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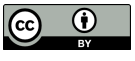

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Decreased lactose percentage in milk associated with quarter health disorder and hyperketolactia, a proxy for negative energy balance, in dairy cows

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ABSTRACT

Several studies have described variations in lactose content (LC) in dairy cows during udder quarter health disorder or negative energy balance (NEB). However, their joint effects on LC have never been described. This was the aim of a longitudinal observational study performed on 5 Quebec dairy farms using automatic milking systems. Quarter milk samples were collected every 14 d from 5 to 300 DIM. Quarter health status was described by combining SCC level (SCC^- or SCC^+: < or ≥100,000 cells/mL, respectively) and infectious status ($Patho^-$ or $Patho^+$: absence or presence of pathogens on a milk culture, respectively). Cows with NEB in early lactation (DIM <70) were identified using milk BHB content: <0.15 m*M* = BHB−; 0.15 to 0.19 m*M* = BHB+; >0.19 m*M* = BHB++. A total of 14,505 quarter cisternal milk samples were collected from 380 lactating cows. The quarter LC was analyzed using a mixed linear regression model with the following fixed effects: quarter health status, parity, time interval between last milking and sampling, quarter milk yield (in kg/d), DIM, and herd. A random quarter intercept with a repeated measures correlation structure and a cow random intercept were also specified. The LC of SCC^+ quarters was lower (−0.17 ± 0.013 percentage points) compared with LC of SCC^- quarters for both primiparous and multiparous cows. Of the 162 bacterial species identified, only 8 species had a prevalence greater than 4.0%, and just 5 of them were associated with a reduction in LC: *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, *Staphylococcus epidermidis*, and *Staphylococcus simulans*. Cows identified as BHB+ and BHB++ in early lactation had a lower LC (−0.05 ± 0.019 and −0.13 ± 0.020 percentage points, respectively) compared with BHB− cows. For BHB++ cows, in both parity groups the decrease in LC (−0.20 ± 0.025 percentage points) was higher in SCC^+ quarters compared with SCC^- quarters. Moreover, the additive effect of the quarter health status and NEB on milk LC was greater with larger increases in BHB. Our findings highlight the necessity to jointly take into consideration both quarter health status and milk BHB concentration when using LC as a biomarker for NEB.

Key words: lactose, udder quarter health, negative energy balance, hyperketolactia, dairy cow

INTRODUCTION

Mastitis remains an important disease in bovine dairy farms with a lactational cumulative incidence, for both clinical and subclinical mastitis, estimated in France at 20% (Barnouin et al., 2005; Coignard et al., 2013). In Canada, clinical mastitis lactational cumulative incidence was estimated at 17% (Olde Riekerink et al., 2008), and subclinical mastitis prevalence was estimated at 32% in Canada, the United States, and Brazil (Kurban et al., 2022). Subclinical mastitis diagnosis is mainly achieved via the determination of SCC. For instance, a SCC threshold of 100,000 cells/mL was previously used to discriminate infected versus healthy quarters (selectivity = 53.1; specificity = 95.7; Sumon et al., 2020). Mastitis risk factors are multiple, and mastitis incidence is high, especially in early lactation and for multiparous cows with a higher milk yield (Barnouin et al., 1994; Jamali et al., 2018). Nutritional status plays an important role in mastitis risk. A higher frequency of mastitis is observed in cows with a negative energy balance (**NEB**), and SCC has been positively associated with the milk BHB concentration (Jánosi et al., 2003; Bach et al., 2021). As a recurrent issue in dairy farming, NEB is particularly common at the onset of lactation, when the cow has a low DMI and is therefore unable to cover its increased energy requirements related to the establishment of milk production. McArt et al. (2013) estimated that, on commercial farms, 40% of early lactation cows are in NEB.

Mastitis affects the milk lactose content (**LC**), which is the main osmoregulator in milk. The variations in LC

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are negatively associated with increases in SCC (Alessio et al., 2021). Several studies have reported a decrease in LC during subclinical mastitis. Bruckmaier et al. (2004) found a decrease of 0.43 percentage points in milk LC from quarters with SCC above 1,000,000 cells/mL of milk compared with healthy quarters in the same udder. Similarly, Nielsen et al. (2005) observed a decrease in LC, regardless of milking time and milking interval, in quarters with mastitis (SCC > 400,000 cells/mL of milk). The decrease in LC appeared to depend on the chosen SCC threshold and on the microbial species causing the inflammation, although there is a limited information on this matter. With regard to microbial species, Antanaitis et al. (2021) reported a greater decrease in LC with *Staphylococcus aureus* and *Streptococcus agalactiae* IMI and no effect when an IMI was caused by *Escherichia coli*. However, Leitner et al. (2006) observed a reduction in the LC in milk from quarters with *Streptococcus dysgalactiae* and *E. coli* IMI, but no difference in LC when quarters were infected by *Staph. aureus*.

Negative energy balance is also known to affect LC. Previous studies have pointed out the correlation between LC and energy balance in early lactation (r = 0.36; $P < 0.001$; Reist et al., 2002). Santschi et al. (2016), using milk BHB as a proxy for NEB, found that LC and lactose yield were decreased by 0.14 ± 0.01 percentage points (mean ± SE) and 0.15 ± 0.01 kg/d, respectively, when the milk BHB was >0.19 mmol/L. In case of extensive underfeeding, such as a basal diet composed of 60% straw, a drop in LC was observed within 24 h (Bjerre-Harpøth et al., 2012).

A few studies reviewed by Costa et al. (2019) have suggested using LC as an indicator of metabolic or udder diseases or to predict NEB. However, no one has considered both quarter health status and metabolic status of cows. We hypothesize that cows experiencing NEB, identified through milk BHB concentration, have an increased risk of inflammation or infection and that cows with inflammation or infection, or both, and with NEB have a lower milk LC. The aim of this investigation was to describe the effect of quarter inflammation or infection, or both, on milk LC over the lactation period (5–300 DIM) and the interaction between quarter inflammation, infection, and cow NEB (using milk BHB concentration as a proxy) on milk LC during early lactation (5–70 DIM).

MATERIALS AND METHODS

Animals

This work is based on a data collected from an observational longitudinal study conducted at the Faculty of Veterinary Medicine of Université de Montréal (Saint-Hyacinthe, QC, Canada). Sampling was conducted on 5 Canadian commercial dairy farms equipped with automated milking systems (**AMS**; Lely Astronaut A4 AMS technology) and located within a 20-km perimeter around the Faculty of Veterinary Medicine. This project was approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine (protocol 18-Rech-1975; Université de Montréal, St-Hyacinthe, QC, Canada). The study was conducted from December 2018 to March 2020 and included 77 primiparous and 303 multiparous (from parity 2 to 8) Holstein cows monitored from 5 to 300 DIM. Cows were recruited systematically as they calved. Milk samples from mammary quarters presenting signs of clinical mastitis (abnormal milk solely, or abnormal milk with an abnormal udder and with or without systemic signs) on a given sampling visit were not analyzed for milk composition nor SCC and thus excluded from subsequent analyses. No other exclusion criteria were used in this project. In all farms, cows were housed in freestalls with free-flow traffic access to the AMS and to water and lying space. The diet consisted of a partial mixed ration formulated to meet or exceed the NRC requirements (NRC, 2001).

