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ORIGINAL ARTICLE

Milk fatty acids as indicators of delayed commencement of luteal activity in dairy cows in early lactation

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Abstract

Excessive mobilization of adipose reserves due to severe negative energy balance in early lactation may be detrimental to dairy cow fertility at individual and herd level. Reproductive efficiency is one of the main factors influencing herd profitability and a strategy for early detection and management of cows with delayed resumption of cyclicity will result higher conception rate, decreased proportion of cows with extended lactation, fewer inseminations per conception and lower culling rates due to reproductive disorders. Using two groups of dairy cows (Holstein $n = 37$, Swedish Red breed [SRB] $n = 49$), we investigated potential differences between cows with different commencement of luteal activity (CLA) and the feasibility of using milk fatty acids (MFAs) as predictors of delayed CLA. Milk samples for progesterone analysis were collected twice weekly during the first six weeks in milk. The concentrations of the MFAs C14:0, C16:0, C18:0 and C18:1 cis-9 in milk (g/100 g milk) and in milk fat (g/100 g fat) were analysed by Fourier transform infrared spectroscopy and individual MFA profiles were calculated by weeks in milk. Commencement of luteal activity was defined as the first day with milk progesterone concentrations >3 ng/ml at two successive measurements. The study population was categorized as early ($n = 42$) or late ($n = 44$) CLA, using the median value of 21 DIM as the cut-off. Analysis of the data revealed that CLA was correlated with the proportion of some specific MFAs, where cows with delayed CLA had lower IGF-1 (92.9 ± 7.9 vs. 114.1 ± 7.9 ng/ml; $p = .05$) and C14:0 levels (10.4 ± 0.2 vs. 11.5 ± 0.2 g/100 fat; $p < .01$) and higher C18:0 (9.6 ± 0.2 vs. 8.9 ± 0.6 g/100 fat; $p < .01$) and C18:1 cis-9 levels (24.9 ± 0.4 vs. 23.5 ± 0.4 g/100 fat; $p < .05$). Delayed CLA (mean 34 days) was predictable for approximately 80% of cows based on C18:0 or C18:1 cis-9 concentrations in week 2 postpartum. Overall, MFAs (C18:0 and C18:1 cis-9) as biomarkers were better indicators than beta-hydroxybutyrate or non-esterified fatty acids in early detection of cows with delayed or normal CLA. The MFA concentrations in milk samples from cows in early lactation can thus be used as a non-invasive method to identify cows at risk of delayed CLA, acting as potential biomarkers for future reproductive performance.

KEYWORDS
biomarkers, dairy cow, fatty acid, Fourier transform spectroscopy, reproduction

1 | INTRODUCTION

The transition period is crucial for the health and reproductive performance of dairy cows (Ferguson, 2005; Pascottini et al., 2020). Body energy demands increase after calving, mostly due to high milk production and low energy intake from consumed feed, placing the cow in a state of negative energy balance (NEB). This leads to adipose tissue mobilization and consequently elevated plasma concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB), which are used by peripheral tissues as a fuel source and by the mammary gland for milk fat synthesis (Goff & Horst, 1997; Trevisi et al., 2012). There are also differences between dairy breeds in terms of nutrient prioritization (Ntallaris, 2018). However, excessive body condition loss during early lactation is associated with severe metabolic imbalance (Mellouk et al., 2019), a greater risk of uterine health problems (Chen et al., 2017), altered gene expression in uterine tissue (Chankeaw et al., 2021) and several endocrine and ovarian dysfunctions, including delayed commencement of luteal activity (CLA) (Abdelli et al., 2017; Wathes et al., 2007).

Early resumption of cyclicity is important for high fertility. Endocrine measurements of fertility, based on progesterone analyses in the interval from calving to CLA, are free from management bias and can be used in herd reproduction management and as endocrine breeding traits (Tarekegn et al., 2019).

Utilization of adipose tissue (lipolysis) is reflected in the content of milk fat (Bauman et al., 2006), in milk fatty acid (MFA) composition and in the relative proportions of individual MFAs in milk (Duchacek et al., 2014). The predominant fat in milk is triacylglycerol, which contains short-chain (C4-C10), intermediate- (C12-C16) or long-chain (C18) fatty acids (FAs). The short-chain FAs are synthesized within the mammary gland from acetate and BHB, the long-chain FAs originate from dietary lipids and from lipolysis of adipose tissue triacylglycerols, and the intermediate-chain FAs derive from both sources (Linn, 1988; Månsson, 2008).

Nutritional metabolites such as NEFA and BHB that are involved in postpartum energy homeostasis and restoration of ovarian activity in cattle play an important role as energy substrates during NEB (Ospina et al., 2013) and can also serve as biomarkers of energy balance. Different biomarkers, such as NEFA and BHB, are commonly used for complete screening of herds (Chapinal et al., 2012) with the aim of discriminating between cows at risk of developing delayed CLA and healthy animals in field conditions. This helps farmers with early detection and minimizes the economic losses. Consequently, identification during early lactation of cows at risk of developing delayed CLA would be useful for decision support on treatment or prevention measures at individual or herd level, minimizing the severity and duration of the period of metabolic imbalance and optimizing cow health, fertility and production.

In a previous study, we demonstrated that MFAs can be used as indicators of NEB (Churakov et al., 2021). In this follow-up study, we investigated potential differences between selected MFAs and blood metabolites in cows with normal or delayed CLA, and the feasibility of using MFAs as predictors of CLA. We also compared MFAs with other plasma biomarkers, such as NEFA and BHB, in terms of their accuracy in identifying cows with delayed CLA during early lactation.

