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Milk component ratios and their associations with energy balance indicators and serum calcium concentration in early-lactation spring-calving pasture-based dairy cows

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ABSTRACT

Indirect assessment of metabolic status using milk samples provides a non-invasive and objective tool for cow-level health monitoring. Milk fat-to-protein ratio (**FPR**) has been commonly evaluated as an indirect measure for negative energy balance (**EB**) in confined dairy cows. However, milk component ratios have not been explored for their association with pasture-based cows' metabolic status. The objectives of this observational study were to 1) describe milk component ratios from 0 to 45 d postpartum, 2) evaluate the associations between milk component ratios [FPR, fat-to-lactose (**FLR**), protein-to-lactose (**PLR**)] and indicators of EB (serum β-hydroxybutyrate (BHB) concentration at 5–45 d postpartum and body condition score (BCS) change during the transition period), and 3) evaluate the associations between milk component ratios and serum Ca concentration 0–4 d postpartum in spring-calving dairy cows from pasture-based commercial farms. Milk component ratios were determined on samples collected before AM or PM milkings from 548 cows at 0–45 d postpartum (n = 970). Serum BHB and Ca determinations were performed in blood samples collected at the time of milk sample collection at 5–45 d postpartum (n = 918) and 0–4 d postpartum (n = 50), respectively; and BCS change was calculated using BCS assigned between 29 d prepartum and 45 d postpartum (n = 851). Cows' calving date, parity (1st, 2nd–3rd or ≥ 4th) and breed (Holstein-Friesian or dairy crossbred) information was obtained from the farm records. Data was analyzed by multiple linear regression. Average milk FPR, FLR and PLR were 0.70, 0.53 and 0.72, respectively. Milk FPR linearly increased while milk FLR linearly decreased postpartum both at a rate of 0.004 units per day; milk PLR decreased 0.05 units per day for the first 30 d postpartum and moderately increased afterward. Milk FPR and FLR were 0.71 and 0.52 units lower before AM than PM milking, respectively; while milk PLR was similar before AM and PM milking. Milk FPR and FLR were 0.07 to 0.10 units higher for 2nd–3rd compared with 1st and ≥ 4th parity cows. Milk PLR was 0.03 units greater for ≥ 4th compared with 2nd–3rd and 1st parity cows. Further, crossbred cows had 0.07, 0.08 and 0.03 higher milk FPR, FLR and PLR than Holstein-Friesian cows, respectively. Moderate to high *P*-values along with moderate to small estimated slopes and wide 95% confidence intervals were observed for the associations between milk component ratios and indicators of EB. A positive linear association was observed between milk FPR and serum Ca concentration within 4 d postpartum; milk FPR increased 0.31 units per each mmol/L increase in serum Ca concentration. Cows with low serum Ca concentration within 4 d postpartum had 0.27 units lower milk FPR compared with cows at or above the threshold (2.12 mmol/L), and tended to have 0.15 units lower milk FPR compared with cows at or above the threshold (2.00 mmol/L). In conclusion, further research is needed to reach conclusions on the association between milk component ratios determined before milking and EB indicators. The potential of milk FPR for monitoring blood Ca status warrants further investigation in early-lactation pasture-based dairy cows.
Key words: Fat-to-protein ratio, milk composition, transition, hypocalcemia, hyperketonemia

INTRODUCTION

Milk composition information from routine testing or new parlor technologies is becoming increasingly available in commercial dairy farms, increasing the interest

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

of its applicability under different production systems. During energy deficient and low intake periods, such as that in early lactation, milk fat concentration increases due to the mobilization of body fat and milk protein concentration decreases due to a diminution of its synthesis (Gross et al., 2011; Gross and Bruckmaier, 2019; Civiero et al., 2021). Thus, milk component ratios are expected to change during lactation in association to cows' energy status, providing a non-invasive alternative for energy balance monitoring in dairy cows. Milk fat-to-protein ratio (**FPR**) has been commonly described as an indicator of energy balance (Duffield et al., 1997; Chandler et al., 2018; Cabezas-Garcia et al., 2021) and disease in confined production systems (Heuer et al., 1999; Toni et al., 2011; King et al., 2019). Alternative milk component ratios [milk fat-to-lactose ratio (**FLR**) and milk protein-to-lactose ratio (**PLR**)], evaluated by fewer studies, have also been associated with negative energy balance and other diseases (Reist et al., 2002; Paudyal et al., 2023). However, to the best of our knowledge milk component ratios have not been tested for their association with mineral balance, despite observations of lower milk protein concentrations but similar fat concentrations in cows with subclinical hypocalcemia (Chamberlin et al., 2013; Rodrigues et al., 2019; Hendriks et al., 2020).

Although limited research is available, milk composition has been shown to change with pasture inclusion in the diet. For instance, compared with TMR-fed cows, higher concentration of fat and protein has been observed in milk from cows fed ryegrass pasture only (O'Callaghan et al., 2016), and lower lactose and protein have been described in milk from cows fed ryegrass pasture and supplemented with concentrates at milking (equivalent to the Irish dairy production system; Timlin et al., 2023). Therefore, milk component ratios could differ and results from studies undertaken in confined production systems may not apply to grazing systems.

Given the higher incidence of negative nutrient balance and disease in early lactation and its association with impaired performance (Kerwin et al., 2022b; Rodríguez et al., 2017; Rodriguez et al., 2022), health monitoring strategies are intensified during this period to guide cow- and herd-level management decisions and ultimately minimize undesirable outcomes (Oetzel, 2004; Mulligan et al., 2006). Common targets of early-lactation cow health monitoring include ketone bodies, such as BHB within 5 to 50 d postpartum and BCS change (Oetzel, 2004; Roche, 2023), due to associations between elevated BHB and negative energy balance, excessive body fat mobilization and impaired performance (Duffield et al., 2009; Roche et al., 2009; Ospina et al., 2010; Civiero et al., 2021). Additionally, recent research suggests that circulating Ca concentration should be monitored within the first 4 d postpartum (Oetzel, 2004; McArt and Neves, 2020) due to low Ca concentration also being been associated with impaired performance (Rodríguez et al., 2017; McArt and Neves, 2020; Valldecabres and Silva-del-Río, 2021). Blood determination of these analytes is labor intensive and potentially stressful for the animals. However, a growing proportion of herds will have milk composition information through inline sensors in milking parlors, the use of milking robots and herd testing in the future. Allowing for automated milk monitoring as a mean to indirectly monitor cow health. Therefore, there is need to assess the validity of milk component ratios as a tool to monitor energy balance and calcemic status in early-lactation grazing dairy cows. The objectives of this study were to 1) describe milk component ratios from 0 to 45 d postpartum, 2) evaluate the associations between indicators of energy balance (serum BHB concentration between 5 to 45 d postpartum and BCS change during the transition period) and milk component ratios (FPR, FLR and PLR), and 3) evaluate the associations between serum Ca concentration within 4 d postpartum and milk component ratios in spring-calving dairy cows from commercial pasture-based farms.

