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Changes in milk ketone and fatty acid concentrations during early lactation in Holstein and Fleckvieh cows

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Abstract: The aim of this study was to compare the changes in milk composition postpartum, especially ketones and milk fatty acids (FAs), in Holstein and Fleckvieh cows kept under identical management conditions. Milk composite samples were collected from 66 cows during afternoon milking, at weekly intervals from one to eight weeks postpartum, and their components were determined by Fourier transform infrared spectroscopy. The Holstein cows had higher ($P < 0.05$) concentrations of long-chain FAs (ranging from 6% to 16% in different weeks), monounsaturated FAs (6% to 12%), and C18:1 (5% to 16%), as well as lower ($P < 0.05$) concentrations of saturated FAs (3% to 8%) and short-chain FAs (7% to 17%) in their milk than the Fleckvieh cows for almost the entire monitored period. These differences can be explained by pronounced lipomobilization, due to a negative energy balance, when mainly long-chain FAs from adipose tissue are incorporated into milk and significantly inhibit the *de novo* synthesis of FAs in the mammary gland. In conclusion, it can be assumed that breed-related metabolic changes during the first weeks of lactation have a large effect on the milk FA composition. This reflection of the metabolic load changes and lipomobilization in differing milk FA profiles would allow for the use of selected milk FAs to detect energy imbalances and their associated diseases in early lactation cows.

Keywords: breed; dairy cattle; periparturient period; milk fatty acids; milk ketones; Fourier transform infrared spectroscopy

During the peripartum period, dairy cows undergo numerous hormonal and metabolic changes which reflect increasing energy requirements caused by foetal growth and the subsequent onset of lactation. In addition, early lactating dairy cows are not able to consume adequate amounts of dry matter to meet the high energy requirements of increased milk production (Roche et al. 2013). Therefore, a certain degree of negative energy balance (NEB) exists in most dairy cows. This NEB leads to the release of fat reserves (lipomobilization), which serve as a source of energy. However, if the NEB is prolonged or extreme, it has a detrimental impact on the dairy cow's health. The NEB can be directly estimated using indicators measured in the blood serum, especially non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), triacylglycerols and cholesterol (Van Saun 2016). However, these blood analyses are invasive, time-consuming, and financially expensive. Therefore, efforts are being made to find reliable indirect indicators of NEB and related diseases. Milk components, especially ketones and fatty acids (FAs), appear to be a suitable diagnostic tool for indirect detecting of energy deficits, and some diseases, in early lactating dairy cows.

The most common method to determine milk FA composition is gas chromatography, which is

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again relatively expensive and time-consuming. An alternative method to predict milk FA composition is Fourier transform infrared spectroscopy (FTIR). Today, FTIR is used worldwide to predict the concentrations of milk fat (MF), protein, urea, and lactose within official milk-recording schemes and milk payment systems (Grelet et al. 2016). Furthermore, several studies undertaken over the last decade have demonstrated the potential of FTIR to predict detailed milk composition (De Marchi et al. 2014), including the FA profile (Soyeurt et al. 2011), the physiological state of the cow (body energy status) (McParland et al. 2011), as well as energy intake and efficiency (McParland et al. 2014). For example, elevated levels of ketone bodies in the milk can be used as a tool for the diagnosis of subclinical and clinical ketosis. Ketogenesis is a common metabolic pathway to provide alternative energy sources for peripheral tissues in the form of ketones (acetone, BHB) during massive lipomobilization in early lactating dairy cows. However, if the synthesis of ketones exceeds the tissue capacity for their use, their concentrations in the blood, milk, and urine increase, and the ketosis develops (Duffield et al. 2009).

Fatty acids can be divided according to their carbon chain length into short-chain FAs (SCFA) with up to 10 carbons, medium-chain FAs (MCFA) with 11 to 16 carbons, and long-chain FAs (LCFA) with 18+ carbons. Furthermore, FAs can be classified according to the degree of saturation (presence and number of double bonds) into saturated FAs (SFA), which do not contain any double bonds, monounsaturated FAs (MUFA) with one double bond, and polyunsaturated FAs (PUFA) with two or more double bonds. Saturated fatty acids account for 70% of all milk FAs, with the quantitatively most important being palmitic acid (C16:0) followed by stearic acid (C18:0) and myristic acid (C14:0). Approximately 25% of milk FAs are MUFAs, the most important of which is oleic acid (C18:1 *cis*-9). The remaining milk FA fraction consists of PUFAs and *trans*-FAs (Lindmark Mansson 2008). Milk FAs originate from four sources (Stoop et al. 2009): (1) approximately 50% is generated from *de novo* synthesis in the mammary gland, (2) 40–45% from feed, (3) < 10% is released by the lipolysis of adipose tissue, and (4) the remainder is formed in the rumen through biohydrogenation, bacterial degradation, and microbial *de novo* synthesis. *De novo* synthesis in the mammary gland produces FAs with an even number of carbons, and with a maximum length of 16 carbons (Chilliard et al. 2000). Part of C16:0, and all LCFA, are derived from feed or lipolysis of tissue triacylglycerols (Lindmark Mansson 2008).