2 | MATERIAL AND METHODS

2.1 | Animals, housing and feeding

A total of 86 dairy cows (Holstein, $n = 37$ and Swedish Red breed [SRB], $n = 49$), both multiparous ($n = 41$) and primiparous ($n = 45$), were used in a study conducted February–April 2016 at Swedish Livestock Research Centre, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden (Figure 1). All animals were housed in a loose housing barn with rubber mats and sawdust-bedded cubicles and were milked twice daily in an automated system (AMR, DeLaval International AB, Tumba, Sweden). A detailed description of the diet, feeding and measurement of dry matter intake (DMI) can be found in a previous publication by our research group (Karlsson et al., 2020). In short, concentrate was dispensed from feeding stations and silage was fed ad libitum to the cows.

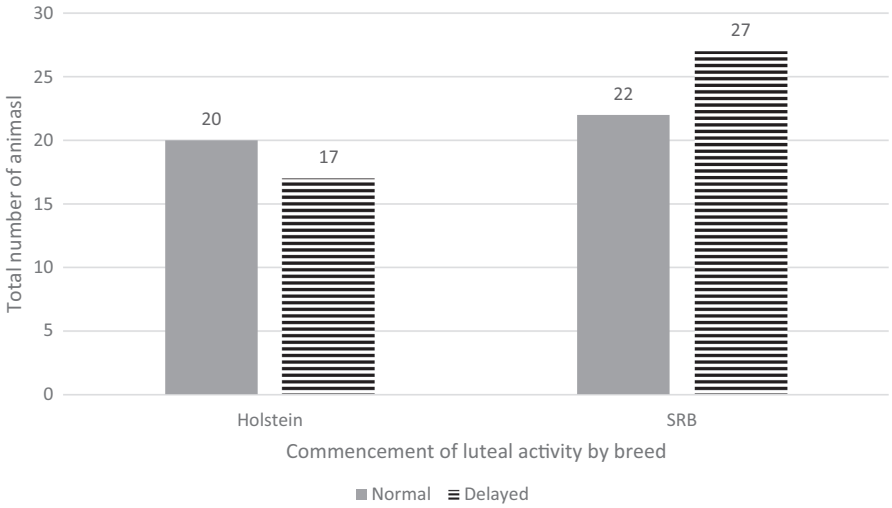

FIGURE 1 Total number of animals with normal ($n = 42$) versus delayed ($n = 44$, >21 days) commencement of luteal activity divided by breed (Holstein $n = 37$ vs. SRB $n = 46$).

2.2 | Recordings and sample collection

Individual forage intake was recorded automatically by the silage troughs (CRFI, BioControl, Ås, Norway), while concentrate dispensers (FSC400, DeLaval International. Tumba, Sweden) automatically recorded daily concentrate intake for each cow. Milk composition analysis was performed for each animal using samples from an afternoon and following morning milking in lactation weeks 2, 4 and 6. Milk from all cows was sampled once daily on two days per week for progesterone analysis. Milk sampling and milk yield measuring were certified by the International Committee for Animal Recording (Rome, Italy), under code MM6 and MM27 (DeLaval International AB, Tumba, Sweden), respectively. All samples were preserved with bronopol and stored at 8°C and analysed within three days of collection.

All cows were weighed automatically after each milking and mean daily body weight (BW) was recorded (originally by DeLaval International AB, Tumba, Sweden, rebuilt by BioControl, Ås, Norway). Body condition scoring (BCS; scale 1–5) was performed automatically with a 3-dimensional camera (DeLaval International AB, Tumba, Sweden) when cows were passing through a sort of gate after milking (Sandgren & Emanuelson, 2016). Weekly mean BW and BCS was calculated from daily mean BW and BCS, respectively. A system certified by the International Organization for Standardization (Geneva, Switzerland) was used for identifying the individual cows at milking, feeding, weighing and BCS.

Blood samples were collected on weeks 2, 4 and 6 postpartum from the coccygeal artery or vein into 10-ml vacuum tubes containing EDTA anticoagulant (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). The blood samples were centrifuged at +4°C for 10 min at 4000 relative centrifugal field within one hour after sampling and stored at −20°C after separation.

2.3 | Chemical analysis and calculations

All analyses were performed by the laboratory at the Department of Animal Nutrition and Management, SLU, Uppsala, Sweden, unless otherwise stated. A detailed description of dry matter (DM) calculations can be found in Karlsson et al. (2020). In short, the DM content of the silage was determined by a two-step procedure comprising drying at 60°C overnight and milling, then drying at 60°C overnight, as described by Akerlind et al. (2011).

The NorFor system (Volden & Nielsen, 2011) was used to calculate energy intake. Energy balance (EB) was calculated as the difference between net energy intake and net energy requirement:

$$EB = NE_{intake} - \left(NE_{maintenance} + NE_{lactation}\right)$$

with NE_{intake}, $NE_{maintenance}$ and $NE_{lactation}$ calculated according to the NorFor system (Volden & Nielsen, 2011).

The severity of NEB was assessed by calculating mean EB at weeks 2, 4 and 6 postpartum. The magnitude of NEB was determined by calculating cumulative energy deficit (area under the curve, AUC) during the first six weeks postpartum (Ntallaris, 2018).

Fourier transform infrared spectroscopy (FTIR; CombiScope 300 HP, Delta Instruments B.V., Drachten, the Netherlands) was used for analysis of the individual milk samples. Statistical analysis was conducted using the mean value of the evening and morning milk samples from each sampling occasion. Fatty acid concentration was analysed by spectroscopy for the four most abundant milk FAs: (C14:0, C16:0, C18:0 and C18:1 cis-9). Lactose was corrected for lactase monohydrate by division by 1.053. Energy-corrected milk (ECM) yield was calculated based on fat, protein and lactose content according to Sjaunja et al. (1990). Milk progesterone was analysed with an enzyme immunoassay method (ELISA M-plate, Ridgeway Science, St Briavels, UK) by Eurofins Milk Testing Sweden AS (Jönköping, Sweden). The progesterone level was analysed for each animal from calving to CLA, with the limit for luteal activity set at a milk progesterone concentration of >3 ng/ml at two consecutive measurements. The study population was categorized as early ($n = 42$) or delayed ($n = 44$) CLA, using the median value of 21 days in milk (DIM) as the cut-off.