MATERIALS AND METHODS

Study Design

This observational study is based on a convenience sample and was conducted as part of a larger study approved by the Teagasc Animal Ethics Committee (Project Reference Number: TAEC2022–343) and the Health Products Regulatory Authority (HPRA) of Ireland (Project Authorization Number: AE19132/P160). Briefly, the larger study enrolled 27 herds randomly chosen from the respondents to a transition cow health and management survey (601 respondents; results yet to be published) who met eligibility criteria (spring-calving, herd size > 60 cows, located within a 2 h drive to Teagasc Moorepark, Irish Cattle Breeding Federation (ICBF) client with a HerdPlus subscription, > 4 milk recordings in 2022, and provision of consent for Teagasc to access their ICBF profile and to be contacted about this study) across 4 milk yield quartiles. The number of cows to be sampled within each herd was determined based on a sample size estimation performed for the larger study blood analytes of interest using the formulae described by Dohoo et al. (2003; Eq 2.5), standard deviation (SD) reported by Spaans et al. (2022), and a finite population correction when the sampling fraction exceeded 20% of the herd size (Dohoo et al., 2003; Eq 2.8). Among the analytes of interest, the largest estimated sample size per herd was 20 cows to obtain a mean herd Ca concentration estimate with 95% confidence interval width of ± 0.1 mmol/L (i.e., a margin of error of 0.1 mmol/L); to this estimate a 25%

surplus was added to account for loss of follow up, thus a maximum number of 25 cows were sampled per farm.

One hundred and 28 primiparous and 420 multiparous cows were enrolled across 27 spring-calving pasture-based commercial dairy farms over 8 counties in the Republic of Ireland (11 to 22 cows per farm) from January to April 2023. A total of 984 milk component results from samples obtained between 0 to 45 d postpartum [mean ± SD: 19 ± 9 d postpartum; one or 2 samples per cow taken approximately 2 weeks apart], 932 serum BHB determinations obtained from 5 to 45 d postpartum (mean ± SD: 20 ± 9 d postpartum; one or 2 determinations per cow taken approximately 2 weeks apart), 863 BCS change observations from −29 to 45 d relative to calving [based on 2 or 3 BCS observations per cow: prepartum (all cows), within approximately 2 weeks postpartum and from approximately 2 to 4 weeks postpartum], and 51 serum Ca determinations within 4 d postpartum (mean ± SD: 3 ± 1 d postpartum; one determination per cow) were available for this observational study. Cows in the enrolled farms were fed grass-silage during the dry period, and a pasture-based diet with concentrates provided during milking after calving. The study farms herd sizes ranged from 61 to 383 milking cows with an average 305-d mature-equivalent milk yield per cow of 6,683 kg during 2022.

Data Collection and Laboratorial Analysis

Cow-level information (breed, calving date, parity and previous lactation 305-d mature-equivalent milk yield) was obtained from the ICBF database. Based on ancestry assignment cows were classified as Holstein-Friesian (>70% of breed fraction attributed to Holstein and/or Friesian breed and < 20% attributed to other dairy or beef breeds) or dairy crossbred [>20% of breed fraction attributed to dairy breeds other than Holstein or Friesian (Jersey, Montbeliarde or Norwegian Red); no purebred cows other than one purebred Norwegian Red were included in this category]. Cows classified as beef cross [>20% of breed fraction attributed to beef breeds (Angus or Shorthorn; n = 6)] or unknown breed (n = 2), and their respective observations (n = 14) were excluded from the study. Parity and breed distribution for observations included in the analyses are presented in Table 1.

Milk component ratios

Milk samples were collected before AM or PM milkings within the first 45 DIM by Teagasc research personnel. Starting with the front teats, teat ends were thoroughly cleaned and disinfected using cotton swabs soaked in a 70% methylated alcohol solution. The first fore strips were discarded and a milk sample pooling the milk from the 4 quarters was collected into a 50 mL sterile milk collection vial without preservative. After collection, samples were transported to the laboratory (Moorepark Milk Quality Laboratory, Fermoy, Ireland) where they were stored at 4°C until analysis. Milk composition was determined by Fourier Transform Infrared within 48 h after sample collection (FTIR; MilkoScan; FOSS Analytics, Hillerod, Denmark). Milk component ratios were calculated as milk fat concentration/milk protein concentration (FPR), milk fat concentration/milk lactose concentration (FLR), and milk protein concentration/milk lactose concentration (PLR) for each milk composition determination. The time of milking distribution for observations included in the analyses is presented in Table 1.

Serum BHB and Ca

Coccygeal blood samples were collected in the milking parlor at the time of milk sample collection into evacuated tubes without anticoagulant (10 mL Serum tubes; Fisher Scientific, Dublin, Ireland). After drawing blood,

Table 1. Description of data sets used in an observational study enrolling pasture-based cows from 27 spring-calving commercial dairy farms in the Republic of Ireland by variable of interest

	Parity, n of cows[1] (n observations/total observations)			Breed, n of cows[1] (n observations/total observations)		Milking time, n of cows[1] (n observations/total observations)		
Variable of interest	1st	2nd – 3rd	≥4th	Holstein-Friesian	Dairy cross	AM	PM	Herds, n
Milk component ratio	128 (224/970)	170 (295/970)	250 (451/970)	420 (455/970)	128 (215/970)	353 (614/970)	195 (356/970)	27
Serum BHB	126 (211/918)	167 (281/918)	244 (426/918)	417 (722/918)	120 (196/918)	343 (579/918)	194 (339/918)	27
BCS change	107 (193/851)	148 (260/851)	221 (398/851)	373 (665/851)	103 (186/851)	310 (559/851)	166 (292/851)	27
Serum Ca	13 (13/50)	14 (14/50)	23 (23/50)	32 (32/50)	18 (18/50)	33 (33/50)	17 (17/50)	21

