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Milk fatty acids as indicators of negative energy balance of dairy cows in early lactation

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ABSTRACT

Most dairy cows experience negative energy balance (**NEB**) in early lactation because energy demand for milk synthesis is not met by energy intake. Excessive NEB may lead to metabolic disorders and impaired fertility. To optimize herd management, it is useful to detect cows in NEB in early lactation, but direct calculation of NEB is not feasible in commercial herds. Alternative methods rely on fat-to-protein ratio in milk or on concentrations of non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHB**) in blood. Here, we considered methods to assess energy balance (**EB**) of dairy cows based on the fatty acid (**FA**) composition in milk. Short- and medium-chain FAs (primarily, C14:0) are typically synthesized *de novo* in the mammary gland and their proportions in milk fat decrease during NEB. Long-chain FAs C18:0 and C18:1 *cis*-9 are typically released from body fat depots during NEB, and their proportions increase. In this study, these FAs were routinely determined by Fourier-transform infrared spectroscopy (**FTIR**) of individual milk samples. We performed an experiment on 85 dairy cows in early lactation, fed the same concentrate ration of up to 5 kg per day and forage *ad libitum*. Daily milk yield and feed intake were automatically recorded. During lactation weeks 2, 4, and 6 after calving, two milk samples were collected for FTIR spectroscopy, Tuesday evening and Wednesday morning, blood plasma samples were collected Thursday morning. Net energy content in feed and net energy required for maintenance and lactation were estimated to derive EB, which was used to compare alternative indicators of severe NEB. Linear univariate models for EB based on NEFA concentration (deviance explained = 0.13) and other metabolites in blood plasma were outperformed by models based on concentrations of metabolites in milk: fat (0.27), fat-to-protein ratio (0.18), BHB (0.20), and especially C18:0 (0.28) and C18:1 *cis*-9 (0.39). Analysis of generalized additive models (GAM) revealed that models based on milk variables performed better than those based on blood plasma (deviance explained 0.46 vs. 0.21). C18:0 and C18:1 *cis*-9 also performed better in severe NEB prediction for EB cut-off values ranging from −50 to 0 MJ NEL/d. Overall, concentrations of C18:0 and C18:1 *cis*-9 in milk, milk fat, and milk BHB were the best variables for early detection of cows in severe NEB. Thus, milk FA concentrations in whole milk can be useful to identify NEB in early-lactation cows.

Implications

Most dairy cows experience negative energy balance in early lactation that can affect their current and future milk production. Concentrations of several blood and milk metabolites have been previously shown to be associated with metabolic disorders. This study showed that concentrations of milk fatty acids C18:0 and C18:1 *cis*-9 in milk determined by Fourier-transform infrared spectroscopy were the best predictors of negative energy balance of dairy cows in the first 6 weeks after calving.

Introduction

In most dairy cows in early lactation, energy intake fails to meet the energy demand for milk production (Drackley, 1999; Grummer et al., 2004), resulting in negative energy balance (**NEB**). The interactions between NEB, fertility, and metabolic diseases are well-established (Wathes et al., 2011; Esposito et al., 2014; Pérez-Báez et al., 2019a). The level of energy balance (**EB**) regulates metabolic and reproductive parameters related to ovarian function (Butler,

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2003; Mellouk et al., 2019). Cows in severe NEB are more susceptible to oxidative stress, metabolic disorders, and impaired fertility (Ingvartsen, 2006; Martin et al., 2015).

Metabolic chambers are considered the gold standard for estimating EB, but this method is expensive and difficult to implement on a large scale. Direct calculations of EB can be performed based on individual milk yield and feed intake, in order to estimate cow energy status. Dedicated software solutions, e.g., the NorFor system (Volden, 2011), also account for nutritional values of diets and cow characteristics such as BW, growth rate, and pregnancy. However, determination of DM intake (**DMI**) in individual cows can rarely be done in commercial herds.

Daily EB may be the best indicator of metabolic load in dairy cows (Coffey et al., 2001), but limited opportunities to determine it in practice have led to the introduction of different indirect markers, such as concentrations of non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHB**) in blood plasma. It is generally agreed that NEFA is correlated to EB. However, several studies (Wylie et al., 2008; Erdmann et al., 2019) reported weak correlations while others (Billa et al., 2020) noted stronger correlation ($r = -0.72$) between plasma NEFA and EB in cows that underwent feed restriction. The use of NEFA did not result in higher precision of prediction of individual and herd-level EB, regardless of sample size (Reist et al., 2002). This creates doubt about the reliability of this particular metabolite as an indicator of EB in early-lactation dairy cows. However, elevated NEFA levels in blood have repeatedly been shown to be related to increased risk of disease and reproductive disturbances (Ospina et al., 2013). Concentration of BHB can be measured quantitatively in both blood and milk. Blood BHB is sometimes used as an indicator of EB (Ospina et al., 2013), but it is weakly related to EB in dairy cows (Erdmann et al., 2019). It may still be useful as a predictor of abomasal displacement, clinical ketosis, metritis, and retained placenta in dairy cows (Ospina et al., 2013). Other markers of EB include changes in BW and body condition score (Thorup et al., 2012; Chebel et al., 2018) and fat-to-protein ratio (**FPR**) in milk (Grieve et al., 1986; Friggens et al., 2007).

Milk can be collected by non-invasive automated techniques and more than 80% of dairy cows in Sweden are test-milked every month. The milk fatty acid (**FA**) C18:1 *cis*-9, which is derived from mobilized body fat resources at NEB, has been suggested as a biomarker for compromised metabolic status in early lactation (Mann et al., 2016). Short- and medium-chain FAs with 16 carbons or less, which are mainly synthesized *de novo* in the mammary gland, decrease during NEB, so the ratio of C18:1 *cis*-9 to these FAs can be expected to be a good early biomarker of NEB (Barbano et al., 2016). Previous studies have shown that long-chain FAs can be used to detect metabolic problems in dairy cows (Jorjong et al., 2015), and to detect both spontaneous and induced NEB (Gross et al., 2011; Billa et al., 2020). The profile of FAs in milk is typically analyzed by gas chromatography, an expensive but precise method that is only suitable for limited numbers of samples. For large-scale studies on commercial herds, analysis of milk samples via Fourier-transform infrared (**FTIR**) spectroscopy is a viable alternative and was used in this study. Milk spectroscopy also permits determination of milk BHB levels, which have been used previously to monitor metabolic status at herd level (Santschi et al., 2016; Tatone et al., 2017).