Plasma glucose, NEFA, insulin, BHB and insulin-like growth factor 1 (IGF-1) concentrations were analysed. An enzymatic colorimetry assay was used to determine plasma concentration of glucose (D-Glucose UV method, R-biopharm AG, Darmstadt, Germany). An enzyme immunoassay method adapted for bovines was used to determine insulin plasma concentration (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden). NEFA concentration was determined using an enzymatic colorimetric method (NEFA-HR, Fujifilm Wako Diagnostics U.S.A. Corporation, CA). The concentration of BHB in plasma was determined using a colorimetric test (MAK041, Sigma-Aldrich, St. Louis, MO), while that of IGF-1 was determined with an enzyme immunoassay (Mediagnost E20, Mediagnost, Reutlingen, Germany).

2.4 | Reproductive traits

Data on reproductive traits were collected from farm records and summarized. The traits considered in this study were: interval from calving to first insemination (CFI), interval from calving to last insemination (CLI), total number of inseminations per conception and calving interval (CI). If an insemination was followed by a new insemination within six days, the first insemination was omitted.

2.5 | Statistical analyses

The experiment was conducted in a 2 × 2 (CLA × breed) factorial design, that is with two groups of cows (Holstein and SRB), which were studied from the first week after calving until 40–42 DIM.

All statistical analyses were performed in SAS 9.3 software (SAS Institute Inc., 2002). Unless otherwise specified, the data were analysed using the MIXED procedure for linear mixed models, with the random effect of cow included in the model. A repeated effect of

week was tested. Correlations between covariance were accounted for by specifying a correlation structure AR (1) among residuals. The residuals from the observations generated from the mixed models were tested for normal distribution and data deviating from a normal distribution (BHB, insulin, NEFA) were log-transformed. However, to improve clarity, avoid redundancy and facilitate interpretation, these log-transformed values are presented as non-transformed least square mean ± standard error of mean (LSMean ± SEM) throughout the paper. The contrast option was used to test individual hypotheses.

The model included the fixed effect of breed (two classes; Holstein, SRB), lactation number (two classes; primiparous, multiparous), week (three classes; weeks 2, 4, 6), CLA (two classes; ≥21 days vs. <21 days), and the two-way interactions between breed and CLA, breed and week, and week and CLA.

All estimated LSMean values from the models were adjusted using Scheffe's adjustment for multiple post-ANOVA comparisons and compared. Differences were considered significant at $p \leq .05$, while values in the range $.05 < p \leq .10$ were considered to indicate a tendency for significance.

A threshold (Q3 quartile) was used on the different biomarkers evaluated for identification of cows with delayed CLA. Above that threshold, animals were classified as positive for delayed CLA, while below that threshold, animals were classified as having normal CLA. The sensitivity, specificity and accuracy rates were calculated using the FREQ procedure in SAS software.

The trapezoidal rule was used to calculate AUC for estimating cumulative energy deficit (NEB) during the first six weeks postpartum.

3 | RESULTS

3.1 | Reproductive performance

Cows with delayed ($n = 44$) CLA resumed cyclicity approximately two weeks later than cows with normal ($n = 42$) CLA (34.2 ± 0.9 vs. 17.2 ± 0.9 days; $p < .0001$) (Table 1). However, no difference in CLA was found between the two breeds (Holstein vs. SRB) or cows with different lactation number (primiparous vs. multiparous).

Overall, CFI interval was not different ($p > .05$) between the CLA groups (delayed vs. normal). Within the delayed CLA group, Holstein cows ($n = 17$) had a longer CFI interval than SRB ($n = 27$) cows (79 ± 2.6 vs. 69 ± 2.0 days; $p < .05$). Within the normal CLA group, Holstein ($n = 20$) had a shorter CFI interval than SRB ($n = 22$) cows (68 ± 2.4 vs. 76 ± 2.3 days; $p < .05$). No difference in CFI interval was found between primiparous and multiparous cows.

Overall, CLI interval was longer in cows with delayed CLA than in cows with normal CLA (131 ± 5.0 vs. 108 ± 5.2 days; $p < .01$). Within the delayed CLA group, Holstein cows had a longer CLI interval than SRB cows (161 ± 7.8 vs. 100 ± 5.7 days; $p < .0001$). In addition, primiparous cows had a longer CLI interval than multiparous cows (129 ± 4.7 vs. 110 ± 5.3 days; $p < .01$).

Total number of inseminations per conception was higher in cows with delayed CLA than in the normal CLA group (2.8 ± 0.2 vs. 2.3 ± 0.2; $p < .05$). Within the delayed CLA group, Holstein cows had a higher total number of inseminations per conception than SRB cows (3.3 ± 0.2 vs. 2.3 ± 0.2; $p < .05$). Total number of inseminations per conception did not differ between primiparous and multiparous cows.

Overall, CI was longer in cows with delayed CLA than in cows with normal CLA (414 ± 5.0 vs. 391 ± 5.3 days; $p < .01$). Within the delayed CLA group, Holstein cows had a longer CI than SRB cows (444 ± 7.9 vs. 385 ± 5.8 days; $p < .0001$). In addition, CI was longer in primiparous than in multiparous cows (411 ± 4.8 vs. 395 ± 5.5 days; $p < .05$).

