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Body fat mobilization in early lactation influences methane production of dairy cows

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Long-chain fatty acids mobilized during early lactation of dairy cows are increasingly used as energy substrate at the expense of acetate. As the synthesis of acetate in the rumen is closely linked to methane (CH₄) production, we hypothesized that decreased acetate utilization would result in lower ruminal acetate levels and thus CH₄ production. Twenty heifers were sampled for blood, rumen fluid and milk, and CH₄ production was measured in respiration chambers in week -4, +5, +13 and +42 relative to first parturition. Based on plasma non-esterified fatty acid (NEFA) concentration determined in week +5, animals were grouped to the ten highest (HM; NEFA > 580 μmol) and ten lowest (LM; NEFA < 580 μmol) mobilizing cows. Dry matter intake (DMI), milk yield and ruminal short-chain fatty acids did not differ between groups, but CH₄/DMI was lower in HM cows in week +5. There was a negative regression between plasma NEFA and plasma acetate, between plasma NEFA and CH₄/DMI and between plasma cholecystokinin and CH₄/DMI in week +5. Our data show for the first time that fat mobilization of the host in early lactation is inversely related with ruminal CH₄ production and that this effect is not attributed to different DMI.

The fermentation of poly- and monosaccharides in the rumen results in the formation of pyruvate which in turn serves as intermediate substrate for the production of short-chain fatty acids, primarily acetate, propionate and butyrate. Propionate synthesis is a hydrogen consuming process, whereas the conversion of pyruvate to acetate or butyrate is accompanied by the production of hydrogen. Methanogenic microbiota utilize the released hydrogen to reduce CO₂ yielding in the formation of CH₄¹. Accordingly, the ruminal acetate or the (acetate + butyrate) : propionate ratio is highly related with daily CH₄ emission (g/d) or CH₄ yield expressed as g per unit of dry matter intake (g/kg DMI)^{2,3}. In the post-absorptive metabolism of dairy cows, propionate is primarily used for hepatic gluconeogenesis, whereas acetate is activated to form acetyl-CoA serving as the main energy-providing substrate for the host. In lactating cows, acetate and butyrate may also serve as precursors for de novo milk fat (C4 – C16) synthesis by the mammary gland. Thus, CH₄ and precursors for de novo milk fatty acids arise from the same biochemical pathway in the rumen. Therefore concentrations of milk fatty acids de novo synthesized from acetate, e.g. C6, C8, C10 and C16 or the sum of these saturated fatty acid concentrations are indicators for the level of acetate production in the rumen and were found to be positively related to CH₄ emission or CH₄ yield^{3–5}.

The (acetate + butyrate) : propionate ratio in the rumen decreases when the diet is supplemented with oilseeds rich in C18 fatty acids, resulting in reduced CH₄ production⁶. The CH₄ suppressing effect of dietary long-chain fatty acids may be due to decreased fiber digestibility or reduced DMI⁷. In lactating animals, C18 milk fatty acid concentrations increased at the expense of shorter chain milk fatty acids upon supplementing the diet with oilseeds^{6,7}. Hence, concentrations of C18 milk fatty acids were found to be negatively related to CH₄ yield^{3–5}. However, neither DMI, feed composition, ruminal short-chain fatty acids nor milk fatty acids alone are sufficient to accurately predict CH₄ emission³, indicating that either the microbial composition or traits of the host may influence methanogenesis in the rumen. Only recently Ricci *et al.*⁸ considered, besides dietary characteristics, the physiological stage of the animal to improve the prediction of CH₄ emissions.

During the transition from late pregnancy to early lactation, high-yielding dairy cows do not ingest enough feed to meet the nutrient and energy requirements for milk production and consequently enter into negative

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energy balance. During this time of lactation provision of energy by dietary derived acetate is lowest and as an adaptive response cows mobilize their body fat reserves leading to increased concentrations of circulating long-chain, non-esterified fatty acids (NEFA). These mobilized NEFA are mainly composed of C16 and C18 and are intensively oxidized to allocate energy but also used by the mammary gland for milk fat synthesis⁹. Plasma NEFA concentrations paralleled the concentrations of milk C18, while the concentration of milk fatty acids synthesized from acetate remained unaffected or were inversely associated with plasma NEFA levels until week 7 after parturition⁹. Thus, the increase in plasma NEFA concentrations reduces the level of acetate utilization, at least in the mammary gland, but whether plasma NEFA concentrations are also negatively related to CH₄ yield remains to be investigated. Herein, we hypothesized that increased body fat mobilization resulting in higher plasma NEFA concentrations would negatively affect metabolic acetate utilization, and as a kind of negative feed-back regulation, acetate production in the rumen and consequently CH₄ production.

Results

Animals and Diets. Per design, animals were grouped according to their plasma NEFA concentrations at the time of the respiration chamber measurements in week 5 ± 0.2 of early lactation in which plasma NEFA concentrations amounted to 811.2 ± 61.9 μmol/L for the ten highest (HM; NEFA > 580 μmol) and 379.1 ± 61.9 μmol/L for the ten lowest (LM; NEFA < 580 μmol) mobilizing cows ($P < 0.001$; Supplemental Fig. 1a). The HM cows had also greater total amounts of plasma NEFA in the time period two weeks before until six weeks after the respiration chamber measurement in week 5 ± 0.2 ($P = 0.007$, Supplemental Fig. 1b), indicating slower increase but longer-lasting fat mobilization compared to LM cows who showed earlier, shorter and less intensive peak concentration far (i.e. 4 weeks) before respiration chamber measurement (702.3 ± 123.7 μmol/L; Supplemental Fig. 1a). The body weight of HM and LM cows ranged from 610 kg to 665 kg 4 weeks before parturition and changed over time ($P < 0.001$), but was not different between groups (Fig. 1a; Supplemental Table S1). From late pregnancy until week 13 post partum (p.p.) body weight declined to 556 ± 112 kg after which it increased again. DMI increased after calving ($P < 0.001$), but without difference between groups at any time point (Fig. 1b; Supplemental Table S1). In week 4 ante partum (a.p.) animals ingested about 7.1 kg of DM per d, which increased to 13.2 kg, 14.5 kg and 15.2 kg in week 5, 13 and 42 p.p., respectively, without difference between groups at times indicated (Supplemental Table S1). Body condition score (BCS) and back fat thickness (BFT) were not different between groups, but changed over time ($P < 0.001$; Fig. 1c,d; Supplemental Table S1). However, the group × time interaction for BFT was significant ($P = 0.02$; Fig. 1d). Precisely, HM cows had a higher BFT at calving ($P = 0.007$) and a higher loss of back fat until week 5 p.p. ($P = 0.006$, Fig. 1d).

Energy corrected milk yield (ECM) increased from week 1 until week 4 p.p. and decreased for all cows over time ($P < 0.001$) from 28.8 ± 1.1 kg in week 5 p.p. to 27.6 ± 0.8 kg in week 13 p.p. to 24.8 ± 0.9 kg in week 42 p.p., but again there was no differences between HM and LM cows (Fig. 1e, Supplemental Table S1). Milk fat decreased from week 5 p.p. to week 13 p.p. in both cow groups and was significantly lower in HM compared to LM cows in week 13 p.p. (HM 3.72 and LM 4.34 ± 0.13%; $P = 0.03$; Supplemental Table S1).

Feed digestibility determined in week 6 p.p. was comparable between groups (77.4 ± 0.3% in HM and LM cows; $P > 0.1$). The mean retention time (MRT) in week 6 p.p. amounted to 27.5 ± 0.6 h in HM cows and to 27.0 ± 0.6 h in LM cows without difference ($P = 0.54$) between groups.

