Research Article

Received: 25 March 2018 Revised: 28 May 2018 Accepted article published: 31 May 2018 Published online in Wiley Online Library: 11 July 2018

(wileyonlinelibrary.com) DOI 10.1002/jsfa.9162

Compositional and structural dynamics of the ruminal microbiota in dairy heifers and its relationship to methane production

Camila S Cunha,[a] Marcos I Marcondes,[a] Cristina M Veloso,[a] Hilário C Mantovani,[b] Luiz Gustavo R Pereira,[c] Thierry R Tomich,[c] Kimberly A Dill-McFarland[d*,§] and Garret Suen[d*]

Abstract

BACKGROUND: Heifers emit more enteric methane (CH_4) than adult cows and these emissions tend to decrease per unit feed intake as they age. However, common mitigation strategies like expensive high-quality feeds are not economically feasible for these pre-production animals. Given its direct role in CH_4 production, altering the rumen microbiota is another potential avenue for reducing CH_4 production by ruminants. However, to identify effective microbial targets, a better understanding of the rumen microbiota and its relationship to CH_4 production across heifer development is needed.

RESULTS: Here, we investigate the relationship between rumen bacterial, archaeal, and fungal communities as well as CH_4 emissions and a number of production traits in prepubertal (PP), pubertal (PB), and pregnant heifers (PG). Overall, PG heifers emitted the most CH_4, followed by PB and PP heifers. The bacterial genus *Acetobacter* and the archaeal genus *Methanobrevibacter* were positively associated, while *Eubacterium* and *Methanosphaera* were negatively associated with raw CH_4 production by heifers. When corrected for dietary intake, both *Eubacterium* and *Methanosphaera* remained negatively associated with CH_4 production.

CONCLUSION: We suggest that *Eubacterium* and *Methanosphaera* represent likely targets for CH_4 mitigation efforts in heifers as they were negatively associated with CH_4 production and not significantly associated with production traits.

Supporting information may be found in the online version of this article.

Keywords: 16S rRNA; greenhouse gases; microbiota; next generation sequencing; rumen microbiology; methane

INTRODUCTION

The digestion process in ruminant animals is based on a complex web of interactions between the animal, its feed, and its rumen microbiota.[1] The bacteria, archaea, and anaerobic fungi that inhabit the rumen are responsible for the majority of the degradation of feed particles.[2] As a result, microbes provide the majority of the nutrients such as volatile fatty acids (VFAs), proteins, and vitamins needed for animal growth, reproduction, and milk production.[3,4] During the degradation of feed components in the rumen, methanogens use hydrogen (H_2) and carbon dioxide (CO_2) released by other microorganisms to produce methane (CH_4).[5] CH_4 production by ruminants is of particular importance because, in addition to being a greenhouse gas with eight-times more global warming potential than CO_2,[6] CH_4 represents a loss of 2–12% of the total energy ingested by the animal.[7]

Hydrogen availability and CH_4 production in the ruminal environment are driven primarily by diet, either by type of feed[8] or forage quality.[9] Different dietary characteristics lead to changes in VFA production in the rumen, resulting in variable proportions of acetate, propionate, and butyrate. With a more fibrous diet, acetate production increases and the preferred microbial pathways to produce this VFA result in more H_2. In contrast, a diet rich in starch leads to more propionate production, which consumes H_2, thereby reducing the availability of this compound to methanogens.[4,10] Diet therefore provides a potentially effective means to reduce CH_4

* *Correspondence to: KA Dill-McFarland, Department of Bacteriology, University of Wisconsin-Madison, 5153 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706, USA. E-mail: kadm@mail.ubc.ca; or G Suen, Department of Bacteriology, University of Wisconsin-Madison, 5159 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706, USA. E-mail: gsuen@wisc.edu*

§ *Current address: Department of Microbiology and Immunology, University of British Columbia, 2504 - 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.*

a *Department of Animal Science, Universidade Federal de Viçosa, Viçosa, Brazil*

b *Department of Microbiology, Universidade Federal de Viçosa, Viçosa, Brazil*

c *Brazilian Agricultural Research Corporation, EMBRAPA Dairy Cattle, Juiz de Fora, Brazil*

d *Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA*

emissions by livestock. However, high-quality, starch-rich feeds are often unavailable or economically infeasible in many production systems, particularly in tropical environments where typical diets are low-quality forages.