3.2 | Milk fatty acids

Cows with delayed CLA had lower C14:0 concentrations in milk (g/100 g milk) and in milk fat (g/100 g fat) than cows with normal CLA, and Holstein cows had lower C14:0 concentrations in milk and

TABLE 1 Reproduction-related variables (LSMean ± SEM[a]) in cows with normal commencement of luteal activity (CLA) (≤21 days) or delayed CLA (>21 days), and significance of differences for breed (two classes; Holstein $n = 37$ and SRB $n = 46$), parity (two classes; primiparous $n = 45$ and multiparous $n = 41$) and CLA class (two classes; delayed $n = 44$ and normal CLA $n = 42$)

			p-values			
	Normal	Delayed	Breed	Parity	CLA class	Breed × CLA class
Variables (days)						
CLA	17.2 ± 0.93	34.2 ± 0.93	.75	.78	<.0001	.67
CFI	71.9 ± 1.7	74.3 ± 1.7	.77	.40	.29	<.0001
CLI	108.2 ± 5.2	130.6 ± 4.9	<.0001	<.01	<.01	<.001
CI	391.4 ± 5.3	414.3 ± 5.0	<.0001	≤.05	<.01	<.01
Total AI (*n*)	2.3 ± 0.2	2.8 ± 0.2	≤.05	.15	≤.05	≤.05

Abbreviations: CFI, interval from calving to first insemination; CI, calving interval; CLI, interval from calving to last insemination (conception); total AI, total number of artificial inseminations needed for conception.

[a]Least square mean and residual error.

milk fat than SRB cows (Table 2). Within the delayed CLA group, Holstein cows had lower C14:0 concentrations in milk and milk fat than SRB cows (Table 2).

Cows with delayed CLA had lower C16:0 concentrations in milk fat (g/100 g fat) than cows with normal CLA (Table 2). In addition, Holstein had lower C16:0 concentrations in milk fat than SRB cows.

Cows with delayed CLA had higher C18:0 concentrations in both milk (g/100 g milk) and milk fat (g/100 g fat) than cows with normal CLA. No difference was found between the breeds, overall or within the delayed CLA group.

Cows with delayed CLA had higher C18:1 cis-9 concentrations in both milk (g/100 g milk) and milk fat (g/100 g fat) than cows with normal CLA. No difference was found between the breeds. However, within the delayed CLA group, Holstein cows had higher C18:1 cis-9 concentrations in milk fat than SRB cows (25.9 ± 0.6 vs. 23.9 ± 0.4 g/100 g milk; $p < .05$).

3.3 | Energy balance and body condition score

No difference in severity or magnitude of NEB was found between the CLA groups or breeds (Figure 2a). However, within the delayed CLA group, the severity and magnitude of NEB were greater in Holstein than in SRB cows (severity: −10.2 ± 3.2 vs. −1.7 ± 2.5 NEL MJ/d; $p < .05$; magnitude: −209.3 ± 65.4 vs. −14.3 ± 51.9 NEL MJ/d; $p < .05$).

Cows with delayed CLA had lower BCS (scale 1–5) than the normal CLA group (3.4 ± 0.03 vs. 3.5 ± 0.03; $p < .01$) (Figure 2b). Within the delayed CLA group, Holstein cows had lower BCS than SRB cows (3.2 ± 0.04 vs. 3.6 ± 0.03; $p < .001$). In addition, BSC losses between weeks 2 and 6 postpartum tended to be more severe for the delayed CLA group than the normal CLA group (−0.28 ± 0.02 vs −0.23 ± 0.02; $p = .09$) (Figure 2c). Multiparous cows had more severe BCS losses than primiparous cows (−0.29 ± 0.02 vs −0.22 ± 0.02; $p < .05$). However, there were no differences in BSC losses between weeks 2 and 4 postpartum in the CLA groups.

3.4 | Blood metabolic analytes

Glucose, BHB, IGF-1 and insulin concentrations increased, while NEFA concentration decreased, during weeks 2–6 postpartum ($p < .01$, $p < .05$, $p < .0001$, $p < .01$ and $p < .01$, respectively) (Table 2).

Overall, cows with delayed CLA had higher blood NEFA (0.31 ± 0.01 vs. 0.26 ± 0.01 mmol/L; $p < .001$) and BHB (0.71 ± 0.03 vs. 0.60 ± 0.03 mmol/L; $p \leq .05$) concentrations, and lower blood IGF-1 (89.4 ± 3.7 vs. 120.0 ± 3.7 ng/ml; $p < .001$) and glucose (3.5 ± 0.05 vs. 3.6 ± 0.05 mmol/L; $p \leq .01$) concentrations, than cows with normal CLA (Table 2). However, insulin blood concentration did not differ between the CLA groups. There were some differences between parities and breeds (see Table 2).

3.5 | Dry matter intake, energy-corrected milk and milk composition

No difference in DMI was observed between the CLA groups (delayed vs. normal). However, within the normal CLA group, Holstein cows had higher DMI than SRB cows (22.2 ± 0.5 vs. 20.6 ± 0.4 kg/d; $p < .01$).

No difference in milk yield was observed between the CLA groups (delayed vs. normal). Within the delayed CLA group, Holstein had higher milk yield than SRB cows (34.6 ± 0.8 vs. 30.5 ± 0.6; kg ECM/d; $p \leq .001$).

Overall, milk fat and lactose did not differ between the CLA groups (delayed vs. normal). However, milk protein was lower in the delayed CLA than in the normal CLA group (3.26 ± 0.02 vs. 3.32 ± 0.02%; $p \leq .05$). Within the delayed CLA group, Holstein cows had lower milk protein than SRB cows (3.16 ± 0.03 vs. 3.35 ± 0.02%; $p < .0001$).