[1]Cows were sampled once or twice.

samples were allowed to clot and placed into polystyrene boxes filled with ice packs. Within a maximum of 6 h after collection, blood samples were centrifuged at 3,000 × *g* for 10 min at room temperature and serum harvested into 1.5 mL aliquots. Harvested serum was stored in a refrigerator set at 4°C until transport in cool conditions to an external laboratory (FBA Laboratories, Cappoquin, Ireland) for serum analyte determination by photoelectric colorimetry using an automated wet chemistry analyzer and Quantum Vet Diagnostics reagents (Woodley InSight DB; Woodley Equipment Company Ltd., Lancashire, UK). Samples were analyzed within 24 h after blood collection. The intra- and inter-assay coefficients of variation (CV) for serum BHB and Ca determinations were 1.9 and 6.9% and 1.3 and 5.2%, respectively.

Body condition score change

Cows were scored for body condition using a 1 to 5 scale with 0.25 increments (Wildman et al., 1982) from −29 to 45 d relative to calving up to in 3 occasions [prepartum (all cows), within approximately 2 weeks postpartum and from approximately 2 to 4 weeks postpartum]. Body condition score change was defined as losing, maintaining, or gaining based on the BCS difference between the BCS assigned at the time of the milk component ratios determination and the BCS assigned at the previous visit. Where a cow was not seen in 2 consecutive farm visits the BCS change variable was not generated. Therefore, up to 2 BCS change observations per cow were available (prepartum to up to approximately 2 weeks postpartum and/or within approximately 2 up to 4 weeks postpartum).

Data Analysis

Statistical analysis was performed using SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). Before data analysis, raw data box plots were generated with the SGPLOT and UNIVARIATE procedures to check for outliers. For further data visualization, milk component ratios were categorized in 0.25 increments from 0.25 to > 1.25. Descriptive statistics were generated using the MEANS and FREQ procedures. Milk component ratios and their associations with cow-level factors (day postpartum, parity, breed and time of milking) were described for samples collected from 0 to 45 d postpartum. The associations between serum BHB concentration and milk component ratios were evaluated in samples collected from 5 to 45 d postpartum (Oetzel, 2004), the associations between BCS change and milk component ratios were evaluated using transition period BCS change records and milk samples obtained from 0 to 45 d postpartum, and the associations between serum Ca concentration and milk component ratios were evaluated in samples obtained within 4 d postpartum (McArt and Neves, 2020).

Multiple linear regression mixed models were used to evaluate the associations between different cow-, lactation- and within lactation-level factors [day postpartum, parity (1st, 2nd – 3rd or ≥ 4th), breed (Holstein-Friesian or crossbred), time of milking (AM or PM)] and milk component ratios (study outcomes) using the MIXED procedure. Repeated measures were modeled using the unstructured covariance structure, and cow nested within herd as the subject in the REPEATED statement. The assumption of a linear relationship between dependent (milk component ratios) and independent continuous variables (serum BHB and Ca concentrations) was graphically assessed with the LOESS option from the SGPLOT procedure; quadratic power terms of continuous variables were added to the models as needed. The likelihood ratio test was then used to assess effects of including power terms on model deviances and confirm the better fit of the model including power terms. Comparisons between independent variable categories associated with the outcomes were performed using the LSMEANS statement and Tukey adjustment. Overall model fit was assessed with final models' residuals plots. Influential observations (studentized residuals > |4|) were evaluated as potential recording or measurement errors; no observations were excluded for these reasons.

Associations between milk component ratios and each energy balance indicator (serum BHB and BCS change) and serum Ca concentration were evaluated by separately including the variable of interest into the models described above, with the exception of the exclusion of the REPEATED statement from the model with Ca concentration as the variable of interest. Day postpartum (ranging from 0 to 45, 5 to 45 or 0 to 4 in the various models), parity, breed and time of milking effects were retained in the models as potential confounders regardless of the *P*-value. Where associations between serum Ca concentration and milk component ratios were observed at $P \leq 0.05$, dichotomized serum Ca variables were created using cut-points commonly accepted by the industry: < 2.00 mmol/L and < 2.12 mmol/L (Oetzel, 2013; Spaans et al., 2022). The categorized Ca variable replaced its continuous form and its association with the respective milk component ratio was quantified using multiple linear regression models as described above. Significance was declared at $P \leq 0.05$ for all results. Results are presented as least squares means (LSM) ± standard error of the mean (SEM) unless otherwise stated. The accompanying figures were created with SigmaPlot (version 14.0; Systat Software Inc., San Jose, CA).

Table 2. Milk component concentrations and ratios determined within 45 (n = 970), within 4 (n = 50) and from 5 to 45 d postpartum (n = 918) on 548, 50 and 537 spring-calving cows from 27 pasture-based commercial dairy herds, respectively

	0 to 45 d postpartum[1]			0 to 4 d postpartum[1]			5 to 45 d postpartum[1]		
Item	LSM	SEM	95% CI	LSM	SEM	95% CI	LSM	SEM	95% CI
Fat, %	2.48	0.067	2.35 - 2.61	2.94	0.311	2.32 - 3.57	2.45	0.067	2.32 - 2.59
Protein, %	3.47	0.023	3.42 - 3.51	5.43	0.359	4.71 - 6.15	3.42	0.014	3.39 - 3.45
Lactose, %	4.80	0.010	4.78 - 4.82	4.25	0.070	4.11 - 4.39	4.80	0.010	4.78 - 4.82
Fat-to-protein ratio	0.70	0.020	0.66 - 0.74	0.62	0.087	0.44 - 0.80	0.71	0.020	0.67 - 0.75
Fat-to-lactose ratio	0.53	0.016	0.50 - 0.56	0.74	0.089	0.56 - 0.91	0.52	0.015	0.49 - 0.55
Protein-to-lactose ratio	0.72	0.007	0.71 - 0.74	1.36	0.126	1.10 - 1.61	0.71	0.003	0.70 - 0.71

[1]Results for a null model intercept accounting for repeated measures and the random effect of cow nested within herd.

