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Variations in methane yield and microbial community profiles in the rumen of dairy cows as they pass through stages of first lactation

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ABSTRACT

Considerable interest exists both from an environmental and economic perspective in reducing methane emissions from agriculture. In ruminants, CH_4 is produced by a complex community of microorganisms that is established in early life but can be influenced by external factors such as feed. Although CH_4 emissions were thought to be constant once an animal reached maturity, recent studies have shown that CH_4 yield significantly increases from early to late lactation in dairy cows. The aim of this study was to test the hypothesis that increases in CH_4 yield over the lactation cycle are related to changes in rumen microbial community structure. Nine cows were monitored throughout their first lactation cycle. Methane and dry matter intake were measured to calculate CH_4 per dry matter intake (CH_4 yield) and ruminal fluid was collected during early, mid, and late lactation. A significant difference in bacterial and archaeal community structure during early and late lactation was observed. Furthermore, when ruminal short-chain fatty acid concentrations were measured, the ratio of acetate and butyrate to propionate was significantly higher in late lactation compared with early lactation. Propionate concentrations were higher in cows with low CH_4 yield during late lactation, but no differences were observed in bacterial or archaeal community structures. *Prevotella* dominated the rumen of cows followed by *Succinclasticum*; *Treponema*, *Fibrobacter*, *Ruminococcus*, and *Bifidobacterium* were also in high abundance relative to other bacterial genera. In general, positive correlations were stronger between the most relatively abundant bacterial genera and acetate and butyrate concentrations in the cows with high CH_4 and weaker between these genera and propionate concentration. This study indicates that increased CH_4 yield in late lactation is reflected in significant changes in microbial community structure.
Key words: dairy cow, methane, rumen microbiome, methanogens, short-chain fatty acids

INTRODUCTION

The microbial ecosystem in the rumen of cattle is highly complex, consisting of many microbial species acting together to convert plant materials into nutrients, primarily short-chain fatty acids (**SCFA**). These fermentation products, predominantly acetate, propionate, and butyrate, are essential for host maintenance and growth (Hobson and Stewart, 1997; Van Houtert, 1993). However, bacterial fermentation also creates by-products, namely hydrogen and carbon dioxide, that cannot be used by the host and are converted to CH_4 by methanogenic archaea (Hobson and Stewart, 1997). The extent of CH_4 produced by archaea is therefore dependent on the level of metabolic by-products formed by other microbial species.

Methane is of no energetic use to the host and is released into the environment through eructation (Dougherty, 1968; Anderson et al., 1987). This is a major environmental problem, as CH_4 is a potent greenhouse gas (**GHG**) that contributes to global warming. Global CH_4 emissions originating from enteric fermentation account for 17% of global CH_4 emissions, whereas dairy cows are estimated to contribute 3.3% to overall anthropogenic GHG emissions (Knapp et al., 2014). In addition, CH_4 represents a 5 to 7% loss of feed energy for dairy cows on commonly fed diets (Arndt et al., 2015), which negatively affects animal productivity. Feed energy loss due to CH_4 is predominantly influenced by the level of feed intake and dietary composition and, to a lesser extent, by feed additives or antimethanogen vaccines (Atakora et al., 2011; Knapp et al., 2014; Sun et al., 2015; Roehe et al., 2016). Implementing strategies to reduce CH_4 emissions from dairy cows would thus be beneficial from both an environmental and economic perspective.

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In the livestock industry, GHG mitigation strategies have largely focused on diet and dietary supplements as a means of reducing CH_4 emissions (de Menezes et al., 2011; Ellison et al., 2014; Cobellis et al., 2016). Although diet has a strong effect on rumen microbial community structure, this effect is often inconsistent and short-lived, making it difficult to create a universal dietary strategy (Yáñez-Ruiz et al., 2015). Furthermore, it is not well understood what type of microbial community profile leads to low CH_4 production. Host genetics have also been shown to influence rumen microbial community structure and CH_4 emissions (Weimer et al., 2010; Goopy et al., 2014). Ranking of sires based on CH_4 yield did not change when they were fed a high concentrate-based diet compared with a medium concentrate-based diet (Roehe et al., 2016), indicating that the influence of host genetics on CH_4 yield did not change with diet.

As microbes in the rumen are responsible for producing the CH_4, information on the factors shaping microbial community composition is needed to inform strategies designed to manipulate these communities to achieve long-term, consistent reduction of enteric CH_4 production. There has been considerable focus on the rumen microbiome of cows in the first year of life, as the rumen microbiome is thought to be relatively stable once mature (Rey et al., 2014; Li et al., 2012; Jami et al., 2013). It has been suggested that early life may represent a window during which the establishing rumen microbiome can be manipulated with long-term effects (Yáñez-Ruiz et al., 2015; Abecia et al., 2014). However, CH_4 emissions vary during different periods of an animal's lifetime; for example, CH_4 levels have been reported to increase by up to 35% from early to late lactation (Bielak et al., 2016; Garnsworthy et al., 2012), but this increase is primarily due to an increase in DMI, the main driver of the CH_4 production. Targeting specific periods of increased CH_4 production may facilitate the development of short-term interventions that can contribute to an overall strategy for the mitigation of CH_4 levels from agriculture. Jewell et al. (2015) reported that bacterial community structure in cows was dynamic over 2 lactation cycles, with specific operational taxonomic units (**OTU**) associated with high and low milk production efficiency; however, those authors did not monitor CH_4 emissions during their study. The aim of the current study was to monitor CH_4 emission per unit of DMI (CH_4/DMI = CH_4 yield), SCFA, and microbial community dynamics of cows during lactation to determine if any observed changes in CH_4 yields across lactation stages were driven by changes in rumen microbiome structure. It was hypothesized that spikes in CH_4 yields would be accompanied by a marked difference in bacterial and archaeal community composition compared with periods when CH_4 yields were lower. Nine cows from the same dairy herd were tracked during their first lactation cycle, with CH_4 production measured in respiration chambers and ruminal fluid samples collected for microbial and SCFA analysis during early, mid, and late lactation (5, 13, and 42 wk postpartum, respectively). In addition, rumen microbial profiles of cows from the dairy herd that were identified as either producing high or low CH_4 yield during the late lactation stage were compared to determine if significant differences in their rumen microbiome could be observed.