In dairy production systems, young dairy heifers contribute significantly to CH_4 production as they produce more CH_4 per unit of feed intake than older heifers[11] or adult cows[12] and are not provided the highest quality diets since they do not contribute to milk production. Thus, other avenues to reduce CH_4 emissions in heifers are of great interest. In particular, altering the rumen microbiota may be effective, as younger animals do not have fully established microbial communities[13] and may be amenable to alterations that reduce CH_4 output. Most research in this area has focused on bacterial and archaeal communities[14–17] since these microorganisms are directly responsible for H_2 and CH_4 production, respectively. However, anaerobic fungal communities are also of interest[3] as these microorganisms serve to expose cellulosic materials to bacteria as well as produce VFAs.[18] Therefore, assessment of all of these communities and their interactions in concert is necessary to determine the best means for manipulating ruminal microbes to attain lower agricultural CH_4 production.

This study aimed to investigate the relationship between bacterial, archaeal, and fungal ruminal communities and enteric CH_4 emissions from dairy heifers at different physiological stages in a tropical production system. Since methanogenesis is dependent on the fermentation activity of rumen microorganisms, we hypothesize that differences in the rumen microbiota of heifers at different physiological stages (age and live weight) correlate with CH_4 emissions. These data will provide microbial targets for influencing the ruminal microbial community to lower CH_4 emissions during the early life of the animal.

MATERIAL AND METHODS

Animals and management

This study was carried out at the Unit for Teaching, Research, and Extension on Dairy Cattle of the Universidade Federal de Viçosa (UFV), Brazil, and at the Department of Bacteriology at the University of Wisconsin-Madison, USA. This project was approved by the Ethics Committee in the Use of Production Animals from the UFV (protocol number 99/2014).

Heifers at different physiological stages were divided into three groups according to their average body weight and gestational condition as follows: prepubertal heifers (PP), 204 ± 14.7 kg ($n = 5$, average age $= 9.5 \pm 1.5$ months), pubertal heifers (PB), 358 ± 11.9 kg ($n = 4$, average age $= 19 \pm 2.8$ months), and pregnant heifers (PG), 473 ± 23.6 kg (90 days post artificial insemination, $n = 5$, average age $= 24 \pm 2.8$ months). Heifers were kept under the same feeding management to which they were already adapted in the production system and were housed in individual tie stalls. The heifers' diet contained 90% corn silage and 10% concentrate based on corn, soybean meal, and minerals (Table S1). Animals were fed twice daily (07:00 and 15:00) and had *ad libitum* access to water. Feed was adjusted daily to keep refusals between 5 and 10% of the total feed provided. At the beginning and end of the trial, the animals were weighed without fasting prior to morning feeding.

The trials were performed separately for each group. The first 5 days of each trial were for adaptation of the animals to tie stall housing and the yokes used for CH_4 evaluation. Gas collections from animal eructation to evaluate CH_4 emissions began on the sixth day. These collections started at 07:00, lasted 24 h, and were performed up to the 10th day. The experiment was designed to obtain at least five high-quality gas samples from each animal. In the case of difficulties arising during the sampling process, the collection of that day was repeated after day 10. Evaluation of voluntary intake was determined from days 6 to 10. Fecal samples were taken on day 6 and rumen samples on day 10.

Feed and fecal sampling

For 5 days after day 6 of each trial, feed offered to and refused by each animal was weighed and subsampled. At day 6, feces were collected during a 24-h period and then subsampled for each animal. Feed offered and refused, as well as fecal samples for each animal were grouped per trial and analyzed for dry matter (DM; method INCT-CA n° G-003/1),[19] crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), non-fiber carbohydrates (NFC), and ash (MM) content.[20] These results were used for the calculation of dry matter intake (DMI), dry matter digestibility (DMD), organic matter intake (OMI), and organic matter digestibility (OMD).[21,22]

Methane emissions measurements

The tracer gas sulfur hexafluoride (SF_6) technique[23] with the adaptations described for this procedure by Oss et al.[24] were used for the evaluation of CH_4 emissions. Briefly, the flow of CH_4 released by the animal was calculated in relation to the flow of SF_6, measured from the SF_6 release rate of a permeation capsule lodged in the rumen and from the concentrations of CH_4 and SF_6 in gas samples.[25] Analysis of CH_4 and SF_6 concentrations were determine by gas chromatography at the Laboratory of Gas Chromatography, EMBRAPA Dairy Cattle, in Juiz de Fora, Minas Gerais, Brazil, as previously described.[24] Raw CH_4 emissions data were corrected for digestible fractions of dry matter intake (DMI) and digestible organic matter intake (OMI) by dividing raw values by DMI and OMI values, respectively.