RESULTS

The data sets available for analysis are described in Table 1. One serum BHB observation (6.9 mmol/L, +11 SD from the mean) and a serum Ca observation (0.6 mmol/L, −4 SD from the mean) were considered outliers and not included in the analyses as animals in the study were clinically healthy at the time of sample collection. Mean (±SD) serum BHB concentration was 0.62 ± 0.54 mmol/L (20 ± 9 d postpartum), BCS change from previous determination (17 ± 4 d apart) was −0.06 ± 0.29 units, and serum Ca concentration was 1.92 ± 0.23 mmol/L (3 ± 1 d postpartum). Milk component concentrations and ratios by postpartum period accounting for the effects of cow and herd are described in Table 2. Descriptive statistics for observed milk component concentrations and ratios, Spearman correlation coefficients for pairwise correlations between milk component ratios, variables of interest (serum BHB, serum Ca and BCS change) and cow-, lactation- and within lactation-level factors (parity, breed, day from calving and milking time), and results for the associations between the study variables of interest and individual milk components are presented in Supplemental Tables (https://doi.org/10.17632/vx466gmfsr.1; Valldecabres, 2024). All slope estimates, LSM, and *P*-values are adjusted for day postpartum, parity, breed and time of milking.

Fat-to-Protein Ratio

Milk FPR increased by 0.004 ± 0.001 units per day postpartum [b (slope coefficient) ± S_b (SE of the slope coefficient); $P = 0.005$; Figure 1A]. Parity and breed were associated with milk FPR; 2nd – 3rd parity (0.89 ± 0.03) and crossbred (0.87 ± 0.03) cows had higher milk FPR compared with 1st (0.79 ± 0.03; $P = 0.04$) and 4th (0.79 ± 0.02; $P = 0.008$) parity and Holstein-Friesian cows (0.78 ± 0.02; $P = 0.02$), respectively; milk FPR was similar for 1st and ≥ 4th parity cows ($P = 1.00$). Milk FPR was lower before AM milking (0.47 ± 0.02) than it was before PM milking (1.18 ± 0.03; $P < 0.001$). Fifteen influential observations were included in the model. Milk FPR distribution (categorized in 0.25 increments) by time of milking is presented in Figure 2A.

Association with energy balance

No evidence of association between serum BHB concentration from 5 to 45 d postpartum and milk FPR was observed; a high *P*-value ($P = 0.57$) along with a small estimated slope (0.016) and a wide 95% CI (−0.04 to 0.07; Table 3) were observed while accounting for the effects of day postpartum ($P = 0.02$), parity ($P = 0.02$), breed ($P = 0.06$) and time of milking ($P < 0.001$). Similarly, no evidence of association between BCS change and milk FPR was observed ($P = 0.46$; BCS gain: −0.030 (−0.10 to 0.04); BCS loss: −0.036 (−0.09 to 0.02); b (95% CI); Table 3), while accounting for the effects of day postpartum ($P < 0.001$), parity ($P = 0.005$), breed ($P = 0.01$) and time of milking ($P < 0.001$).

Association with serum Ca

Serum Ca concentration from 0 to 4 d postpartum was linearly associated with milk FPR. Milk FPR increased 0.31 ± 0.14 units per each mmol/L increase in serum Ca concentration ($P = 0.04$; Table 3; Figure 3), while accounting for the effects of day postpartum ($P = 0.62$), parity ($P = 0.64$), breed ($P = 0.73$) and time of milking ($P < 0.001$). The dichotomized serum Ca concentration variable defined using the 2.12 mmol/L cut-point was associated with milk FPR ($P = 0.007$); cows with serum Ca < 2.12 mmol/L (n = 39) within the first 4 d postpartum had 0.27 units lower milk FPR compared with cows above the threshold (0.61 ± 0.05 vs. 0.88 ± 0.08 units, respectively); while accounting for the effects of day postpartum ($P = 0.43$), parity ($P = 0.71$), breed ($P = 0.88$) and time of milking ($P < 0.001$). Additionally, the dichotomized serum Ca concentration variable defined using the 2.00 mmol/L cut-point tended to be associated with milk FPR ($P = 0.09$; n = 29) where cows with serum Ca < 2.00 mmol/L tended to have lower milk FPR than