MATERIALS AND METHODS

Animals and Feed

A group of 9 German Holstein dairy cows of the same age in first lactation were used for the present study. All animals were treated in accordance with the State Government guidelines for the use of animals as experimental subjects 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).

Animals were kept in a freestall of the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany, with ad libitum access to fresh water and feed offered as TMR. To investigate whether the microbial community profile changes over the course of lactation and not in response to an altered ration composition, the diet was formulated to ensure a constant nutrient and energy composition over the course of lactation. Individual daily feed intake was recorded via automated weighing troughs and the TMR was sampled at each respiration chamber measurement for the determination of DM and nutrient composition. Feed analysis was performed by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) in Rostock, Germany. Dietary ingredient and nutrient compositions are listed in Supplemental Table S1 (https://doi.org/10.3168/jds.2017-14200).

Methane Measurements in Respiration Chambers

Cows calved between October 2014 and April 2015. In wk 5, 13, and 42 (±0.2 wk; SE) of lactation animals were transferred from the freestall into open-circuit respiration chambers as described previously (Bielak et al., 2016). Before the actual gas exchange measure-

ments, the animals were adapted several times to the rubber mat-floored chambers to ensure normal feeding and lying behavior. Prior to entering the chamber, cows were weighed on an electronic scale. Animals were transferred into the chambers around midday to allow overnight gas equilibration. On the next day, gas exchange measurements began at 0700 h and lasted for the next 48 h. The daily mean of the measuring period lasting from 0700 to 0659 h of the next day was calculated. Two adjacent chambers separated by a common glass window ensured visual contact with the neighboring animal. In the chambers, cows were tied and feed intake was measured by feed disappearance from a feeding trough connected to an electronic scale. Cows had ad libitum access to water. Animals were fed twice daily at 0730 and 1500 h and milked at 0700 and 1700 h. Milk samples from the preceding evening and the morning milking were pooled and milk composition was analyzed by the Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern e.V. (Güstrow, Germany). Energy-corrected milk yield was calculated as

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

The temperature within the chamber was kept at 15°C with a dark–light cycle from 0600 to 1900 h. Data collection was automatically conducted every 6 min. The CH_4 concentration in the air flow out of the chambers was measured by infrared absorption (Sidor, Sick Maihak GmbH, Reute, Germany) as described recently (Derno et al., 2013). The recovery test for CH_4 revealed 99.8%.

Rumen Fluid Sampling and Analyses

Rumen fluid sampling was conducted as described by Bielak et al. (2016). About 2 h before the transfer into respiration chambers, animals were sampled for rumen fluid via an esophageal tube system connected to a vacuum pump. The first ~100 mL was discarded to eliminate potential contamination with saliva. The subsequent 200 mL of fluid was collected and immediately stored on ice until processing. After sieving (mash size = 0.7–1.0 mm) and pH measurement using a glass electrode (Roth, Karlsruhe, Germany), the filtrate was centrifuged at 4,000 × g and 4°C for 10 min and the resulting pellet was immediately frozen at −80°C. The ruminal fluid sample was freeze-dried before DNA extraction. In the supernatant, SCFA concentrations were analyzed using a GC (GC-FID, Series 17A, Shimadzu Corp., Kyoto, Japan) equipped with a 25-m free fatty acid phase column according to (Ryan, 1980).

DNA Extraction and Amplicon Sequencing

The DNA of the rumen samples was extracted from all 9 animals at sampling time points at wk 5, 13, and 42 postpartum (27 samples in total) using the PCSA method described by Lueders et al. (2004), with phenol chloroform added before the bead beating step. The DNA concentration and purity were assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and DNA was stored at −20°C until needed. Bacterial and archaeal community structures were determined using amplicon sequencing on an Illumina Miseq platform (Illumina, San Diego, CA), using the method described by Kozich et al. (2013). Briefly, once DNA was extracted and purified, 1 μL of target DNA was added to a well on a 96-well PCR plate already containing 17 μL of Accuprime Pfx Supermix (Invitrogen, Thermo Fisher Scientific, Dublin, Ireland) and 2 μL of a primer set targeting the V4 region of the 16S rRNA gene (Kozich et al., 2013). The PCR conditions consisted of a hot start at 95°C for 2 min, followed by denaturation at 95°C for 20 s, annealing at 55°C for 15 s, and extension at 72°C for 5 min (30 cycles) with a final extension at 72°C for 10 min. The PCR products were visualized on a 1.2% (wt/vol) agarose gel (Roche Diagnostic, Basel, Switzerland); PCR products were then purified and concentrations normalized using the SequalPrep Normalization Plate Kit (Invitrogen) according to manufacturer's instructions (www.thermofisher.com/order/catalog/product/A1051001) and an amplicon pool was created using 5 μL of PCR product from each sample. The concentration of the pool was then determined using a Qubit fluorometer and dsDNA kit (Thermo Fisher Scientific) to ensure that at least 100 ng of DNA was present. The amplicon pool was then sent to the Centre for Genomic Research, University of Liverpool (Liverpool, UK), for sequencing on an Illumina Miseq platform. Sequence files associated with each sample have been submitted to the NCBI Sequence Read Archive (Accession no. PRJNA393984).