Plasma, Milk and Rumen fluid. Plasma NEFA concentrations were greater in HM than LM cows not only in week 5 ($P < 0.001$), but still tended to be higher in week 13 after parturition ($P = 0.1$, Fig. 2a). Plasma beta-hydroxybutyrate (BHBA) concentrations increased after parturition ($P < 0.01$), but there were no differences ($P = 0.18$) between groups (Fig. 2b). Plasma acetate concentrations continuously increased over time ($P < 0.001$) and were lower in HM compared to LM cows (0.67 and 0.85 ± 0.06 mmol/L, respectively) in week 5 p.p. ($P = 0.04$; Fig. 2c). Accordingly, plasma NEFA and plasma acetate concentrations showed a significant ($P < 0.001$) inverse relationship in week 5 p.p. (Fig. 2d). Plasma cholecystokinin (CCK) concentrations increased after parturition ($P < 0.01$) and tended to be lower in HM compared to LM cows in week 5 p.p. ($P = 0.1$; Fig. 2e). Ruminal acetate concentrations varied with time (each $P < 0.05$) and pair-wise comparison for each time point revealed that HM cows tended ($P = 0.087$) to have higher rumen acetate concentrations in week 4 a.p. only (Fig. 3a). Ruminal propionate and butyrate, the (acetate + butyrate) : propionate ratio and ruminal pH were not influenced by time or group (Fig. 3b–e). In addition, linear regression between ruminal and plasma acetate was not significant (Fig. 3f; $P = 0.57$).

Methane, Fat and Carbohydrate Oxidation. Total CH₄ production increased over time ($P < 0.001$), but was not different between groups (Fig. 4a) and CH₄ yield did not change over time (Fig. 4b). However, in week 5 p.p. HM cows tended to have lower CH₄ emission when related to DMI (29.7 and 32.8 ± 1.3 L/kg, respectively; $P = 0.1$; Fig. 4b) or to neutral detergent fiber (NDF) (86.0 and 95.3 ± 3.5 L/kg, respectively; $P = 0.08$; Fig. 4c) than LM cows. But when CH₄ emission was related to ME intake, groups did not differ at any time point (Fig. 4d). Methane emission per kilogram ECM decreased over time ($P < 0.01$), but was not different between groups (Fig. 4e).

Net carbohydrate oxidation (COX) continuously increased until week 42 of lactation and tended to be different ($P = 0.09$) between cow groups in week 13, but not in week 5 p.p. (Fig. 5a). After parturition net fat oxidation (FOX) decreased over time in both groups ($P < 0.001$; Fig. 5b). The pair-wise comparison in week 5 p.p. showed that HM cows tended to have a higher fat oxidation than LM cows (1783 and 1352 ± 140 g/d, respectively; $P = 0.058$; Fig. 5a), but there was no significant regression between net FOX and CH₄ ($P = 0.37$) or CH₄/DMI ($P = 0.78$), respectively (Fig. 5c,d).

Linear regression between CH₄ production and plasma NEFA concentration in week 5 p.p. was not significant (Fig. 6a; $P = 0.17$), whereas we found an inverse relationship between CH₄ yield expressed as CH₄/DMI ($P = 0.002$) or CH₄/NDF ($P = 0.005$) with plasma NEFA concentrations of individual cows in week 5 p.p.

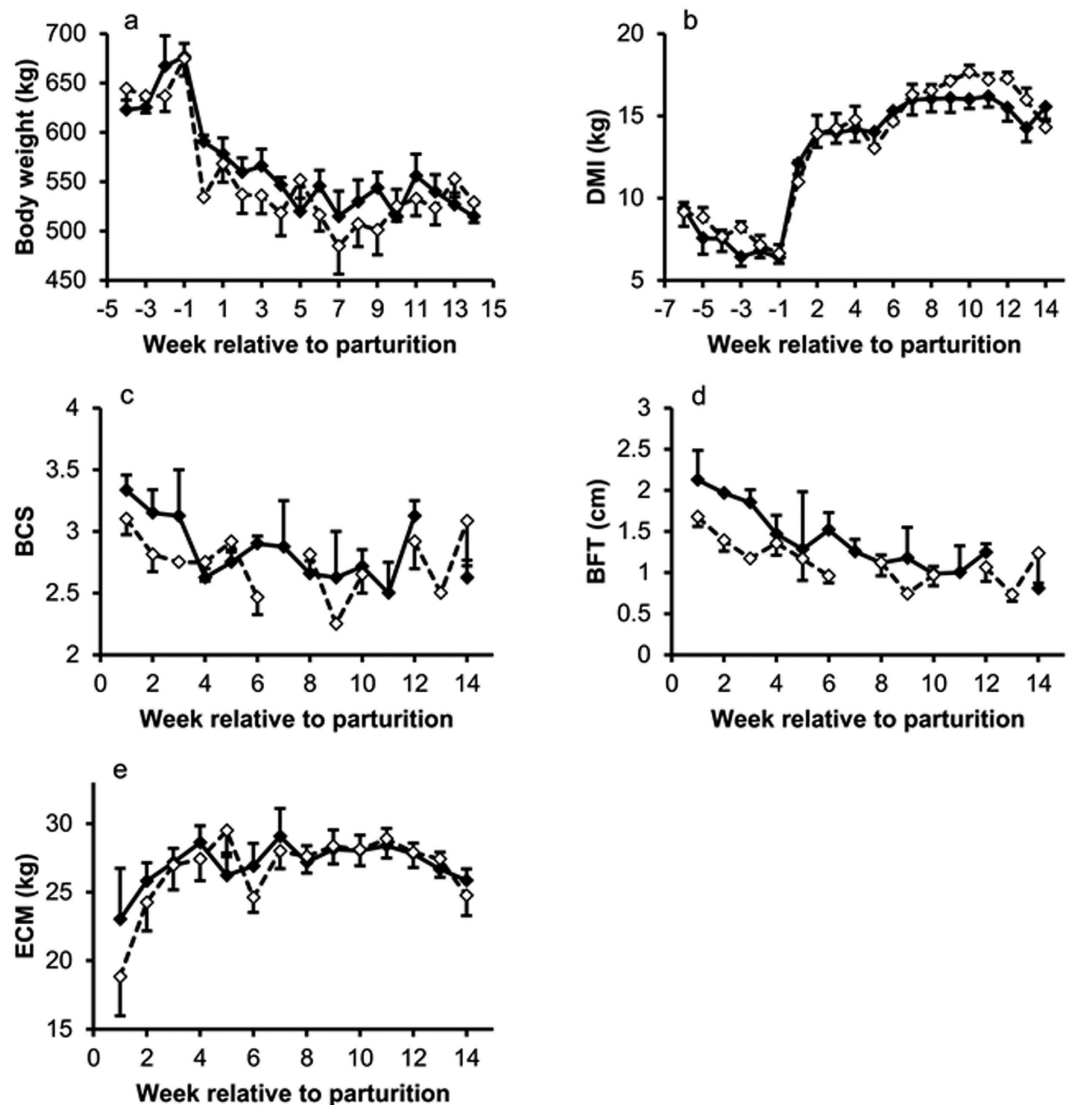


Figure 1. Body weight, DMI, BCS, BFT and ECM of high (\blacklozenge , solid line; $n = 10$) and low mobilizing (\diamond , dashed line; $n = 10$) cows during the period of first lactation. ANOVA calculated time \times group interactions were: (a) $P = 0.18$, (b) $P = 0.04$, (c) $P = 0.60$, (d) $P = 0.02$, (e) $P = 0.15$. Data are shown as LSM \pm SE.

(Fig. 6c,e). These regressions were not evident in week -4 , $+13$ and $+42$ relative to parturition (data not shown). Furthermore, we were not able to detect any significant relationship between CH_4 production and ruminal acetate concentrations (Supplemental Fig. 2).

The best fitted curve for a relationship between CCK and CH_4 , CH_4/DMI or CH_4/NDF was a two parametrical exponential function, but the coefficients of determination only reached $R^2 = 0.06$, $R^2 = 0.23$ and $R^2 = 0.17$, respectively (Fig. 6b,d,f).