Collection of Milk Samples and Analyses

Every 14 d, each quarter of all recruited cows was sampled aseptically after cleaning and disinfection of the teats. Briefly, approximately 60 mL of milk was discarded, and then 50 mL were collected (later described as cisternal milk). The hour of sample collection was recorded. After collection, all milk samples were placed in coolers with icepacks. Within 6 h of collection, in our laboratory, a 10-mL aliquot was set aside and frozen at −20°C for future bacteriological analyses. Bronopol was then added to the remaining part of the samples (≈40 mL), which were immediately cooled to 4°C. Within 24 to 96 h, the quarter cisternal milk samples were analyzed to determine SCC and milk composition. More precisely, fat content (**FC**), protein content (**PC**), LC, and BHB (mmol/L) were analyzed at Lactanet (Ste-Anne-de-Bellevue, QC, Canada) by midinfrared spectroscopy using a MilkoScan FT6000 (Foss Electric Analytical Solutions, Hillerød, Denmark). On a given milk sample, parameters with values outside of the Milkoscan calibration range were recorded as missing information (the number of samples with missing values was as follows: fat =1,280; protein = 300; lactose = 867, and BHB = 1,146). The calibration values obtained from Foss were as follows: 1.44% to 10.38% for fat, 2.59% to 4.60% for protein, 3.76% to 5.53% for lactose, and 0.04 to 0.60 m*M* for BHB. Somatic cell count was determined by flow cytometry with the Fossomatic 7 DC (Foss Analytics).

To define the infectious status, milk samples were thawed and bacteriological culturing was performed using a disposable microbiological loop by smearing 0.01

mL of milk on a blood agar containing 5% defibrinated sheep blood. Agars were then incubated for 24 to 48 h, and bacterial enumeration (up to 10 cfu) was performed for each colony type. The identification of microbial species was performed using the MALDI Biotyper CM System (the mass spectrometry method used was MALDI-TOF MS). All samples were analyzed within 6 mo of collection. When 3 or more colony types were observed (5.5% of the samples collected), the sample was considered "contaminated" and deemed as "noninformative;" thus, it was excluded from further analysis.

The milking time and milk yield of each quarter at each milking of all enrolled cows were extracted via the software of the AMS on each farm for the whole sampling period. A rolling 3-d average of the milking production of each quarter was calculated for each milk sampling (Deluyker et al., 1990). Because the milking interval can affect some of the milk parameters, time since last milking was computed for each sampling. On average, cows were sampled 4.7 ± 3.2 h (mean ± SD) following a previous milking (interquartile range: 2.3–6.4 h).

The quarter health status was determined for the 14,505 quarter samples collected by a combination of the inflammatory and infectious status of the quarter. The inflammatory status was defined by SCC using a threshold of 100,000 cells/mL of milk to guarantee the selection of a healthy quarter (Bansal et al., 2005; Leitner et al., 2006; Schwarz et al., 2010). The infectious status was determined by whether a pathogen was detected or not. The health status of the quarter was therefore defined using a 4-category variable: low SCC and no pathogen (SCC^-_$Patho^-$), high SCC and no pathogen (SCC^+_$Patho^-$), low SCC and pathogen (SCC^-_$Patho^+$), and high SCC and pathogen (SCC^+_$Patho^+$; Table 1). The effects of pathogen species from 5 to 300 DIM were studied by examining the most common bacterial species (i.e., species with prevalence >1.5% among the 14,505 quarter samples): *Aerococcus viridans*, *Corynebacterium bovis*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Staphylococcus haemolyticus*, and *Staphylococcus simulans*. The remaining identified species were classified as "other species" (Table 2). Samples with no growth were identified as such. In 1,858 of the collected milk samples (12.8%), 2 bacterial species were recovered, indicating the presence of a dual infection. If one of the 2 species was one of the 8 most frequent bacterial species identified, the IMI was considered as belonging to this species; if not, the sample was classified as "other species." When 2 frequent species were identified (130 samples), the IMI was considered as one of the species with the largest cfu number. When the cfu number was the same (45 samples), the bacterial species was randomly selected between the 2 species with a random number generator.

Table 1. Description of quarter health status for primiparous and multiparous cows sampled between 5 and 300 DIM[1]

			Primiparous			Multiparous		
Quarter health	SCC (cells/mL milk)	Pathogen	Number of quarters affected[2]	Number of cows affected[2]	Mean number of cfu/0.01 mL	Number of quarters affected[2]	Number of cows affected[2]	Mean number of cfu/0.01 mL
SCC^--$Patho^-$	<100,000	Not detected	260	76	0	935	292	0
SCC^--$Patho^+$	<100,000	Detected	224	74	3.03 ± 3.71	831	292	3.63 ± 4.05
SCC^+-$Patho^-$	≥100,000	Not detected	160	62	0	622	249	0
SCC^+-$Patho^+$	≥100,000	Detected	104	59	8.04 ± 5.09	632	250	7.49 ± 5.43

[1]Primiparous cows = 306 quarters from 77 cows; multiparous cows = 1,183 quarters from 303 cows; SCC^- = < 100,000 cells/mL; SCC^+ = > 100,000 cells/mL; $Patho^-$ = no microorganisms detected; $Patho^+$ = microorganisms detected; cfu = colony-forming units; cfu values are given as mean ± SD.
[2]Number of quarters or cows classified within a given category at least once between 5 and 300 DIM.

Table 2. Microbial species and their prevalence in cisternal milk quarter[1]

Category of bacterial species	Number of samples	Prevalence 1 (%)	Prevalence 2 (%)
No growth	8,694	59.9	—
Another species	2,618	18.0	45.1
Aerococcus viridans	636	4.4	10.9
Corynebacterium bovis	618	4.3	10.6
Staphylococcus aureus	233	1.6	4.0
Staphylococcus chromogenes	567	3.9	9.8
Streptococcus dysgalactiae	251	1.7	4.3
Staphylococcus epidermidis	373	2.6	6.4
Staphylococcus haemolyticus	273	1.9	4.7
Staphylococcus simulans	242	1.7	4.2

[1]The percentages for prevalence 1 were across all 14,505 samples and the percentages for prevalence 2 were across the 5,811 samples in which microorganisms were detected.

In our study, the only parameter that could be used to describe NEB was the milk BHB concentration. Therefore, an elevated milk BHB concentration (hyperketolactia) was used as a proxy for NEB in early lactation (DIM < 70). For this purpose, a cow-level BHB value was computed using the milk-yield weighted content of the 4 quarters to describe the nutritional status of the cow because we did observe some BHB variation between quarters, on a given sampling within the same cow (mean CV = 20%). This cow-level value was then used to classify cows using the following thresholds: <0.15 mmol/L = BHB−; 0.15–0.19 mmol/L = BHB+; >0.19 mmol/L = BHB++ according to Denis-Robichaud et al. (2014).

