (fat and casein) and of milk minerals (calcium, phosphorus, sodium, potassium and magnesium).

	Milk components, %		Minerals, mg/L				
	Fat	Casein	Ca	P	Na	K	Mg
Descriptive statistics:							
Mean	4.23	2.65	1,223	1,014	357	1,694	103
±SD	2.00	0.42	132	114	46	109	14
Random factors (%[1]):							
Herd	11.2	11.4	9.8	26.7	13.7	11.8	15.1
Breed	16.1	45.7	3.2	23.9	7.3	1.2	15.0
Fixed factors (*F-values*):							
DIM	0.8	6.5^{***}	3.0^{*}	2.5^{*}	4.0^{***}	2.0	2.0
Parity	0.5	0.5	1.8	6.7^{**}	19.9^{***}	0.7	9.0^{***}
Fat	-	0.3	4.5^{*}	0.3	7.6^{**}	3.3	5.5^{*}
Casein	1.5	-	19.9^{***}	56.5^{***}	0.2	25.4^{***}	9.1^{***}
Ca	0.7	3.9^{**}	-	5.4^{***}	1.2	1.6	2.3
P	1.1	13.4^{***}	6.3^{***}	-	2.4	5.2^{***}	1.3
Na	0.7	1.8	1.1	1.1	-	2.6^{*}	6.5^{***}
K	0.8	6.4^{***}	0.5	8.2^{***}	2.1	-	1.4
Mg	1.7	2.4	2.4^{*}	2.3	10.1^{***}	1.2	-
RMSE[2]	0.9	0.2	80.4	58.5	32.6	84.4	8.4

[1] The variance of each random factor is expressed as percentage of the sum of variances of all random factors (including residual variance); [2] RMSE = Root Mean Square Error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2. Descriptive statistics (mean ± SD) and analysis of variance of traditional milk coagulation properties (MCP) and of curd firming over time (CF_t) equation parameters.

	Traditional milk coagulation properties (MCP)[1]:					Curd firming (CF_t) equation parameters[2]:				
	RCT min	k_{20} min	a_{30} mm	a_{45} mm	a_{60} mm	RCT_{eq}, min	k_{CF} %/min	k_{SR} %/min	CF_P mm	t_{max} min
Descriptive statistics:										
Mean	15.8	3.58	45.2	51.5	51.6	16.0	9.15	0.74	73.1	46.8
±SD	5.4	1.63	16.3	12.5	12.0	5.42	2.68	0.26	15.6	9.2
Random factors (%[3]):										
Herd	12.6	8.4	18.0	12.6	11.4	13.5	3.1	0.5	13.3	6.3
Breed	9.1	0.2	6.9	1.0	0.6	9.5	4.0	3.2	1.5	7.7
Fixed factors (*F-value*):										
DIM	2.8^{*}	0.9	3.0^{**}	1.3	1.3	3.0^{**}	1.2	1.1	1.6	1.4
Parity	2.4	1.9	2.6	4.1^{*}	2.5	2.5	2.0	2.5	4.0^{*}	4.3^{*}
Fat	0.1	1.1	1.0	1.8	2.2	0.1	0.8	2.3	0.8	0.4
Casein	0.2	14.5^{***}	13.2^{***}	24.9^{***}	25.6^{***}	0.3	1.4	0.7	38.5^{***}	0.0
Ca	2.6^{*}	2.4	2.5^{*}	1.3	1.5	2.7^{*}	3.1^{*}	1.7	1.7	2.7^{*}
P	1.6	1.5	1.3	0.9	1.0	1.5	1.1	0.7	0.8	0.7
Na	2.0	1.9	2.0	0.5	0.5	2.2	1.3	1.4	0.3	3.1^{*}
K	1.5	2.2	1.4	0.4	0.9	1.7	1.6	0.9	0.7	1.7
Mg	0.6	2.5^{*}	1.5	2.1	1.4	0.7	0.1	0.1	1.9	0.8
RMSE[4]	4.5	1.3	12.2	9.6	9.3	4.5	2.3	0.3	10.8	8.1

[1] RCT = measured rennet gelation time; k_{20} = time interval between gelation and attainment of curd firmness of 20 mm; a_{30}, a_{45} and a_{60} = curd firmness 30, 45 and 60 min after rennet addition;

[3] RCT_{eq} = rennet coagulation time estimated by CF_t modeling; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_P = asymptotic potential curd firmness; t_{max} = time at achievement of maximum curd firmness (CF_{max}).

