Intravenous lipid infusion affects dry matter intake, methane yield, and rumen bacteria structure in late-lactating Holstein cows

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ABSTRACT

Increasing the dietary fat content of ruminant diets decreases methane (CH₄) production. This effect is caused by the toxic properties of fatty acids on rumen microbial populations, coating of feed particles diminishing the accessibility for microbes, and a reduction in dry matter intake (DMI). The latter effect is caused by postabsorptive long-chain fatty acids eliciting anorexic signaling; however, whether circulating long-chain fatty acids affect rumen CH₄ production alike is unknown. To approach this question, 5 rumen-cannulated Holstein cows in late lactation received 2 jugular catheters and were kept in respiration chambers to measure CH₄ production and DMI for 48 h. In a crossover design, cows were intravenously infused with a 20% lipid emulsion (LIPO) or 0.9% NaCl (CON). The LIPO cows received 2.1 kg of triglycerides/d [0.152 ± 0.007 g of triglycerides/(kg of BW $\times$ h)⁻¹] consisting of 12.1% palmitic acid, 4.2% stearic acid, 31.1% oleic acid, and 52.7% linoleic acid. Blood and rumen fluid samples were taken hourly during the day. Results showed that LIPO compared with CON infusion increased plasma triglyceride as well as free fatty acid and serotonin concentrations but reduced the proportion of de novo synthesized milk fatty acids (sum of C6 to C16). Daily CH₄ production and DMI were lower, whereas daily CH₄ yield (CH₄/DMI) was greater in LIPO than CON cows, although CH₄ yield decreased from d 1 to d 2 by 2 to 14% in LIPO-infused cows only. This effect was as-

sociated with a higher (acetate + butyrate)/propionate ratio, tending lower propionate concentrations between 24 and 34 h of infusion, reduced relative abundances of genera belonging to *Succinivibrio*, *Ruminococcaceae*, and *Ruminiclostridium*, and greater relative *Bacteroidetes* genus abundances in the rumen.

Key words: lipid infusion, milk fat synthesis, methane, microbiome, dairy cow

INTRODUCTION

Daily methane (CH₄) production from lactating dairy cows was shown to be directly related to the concentration of de novo-synthesized milk fatty acids (individual or the sum of C8:0, C10:0, C12:0, C14:0, and C16:0) but negatively related to C18 milk fatty acids (individual or the sum of C18, the sum of C18:1 *trans*, or the sum of C18:1 *cis*; Chilliard et al., 2009). Dietary supplementation with oilseeds rich in C18 UFA such as different linseed products, cottonseeds, or canola oilseeds increased total C18 but reduced C6 to C16 fatty acid contents in milk while diminishing enteric CH₄ production (Johnson et al., 2002; Martin et al., 2008; Chilliard et al., 2009). The reduction of enteric CH₄ production in response to dietary oilseeds is due to decreased accessibility of feed particles for microbes, direct toxic effects on microbial populations primarily on protozoa, and a reduction in DMI (Machmüller and Kreuzer, 1999; Chilliard et al., 2009). Apart from the dietary effects on microbes, circulating long-chain fatty acids may disturb the intestinal barrier integrity and affect bacterial composition in the lumen (Hodin et al., 2012; Harris et al., 2014; Lavalley et al., 2016), suggesting that CH₄ production may also be influenced by systemic fatty acids of the host. Furthermore, circulating long-chain fatty acids can be sensed by enterocytes or the liver to signal satiety (Leonhardt and Langhans,

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2004), suggesting that the long-chain fatty acid-induced decline in DMI and the accompanying reduction in CH₄ production may also be influenced by fatty acid metabolism of the host, although intravenously infused lipids may not affect DMI of dairy cows (Stocks and Allen, 2014).

During the course of lactation, DMI increases from early to peak lactation and slowly declines afterward, and CH₄ production of cows in second or higher lactation usually follows the course of DMI (Garnsworthy et al., 2012). As first lactating cows still grow, daily CH₄ production increases from early (e.g., wk 5) to peak (e.g., wk 13) to late (e.g., wk 42) lactation, at least when cows are fed the same diet throughout the entire lactation period (Bielak et al., 2016). However, all high-yielding dairy cows mobilize endogenous fatty acids from adipose tissue in the early lactation period, thereby increasing the amount of C18:0 secreted with milk (Lerch et al., 2015). In late lactation, endogenous long-chain fatty acids are less mobilized and therefore used only to a marginal extent for milk fat synthesis (Lerch et al., 2015), underlining that the inverse relationship between the sum of C18 milk fatty acids and CH₄ production is valid for different stages of lactation and independent of dietary fat supplementation (Chilliard et al., 2009). Also, CH₄ yield (CH₄/DMI) in early but not late lactation was found to be lower in cows that mobilized high fat compared with less fat (Bielak et al., 2016). The plasma concentration of long-chain fatty acids including C18:0 reflects the extent of fat mobilization and was found to be directly related to C18:0 milk fatty acids (MFA; Lerch et al., 2015). It has been proposed that circulating long-chain fatty acids affect rumen-intestinal motility, which in turn could influence the retention time of the digesta in the rumen and, consequently, CH₄ yield; however, this assumption was deduced only from an association study (Bielak et al., 2016). Various rumen-intestinal hormones and neurotransmitters such as ghrelin, cholecystokinin, and serotonin are involved in the regulation of rumen motility, and their release from entero- and neuroendocrine cells is closely related to long-chain fatty acid metabolism (Plaza et al., 1996; Börner et al., 2013b; Bielak et al., 2016). Therefore, the aim of the present study was to test the hypothesis of whether increased systemic lipid levels rich in C18 fatty acids reduce CH₄ production and CH₄ yield from dairy cows and whether this effect is accompanied by changes in the rumen microbial community composition. This hypothesis was tested by an intravenous infusion of C18-rich triglycerides (TG), which are converted in vivo by lipases forming free C18 fatty acids.