Preliminary results from this study have been previously presented at the ISRP 2019 conference and the abstract has been published in the conference proceedings (Churakov et al., 2019).

The aim of this study was to compare concentrations of the most abundant milk FAs in milk with other milk and blood variables in terms of their ability to estimate EB and predict severe NEB in early-lactation cows.

Material and methods

Experimental design

The study was conducted at the Swedish Livestock Research Centre, Uppsala, Sweden, from February 2016 to July 2016, after approval by the Uppsala Ethics Committee for Animal Research, Uppsala, Sweden (diary number C98/15). Procedures for sample collection and chemical analyses are similar to those from another study performed in parallel at the same research farm (Karlsson et al., 2020a).

The study included 85 lactating dairy cows that were fed the same concentrate ration. The cows were of breeds Holstein ($n = 37$) and Swedish Red ($n = 48$), and were both primiparous ($n = 45$) and multiparous ($n = 40$). The cows entered the study during the first week after calving and stayed until 42 days in milk.

All cows were housed in an insulated loose housing barn with rubber mats and sawdust-bedded cubicles. The cows were milked morning and evening in an automatic milking rotary (AMR, DeLaval International AB, Tumba, Sweden). Forage intake for each individual animal was recorded automatically at 22 forage troughs (CRFI, BioControl, Ås, Norway), as was daily concentrate intake fed from four dispensers (FSC400, DeLaval International. Tumba, Sweden). The cows were not offered any feed during milking, but entered the feeding area directly after milking.

Concentrate ration was gradually increased by 0.4 kg DM/d from 2.6 kg DM/d at calving to 4.4 kg DM/d for multiparous cows, while primiparous cows were given a maximum of 3.5 kg DM/d. The daily concentrate ration was automatically distributed over several smaller portions, with the portion size set at maximum 2 kg and minimum 0.5 kg. The concentrate was pelleted and by-product based, while the forage was a grass silage fed *ad libitum*. The silage from one silo did not last the whole study period, so from June 2, silage was taken from another silo. All silage was first-cut silage with less than 25% clover, harvested in the beginning of June 2015 and preserved using an acid-based additive (Promyr NT 570, Perstorp, Sweden). The herbage was precision-chopped, wilted, and stored in bunker silos. A stationary feed mixer, conveyor belt, and automatic feed wagon (DeLaval International AB, Tumba Sweden) were used to distribute the forage to the cows up to seven times per day, to ensure *ad libitum* access. Salt (50 g/cow/d) was mixed with the silage before distribution into the forage troughs. The cows had free access to water offered in drinking cups.

The cows were weighed automatically at least twice a week on passing through a sorting gate after milking, and mean daily BW was recorded. The weight scale was calibrated monthly.

Sample collection

Milk samples were collected from individual cows at two consecutive milkings, on Tuesday afternoon (15:30–19:30) and the following Wednesday morning (05:30–9:30) in lactation weeks 2, 4, and 6. The equipment used for milk sampling (MM6, DeLaval International AB, Tumba, Sweden) and measuring milk yield has been certified by the International Committee for Animal Recording (Rome, Italy). Milk samples were preserved with bronopol, stored at 8 °C, and analyzed within 3 days.

Blood was sampled on Thursday mornings (9:00–10:00) in lactation weeks 2, 4, and 6. Blood was harvested from the coccygeal artery or vein into 10-mL vacuum tubes with lithium heparin as anticoagulant (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). The blood samples were stored on ice between sampling and centrifugation. The blood samples were centrifuged at +4 °C for 10 minutes, at 4 000 relative centrifugal field, within

1 h after sampling. After separation, plasma samples were frozen and stored at −20 °C.

Chemical analysis and calculations

Milk samples were individually analyzed for composition of fat, protein, lactose, FAs, BHB, and other parameters using FTIR spectroscopy (CombiScope FTIR 300 HP, Delta Instruments B.V., Drachten, the Netherlands) according to Parameter Application document (version 1.6, 2018-06-11). Average values for the two consecutive milk samples from each sampling occasion were used in the statistical analysis. The content in milk (g/100 g milk) of four FAs (C14:0, C16:0, C18:0, and C18:1 *cis*-9) was estimated by FTIR spectroscopy. The FA concentration is often measured in milk fat, but in this study, we measured the concentration in milk. To calculate FA in milk fat, we divided FA in milk by milk fat concentration. Lactose was corrected for lactase monohydrate (i.e., divided by 1.053). Energy-corrected milk (**ECM**) yield was calculated based on fat, protein, and lactose content according to (Sjaunja et al., 1990) and used for EB calculation.

Net energy content in feed and energy intake were estimated according to the NorFor system (Volden, 2011). Energy balance was then calculated as EB = NE_{intake} − ($NE_{maintenance}$ + NE_{milk}), with NE_{intake}, $NE_{maintenance}$, and NE_{milk} also derived in accordance with the NorFor system.

Blood plasma was analyzed enzymatically for glucose (D-Glucose UV-method, R-biopharm AG, Darmstadt, Germany). Insulin concentration in blood plasma was estimated using an enzyme immunoassay method adapted for bovines (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden), and the concentration of NEFA using an enzymatic colorimetric method (NEFA-HR, FujifilmWako Diagnostics U.S.A. Corporation, CA). The concentration of BHB in plasma was assessed with a colorimetric test (MAK041, Sigma-Aldrich, St. Louis, MO), while insulin-like growth factor 1 (IGF-1) concentration was analyzed with an enzyme immunoassay (Mediagnost E20, Mediagnost, Reutlingen, Germany). The intra-assay coefficients of variability (CV) were below 6.46% for all blood assays.