Statistical Analyses

All statistical analyses were performed using R (version 4.0.4). To ensure reproducibility of the analyses, the assembled data set and the R scripts used for analyses are available as Supplemental Files S1, S2, S3, S4, S5, S6, and S7 (see Notes). Descriptive statistics about effect of parity and interval between milk sampling and previous milking on milk components were studied through univariable ANOVA. Three models were used to study the effects of 3 main predictors on a given milk component: (1) the quarter health status (between 5 and 300 DIM; 14,505 samples), (2) the quarter infectious status (between 5 and 300 DIM; 14,505 samples), and (3) the cow milk BHB concentration and the simultaneous effect of the cow milk BHB concentration and the quarter health status (between 5 and 70 DIM; 4,628 samples). Models were run with these different variables as the main predictors and with some adaptations (described in the following sections). The general model was as follows:

$$\text{Component}_{ijk} = \beta_0 + \beta_1\text{Main Predictor}_{ijk} + \boldsymbol{\beta}_{\mathbf{x}}\text{Poly DIM}_{ijk} + \boldsymbol{\beta}_{\mathbf{z}}\text{Main Predictor}_{ijk} \times \text{Poly DIM}_{ijk} + \boldsymbol{\beta}_{\mathbf{cov}}\text{Covariates}_{ijk} + \varepsilon_{ijk} + u_{jk} + v_k.$$

In the formula, Component_{ijk} is the content of a given component in milk (LC, FC, or PC) of the ith milk sample (i = 14,505) from the jth quarter (j = 1,489) from the kth cow (k = 380); β_0 is the mean component content when all predictor values were set to zero or to the reference value (for categorical variables); β_1 is the effect of the main predictor; $\boldsymbol{\beta}_{\mathbf{x}}$ is a vector of coefficients associated with a set of second-degree polynomial terms used to represent the association between DIM at sample collection and the milk component concentration; $\boldsymbol{\beta}_{\mathbf{z}}$ is a vector of coefficients for the interaction terms between the main predictor and the DIM polynomial terms (thus allowing to describe the effect on the milk component concentration of the main predictor as a function of DIM in a curvilinear fashion); and $\boldsymbol{\beta}_{\mathbf{cov}}$ is a vector of the coefficient representing the association between other important extraneous covariates and the milk component analyzed to adjust the estimated component concentration for the latter covariates. Also, $\varepsilon \sim N\left(0, \sigma^2_{ijk}\right)$, $u_{jk} \sim N\left(0, \sigma^2_{jk}\right)$, and $v_k \sim N\left(0, \sigma^2_k\right)$. In all models, the covariates used to adjust the estimated milk component concentration were as follows: the time between the last milking and milk sample collection (in hours), the quarter preceding 3-d average milk yield (in kilograms/day), the cow parity (primiparous vs. multiparous), and the herd identification to account for the clustering of cows by herd. Moreover, a random quarter intercept (u_{jk}) was used to account for clustering of samples by quarter. For the latter intercept, a spatial exponential correlation structure was used to account for the greater correlation between samples collected closer in time. Finally, a random cow intercept (v_k) was used to account for the clustering of quarters by cow. For the latter, a compound symmetry correlation structure was used, thus assuming similar correlation between pairs of quarters from a same cow. The sample- (ε_{ijk}), quarter- (u_{jk}), and cow- (v_k) level residuals were assumed to follow normal distributions centered on zero and with a constant variance (σ^2_{ijk}, σ^2_{jk}, σ^2_k, respectively).

Homoscedasticity and normality of the residuals were checked using plots of the residuals at the lowest level of the hierarchy. Adjustments for multiple comparisons were made using the Tukey–Kramer adjustment. All samples with missing values were excluded from the model.

To study the effect of the bacteriological status of the quarter (Table 2), the quarter SCC level (low or high) was not considered because it was hypothesized to be determined, in large part, by the bacterial species itself.

To study the effect of the cow BHB status and the combined effect of health quarter status and cow BHB status on milk composition, the interaction between DIM and the main predictors (cow BHB status and health quarter status) was initially included in the model, but removed if statistically not significant. The combined effect of the quarter health status and of the cow's BHB status on milk composition was studied by including these 2 variables and their interaction in the model. A chi-squared test was also realized to explore the correlation between cow BHB status and health quarter status.

Post Hoc Power Calculation

For the current study, we used data collected for another project (France et al., 2022) aiming at elucidating the quarter-level milk losses associated with presence of various bacterial species. The sample size of the current study was, thus, limited by the power consideration of that latter study. Nevertheless, we conducted a post hoc power calculation to estimate the difference in LC that could be detected with the available sample size. When considering in multiparous cows, comparisons between LC of the category with the largest number of observations (SCC^-_$Patho^-$ and BHB^-; n ≈1,100 samples; mean LC of 4.5% and SD of 0.25%) to categories with substantially less observations (e.g., SCC^-_$Patho^-$ and BHB+; n ≈ 80; mean LC of 4.4 and SD of 0.25%), we estimated that our study had >95% power to detect a 0.11 percentage point reduction in LC. For this calculation, however, neither the structure of the dataset (i.e., repeated samples per quarter, quarters grouped by cows, and cows clustered by farms) nor the multiple comparisons issues were considered. This estimation should, therefore, be considered as a best-case scenario (hence the choice of setting power at >95%). When comparing 2 of the quarter health and NEB categories with the smallest number of samples, our study had >95% power to detect a 0.15 percentage points reduction in LC.

Using the primiparous cows' dataset, our study had >95% power to detect LC reduction ranging from 0.15 to 0.21 percentage points when comparing, respectively, the largest quarter health and NEB category (n ≈ 400) with the smallest one (n ≈ 40), or 2 of the smallest categories against each other (n ≈ 40 vs. n ≈ 40). Again, given that neither the structure of the data nor the multiple comparisons issue were considered, these should be considered as optimistic scenarios.

RESULTS

Variations in Cisternal Milk Composition Throughout the Lactation Period (5–300 DIM)

In total, among the 380 cows, 1,489 quarters were recruited with an average of 10 samples per quarter collected throughout the lactation (range: 1–20), resulting in the collection of 14,505 samples. The milk content was slightly different between primiparous and multiparous; the mean LC was higher for primiparous than for multiparous cows: 4.56% ± 0.269% vs. 4.40% ± 0.264% (±SD; $P < 0.001$), whereas the mean FC was lower for primiparous compared with multiparous cows: 4.48% ± 2.120% vs. 5.04% ± 2.360% (±SD; $P < 0.001$; Figure 1). The PC was not affected by parity ($P = 0.94$).

The milking interval between sampling and previous milking varied and had an effect on the milk composition in both primiparous and multiparous cows. The LC was slightly lower throughout the lactation period (on average, −0.21% ± 0.257%; $P < 0.001$), the PC was slightly reduced (−0.16% ± 0.314%; $P < 0.001$), whereas the FC was much higher (+2.75% ± 2.160%; $P < 0.001$) when the interval between milk sampling and previous milking was short (<6 h; Figure 1). Regardless of the interval between milk sampling and previous milking, the LC was largely constant throughout the lactation period for both primiparous and multiparous cows. The mean LC in milk sampled more than 6 h after the previous milking was highest at approximately 30 to 35 DIM (4.92% ± 0.032% and 4.93% ± 0.109%, respectively, for primiparous and multiparous cows; mean ± SD) and then decreased to a minimum of 4.58% ± 0.284% and 4.43% ± 0.326% toward the end of lactation (between 250 and 300 DIM). Conversely, the FC and PC decreased from the beginning of the lactation to around 100 DIM and then increased steadily up to the end of lactation, regardless of the interval between milk sampling and previous milking.