MATERIALS AND METHODS

Animals, Experimental Design, and Infusions

The experimental protocol was approved by the local animal ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; approval no. 7221.3-1-056/13). Six rumen-fistulated German Holstein cows were randomly assigned to a crossover design with 2 infusions per cow in 3 blocks. One cow suffered from fever during the second infusion and was removed from the trial and data evaluation. The remaining 5 cows (4 in second lactation and 1 in third lactation) were between 270 and 310 DIM and had a BW between 515 and 667 kg. Infusions consisted of 48-h intravenous infusions of either 0.9% NaCl (B. Braun, Melsungen, Germany; **CON**) or a long-chain TG emulsion (Intralipid, Fresenius Kabi, Bad Homburg, Germany, or Lipofundin N, B. Braun; **LIPO**), both containing 20.0% soybean oil, 1.2% egg lecithin, 2.5% glycerol, and 76.3% water) to elevate plasma nonesterified fatty acids (**NEFA**). The fatty acid composition of LIPO was 12.1% palmitic acid (C16:0), 4.2% stearic acid (C18:0), 31.1% oleic acid (C18:1 *cis*-9), and 52.7% linoleic acid (C18:2 *cis*-9, *cis*-12) as determined by GC-MS (see below). Infusions were administered to the right jugular vein via a peristaltic pump at a targeted rate of 7.4 mL/min for NaCl and LIPO. Each cow received 2.1 kg of TG/d intravenously [0.152 ± 0.007 g of TG/(kg of BW $\times$ h)⁻¹]. A washout of at least 4 d (4–10 d) between 2 treatments prevented carryover effects because plasma NEFA concentrations declined to basal level within 24 h after the end of infusion and DMI returned to the preinfusion period level within 48 h. Four days before the first treatment and during the washout phase, cows were kept in tiestalls at 15°C with ad libitum feeding.

Measurements and Sample Collections

Twenty hours before initiation of infusion, animals were equipped with indwelling catheters (Certofix mono; B. Braun) inserted in the right and left jugular veins. Patency of the catheters was maintained by flushing with 5 mL of heparinized saline (10 IU/mL) before and between blood samplings. The BW was measured and animals in pairs of 2 (1 CON and 1 LIPO) were transferred to 2 adjacent open-circuit respiration chambers (each with a CO₂ recovery of 99.9%) separated by a window through which the animals could see each other (Derno et al., 2013). A light cycled on from 0600 to 1900 h. The airflow through the chamber was approximately 30 m³/h and was measured by a differ-

ential pressure-type V cone flow meter (McCrometer, Hemet, CA). After equilibration of gas concentrations for 4 h, concentrations of CO₂ and CH₄ in the chamber were analyzed by infrared absorption (UNOR 610, Maihak, Hamburg, Germany) and the concentration of O₂ paramagnetically (Oxygor 610, Maihak, Hamburg, Germany) every 6 min. As total production (V) of CO₂ production (V_{CO₂}) is the sum of fermentative CO₂ (CO₂ _{ferm}) and metabolic CO₂ (CO₂ _{metab}), CO₂ _{ferm} was estimated according to Chwalibog et al. (1996):

$$V_{\text{CO}_2 \text{ ferm}} (\text{L}) = 1.7 \times V_{\text{CH}_4} (\text{L}), \quad [1]$$

in which the factor 1.7 is constant for a variety of diet compositions. Accordingly, V_{CO₂metab} was calculated by subtracting V_{CO₂ferm} from V_{CO₂}. Net carbohydrate oxidation (COX), net fat oxidation (FOX), and energy expenditure (EE) were calculated according to the following equations:

$$\text{FOX} (\text{g}) = 1.69 V_{\text{O}_2} (\text{L}) - 1.69 V_{\text{CO}_2 \text{ metab}} (\text{L}) - 2.03 N_{\text{u}} (\text{g}), \quad [2]$$

$$\text{COX} (\text{g}) = 4.75 V_{\text{CO}_2 \text{ metab}} (\text{L}) - 3.23 V_{\text{O}_2} (\text{L}) - 2.60 N_{\text{u}} (\text{g}), \quad [3]$$

$$\begin{aligned} \text{EE} (\text{kJ}) = & 16.18 V_{\text{O}_2} (\text{L}) - 5.02 V_{\text{CO}_2 \text{ metab}} (\text{L}) \\ & - 2.17 V_{\text{CH}_4} (\text{L}) - 5.99 N_{\text{u}} (\text{g}). \end{aligned} \quad [4]$$

Urine N excretion (N_u) was not measured, but to make FOX, COX, and EE data comparable with our previous studies (Börner et al., 2013b; Derno et al., 2013; Bielak et al., 2016), N_u was set to 50 g/d. Although real N_u may amount to higher values (e.g., 75–150 g/d) when estimated using different regression equations (Dijkstra et al., 2013), we hereby accepted an error of about 10% in the absolute values of COX and FOX and 0.4% in the absolute values of EE. Gas exchange data were recorded for 12 h before the initiation of infusion and for 48 h (2 d) during infusion.