Discussion

We hypothesized that increased body fat mobilization in early lactation associated with reduced acetate utilization of the host would negatively affect ruminal acetate concentration and CH_4 production. To examine this hypothesis we retrospectively grouped the cows according to their plasma NEFA concentrations in early lactation (i.e. week 5) to high and low mobilizing cows and determined CH_4 production at different physiological stages throughout the lactation cycle. Both HM and LM cows had a continuous increase in daily CH_4 production from one month before parturition to lactation week 42. This course does not parallel the biphasic course of CH_4 production predicted by mid-infrared spectra validated by the SF_6 technique¹⁰. A decrease in CH_4 production in late lactation however, may be initiated by a diet change often applied during that time, while our cows were kept on the same diet throughout lactation. Alterations in CH_4 emission during the lactation cycle can be due to the increasing rumen capacity during early lactation, accompanied by a passage rate that is decoupled from DMI, as well as changes in the digestibility of feed¹¹. In agreement with their greater plasma NEFA concentrations, HM cows had the greater loss of back fat thickness during the first five weeks of lactation. Recent studies showed that elevated plasma NEFA concentrations around parturition are utilized for about 40% of milk fat synthesis during

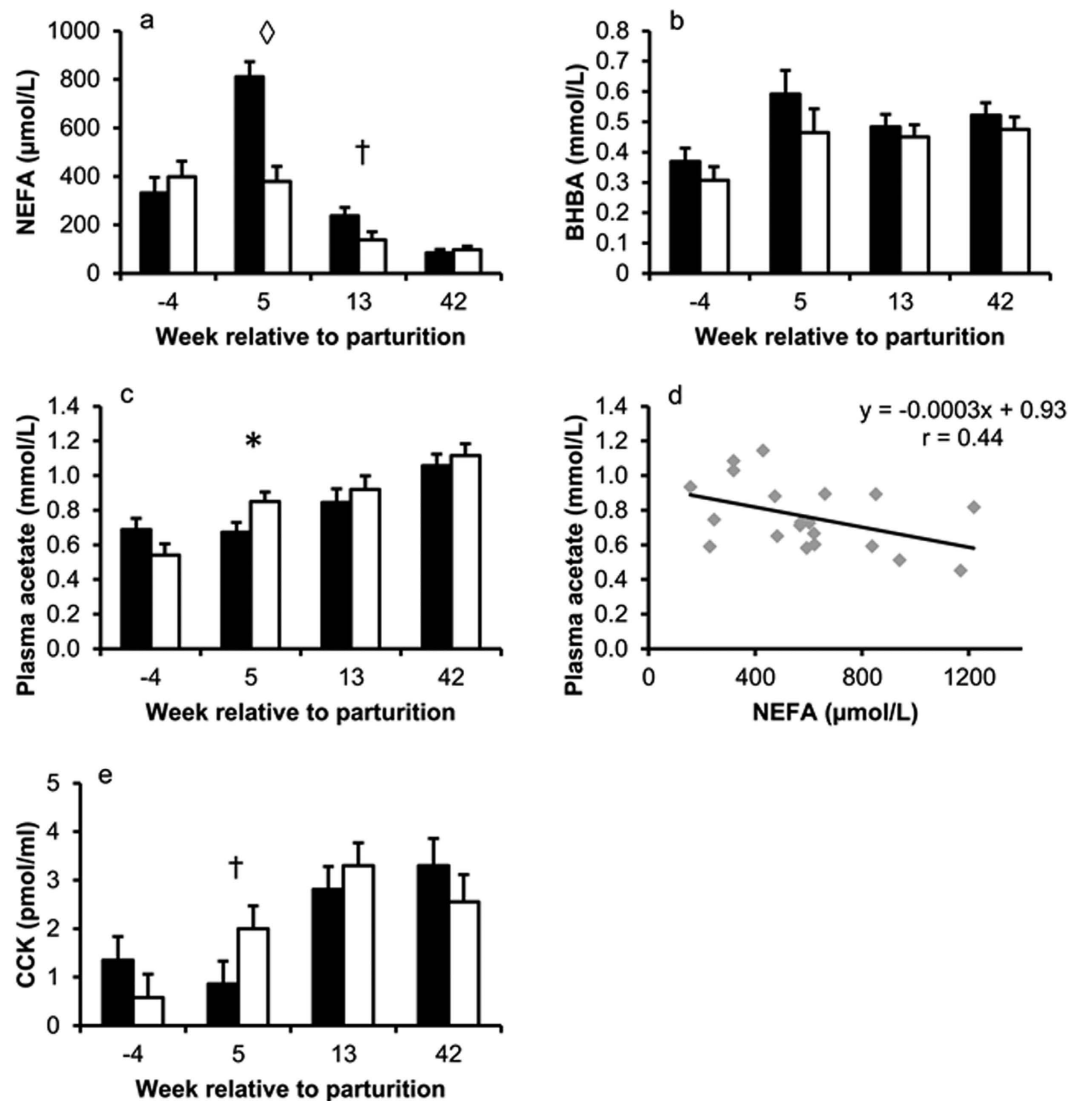


Figure 2. Plasma NEFA, BHBA and acetate concentrations at different time points, and regression between plasma acetate and plasma NEFA concentrations in week 5 p.p. Cows grouped as high mobilizing are marked ■ (n = 10), cows grouped as low mobilizing are marked □ (n = 10). (a) ◇ Indicates the time point of grouping the animals. (b) Time $P=0.01$, group $P=0.18$, time \times group $P=0.87$, ANOVA. (c) Time $P<0.001$, group $P=0.32$, time \times group $P=0.13$, ANOVA; * indicates $P=0.04$, Tukey-Test. (d) Slope $P=0.053$, $n=20$. (e) Time $P=0.003$, group $P=0.95$, time \times group $P=0.12$, ANOVA; † indicates $P=0.1$. Data in bar charts are shown as LSM \pm SE.

early lactation^{12,13}. Other authors discovered that cows with greater fat mobilization produce higher milk fat contents and thus a higher ECM in early lactation¹⁴. In our study, ECM and milk fat content did not differ between groups in week 5 p.p. Reasons for this may be the difference in the dynamic of fat mobilization with LM cows showing an early but short and less intensive peak NEFA concentration while HM cows had slowly increasing but longer-lasting fat mobilization (Supplemental Fig. 1). Another reason may be because the extent of fat mobilization reflected by plasma NEFA concentrations is much lower in first lactating cows as compared to cows with more parities. It is well established that increased utilization of NEFA for milk fat synthesis accounts not only for an increased total milk fat content but also for a higher proportion of long-chain fatty acids on the expense of short- and medium-chain fatty acids *de novo* synthesized from acetate or butyrate¹³. Hence, the mammary gland utilizes less acetate and as a consequence one might expect rising plasma acetate concentrations. Instead we observed lower plasma acetate concentrations in HM cows in week 5 p.p. which argues against our hypothesis proposed above - higher plasma NEFA concentrations would negatively affect metabolic acetate utilization - and led us to waive milk fatty acid profile analysis.