Data preparation

Data on cows (BW, breed, etc.) were collated with FTIR results, blood sample results, and EB estimation, by matching cow IDs and dates. Each cow should have had two milk FTIR results per each study week, but for some cows-weeks data were missing: on 19 occasions, there was only one milk sample, on three occasions, there were no samples. If more than one sample was obtained per cow and per lactation week, the mean value was used for the weekly analysis. The final dataset included information on 85 cows and had three missing data points (one cow in week 4 and two cows in week 6), resulting in 252 observations in total.

Statistical analysis

Data management and statistical analysis were performed in R statistical software (R Core Team, 2019). Data from milk and blood samples were analyzed in relation to calculated EB, which was used as reference. First, we examined Spearman correlation between potential EB predictors and EB estimates, to obtain an indication of possible interactions between variables. To estimate EB, we used linear and spline, i.e., univariate generalized additive models (**GAM**), for each individual variable and assessed how they fitted the observed data by comparing their performance using deviance explained. We used multivariate GAM models to account for several variables. In this, we used the Restricted Maximum Likelihood method, did not transform the predictions, and used the shrinkage procedure to select significant variables and to remove variables that do not improve the model fit, i.e., those that have effective degrees of freedom close to zero. The shrinkage procedure helps with a common problem of multicollinearity by eliminating highly correlated variables. We assessed model performance using Akaike information criterion (**AIC**) and deviance explained, which equals R^2 for linear models and is a preferred alternative to R^2 for GAM models.

To predict cows in severe NEB, we performed receiver operating characteristic (**ROC**) curve analysis to compare each variable's performance in predicting severe NEB status (i.e., net energy below a predefined value, e.g., −30 MJ NEL/d). We used various severe NEB cut-off levels to divide cows into two classes, and assessed area under the ROC curve (**AUC**) to compare predictors. AUC is a 'class separation capacity' that shows each variable's capability to distinguish between two classes. Ideally, the AUC value should be close to 1. An AUC value of 0.5 means that the model has no discriminative capacity, while values below 0.5 mean that the model separates classes well, but lacks the correct attribution (i.e., predicts severe NEB for cows that are not in severe NEB and vice versa).

Finally, we explored optimal thresholds for each variable that maximized sensitivity and specificity to detect EB below −30 MJ NEL/d.

Results

Estimated energy balance

We present a summary of production, blood, and milk variables in Table 1. The mean BW was higher in lactation week 2 compared to lactation weeks 4 and 6 (mean BW ± SD for lactation weeks 2, 4, and 6: 644 ± 71, 633 ± 73, and 633 ± 71 kg, respectively), but cows showed increased DMI (19.5 ± 4.9, 22.2 ± 5.2, 22.3 ± 4.6 kg) and milk yield (29.2 ± 7.3, 32.2 ± 7.7, 33.8 ± 7.9 kg) during the study period. Estimated EB increased from a median of −21 MJ NEL/d in week 2 to −7.4 in week 4 and −1.8 in week 6 (Fig. 1), showing gradual recovery from NEB. However, the majority of cows (46 out of 83 or 55%) still experienced NEB in week 6.

NEFA levels in blood dropped from 0.36 ± 0.17 mmol/L in week 2 to 0.28 ± 0.13 in week 4 and 0.27 ± 0.14 in week 6. Other blood indicators of EB are presented in Table 1.

Composition of milk fatty acids

Fig. 2 shows the pattern of change in concentrations of individual FAs in milk over time. Long-chain preformed FAs C18:0 and C18:1 *cis*-9 decreased within the first 6 weeks after calving (Mann-Whitney paired test $P < 0.001$ for all pairs of weeks), while production of C14:0 and C16:0 was relatively stable ($P = 0.018$ and $P = 0.09$, respectively, between weeks 2 and 4; $P = 0.013$ for C14:0 between weeks 2 and 6; $P < 0.01$ for other pairs of weeks).

Energy balance estimation: comparison of predictors

The next step in assessing variables in terms of their relation to EB was to explore univariate linear and spline models, and compare their performance using deviance explained. The results are summarized in Table 2. Performance of blood NEFA was relatively moderate compared with that of BHB, fat, FPR, and C18:0 and C18:1 *cis*-9 in milk, which showed the highest deviance explained. Models based on concentrations of FAs in milk fat performed relatively well, but among all four FAs, only C14:0 in milk fat was slightly better than measures based on absolute FA content in milk. Therefore, we focused on FA concentrations in milk rather than in milk fat for further analysis.

Table 1
Summary of cows' production variables and biomarkers of energy balance for weeks 2, 4, 6 and the total study period (n = 252 observations).