The feed was prepared as one batch that was sufficiently available for 4 d before the start of infusion, the duration of both infusions (LIPO and CON), and the washout period, and was stored at 4°C as a mixed ration (Table 1). The average daily feed intake of the 3 d before animals entered the chamber was calculated. From 0700 to 1900 h, 1/12 portions of the mean intake were offered in 2-h intervals to minimize sorting of feed by the animals. At 1900 h, the other half of the daily ration was provided. The amount of feed consumed was

Table 1. Ingredients and nutrient composition of diet

Item	Value
Ingredient (g/kg of DM)	
Corn silage	325
Grass silage	181
Hay	32
Straw	38
Corn kernels	70
Concentrate ¹	259
Extracted soy meal	21
Extracted canola meal	38
Molassed sugar beet pulp	23
Minerals ²	9
Lime	4
Nutrient composition (g/kg of DM)	
Crude fat	31
CP	152
Starch	247
Sugars	26
ADF	217
NDF	367
Ash	69
Energy content (MJ/kg of DM)	
Gross energy	17.0
ME	11.2
NE _L	7.0

¹MF 2000 (Vollkraft, Güstrow, Germany): 33% extracted soy meal, 20% corn, 17% wheat gluten, 13% wheat, 8% extracted rapeseed meal, 5% sugar beet pulp, 2% sodium hydrogen carbonate, 1.3% calcium carbonate, 0.2% sodium chloride, 8.0 MJ of NE_L/kg of DM, 204 g of utilizable protein/kg of DM.

²ATR R 7256 (ATR, Ratzeburg, Germany): 15.30% calcium, 13.40% sodium, 5.20% magnesium, 0.85% phosphorus, vitamins.

determined every 6 min by feed disappearance from the trough placed on an electronic scale.

Blood samples were collected from the left jugular vein hourly between 0 and 10 h (d 1) and 24 and 34 h (d 2), and 1 sample was collected at 48 h (after 2 d) relative to initiation of infusion. Blood was sampled into 9-mL monovettes containing EDTA (Sarstedt, Nümbrecht, Germany). Monovettes were immediately placed on ice and centrifuged at 1,570 × *g* for 20 min at 4°C to obtain plasma, which was stored at –80°C before analysis. In parallel to blood samplings, 60 mL of rumen fluid was drawn with a syringe from a tube attached to a sieve probe, which was introduced to the saccus ventralis and fixed at the fistula to ensure withdrawal of fluid from the same position in the rumen. Rumen fluid was sieved (mesh size: 0.7–1.0 mm), and the filtrate was centrifuged at 4°C for 10 min at 4,000 × *g*. The supernatant was removed and stored at –20°C, whereas the microbial pellet was immediately frozen at –80°C.

Animals were milked in the chamber at 0700 and 1630 h. Milk yield was recorded, and subsamples were stored at –20°C for MFA analyses. A further subsample was sent to the Milk Testing Laboratory (Güstrow,

Germany) for the analyses of milk fat, protein, lactose, and urea using infrared spectrophotometry (MilkoScan; Foss GmbH, Rellingen, Germany) to calculate ECM yield as follows:

$$\text{ECM (kg)} = [0.038 \times \text{fat (g)} + 0.024 \times \text{protein (g)} + 0.017 \times \text{lactose (g)}] \times \text{milk (kg)} / 3.14. \quad [5]$$

Chemical Analyses

Feed samples were dried at 60°C for 72 h and ground through a 1-mm screen. Dry matter weight was calculated after drying samples at 110°C overnight. Samples were analyzed for CP (6.25 N; FP-428 nitrogen determinator; Leco, St. Joseph, MI), NDF, ADF and ADL (Ankom filter bag technique; Ankom, Macedon, NY), crude fat (petrol ether extraction), starch and sugar (polarimetric), and ash after heating to 550°C according to Naumann and Bassler (2006).

Plasma NEFA, TG, glucose, and BHB concentrations were analyzed photometrically (Abx Pentra 400; Horiba, Kyoto, Japan) using kit NEFA-HR(2) (Wako Chemicals, Neuss, Germany) for NEFA, no. A11A01640 (Horiba) for TG, no. RB 1008 (Labor+Technik Lehmann, Berlin, Germany) for BHB, and no. A11A01667 (Axon Lab, Reichenbach, Germany) for glucose. Plasma serotonin concentrations were determined in duplicate after protein precipitation on an HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany). Protein precipitation was achieved by adding 25 µL of 2 M perchloric acid to 250 µL of plasma. Samples were shaken for 30 s, incubated for 5 min on ice, and centrifuged 2 times at $37,000 \times g$ for 10 min at 4°C. Supernatants were filtered using 0.2-µm polytetrafluoroethylene filters (Millex-LG, Millipore, Darmstadt, Germany) and injected directly into the HPLC system. Serotonin was analyzed with fluorescence detection as described recently (Stracke et al., 2017). Plasma acetate concentrations were determined as chloroethyl ester derivative on a GC-flame ionization detector instrument (Series 2010, Shimadzu Corp., Kyoto, Japan) on a 25-m RTX-1701 column according to Kristensen et al. (2000). Due to spoilage of the rumen samples from 1 cow, the analyses could be performed for only 4 cows. Rumen fluid short-chain fatty acid concentrations were measured using a GC equipped with a flame ionization detector (Series 20, Shimadzu Corp.) and a 25-m free fatty acid phase column according to Ryan (1980). Milk fatty acids were analyzed as described previously (Börner et al., 2013a) as methyl ester derivatives on an HP-Ultra 2 column (50 m, 0.32 i.d.; 0.52 µm) by GC-coupled MS (GC-MS-QP2010, Shimadzu Corp.).