The lower plasma acetate concentrations in HM cows in week 5 p.p., but not at the other time points investigated, suggest that HM and LM cows differ in their acetate oxidation, acetate absorption or ruminal acetate production rates, and that these differences are specific for the early lactation period only. Acetate originates from carbohydrate fermentation and thus COX can be seen as an estimate for acetate oxidation. We found no

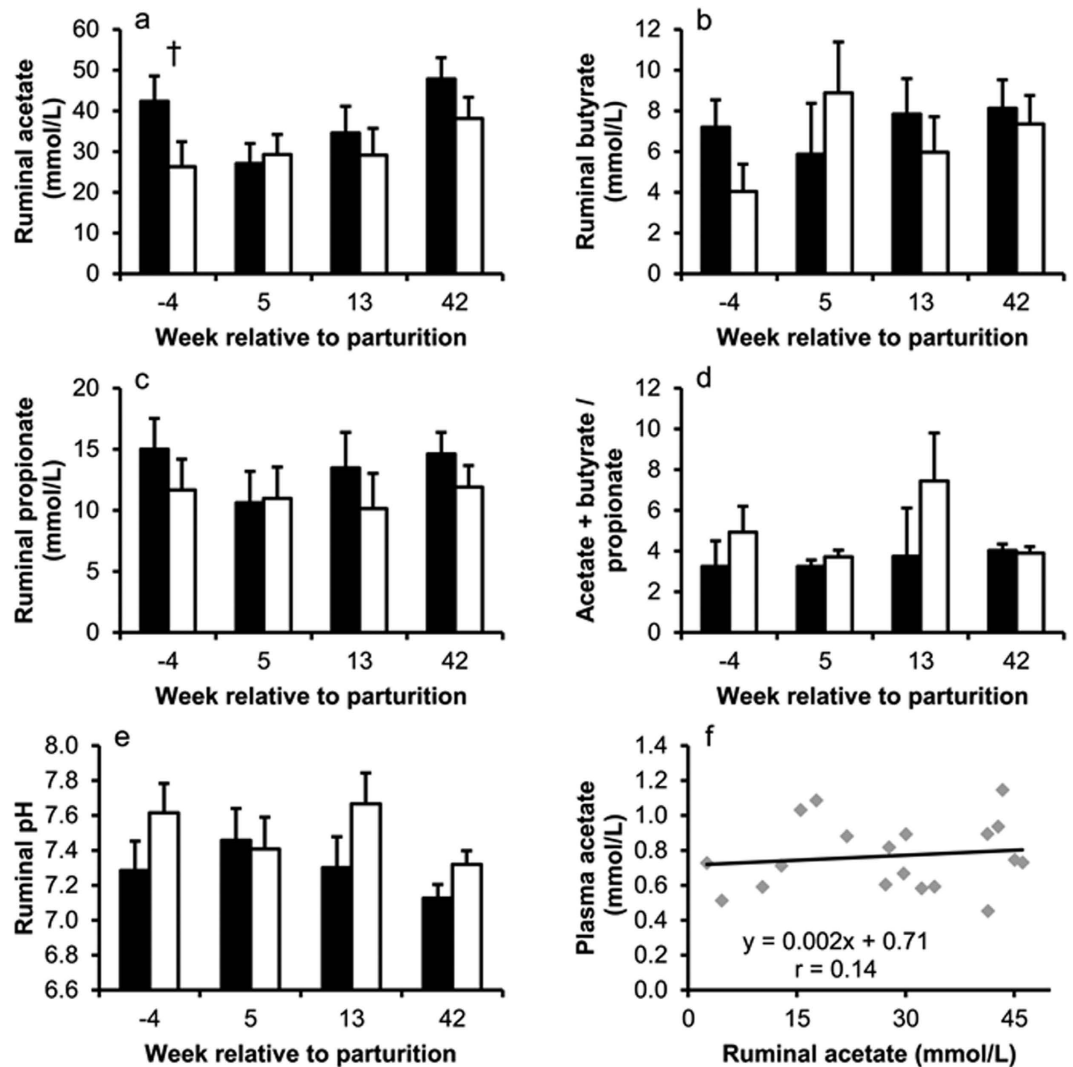


Figure 3. Ruminal variables as group means at the times of respiration chamber measurements in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p. and regression between plasma and ruminal acetate concentrations measured in week 5 p.p. (a) Time $P = 0.045$, group $P = 0.045$, time \times group $P = 0.44$, ANOVA; † indicates $P = 0.09$; Tukey-Test. (b) Time $P = 0.71$, group $P = 0.55$, time \times group $P = 0.61$, ANOVA. (c) Time $P = 0.61$, group $P = 0.25$, time \times group $P = 0.81$, ANOVA. (d) Time $P = 0.17$, group $P = 0.11$, time \times group $P = 0.48$, ANOVA. (e) Time $P = 0.16$, group $P = 0.12$, time \times group $P = 0.57$, ANOVA. (f) Slope $P = 0.57$. Data in bar charts are shown as LSM \pm SE.

differences in COX between groups in week 5 p.p., suggesting that metabolic acetate oxidation should be similar between HM and LM cows at this time. In addition, comparable COX between groups in week 5 p.p. also reflects the same level of DMI of HM and LM cows.

The absorption of acetate through the ruminal epithelium into the blood stream follows partly mass action diffusion and partly active protein mediated transporters^{15,16}. The former depends largely on ruminal pH, capillary blood flow to the epithelium and the size of the epithelial surface area. Equally sized and aged cows fed the same diet are also expected to have a comparable rumen size and epithelial surface area. Also, the comparable rumen pH of HM and LM cows argues against a different acetate diffusion rate. Therefore, different active acetate transport rates in the two cow groups influenced by increased circulating NEFA concentrations are a credible feasibility, but this assumption remains to be investigated. The fact that we did not observe a significant relationship between acetate concentrations in plasma and in ruminal fluid enforces the idea that diminished active transport of acetate across the rumen wall contributes to reduced plasma acetate concentration in HM cows during early lactation. This assumption is supported by Dijkstra *et al.*¹⁷, who reported that ruminal concentrations of short chain fatty acids do not reflect their production rates. Other factors such as absorption rates, ruminal pH and the microbial populations account for this discrepancy¹⁷. Ultimately, we can only speculate whether the ruminal acetate production rate or the microbial composition differed temporally between HM and LM cows in week 5 p.p.