Ruminal sampling and fermentation pattern

On the 10th day of each trial, ruminal contents were sampled 4 h after feeding using the stomach tube technique.[18,26,27] The initial 200 mL of collected fluid was discarded to avoid saliva contamination. Because of the collection method, rumen samples represent only the liquid phase of the ruminal content. A sample of approximately 70 mL was filtered through four layers of cheesecloth and split into two aliquots. The first was used for DNA extraction (50 mL) and the second (20 mL) for analysis of volatile fatty acid (VFA) concentrations. Both samples were frozen at −80 °C until the time they were analyzed.

Volatile fatty acids (VFA) concentrations were analyzed using high-performance liquid chromatography (HPLC) as previously described. Briefly, after centrifugation, 1.5 mL of a cell-free supernatant was treated as described[28] and analyzed in a Dionex Ultimate 3000 Dual chromatograph (Dionex Corporation, Sunnyvale, CA, USA) with a Shodex RI-101 refractive index detector at 45 °C, using a Phenomenex Rezex ROA 300 × 7.8 mm ion-exclusion column, also at 45 °C. The mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.7 mL min^{-1}.

DNA extraction, amplicon library preparation, and sequencing

DNA extractions were performed on 25 mL of rumen samples as described by Stevenson and Weimer,[29] and then treated with RNAse A (10 mg mL^{-1}). Total DNA was quantified using a Qubit 2.0

Fluorometer (Invitrogen, San Diego, CA, USA). A two-step PCR was employed to amplify the V3 and V4 regions of the 16S ribosomal RNA gene for bacteria, the V6 through V8 regions of the16S rRNA gene for archaea, and the ITS1 gene for fungi.[30] PCR, clean-up and sequencing were performed as described.[31]

Briefly, PCR was performed using 5–200 ng of DNA or 5 μL of cleaned PCR1 product, 0.4 μmol of each primer, 1 × KAPA HiFi HotStart ReadyMix (Kapa Biosystems®, Wilmington, MA, United States), and ultrapure water to 25 μL. Reactions were amplified at 95 ° C for 3 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. A total of 25 cycles were performed for bacterial and archaeal amplicons, 35 cycles for fungal, and 8 cycles for the second PCR for all amplicons. The first PCR product was purified using an Invitrogen PureLink Pro96 PCR Purification Kit, and the second PCR product was purified using a ZR-96 Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). DNA was then quantified using a Qubit® 2.0 Fluorometer, equimolar pooled, and sequnced on an Illumina MiSeq using the 2 × 300bp v3 kit following manufacturer's guidelines.

DNA sequence processing analysis

The mothur program, version 1.36.1,[32] was used to analyze the obtained sequences, following a protocol adapted from Kozich *et al.*[33] Sequences that could not be aligned, were chimeric sequences, or were contaminants were removed. Sequences generated using either bacterial or archaeal primers were aligned against the SILVA 16S rRNA gene database[34] and classified using the Green Genes database.[35] For anaerobic fungal analysis, sequences were classified using the UNITE database[36] and any contaminants were also removed. A sequence similarity of 97% was used as a cut-off to classify operational taxonomic units (OTUs) at the species level. Taxonomy assignment of OTU's was performed in mothur. Each sample within a microbial community type was then normalized to the sample with the lowest sequence count within that microbial community type before further analyses (bacteria: 5838; archaea: 1896; fungi: 77 sequences per sample). All sequences used for this study were deposited in the NCBI's Short Read Archive and are publicly available under BioProject PRJNA380769.