MiSeq sequencing data were initially processed using the mothur program v.1.32.1 developed by Schloss et al. (2009). Illumina adapter sequences were trimmed by cutadapt ver.2.1.1 using option -O 3 (Martin, 2011). Sickle ver.1.2 (Joshi and Fass, 2011) was used to further trim the data with a quality score of ≥20. Reads <10 bp after trimming were removed. Each read was then trimmed to a maximum of 275 bp and ambiguous

Table 1. Methane emission and its relation to input and output traits of 9 dairy cows measured in wk 5, 13, and 42 postpartum

Item	wk 5	wk 13	wk 42	*P*-value
CH_4 (L/d)	434.3 ± 50.3[a]	450.9 ± 56.0[a]	540.5 ± 48.4[b]	0.001
CH_4/DMI (L/kg)	32.2 ± 2.3[a]	33.8 ± 3.7[a]	36.7 ± 3.6[b]	0.044
DMI (kg/d)	13.6 ± 0.6[a]	13.41 ± 0.53[a]	14.80 ± 0.40[a]	0.082
ECM (kg/d)	29.47 ± 1.34[a]	27.07 ± 1.11[a]	25.12 ± 1.05[a]	0.019
ECM/DMI (kg/kg)	2.23 ± 0.18	2.05 ± 0.12	1.71 ± 0.10	0.001
CH_4/ECM (L/kg)	15.10 ± 1.01[a]	16.89 ± 0.92[a]	21.98 ± 1.22[b]	<0.001
CH_4/NDF (L/kg)	92.6 ± 8.3[a]	97.2 ± 12.0[a]	75.1 ± 8.5[b]	<0.001
BW (kg)	575.75 ± 12.65[a]	571.28 ± 14.34[a]	647.78 ± 15.99[b]	<0.001

[a,b]Different superscript letters within row indicate significance ($P < 0.05$, Tukey honestly significant difference).

bases were removed. Sequences which contained homopolymer runs >8 bases were discarded. After trimming, identical sequences were grouped into unique sequences. Chimeric sequences were identified using the UCHIME algorithm (Edgar et al., 2011) within mothur and were then removed. Sequences were assigned to OTU using the cluster command and the average neighbor algorithm. All subsequent OTU-based analyses were performed using a cutoff of 0.03. Taxonomy was assigned to all remaining aligned sequences by comparing processed data to the silva128 databases for bacteria and archaea independently (arb-silva.de/silva-license-information; Quast et al., 2013). A rarefaction curve was constructed to ensure sufficient sequencing depth had been achieved (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-14200).

Statistical Analysis

Means for each of the 3 lactation stages were calculated for CH_4 yield, SCFA concentrations, ECM, and CH_4/ECM. An ANOVA and post hoc Tukey HSD tests were carried out within the R software environment (www.r-project.org) using the lsmeans package to analyze differences between means. Multivariate analysis was carried out using Primer-E v.7 software with the Permanova add-on (Quest Research Limited, Auckland, New Zealand). Similarity matrices were constructed for samples using Bray-Curtis similarities on standardized, fourth root-transformed abundance data. Distance-based permutational multivariate ANOVA (Anderson et al., 2001) was then performed to test the null hypothesis that no differences existed in microbial community structure across time or CH_4 yield group at a significance level of $\alpha = 0.05$ based on 9,999 possible permutations. The nonmetric multidimensional scaling plots were constructed to visualize the data. Similarity percentages were calculated using Bray-Curtis similarities to evaluate the level to which each genus contributed to the difference in community structures in the rumen across time.

RESULTS

CH_4 Yield and Performance Parameters

Individual DMI and CH_4 production were measured for each cow (n = 9) during early (5 wk postpartum), mid (13 wk postpartum), and late lactation (42 wk postpartum) to calculate CH_4 yield. Although DMI did not change over time (Table 1), methane yield differed significantly between stages ($P = 0.001$), with highest levels (40.96 L/kg of DMI) recorded during late lactation. Changes in CH_4 yields were observed between early and late stage lactation ($P = 0.002$, Tukey HSD) and between mid and late stage lactation ($P = 0.009$, Tukey-HSD). We found no significant difference between early and mid lactation; therefore, we focused on the mid and late lactation stages to determine possible explanations for the shift in CH_4 yield between these 2 time points (Table 1). The pattern of higher CH_4 yields in mid and late lactation was still apparent when CH_4 was corrected for ECM yield (expressed as CH_4/ECM, L/kg; Table 1). Energy-corrected milk yield and gross feed efficiency (expressed as ECM/DMI) decreased, whereas BW and methane emission intensity (defined as CH_4/ECM) increased from early to late lactation (each $P < 0.05$; Table 1).

Rumen Microbial Community Structures

The DNA was extracted from rumen fluid collected from all cows during early, mid, and late stage lactation and amplicon libraries constructed based on the V4 region of the 16S rRNA gene and sequenced using the Illumina MiSeq platform. Following initial processing, an average of 315,617 good-quality sequences were obtained, with sequence numbers per sample ranging from 45,771 to 1,607,531 (median = 262,413). A total of 346 unique OTU that could be taxonomically classified to genus level were identified across all animals. Twenty-three distinct bacterial phyla were identified in the rumen of cows during all stages of lactation. *Bacteroidetes* (42–48%) and *Firmicutes* (30–31%) dominated