Relationship Between Quarter Health Status and Cisternal Milk Component (5–300 DIM)

The prevalence values of quarters with ≥1 milk samples in a given health status category were 73.5%, 53.3%, and 35.0%, respectively, for SCC^-_$Patho^+$, SCC^+_$Patho^-$, and SCC^+_$Patho^+$ for primiparous cows (303 quarters) and 70.5%, 53.3%, and 53.8% for multiparous cows (1,183 quarters; Table 1). All primiparous and almost all (96%) multiparous cows had at least 1 SCC^-_$Patho^-$ milk

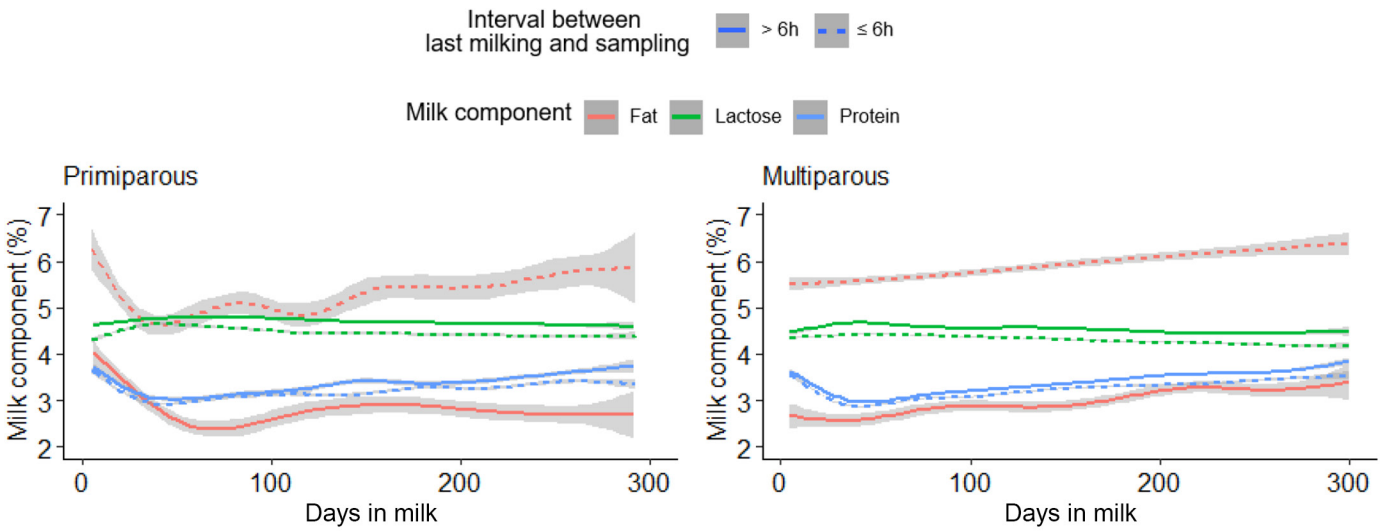

Figure 1. Lactose, fat, and protein quarter cisternal milk composition described according to parity (primiparous = 77; multiparous = 303), DIM (5–300), and time between milk sampling and previous milking. The figure was generated using a generalized additive mode smoothing. Data are presented as the means estimated by the statistical model ± SE (gray shading).

sample between 5 and 300 DIM. Only 25 cows never had quarter SCC$^+$ (7 primiparous and 18 multiparous), 156 cows (37 primiparous and 119 multiparous) had between 1 and 5 occurrences of a quarter SCC$^+$, and 129 cows (89 primiparous and 40 multiparous) had between 6 and 10 occurrences of a quarter SCC$^+$. Finally, 76 cows (13 primiparous and 63 multiparous) had between 11 and 15 occurrences of a quarter SCC$^+$, and 19 cows (5 primiparous and 14 multiparous) had between 16 and 20 quarter SCC$^+$ occurrences.

When quarters were Patho$^+$, the number of cfu was, on average, higher in quarters SCC$^+$_Patho$^+$ than in quarters SCC$^-$_Patho$^+$ (Table 1; $P < 0.001$). Regarding milk composition, the LC was lower in milk of quarters that were SCC$^+$, regardless of their infection status (Patho$^-$ or Patho$^+$; −0.17 ± 0.013 percentage points; $P < 0.001$; Table 3). The FC was only higher in milk of SCC$^+$_Patho$^-$ quarters (+0.30 ± 0.081 percentage points compared with SCC$^-$_Patho$^-$ quarters; $P < 0.01$; Table 3). The PC did not vary according to quarter health status ($P = 1.00$). There were no differences in FC, PC, or LC between Patho$^-$ and Patho$^+$ in quarters with high SCC (FC: $P = 0.12$; PC: $P = 0.99$; and LC: $P = 0.50$).

Relationship Between Quarter Milk Bacteriological Status and Cisternal Milk LC (5–300 DIM)

Regarding the prevalence of bacterial species over the 5,811 infected quarter samples, *Aerococcus viridans* (10.9%) was the predominant species, followed by *Corynebacterium bovis* (10.6%), *Staph. chromogenes* (9.8%), *Staph. epidermidis* (6.4%), *Staph. haemolyticus* (4.7%), *Strep. dysgalactiae* (4.3%), *Staph. simulans* (4.2%), and *Staph. aureus* (4.0%); 45.1% of the IMI were classified as "other species." Of all milk components studied, only LC was affected by bacterial species, and this effect was dependent on DIM (Figure 2). Only 5 of the 8 most frequent species had an effect on the LC. Quarters infected by *Staph. epidermidis* and *Staph. chromogenes* had a mean LC 0.06 to 0.10 percentage points ± 0.001

Table 3. Least squares means estimates (± SE) of cisternal milk component in relation to quarter health status in primiparous and multiparous cows from 5 to 300 DIM

	Primiparous (n = 77)			Multiparous (n = 303)		
Quarter health[1]	Fat (%)	Protein (%)	Lactose (%)	Fat (%)	Protein (%)	Lactose (%)
SCC$^-$_Patho$^-$	4.57 ± 0.113^a	3.10 ± 0.023^a	4.61 ± 0.016^a	5.02 ± 0.065^a	3.16 ± 0.012^a	4.43 ± 0.009^a
SCC$^-$_Patho$^+$	4.53 ± 0.120^a	3.09 ± 0.023^a	4.61 ± 0.017^a	4.98 ± 0.074^a	3.15 ± 0.013^a	4.42 ± 0.010^a
SCC$^+$_Patho$^-$	4.86 ± 0.129^b	3.10 ± 0.024^a	4.45 ± 0.018^b	5.31 ± 0.086^b	3.16 ± 0.014^a	4.26 ± 0.011^b
SCC$^+$_Patho$^+$	4.63 ± 0.122^a	3.10 ± 0.023^a	4.43 ± 0.017^b	5.08 ± 0.074^a	3.16 ± 0.013^a	4.25 ± 0.010^b

^{a,b}Means with different letters within the same column are significantly different ($P \leq 0.05$).