Statistical analysis

Relative abundances were calculated as the number of sequences in each taxon divided by the number of total sequences present in the sample multiplied by 100. Alpha diversity indexes for richness (Chao1), diversity (Shannon), and Good's Coverage were calculated in mothur. The statistical program R[37] was used to test differences in CH_4 emissions from each group using analysis of variance (ANOVA) followed by Tukey's honest significance test (TukeyHSD) for multiple comparisons, when necessary. Based on the CH_4 emission data, heifers were split into groups by CH_4 emission level (high or low) using the NbClust package[38] in R. Thus, heifers were evaluated by both physiological state and CH_4 emission level group. To test for differences in richness and diversity of bacterial, archaeal, and anaerobic fungal communities, ANOVA and Tukey's tests were also performed.

Bacterial, archaeal, and anaerobic fungal communities were visualized using non-metric multidimensional scaling (nMDS) plots of the Bray–Curtis dissimilarity index calculated using the vegan package[39] in R. An Analysis of Similarity (ANOSIM)[40] was performed to determine the overall differences in microbial communities by groups of interest (e.g. PP, PB, and PG heifers, and CH_4 level). Specific bacterial taxa of interest, as well as all genera for the archaeal and anaerobic fungal communities, were tested using the Kruskal–Wallis (Agricolae package[41]) analysis to assess differences in relative abundances of taxa in physiological groups, and the Wilcoxon Rank Sum Test was used to determine differences between CH_4 groups. For these tests, $P < 0.10$ was accepted as significantly different.

To investigate if microbial community data and VFA concentrations co-varied, a Mantel test[42] was performed. A Mantel test was also used to evaluate if bacterial, archaeal, and anaerobic fungal communities co-varied. All analyses were performed in the vegan package in R. For these tests, *P*-values < 0.05 were accepted as statistically significant. Spearman's rank correlation was used to correlate continuous variables [CH_4 emissions, digestible dry matter intake (dDMI), digestible organic matter intake (dOMI), average body weight (BW)] and the relative abundances of bacterial, archaeal, or anaerobic fungal families or genera using the corrplot package[43] in R. All families and genera were tested, and taxa that showed significant correlations at $|r| > 0.45$ and/or $P < 0.1$ for at least one variable were visualized in a correlogram.

RESULTS

Methane emissions and production traits differ by physiological stage

Prepubertal (PP), pubertal (PB), and pregnant (PG) heifers were assessed for raw and energy-corrected CH_4 emissions (Table S2). PB and PG heifers had significantly higher digestible dry matter intake (dDMI) and digestible organic matter intake (dOMI) than PP heifers (ANOVA, Tukey HSD $P < 0.05$; Table 1, Table S3). However, PP heifers were less efficient, with higher DMI relative to total body weight (ANOVA, Tukey HSD $P < 0.05$). Raw CH_4 emissions in g day^{-1}, as well as emissions corrected for feed intake in g CH_4 kg^{-1} of DMI, increased with animal age, with PG heifers producing the highest amounts of CH_4 (ANOVA, Tukey HSD $P < 0.10$; Table 1, Table S3). When corrected for dDMI and dOMI, similar trends were observed, although increases were no longer significant from PB to PG heifers (Table 1). Based on these data, heifers were designated as either high or low CH_4 emitters, independent of physiological stage (Table S2).

Total volatile fatty acid concentrations (Table S2) were lower in PB than either PP or PG heifers (ANOVA, Tukey HSD $P < 0.05$; Table 2, Table S3). Acetate, propionate and butyrate concentrations were similar in PP and PG heifers (ANOVA, Tukey HSD $P > 0.05$) and both had higher concentrations than in PB heifers (ANOVA, Tukey HSD $P < 0.05$). The acetate:propionate ratio in PB and PG heifers did not show differences (ANOVA, Tukey HSD $P = 0.99$), and both had higher ratios than in PP heifers (ANOVA, Tukey HSD $P < 0.10$).

Rumen microbiota characterization

After quality filtering, 164 421 sequences were obtained for bacteria (mean = 11 744 ± 6219), 59 645 for archaea (mean = 4260 ± 1935), and 9039 for anaerobic fungi (mean = 646 ± 886). For all amplicons, Good's Coverage of each sample was 0.99 or greater.