16S rRNA Profiling

As the rumen fluid pellets from 1 cow spoiled, DNA could be obtained from only 4 LIPO and corresponding CON animals for 3 time points (0, 24, and 48 h relative to initiation of infusion; 24 samples total) using the PowerLyzer PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). The procedure followed the manufacture's recommendations with additional incubation steps for 10 min at 70°C and 10 min at 95°C before beating. Based on purified DNA, the hypervariable region V4 of the 16S rRNA gene was amplified using specific primer 515'F and 806R including adapters and barcodes (Kozich et al., 2013; Hugerth et al., 2014). Polymerase chain reaction was carried out in duplicate with an initial denaturation at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 55°C for 60 s, and 72°C for 90 s, and a final extension for 10 min at 72°C using the 5 Prime HotMasterMix (5 Prime, Hamburg, Germany). Duplicates were combined and purified using magnetic beads (Agencourt AMPure XP, Beckman Coulter, Krefeld, Germany). All PCR products were mixed equimolarly and sequenced on a HiSeq2500 (Illumina, San Diego, CA) generating 250-bp paired-end reads. Raw data were filtered, trimmed, and analyzed using mothur software (version 1.37.4; Schloss et al., 2009). Sequences were globally aligned to the Silva reference database (release 123; <https://www.arb-silva.de/>, accessed May 13, 2016). Putative chimeric sequences were removed using Uchime (Edgar et al., 2011). Sequences were clustered into operational taxonomic units considering a sequence identity of $\geq 97\%$. Taxonomic annotations for operational taxonomic units were retrieved from the Silva database (release 123).

Statistical Analyses

Repeated measures data were analyzed for the effects of infusion (LIPO or CON), time (0–10, 24–36, and 48 h), order of infusion (LIPO first and NaCl second, or vice versa), and their interactions as a completely randomized design using PROC MIXED with a repeated measurements analysis with treatment and infusion time as the repeated effects (SAS Institute, Cary, NC). We did not consider the block effect (animals tested at the same time) in our statistical model because 1 of the 6 animals was removed from the trial so that only 2 pairs of cows remained, which is too few for a reliable estimate of this effect. To evaluate the effect of period, means of hourly plasma and rumen metabolite values from 0 to 24 h (d 1) and 25 to 48 h (d 2) were calculated, and the effects of period (d 1 and 2), infusion, order of infusion, and their interactions with treatment

and period as repeated effects were tested accordingly (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14101>). The covariance structure for data on milk yield and constituents, gases, DMI, and rumen and plasma metabolites was modeled as unstructured for treatment factor and as autoregressive or compound symmetry for time effect. For statistical comparison of 16S rRNA profiles between LIPO and CON cows, analysis of count data was carried out with the DESeq2 package using the negative binominal Wald test (Love et al., 2014). To exclude taxa with very low abundance, observations with fewer than 5 read counts in at least 10 samples were discarded. The design used for DESeq2 considered treatment as a fixed effect while controlling for the effects of animal and time. *P*-values were adjusted for multiple testing following the Benjamini-Hochberg method, and differences were considered significant at an adjusted *P*-value of <0.05.

RESULTS

Plasma, Rumen, and Milk Metabolites

The LIPO but not CON infusion increased plasma TG and NEFA during the first day of infusion ($P_{\text{infusion}} \leq 0.01$) with increased infusion time ($P_{\text{infusion} \times \text{time}} < 0.01$; Figure 1a and b). During h 24 to 34 and after the 48-h infusion period, plasma TG and NEFA concentrations were ($P_{\text{infusion}} \leq 0.01$) or tended to be ($P_{\text{infusion}} < 0.10$) higher in LIPO cows than CON cows. There was no LIPO effect on plasma glucose and BHB concentrations, but plasma BHB increased during d 2 ($P_{\text{time}} < 0.01$), whereas glucose was unaltered on d 1 and changed with time on d 2 of infusion ($P_{\text{time}} < 0.05$) in both groups (Figure 1c and d). Plasma acetate concentrations did not change during the first day of infusion but were greater in CON cows than in LIPO cows during d 2 and after 48 h of infusion ($P_{\text{infusion}} \leq 0.05$; Figure 1e).

Rumen acetate was not affected by infusion, but rumen propionate tended to be lower during LIPO treatment compared with CON treatment on both days of infusion ($P_{\text{infusion}} < 0.10$; Figure 2a and b). Rumen butyrate tended to be lower on d 1 but not on d 2 in LIPO-infused cows ($P_{\text{infusion}} < 0.10$) and tended to vary over time during d 2 only ($P_{\text{time}} < 0.1$; Figure 2c). Accordingly, the rumen (acetate + butyrate)/propionate ratio was greater in LIPO cows than in CON cows on d 2 ($P_{\text{infusion}} < 0.05$) but not on d 1 and at 48 h of infusion (Figure 2d). However, none of the mean plasma and rumen metabolite concentrations changed from d 1 to d 2 of infusion ($P_{\text{period}} > 0.1$; Supplemental Table S1, <https://doi.org/10.3168/jds.2017-14101>).