[1]SCC$^-$ = < 100,000 cells/mL; SCC$^+$ = > 100,000 cells/mL; Patho$^-$ = no microorganism detected; Patho$^+$ = microorganisms detected.

lower than uninfected quarters from approximately 60 to 220 DIM ($P < 0.01$). *Staphylococcus simulans*, when detected between 45 and 255 DIM, and *Staph. aureus*, when detected between 15 and 225 DIM, respectively, were associated with a reduction in LC of −0.14 to −0.20 percentage points ± 0.001 ($P < 0.01$) compared with uninfected quarters. *Streptococcus dysgalactiae* was correlated with the greatest LC diminution across lactation, with the largest reduction in late lactation: −0.35 percentage points ± 0.001 ($P < 0.01$). Quarters infected by "other species" had, on average 0.02 to 0.03 percentage points ± 0.001 lower LC between 120 and 210 DIM ($P < 0.05$). No significant effect on LC was found for *A. viridans* ($P = 0.51$) or *C. bovis* ($P = 0.09$).

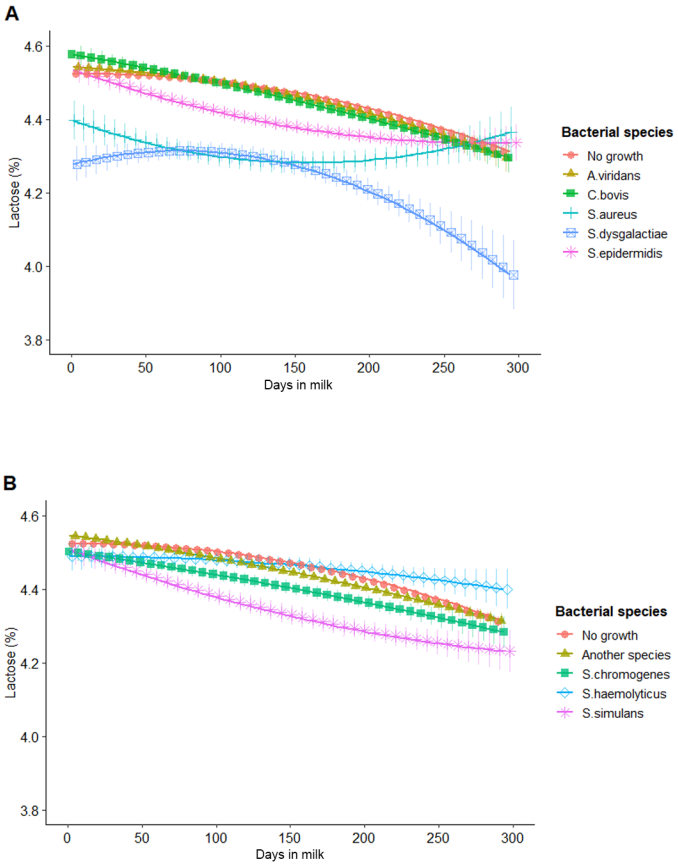

Figure 2. Least squares means estimates of the lactose content of quarter cisternal milk samples in relation to bacteriological status for both (A) primiparous cows (n = 306 quarters from 77 cows) and (B) multiparous cows (1,183 quarters from 303 cows) sampled between 5 and 300 DIM. The figure is separated into 2 plots to facilitate viewing; quarters with no bacterial growth (red bullets/line) are presented on both plots as a reference. The bacterial species included *Aerococcus viridans*, *Corynebacterium bovis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, and *Staphylococcus simulans*. Error bars represent the SE.

Relationship Between Cow NEB Status, Quarter Health Status, and Cisternal Milk LC (5–70 DIM)

In the current study, out of 380 cows, 74 were identified as BHB++ (>0.19 m*M*), comprising 16 primiparous and 58 multiparous cows. The threshold of 0.19 m*M* was exceeded once for 54 cows (13 primiparous and 41 multiparous), twice for 15 cows (3 primiparous and 12 multiparous), and thrice for 5 multiparous cows. Out of the 380 enrolled cows, 67 were classified as BHB+ (0.15 < BHB <0.19 m*M*). The threshold was exceeded twice and thrice for 10 cows (2 primiparous and 8 multiparous ones) and 2 multiparous cows, respectively. First, regarding the only effect of NEB on milk from healthy quarters (SCC^-_$Patho^-$), FC was higher in cows that were BHB+ and BHB++, irrespective of parity, with +0.51 ± 0.160 percentage points ($P = 0.003$) and +1.58 ± 0.138 percentage points, respectively ($P < 0.001$; Figure 3). The PC did not vary in relation to NEB cows' status (BHB+: $P = 0.12$; BHB++: $P = 1.00$; Figure 4). The LC was lower in milk from BHB+ (−0.05 ± 0.019 percentage points; $P = 0.043$) and BHB++ (−0.13 ± 0.020 percentage points; $P < 0.001$) cows compared with milk from BHB- cows (Figure 5). The combined effects of quarter health and cow NEB status on LC were similar for primiparous and multiparous cows (Figure 5). Furthermore, the interaction between quarter health, cow BHB status, and DIM was not statistically significant, which suggests that effect of quarter health and NEB on LC did not vary as function of DIM during the 5 to 70 DIM period. It appears that quarter health status affected LC slightly more than cow NEB. Quarters SCC^+ from cows with a BHB++ status (primiparous and multiparous) exhibited a lower milk LC (−0.15 ± 0.019 percentage points) compared with quarters SCC^+ from cows with a BHB− status (Figure 5; $P < 0.001$). The chi-squared test showed an augmentation of quarter SCC^+ frequency for cows BHB+ and BHB++ ($P < 0.001$). Similarly, the frequency of infection by *Staph. aureus* and *Strep. dysgalactiae* was also higher for cows in NEB (BHB++ cows: +228% quarters infected by *Strep. dysgalactiae* and + 260% quarters infected by *Staph. aureus*; $P < 0.01$).

DISCUSSION

The aim of this investigation was to describe the effect of quarters with high SCC or infection, or both, on milk LC throughout the lactation period (5–300 DIM) and the simultaneous effect of quarter health status and cow NEB (using milk BHB concentration) on milk LC during early lactation (5–70 DIM). The observed interaction highlights the necessity and value of further investigating how NEB and quarter health are related and their joint effect on milk LC.