3.6 | Sensitivity and specificity

Overall, C18:0 and C18:1 cis-9 MFAs showed higher sensitivity, specificity and accuracy than C14:0, C16:0, BHB, NEFA, IGF-1 and NEB in early detection (week 2 after calving) of cows with delayed or normal CLA (Table 3). When measured in milk (g/100 g milk), C18:1 cis-9 and C18:0 MFAs had 64% accuracy in detecting 4.1 cows out of 5 with delayed CLA and 2.9 cows out of 5 with normal CLA.

4 | DISCUSSION

In our previous study (Churakov et al., 2021), we demonstrated a correlation between MFAs and NEB. In this follow-up study, we compared the concentrations of MFAs in whole milk and milk fat from cows in early lactation and the concentrations of blood variables for the same cows in terms of their accuracy in predicting future delayed CLA. We also compared the metabolic profile of animals with normal or delayed CLA, taking into consideration the effects of breed (Holstein, SRB) and lactation number (primiparous, multiparous). The results showed that cows with delayed CLA typically had lower levels of IGF-1 and short-chain MFAs (C14:0), which are synthesized de novo in the mammary gland, while their levels of long-chain MFAs (C18:0 and C18:1 cis-9), mainly deriving from lipolysis of adipose tissue, were higher. Already in the second week postpartum, levels of long-chain MFAs were able to predict delayed first ovulation and luteal phase in 80% of cows. Compared with NEFA, BHB and IGF-1 analysed in blood, C18:0 and C18:1 cis-9 analysed in milk were better biomarkers for early detection of cows with delayed or normal CLA. The plasma IGF-1 profile and the associated changes in NEFA, BHB and BCS were within the scope of the somatotropic axis in anticipating the energetic demands of high milk production. To our knowledge, this is the first

TABLE 2 Production variables and biomarkers (LSMean ± sem[a]) of commencement of luteal activity (CLA) for weeks 2, 4 and 6 in cows with normal (<21 days) CLA or delayed (>21 days) CLA, and significance of differences for breed (two classes; Holstein $n = 37$ and SRB $n = 49$), parity (two classes; primiparous $n = 25$ and multiparous $n = 41$) and CLA class (two classes; delayed $n = 42$ and normal CLA $n = 46$)

	Week 2		Week 4		Week 6		*p*-values				
	Normal	Delayed	Normal	Delayed	Normal	Delayed	Breed	Parity	CLA class	Week	Breed × CLA class
Production variables											
BW (kg)	638 ± 9.3	638 ± 9.3	632 ± 9.3	626 ± 9.3	631 ± 9.3	625 ± 9.3	≤.05	<.0001	.60	.49	.93
ECM (kg/day)	30.9 ± 0.9	31.8 ± 0.9	31.4 ± 0.9	32.8 ± 0.9	31.8 ± 0.9	33.2 ± 0.9	<.001	<.0001	.09	.40	≤.05
Total DMI (kg/day)	19.6 ± 0.6	18.5 ± 0.5	21.8 ± 0.6	22.0 ± 0.5	22.90.6	23.2 ± 0.6	<.01	<.0001	.63	<.0001	.51
SCC (10^3)	134 ± 25.8	121 ± 25.7	85 ± 25.8	94 ± 25.4	72 ± 26.4	101 ± 25.4	<.001	<.001	.25	<.01	≤.05
Energy balance											
NEB (MJ)	−11.5 ± 3.5	−1.7 ± 3.5	5.5 ± 3.7	−20.2 ± 3.4	−0.002 ± 3.4	2.1 ± 3.5	.55	.49	.23	<.0001	≤.05
BCS (scale 1–5)	3.6 ± 0.04	3.5 ± 0.04	3.4 ± 0.04	3.5 ± 0.04	3.4 ± 0.04	3.3 ± 0.04	<.0001	<.0001	≤.05	<.0001	.80
Biomarkers in blood plasma											
NEFA (mmol/L)	0.331 ± 0.02	0.3567 ± 0.02	0.2316 ± 0.02	0.306 ± 0.02	0.226 ± 0.02	0.277 ± 0.02	.11	<.01	<.001	<.0001	≤.05
BHB (mmol/L)	0.538 ± 0.05	0.603 ± 0.05	0.637 ± 0.05	0.810 ± 0.05	0.612 ± 0.05	0.713 ± 0.05	.36	<.0001	≤.05	≤.05	.57
IGF-1 (ng/ml)	110.1 ± 6.4	73.5 ± 6.2	123.4 ± 6.3	92.9 ± 6.2	125.2 ± 6.6	101.6 ± 6.2	<.05	<.0001	<.0001	<.01	.21
Glucose (mmol/L)	3.54 ± 0.08	3.35 ± 0.08	3.6 ± 0.08	3.4 ± 0.08	3.7 ± 0.08	3.6 ± 0.08	.87	<.0001	<.01	<.01	.65
Insulin (μg/ml)	0.202 ± 0.03	0.165 ± 0.03	0.268 ± 0.03	0.207 ± 0.03	0.346 ± 0.03	0.216 ± 0.03	.09	<.0001	.14	<.01	.77
Biomarkers in milk (g/100 g milk)											
Fat	4.5 ± 0.1	4.8 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	.56	.11	.60	<.0001	.97
Protein	3.6 ± 0.03	3.6 ± 0.03	3.2 ± 0.03	3.1 ± 0.03	3.1 ± 0.03	3.1 ± 0.03	<.0001	≤.05	≤.05	<.0001	≤.05
Lactose	4.5 ± 0.02	4.4 ± 0.02	4.6 ± 0.02	4.5 ± 0.02	4.6 ± 0.02	4.6 ± 0.02	≤.05	<.0001	≤.05	<.0001	.30
Fat: protein ratio	1.25 ± 0.03	1.34 ± 0.03	1.34 ± 0.03	1.36 ± 0.03	1.31 ± 0.03	1.32 ± 0.03	.22	.55	.16	.27	.32
C14:0	0.50 ± 0.01	0.48 ± 0.01	0.49 ± 0.01	0.44 ± 0.01	0.46 ± 0.01	0.44 ± 0.01	<.001	.77	<.01	<.01	.15
C16:0	1.19 ± 0.03	1.19 ± 0.03	1.18 ± 0.03	1.10 ± 0.03	1.11 ± 0.03	1.07 ± 0.03	.15	.22	.11	≤.05	.37
C18:0	0.47 ± 0.02	0.55 ± 0.02	0.38 ± 0.02	0.40 ± 0.02	0.32 ± 0.02	0.36 ± 0.02	.28	<.01	<.001	<.0001	.08
C18:1 cis-9	1.08[a] ± 0.03	1.25[a] ± 0.03	0.98 ± 0.03	1.02 ± 0.03	0.91 ± 0.03	0.97 ± 0.03	.19	.15	<.001	<.0001	.09
Biomarkers in milk fat (g/100 g fat)											
C14:0	11.44 ± 0.34	10.02 ± 0.34	11.2 ± 0.35	10.48 ± 0.34	11.59 ± 0.35	10.91 ± 0.35	<.001	.11	<.001	.31	.52
C16:0	27.43 ± 0.8	24.32 ± 0.8	27.18 ± 0.8	26.24 ± 0.8	27.70 ± 0.8	26.50 ± 0.8	.06	.45	≤.05	.30	.91
C18:0	9.11 ± 0.66	11.43 ± 0.66	8.80 ± 0.67	9.7 ± 0.66	8.03 ± 0.67	8.98 ± 0.66	.12	.09	≤.05	≤.05	.76
C18:1 cis-9	25.06 ± 0.59	25.80 ± 0.59	22.87 ± 0.59	24.77 ± 0.59	22.61 ± 0.60	24.13 ± 0.59	.16	.49	<.01	<.01	≤.05