[3] The variance of each random factor is expressed as percentage of the sum of variances of all random factors (including residual variance);

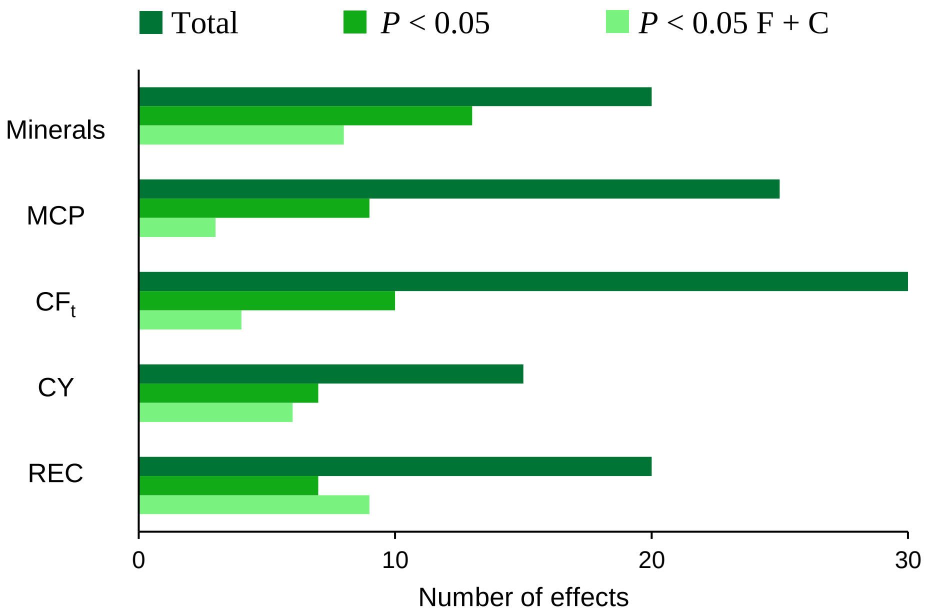
[4] RMSE = Root Mean Square Error. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 3. Descriptive statistics (mean±SD) and analysis of variance of cheese yield (CY) measures and nutrient recovery traits (REC).

	Cheese yield (CY), %			Nutrients recovery (REC), %			
	CY_{CURD}	$\text{CY}_{\text{SOLIDS}}$	CY_{WATER}	REC_{FAT}	$\text{REC}_{\text{PROTEIN}}$	$\text{REC}_{\text{SOLIDS}}$	$\text{REC}_{\text{ENERGY}}$
Descriptive statistics:							
Mean	15.7	8.42	7.24	85.1	79.4	53.4	69.0
±SD	3.0	1.69	1.38	4.32	1.9	5.0	4.2
Random factors (%[1]):							
Herd	15.8	15.6	19.5	6.6	3.1	15.7	6.6
Breed	6.1	6.2	1.7	10.8	3.9	2.3	7.0
Fixed factors (*F-value*);							
DIM	0.6	0.6	0.4	1.1	0.7	0.5	0.8
Parity	0.3	0.2	0.6	1.8	0.7	0.2	0.6
Fat	286.4***	1891.5***	26.6***	2.0	8.6**	344.6***	153.8***
Casein	169.0***	248.0***	102.4***	1.4	8.8**	55.8***	9.6**
Ca	3.8**	2.4	3.3*	4.0**	1.5	2.5*	3.9**
P	0.7	3.6**	0.2	4.4**	4.0**	3.6**	3.9**
Na	1.7	1.1	1.5	2.1	8.9***	2.6*	1.9
K	1.5	0.9	1.6	1.3	1.3	0.8	0.8
Mg	3.1*	2.6*	2.5*	1.2	1.0	2.4	2.1
RMSE[2]	0.8	0.2	0.7	3.6	1.4	1.8	2.1

[1] The variance of each random factor is expressed as percentage of the sum of variances of all random factors (including residual variance); [2] RMSE = Root Mean Square Error. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 1.



Minerals: milk content of Ca, P, Na, K and Mg (5 traits);
MCP: milk traditional coagulation properties (5 traits);
CF_t: parameters of the curd firming equation (6 traits);
CY: cheese yields (3 traits);
REC: recovery of milk nutrients in the curd (4 traits).

Figure 2.

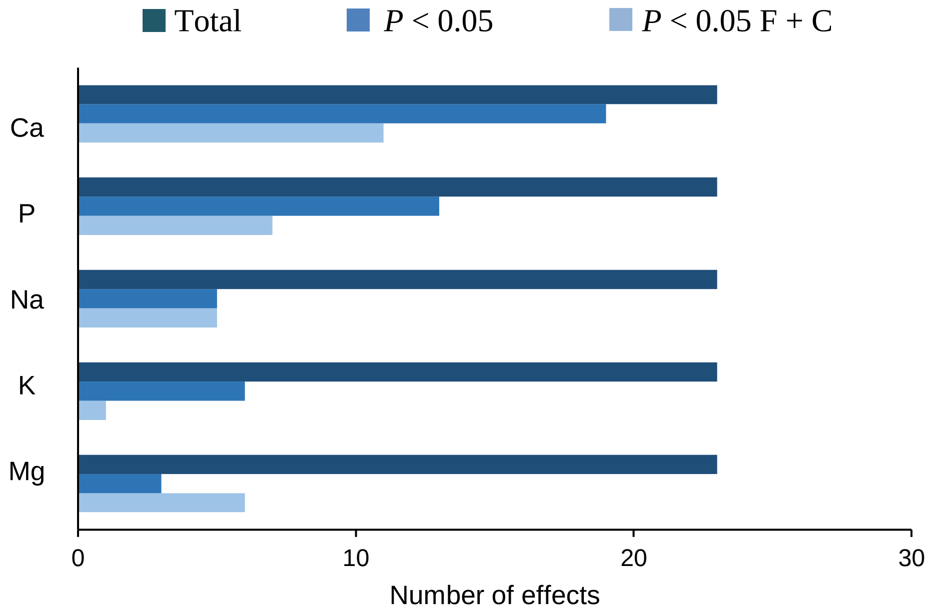


Figure 3.