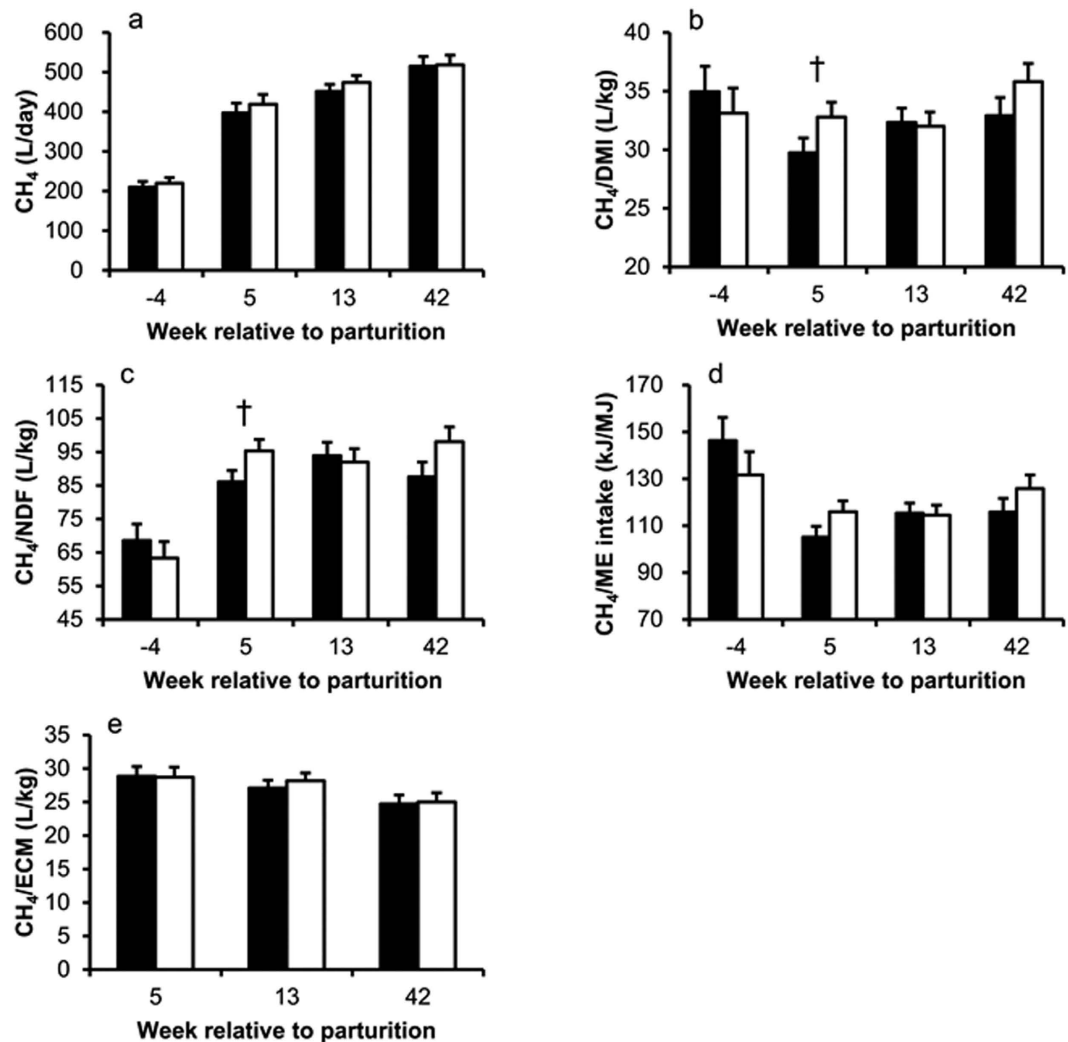


Figure 4. Daily CH₄ production, CH₄ per dry matter intake (CH₄/DMI), CH₄ per neutral detergent fiber (CH₄/NDF), CH₄ per metabolizable energy (ME) intake (CH₄/ME intake), and CH₄ per energy corrected milk yield (CH₄/ECM) in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p. (a) Time $P < 0.001$, group $P = 0.51$, time \times group $P = 0.91$, ANOVA. (b) Time $P = 0.17$, group $P = 0.54$, time \times group $P = 0.14$, ANOVA; † indicates $P = 0.1$, Tukey-Test. (c) Time $P < 0.001$, group $P = 0.66$, time \times group $P = 0.91$, ANOVA; † indicates $P = 0.08$, Tukey-Test. (d) Time $P < 0.001$, group $P = 0.44$, time \times group $P = 0.09$, ANOVA. (e) Time $P < 0.023$, group $P = 0.82$, time \times group $P = 0.14$, ANOVA. Data in bar charts are shown as LSM \pm SE.

Interestingly we did not find a relationship between the ruminal acetate concentration and CH₄ emission in the present study. This is in accordance with findings in sheep¹⁸, although other authors described a positive relationship between CH₄ yield and ruminal acetate concentration or CH₄ yield and acetate : propionate ratio^{19–21}. However, the latter studies involved different diets that were fed to the animals, whereas all cows in the present study received the same diet at each time point investigated. Thus, a diet effect accounting for the rumen acetate to CH₄ yield relationship described^{19–21} cannot be excluded.

As our initial hypothesis - plasma NEFA concentrations would negatively affect metabolic acetate utilization - could not be confirmed, we examined whether the extent of fat mobilisation is associative with different passage rates accounting for the different CH₄ yield between in HM and LM cows. Increase in DMI usually increases the passage rate of the digesta through the gastro-intestinal tract, or vice versa decreases MRT, which in turn reduces feed digestibility and CH₄ emission per unit feed ingested^{22–24}. Conversely, CH₄ within the gut has been shown to slow digesta transit and influence gut motility in dogs, guinea pigs and humans²⁵. We found comparable MRT in the rumen-intestinal tract and feed digestibility in LM and HM cows, which is in line with the comparable DMI of both groups. The small differences in CH₄ yield between cow groups in early lactation can therefore not be explained by MRT measured over the whole rumen-intestinal tract. Different studies have shown that the passage rate through the entire gastro-intestinal tract is not proportional to the reticulorumen passage of indigestible NDF in Holstein cows fed a corn silage based diet^{26,27}. The precise measurement of the reticulorumen passage rate, however, requires the use of cannulated animals, which could not be realized in the present study. Goopy *et al.*²⁸ described that sheep with a lower MRT of particulate and liquid matter in the rumen produced less CH₄ despite

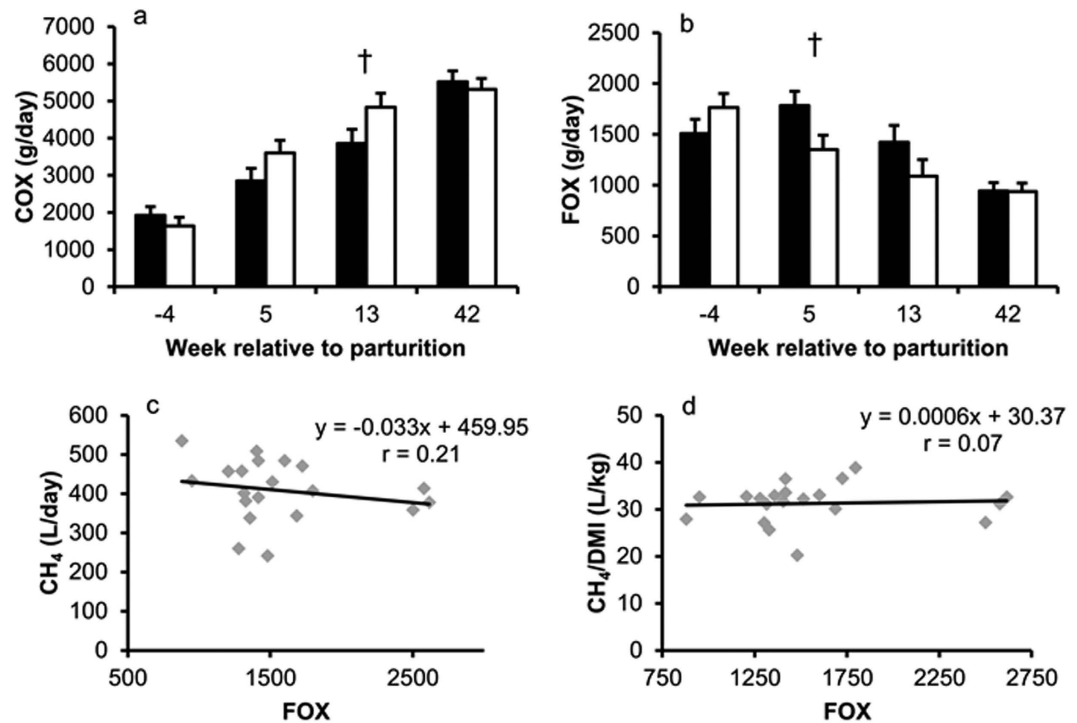


Figure 5. Carbohydrate oxidation (COX) and fat oxidation (FOX) at different time points in cows with high (■; n = 10) and low (□; n = 10) plasma NEFA concentrations in week 5 p.p.; and linear regression of FOX and CH₄ yield (n = 20). (a) Time $P < 0.001$, group $P = 0.24$, time \times group $P = 0.08$, ANOVA; † indicates $P = 0.09$; Tukey-Test. (b) Time $P < 0.001$, group $P = 0.14$, time \times group $P = 0.22$, ANOVA; † indicates $P = 0.06$; Tukey-Test. (c) Slope $P = 0.37$. (d) Slope $P = 0.78$. Data in bar charts are shown as LSM \pm SE.

the fact that their DMI was comparable with sheep possessing a higher ruminal MRT and CH₄ yield. Sheep with lower CH₄ yield had smaller rumen volumes²⁸, however, general differences in the rumen size of HM and LM cows are unlikely because differences in CH₄ yield between the two groups occurred only in week 5 p.p. and did not persist throughout whole lactation.