Variable	Week 2 (mean ± SD)	Week 4 (mean ± SD).	Week 6 (mean ± SD).	Total (mean ± SD)
Production variables				
BW (kg)	644 ± 71	633 ± 73	633 ± 71	637 ± 71
Milk yield (kg/day)	29.2 ± 7.3	32.2 ± 7.7	33.8 ± 7.9	31.7 ± 7.8
ECM (kg/day)	33.0 ± 8.5	34.0 ± 9.0	33.1 ± 7.6	33.4 ± 8.4
Total DMI (kg/day)	19.5 ± 4.9	22.2 ± 5.2	22.3 ± 4.6	21.3 ± 5.1
Forage DMI (kg/day)	15.8 ± 4.6	18.3 ± 4.9	18.5 ± 4.4	17.5 ± 4.8
Concentrate DMI (kg/day)	3.7 ± 0.7	3.9 ± 0.4	3.8 ± 0.5	3.8 ± 0.5
In blood plasma				
NEFA (mmol/L)	0.36 ± 0.17	0.28 ± 0.13	0.27 ± 0.14	0.30 ± 0.15
BHB (mmol/L)	0.59 ± 0.19	0.75 ± 0.43	0.69 ± 0.38	0.67 ± 0.35
IGF-1 (ng/mL)	93.9 ± 59.9	108 ± 51.4	113 ± 53	105 ± 55.2
Glucose (mmol/L)	3.45 ± 0.60	3.46 ± 0.64	3.67 ± 0.59	3.53 ± 0.61
Insulin (μg/mL)	0.19 ± 0.20	0.22 ± 0.18	0.28 ± 0.25	0.23 ± 0.21
In milk				
Fat (g in 100 g milk)	5.01 ± 1.12	4.7 ± 1.52	4.17 ± 0.98	4.63 ± 1.27
Protein (g in 100 g milk)	3.65 ± 0.23	3.15 ± 0.23	3.07 ± 0.34	3.29 ± 0.37
Lactose (g in 100 g milk)	4.69 ± 0.13	4.77 ± 0.29	4.78 ± 0.37	4.75 ± 0.28
Fat-to-protein ratio (FPR)	1.38 ± 0.35	1.50 ± 0.50	1.36 ± 0.30	1.41 ± 0.40
BHB in milk (mmol/L)	0.17 ± 0.05	0.19 ± 0.06	0.18 ± 0.05	0.18 ± 0.06
C14:0 (g/100 g milk)	0.52 ± 0.13	0.51 ± 0.19	0.47 ± 0.11	0.50 ± 0.14
C16:0 (g/100 g milk)	1.25 ± 0.29	1.25 ± 0.39	1.13 ± 0.26	1.21 ± 0.32
C18:0 (g/100 g milk)	0.56 ± 0.19	0.45 ± 0.22	0.37 ± 0.14	0.46 ± 0.20
C18:1 *cis*-9 (g/100 g milk)	1.22 ± 0.29	1.09 ± 0.29	0.96 ± 0.22	1.09 ± 0.29
In milk fat				
C14:0 (g/100 g fat)	10.4 ± 1.6	10.9 ± 1.3	11.3 ± 1.2	10.9 ± 1.4
C16:0 (g/100 g fat)	25.0 ± 2.5	26.6 ± 2.4	27.3 ± 2.5	26.3 ± 2.6
C18:0 (g/100 g fat)	11.1 ± 1.9	9.45 ± 2.03	8.59 ± 1.97	9.71 ± 2.23
C18:1 *cis*-9 (g/100 g fat)	24.6 ± 3.3	23.6 ± 3.1	23.1 ± 2.5	23.8 ± 3.04

ECM = energy-corrected milk yield, DMI = DM intake, NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate.

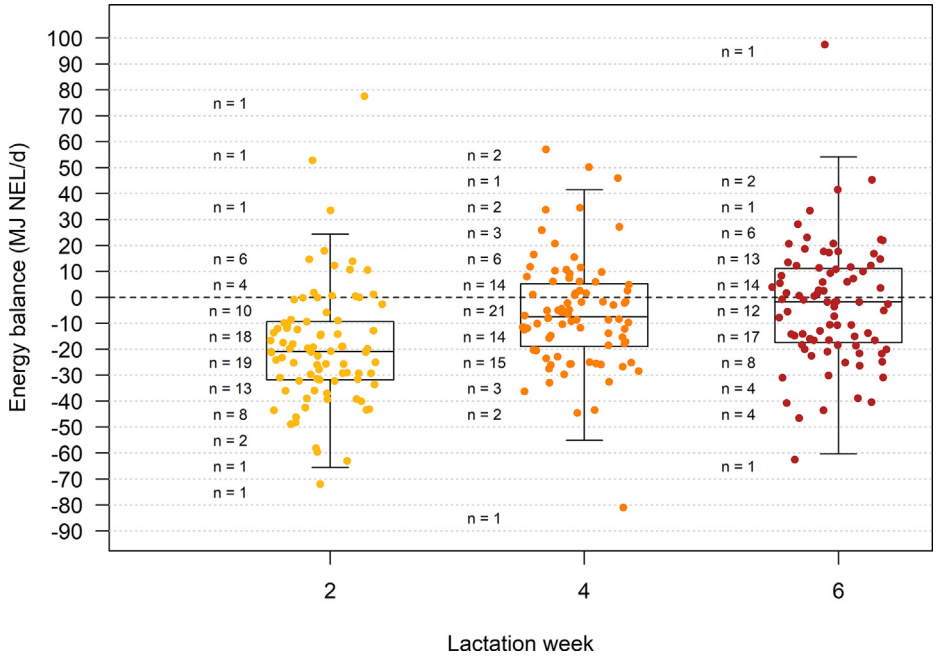

Fig. 1. Energy balance estimated from milk yield and feed intake for each cow (n = 85) in weeks 2, 4, and 6 after calving. Boxplots show medians and interquartile ranges for each week. NEL = net energy for lactation.

Energy balance estimation: generalized additive models

We tested various GAM models that accounted for several variables (Table 3). These models were based on blood variables (NEFA, BHB, glucose, insulin, IGF-1), standard milk measures (fat, protein, lactose), concentrations of milk FAs (C14:0, C16:0, C18:0, C18:1 *cis*-9) and BHB in milk, and their combinations. AIC and deviance explained were used to assess the predictive performance of the models.