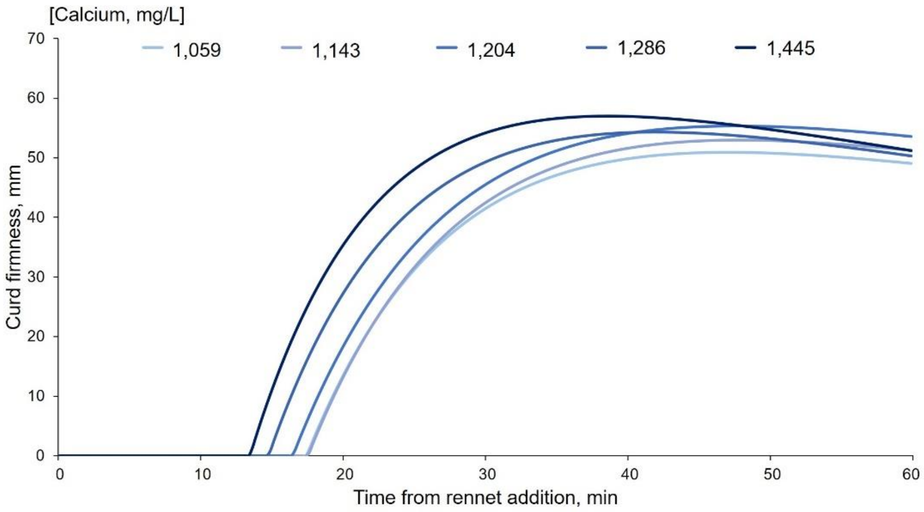

[Calcium, mg/L]
1,059
1,143
1,204
1,286
1,445
Curd firmness, mm
0
10
20
30
40
50
60
70
Time from rennet addition, min
0
10
20
30
40
50
60

Figure 4.

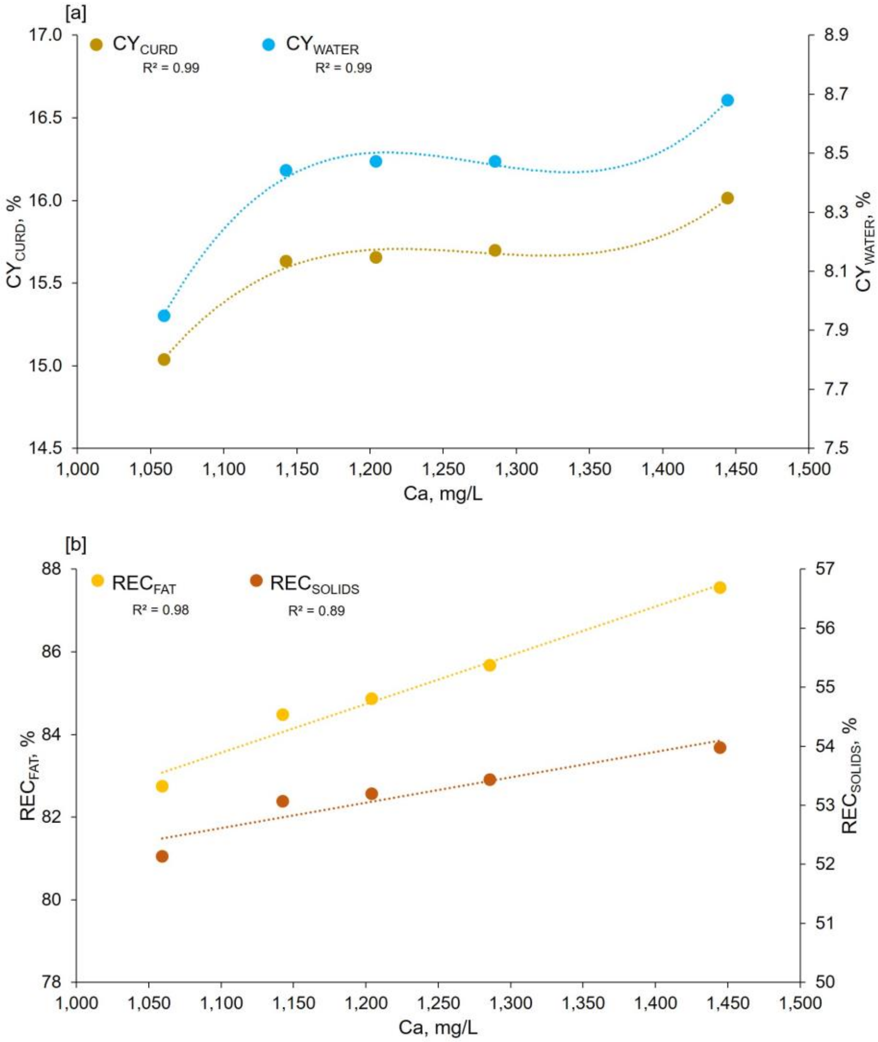

[a]
CY_CURD
R² = 0.99
CY_WATER
R² = 0.99
CY_CURD, %
CY_WATER, %
17.0
16.5
16.0
15.5
15.0
14.5
8.9
8.7
8.5
8.3
8.1
7.9
7.7
7.5
1,000
1,050
1,100
1,150
1,200
1,250
1,300
1,350
1,400
1,450
1,500
Ca, mg/L
[b]
REC_FAT
R² = 0.98
REC_SOLIDS
R² = 0.89
REC_FAT, %
REC_SOLIDS, %
88
86
84
82
80
78
57
56
55
54
53
52
51
50
1,000
1,050
1,100
1,150
1,200
1,250
1,300
1,350
1,400
1,450
1,500
Ca, mg/L