There was no change in milk yield, lactose, or milk protein concentrations between CON and LIPO infusions, but milk urea concentrations were higher and milk protein concentrations tended to be lower in LIPO cows ($P_{\text{infusion}} = 0.03$ and 0.07 , respectively; Table 2). Milk fat content, milk fat yield, and milk urea concentration increased with progressing time of LIPO infusion ($P_{\text{infusion} \times \text{time}} \leq 0.01$), whereas ECM tended to decrease over time in CON cows only ($P_{\text{infusion} \times \text{time}} = 0.06$). Both concentration and yield of de novo-synthesized MFA (sum of C6:0, C8:0, C10:0, C12:0, C14:0, C14:1 *cis*-9, C16:0, and C16:1 *cis*-9) was reduced, whereas the sum of C16 and C18 MFA increased in LIPO cows but not CON cows ($P_{\text{infusion}} < 0.01$) at both days of infusion (Table 2). Specifically, levels of C18:2 *cis*-9,*cis*-12 and the sum of C18:1 *cis*, which are also present in both lipid emulsions, were 9-fold or tended to be 1.1-fold greater ($P_{\text{infusion}} < 0.01$ and $= 0.07$, respectively), whereas C18:0 and the sum of C18:1 *trans* in milk fat was or tended to be 0.6- to 0.4-fold lower ($P_{\text{infusion}} < 0.01$ and $= 0.06$, respectively) in LIPO relative to CON animals (Table 2).

DMI, CH₄ Production, and Metabolic Oxidation

The amount of DMI on the day before transfer to the chamber did not differ between LIPO and CON cows (14.9 ± 0.5 kg and 15.0 ± 0.5 kg, respectively; $P_{\text{infusion}} = 0.78$; $P_{\text{order}} = 0.31$). In the 12-h nocturnal preinfusion period, DMI amounted to 3.3 ± 0.5 kg for CON and 3.8 ± 0.5 kg for LIPO cows and was not different between treatments ($P_{\text{infusion}} = 0.39$; $P_{\text{order}} = 0.60$). Also, CH₄ production, DMI, and CH₄ yield were not different between CON and LIPO cows within the 12-h interval before the start of infusion (data not shown). The LIPO treatment but not the CON treatment reduced DMI ($P_{\text{infusion}} < 0.01$; Figure 3a), which was associated with a shorter daily eating time in LIPO compared with CON animals ($P_{\text{infusion}} = 0.02$; Figure 3b), whereas the number of meals per day and the intermeal interval were not affected (data not shown). When expressed on a gross energy basis, LIPO cows ingested 54.4 and 49.3 MJ less than CON cows on d 1 and d 2, respectively, whereas LIPO infusion provided 84 MJ/d. According to the reduction in DMI, CH₄ production was lower in LIPO cows than in CON cows ($P_{\text{infusion}} = 0.01$) and was not different between d 1 and 2 of infusion ($P_{\text{time}} = 0.93$; Figure 3c). The CH₄ emission normalized to ECM was lower in LIPO-infused animals ($P_{\text{infusion}} = 0.02$; Figure 3d). When CH₄ production on d 1 was divided by DMI on d 1 (and when CH₄ production on d 2 was divided by DMI on d 2), there was an overall effect of LIPO infusion on CH₄ yield ($P_{\text{infusion}} = 0.03$). However,

Figure 1. Plasma (a) triglyceride (TG), (b) nonesterified fatty acid (NEFA), (c) BHB, (d) glucose, and (e) acetate (each $n = 5$) and (f) percentage change of serotonin ($n = 4$) concentrations of cows intravenously infused with lipofundin (LIPO, ■) or saline (CON, □) for 48 h. Data are shown as mean $\pm$ SE. For each time interval, * indicates $P_{\text{infusion}} \leq 0.05$, § indicates $P_{\text{infusion}} \leq 0.10$, and # indicates $P_{\text{time}} \leq 0.05$ (ANOVA). P -values for infusion $\times$ time, order, infusion $\times$ order, time $\times$ order, and infusion $\times$ time $\times$ order are presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-14101>).

because CH_4 yield of LIPO-infused cows decreased from d 1 to d 2 on average by 8% (2–14%), CH_4/DMI was significantly greater on d 1 ($P < 0.05$, Tukey-Kramer) but not on d 2 ($P = 0.63$, Tukey-Kramer) with LIPO infusion (Figure 3e).

Because part of the CH_4 production measured originates from feed intake of the preceding day, we also calculated CH_4 yield from CH_4 production on d 2 (24–48 h) divided by DMI ingested on d 1 (0–24 h) of infusion, thereby accounting for a 24 h-delay between CH_4 production and the preceding DMI. The resulting

24-h offset CH_4 yield differed between LIPO and CON cows ($P_{\text{infusion}} = 0.03$; Figure 3f) and was intermediate to the CH_4 yield calculated from CH_4 production and DMI on d 1 and d 2, respectively (Figure 3e). In contrast, it remained constant in CON cows, indicating that the high CH_4 yield on d 1 of LIPO cows is affected by feed consumed within the 24-h preinfusion period.

Although CH_4 production and CH_4 yield were different in LIPO and CON cows, there was no change in the composition of the most abundant phyla between treatments (Figure 4). However, slight alterations in

Figure 2. Ruminal (a) acetate, (b) propionate, and (c) butyrate concentrations and (d) the (acetate + butyrate)/propionate ratio of cows intravenously infused with lipofundin (LIPO, ■) or saline (CON, □) for 48 h. Data are shown as mean $\pm$ SE. For each time interval, * indicates $P_{\text{infusion}} < 0.05$, § indicates $P_{\text{infusion}} \leq 0.10$, and # indicates $P_{\text{time}} \leq 0.10$ (ANOVA). P -values for infusion $\times$ time, order, infusion $\times$ order, time $\times$ order, and infusion $\times$ time $\times$ order are presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-14101>).