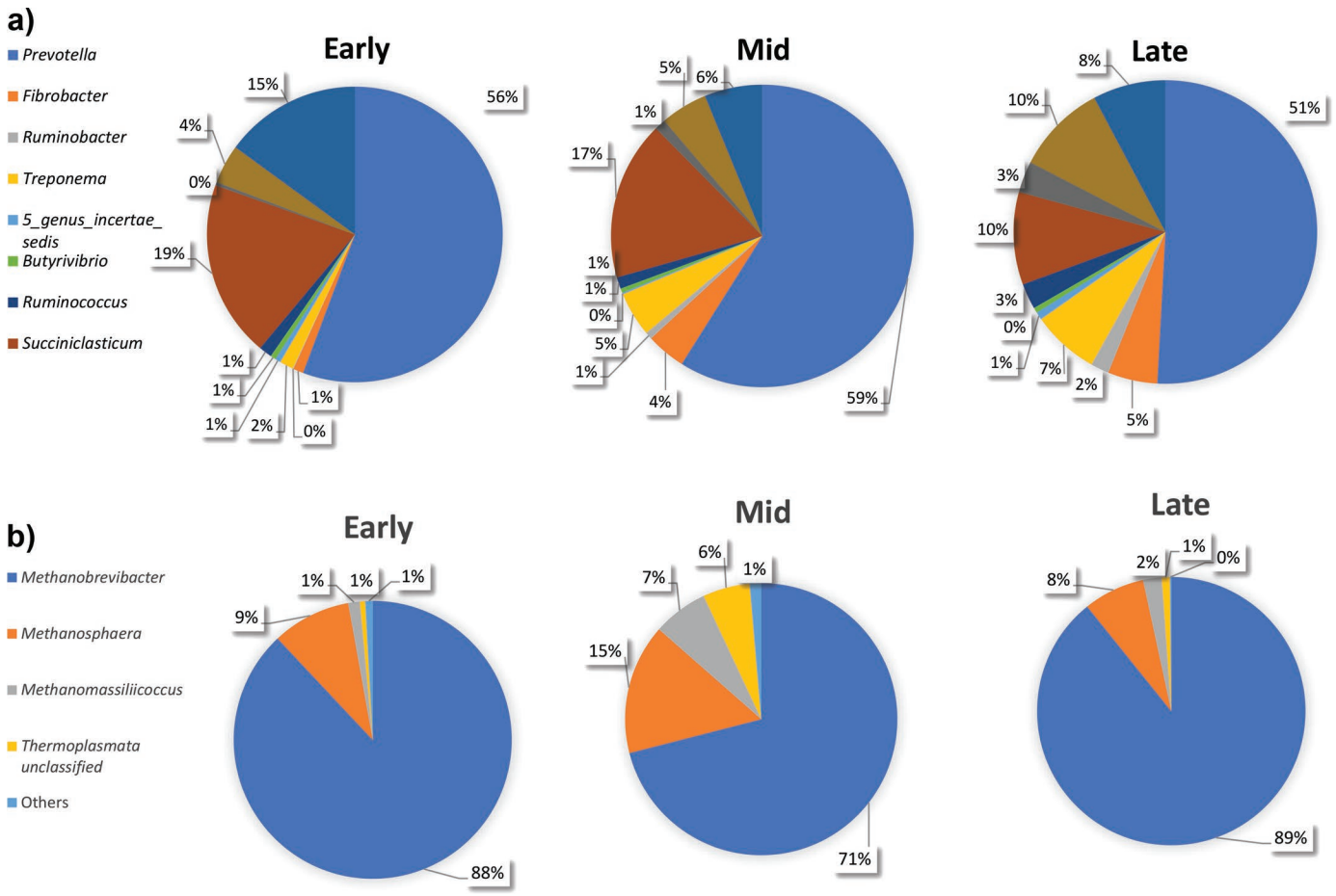

Figure 1. Relative abundance of (a) bacterial and (b) archaeal genera in the rumen of cows in early-, mid-, and late-phase lactation. Similarity percentages analysis of these data (Table 2 and Supplemental Table S4; https://doi.org/10.3168/jds.2017-14200) identified which genera contributed to dissimilarity between bacterial community structures of cows in mid- and late-stage lactation. Color version available online.

the rumen microbiome of cows at all lactation stages. *Proteobacteria* were also present in relatively high abundance (5–11%) across all stages (Figure 1a). Of the bacterial genera detected, *Prevotella* was by far the relatively most abundant genus, accounting for 51 to 59% of total bacterial sequences, with *Succiniclasticum* accounting for a further 10 to 19%. The relative abundances of *Treponema*, *Fibrobacter*, *Ruminococcus*, and SR1 genus *incertae sedis* were also high compared with other bacterial genera. It should be noted that 1.9% of sequences detected in the rumen of cows could not be taxonomically assigned at any level and differences observed were not due to any bias associated with DNA recovery levels.

Only 8 genera of archaea were identified in the rumen of cows across all lactation stages. *Methanobrevibacter* was by far the most relatively abundant (71–89%), followed by *Methanosphaera* (8–15%). The relative abundance of *Methanomassiliicoccus* and an unclassified genus of *Thermoplasmata* appeared to increase as lactation stage advanced (Figure 1b). The remaining sequences were assigned to unclassified genera of *Methanobacteriaceae*, *Methanosarcinaceae*, *Euryarchaeota*, and *Nitrososphaera*.

Changes in Rumen Microbiome Profile and SCFA Concentrations over Lactation Cycle

Although the rumen microbiome was dominated by similar bacterial and archaeal genera throughout the lactation cycle, the overall microbial community structure did change over time. Differences in bacterial community structure were identified at all taxonomic levels but became most pronounced at genus level. Shannon diversity indices differed significantly between mid and late lactation ($P = 0.001$; Supplemental Table S2; https://doi.org/10.3168/jds.2017-14200) and permutational multivariate ANOVA analysis revealed significant ($P = 0.0014$) differences in bacterial community structure in the rumen of cows as they passed through

Table 2. Similarity percentage analysis of bacterial genera accounting for 19.34% of dissimilarity calculated between the ruminal community structures of cows in mid- and late-stage lactation