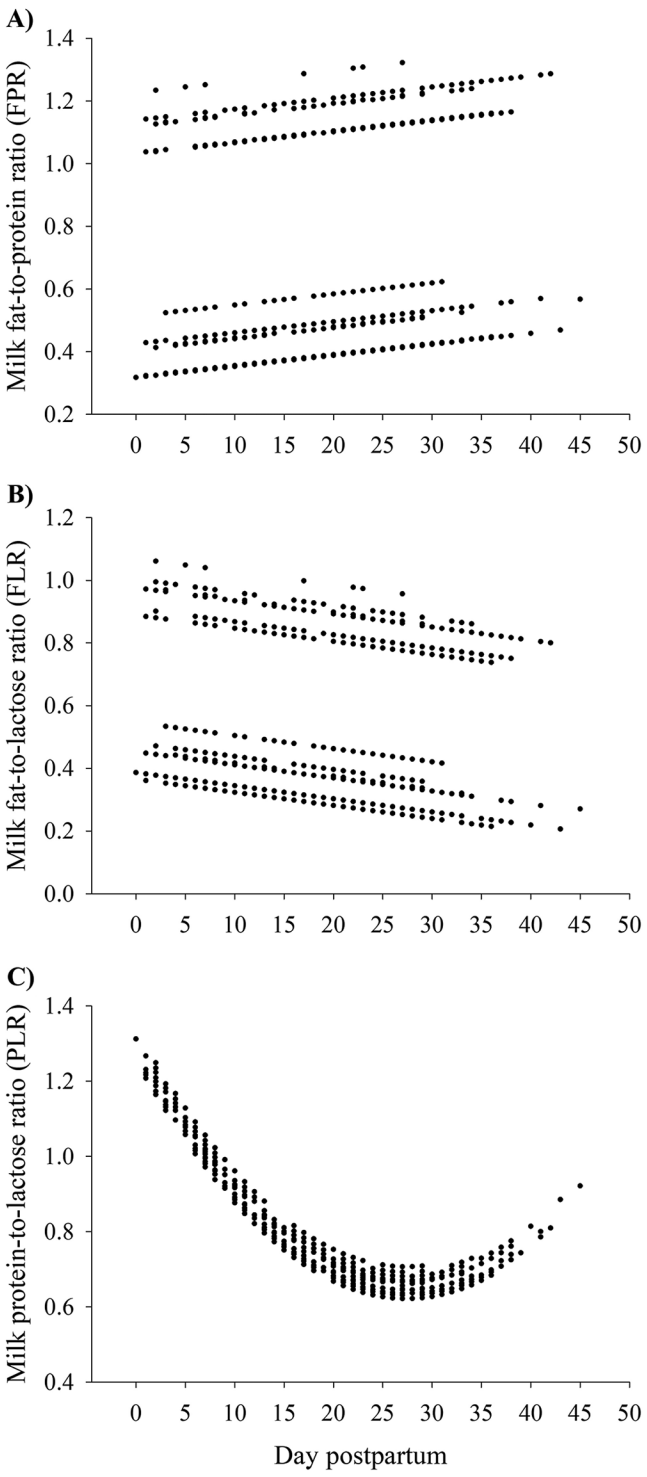

Figure 1. Scatterplots for the association between milk component ratios [fat-to-protein (Panel A), fat-to-lactose (Panel B) and protein-to-lactose (Panel C)] and day postpartum as predicted by multiple linear regression models accounting for the effects of day postpartum, parity, breed and time of milking, as well as the random effect of cow nested within herd for 548 spring-calving cows from 27 pasture-based commercial dairy herds. The determining variable for overall upper and lower fitted values' clusters in panels A and B was time of milking (PM and AM, respectively).

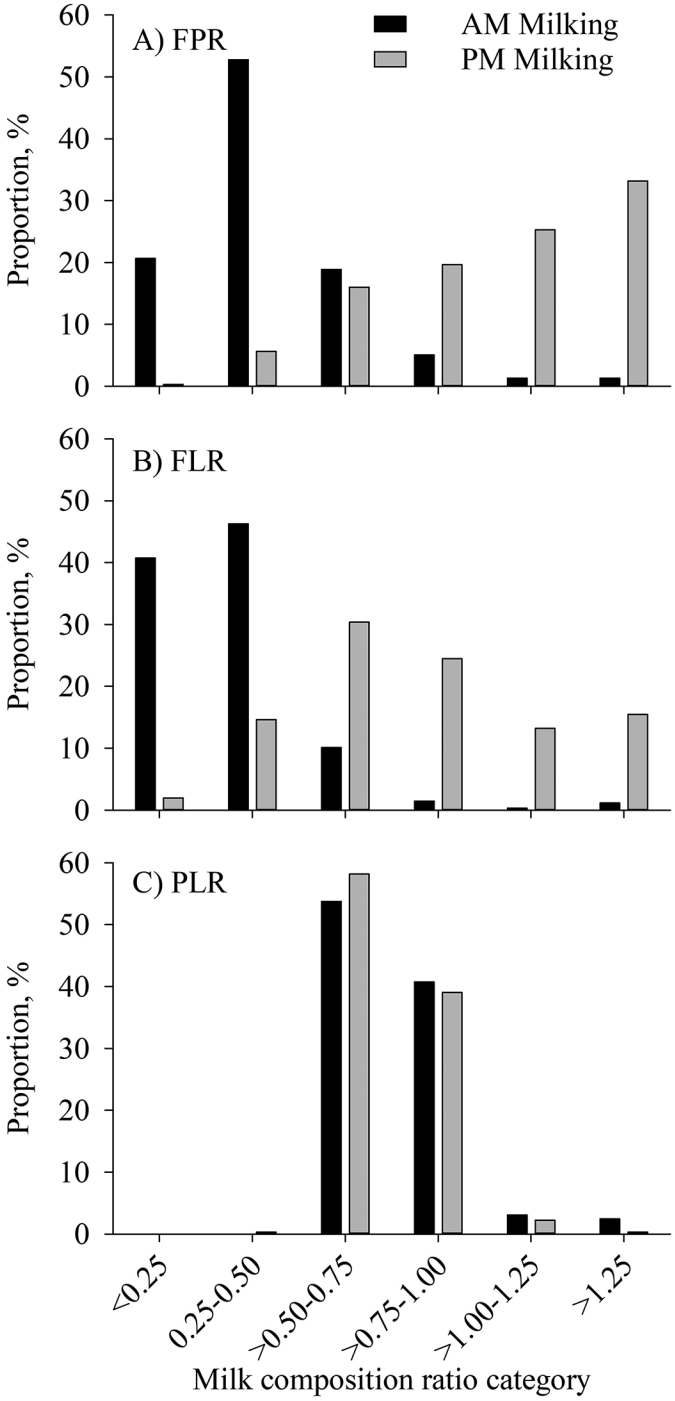

Figure 2. Distribution of milk fat-to-protein ratio (FPR; Panel A), fat-to-lactose ratio (FLR; Panel B) and protein-to-lactose ratio (PLR; Panel C) observations by time of milking for 548 spring-calving cows from 27 pasture-based commercial dairy herds sampled before AM (n = 614 samples) or PM milking (n = 356 samples) from d 0 to 45 postpartum.

cows above this threshold (0.62 ± 0.06 vs. 0.77 ± 0.06 units, respectively); while accounting for the effects of

Table 3. Estimated associations between milk composition ratios and each of energy balance indicators (BHB from 5 to 45 d postpartum and BCS change during the transition period) and serum Ca concentration within 4 d postpartum in spring-calving grazing dairy cows enrolled in an observational study

	Fat to protein ratio (FPR)			Fat to lactose ratio (FLR)			Protein to lactose ratio (PLR)		
Item[1]	Estimate	95% CI	*P*-value[2]	Estimate	95% CI	*P*-value[2]	Estimate	95% CI	*P*-value[2]
Energy balance									
BHB, mmol/L	0.016	−0.04 to 0.07	0.57	0.0004	−0.04 to 0.04	0.98	−0.009	−0.02 to 0.002	0.11
BCS change[3]			0.46			0.20			0.63
Gain	−0.030	−0.10 to 0.04		−0.019	−0.06 to 0.022		−0.008	−0.03 to 0.01	
Loss	−0.036	−0.09 to 0.02		−0.032	−0.07 to 0.003		−0.006	−0.02 to 0.01	
Maintain	Referent	—		Referent	—		Referent	—	
Ca, mmol/L	0.310	0.02 to 0.60	0.04	0.301	−0.35 to 0.96	0.36	−0.012	−0.91 to 0.88	0.98

[1]Number of observations by item was: serum BHB (n = 918), BCS change (n = 851; Gain: n = 203; Loss: n = 333; Maintain: n = 315), serum Ca (n = 50).