Abbreviations: BCS, body condition score; BHB, beta-hydroxybutyrate; BW, body weight; DMI, dry matter intake; EB, energy balance; ECM, energy-corrected milk yield; NEFA, non-esterified fatty acids; SCC, somatic cell count.

[a]Least square mean and residual standard error of mean.

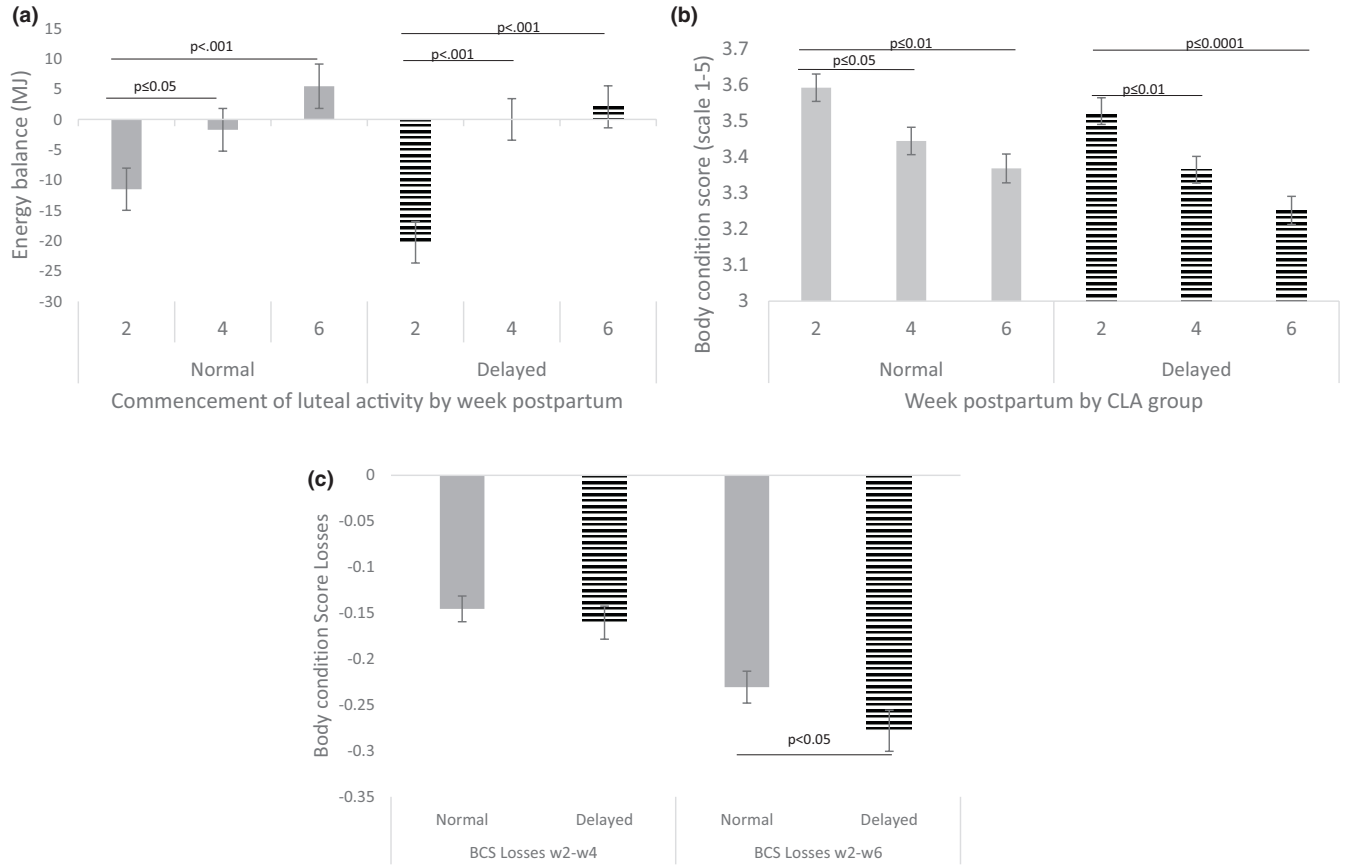

FIGURE 2 (a) Energy balance (MJ) and (b) body condition score in weeks 2–6 postpartum and (c) body condition score loss between weeks 2–4 and weeks 2–6 postpartum in cows with normal ($n = 42$) versus delayed ($n = 44$, >21 days) commencement of luteal activity (CLA). Values shown are least square means, lines on bars indicate residual standard error of the mean.

scientific study to demonstrate that MFA concentrations (g/100 g milk) are better biomarkers than commonly measured blood metabolic analytes and have better potential to identify cows with delayed CLA.

Further analysis of the results showed that MFA content in milk was a better predictor of CLA than MFAs expressed as a fraction of total milk fat. To our knowledge, no previous published study examining the relationship between MFAs and CLA has expressed the MFA content either as a fraction of milk fat or as a fraction of milk. Several studies have shown that NEB causes mobilization of the animal's own reserves, resulting in an increase in the fat content in milk (de Vries & Veerkamp, 2000; Erdmann et al., 2019; Stoop et al., 2009). Thus the concentration of a specific FA in milk will depend on both the milk fat content and the proportion of that specific FA in milk fat. In the present study, the C14:0 and C16:0 concentrations in milk fat were negatively correlated with CLA. In terms of early CLA prediction, the concentrations of C18:0 and C18:1 cis-9 in milk fat showed higher sensitivity and specificity than C14:0 and C16:0.

The accuracy of prediction of delayed CLA varied between lactation weeks. Both C18:1 cis-9 and C18:0 in milk had over 80% sensitivity in week 2 and values above 70% for subsequent weeks, making them the best indicators for early detection of delayed CLA. In contrast, the C14:0 and C16:0 concentrations in milk fat in week 2 could not be used for differentiating between cows with delayed or normal CLA. In addition, the NEFA and BHB concentrations in weeks 2 and 6 were not helpful for differentiating between cows with delayed or normal CLA. The high overall predictive performance of C18:0 and C18:1 cis-9 concentrations in milk in week 2 is positive from