Genus	Contribution to community dissimilarity (%)	Relative abundance (%)	
		Mid lactation	Late lactation
Acetobacter	1.10	0.54	0.12
Bifidobacterium	1.08	0.74	0.79
Lactobacillus	1.03	0.68	0.24
Arthrobacter	0.95	0.52	0.16
Clostridium IV	0.94	0.52	0.82
Chloroflexi unclassified	0.90	0.26	0.29
Streptococcus	0.88	0.75	0.43
SR1 genus *incertae sedis*	0.88	1.01	1.31
Weissella	0.87	0.47	0.11
Sharpea	0.85	0.37	0.41
Rheinheimera	0.84	0.42	0.15
Treponema	0.81	1.24	1.34
Ruminobacter	0.80	0.99	0.88
Fusobacterium	0.79	0.42	0.16
Pediococcus	0.78	0.34	0.00
Acidaminococcus	0.77	0.34	0.43
Lactococcus	0.76	0.38	0.05
Leuconostoc	0.74	0.33	0.03
Flavobacterium	0.73	0.37	0.09
Escherichia/Shigella	0.72	0.25	0.18
Brevundimonas	0.72	0.32	0.03
Devosia	0.70	0.31	0.04
5 Genera *incertae sedis*	0.69	0.85	1.01

stages of lactation. Bacterial community structures in the rumen of mid-lactation cows were significantly ($P = 0.0007$) different from those in late lactation, as were those present in the rumen during early lactation and late lactation ($P = 0.02$; Supplemental Table S3; https://doi.org/10.3168/jds.2017-14200). A similar difference was seen with archaeal community structure, which differed significantly ($P = 0.042$) in the rumen of cows in early lactation compared with those in late lactation. Similarity percentage analysis indicated that the top 5 contributors to the dissimilarity between bacterial community profiles in cows at different stages of lactation were *Acetobacter*, *Bifidobacterium*, *Lactobacillus*, *Arthrobacter*, *and Clostridium* IV spp. (Table 2).

In early, mid, and late lactation, rumen acetate (32.2 ± 3.4, 34.8 ± 7.5, and 45.9 ± 6.7 mmol/L), propionate (11.8 ± 1.6, 11.3 ± 2.2, and 12.7 ± 2.3 mmol/L), and butyrate (9.7 ± 3.3, 7.2 ± 1.8, and 8.4 ± 1.7 mmol/L) concentrations, respectively, did not differ by stage of lactation; however, a significant ($P = 0.012$) difference was observed in the ratio of acetate and butyrate to propionate between cows in mid and late stage lactation (Figure 2).

Pearson correlation coefficient (r) was used to determine if any significant correlations could be observed between SCFA profiles in the rumen and the relative abundance of any of the 9 most relatively abundant bacterial genera (Table 3). In general, the relative abundance of all bacterial genera was strongly negatively correlated with acetate, butyrate, and propionate in early lactation, and this correlation weakened as cows progressed through the lactation cycle. *Ruminococcus* was an exception, as the relative abundance of this genus displayed a weak positive correlation to acetate, butyrate, and propionate in early lactation, but this changed to a stronger positive correlation in late lactation, particularly with butyrate.

Rumen Microbiome Structure and SCFA Concentrations in Low Versus High CH_4-Yielding Cows

Although CH_4 yield increased as the lactation cycle progressed (Table 1), no individual cow could be identified as a persistently extremely low or high CH_4-emitter across all lactation stages. However, clear differences could be observed in CH_4 yields from different cows during late lactation (42 wk postpartum), when CH_4 yields were highest. Therefore, 3 cows that produced low (mean 29 L/kg of DMI) and 3 that produced high (mean 38 L/kg of DMI) CH_4 yields during this stage of lactation were selected for SCFA and rumen microbial community structure analyses to investigate whether any differences could be observed between the 2 cohorts of animals from the same herd. A significant ($P = 0.032$) difference in propionate concentration was observed between the high and low CH_4-yielding cows, with a higher concentration of this SCFA detected in

the rumen of cows emitting less CH_4 yield (Figure 3a). A decrease in the acetate and butyrate-to-propionate ratio was noted in cows emitting lower levels of CH_4, although this difference was not significant ($P = 0.08$; Figure 3b). No differences in acetate or butyrate concentrations were detected between high and low CH_4 yielders. Despite the differences observed in CH_4 yields between the low and high CH_4-emitting cows, no differences were observed in bacterial ($P = 0.3$) or archaeal ($P = 0.8$) community structures. *Prevotella* (55.96%) dominated the rumen of cows, with *Succinclasticum* (15.35%) *Treponema*, *Fibrobacter*, *Ruminococcus*, and *Bifidobacterium* also in high abundance relative to other bacterial genera. Strong positive correlations were observed between the relative abundance of *Fibrobacter*, *Treponema*, and SR1 genus *incertae sedis* and all 3 SCFA in the rumen of cows emitting lower levels of CH_4 (Table 4). Furthermore, in this group, strong negative correlations were detected between the relative abundance of *Succiniclasticum* and all 3 SCFA. In the rumen of cows emitting high levels of CH_4, strong negative correlations existed between *Bifidobacterium* and acetate and butyrate concentrations, whereas *Fibrobacter* and *Succiniclasticum* were positively correlated with all 3 SCFA and *Prevotella* had a strong positive correlation with propionate concentration. Although some exceptions were noted, in general positive correlations were stronger between the most relatively abundant bacterial genera and concentrations of acetate and butyrate in higher emitters, whereas correlations were weaker between bacterial genera and concentrations of propionate.

DISCUSSION

Are Changes in CH_4 Yield Driven by Shifts in Rumen Microbial Community Structure?

For beef cattle, it has been shown that repeatability of CH_4 measurements decreases with increasing time between 2 measurements (Donoghue et al., 2016). Therefore, in the current study, each cow was measured at the same 3 time points [i.e., early (5 wk postpartum), mid (13 wk postpartum), and late (42 weeks postpartum) lactation] by using the most accurate method, respiration chambers. Although respiration chambers can be prohibitively expensive, they are the most reliable tool available for measuring enteric CH_4 emissions and are widely used (Yan et al., 2010; Hellwing et al., 2012). Similar to previous studies (Garnsworthy et al., 2012; Bielak et al., 2016), we showed that CH_4 yields from dairy cows are not constant during the lactation