[2]Models were adjusted for the effects of day postpartum (serum BHB: 5 to 45; serum Ca: 0 to 4), parity (1st, 2nd – 3rd or ≥ 4th), breed (Holstein-Friesian or dairy crossbred) and time of milking (AM or PM). Energy balance models for PLR also included the quadratic effect of day postpartum.

[3]Based on two or three BCS observations per cow: prepartum (all cows), within approximately two weeks postpartum and from approximately two to four weeks postpartum.

day postpartum ($P = 0.88$), parity ($P = 0.73$), breed ($P = 0.47$) and time of milking ($P < 0.001$).

Fat-to-Lactose Ratio

Overall, milk FLR decreased by 0.004 ± 0.001 units per day postpartum (b ± S_b; $P < 0.001$; Figure 1B). Milk FLR was lower before AM milking (0.37 ± 0.02) than it was before PM milking (0.89 ± 0.02; $P < 0.001$) and for Holstein-Friesian (0.58 ± 0.01) compared with crossbred cows (0.68 ± 0.03; $P = 0.001$). Milk FLR was higher for 2nd – 3rd (0.68 ± 0.02) compared with 1st (0.59 ± 0.03; $P = 0.02$) and ≥ 4th parity cows (0.61 ± 0.02; $P = 0.04$), while similar for 1st and ≥ 4th parity cows ($P = 0.76$). Ten influential observations were included in the model. Milk FLR distribution (categorized in 0.25 increments) by time of milking is presented in Figure 2B.

Association with energy balance

No evidence of association between serum BHB concentration from 5 to 45 d postpartum and milk FLR was observed; a high *P*-value ($P = 0.98$) along with a small estimated slope (0.0004) and a wide 95% CI (−0.04 to 0.04; Table 3) were observed, while accounting for the effects of day postpartum ($P = 0.01$), parity ($P = 0.04$), breed ($P = 0.002$) and time of milking ($P < 0.001$). Similarly, no evidence of association between BCS change and milk FLR was observed ($P = 0.20$; BCS gain: −0.019 (−0.06 to 0.02); BCS loss: −0.032 (−0.07 to 0.003); b (95% CI); Table 3); while accounting for the effects of day postpartum ($P = 0.04$), parity ($P = 0.28$), breed ($P < 0.001$) and time of milking ($P < 0.001$).

Association with serum Ca

No evidence of association between serum Ca concentration and milk FLR was observed ($P = 0.36$; Table 3); a high *P*-value ($P = 0.36$) along with a moderate estimated slope (0.301) and a wide 95% CI (−0.35 to 0.96; Table 3) were observed, while accounting for the effects of day postpartum ($P = 0.36$), parity ($P = 0.15$), breed ($P = 0.67$) and time of milking ($P < 0.001$).

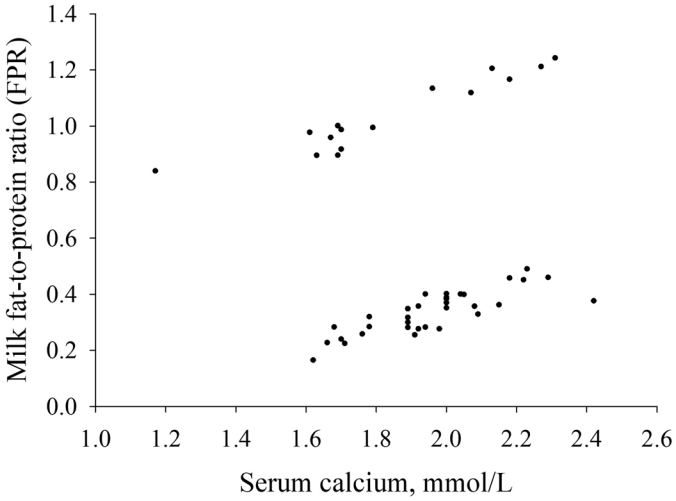

Figure 3. Scatterplot for the association between milk fat-to-protein ratio and serum Ca concentration as predicted by a multiple linear regression model accounting for the effects of day postpartum, parity, breed and time of milking, as well as the random effect of cow nested within herd for 49 spring-calving cows from 20 pasture-based commercial dairy herds. The determining variable for overall upper and lower fitted values' clusters was time of milking (PM and AM, respectively).

Protein-to-Lactose Ratio

A quadratic association was observed between day postpartum and milk PLR; milk PLR decreased 0.05 ± 0.003 units per day up to 30 d postpartum whereas it moderately increased (0.001 units per day) afterward (b ± S_b; $P < 0.001$; Figure 1C). Parity was associated with milk PLR; cows of parity ≥ 4th (0.78 ± 0.005) had higher milk PLR compared with 2nd – 3rd (0.75 ± 0.006; $P = 0.007$) and 1st (0.75 ± 0.007; $P = 0.006$) parity cows, while milk PLR was similar for 1st compared with 2nd – 3rd parity cows ($P = 0.96$). Breed tended to be associated with milk PLR; crossbreds (0.80 ± 0.014) tended to have higher milk PLR compared with Holstein-Friesian cows (0.77 ± 0.008; $P = 0.09$). No evidence of association was observed between time of milking and milk PLR (before AM milking: 0.79 ± 0.009; before PM milking: 0.78 ± 0.012; $P = 0.26$). Seven influential observations were included in the model. Milk PLR distribution (categorized in 0.25 increments) by time of milking is presented in Figure 2C.

Association with energy balance

No evidence of association between serum BHB concentration and milk PLR was observed; a moderate P-value ($P = 0.11$) along with a small estimated slope (−0.009) and a moderately narrow 95% CI (−0.02 to 0.002; Table 3) were observed, while accounting for the effects of day postpartum (linear and quadratic $P < 0.001$ for both), parity ($P < 0.001$), breed ($P < 0.001$) and time of milking ($P = 0.33$). Similarly, no evidence of association between BCS change and milk PLR was observed ($P = 0.63$; BCS gain: −0.008 (−0.03 to 0.01); BCS loss: −0.006 (−0.02 to 0.01); b (95% CI); Table 3); while accounting for the effects of day postpartum (linear and quadratic $P < 0.001$ for both), parity ($P = 0.005$), breed ($P = 0.002$) and time of milking ($P = 0.28$).