It might be that differences in CH₄ yield between HM and LM cows in week 5 p.p. are owed to temporary differences in the MRT of the reticulorumen only, and not of the gastrointestinal tract as a whole, although we were not able to measure the reticulorumen-specific MRT in the present study. Dias *et al.*²⁹ and Oshita *et al.*³⁰ have shown that the fractional outflow rate of particulate matter from the reticulorumen was positively correlated with total chews, and that the decrease in particle size caused by chewing facilitates particle flow through the digestive tract. We did not evaluate chewing behavior of the animals, but because CH₄ yield tended to differ only temporarily between HM and LM cows and both groups received the same diet, it seems unlikely that differences in chewing account for different MRT of the reticulorumen and therefore the statistical trend observed for CH₄ yield in week 5 pp.

Possible causes for the approximately 10% different CH₄ yield in HM and LM cows during early lactation may be temporary differences at the systemic side of the rumen-intestinal tract, e.g. differences in fat metabolism of the cows which affect the MRT of the reticulorumen and thus digestive processes. Several previous studies have shown that hormones related to fat metabolism and fat accretion can directly or indirectly influence gut motility. For example ghrelin and motilin stimulate gastric motility, accelerate gastric emptying and small intestinal transit time^{31,32}. In an earlier study we have shown that HM cows have greater preprandial ghrelin concentrations than LM cows, and that this difference was particularly prominent during early lactation and less in late pregnancy¹⁴. This data indicates that HM cows have a higher motility at the proximal rumen-intestinal tract, conclusively reduced feed retention time in the rumen and therefore tend to have less CH₄ yield in week 5 p.p. Moreover, 3rd to 5th lactating HM cows investigated in the earlier study¹⁴ had significantly less CH₄ yield as compared to their LM counterparts in week 2 p.p. but not in week 6 a.p. (data not published yet) supporting the findings of the present study. However, whether administration of ghrelin directly affects CH₄ emission remains to be examined in future studies. In contrast to ghrelin, intravenous infusion of cholecystokinin has been shown to depress frequency of reticular contractions and rumen motility^{33,34}. Thus, lower CCK plasma concentrations in HM compared to LM cows in week 5 p.p. further underscores greater rumen motility which should promote an acceleration of ruminal passage rate in cows with greater fat mobilisation in early lactation.

Leptin, a hormone secreted by adipose tissue, interacts with the vagus nerve and the release of CCK and has a complex effect on motility of the gastrointestinal tract, e.g. by delaying gastric emptying and transit time of the ingesta through the small intestine³¹. It has been described that LM cows have lower plasma leptin concentrations than HM cows, but only before and not after parturition³⁵. Thus, different CH₄ yields observed as a trend between groups in week 5 p.p. can therefore not be explained by different plasma leptin concentrations.

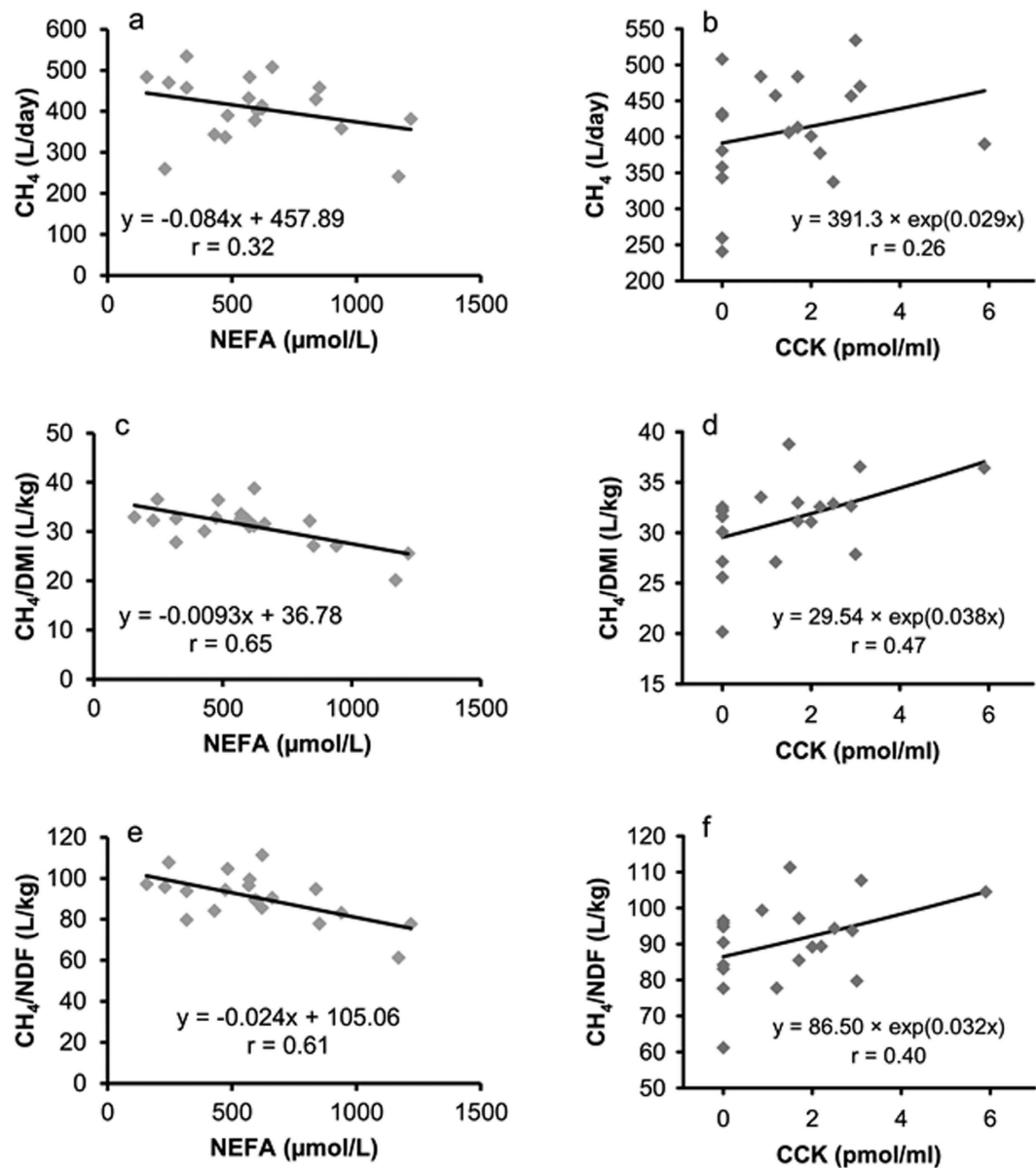


Figure 6. Linear regression between plasma NEFA concentration and daily CH₄ production either expressed as L/d, L/kg DMI, or L/kg NDF, respectively and 2 parametrical exponential regression ($y = a \times e^{(bx)}$) between plasma CCK concentrations and CH₄ production (n = 20). (a) Slope $P = 0.17$. (b) $P < 0.0001$ for a; $P = 0.28$ for b. (c) Slope $P = 0.002$. (d) $P < 0.0001$ for a; $P = 0.03$ for b. (e) Slope $P = 0.005$. (f) $P < 0.0001$ for a; $P = 0.07$ for b.

Intravenous infusion of acetate did not modify gastric motility³⁶ and conclusively it seems unlikely that differences in plasma acetate concentrations of HM and LM cows p.p. would affect motility of the gastrointestinal tract. The effect of circulating long-chain fatty acids on rumen or gut motility is not known, however, enterocytes are able to oxidize long-chain fatty acids to activate enteric neurons signaling to reduce feed intake³⁷. Greater plasma NEFA concentrations and FOX rates in HM cows during early lactation argue against this mechanism because DMI is comparable to LM cows. This opens the question whether circulating NEFA may influence the motility of the gastrointestinal tract and consequently CH₄ yield.