Models based on milk variables (models 2–4) and especially on FAs (model 3) outperformed blood variables (model 1) in terms of deviance explained (Table 3). As milk FAs were highly correlated,

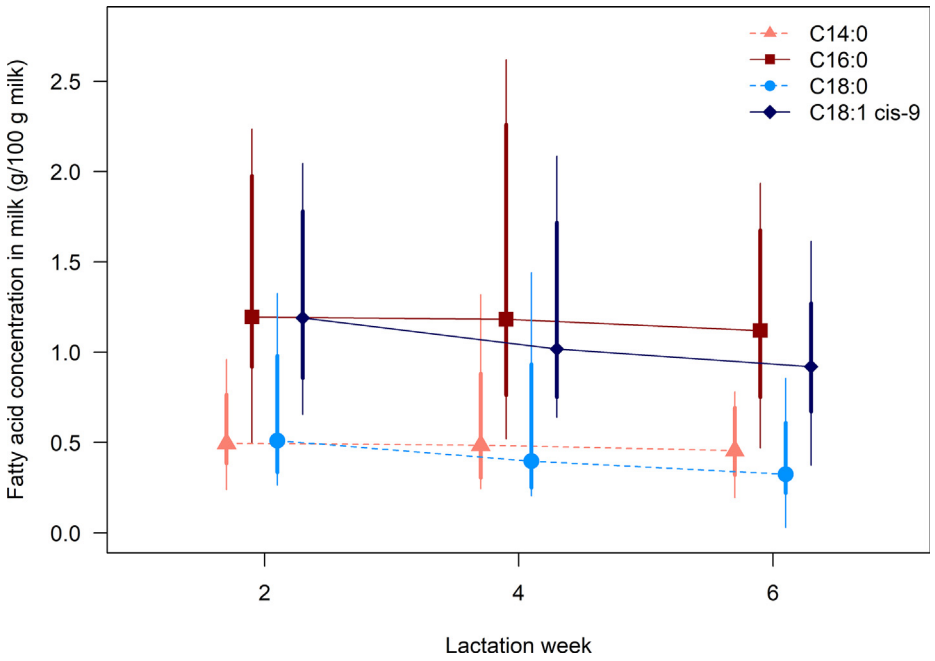

Fig. 2. Fatty acid concentrations in cows' milk over time. Overall range (fine vertical line) for each week and 5–95% quantiles (solid vertical line) along the median values (point symbols) connected by corresponding lines for each fatty acid.

Table 2
Comparison of cows' energy balance predictors. Performance of linear and spline models (n = 252 observations) was compared using deviance explained (dev. expl.).

Variable	Spearman correlation coefficient	Linear dev. expl.	Spline dev. expl.
In blood plasma			
NEFA (mmol/L)	−0.38	0.13	0.14
BHB (mmol/L)	0.02	< 0.01	< 0.01
IGF-1 (ng/mL)	0.35	0.09	0.09
Glucose (mmol/L)	0.07	< 0.01	0.02
Insulin (μg/mL)	0.18	0.03	0.03
In milk			
Fat (g in 100 g milk)	−0.53	0.27	0.38
Protein (g in 100 g milk)	−0.18	0.07	0.11
Lactose (g in 100 g milk)	0.21	0.02	0.16
Fat-to-protein ratio	−0.44	0.18	0.21
BHB in milk (mmol/L)	−0.43	0.20	0.21
C14:0 (g/100 g milk)	−0.19	0.09	0.16
C16:0 (g/100 g milk)	−0.30	0.15	0.19
C18:0 (g/100 g milk)	−0.55	0.28	0.35
C18:1 *cis*-9 (g/100 g milk)	−0.64	0.39	0.42
In milk fat			
C14:0 (g/100 g fat)	0.46	0.20	0.20
C16:0 (g/100 g fat	0.45	0.17	0.17
C18:0 (g/100 g fat)	−0.41	0.17	0.22
C18:1 *cis*-9 (g/100 g fat)	−0.26	0.03	0.04

NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate.

only C18:1 *cis*-9 was retained in model 3, as it was the most significant variable. C18:1 *cis*-9 was also retained and significant in combined models.

Severe negative energy balance prediction

Another task was to classify each cow based on predictive variables into two categories: severe NEB or above severe NEB threshold. We explored a range of thresholds from −50 to 0 MJ NEL/d that divided cows into two categories. We calculated performance (true positive and false positive rates) of various energy balance indicators for predicting severe NEB, which allowed us to obtain AUC values for each predictor and for each cut-off net energy level (Fig. 3).

We performed the same analysis on separate weeks (Fig. 4) to compare how NEB predictions differ between weeks. We found that performance of NEFA was poor for weeks 4 and 6. Blood BHB, glucose, milk protein, and lactose performed poorly for all weeks. C14:0 and C16:0 showed modest results for week 2, but performed better for weeks 4 and 6, while milk BHB, milk fat, FPR, C18:0 and C18:1 *cis*-9 were relevant measures of EB throughout the study period. Non-esterified fatty acids, C14:0 and C16:0, were not suitable for identifying cows in severe NEB in week 2, while milk BHB, milk fat, FPR, C18:0 and C18:1 *cis*-9 proved to be suitable indicators.

Thresholds for severe negative energy balance classification

For a selected NEB cut-off level, the optimal threshold that maximizes sensitivity and specificity of classification can be identified using any EB indicator. Table 4 presents the results for classification of cows below/above −30 MJ NEL/d energy level. Some indicators had high sensitivity, but were not reliable as their specificity was too low (e.g., glucose, protein, lactose), while others had high specificity but were not sensitive (e.g., NEFA, IGF-1, C14:0, C16:0). Among all variables, milk BHB, milk fat and especially C18:0 and C18:1 *cis*-9 in milk were the most reliable predictors of EB below −30 MJ NEL/d.

Discussion

In this study, we compared concentrations of milk FAs in whole milk with other milk and blood variables for their ability to estimate EB and predict severe NEB in early-lactation cows. The complexity of EB determination in lactating cows suggests that there is no clear-cut definition of severe NEB. However, it is generally accepted that severe NEB is associated with reduced fertility and immune function, along with several metabolic problems including fatty liver and displaced abomasum (Kim and Suh, 2003; Wathes et al., 2007; McArt et al., 2012). In several studies, NEB

Table 3
Performance of generalized additive models in estimating energy balance of early-lactation cows. Variables for each model are divided into significant ($P < 0.05$) and non-significant, and variables excluded by the shrinkage procedure are crossed out. All milk variables are concentrations in milk measured by Fourier-transform infrared spectroscopy (FTIR).