Figure 5.

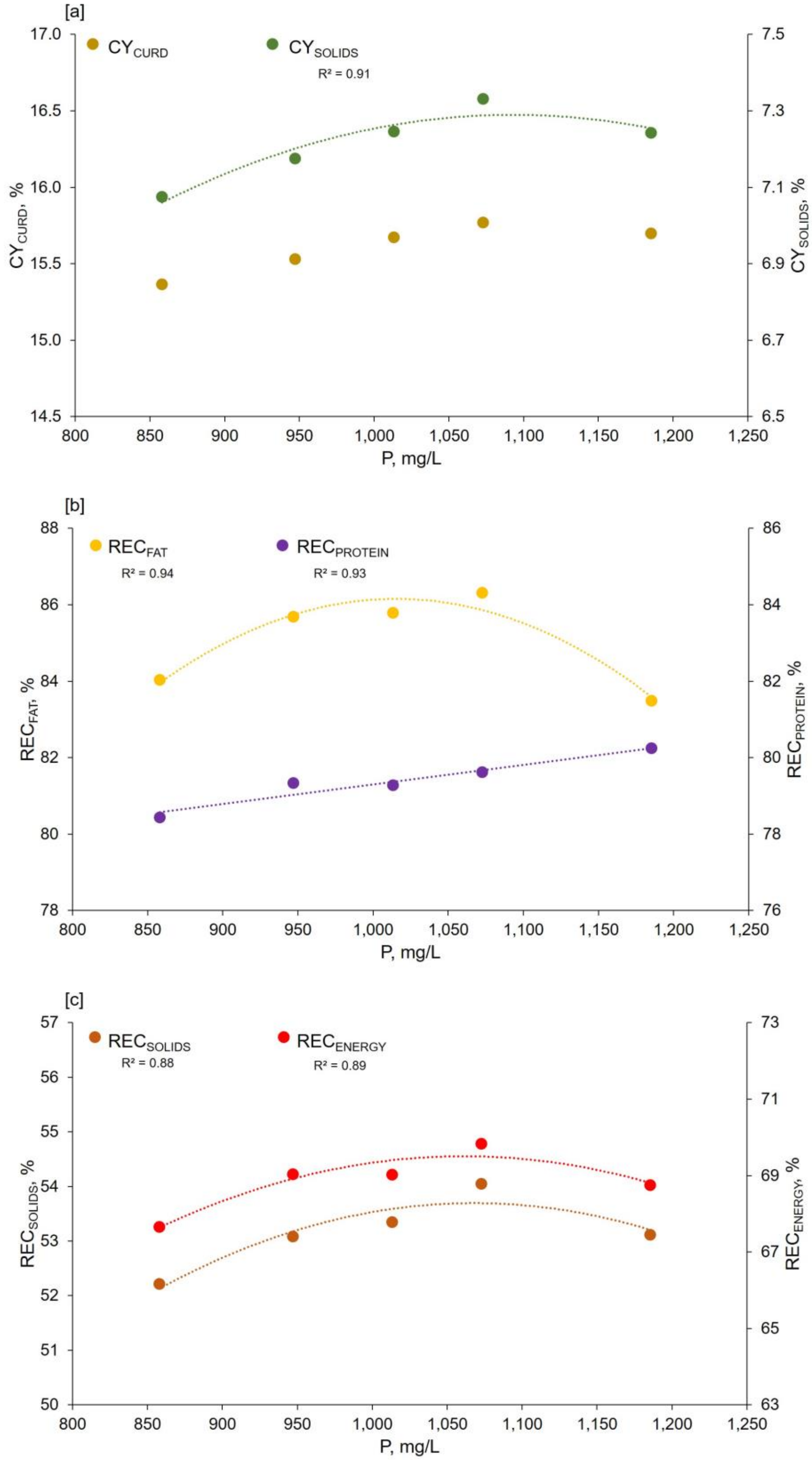

[a]
CY_{CURD}
CY_{SOLIDS}
R² = 0.91
CY_{CURD}, %
CY_{SOLIDS}, %
17.0
16.5
16.0
15.5
15.0
14.5
7.5
7.3
7.1
6.9
6.7
6.5
800
850
900
950
1,000
1,050
1,100
1,150
1,200
1,250
P, mg/L
[b]
REC_{FAT}
R² = 0.94
$REC_{PROTEIN}$
R² = 0.93
REC_{FAT}, %
$REC_{PROTEIN}$, %
88
86
84
82
80
78
86
84
82
80
78
76
800
850
900
950
1,000
1,050
1,100
1,150
1,200
1,250
P, mg/L
[c]
REC_{SOLIDS}
R² = 0.88
REC_{ENERGY}
R² = 0.89
REC_{SOLIDS}, %
REC_{ENERGY}, %
57
56
55
54
53
52
51
50
73
71
69
67
65
63
800
850
900
950
1,000
1,050
1,100
1,150
1,200
1,250
P, mg/L

Figure 6.