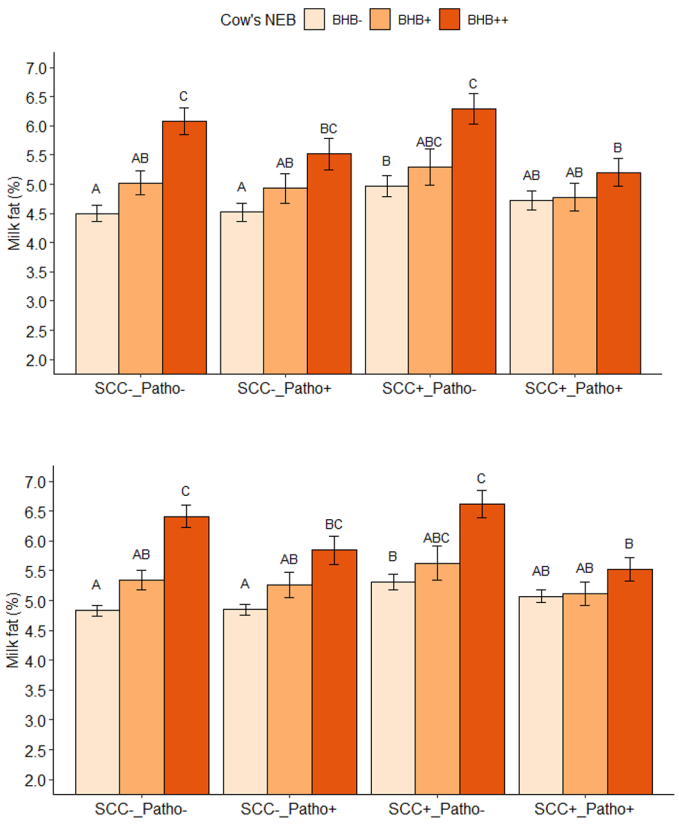

Figure 3. Least squares means estimates of the fat content of cisternal milk in relation to quarter health and cow's NEB status in primiparous (n = 77; top) and multiparous (n = 303; bottom) cows during the first 70 d of lactation (5–70 DIM). Bars within a plot with different superscripts (A–C) have significantly different ($P \leq 0.05$) means after adjusting for multiple comparisons. SCC^- = <100,000 cells/mL; SCC^+ = >100,000 cells/mL; $Patho^-$ = no microorganism detected; $Patho^+$ = microorganisms detected; BHB− = <15 m*M*; 0.15 ≤ BHB+ < 0.19 mM; and BHB++ = >0.19 m*M*. Error bars represent the SE.

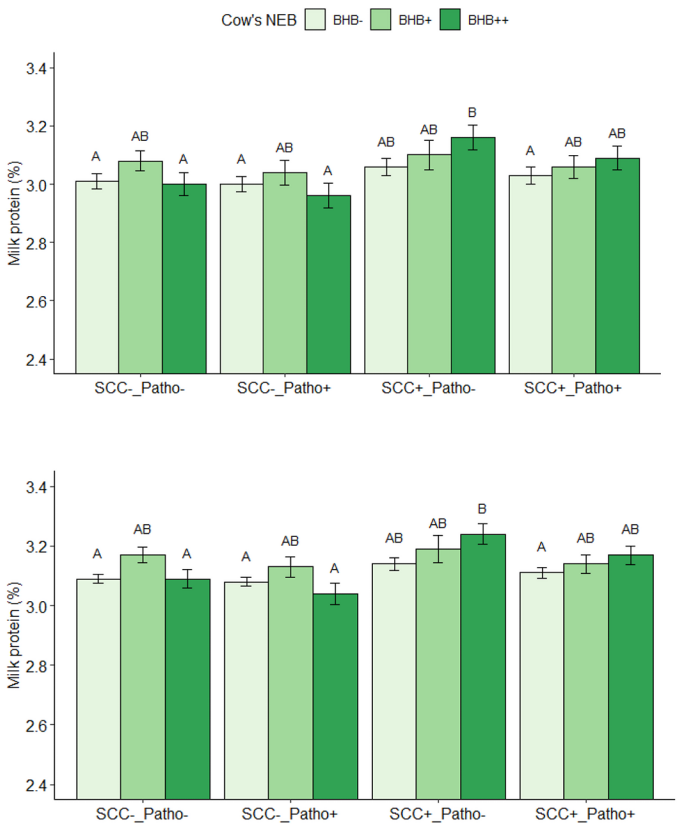

Figure 4. Least squares means estimates of the protein content of cisternal milk in relation to quarter health and cow's NEB status in primiparous (n = 77; top) and multiparous (n = 303; bottom) cows during the first 70 d of lactation (5–70 DIM). Bars within a plot with different superscripts (A,B) have significantly different ($P \leq 0.05$) means after adjusting for multiple comparisons. SCC^- = <100,000 cells/mL; SCC^+ = >100,000 cells/mL; $Patho^-$ = no microorganisms detected; $Patho^+$ = microorganisms detected; BHB− = <15 m*M*; 0.15 ≤ BHB+ < 0.19 m*M*; BHB++ = >0.19 m*M*. Error bars represent the SE.

Sampling was performed on cisternal milk, which corresponds to the milk fraction removed at the beginning of milking before hormonal stimulation. The composition of this cisternal milk is specific because it is composed of milk residuals from the last milking but also of newly synthesized milk. The shorter the interval between the last milking and sampling, the greater the proportion of residual milk at sampling. The dairy farms sampled in this study were equipped with AMS, inducing variations of this interval. To verify the ability of the statistical model to describe changes in milk components, especially FC and LC, which depend on milk fraction and delay between milkings (Nielsen et al., 2005), the lactation curves for FC, PC, and LC from cisternal milk quarters were described. The curves were similar to the findings in the literature. Generally, FC and PC drop at the beginning of lactation and then slowly increase throughout the lactation period (Coulon et al., 1991; Silvestre et al., 2009). In contrast, LC increases in early lactation to reach a maximum around 65 DIM for primiparous and 45 DIM for multiparous cows, followed by a slight decrease beyond that stage. The lactation curves were similar between primiparous and multiparous cows, except that primiparous cows have a greater LC than multiparous cows as described by Miglior et al. (2006).

Relationship Between Quarter Health and Cisternal Milk Component

In this study, we observed lower LC values of −0.17 ± 0.013 percentage points in quarter SCC^+. Bansal et al. (2005) also observed a decrease in LC of −0.31 ± 0.27 percentage points in cisternal milk from quarters with an SCC above 100,000 cells/mL. Similarly, Berglund et al. (2007) reported an LC of −0.06 ± 0.04 and −0.17 ± 0.04 percentage points in milk composite from quarters with an SCC >100,000 cells/mL on 1 and >1 sampling occasion, respectively. However, Forsbäck et al. (2010)