Model	Significant variables	Non-significant variables	AIC	Dev. expl.
1. Blood variables, mmol/L or stated otherwise	NEFA***, IGF-1, ng/mL***, Glucose***	~~BHB,~~ ~~Insulin, μg/L~~	2 138	0.21
2. Standard milk measures, g/100 g milk	Fat***, Lactose*	~~Protein~~	2 211	0.35
3. Milk FAs, g/100 g milk	C18:1 *cis*-9***	~~C14:0,~~ ~~C16:0,~~ ~~C18:0~~	2 178	0.42
4. Milk variables, g/100 g milk	Lactose**, C18:0*, C18:1 *cis*-9***	~~Fat,~~ Protein, mBHB, ~~C14:0,~~ ~~C16:0~~	2 168	0.46
5. All variables	IGF-1***, Glucose***, Protein**, Lactose*, mBHB*, C14:0**, C18:1 *cis*-9***	NEFA, ~~BHB,~~ Insulin, Fat, ~~C16:0,~~ ~~C18:0~~	1 999	0.59

AIC = Akaike information criterion, Dev. expl. = deviance explained, FA = fatty acid, NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate (in blood), mBHB = BHB concentration in milk.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

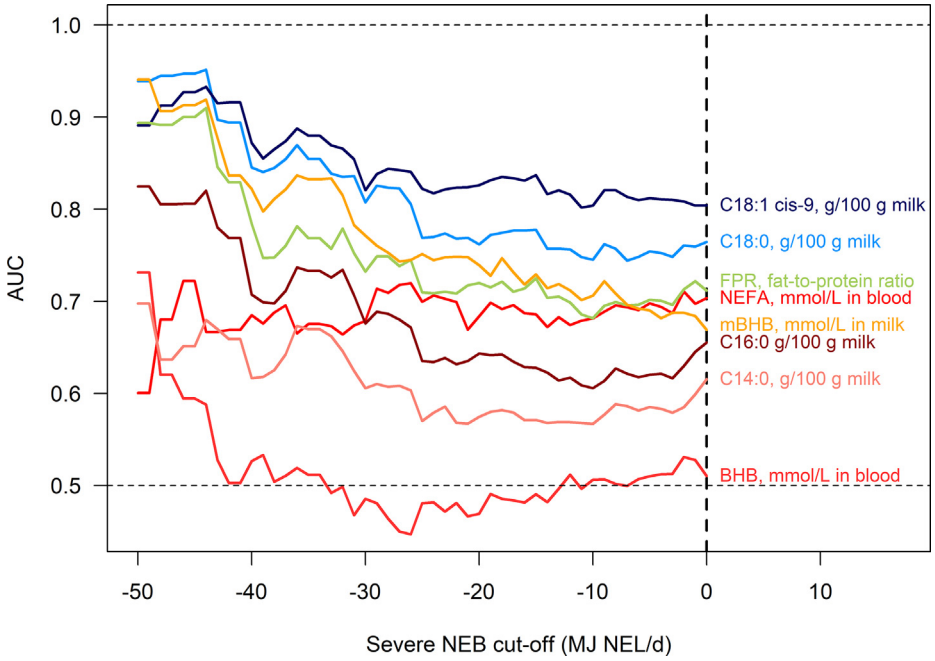

Fig. 3. Performance assessment of negative energy balance (NEB) prediction in dairy cows via area under the curve (AUC) values for different severe NEB cut-off values. NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate (in blood), mBHB = BHB concentration in milk, NEL = net energy for lactation.

has been induced by feed restriction (Gross et al., 2011; Billa et al., 2020). The resulting NEB, which apparently reached values below −30 MJ NEL/day, affected mammary metabolism and response to inflammation, prolonged the time required for uterine recovery, and compromised subsequent fertility (Wathes et al., 2009; Buttchereit et al., 2011). Metritis in dairy cows was associated with a postpartum nadir of EB around −40 MJ NEL/day (Pérez-Báez et al., 2019b). NEB of −30 MJ NEL/day corresponds to a daily BW loss of 1.2 kg (Volden, 2011).

We calculated EB values using the NorFor system (Volden, 2011), based on estimates of energy intake and requirements. We observed significant improvement in cow energy status by week 6 (Fig. 1), but a number of cows were still experiencing severe energy shortage at that time.