Milk FA composition is influenced by several factors, the main being breed, stage of lactation, nutrition, individual variability, and metabolic status of the animal. Differences between cattle breeds have been studied, mainly between Holstein and Jersey cows, as well as between Brown Swiss or Simmental cows (Soyeurt et al. 2006; Gottardo et al. 2017; Mlynek et al. 2021). Some authors (Kelsey et al. 2003; Soyeurt et al. 2006) claim that there is greater variability in the composition of FAs between individual dairy cows (within the breed) than between breeds. Individual variability may be due to differences in metabolic stress, especially at the beginning of lactation, with the NEB that may occur during this period being largely responsible for the changes in the milk FA composition (Gross et al. 2011). The NEB induces excessive lipolysis of adipose tissue, through which LCFA are released into the bloodstream and are incorporated into MF, while inhibiting *de novo* synthesis of SCFA and MCFA (Palmquist et al. 1993; Stoop et al. 2009; Gross et al. 2011).

The aim of this study was to compare the changes in milk composition during early lactation (the first 60 days), especially ketones and FAs, as determined by FTIR, of Holstein (H) and Fleckvieh (F) cows kept under identical management conditions.

MATERIAL AND METHODS

Animals, diets, and experimental design

All experimental procedures involving animals were approved by the Animal Care Committee of the Ministry of Agriculture of the Czech Republic (No. 14608/2016-MZE-17214). The study was carried out at the experimental station belonging to the Institute of Animal Science in Prague, Czech Republic. The methodology of this study follows Stolcova et al. (2020), where further details may be found regarding diet composition, blood biochemical analyses, and milk analyses. Briefly, cows were selected from a herd of approximately 200 cows which includes H (dairy) and F (dual-purpose) cattle, kept under identical management conditions.

https://doi.org/10.17221/122/2021-CJAS

A total of 66 primiparous and multiparous (up to the ninth lactation) cows which calved between November and March were included in the experiment (Table 1). All the cows entering the experiment were evaluated in advance and deemed to be healthy. They were statistically blocked by parity into three groups (primiparous, 2nd and 3rd parity, and 4+ parities). The experimental period began 14 days before the expected date of calving and continued until eight weeks postpartum (week 1 to 8). The cows were milked in a 2 × 5 tandem milking parlour (AfiMilk®; S.A.E. Afikim – Computerized Dairy Management Systems, Kibbutz Afikim, Israel) twice per day (04:00 and 16:00). Milk production data were automatically recorded during each milking using the AfiFarm™ herd management software version 4.1 (S.A.E. Afikim – Computerized Dairy Management Systems, Israel). Cows were fed *ad libitum* twice per day (04:00 and 16:00) a total mixed ration, differing in its composition according to the lactation and reproduction cycle phase. Close-up dry cows were fed a close-up diet, starting three weeks prior to the expected date of calving. After calving, cows were given an early-lactation diet to meet the energy demands for high milk production yields (approximately 40 l per day). The total mixed rations included maize silage, lucerne haylage, hay, wheat straw, concentrates, and a mineral-vitamin supplement. The close-up diet contained 465 g dry matter (DM)/kg on an as is basis, 6.5 MJ/kg DM net energy of lactation (NEL), 142 g/kg DM crude protein, and 208 g/kg DM crude fibre. The early-lactation diet contained 450 g DM/kg on an as is basis, 7.7 MJ/kg DM NEL, 178 g/kg DM crude protein, and 148 g/kg DM crude fibre.