a practical perspective, as it enables early intervention, care and treatment for individual cows at risk, so that the predicted delay in CLA may be avoided. Early indications of disturbances in individual cows also allow for appropriate preventive measures at herd level to help all cows cope with the transition period, making the period of NEB as short and mild as possible.

The concentrations of individual MFAs changed over time, following previously established patterns (Palmquist et al., 1993). Long-chain MFAs decreased with time after calving, reflecting a decline in mobilization of adipose tissue, while short-chain MFAs increased with time. Comparable patterns of MFAs in early-lactation cows have been reported previously (Gross et al., 2011; Karlsson et al., 2020), leading to the conclusion that MFA profiles and the interrelationship between individual FAs reflect changes in energy balance in dairy cows.

A correlation between specific MFAs (C18:0 and C18:1 cis-9) and adipose tissue lipolysis, and thus NEB, has been demonstrated

TABLE 3 Sensitivity, specificity and accuracy of different biomarkers analysed in weeks 2, 4 and 6 postpartum for identification of cows ($n = 86$) for delayed commencement of luteal activity (CLA) (>21 days postpartum)

	Threshold	Sensitivity (%)	Specificity (%)	Accuracy (%)
	Week 2			
Variable				
EB (MJ)	−4.7	38.1	44.6	43
In blood plasma				
NEFA (mmol/L)	0.4	57.1	50.8	52.3
BHB (mmol/L)	0.7	57.1	50.8	52.3
IGF-1	122	40.0	45.5	44.2
In milk (g/100 g milk)				
C14:0	0.5	47.8	47.6	47.7
C16:0	1.3	47.6	47.7	47.7
C18:0	0.6	81.0	58.5	64.0
C18:1 cis-9	1.3	81.0	58.5	64.0
In milk fat (g/100 g fat)				
C14:0	11.9	33.3	43.1	40.7
C16:0	26.8	38.1	44.6	43.0
C18:0	12.1	76.2	56.9	61.6
C18:1 cis-9	27.3	66.7	53.9	57.0
	Week 4			
Variable				
EB (MJ)	9.7	54.6	50.0	52.3
In blood plasma				
NEFA (mmol/L)	0.3	71.4	55.4	59.3
BHB (mmol/L)	0.8	57.1	50.8	52.3
IGF-1	145.0	42.9	46.2	45.3
In milk (g/100 g milk)				
C14:0	0.5	42.9	46.2	45.3
C16:0	1.3	52.4	49.2	50.0
C18:0	0.5	57.1	50.8	52.3
C18:1 cis-9	1.2	61.9	52.3	54.7
In milk fat (g/100 g fat)				
C14:0	11.8	28.6	41.5	38.4
C16:0	28.3	42.9	46.2	45.3
C18:0	9.9	66.7	53.9	57.0
C18:1 cis-9	25.3	71.4	55.4	59.0
	Week 6			
Variable				
EB (MJ)	14.0	47.6	47.7	47.7
In blood plasma				
NEFA (mmol/L)	0.3	60.0	51.5	53.5
BHB (mmol/L)	0.8	60.0	51.5	53.5
IGF-1	157.0	45.0	47.0	46.5
In milk (g/100 g milk)				
C14:0	0.5	47.6	47.7	47.7
C16:0	1.2	47.6	47.7	47.7
C18:0	0.4	76.2	56.9	61.6
C18:1 cis-9	1.1	70.0	54.6	58.1