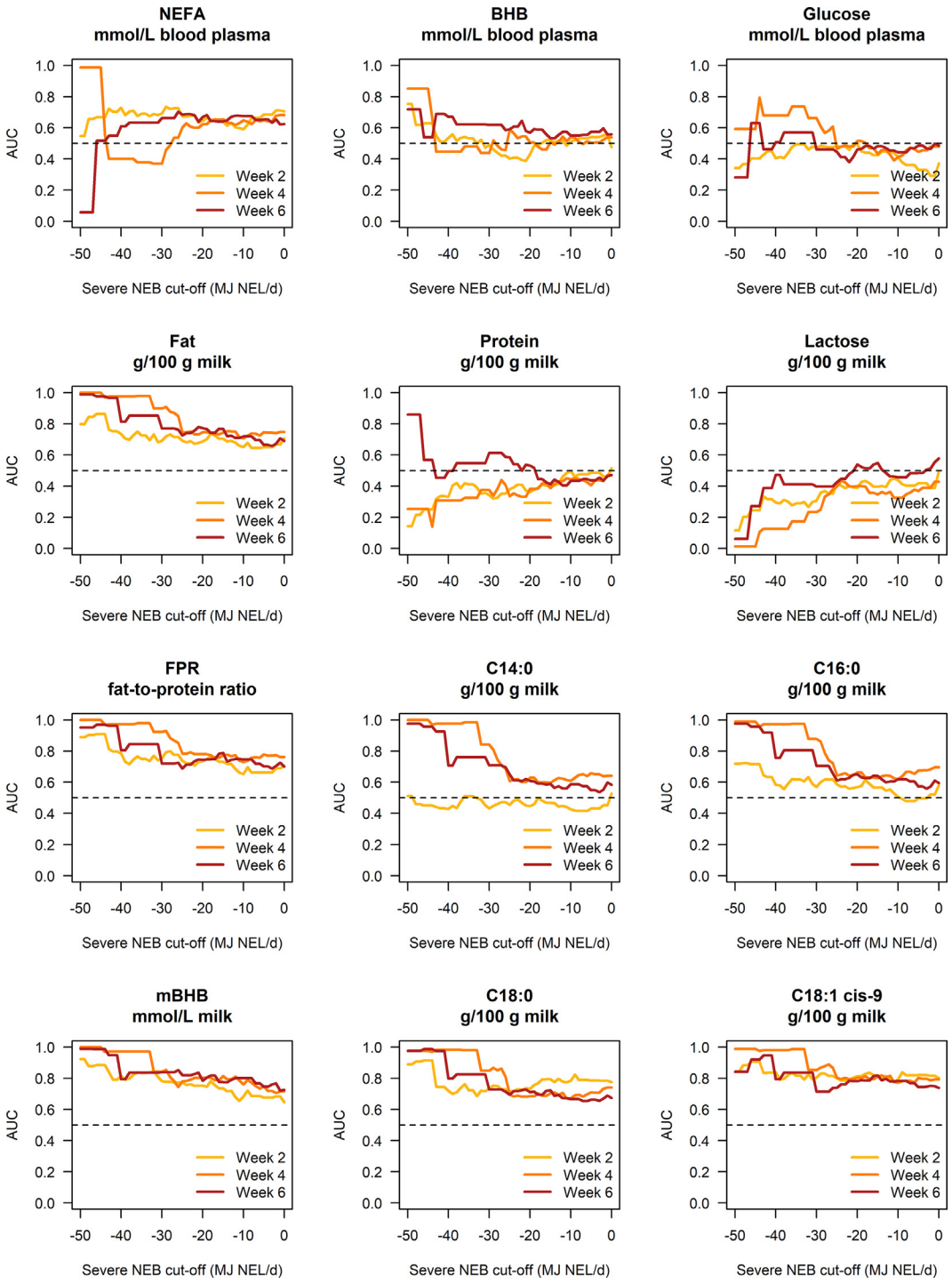

Fig. 4. Comparison of prediction performance of severe negative energy balance (NEB) (defined by various cut-off levels) of dairy cows in individual weeks 2, 4 and 6, based on area under the curve (AUC) values. NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate (in blood), mBHB = BHB concentration in milk, NEL = net energy for lactation.

The concentrations of individual FAs in milk changed over time (Fig. 2), following previously established patterns (Palmquist et al., 1993). The concentrations of long-chain preformed FAs C18:0 and C18:1 *cis*-9 in milk decreased with time after parturition, reflecting a decline in mobilization of adipose tissue. The concentrations of C14:0 and C16:0 in milk remained fairly stable. Similar temporal patterns of FA in early-lactation cows have been reported previously (Gross et al., 2011; Karlsson et al., 2020b), leading to the conclusion that milk FA profiles reflect changes in energy balance.

Spearman correlation, linear models, and spline models were used to estimate the relationship between blood and milk constituents and EB (Table 2). The concentration of C18:1 *cis*-9 in milk showed the strongest negative relationship with EB, followed by the concentration of C18:0 in milk. The blood plasma components showed relatively weak relationships with EB.

Table 4
Optimal thresholds and performance (sensitivity and specificity) of predictors of energy balance (EB) of dairy cows below −30 MJ NEL/d. For insulin and C14:0 in milk fat, sum of sensitivity and specificity was <1 for every possible threshold.

Variable	AUC for −30 MJ NEL/d	Threshold	Sensitivity	Specificity
In blood plasma				
NEFA	0.68	0.41 mmol/L	0.45	0.87
BHB	0.49	0.55 mmol/L	0.70	0.39
IGF-1	0.33	256 ng/mL	0.03	0.99
Glucose	0.49	3.11 mmol/L	0.83	0.27
Insulin	0.42	–	–	–
In milk				
Fat	0.79	4.47 g/100 g milk	0.90	0.57
Protein	0.61	3.25 g/100 g milk	0.73	0.47
Lactose	0.29	4.23 g/100 g milk	1.00	0.01
Fat-to-protein ration	0.73	1.52	0.53	0.86
BHB in milk	0.77	0.19 mmol/L	0.70	0.79
C14:0 in milk	0.61	0.62 g/100 g milk	0.33	0.92
C16:0 in milk	0.68	1.62 g/100 g milk	0.30	0.96
C18:0 in milk	0.81	0.47 g/100 g milk	0.85	0.69
C18:1 *cis*-9 in milk	0.82	1.16 g/100 g milk	0.78	0.76
In milk fat				
C14:0 in fat	0.28	–	–	–
C16:0 in fat	0.26	32 g/100 g fat	0.05	0.99
C18:0 in fat	0.73	9 g/100 g fat	0.80	0.67
C18:1 *cis*-9 in fat	0.57	26 g/100 g fat	0.45	0.79

AUC = area under the curve, NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate, NEL = net energy for lactation.

We found that FA content in milk was a better predictor of EB than FA expressed as a fraction of total milk fat (Table 2). Other published studies examining the relationship between FAs and EB have presented the FA content as a fraction of milk fat (Gross et al., 2011; Barbano et al., 2016), and not as a fraction of milk. The concentration of a specific FA in milk is determined by both the fat content in the milk and the proportion of that FA in milk fat. It is well-established that the milk fat concentration is elevated in cows in NEB (De Vries and Veerkamp, 2000; Stoop et al., 2009; Erdmann et al., 2019). Thus, the relationship between EB and the concentration of preformed FAs in milk observed in this study reflected increased milk fat content, but also a relatively higher proportion of the preformed FAs in the milk. The C14:0 and C16:0 concentrations in milk fat were positively related to EB, which is in line with previous studies (Gross et al., 2011). In terms of NEB prediction, concentrations of C18:0 and C18:1 *cis*-9 in milk fat had AUC below 0.75 for all cut-off levels between −50 and 0 MJ NEL/d (results not shown), while AUC values for their concentrations in milk were consistently above this level (Fig. 3).