Milk sampling and analysis

Eight milk composite samples (pooling milk from all four quarters of the udder during the afternoon milking) were collected from each cow. The first samples were collected seven (± 3) days postpartum, and thereafter at weekly intervals until the end of the experiment (total of eight weeks postpartum). The samples were collected in plastic tubes (D&F Control System, Inc., Dublin, CA, USA), containing a combination of Bronopol and Natamycin (Broad Spectrum Microtabs II®; D&F Control System, Inc., Dublin, CA, USA) and transported to a certified Laboratory for Milk Analysis in Buštěhrad, Czech Republic.

Table 1. Numbers of Fleckvieh and Holstein cows, according to parity number, included in the experiment

Parity	Fleckvieh	Holstein
1	6	17
2 and 3	8	14
4 and more	10	11
Total	24	42

All milk constituents were determined using a MilkoScan™ FTIR analyser (Foss Electric, Hillerød, Denmark). The concentrations of the following milk components were determined: fat (%), protein (%), lactose (%), ketones (acetone, BHB; mmol/l), and FAs (g/100 g of milk). The FAs were classified (1) according to the length of the carbon chain (based on Application Note 64 for MilkoScan): SCFA with C4–C10, MCFA with C12–C16 and LCFA with C18 and more; (2) and according to the number of double bonds: SFA without double bonds, MUFA with one and PUFA with two or more double bonds. Furthermore, the concentrations of individual milk FAs (palmitic, stearic and octadecenoic) were also determined. The FA concentrations were converted from g/100 g of milk to g/100 g of MF according to the following equation:

$$\text{FAs (g/100 g of MF)} = \text{FAs (g/100 g of milk)} \times \times 100/\text{MF (\%)} \quad (1)$$

Calculation and statistical analysis

Each dependent variable (milk yield and milk composition results) was tested for normality and the presence of outliers using Anderson-Darling, Cramer-von Mises, and Kolmogorov-Smirnov tests, following the UNIVARIATE procedure in SAS (v9.3; SAS Institute Inc., Cary, NC, USA). Cows with fewer than five sampling day records were removed from the data set. The complete data set thus consisted of 494 records from 66 cows. The data were evaluated using a mixed linear model with repeated measures. Parameters were estimated following the REML method using the MIXED procedure in SAS.

The initial model was structured to determine the fixed effect of breed, week of lactation, parity, and their two-way interactions. Effects that were

https://doi.org/10.17221/122/2021-CJAS

not significant were subsequently removed from the model by backward elimination. The final statistical model included the fixed effects of the combined effect of breed and week of lactation, parity within breed, and the random effect of sampling day.

Due to the repeated measures within each cow, random covariances between weeks were summarized by the residual **R** matrix, which was assumed to be a block diagonal with identical 8 × 8 submatrices. As alternatives, the compound symmetry, unstructured, first-order autoregressive and Toeplitz covariance structures were compared. The first-order autoregressive covariance structure with the random effect for cow was found to be the most appropriate, in accordance with the Akaike information criterion and Schwarz Bayesian criterion (Littell et al. 2000). The least squares means were calculated and multiple comparisons were made, with *P*-values adjusted according to Tukey's procedure. Significant differences are reported at $P < 0.05$.

RESULTS

The descriptive statistics of milk ketone (acetone, BHB) and milk FA concentrations of all cows included in the experiment are reported in Table 2.

The breed effects on the milk yield and basic milk constituents during the first eight weeks of lactation for the experimental animals may be found in Stolcova et al. (2020). The effect of breed on the milk ketone concentrations is reported in Figure 1, with the concentrations of acetone and BHB in milk being similar in both breeds. It is evident that the milk ketone levels were higher in the first week after calving than in the following weeks of the experimental period in both breeds.

The FA concentrations for SCFA, MCFA, and LCFA are reported in Figure 2, while the concentrations of SFA and unsaturated FA groups (MUFA and PUFA) are shown in Figure 3. The milk SCFA concentration was higher ($P < 0.05$) in F cows compared to H cows over the experimental period, except for week 8, when the concentrations for both breeds were comparable. In contrast, milk LCFA contents were higher in H cows, with differences being significant during weeks 1, 2, 3, and 5 ($P < 0.05$). However, MCFA concentrations did not differ between the breeds throughout the analysed period. The SFA concentration was higher ($P < 0.05$) in F cows compared to H cows during the first five weeks after calving. The MUFA levels were higher ($P < 0.05$) in the milk from H cows during the first five weeks after calving. Milk PUFA concentrations were similar in both breeds, except for week 5, when they were significantly higher in the milk from H cows