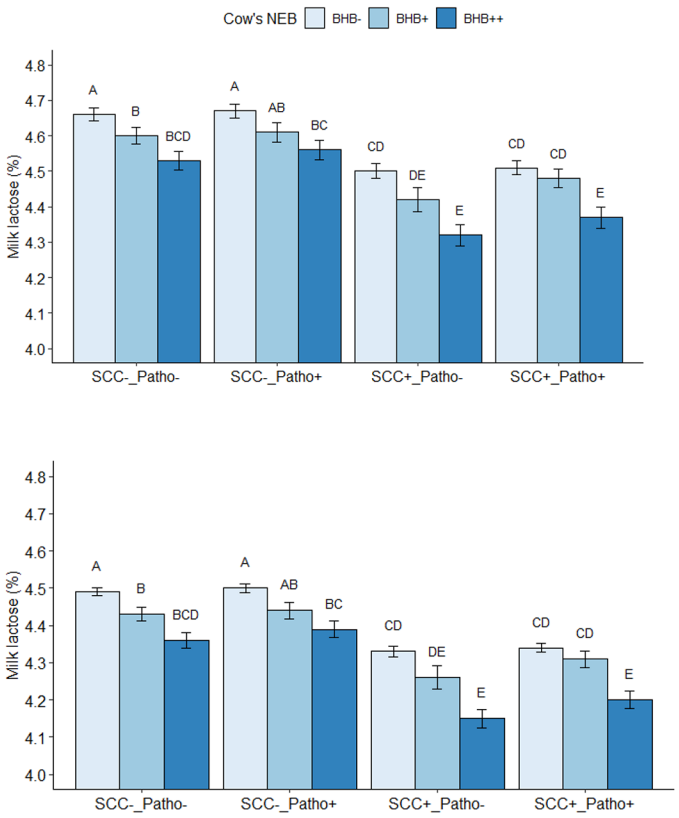

Figure 5. Least squares means estimates of the lactose content of cisternal milk in relation to quarter health and cow's NEB status in primiparous (n = 77; top) and multiparous (n = 303; bottom) cows during the first 70 d of lactation (5–70 DIM). Bars within a plot with different superscripts (A–E) have significantly different ($P \leq 0.05$) means after adjusting for multiple comparisons. SCC^- = <100,000 cells/mL; SCC^+ = >100,000 cells/mL; $Patho^-$ = no microorganisms detected; $Patho^+$ = microorganisms detected; BHB− = <15 m*M*; 0.15 ≤ BHB+ < 0.19 m*M*; BHB++ = >0.19 m*M*. Error bars represent the SE.

have reported no difference in LC between unhealthy and healthy quarters (criteria: SCC > 100,000 cells/mL and negative test for bacteria), but a larger day-to-day variation in LC among healthy vs. unhealthy quarters (CV = 0.9% vs. 1.5%). In our study, we did not observe larger day-to-day variation in LC between SCC^- and SCC^+ quarters (CV of 0.05 vs. 0.04), possibly because sampling was conducted every 14 d. Regarding the difference in LC in healthy versus unhealthy quarters reported in the literature, these are not always detected possibly because of the use of quarter- or cow-level analyses, the sampling of cisternal milk or composite milk, or the different criteria used to defined healthy versus unhealthy quarters (e.g., only SCC or both SCC and bacterial detection). In our study, we selected a threshold of 100,000 cells/mL, which is relatively low compared with the SCC amplitude in this data set (1,096 quarter samples with SCC > 1,000,000 cells/mL). However, the purpose of the threshold of 100,000 cells/mL was to guarantee the selection of healthy quarters with normal milk secretion and composition, free from inflammation or infection or any other disease, as documented by Leitner et al. (2000), Bansal et al. (2005), and Schwarz et al. (2010). We also ensured that similar results were observed with a threshold of SCC of 200,000 cells/mL (results not shown). Describing the effect of the exact SCC values on LC was not the aim of this study and has already been well documented in previous works (Bruckmaier et al., 2004; Bansal et al., 2005; Berglund et al., 2007; Forsbäck et al., 2010; Gonçalves et al., 2016). In this context, it appears that there is a reduction in LC in quarters with high SCC, but no additional changes in infected quarters. In fact, immune activation and the production of inflammatory molecules could lead to mammary epithelium damage, which might induce alteration in lactose secretion and impair tight junction permeability (loss of lactose in blood; Nguyen and Neville, 1998; Gonçalves et al., 2016).

Also, in case of infection, LC may also be used as a substrate for the microorganisms leading to its diminution in milk (Nielsen et al., 2005), but in this study we did not find a difference in LC between infected versus uninfected quarters, similarly to Bansal et al. (2005). Thus, no association was found between the overall detection of microorganism and LC. However, it appears, that some microbial species, when detected, were correlated with lower LC. The effect of specific microorganism species on LC is not well documented. Although we identified a variety of different species, with some having a low prevalence, our findings on relationships between the most common pathogen species and milk components does provide additional novel information. In the present study, of the 8 predominant species, 5 were associated with a reduction in LC when they were detected. Some of these pathogens are related to a decrease in LC only at a specific stage of lactation, such as *Staph. aureus*, which is linked with a decrease in LC only at early and mid lactation, or *Staph. epidermidis*, which is correlated with a LC reduction only at mid lactation. These observations have never been reported in the literature. Further investigations are necessary while taking into account the fact that the prevalence of the microorganism throughout the lactation period could be variable.

The largest decreases in milk LC were found in quarters infected by *Staph. aureus* and *Strep. dysgalactiae*. These species are among the most dominant pathogens causing mastitis. Leitner et al. (2006) found a reduction of −0.72 ± 1.369 percentage points in LC in quarters infected by *Strep. dysgalactiae* but no change in LC when *Staph. aureus* was detected. This reduction in LC by *Strep. dysgalactiae* is 2 times higher than that found in the current study (−0.35 percentage points ± 0.001). This difference is likely related to the selection of chronically infected cows in the study by Leitner et al. (2006) as op-

posed to the inclusion, in our study, of apparently normal milking cows. On the other hand, Antanaitis et al. (2021) identified *Staph. aureus* as one of the bacterial species, along with *Strep. agalactiae*, correlated with the greatest LC drop (*Staph. aureus*: −0.05 ± 0.009 and *Strep. agalactiae*: −0.10 ± 0.16 percentage points). The following microbial species were also associated with LC reduction in our study: *Staph. chromogenes*, *Staph. epidermidis*, and *Staph. simulans*. However, others did not observe a similar effect for these bacterial species on milk LC (Leitner et al., 2006; Antanaitis et al., 2021).

Only a few previous studies have reported the effect of specific bacteria species on milk composition. In the current study, the overall detection of microorganism appears to have no effect on LC and did not provide any additional information to that already provided by the SCC. It seems that the effect of the bacterial species on LC was mainly dependent on the inflammatory reaction it induced and not on other infectious reactions.