A further question to be answered is concerning the fate of the hydrogen in HM cows producing less CH₄ in week 5 p.p. Possibly, hydrogen is increasingly transferred towards propionate although ruminal propionate concentrations were not different between groups. But keeping in mind that the total amount of propionate produced in the rumen exceeds the total amount of CH₄ by far, conclusions on the hydrogen transfer towards propionate are difficult to draw. It is also conceivable that in HM cows less hydrogen is transferred to the butyrate producing pathway as indicated by the numerically lower ruminal butyrate concentrations in HM cows in week 5 p.p. and that this hydrogen is released via eructation.

Component (g/kg DM)	Far-off	Close-up	Lactation		
Grass silage	793	160	181		
Corn silage		432	325		
Grass hay	66	102	32		
Barley straw	133	63	38		
Corn kernels		45	70		
Grain mix			39		
Extracted soy meal		54	21		
Extracted canola seed meal		68	38		
Feed lime			4		
MF 2000 (concentrate) ¹		65	220		
Mineral 9522 ²			9		
Prenatal TR40 ³	11	11			
Chemical analysis		4 weeks a.p.	5 weeks p.p.	13 weeks p.p.	42 weeks p.p.
Crude ash	(g/kg DM)	86	69	68	73
Crude protein	(g/kg DM)	177	162	157	156
Crude fiber	(g/kg DM)	231	167	171	166
Crude fat	(g/kg DM)	21	31	30	32
Sugar	(g/kg DM)	10	26	29	21
Starch	(g/kg DM)	100	255	252	254
NDF	(g/kg DM)	501	345	347	371
ADF	(g/kg DM)	296	208	211	210
N free extracts	(g/kg DM)	407	494	494	494
ME	(MJ/kg DM)	9.6	11.3	11.2	11.3
NE _L	(MJ/kg DM)	5.7	7.0	6.9	7.1

Table 1. Diet components and analysis of the total mixed ration (TMR) ingested during stays in respiration chamber. ¹MF 2000 (Vollkraft Mischfutterwerke GmbH, Güstrow, Germany): 7.1 MJ NE_L/kg, 24% crude protein, 3.3% crude fat, 6.2% crude fiber, 8.4% crude ash, 0.7% calcium, 0.5% phosphorus, 0.65% sodium, vitamins A, D₃, E, calcium iodate, cobalt carbonate, manganese oxide, zinc oxide, sodium selenite. ²Rinderstolz 9522 (Salvana GmbH, Sparrieshoop, Germany): 92% crude ash, 20% calcium, 5% phosphorus, 8% sodium, 6% magnesium, vitamins A, D₃, E, copper sulfate, zinc oxide, manganese oxide, calcium iodate, sodium selenite, cobalt carbonate. ³Prenatal TR 40 (Salvana GmbH, Sparrieshoop, Germany): 75% crude ash, 4% calcium, 6% phosphorus, 15% magnesium, 8% sodium, vitamins A, D₃, E, zinc oxide, manganese oxide, copper sulfate, calcium iodate, sodium selenite, cobalt carbonate.

Materials and Methods

Animals, feeding and milking. Twenty pregnant German Holstein heifers were kept in a free stall at the Leibniz-Institute for Farm Animal Biology (FBN), Dummerstorf, Germany and monitored until 291 DIM (SE ± 1.5) of their first lactation. 6 weeks prior to their expected calving date heifers were transferred to the straw bedded calving box and returned to the free stall on the first day after calving. All animals were treated in accordance with the guidelines for the use of animals as experimental subjects of the State Government in Mecklenburg-Western Pomerania. All experimental protocols were approved by the local animal ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; approval No. 7221.1-1.-053/13).

Heifers had free access to water and the diet was offered as a standard ruminant total mixed ration (TMR) for ad libitum intake. 21 days before expected calving date animals were switched from the far-off to the close-up diet, and after parturition they received the lactation diet (Table 1). Individual daily feed intake was recorded as disappearance of feed from troughs connected to an electronic scale to which access was controlled by individual transponder (Roughage Intake Control, Insentec, Marknesse, The Netherlands). Based on the analysis of the individual TMR ration components, diet compositions for the far-off, close-up and lactation period were formulated and calculated according to the feeding standards of the German Society of Nutrition Physiology (GfE)³⁸. An additional TMR sample was taken at the time of respiration chamber measurements (see below) for the determination of dry matter (DM) and diet composition. Analyses were conducted by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUF) in Rostock, Germany. Chemical composition of TMR samples are shown in Table 1.

Feed energy was calculated according to Boguhn *et al.*³⁹ and GfE³⁸:

$$GE \text{ (MJ/kg DM)} = 0.0239 XP + 0.0398 XL + 0.0201 XF + 0.0175 XX \quad (1)$$

$$ME \text{ (MJ/kg DM)} = 6.0756 + 0.19123 XL + 0.02459 XP - 0.000038 XF^2 - 0.002139 XL^2 - 0.000060 XP^2 \quad (2)$$

$$NE_L (\text{MJ/kg DM}) = 0.6 [1 + 0.004 (q - 57)] \text{ ME}, \quad (3)$$

where GE is gross energy, ME is metabolizable energy, NE_L is net energy lactation, XP is crude protein, XL is crude fat, XF is crude fibre, XX is N free extracts and $q = \text{ME}/\text{GE} \times 100$.

Body weight of the animals was measured automatically after milking and recorded as weekly means, and immediately before each indirect calorimetric measurement.

BFT measurements were conducted ultrasonographically (Titan Ultrasound System, SonoSite Inc., USA) from 1 until 14 weeks after calving at 14-day intervals. Additionally, BCS was determined at the same day on a 5 point scale according to Schroder and Staufienbiel⁴⁰. Animals were milked twice daily at 04:30 h and 16:30 h and milk yield was recorded automatically. Milk samples from evening and morning milking were pooled and analyzed weekly by the Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern e.V. for milk composition. To calculate the ECM the following formula according to Reist *et al.*⁴¹ was used:

$$\text{ECM (kg)} = [0.038 \times \text{crude fat (g)} + 0.024 \times \text{crude protein (g)} + 0.017 \times \text{lactose (g)}] \times \text{milk (kg)}/3.14. \quad (4)$$

Indirect Calorimetry. For CH_4 measurement animals were transferred into open-circuit respiration chambers⁴² in week 4 a.p. and in week 5, 13 and 42 p.p. ($\text{SE} \pm 0.2$ weeks). Animals were halter-trained and well adapted before measurements in the chamber, meaning habituation at least three times until the animal appeared relaxed and displayed regular behavior such as eating, ruminating and lying down. Within the chambers animals were kept in tie-stall at 15 °C, a dark–light cycle from 06:00 h to 19:00 h, milked twice daily if lactating and had access to fresh water. After an overnight stay allowing gas exchange equilibration, measurement of gas concentrations started at 07:00 h and lasted for 24 h. The CH_4 and CO_2 concentrations in the chamber were analyzed by infrared absorption and the O_2 concentration was measured paramagnetically (SIDOR, SICK MAIHAK GmbH, Reute, Germany) as described recently by Derno *et al.*⁴². Air flow through the chambers was recorded with a differential-pressure type V-cone flow meter (McCrometer, Hemet, CA, USA). Fermentative CO_2 ($C_{\text{CO}_2 \text{ ferm.}}$) production was estimated from CH_4 production according to Chwalibog *et al.*⁴³:

$$V_{\text{CO}_2 \text{ ferm.}} (\text{L}) = 1.7 \times V_{\text{CH}_4} (\text{L}) \quad (5)$$

in which the factor 1.7 is constant for a variety of diet compositions⁴⁴.