Table 2. Descriptive statistics of milk ketone and fatty acid concentrations of all measurements (n = 494) included in the experiment

Parameter	Mean	Standard deviation	Minimum	Maximum
Milk ketones (mmol/l)				
Acetone	0.22	0.254	0.01	3.30
Milk BHB	0.89	0.072	0.01	0.60
Milk fatty acids (g/100 g of milk fat)				
SCFA	9.7	1.42	3.3	14.9
MCFA	38.8	5.69	20.5	56.1
LCFA	39.1	5.75	24.5	55.7
SFA	62.8	4.51	51.0	75.5
MUFA	32.1	4.13	21.0	44.1
PUFA	6.6	1.26	3.8	14.0
C16:0	31.1	3.30	20.9	41.7
C18:0	14.2	1.57	8.1	20.5
C18:1	29.0	4.21	19.0	41.9

BHB = β-hydroxybutyrate; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = octadecenoic acid; LCFA = long-chain fatty acids; MCFA = medium-chain fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SCFA = short-chain fatty acids; SFA = saturated fatty acids

https://doi.org/10.17221/122/2021-CJAS

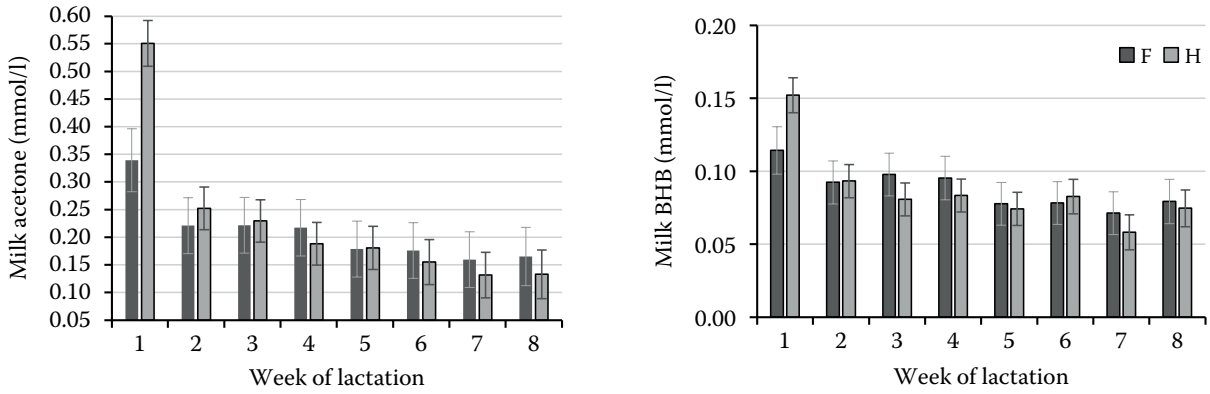

Figure 1. Means and standard errors of milk acetone and milk β-hydroxybutyrate (BHB) concentrations in Fleckvieh (F) and Holstein (H) cows

*Significant differences between breeds ($P < 0.05$)