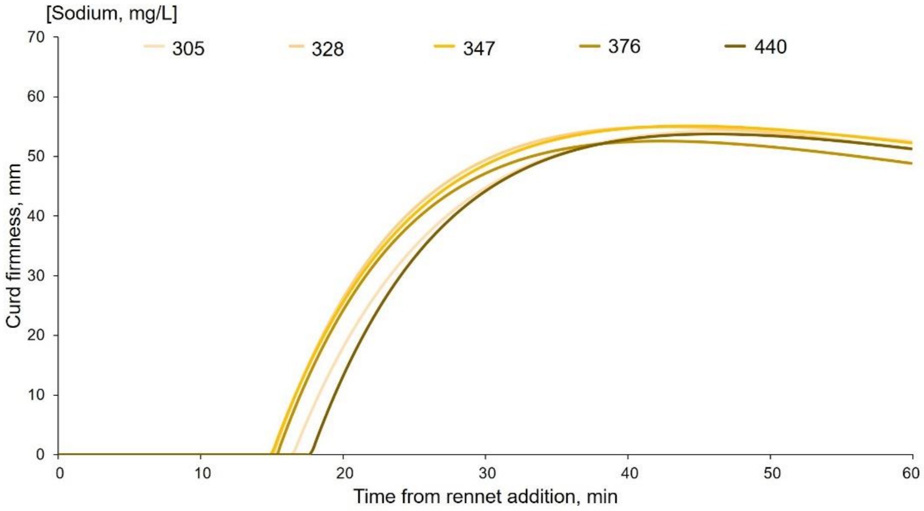
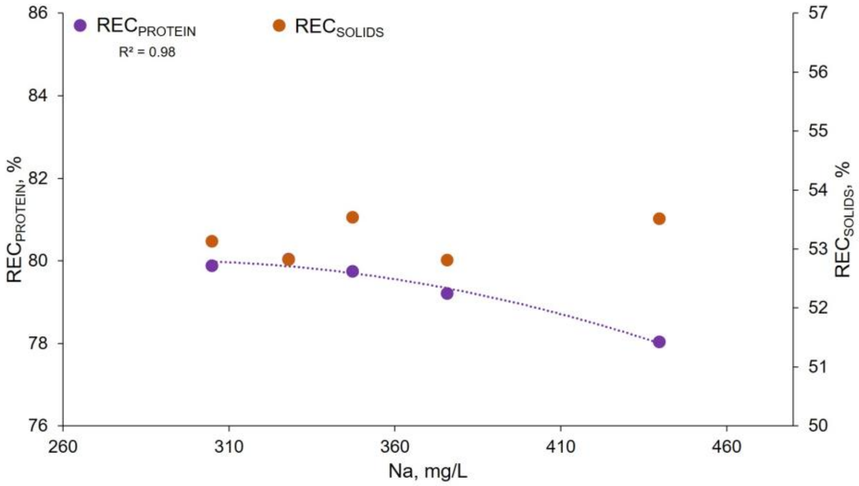

[Sodium, mg/L]
305
328
347
376
440
Curd firmness, mm
0
10
20
30
40
50
60
70
Time from rennet addition, min
0
10
20
30
40
50
60

Figure 7.



REC$_{PROTEIN}$
R² = 0.98
REC$_{SOLIDS}$
REC$_{PROTEIN}$, %
REC$_{SOLIDS}$, %
Na, mg/L
86
84
82
80
78
76
57
56
55
54
53
52
51
50
260
310
360
410
460

Figure captions

Figure 1. Total number of effects of minerals on minerals and milk technological traits tested, number of effects significant in the base model ($P < 0.05$), and number of effects significant in the model including the covariate of milk fat and casein contents ($P < 0.05$ F+C).

Figure 2. Total number of effects of each individual mineral on the 23 milk technological traits tested, number of effects significant in the base model ($P < 0.05$), and number of effects significant in the model including the covariate of milk fat and casein contents ($P < 0.05$ F+C).

Figure 3. Effect of Ca on gelation, curd-firming and syneresis of individual milk samples. Mineral concentrations are reported as mean values (mg/L) of each quintile of the distribution.

Figure 4. Effect of Ca on CY_{CURD} and CY_{WATER} [a], REC_{FAT} and REC_{SOLIDS} [b] of individual milk samples, and the coefficient of determination (R^2) of the regression.

Figure 5. Effect of P on CY_{CURD} and CY_{SOLIDS} [a], REC_{FAT} and $REC_{PROTEIN}$ [b], REC_{SOLIDS} and REC_{ENERGY} [c] of individual milk samples, and the coefficient of determination (R^2) of the regression.