For the bacterial community, 21 phyla were identified, but only 9 were present in all animals. The most abundant phyla were the Firmicutes (51.27% ± 9.76), Bacteroidetes (26.44% ± 11.52), TM7 (11.67% ± 12.83), Proteobacteria (2.49% ± 2.66), and SR1 (2.36% ± 4.16, Fig. 1A). The remaining phyla each had relative abundances lower than 2%. Within the Firmicutes, 76.47% of

Table 1. Methane emission, voluntary intake, dry matter digestibility, and live body weight of growing heifers

Group	CH_4 (g day^{-1})	CH_4 (g kg^{-1} DMI)	CH_4 (g kg^{-1} dDMI)	CH_4 (g kg^{-1} dOMI)	dDMI (kg day^{-1})	dOMI (kg day^{-1})	BW (kg)	DMI/BW (Kg)
PP	83.43 ± 15.93*C*	18.54 ± 2.33*C*	27.38 ± 4.98*B*	28.30 ± 4.97*B*	3.09 ± 0.55*B*	2.98 ± 0.51*B*	204 ± 36.04*C*	0.022 ± 0.0015*A*
PB	173.60 ± 42.06*B*	24.58 ± 2.70*B*	35.82 ± 6.55*AB*	37.10 ± 6.00*A*	5.03 ± 1.62*A*	4.81 ± 1.48*A*	358.5 ± 26.54*B*	0.019 ± 0.0024*B*
PG	216.47 ± 13.21*A*	31.02 ± 3.67*A*	43.84 ± 6.18*A*	45.10 ± 5.93*A*	5.00 ± 0.65*A*	4.86 ± 0.60*A*	473 ± 52.74*A*	0.015 ± 0.0011*C*

Means + standard deviation followed by different letters in a column are different by Tukey's HSD ($P < 0.10$).
CH_4, methane; DMI, dry matter intake; dDMI, digestible dry matter intake; dOMI, digestible organic matter intake; BW, average body weight; PP, prepubertal heifers; PB, pubertal heifers; PG, pregnant heifers.

Table 2. Volatile fatty acid concentrations in the rumens of growing heifers

Group	Total VFA (mmol L^{-1})	Acetate (mmol L^{-1})	Propionate (mmol L^{-1})	Butyrate (mmol L^{-1})	A:P
PP	91.89 ± 5.93*A*	59.10 ± 4.01*AB*	19.15 ± 1.74*A*	8.91 ± 0.45*A*	3.10 ± 0.22*B*
PB	79.54 ± 6.07*B*	54.12 ± 3.84*B*	16.05 ± 0.88*B*	6.42 ± 0.51*B*	3.37 ± 0.10*A*
PG	98.22 ± 9.37*A*	65.59 ± 6.23*A*	19.42 ± 2.14*A*	8.80 ± 1.26*A*	3.38 ± 0.11*A*

VFA volatile fatty acid (acetate, propionate, butyrate, lactate, isobutyrate, isovalerate, valerate); A:P, acetate:propionate ratio; PP, prepubertal heifers; PB, pubertal heifers; PG, pregnant heifers; Means + standard deviation followed by different letters in a column are different by Tukey's HSD ($P < 0.10$).

the sequences were not classified at the genus level, although 9.88% of the sequences belong to the genus *Ruminococcus*, 4.50% to *Butyrivibrio*, 3.03% to *Coprococcus*, and 2.50% to *Clostridium*. The remaining genera were each responsible for less than 1% of the total sequences. In the phylum Bacteroidetes, 60.78% of the sequences belonged to the genus *Prevotella*, 4.07% to the candidate genus CF231 (family Paraprevotellaceae), and 32.01% of the sequences were not classified to the genus level.

All archaeal community sequences were attributed to the phylum Euryarchaeota. The relative abundance at the genus level was dominated by the *Methanobrevibacter* (95.03% ± 1.89), followed by *Methanosphaera* (4.32% ± 1.76) (Fig. 1B). Sequences identified as belonging to the genera *Vadin CA11* and *Methanomicrococcus* had relative abundances lower than 1% (0.62% ± 1.24 and 0.03% ± 0.11, respectively).