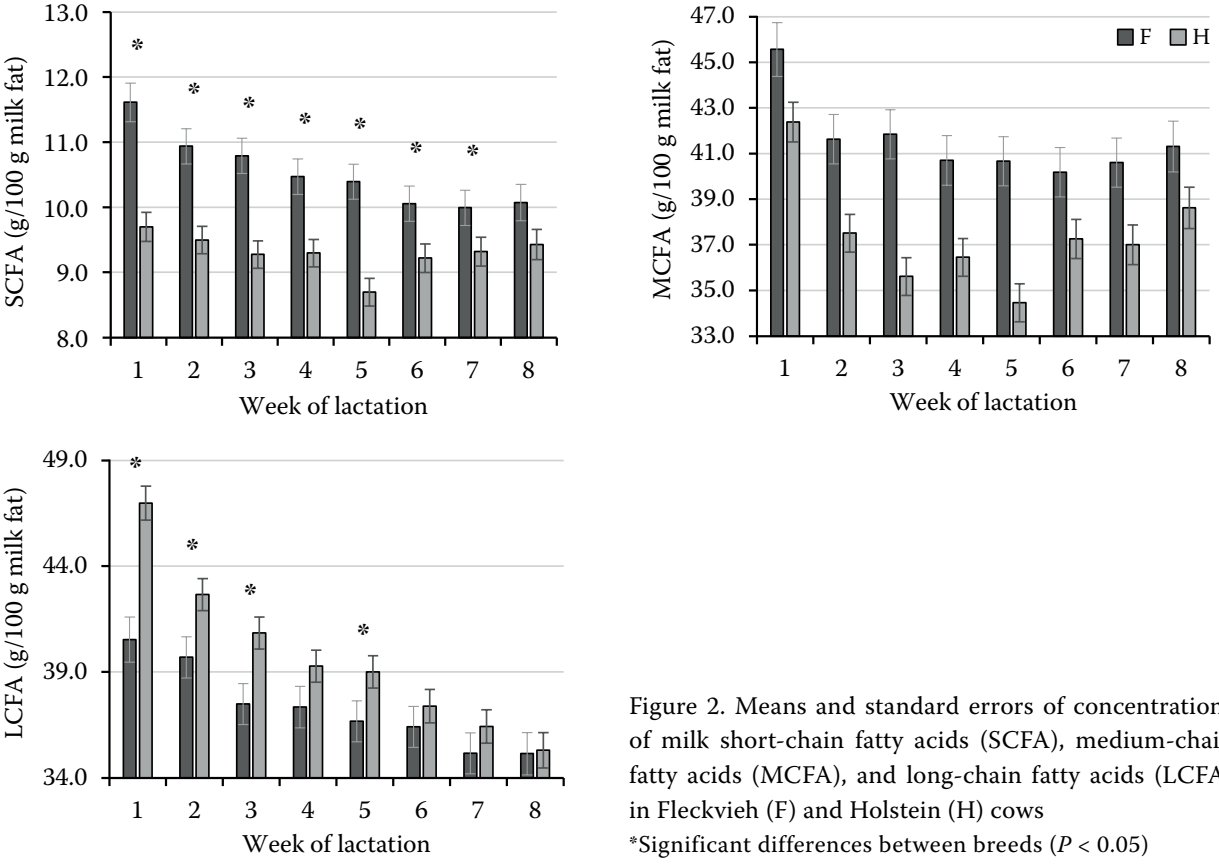

Figure 2. Means and standard errors of concentrations of milk short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), and long-chain fatty acids (LCFA) in Fleckvieh (F) and Holstein (H) cows

*Significant differences between breeds ($P < 0.05$)

compared to that of F cows (7.2 g/100 g of MF vs 6.3 g/100 g of MF, respectively; $P < 0.05$).

The development over time of C16:0, C18:0 and C18:1 is shown in Figure 4. Significantly higher concentrations of C16:0 were reported in the milk from F cows than in that of H cows during week 1 (29.2 g/100 g of MF vs 26.9 g/100 g of MF, respectively; $P < 0.05$), whereas no breed differences were seen for the remainder of the experimental period. While the milk concentrations of C18:0 were higher in H cows than in F cows (16.0 g/100 g of MF vs 15.0 g/100 g of MF, respectively; $P < 0.05$), the inverse tendency was observed at the end of the experimental period (weeks 6, 7, and 8). The H cows had markedly higher ($P < 0.05$) milk C18:1 concentrations compared to F cows throughout the entire experimental period, except for week 8 when they were comparable.

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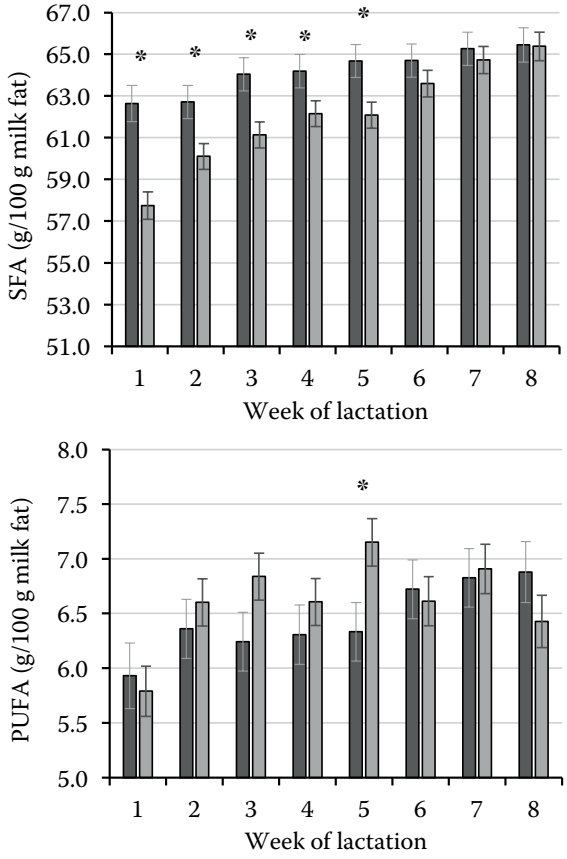