Association with serum Ca

No evidence of association between serum Ca concentration and milk PLR was observed); a high P-value ($P = 0.98$) along with a small estimated slope (−0.012) and a wide 95% CI (−0.91 to 0.88; Table 3) were observed, while accounting for the effects of day postpartum ($P < 0.001$), parity ($P = 0.66$), breed ($P = 0.22$) and time of milking ($P = 0.03$).

DISCUSSION

Our study described milk component ratios and evaluated their associations with markers of energy balance (serum BHB concentration from 5 to 45 d postpartum and BCS change during the transition period) and serum Ca concentration (within 4 d postpartum) in spring-calving pasture-based dairy cows. We hypothesized that milk component ratios would be associated with markers of energy balance and serum Ca concentration and could serve as an indirect tool to assess these conditions in early-lactation pasture-based dairy cows. While previous research has evaluated the associations between circulating BHB concentrations and milk FPR and FLR (often determined at different days within a study), we are unaware of studies evaluating the associations of serum Ca concentration and milk component ratios. Also, in contrast to our study, previous research used various types of categorizations of the continuous variables instead of reporting the data on a continuous scale. To the author's view, the categorization of continuous variables, although aiding on results interpretation, could potentially lead to the loss of information as it is assuming a constant relationship between the outcome and the values within each category. Since we did not find evidence of association between markers of energy balance and milk component ratios in a continuous scale, we did not explore the association between categorical forms of these variables. A limitation for the extrapolation and comparison of findings from this study could be the nature of the milk samples which were collected before AM or PM milkings, and therefore, are not representative of daily composite milk samples (i.e., milk recording samples) as milk composition is known to vary during milking and by milking interval (AM or PM milking). Although commercial alternatives are available and milk recording is becoming more popular, most dairy farmers in Ireland (average herd size = 93 cows; Teagasc, 2022) do not have the required technology to obtain daily milk composition samples at their own convenience, and therefore, using samples from the start of individual milkings was deemed more practical if study results were applied on farm. We also decided to use milk component ratios as dependent variables instead of the individual milk components based on previous reports for the association between milk component ratios and negative energy balance (Gross et al., 2011; Civiero et al., 2021) and disease (Toni et al., 2011; King et al., 2019; Paudyal et al., 2023). Therefore, it has to be noted that the association between the evaluated milk component ratios and an independent variable reflects the combined effects of the independent variable on each of the ratio components.

Average milk FPR (0.70) from both AM and PM milkings within 45 d postpartum observed in our study was ~2-fold lower compared with that reported from daily representative milk samples [Heuer et al., 1999: 1.38 for 1,046 cows within 40 d postpartum in 16 herds; Paudyal et al., 2023: 1.19 for 198 cows within 60 d postpartum in 1 herd], and ~3-fold lower than morning milk samples

obtained from housed cows (Toni et al., 2011; 1.82 for 1,498 cows from 3 herds within 1 wk postpartum). Similarly, average milk FLR (0.53) observed in our study was lower compared with that reported by Paudyal et al. (2023; 0.81); while the smallest difference was seen with reported milk PLR values (0.69; our study: 0.72). It is well known that milk composition changes relative to calving (e.g., during colostrogenesis), during milking, and with the milking interval, which may explain, in part, variation between studies. Milk fat concentration increases while protein concentration tends to decrease during milking (Rico et al., 2014), and fat concentration decreases with milking interval while milk protein concentration varies less in association to the milking interval in cisternal milk (Ayadi et al., 2004). In our study, samples were collected before milking (likely samples of milk contained in the mammary gland cistern) and more often before AM milking which is preceded by a larger interval than the PM milking; thus, overall lower fat concentration result of the study sampling protocol could have led to lower milk FPR and FLR. Nevertheless, dietary differences intrinsic to the intensive grazing system could also explain the differences between milk component ratios observed in our study compared with those from TMR-fed cows (O'Callaghan et al., 2016; Timlin et al., 2023). Fresh cow diets were not characterized in our study, thus, although perennial ryegrass is the most common pasture in Irish dairy farms, our results cannot be fully discussed under that umbrella. Research comparing milk composition of cows grazing perennial ryegrass only or perennial ryegrass and clover pastures is inconclusive; Enriquez-Hidalgo et al. (2014) and Egan et al. (2018) observed similar milk composition while O'Callaghan et al. (2016) reported higher fat concentration for cows under ryegrass only compared with those fed grass-clover pasture. Further, under common Irish management, fresh cows will be housed and fed grass silage when the weather does not allow for outdoor grazing, nevertheless this study did not record the silage feeding periods for fresh cows, which would also differ by farm given the geographic distribution of the study farms.

Milk component ratios varied from calving to 45 d postpartum in line with expected lactation changes in milk components. A sharper drop on milk protein concentration compared with milk fat concentration would explain the increase in milk FPR while the sharper increase in lactose concentration would explain the linear decrease in milk FLR and overall decrease PLR during the study period (Quinn et al., 2006; Cabezas-Garcia et al., 2021). Also in agreement with previous reports on breed effects on milk components (Prendiville et al., 2011), milk component ratios were higher for dairy crossbred compared with Holstein-Friesian cows. Parity effects were observed for milk PLR, however, these were driven by small differences between parities. In the milk FPR lactation profiles reported by Buttchereit et al. (2010) and Cabezas-Garcia et al. (2021) different patterns are observed by parity group, however, the early lactation parity effects were not quantified in these studies which include observations from 1 week postpartum. Last, the observed effect of time of milking on milk FPR and FLR is likely the reflection of the milking interval effect on milk fat concentration (Ayadi et al., 2004). Overall, what these associations indicate is that cow-, lactation- and within lactation-level factors account for some of the variation observed in milk component ratios, and that its consideration is warranted when interpreting milk component ratios and its association with cows' energy balance and performance.