For the anaerobic fungal community, 90.23% ± 5.97 of the sequences belonged to the phylum Chytridiomycota, while the remaining 9.77% ± 6.18 were unclassified. At the genus level, the relative abundance was 52.23% ± 26.34 for unclassified sequences, 45.36% ± 26.04 for *Caecomyces*, 2.04% ± 2.13 for *Orpinomyces,* and 0.37% ± 1.33 for *Neocallimastix* (Fig. 1C).

A total of 38 bacterial OTUs were found in 100% of the samples, including OTUs within the genera *Prevotella*, *Ruminococcus*, *Coprococcus*, *Butyrivibrio*, *Clostridium*, *Shuttleworthia*, and candidate genera SHD-231, CF231, and p-75-a5. Archaeal genera shared among all samples include the *Methanobrevibacter* and *Methanosphaera*. For anaerobic fungi, only one OTU appeared in all samples, which was classified as *Caecomyces communis*.

The rumen microbiota varies by physiological stage

The overall bacterial community appeared to change from PB to PP and PG heifers, with trends toward increasing CH_4 emissions, dDMI, dOMI, BW, and acetate:propionate in older animals (Fig. 2). PB heifers had a greater bacterial diversity (ANOVA, Tukey HSD $P = 0.02$) than PP heifers (Table S4, Table S5), but the bacterial community structure (Bray–Curtis) and composition (Jaccard) did not significantly differ (ANOSIM $P < 0.05$; Table S6) among PP, PB, and PG heifer groups or between CH_4 groups. Bacterial species richness (Chao1) and diversity (Shannon) also did not differ between high- and low-CH_4 -production groups (CH_4, ANOVA P = 0.28; Table S4, Table S5).

With respect to the relative sequence abundance, those belonging to the phylum Firmicutes did not differ by physiological stage or CH_4 group (Kruskal–Wallis and Wilcoxon test $P > 0.05$; Table 3, Table S7). In contrast, the sequence abundance of the Bacteroidetes was lower in PP than in PB heifers (Kruskal–Wallis $P = 0.04$) and tended to be lower in low CH_4 emitters (Wilcoxon test $P = 0.08$). Overall, the ratio of Firmicutes to Bacteroidetes was greater in PP than in PB heifers (Kruskal–Wallis $P = 0.04$), tended to be greater in PP than in PG heifers (Kruskal–Wallis $P = 0.08$) and was greater in low CH_4 emitters (Wilcoxon test $P = 0.04$). At the genus level, sequences classified to *Acetobacter* were found to be more abundant in PB and PG heifers, and also in the high CH_4 emission group (Kruskal–Wallis and Wilcoxon test $P < 0.05$). Sequences classified to the genus *Eubacterium* were more abundant in PP heifers and low CH_4 emissions heifers (Kruskal–Wallis and Wilcoxon test $P < 0.05$).

For the archaeal community, diversity and richness did not differ between PP, PB, and PG heifers, or by CH_4 group (ANOVA $P > 0.05$; Table S4, Table S5). However, archaeal community composition and structure differed according to physiological stage (ANOSIM $P < 0.05$) and CH_4 group (ANOSIM $P < 0.01$; Table S6). The relative abundance of sequences belonging to the genus *Methanosphaera* was different (Kruskal–Wallis $P < 0.05$; Table 3, Table S7) between PP, PB, and PG heifers, and was found to decrease as heifers aged and gained body weight (Fig. 1B). The relative abundance of sequences belonging to the *Methanosphaera* was 1.85 times greater in low CH_4-emitting heifers (Wilcoxon test $P = 0.01$), whereas the relative abundance of sequences belonging to the *Methanobrevibacter* was only 1.02 times greater in high CH_4 emitters (Wilcoxon test $P = 0.04$) and trended toward lower abundances in PP than in PB and PG heifers (Kruskal–Wallis $P = 0.08$). Sequences belonging to the candidate genus Vadin CA11 were found to be more highly abundant in PB (Kruskal–Wallis $P = 0.05$) and PP (Kruskal–Wallis $P = 0.03$) heifers when compared with PG heifers (Table 3, Table S7).