Metabolic CO_2 ($\text{CO}_2 \text{ metab.}$) was calculated as difference between total and fermentative CO_2 production:

$$V_{\text{CO}_2 \text{ metab.}} = V_{\text{CO}_2} - V_{\text{CO}_2 \text{ ferm.}} \quad (6)$$

COX and FOX were calculated as described by Derno *et al.*⁴²:

$$\text{COX (g)} = (4.75 V_{\text{CO}_2 \text{ metab.}} (\text{L}) - 3.23 V_{\text{O}_2} (\text{L}) - 2.60 N_{\text{U}} (\text{g})), \quad (7)$$

$$\text{FOX (g)} = (1.69 V_{\text{O}_2} (\text{L}) - 1.69 V_{\text{CO}_2 \text{ metab.}} (\text{L}) - 2.03 N_{\text{U}} (\text{g})), \quad (8)$$

where N_{U} is urine N excretion. N_{U} was not measured and set to zero accepting an error of about 10%⁴³ for both COX and FOX.

Feed was given twice at 07:30 h and 15:00 h. Feed intake in the chamber was determined by feed disappearance measured by using a scale connected to an electronic registration device. Data was collected every 6 min for 24 consecutive hours. Animals were milked in the chamber at 07:00 h and 16:30 h and the milk yield was recorded.

Blood sampling. Animals were blood sampled at 07:00 h in the morning immediately before transferring into the respiration chambers by puncture of the Vena jugularis externa using BD Vacutainers containing potassium ethylenediaminetetraacetate (Greiner bio-one, Frickenhausen, Germany). Additional blood samples were taken weekly from week –3 to +12 relative to parturition (Supplemental Figure S1). Immediately after collection the vials were processed in a centrifuge at 4 °C for 20 minutes at 1,300 × g. Plasma was harvested and stored at –20 °C until analysis. Plasma concentrations of NEFA and BHBA were analyzed photometrically (Abx Pentra 400, Horiba ABX SAS, Montpellier, France) using kit no. 436-91995 for NEFA (Wako Chemicals GmbH, Neuss, Germany) and kit RB 1008 (Labor und Technik, Berlin, Germany) for BHBA. Plasma acetate concentrations were determined as chloroethyl ester derivative on a gas chromatography–flame ionization detector instrument (GC-FID, Series 2010, Shimadzu Corp., Kyoto, Japan) on a 25 m RTX-1701 column according to Kristensen *et al.*⁴⁵. CCK was measured using a double antibody radioimmuno assay (Wizard 1470 Automatic Gamma Counter, Perkin Elmer, Waltham, USA) according to Relling and Reynolds⁴⁶.

Ruminal fluid. Ruminal fluid was collected immediately before animals were transferred into respiration chambers using an esophageal tube system attached to a vacuum pump. Rumen fluid was sieved (mesh size 0.7–1.0 mm) and pH was determined using a glass electrode (Roth, Karlsruhe, Germany). The filtrate was centrifuged at 4 °C for 10 min at 4,000 g. Rumen fluid short-chain fatty acid concentrations were measured in the supernatant using a gas chromatograph (GC-FID, Series 17A, Shimadzu Corp., Kyoto, Japan), equipped with a 25 m FFAP column according to Ryan⁴⁷.

Gastrointestinal passage rate and digestibility. Analysis of gastrointestinal passage rate was performed in week 6 p.p. Cows were fed 15 g titanium dioxide (TiO₂; pelleted with corn meal in a ratio of 1:2) twice daily for five days. Starting four days after begin of TiO₂ application, feces samples (approx. 600 g) were taken twice daily at 08:30 h and 16:00 h for five days. Daily samples were pooled and stored at -20 °C until drying and analysis. Feces DM was determined after drying the samples at 65 °C for 72 h. TiO₂ was analyzed according to the method described by Brandt and Allam⁴⁸. For the calculation of the MRT of digesta we used the formula described by Voigt *et al.*⁴⁹:

$$MRT = \frac{\sum_{i=1}^n (t_i x m_i)}{\sum_{i=1}^n m_i} \quad (9)$$

where m_i is the amount of TiO₂ excreted at the i th sample and t_i is the time elapsed between dosing and the mid-point of the i th collection interval.

The Kjeldahl procedure was used to determine fecal N content with small modifications⁵⁰. Fecal CP was calculated on an organic matter (OM) basis:

$$CP \text{ (g/kg OM)} = N \text{ (g/kg OM)} \times 6.25. \quad (10)$$

The OM digestibility of the ration was estimated using the formula of Lukas *et al.*⁵¹:

$$OM \text{ digestibility, \%} = 79.76 - 107.7^{(-0.01515 * \text{fecal CP (g/kg OM)})}. \quad (11)$$

Data handling and Statistics. Animals were grouped according to their plasma NEFA concentrations at the time of the respiration chamber measurements in early lactation (week 5 ± 0.2 p.p.) to the ten highest mobilizing (HM; plasma NEFA > 580 μmol/L) and the ten lowest mobilizing (LM; plasma NEFA < 580 μmol/L) cows. Weekly means of BFT and BCS were calculated from two consecutive measurements performed at 14 day intervals.

The data analysis was generated using SAS software, Version 9.3 for Windows, SAS Institute Inc., Cary, NC, USA. Variables BCS, BFT, DMI, CH₄, milk, rumen fluid and plasma parameters were analyzed by repeated measurement ANOVA with the MIXED procedure of SAS/STAT software. The ANOVA models contained the fixed factors group (levels: HM, LM), time and the interaction group × time. The levels of the repeated variable time for milk data are weeks 5 p.p., 13 p.p., 42 p.p. and, for all other variables weeks 4 a.p., 5 p.p., 13 p.p., 42 p.p. Additionally, the levels of the repeated variable time for the variable DMI are weekly from 6 weeks a.p. to 14 weeks p.p., for the variable BW weekly from 4 weeks a.p. to 14 weeks p.p., and for the variables BCS, BFT, ECM are weekly from 1 week to 14 weeks p.p. Repeated measures on the same animal were taken into account by the REPEATED statement of the MIXED procedure and the type for the block diagonal residual covariance matrix was unstructured for the calculations with three or four time points and compound symmetry for the weekly calculations. Least-squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the models, and all pairwise differences of LS-means were tested by the Tukey-Kramer procedure. The SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LS-means for the interaction group × time. The MIXED procedure was also used to test the variable area under the curve (AUC) for the fixed factor group.

Linear relationships between variables NEFA, ruminal acetate and plasma acetate, CH₄, CH₄/DMI and CH₄/NDF in week 5 p.p. were estimated and tested with the REG procedure of SAS/STAT software. Exponential relationships between variables CCK and CH₄, CH₄/DMI, CH₄/NDF in week 5 p.p. were estimated and tested with the NLIN procedure of SAS/STAT software using the model formula $y = a + b^{(cx)}$. Effects and differences were declared significant if $P < 0.05$ and trends as $P < 0.1$.

Conclusions

We investigated the individual CH₄ production of first lactating dairy cows to characterize the impact of body fat mobilization during the exceptional metabolic state in early lactation on ruminal fermentation characteristics. While ruminal acetate concentrations proved to be unrelated to levels of CH₄ production, cows with high body fat mobilization and low plasma acetate concentrations tended to have lower CH₄/DMI and CH₄/NDF production rates in early lactation than less mobilizing cows. Lower plasma CCK concentrations in early lactation accounts for increased rumen motility and a faster digesta passage through the rumen (without affecting whole rumen-intestinal MRT) and therefore the lower CH₄ yield of high mobilizing cows. The direct relationship between plasma CCK concentrations and CH₄ yield as well as the inverse relationship between plasma NEFA concentrations and CH₄ yield offers a new perspective on the interaction between host metabolism and rumen fermentation.

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Author Contributions

A.B. and B.K. wrote the main manuscript text. Figures and tables were created by A.B., H.M.H. and B.K. designed the study and performed plasma and rumen fluid analyses. M.D. performed the methane measurements, A.S. analyzed the passage rate and digestibility, and A.T. performed the statistical calculations.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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