We did not find evidence of association between markers of energy balance and milk component ratios. The limited prevalence of high serum BHB values (interquartile range = 0.32 to 0.73 mmol/L; BHB $\geq$ 1.2 mmol/L: 9.0%) and BCS loss (interquartile range = 0.50 to 0.25; > 0.5: 12.0%) in our study population could have limited our ability to detect associations with milk component ratios. In cows under automated milking systems, King et al. (2019) reported a 35% odds increase for hyperketonemia per each 0.1 milk FPR unit increase over the mean (1.16) within 3 weeks postpartum, and milk FPR > 1.5 has been commonly associated with higher blood BHB concentration and risk of hyperketonemia (BHB $\geq$ 1.2 mmol/L), as well as with BCS loss (>0.5) during early lactation (Duffield et al., 1997; Heuer et al., 1999; Cabezas-Garcia et al., 2021). However, using milk FPR > 1.5 as a decision tool for propylene glycol treatment within 4 weeks postpartum did not lower the incidence of clinical ketosis nor improved milk production in a study with 158 Holstein-Friesian cows (Jenkins et al., 2015). Paudyal et al. (2023) compared milk FPR, FLR, and PLR for the diagnosis of ketosis and concluded that milk FLR was better at identifying the disease. The 95% CI accompanying the regression coefficients for the energy balance variables in our study include the null value, not supporting the hypothesis that an association exists between these variables and the evaluated milk component ratios; however the width of these intervals indicates that the true regression coefficient value may point to associations between the parameters of interest in the population represented by our study sample. The widest 95% CI are observed for the associations between milk FPR and energy balance variables. For instance, the interpretation of the true value for the regression coefficient for the association of milk FPR and circulating BHB lies between a 0.04 units decrease to 0.07 units increase in milk FPR per each mmol/L increase in serum BHB concentration. Thus, we cannot conclude that milk FPR is not associated

with serum BHB concentration and that the former does not have diagnostic value for hyperketonemia. It has to be noted that although based on statistically significant associations, previously reported prediction ability of milk FPR for same-day hyperketonemia was very low (R^2 = 0.07; King et al., 2019) and Pearson correlation between circulating BHB and milk FPR moderate (r = 0.34; Benedet et al., 2019). The Spearman's correlation coefficient for circulating BHB and milk FPR indicated a weak correlation between the 2 variables in our study (ρ = 0.21; Supplemental Table S2). Thus, it is plausible that the relationship between serum BHB concentration and milk component ratios is intrinsically weak. Recent research suggests that milk BHB determined by infrared spectra and models combining test-day and performance variables may be better predictors for circulating BHB concentration than milk component ratios (Chandler et al., 2018; Benedet et al., 2019; Bonfatti et al., 2019). When looking at the association between markers of energy balance (serum BHB and change in BCS) and individual milk components (Supplemental Table S3), our results suggest that milk protein concentration linearly decreases by 0.001 to 0.09 units per each mmol/L increase in serum BHB concentration; while they do not support the hypothesis that an association exists between any of the other evaluated markers of energy balance and individual milk components. Nevertheless, it has to be noted that the 95% CI accompanying the regression coefficients for these associations are wide, indicating that further information is needed if conclusions in this regard are to be reached. As previously discussed, it is plausible that the nature of the milk samples used in this study has limited our ability to detect associations. However, in agreement with our findings, previous research evaluating cows' physiological adaptation to negative energy balance report lower milk protein concentration and similar milk fat concentration for affected and control cows (Gross et al., 2011; Civiero et al., 2021).

The 95% CI for the association between milk FPR and serum Ca concentration, along with the efforts devoted to minimize bias in our study, indicate that per each unit increase in serum Ca concentration milk FPR would, with 95% certainty, increase within the range of 0.02 to 0.60 units. That, along with the limited prior information about this association and the 95% CI accompanying the mean difference on milk FPR among cows above and below the 2.12 mmol/L threshold (0.08 to 0.46 units) suggest that a meaningful association between milk FPR and serum Ca concentration may exist and warrants further investigation. Calcium is a main component of colostrum and milk, where among others, it is required for casein micelles' formation (Farrell, 1973; Gaucheron, 2005; Valldecabres and Silva-del-Río, 2022); however, few studies have evaluated the association between cows' mineral status and milk composition, and to the best of our knowledge, none have evaluated the association of serum Ca concentration and milk component ratios determined in samples collected at the same time point. The positive linear association between serum Ca concentration and milk FPR and the lower milk FPR in cows with low Ca (<2.12 mmol/L) observed in our study could be biologically explained by an association between circulating Ca availability for mammary gland uptake and protein synthesis (Gaucheron, 2005). However, Valldecabres and Silva-del-Río (2022) reported no association between serum Ca concentration and colostrum Ca yield or concentration; although, this may be influenced by breed as the study evaluated these associations in 100 multiparous Jersey cows. Further, samples in our study could be considered transition milk but not colostrum, as none were taken as part of the cows' first milking; it is plausible that the shorter process of milk compared with colostrum synthesis, facilitates the association of milk composition and serum Ca concentration. It should be noted that cows with low Ca are potentially also experiencing a degree of negative energy balance which has been associated with lower milk protein concentration, physiologically explained based on the lower availability of energy for microbial and mammary gland protein synthesis (Nousiainen et al., 2004; Rius et al., 2010). Thus, it is uncertain if circulating Ca levels are directly interfering with milk protein concentration. When looking at the association between serum Ca concentration and individual milk components (Supplemental Table S3), our results do not support the hypothesis that an association exists between them nor allow to reject the null hypothesis given that either end of the 95% CI holds potentially meaningful true values for these associations. Interestingly, other studies evaluating the association between low Ca around calving and milk protein concentration further into the lactation reported lower milk protein concentrations for cows with low Ca, suggesting that there may be longer term factors involved in that association (Chamberlin et al., 2013; Rodrigues et al., 2019; Hendriks et al., 2020). Therefore, further research is needed to understand the association between blood mineral status and milk composition, as well as other factors that influence its variation, so that conclusions can be drawn on the possibility of the last being a tool for monitoring blood mineral status.

CONCLUSION

Associations between markers of energy balance (serum BHB concentration from 5 to 45 d postpartum and BCS change during the transition period) and milk fat-to-protein (FPR), fat-to-lactose (FLR) and protein-to-lactose (PLR) ratios were characterized by moderate

to high *P*-values along with moderate to small estimated slopes and wide 95% CI, while accounting for the effects of day postpartum, parity, breed and time of milking, which were contributors to the variability observed in the milk component ratios. In contrast, a positive linear association was observed between serum Ca concentration within 4 d postpartum and milk FPR, and lower milk FPR was observed in cows with low serum Ca, suggesting that milk FPR could serve as an indirect tool to monitor Ca balance in early-lactation spring-calving pasture-based dairy cows.

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