The GAM analysis allowed combinations of potential individual EB predictors to be explored. While model performance was strongly dependent on choice of predictors, we found that models based on milk variables performed better, in terms of deviance explained, than those based on blood plasma indicators (Table 3). Inclusion of C18:1 *cis*-9 in milk in the models consistently improved their fit. This can be explained by the fact that the concentration of C18:1 *cis*-9 in milk is related to adipose tissue lipolysis, and in turn EB. Interestingly, the combined model that included both plasma and milk variables increased deviance explained by up to 59%. This result is promising, especially since the EB calculation as such involves recording errors (Erdmann et al., 2019). However, blood sampling is an invasive procedure that is difficult to implement routinely in commercial dairy herds, which is a limiting factor for use of combined milk and blood models in practice. In addition, laboratory analysis of blood samples is costly and requires extra labor.

To predict cows in severe NEB, we explored various cut-off levels for NEB (Fig. 3). C18:0 and C18:1 *cis*-9 in milk, fat content, FPR, and BHB in milk showed high overall performance. Blood BHB showed modest results for very low cut-off values, but otherwise could not predict severe NEB. The results were consistent for a wide range of possible definitions of severe NEB for most variables apart from NEFA, which performed poorly for cut-offs below −30 MJ NEL/d. The AUC values across all variables were predominantly stable for cut-off levels below −25 MJ NEL/d, suggesting that meaningful definitions of severe NEB should be below this threshold.

Among blood indicators, only NEFA and, to a lesser extent, IGF-1 were weakly correlated with EB (Table 2). BHB has previously been shown to be a poor indicator of EB in early-lactation cows (Erdmann et al., 2019). Additionally, BHB and NEFA are known to be weakly correlated to each other during the transition period (McCarthy et al., 2015). NEFA and other blood variables are not reliable indicators of EB, but they are still relevant indicators of metabolic imbalances and fertility disturbances that are not necessarily related to EB (Ospina et al., 2013). (Piantoni et al., 2015) have shown that NEFA and other blood plasma metabolites were related to feeding when lactating cows were fed once daily. In the present study, cows were offered silage seven times over the 24 h cycle. The cows could only consume small amounts of concentrate at each occasion. Thus, intake-related effects on the plasma parameters were probably limited. We found that milk BHB was a reasonable predictor of severe NEB for a range of EB cut-off levels (Figs. 3 and 4), especially for EB below −30 MJ NEL/d. However, these results should be interpreted with caution and validated by further studies.

Results of severe NEB predictions varied between lactation weeks (Fig. 4). C14:0 and C16:0 in milk in week 2 were practically useless for differentiating between cows in severe and moderate NEB. NEFA performed poorly for weeks 4 and 6. Milk fat, FPR, C18:0, C18:1 *cis*-9 in milk, and milk BHB had relatively high AUC, especially below −30 MJ NEL/d. However, C18:1 *cis*-9 in milk had stable AUC values over 0.8 for cut-offs below −30 MJ NEL/d for each week, which makes it the best indicator for early detection of severe NEB.

In Table 4, we presented optimal thresholds that maximize the sum of sensitivity and specificity to detect cows in EB below −30 MJ NEL/d. These thresholds need to be validated by further large-scale studies, but provide a rough idea of abnormal values that tend to be indicative of NEB issues. This information has strong practical implications and may be useful for on-farm detection of severe NEB. Despite the fact that our models were based on

averaged variables from afternoon and morning milk samples and that there might be a diurnal variation in milk composition (Forsbäck et al., 2010), the variation in milk FA composition appears to be virtually negligible as shown by Rico et al. (2014), which makes FA concentrations a suitable measure even for routine one-milking sampling.

We did not use ECM yield in the predictive models in this study, since it was used to calculate EB. However, dairy farmers have this information, and ECM yield can potentially be used along with FA composition to improve model performance, due to its impact on EB.

Limitations

This study was carried out on only one dairy herd and all participating cows had the same feeding regime, so we were unable to assess effects of additional variations in milk FA composition potentially introduced by differences in feed ration, individual characteristics of cows, and herd management. We also did not account for breed and parity, due to the small sample size. Therefore, our findings should be validated in large-scale studies that also account for the effects of diet and breed.

Larger datasets would also help to create more robust predictive models. Due to the relatively small sample size (n = 252), we were not able to perform cross-validation of our models. All milk samples in our study were analyzed on the same FTIR equipment. Raw spectral data for the same sample might differ between different instruments. However, most companies on the market of dairy analyzers offer calibration packages for the actual fatty acids. Thus, FTIR measurements and the presented results should be valid if another equipment is used for analysis.

We estimated EB based on the NorFor equations (Volden, 2011). It has recently been shown that other common equations overestimate EB compared with direct calorimetry measurements (Erdmann et al., 2019). However, we found clear indications that milk FA concentrations in whole milk determined by spectroscopy are strongly related to EB in cows in early lactation.

Methods that utilize all information from raw FTIR spectral data on milk samples, instead of some derived variables, could potentially further improve EB predictions.

Conclusion

The results in this study suggest that concentrations of FAs in milk can be used as a biomarker for NEB. The concentrations in milk of C18:0 and especially C18:1 *cis*-9 were the most suitable variables for early detection of cows in severe NEB, which is important for herd management. Overall, milk biomarkers proved to be more useful for severe NEB prediction than blood plasma biomarkers. Moreover, it is difficult to routinely collect blood from each animal on a farm, while milk FTIR spectroscopy data may be readily available to dairy farmers. This makes milk biomarkers potentially applicable for screening the energy balance of individual dairy cows.

Ethics approval

The study was approved by the Uppsala Ethics Committee for Animal Research, Uppsala, Sweden (diary number C98/15).

Data and model availability statement

None of the data were deposited in an official repository. The model was not deposited in an official repository. The code for analysis is available upon request.

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Declaration of interest

None.

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