Table 2. Least squares means and SE of milk and ECM yield, milk constituents, and milk fatty acids (FA) before (0 h) and after 24 and 48 h of either 0.9% NaCl as control (CON) or lipofundin (LIPO) infusion (n = 5 cows)

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Figure 3. Daily (a) DMI, (b) eating time, (c) CH₄ production, (d) CH₄ per ECM yield, (e) CH₄ per DMI, and (f) 24 h-offset CH₄ yield of cows intravenously infused with lipofundin (LIPO, ■) or saline (CON, □) for 48 h (each n = 5). Data are shown as mean ± SE; * indicates $P < 0.05$ and § indicates $P < 0.1$ (Tukey-Kramer). (a) $P_{\text{infusion}} < 0.01$, $P_{\text{time}} = 0.68$. (b) $P_{\text{infusion}} = 0.02$, $P_{\text{time}} = 0.92$. (c) $P_{\text{infusion}} = 0.01$, $P_{\text{time}} = 0.93$. (d) $P_{\text{infusion}} = 0.02$, $P_{\text{time}} = 0.42$. (e) $P_{\text{infusion}} = 0.03$, $P_{\text{time}} = 0.22$. (f) $P_{\text{infusion}} = 0.03$. P -values for infusion × time, order, infusion × order, time × order, and infusion × time × order are presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-14101>).

Figure 4. Mean prevalence of the 10 most abundant phyla as group means before (0 h) and after 24 and 48 h of either lipofundin (LIPO) or saline (CON) infusion (each $n = 4$). Statistical evaluations of taxa relative abundances are presented in Table 3 and Supplemental Table S3 (<https://doi.org/10.3168/jds.2017-14101>). Color version available online.

the microbial composition were observed at the level of low abundant phyla represented by *Chloroflexi* (Table 3). At the genus level, 3 genera, specifically *Ruminococcaceae_UCG-001*, *Succinivibrio*, and *Ruminiclostridium_9*, were found to be less abundant in the rumen of LIPO-infused cows, whereas an unclassified genus of *Bacteroidetes* was more abundant compared with CON cows. However, the relative abundances of these genera did not significantly change from d 1 to d 2 of infusion.

The concentration of plasma serotonin, which is known to reduce rumen motility, increased with increasing time of infusion in both groups ($P_{\text{time}} = 0.02$) but was significantly higher with LIPO compared with CON infusion ($P_{\text{infusion}} < 0.01$; Figure 1f). In parallel to the lower DMI, COX was reduced and FOX increased (each: $P_{\text{infusion}} < 0.05$) during LIPO relative to CON infusion (Figures 5a and b), whereas EE was comparable between CON- and LIPO-infused animals ($P_{\text{infusion}} =$

Table 3. Mean and differential relative abundances of taxa in the rumen of cows receiving intravenous lipofundin (LIPO) or saline (CON) infusions ($n = 4$ cows)

Taxon	Mean relative abundance (%)	Fold change CON vs. LIPO	Nominal P -value	Adjusted P -value ¹
Phylum				
<i>Chloroflexi</i>	0.88	-1.26	<0.01	<0.05
Genus ²				
<i>Ruminococcaceae_UCG-001</i>	0.05	2.42	<0.01	<0.01
<i>Bacteroidetes_BD2-2_unc.</i>	0.05	-1.90	<0.01	<0.01
<i>Succinivibrio</i>	0.24	1.91	<0.01	<0.01
<i>Ruminiclostridium_9</i>	0.03	2.58	<0.01	<0.01

¹Based on Benjamini-Hochberg corrections for multiple testing. Adjusted P -values < 0.05 were considered to be statistically significant. Additional taxa comparisons exceeding the threshold value are reported in Supplemental Table S3 (<https://doi.org/10.3168/jds.2017-14101>).

²The lowest possible level of classification is given, followed by "unclassified" (unc.).

Figure 5. (a) Carbohydrate oxidation (COX), (b) fat oxidation (FOX), and (c) energy expenditure (EE) of cows intravenously infused with lipofundin (LIPO, ■) or saline (CON, □) for 48 h. Data are shown as mean $\pm$ SE. (a) $P_{\text{infusion}} = 0.02$, $P_{\text{time}} = 0.48$. (b) $P_{\text{infusion}} = 0.04$, $P_{\text{time}} = 0.89$. (c) $P_{\text{infusion}} = 0.38$, $P_{\text{time}} = 0.99$. P -values for infusion $\times$ time, order, infusion $\times$ order, time $\times$ order, and infusion $\times$ time $\times$ order are presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-14101>).

0.38; Figure 5c). There was no change in COX, FOX, or EE from d 1 to d 2 of infusion.