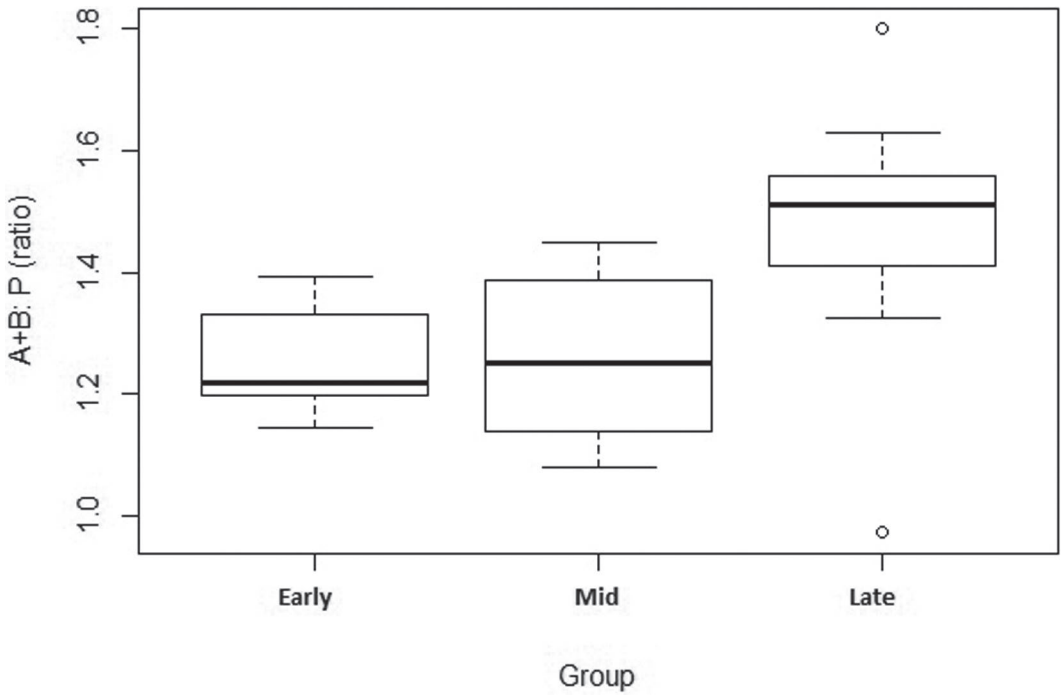

Figure 2. Acetate and butyrate-to-propionate ratio (A+B:P) measured in rumen fluid of cows during early, mid and late lactation. Each box depicts the interquartile range of the data; the horizontal line in each box shows the median, which separates the upper quartile (25%) from the lower quartile (25%). The whiskers (vertical capped lines) represent a further upper and lower 25%, and the circles represent outliers in the data set.

Table 3. Pearson correlation coefficients[1] between the relative abundance of sequences assigned to each bacterial genus and short-chain fatty acid concentrations (acetate, butyrate, and propionate) in the rumen of cows during early, mid, and late lactation

	Early			Mid			Late		
Genus	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate
Prevotella	−0.529	−0.418	−0.610	−0.398	−0.340	−0.498	−0.302	−0.174	−0.179
Fibrobacter	−0.341	−0.307	−0.397	−0.040	0.055	−0.165	−0.017	0.181	0.110
Ruminobacter	0.305	0.097	0.203	0.516	0.606	0.418	0.472	0.661	0.420
Treponema	−0.518	−0.492	−0.625	−0.212	−0.151	−0.266	−0.486	−0.293	−0.341
5 Genera *incertae sedis*	−0.032	−0.358	−0.161	−0.198	−0.292	−0.236	−0.226	−0.219	−0.367
Butyrivibrio	−0.425	−0.397	−0.478	−0.378	−0.431	−0.437	−0.419	−0.251	−0.354
Ruminococcus	−0.499	−0.286	−0.457	−0.450	−0.439	−0.519	−0.153	−0.081	−0.015
Succiniclasticum	−0.537	−0.577	−0.655	−0.298	−0.189	−0.389	−0.245	−0.126	−0.177
SR1 genus *incertae sedis*	−0.269	−0.309	−0.327	−0.040	0.014	0.097	−0.282	−0.183	−0.300
Unclassified phylum	−0.681	−0.519	−0.717	−0.361	−0.302	−0.421	−0.485	−0.279	−0.288

[1]Calculated at a 95% confidence interval.

period, but increase from early to late lactation. This is in contrast to the relatively constant CH_4 yields measured in nonlactating adult sheep (Shi et al., 2014).

Although energy nutrient composition of the diet was constant over the course of lactation, a significant difference in bacterial community structure was detected ($P = 0.001$), and this shift was most pronounced between mid and late lactation. This is in contrast to the widely held opinion that, once established, the rumen microbiome is relatively stable in adult cows except in cases of major disruptions caused by antibiotics or major feed alterations (Rey et al., 2014). Michelland et al. (2011) reported that ruminal bacterial communities never appeared to be in a steady state in nonlactating cows, whereas Jewell et al. (2015) similarly reported that the rumen bacterial community composition in

Figure 3. Concentration of VFA (a) propionate and (b) acetate and butyrate-to-propionate ratio (A+B:P) measured in the rumen of cows producing high (mean 38 L/kg of DMI) levels of CH_4 (high emitters) and cows producing low (mean 29 L/kg of DMI) levels of CH_4 (low emitters). Each box depicts the interquartile range of the data; the horizontal line in each box shows the median, which separates the upper quartile (25%) from the lower quartile (25%). The whiskers (vertical capped lines) represent a further upper and lower 25%.