Figure 3. Means and standard errors of concentrations of milk saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in Fleckvieh (F) and Holstein (H) cows

*Significant differences between breeds ($P < 0.05$)

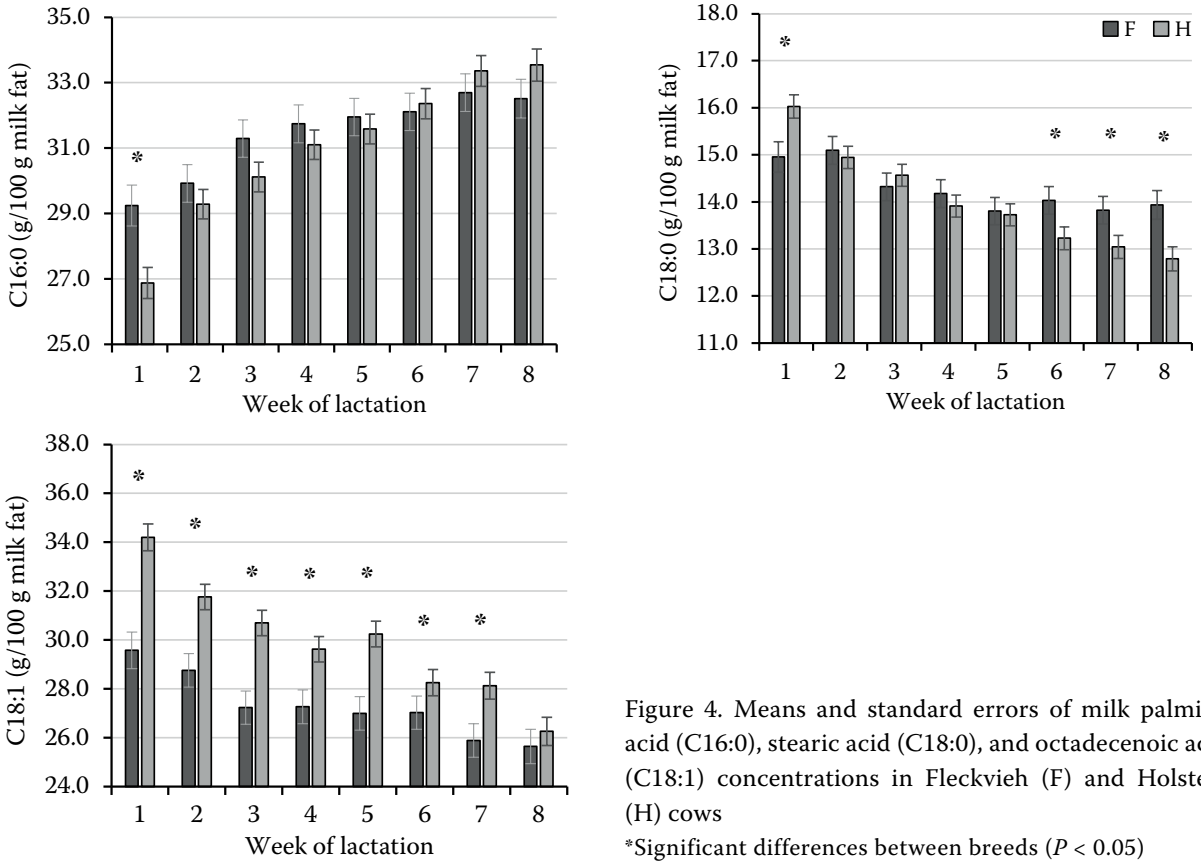

Figure 4. Means and standard errors of milk palmitic acid (C16:0), stearic acid (C18:0), and octadecenoic acid (C18:1) concentrations in Fleckvieh (F) and Holstein (H) cows