DISCUSSION

Plasma Metabolites

Triglycerides intravenously infused to dairy cows are converted by lipases, resulting in the formation of free fatty acids and glycerol. The concentrations of TG and free fatty acids at plateau depend on multiple factors involving the concentration of the lipid emulsion, rate and duration of infusion, BW, lactation status, milk yield, and activities of lipase and fat oxidation

enzymes. Intrajugular 20% Intralipid infusion for 3 h increased plasma NEFA concentrations to 1,500 μM in late-lactating cows with a BW of 685 kg and a milk yield of 23 L/d (Chelikani et al., 2003). Although cows investigated in the present study were comparable in stage of lactation and milk yield and weighted on average 100 kg less than cows in the Chelikani et al. (2003) study, the NEFA plateau of our cows reached only 380 to 400 μM after the second hour of infusion, which may be due to different infusion rates; however, Chelikani et al. (2003) provided no data on this. On the other hand, Pires et al. (2007) infused 0.1 g of TG/kg of BW per hour to nonlactating, nongestating cows and reached an NEFA plateau at 300 to 370 μM , which is

comparable with the infusion rate of 0.15 g of TG/kg of BW per hour and an NEFA plateau at 400 to 500 μ M observed in the present study. Continuous LIPO infusion lasting for 4 (Bareille and Faverdin, 1996) or 6 h (Chelikani et al., 2003) did not affect plasma BHB and glucose concentrations of cows that were 92 or 249 DIM, and our study extends these findings to a 48-h continuous infusion period. Furthermore, when TG emulsion was administered for 20 to 30 min every 4 h throughout a 4-d fasting period of nonlactating cows, plasma BHB concentrations continuously increased over time (Mashek et al., 2005). The latter result likely was caused by the negative energy balance as TG infusions to early-lactating cows increased plasma BHB concentrations (Chelikani et al., 2003). The significant effect of order of infusion on plasma TG, NEFA, and glucose concentrations (P_{order} ; Supplemental Table S2, <https://doi.org/10.3168/jds.2017-14101>) is not due to potential carryover effects, as higher values were observed in cows first receiving CON compared with cows first receiving LIPO. As CON infusion did not increase plasma TG, NEFA, and glucose concentrations, it seems that the 2 cows first receiving a CON infusion had intrinsically higher basal concentrations of these metabolites.

Milk Yield and Constituents

The ECM tended to decline from the preinfusion period to the end of infusion in CON cows only ($P_{\text{infusion} \times \text{time}} = 0.06$). As the DMI of CON cows was comparable between the day before chamber transfer and d 1 and d 2 of infusion, this effect cannot be caused by a reduction of DMI, which normally is observed when free-ranging cows are transferred into a respiration chamber. It rather appears that both LIPO and CON cows experienced a similar level of stress but that LIPO infusion compensated for the loss of ECM observed in CON cows. Percentage of milk protein tended to decrease with LIPO infusion, likely because of the decreased supply of rumen fermentable energy reducing microbial growth in the rumen. On the other hand, milk urea concentrations were greater in LIPO cows and increased over time in LIPO but not CON cows. It has been shown that feed restriction diminishes milk urea concentrations (Bjerre-Harpøth et al., 2012), indicating that the lower DMI of LIPO cows does not account for the observed increase in milk urea concentration. As LIPO infusion causes insulin resistance in dairy cows (Pires et al., 2007), it seems that AA catabolism predominates anabolism during LIPO treatment, thereby increasing milk urea levels.

Arterial fatty acid concentrations determine the uptake, synthesis, and desaturation of fatty acids by the mammary gland. The transfer efficiency of intrave-

nously administered linoleic acid, the major fatty acid of the lipid emulsion, into milk of dairy cows on d 235 of lactation has been determined to be as high as 38% (Stamey Lanier et al., 2013). Accordingly, we found that the highest increase of MFA for C18:2 *cis*-9,*cis*-12 and daily production of C16 plus C18 MFA increased with LIPO infusion. By contrast, the sum of daily produced de novo-synthesized MFA (sum of C6 to C16) decreased, suggesting inhibition of the de novo MFA synthesis and a sparing of acetate. However, saving acetate in milk fat synthesis could result in accumulating plasma acetate concentrations if the amount of acetate absorption from the rumen remains constant. However, contrary to our assumption, plasma acetate concentrations were not different from CON infusion on the first day but rather tended to be lower in LIPO than CON cows on the second day of infusion. This result might be due to the reduction in DMI decreasing the acetate absorption under the influence of LIPO treatment.

Effect of Lipid Infusion on DMI

It appears that the order of infusion had an effect on DMI ($P_{\text{order}} = 0.05$). However, any carryover effects can be excluded as the 3 cows first receiving LIPO had intrinsically higher DMI levels before infusion. The present study further showed that LIPO infusion reduced DMI, whereas Stocks and Allen (2014) recently reported that a 24-h intravenous lipid infusion did not affect DMI of dairy cows that were 81 to 252 DIM. Comparable with our results, intravenous infusion of a 20% TG emulsion has been reported to reduce DMI in cows of 92 DIM (Bareille and Faverdin, 1996) and 249 DIM (Chelikani et al., 2003). The LIPO-induced reduction in DMI was attributed primarily to a reduction in eating time, whereas cows infused with TG on d 92 postpartum responded with a reduction in eating rate but not with an altered eating time (Bareille and Faverdin, 1996). It is highly likely that the anorexic effect of TG is elicited by free fatty acids resulting from TG cleavage, as intravenous long-chain fatty acid infusion to sheep reduced DMI during infusion (Vandermeersch-Doizé and Paquay, 1984). Whether long-chain fatty acids act directly at anorexic centers in the brain, trigger for anorexic signaling through hepatic β -oxidation (Friedman et al., 1999), or both remains speculative. It seems that fatty acids accumulating with LIPO infusion are primarily metabolized by extrahepatic organs as the infusion of tallow emulsion did not affect total or peroxisomal oxidation in liver homogenates (Mashek et al., 2005). Therefore, the anorexic response elicited by LIPO seems not to be triggered by hepatic β -oxidation pathways. Moreover, our recent finding elucidating the dynamic association

between whole-body fat oxidation and feed intake of late-lactating dairy cows rather shows that feed intake affects FOX but that FOX has only a minor effect on feed intake (Derno et al., 2013). The increase in FOX and the reduction in DMI reducing COX is likely the attempt of the host to maintain energy homeostasis, as indicated by a constant EE during LIPO treatment.