Relationship Between Cow NEB Status and Cisternal Milk Component

Consistent with previous studies, cows in early lactation showed elevated milk BHB concentration, which could indicate a NEB. In the present experiment, 17.0% of the 380 cows in early lactation (5–70 DIM) had a milk BHB greater than 0.15 m*M* at least once. This number was slightly lower than the prevalence of 22.6% of NEB found by Santschi et al. (2016), also diagnosed by milk BHB. In contrast to our study, the results from Santschi et al. (2016) were obtained using udder-composite milk samples from 3,349 cows between 5 and 35 DIM. This difference in the results could be explained by the different period of the lactation when the 2 respective studies were conducted. Indeed, BHB release in blood is usually higher between 1 and 25 DIM compared with 25 to 70 DIM (Gross et al., 2015). The difference in the prevalence of high BHB between Santschi et al. (2016) and the present experiment might also be due to the difference in milking sampling (milk composite vs. cisternal milk). Furthermore, indication about the time between milk samples and feeding was not provided, and the capture of BHB by the mammary gland and the transfer in milk largely depends on this delay (Santschi et al., 2016; Bach et al., 2021). The NEB prevalence reported in the literature varies depending on the detection method used, the proxy measured (nonesterified fatty acids, fatty acids, or BHB), the fluid tested (serum or milk), the DIM at testing, and the type of NEB (induced by physiological status or by feeding restriction). In the present study, we focused on the onset of the lactation, when naturally NEB occurred for most of the cows, whereas in the majority of the previously cited studies, the effect of NEB on LC was mainly studied on cows undergoing feed restriction (qualitative or quantitative) in early, mid, or late lactation. Nevertheless, the mechanism of adaptation during NEB is dependent on the lactation stage, particularly in early lactation (Bjerre-Harpøth et al., 2012). After parturition, homeorhetic regulation prioritizes the milk production over basal metabolism and other energy expenditures (e.g., growth, gestation). Lactose synthesis is greatly affected by blood glucose availability, and blood serum glucose concentrations tend to be lower when cows are experiencing NEB (Larsen and Moyes, 2015). In the case of NEB, the activation of lipolysis leads to elevated blood nonesterified fatty acids levels, inducing fat oxidation. The saturation of the liver is related to BHB production and leads to its elevation in blood and in milk, which could allow the detection of NEB in the case of metabolic disorders (Bjerre-Harpøth et al., 2012; Santschi et al., 2016). We observed an LC of −0.13 ± 0.020 percentage points lower in cows with elevated milk BHB (cows that were BHB++). Similarly Santschi et al. (2016) reported a lower LC by −0.14 ± 0.01 percentage points. Larsen and Moyes (2015) found a negative phenotypic association ($r = -0.17$) between LC and milk BHB. Likewise, the energy balance was positively correlated with LC at $r = 0.363$ ($P < 0.001$) from 1 to 70 DIM in multiparous cows (Reist et al., 2002).

In summary, in the present study we observed lower LC levels when quarters exhibited high SCC as supported by the literature (Bansal et al., 2005; Berglund et al., 2007; Forsbäck et al., 2010). We also observed a decrease in LC for cows with high milk BHB concentration, which could be related to NEB, as reported previously (Reist et al., 2002; Larsen and Moyes, 2015; Santschi et al., 2016). These findings prompt the question of the combined effect of quarter health and NEB on LC.

Combined Effect of Quarter Health and Cow NEB Status on LC

The combined effect of inflammation or infection, or both, and NEB, measured through milk BHB concentration, on LC has never been investigated and was the objective of this research. When quarter health and energy balance were analyzed concomitantly, we found a larger decrease in LC in quarters with high SCC from cows with high milk BHB concentration at the beginning of lactation (5–70 DIM). In addition to the 0.16 ± 0.014 percentage points reduction in LC associated with high SCC in a quarter, we observed an additional LC drop of −0.16 ± 0.02 percentage points when a cow had also high milk BHB concentration in early lactation. Several mechanisms could potentially explain the additive effect

of quarter health and metabolic status on LC, and it could result from cumulative mechanisms specific to each one of them: (1) microorganism action and inflammatory reaction in mammary tissues for unhealthy quarter (Bansal et al., 2005) and (2) lower glucose availability for the mammary glands of cows experiencing NEB (Cant et al., 2002). The effect of immune mechanisms on mammary tissues involves the alteration of lactose synthesis by the mammary epithelial cells, the transfer of lactose from milk to blood, the use of lactose as a substrate by the microorganisms, and the use of glucose (lactose precursor) to activate and support local immune reactions (Enger, 2019). Another hypothesis is that the decrease in LC and the higher concentration of BHB in milk could be linked to compromised permeability of the mammary epithelium (Nguyen and Neville, 1998). For instance, quarter inflammation or infection might lead to lactose transfer from the mammary gland to the blood, and conversely, BHB may transfer from the blood to the mammary gland because its molecular weight is low (104 g/mol vs. 342 g/mol for lactose). Accordingly, Nielsen et al. (2005) found a significantly higher BHB concentration in unhealthy quarters (detection via the California Mastitis Test) regardless of milking intervals and milk fraction (+0.13 ± 0.02 m*M*; $P < 0.01$). However, a low correlation ($r = 0.09$, $P < 0.001$) exists between milk BHB and SCC according to Larsen and Moyes (2015).

Last, we observed an increase in the number of quarters with high SCC and higher identification of *Staph. aureus* and *Strep. dysgalactiae* for cows experiencing NEB. This could be explained by the dysfunction of the immune system in the case of elevated milk BHB concentration. In fact, Suriyasathaporn et al. (1999) conducted in vitro studies, examining the effects of BHB on immune cells and observed a decrease in the phagocytic capacity of polymorphonuclear cells and macrophages, a reduction in the production of chemoattractant molecules, and impaired leukocyte migration to the mammary gland. In such cases, the risk of inflammation or the infection by some microorganisms could be elevated for cows experiencing NEB, possibly leading to higher frequency of cows undergoing both IMI and NEB. The association between metabolic disorder, such as subclinical ketosis, and high SCC (SCC thresholds of 250,000 or 400,000 cells/mL) has already been reported with a mean risk (±SD) of 1.46 ± 0.2 in a meta-analysis by Raboisson et al., 2014. Moreover, as observed in our study, the cumulative effect of NEB and IMI could alter more severely the LC of milk. Thus, further investigations are needed to better understand the relationship between IMI and NEB and to identify the physiological mechanisms leading to the observed association of lower LC in unhealthy quarters from cows experiencing NEB.

CONCLUSIONS

The results of this study highlight milk LC diminution throughout the lactation period when the quarter is unhealthy. This was mainly associated with the inflammatory reaction because the presence or absence of pathogens did not affect LC, even if microbial species such as *Staph. epidermidis*, *Staph. chromogenes*, *Staph. simulans*, *Staph. aureus*, and *Strep. dysgalactiae* were associated with a milk LC reduction. The LC was also reduced in the case of NEB in early lactation, and the frequency of quarters with high SCC was higher for cows in NEB. The most important finding of this study is the highest reduction in milk LC in inflamed quarters from cows experiencing NEB. These findings highlight the complexity of LC variation in milk, which is a function of many factors (the milk fraction, cow variability, and DIM). They illustrate the necessity of considering both quarter health and a cow's NEB to use LC as an indicator. The interest in LC is currently growing as it can easily and cost-efficiently be measured using the infrared spectroscopy technique in the Dairy Herd Improvement routine analysis of milk. Further studies are needed to confirm the possibility of using LC as a health indicator on udder-composite milk samples and with constant milking intervals.

NOTES

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Abbreviations used: AMS = automated milking system; BHB− = <15 m*M*; BHB+ = 0.15 ≤ BHB+ < 0.19 m*M*; BHB++ = >0.19 m*M*; FC = fat content; LC = lactose content; NEB = negative energy balance; Patho^{-} = no microorganisms detected; Patho^{+} = microorganisms detected; PC = protein content; SCC^{-} = <100,000 cells/mL; SCC^{+} = >100,000 cells/mL.

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