Table 4. Pearson correlation coefficients[1] between the relative abundance of sequences assigned to each bacterial genus and short-chain fatty acid concentrations (acetate, butyrate, and propionate) in the rumen of cows producing high (mean 38 L/kg of DMI) levels of CH_4 (high emitters) and cows producing low (mean 29 L/kg of DMI) levels of CH_4 (low emitters)

	High emitters			Low emitters		
Item	Acetate	Butyrate	Propionate	Acetate	Butyrate	Propionate
Bifidobacterium	−0.667	−0.704	−0.099	0.517	0.215	0.457
Prevotella	0.528	0.484	0.927	0.467	0.157	0.405
Fibrobacter	0.959	0.943	0.940	0.795	0.948	0.835
Treponema	0.125	0.075	0.687	0.882	0.986	0.912
5 Genera *incertae sedis*	0.680	0.716	0.115	0.411	0.095	0.347
Butyrivibrio	0.706	0.740	0.151	0.364	0.045	0.299
Succiniclasticum	0.834	0.805	0.99	−0.993	−0.976	−0.999
SR1 genus *incertae sedis*	0.670	0.707	0.103	0.993	0.902	0.982
Unclassified phylum	0.801	0.831	0.294	0.660	0.383	0.607

[1]Calculated at a 95% confidence interval.

dairy cows was dynamic over the course of lactation and suggested that certain profiles favor high production efficiency in cows; however, CH_4 yield as a component determining feed energy utilization efficiency by the host was not measured. Gross feed efficiency of cows in the present study declined from early to late lactation, but whether correlation exists between the bacterial community structure and the numerously known feed efficiency indicators such as ECM/DMI, NE_L/ECM, or (NE_L − ECM)/metabolic BW needs to be determined in future studies. However, the level of ECM production in early lactation is in part decoupled from DMI, as cows produce milk to some extent from mobilized body reserves during this time (Bielak et al., 2016).

Although the concentrations of acetate, butyrate, and propionate in the rumen did not appear to change significantly between mid and late lactation, the ratio of acetate and butyrate to propionate was significantly higher in late lactation. This correlates with the sharp increase in CH_4 emissions measured in late lactation. Mohammed et al. (2011) also reported a strong correlation between enteric CH_4 emissions and the ratio of acetate and butyrate to propionate in the rumen of dairy cows and concluded that the ratio could be used as a predictor of CH_4 production. Microbial metabolism resulting in acetate and butyrate production creates hydrogen as a by-product, whereas synthesis of propionate consumes hydrogen (Van Soest, 1994). Increased levels of acetate and butyrate compared with propionate could result in overall increased levels of hydrogen in the rumen and, therefore, increased substrate availability for CH_4 production by methanogens.

Even though the same 6 bacterial genera (*Prevotella*, *Succiniclasticum*, *Treponema*, *Fibrobacter*, SR1 genus *incertae sedis*, *and Ruminococcus*) were the most relatively abundant in the rumen of cows in both mid and late lactation, their distribution changed in late lactation when CH_4 yields were higher. These bacterial genera are commonly found in the rumen, with *Prevotella*, *Fibrobacter*, *Succiniclasticum*, and *Ruminococcus* considered part of the core bovine rumen microbiome (Henderson et al., 2015; Lima et al., 2015). *Ruminococcus albus* and *Ruminococcus flavefaciens*, both cellulolytic bacteria, are the most abundant species of *Ruminococcus* present in the rumen. In pure culture, *R. albus* is known to produce ethanol, acetate, formate, and hydrogen during cellulose metabolism, whereas *R. flavefaciens* forms similar metabolites but produces succinate instead of ethanol (Russell et al., 2008). *Acetobacter*, a bacterial genus which also produces acetate by oxidizing sugars (Balch et al., 1977), was one of the top contributors to dissimilarity and showed largest relative abundances in the rumen of cows in mid lactation compared with late lactation. However, acetate concentrations were not significantly different in mid and late lactation, suggesting that these genera and their metabolites are not solely responsible for the increased CH_4 yield seen in late lactation. Members of the candidate phylum SR1 have only recently been discovered in the rumen (Lima et al., 2015). Animals fed a dietary supplement (linseed oil) designed to decrease CH_4 production were shown to have lower abundances of SR1 genus *incertae sedis* in their rumen (Veneman et al., 2015). *Bifidobacterium* was also a significant contributor to differences in bacterial community structures, with a higher relative abundance appearing in late lactation. This genus is known to confer numerous health benefits, such as vitamin production, modulation of immune system response, pathogen inhibition, and regulation of intestinal homeostasis (Mayo and Van Sinderen, 2010). No clear association between *Bifidobacterium* and CH_4 levels has been reported, and the relative increase may merely reflect a return to host homeostasis at the end of lactation. However, this bacterial genus is important for dietary carbohydrate degradation and produces lactic acid (Pokusaeva et al., 2011), which is further

metabolized to either propionate or acetate by microbial species in the rumen (Ladd and Walker, 1965). An increased relative abundance of *Bifidobacterium* may therefore result in increased levels of these SCFA. *Lactobacillus* and *Arthrobacter* were both relatively less abundant in late lactation, whereas *Clostridium* IV was relatively more abundant. Similar to *Bifidobacterium*, lactobacilli produce lactic acid that is subsequently converted to propionate or acetate (Ladd and Walker, 1965; Han et al., 2014), whereas *Clostridium* IV are butyrate producers (Lopetuso et al., 2013). Hydrogen availability is the limiting factor for CH_4 production by methanogens (Baker, 1999), and acetate and butyrate synthesis leads to the production of hydrogen whereas propionate synthesis consumes hydrogen. (Van Soest, 1994). Therefore, increases in the relative abundance of microbial species producing propionate and decreases in the relative abundance of microbial species producing metabolites leading to acetate or butyrate would have a knock-on effect on overall CH_4 yield.

None of the dominant bacterial genera appeared to be positively correlated with propionate. In fact, all genera, with the exception of *Ruminococcus*, were negatively correlated with acetate, butyrate, and propionate throughout lactation, although this correlation did weaken in late lactation. *Ruminococcus* are cellulolytic bacteria capable of producing both acetate and succinate, a propionate precursor (Ladd and Walker, 1965). The predominately negative correlations observed may indicate that these bacterial genera are using the SCFA or competing for nutrients with acetate, butyrate, and propionate producers. Although some studies have proposed roles for specific bacteria based on observed correlations (Sandri et al., 2014), it is difficult to draw conclusions based on correlations given the complex nature of the rumen microbial community and the lack of information concerning metabolic pathways employed by particular microbes. Furthermore, rumen fungi and ciliate protozoa also produce acetate, butyrate, and propionate during anaerobic digestion of feed (Martin et al., 2010). Although most research on this topic has focused on bacterial community composition, as they are typically the most abundant and metabolically diverse members of the rumen microbiome, ciliate protozoa can account for up to half of rumen microbial biomass and therefore strongly influence overall VFA production (Newbold et al., 2015); these organisms no doubt have contributed to the concentrations of acetate, butyrate, and propionate produced in the current study.