Figure 6. Effect of Na on gelation, curd-firming and syneresis of individual milk samples. Mineral concentrations are reported as mean values (mg/L) of each quintile of the distribution.

Figure 7. Effect of Na on $REC_{PROTEIN}$ and REC_{SOLIDS} of individual milk samples, and the coefficient of determination (R^2) of the regression.

SUPPLEMENTAL MATERIAL

Supplemental Table S1. Ranges of mineral contents per each quintile of their distribution. The number of cows per each quintile is reported superscript in parentheses.

Mineral	Quintile, mg/L				
	1st	2nd	3rd	4th	5th
Ca	944-1,110 $^{(47)}$	1,111-1,168 $^{(48)}$	1,169-1,242 $^{(48)}$	1,243-1,331 $^{(48)}$	1,334-1,594 $^{(46)}$
P	741-909 $^{(47)}$	911-982 $^{(48)}$	983-1,047 $^{(48)}$	1,048-1,100 $^{(48)}$	1,103-1,329 $^{(46)}$
Na	281-320 $^{(47)}$	321-337 $^{(48)}$	338-357 $^{(48)}$	358-395 $^{(48)}$	397-488 $^{(44)}$
K	1,375-1,599 $^{(46)}$	1,602-1,671 $^{(48)}$	1,672-1,724 $^{(48)}$	1,725-1,789 $^{(48)}$	1,792-1,975 $^{(46)}$
Mg	70-91 $^{(47)}$	92-99 $^{(48)}$	100-106 $^{(48)}$	107-114 $^{(48)}$	115-143 $^{(44)}$

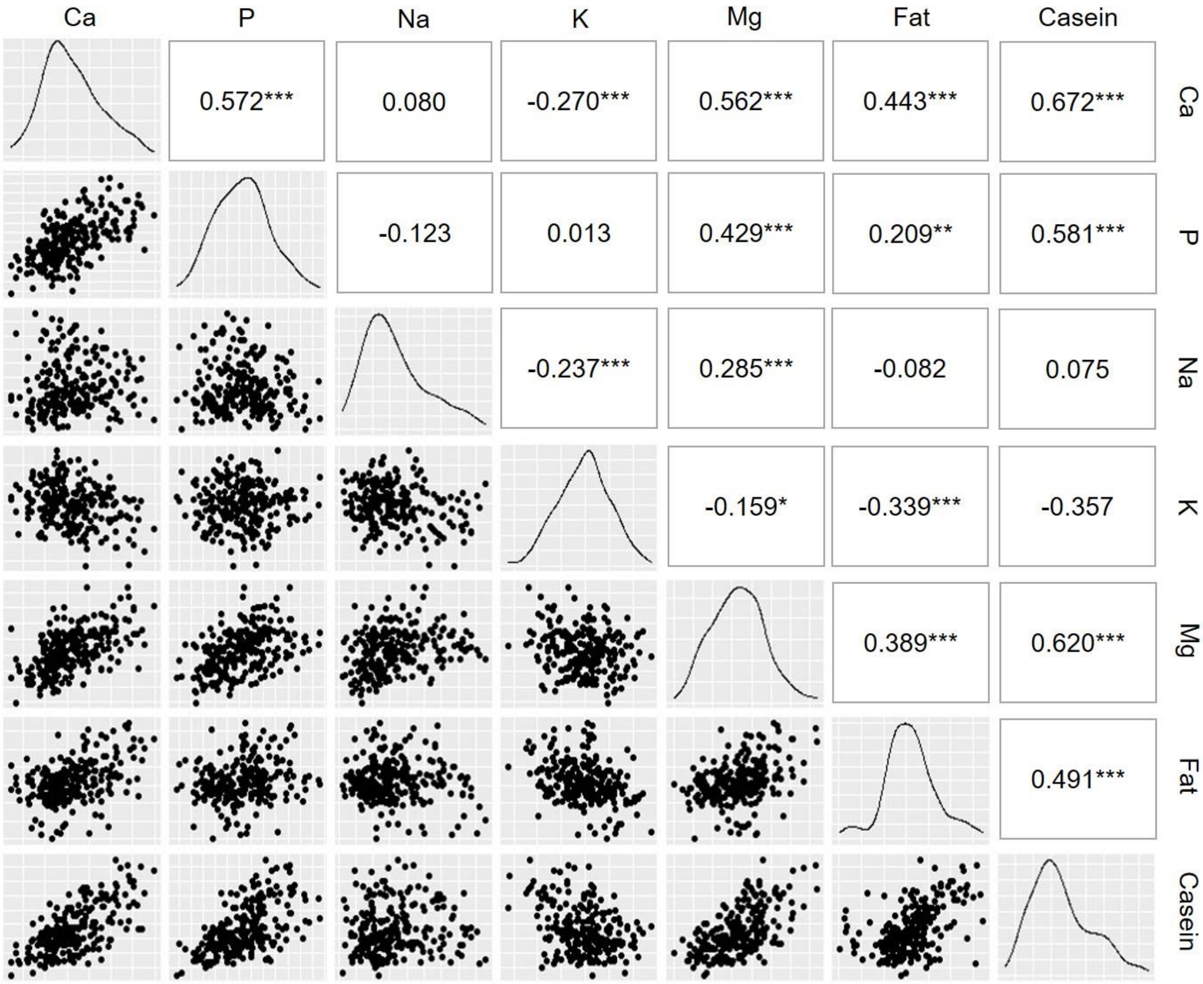
Supplemental Table S2. Analysis of variance from model M2 (no fat and casein; contemporary inclusion of minerals) for fat, casein, mineral contents, coagulation and cheese-making traits with *F*-value and significance for fixed factors and the proportion of variance (in percentage) explained by random factors.