*Significant differences between breeds ($P < 0.05$)

https://doi.org/10.17221/122/2021-CJAS

DISCUSSION

Milk ketone contents

The measurement of milk ketone levels could be used for the early detection of dairy cows with associated high levels of BHB in the blood (hyperketonaemia; serum BHB > 1.2 mmol/l, McArt et al. 2012) and thus may assist in the diagnosis of subclinical ketosis (SCK). The threshold values for the identification of dairy cows with hyperketonaemia, utilizing milk BHB as an indicator, range from 0.14 mmol/l to 0.20 mmol/l in current literature (Denis-Robichaud et al. 2014; Renaud et al. 2019), were not exceeded in the present study. The threshold values for the determination of hyperketonaemia using milk acetone, as reported in literature, have a large variance which needs to be further investigated. According to De Roos et al. (2007), the threshold value for the determination of SCK using milk acetone in H cows in the first three weeks of lactation is 0.15 mmol/l. In the present study, this threshold value was markedly exceeded during the first week after calving, when the milk acetone content in H cows was 0.55 mmol/l. The possible presence of SCK is also supported by the serum BHB levels, which exceeded the SCK threshold value in 11 out of 42 H cows from the same experiment, during the first two weeks post-calving (Stolcova et al. 2020). However, according to a study by Gustafsson and Emanuelson (1996), not even these elevated milk acetone concentrations would likely affect the production and reproductive performance of Holstein cows, as these negative effects should be expected only after exceeding 0.7 mmol/l. The afore-mentioned study also investigated the Swedish Red and White breed, which was found to be more sensitive to elevated concentrations of acetone in milk, and resulted in a more significant milk yield reduction in the cows exceeding the acetone level of 0.7 mmol/l milk. However, when the content of milk acetone was higher than 1.4 mmol/l, the decrease in milk production was similar in both breeds, indicating that at a very high acetone concentration the physiological response of both breeds was the same. According to Chandler et al. (2018), however, the contents of ketones in the milk alone are rather unsuitable for the prediction of hyperketonaemia in dairy cows, due to a relatively high proportion of false-positive results. In the current authors' personal experience, the values of milk ketone contents vary considerably, which makes the direct diagnosis of subclinical ketoses quite difficult. It is thus recommended that dairy cows with abnormally high levels, or with significant changes, of milk ketone concentrations should be marked as suspicious and standard ketonaemia tests should be performed.

Selected milk fatty acids

There are few studies available comparing the milk FA composition between dairy and dual-purpose breeds. To the best of our knowledge, relevant scientific studies directly related to the comparison of Holstein and Fleckvieh breeds are not currently available. The most frequently studied comparisons are between H and Jersey, Brown Swiss (BS), Simmental (SI), Alpine Grey (AG), Meuse-Rhine-Yssel (MRY), and Montbéliarde. The most similar to Fleckvieh cattle in terms of milk yield (based on the comparison of average milk yield, including the percentage of MF and protein according to ICAR 2021) are the BS, SI, and MRY cattle. Another factor limiting the comparison of milk FA composition between studies is the use of different analytical methods for the determination of FAs and the expression of their concentrations. Many studies use gas chromatography for the analysis of milk FAs, which is considered to be the reference method for this purpose. Other studies, including this work, use the FTIR method, which is commonly used in milk recording laboratories. Gottardo et al. (2017) observed differences in the composition of milk FAs between four breeds during the entire lactation period, and the results were similar to those obtained in the present study. The H cows had the lowest SCFA and the highest C18:1 concentrations in comparison with the BS, SI, and AG breeds. The concentrations of LCFA and MUFA were higher in the H breed, only when compared to BS. In contrast, no milk FA composition differences were observed throughout the lactation period between H and MRY breeds (Soyeurt et al. 2006; Maurice-Van Eijndhoven et al. 2013). Kelsey et al. (2003) compared H and BS breeds over the entire lactation, and the concentrations of important FAs obtained using the gas chromatography were also similar in both breeds.