Archaeal community structure, at genus level, also changed significantly ($P = 0.04$) in the rumen of dairy cows between mid and late lactation. Although archaea with diverse metabolisms exist and are found in a range of habitats, those detected in the rumen thus far are all strictly anaerobic methanogens (Janssen and Kirs, 2008). In a pattern similar to that observed with bacterial communities, the most abundant archaeal genera remained the same, but the distribution shifted. *Methanobrevibacter* were found to be in a higher relative abundance in the rumen of cows in late lactation, whereas *Methanosphaera*, *Methanomassilicoccus*, and an unclassified genus of *Thermoplasmata* were all in a lower relative abundance. *Methanobrevibacter* have previously been associated with high CH_4 production. Zhou et al. (2011) reported high abundances of certain species of *Methanobrevibacter* in animals producing high CH_4 yields; however, this may reflect species-level differences and the current study only has information at genus level. As methanogens rely on metabolites produced by other microbial species in the rumen, changes observed in archaeal community structure may be a consequence of the changes occurring in bacterial community composition and changes in protozoal or fungal abundance or species richness.

Does the Rumen Microbial Profile Differ Between Low and High CH_4-Yielding Cows in Late Lactation?

Similar to previous studies (Garnsworthy et al., 2012; Bielak et al., 2016), overall CH_4 emissions from the dairy herd used in the current study increased from early to late lactation. Increased rumen volume and particulate passage rate have been associated with higher CH_4 yields (Goopy et al., 2014). Although rumen volume and particulate retention time were not measured in our study, rumen DM volume and rumen capacity have been shown to increase in cows up to 30 d postpartum compared with late lactation (72 d preparturition) volumes (Park et al., 2001; Reynolds et al., 2004). Increasing CH_4 yields throughout lactation could therefore, in part, be explained by increased rumen volume. Although overall CH_4 emissions were lower in early lactation and higher in late lactation, individual cows emitted varying CH_4 yields and no group of cows produced consistently lower amounts of CH_4 yields at all time points throughout the lactation cycle when compared with the herd as a whole. Comparison of the rumen microbiome of cows producing low versus high CH_4 yields during late lactation revealed no significant differences in bacterial or archaeal community structures. Differences were observed in the relative abundance of some bacterial genera. *Succiniclasticum*, which have been reported to convert succinate to propionate in the rumen (van Gylswyk, 1995), appeared to be relatively more abundant in cows producing lower levels of CH_4. In line with our result, Wallace et al. (2015) found that *Succinivibrionaceae* was 4-fold less abundant in high CH_4-emitting animals. Increased lev-

els of propionate have been linked to lower levels of CH_4 production, and propionate concentrations were found to be significantly ($P = 0.03$) higher in the rumen of cows producing low levels of CH_4 in the current study. In addition, the ratio of acetate and butyrate to propionate concentrations appeared to be reduced in the rumen of low emitters, although this reduction was not significant ($P = 0.08$). Despite its reported link with propionate production, no clear relationship between *Succiniclasticum* with propionate could be seen; this genus was negatively correlated with all 3 SCFA in cows that produced low levels of CH_4 and positively correlated with cows that produced high levels of CH_4. This finding again highlights the complexity of metabolism in the rumen and the difficulty in identifying the role(s) specific bacteria may be playing based on relative abundance alone and the possible role of protozoa and fungi. The rumen microbiome contains a high level of microbial diversity and therefore harbors a high level of functional redundancy, with many species carrying out overlapping activities (Weimer, 2015). Similar phylogenetic profiles in the rumen may be misleading, as the functional profiles may be decidedly different. Shi et al. (2014) performed metagenomic and metatranscriptomic sequencing of rumen samples from 4 high versus 4 low CH_4-yielding sheep and found that methanogen abundance was similar in both groups, but transcription of methanogenesis pathway genes was significantly increased in sheep with high CH_4 yield. This may explain why microbial communities appear largely unchanged in the rumen of dairy cows even when they produce higher levels of CH_4 yield.

It is known that host genetics influence rumen microbiome structure and that animal-to-animal variation exists (Li et al., 2009; Henderson et al., 2015; Roehe et al., 2016). Therefore, when examining potential drivers of low or high CH_4 production, it is important to examine overall trends in the herd rather than focusing on individual animals. Because CH_4 yield varies considerably between individuals and increased significantly in late lactation, we focused on the average change over time. Short-term dietary interventions in early life aimed at manipulating the microbiome to create stable, desirable community compositions that persist throughout life are already being investigated and are yielding encouraging results (Abecia et al., 2014; Yáñez-Ruiz et al., 2015; Lyons et al., 2017). The identification of significantly higher CH_4 yield in late lactation in the current study could mark this period as an additional target for interventions designed to reduce overall CH_4 emissions from the livestock sector. Dietary interventions are expensive, and it would be largely impractical to implement them throughout the animal production cycle; therefore, identifying periods where short-term dietary intervention results in meaningful CH_4 reductions could be a better approach for mitigation strategies in this sector.

In conclusion, CH_4 yield increased significantly in late lactation and was accompanied by a shift in bacterial and archaeal community structure and propionate concentration in the rumen. This suggests that changes in the microbial community structure, which cause changes in the SCFA profile contribute to increases in CH_4 yield. However, due to the complex nature of the rumen microbiome, individual microorganisms cannot be singled out as solely accountable for changes in CH_4 yield. Rather, shifts in the microbial communities as a whole appear to be responsible. Furthermore, our study highlights late lactation as a critical period where short-term intervention could be useful to reduce overall CH_4 emissions from the dairy sector.

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