	Random factors (% on total variance)		Fixed factors (*F*-value and significance)							RMSE[1]
Trait	Herd	Breed	DIM	Parity	Ca	P	Na	K	Mg	
Milk components, %										
Fat	11.9	20.1	0.8	0.5	0.9	0.8	0.6	0.9	1.7	32.8
Casein	13.1	43.7	6.8***	0.2	4.3**	13.2***	1.6	6.9***	2.2	57.2
Minerals, mg/L										
Ca	8.55	17.1	2.8*	1.8	-	13.2***	1.7	2.3	3.8*	83.5
P	32.3	11.4	2.3*	5.7**	12.0***	-	2.2	3.8**	3.5**	65.8
Na	11.5	12.5	3.9**	18.2***	1.8	2.9*	-	1.8	9.1***	33.2
K	10.6	10.4	3.3**	0.4	3.1*	2.7*	2.5*	-	0.4	88.2
Mg	20.7	5.94	3.7**	8.5***	4.6**	3.2*	6.5***	0.9	-	8.42
Traditional MCP[2]										
RCT, min	13.2	8.60	3.4**	2.8	2.7*	1.5	2.2	1.5	0.7	4.45
k_{20}, min	7.09	2.92	0.2	1.6	4.0**	2.8*	2.0	3.6**	2.2	1.36
a_{30}, mm	15.7	8.18	1.9	2.3	3.8**	1.5	2.0	2.9*	0.8	12.6
a_{45}, mm	10.8	5.53	0.8	3.0	2.8*	3.7**	0.4	1.6	1.4	10.0
a_{60}, mm	10.5	4.67	1.5	1.8	2.4	4.2**	0.3	1.7	0.9	9.70
CF_t parameters[3]										
RCT_{eq}, min	14.2	9.00	3.7**	2.9	2.7*	1.5	2.4	1.6	0.8	4.43
k_{CF}, %/min	5.26	4.10	0.8	2.3	4.5**	0.7	1.5	2.5*	0.1	2.32
k_{SR}, %/min	2.46	4.47	0.9	2.7	2.4*	0.7	1.3	1.4	0.1	0.25
CF_P, mm	10.2	9.77	1.4	2.8	3.4*	4.4**	0.2	2.4	1.1	11.5
CF_{max}, mm	10.2	9.77	1.4	2.8	3.4*	4.4**	0.2	2.4	1.1	8.56
t_{max}, min	7.44	6.07	1.4	5.2**	3.3*	0.6	3.5**	2.1	0.9	8.03

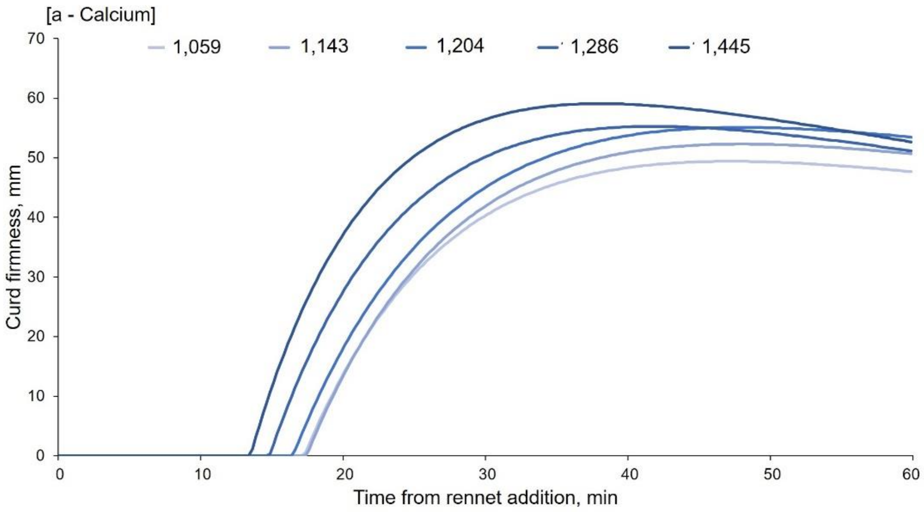
Cheese yields, %										
CY$_{CURD}$	21.6	34.9	3.5**	0.1	6.5***	2.9*	0.8	3.4*	1.3	1.37
CY$_{SOLIDS}$	21.1	34.0	1.9	0.3	3.4*	1.3	0.5	1.8	1.6	0.78
CY$_{WATER}$	12.9	32.5	3.2**	0.5	7.2***	4.0**	0.8	3.9**	0.9	0.82
Nutrients Recovery %										
REC$_{FAT}$	6.76	10.2	0.9	1.7	4.6**	4.4**	2.1	1.6	1.1	3.60
REC$_{PROTEIN}$	5.48	4.30	0.9	0.8	0.5	6.9***	9.7***	1.1	0.6	1.47
REC$_{SOLIDS}$	13.4	18.8	1.9	0.6	4.6**	2.0	0.6	1.7	0.8	3.13
REC$_{ENERGY}$	7.00	13.0	1.8	1.2	5.5***	2.0	3.0*	2.3	2.0	2.88