TABLE 3 (Continued)

	Threshold	Sensitivity (%)	Specificity (%)	Accuracy (%)
In milk fat (g/100 g fat)				
C14:0	12.2	33.3	43.1	40.7
C16:0	28.5	45.0	47.0	46.5
C18:0	9.1	70.0	54.6	58.1
C18:1 cis-9	24.4	71.4	55.4	59.3

Abbreviations: BHB, beta-hydroxybutyrate; EB, energy balance; IGF-1, insulin growth factor 1; NEFA, non-esterified fatty acids.

previously by our research group (Churakov et al., 2021). This correlation can be used as a predictive tool since EB calculations may involve recording errors and EB is difficult to estimate directly in dairy herds (Erdmann et al., 2019). In addition, although blood sampling is low-risk and is generally quick, it is an invasive and costly procedure that is difficult to implement routinely in commercial dairy herds as it requires extra labour.

Early resumption of cyclicity is one of the most important events in maintaining maximum reproductive potential in dairy cows following parturition (Opsomer et al., 2000). Even though CLA differed between the two CLA-based groups in this study, the time from calving to first AI did not differ between these groups, probably because time of the first AI after calving is influenced by management practices and breeding decisions. However, the interval from calving to conception was different between the CLA groups, following previously established patterns (Ratnayake et al., 1998). Irrespective of whether herd health advisors recommend a conventional, short calving interval of 12–13 months or a longer interval better adapted for high-producing cows with high lactation persistency (Andrée O'Hara et al., 2019), in both cases it is beneficial for fertility to have early onset of cyclicity (Crowe et al., 2014), with early CLA followed by normal cycles, optimal oestrus detection rates and conception rates.

Among the blood parameters tested, BHB, NEFA and to some extent IGF-1 were found to be weak indicators of delayed CLA. BHB has been shown previously to be a poor indicator of CLA in early-lactation cows (Jackson et al., 2011). Additionally, McCarthy et al. (2015) showed that BHB and NEFA do not have a strong correlation to each other during the transition period. Thus the reliability of blood metabolites such as NEFA, BHB, insulin and IGF-1 as diagnostic tools for detection of NEB may vary, although they are still relevant useful indicators of metabolic and fertility disorders. It has been shown that BHB and NEFA blood concentrations can be influenced when lactating cows are fed once daily (Piantoni et al., 2015). In the present study, the cows were offered silage ad libitum and could consume small amounts of concentrate on different occasions, so the effects of feeding on plasma parameters were probably limited.

In this study, we used the third quartile as a threshold for the sum of sensitivity and specificity in identifying cows with delayed CLA (>21 days). The threshold can be improved with the use of receiver operating characteristic (ROC) curves and validated by further large-scale studies, but provides a guideline indicative of delayed CLA. This finding has strong practical implications, as it can be useful for on-farm detection of delayed CLA.

The risk of diurnal variation in milk composition (Forsbäck et al., 2010) was low in our samples, as shown by Rico et al. (2014), making MFA concentrations suitable for routine sampling. Ideally, automated, in-line analysis of MFAs should be developed. Existing tools for chemical analyses of milk, such as DeLaval's Herd Navigator, have proven to be very beneficial for monitoring reproductive functions, metabolic status and udder health. At least two companies (AfiLab and Lely) now offer commercial products for analysing, for example fat and protein in-line using near-infrared reflectance (NIR), but these devices do not appear to be precise enough to be accepted by ICAR (Kaniyamattam & De Vries, 2014; King et al., 2019). Besides identification of risk cows in need of special care, it is of great value that all normal and healthy cows can be identified as normal, which will save labour and time and allow farm staff to allocate their time to better purposes.

4.1 | Limitations

This study accounted for the effects of breed and lactation number, but the experiment was conducted on only one dairy herd and all animals consumed the same feed ration. Different feed rations and herd management routines might cause variations in milk FA composition. All milk samples were analysed by the same FTIR equipment, but raw data on frequency spectrum in a sample might differ between devices unless the spectroscopy equipment used for analysis is calibrated for the actual FAs.

5 | CONCLUSIONS

This study provided proof of concept that concentrations of long-chain FAs in milk, detected by FTIR spectroscopy, can be used as biomarkers for early discrimination of dairy cows with future delayed or normal CLA. Concentrations of C18:0 and C18:1 cis-9 measured in milk were found to be the most reliable variables for early prediction of cows with delayed CLA, which is important for herd management and decisions on treatment and prevention at individual and herd level.

Overall, MFAs were more accurate in prediction of cows with delayed CLA than blood plasma biomarkers (NEFA). Moreover, milk sampling and milk FTIR spectroscopy are more practically

convenient than routine blood sampling on individual cows, making FTIR spectroscopy for milk biomarkers practically applicable in monitoring for delayed CLA at the individual cow level.

AUTHOR CONTRIBUTIONS

Theodoros Ntallaris involved in data curation, conceptualization, formal analysis, methodology, software, writing the original draft, review and editing. **Johanna Karlsson** involved in data curation, investigation, project administration, review and editing. **Renée Båge** involved in conceptualization, investigation, methodology, supervision, review and editing. **Kjell Holtenius** involved in conceptualization, funding acquisition, investigation, methodology, project administration, review and editing. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

None of the data and none of the models have been deposited in an official repository. The code for analysis is available upon request.

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