https://doi.org/10.17221/122/2021-CJAS

The experimental period is another important factor that may limit the comparison of the current results with other studies. While most publications aimed to investigate the FA composition over the entire lactation period, the present research focused only on the first eight weeks of lactation, when dairy cows undergo essential metabolic and hormonal changes that can potentially affect milk FA proportions. In the present study, the milk from H cows had significantly higher concentrations of LCFA, MUFA, and C18:1, but lower concentrations of SCFA for almost the entire monitored period compared to F cows. These differences can be explained by pronounced lipomobilization and by the resulting NEB, where primarily C16:0, LCFA, and MUFA (especially C18:1) from the adipose tissue are incorporated into milk. These FAs significantly inhibit the *de novo* synthesis of SCFA and MCFA in the mammary gland, as they reduce the activity of acetyl-coenzyme A carboxylase, which is the primary regulatory step in FA synthesis (Palmquist et al. 1993; Stoop et al. 2009; Gross et al. 2011). Indeed, lower SCFA in the milk from Holsteins were also observed in the present study. Similar results were obtained by Mlynek et al. (2021), who compared the milk FA composition between, *inter alia*, H and SI breeds during the first 100 days of lactation. The H cows had higher concentrations of milk LCFA and C18:1, and lower concentrations of SCFA. The authors argued that the differences were primarily due to metabolic load, especially lipomobilization. Lipomobilization was also confirmed in cows in the present study. A limitation of this study is that the individual dry matter intakes could not be measured, and thus the energy balance based on estimates of energy intake and requirements could not be determined. However, as revealed by results of blood analyses, the threshold value of serum NEFA (NEFA > 0.6 mmol/l) for detecting NEB and its associated problems was exceeded at least once in 21 H cows (out of 42) and seven F cows (out of 24) during the first two weeks after calving (Stolcova et al. 2020). Despite the fact that both H and F cows received the same early-lactation diet which was adequate for H but may have led to overfeeding of F, a number of postpartum cows were unable to consume sufficient nutrients to meet their lactation requirements. As a consequence, tissue reserves were likely mobilised and the cows entered a state of NEB.

The metabolic level differs between specialised dairy and dual-purpose breeds, as investigated by Knob et al. (2021), who compared the energy balance among H and SI cows as well as their crossbreds. They found that purebred SI and crossbreds with 1–49% proportion of H dealt better with the NEB after calving. These genetic groups lost less back fat and body weight than other genetic groups with greater H proportions. These findings also correspond to the current results, as more H cows (50%) than F cows (21%) entered a state of NEB. The differences in milk composition may be attributed to different metabolic loads in breeds with a contrasting production purpose to some extent. The dual-purpose F cows, belonging to the family of SI breeds, are selected for milk and meat production, whereas the H breed is selected only for milk production. Duchacek et al. (2020) aimed to evaluate the influence of NEB on the milk FAs of F cows after calving. Body condition scores and milk citric acid concentrations were used as NEB indicators. In agreement with the current results, the authors observed that the cows with larger NEBs produced milk with higher proportions of unsaturated fatty acids (UFA) and lower concentrations of SFAs. Additionally, in a similar study, Duchacek et al. (2014) concluded that H cows in a NEB also had lower SFA and higher UFA concentrations.

The differences in milk C18:0 and C16:0 concentrations between the two breeds were negligible in the present study. This may be a result of the fact that a large proportion of C18:0 is desaturated in the mammary gland to C18:1, primarily to maintain the optimal fluidity of milk. If the concentrations of FAs with high melting points (C16:0 and C18:0) increase during the period of NEB, the structure of newly formed triacylglycerols in the milk stimulates the occupation of glycerol positions by FAs with low melting points (C4:0 and C18:1). The melting temperature of the resulting triacylglycerols is thus ideal for maintaining the fluidity of milk (Loften et al. 2014). It is quite complicated to quantify the transition of C16:0 originating from fat reserves to milk, as a part of C16:0 is also formed by *de novo* synthesis, which is, however, inhibited during lipomobilization, as explained above. Simultaneously, the oxidation of palmitate decreases and C16:0 accumulates in the liver during overfeeding in the dry period, and is not incorporated into milk in the subsequent lactation (Litherland et al. 2011).

https://doi.org/10.17221/122/2021-CJAS

CONCLUSION

Breed differences were found in the concentrations of milk FA groups and individual FAs despite the same feeding conditions. The most significant differences were observed for the FAs which are transported from the adipose tissue to milk during lipomobilization during early lactation (especially C18:1, which is part of LCFA and MUFA), and for SCFA, whose *de novo* synthesis is inhibited by blood-derived LCFA. Similar patterns of changes in these parameters were recorded in both monitored breeds. The reflection of metabolic changes and lipomobilization in the milk FA profile may allow for the use of such selected milk FA to detect an energy imbalance and metabolic disease risk in early lactation cows. Further research is needed to verify the association of the differing milk FA composition and the energy balance of dairy cows using a larger set of observations within individual breeds and under different management conditions.

Conflict of interest

The authors declare no conflict of interest.

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