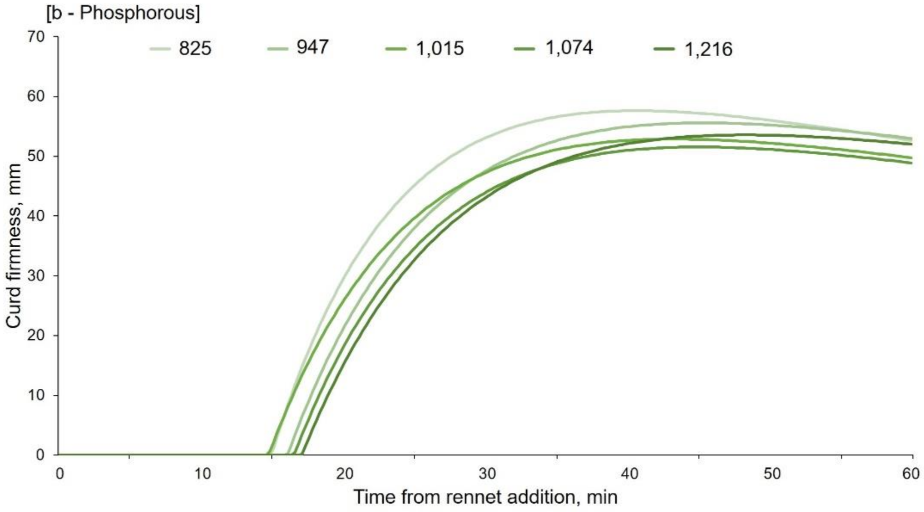
[1] RMSE = Root Mean Square Error; [2] RCT = measured rennet gelation time; k_{20} = time interval between gelation and attainment of curd firmness of 20 mm; a_{30}, a_{45} and a_{60} = curd firmness 30, 45 and 60 min after rennet addition; [3] RCT$_{eq}$ = rennet coagulation time estimated by CF$_t$ modeling; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF$_P$ = asymptotic potential curd firmness; CF$_{max}$ = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF$_{max}$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

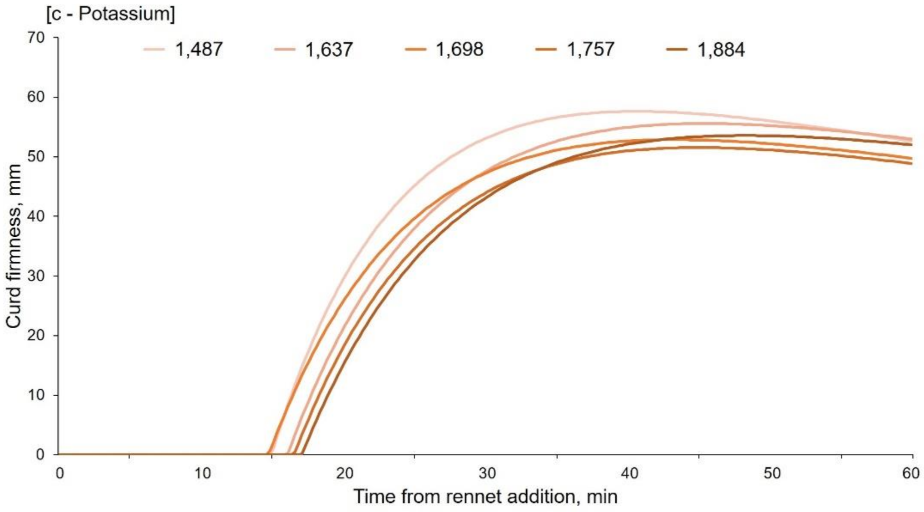
Supplemental Figure S1. Scatter plots of the Pearson's correlations among milk minerals, fat and casein, and their coefficients with significance (* *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001).



Supplemental Figure S2. Effect of Ca [a], P [b], and K [c] [reported as mean values (mg/L) of each quintile of the distributions] on gelation, curd-firming and syneresis of individual milk samples using model M2 (no fat and casein; contemporary inclusion of minerals).


[a - Calcium]
1,059
1,143
1,204
1,286
1,445
Curd firmness, mm
0
10
20
30
40
50
60
70
Time from rennet addition, min
0
10
20
30
40
50
60


[b - Phosphorous]
825
947
1,015
1,074
1,216
Curd firmness, mm
0
10
20
30
40
50
60
70
Time from rennet addition, min
0
10
20
30
40
50
60


[c - Potassium]
1,487
1,637
1,698
1,757
1,884
Curd firmness, mm
0
10
20
30
40
50
60
70
Time from rennet addition, min
0
10
20
30
40
50
60