Effect of Lipid Infusion on Fermentation Characteristics, Bacteria Structure, and CH₄ Production

It is important to note that DMI during LIPO treatment declined to approximately 80% relative to CON animals on both days of infusion. The reduction of feed intake is usually accompanied by prolonged digesta retention time in the rumen-intestinal tract, an increased molar acetate:propionate ratio in the rumen (Voigt et al., 2000), and an increase in CH₄ yield (Benchaar et al., 1998). We found increased plasma serotonin concentrations with LIPO infusion, and intravenous application of serotonin has been shown to induce hypomotility in the rumen (Plaza et al., 1996), indicating that a LIPO-induced increase in serotonin may account for a longer digesta retention time in the rumen. A longer retention time would intensify fiber fermentation, resulting in a greater (acetate + butyrate)/propionate ratio. Accordingly, the ratio was found to be significantly higher in LIPO cows than in CON cows on d 2 but not on d 1 or at 48 h of infusion. Thus, it seems that the reduction in DMI accounts for the increased molar (acetate + butyrate)/propionate proportion and the greater CH₄ yield in LIPO cows. However, the transient increase in the (acetate + butyrate)/propionate ratio apparently depends on the duration of infusion and the time during the 24-h cycle and was surprisingly not due to an increase in rumen acetate concentrations, as described for ruminants on a reduced nutrition level (Voigt et al., 2000). Nevertheless, one genus among *Bacteroidetes* (*Bacteroidetes*_BD2-2) producing mainly acetate was upregulated in LIPO cows. There is evidence that the relative abundance of *Bacteroidetes* is affected by both fasting (Crawford et al., 2009) and intravenous lipid supply (Hodin et al., 2012). Hodin et al. (2012) showed that the portion of *Bacteroidetes* in total bacteria increased when rats received an infusion of total parenteral nutrition solution containing 20% Intralipid. However, these effects on the level of phylum were observed after an infusion period of 14 d, whereas the differences induced by a 2-d treatment between CON and LIPO in the current study were visible only at the *Bacteroidetes*_BD2-2 genus. The total relative abundance of *Bacteroidetes* did not change significantly between LIPO and CON cows.

The increased (acetate + butyrate)/propionate ratio in LIPO cows was primarily caused by a trend toward declining rumen propionate concentrations in LIPO cows. This finding can be at least in part attributed to the lower relative abundance of *Succinivibrio* because this genus is known to facilitate ruminal starch digestion and propionate formation (Hespell, 1992). Interestingly, relative gene abundance of *Succinivibrio* was found to be positively correlated with the relative abundance of *Methanobrevibacter* in cows (Liu et al., 2017) and with CH₄ yield in sheep (Kamke et al., 2017), whereas *Succinivibrionaceae* were reduced, but the *Methanobrevibacter gottschalkii* clade increased in feed-restricted cattle (McCabe et al., 2015). Furthermore, the fibrolytic species *Ruminococcaceae* and *Ruminiclostridium*_9 were less abundant in LIPO than CON cows, and, comparably, intravenous infusion of a lipid emulsion reduced at least the relative abundance of *Clostridiaceae* in the intestine of neonatal piglets (Lavalée et al., 2016). Both *Ruminococcaceae* and *Ruminiclostridium*_9 degrade lignocellulose and produce H₂, suggesting that the intermediary H₂ production, the precondition for CH₄ synthesis, and interspecies H₂ transfer were negatively affected in LIPO cows. Thus, the lower relative abundances of *Succinivibrio*, *Ruminococcaceae*, and *Ruminiclostridium*_9 may explain the trend toward reduced rumen propionate concentrations and lower CH₄ production of LIPO cows. However, differences in the relative abundance of methanogens between treatments could not be determined.

In conclusion, our study demonstrates that increased concentrations of circulating lipids and long-chain fatty acids increase the serotonin tone, presumably leading to hypomotility of the rumen, reduction in DMI, and, consequently, CH₄ production in late-lactating dairy cows. Although DMI was reduced to the same extent on both days of LIPO infusion, CH₄ yield was 8% lower on d 2 compared with d 1 of LIPO infusion, indicating that CH₄ yield on d 1 was still affected by feed consumed in the 24-h preinfusion period. However, the greater 24-h offset CH₄ yield of LIPO cows compared with CON cows suggests that the lower intake rather than the intravenously administered TG per se leads to an increased CH₄ yield. This result led us to conclude that the lower CH₄ yield of cows that mobilized high fat compared with less fat (Bielak et al., 2016) was not due to higher NEFA concentration in early lactation. Furthermore, the tendency for lower ruminal propionate concentration as well as relative abundances of *Succinivibrio*, *Ruminococcaceae*, and *Ruminiclostridium* in the rumen indicate that less intermediary H₂ is formed with reduced DMI or LIPO infusion, respectively. To distinguish between the effect of DMI and in vitro LIPO infusion, future pair-feeding experiments with longer

infusion times than applied in the current study need to be performed to test whether CH₄ yield decreases below the level of pair-fed controls.

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