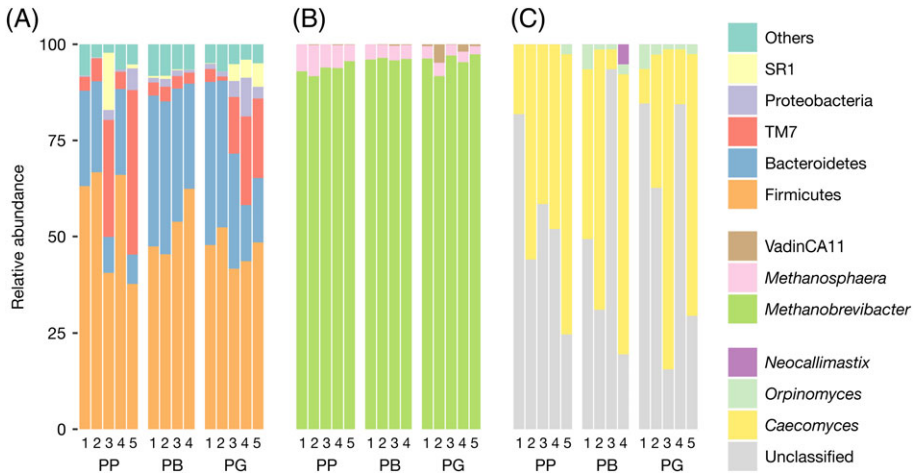

Figure 1. Relative abundances of bacterial (A), archaeal (B), and anaerobic fungal (C) taxa in the rumens of growing heifers. Bacteria are displayed at the phylum level, while archaea and fungi are at the genus level. PP, prepubertal heifers; PB, pubertal heifers; PG, pregnant heifers.

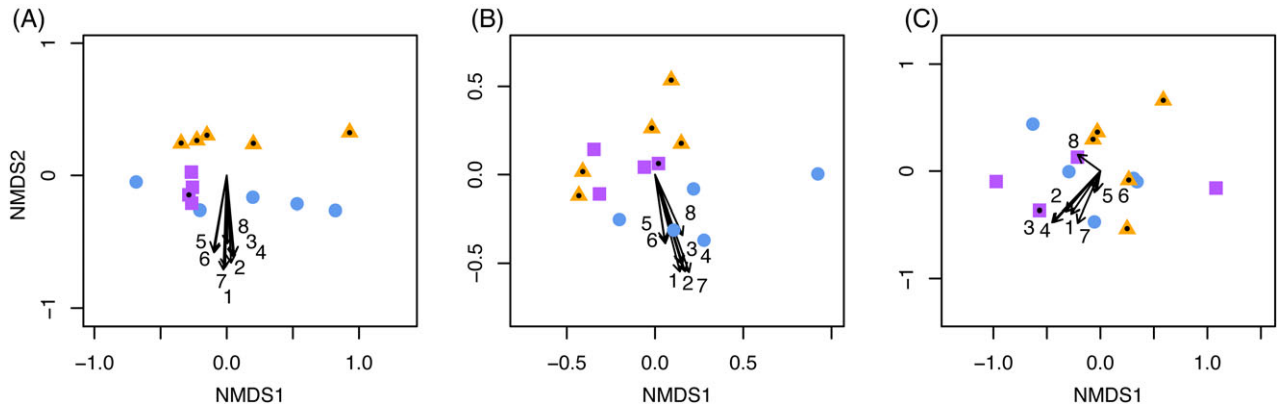

Figure 2. Non-metric multidimensional scaling plot of the Bray–Curtis dissimilarity index of (A) bacterial, (B) archaeal, and (C) fungal community structure. Animals are represented by orange triangles (prepubertal heifers, PP), purple squares (pubertal heifers, PB), and blue circles (pregnant heifers, PG). Those with black dots are low-emitting animals. Production metrics fitted by maximum correlation (envfit) and scaled to 0.75, 0.75, and 1.5, respectively, to fit plots. 1, CH_4 g day^{-1}; 2, CH_4 g kg^{-1} DMI; 3, CH_4 g kg^{-1} dDMI; 4, CH_4 g kg^{-1} dOMI; 5, dDMI; 6, dOMI; 7, BW; 8, acetate:propionate ratio.

For the anaerobic fungal microbiota, both community structure (Bray–Curtis) and composition (Jaccard) did not differ (ANOSIM $P < 0.05$; Table S6) among PP, PB, and PG heifers or between CH_4 groups. PB heifers had a trend toward greater fungal richness relative to PP heifers (ANOVA, Tukey HSD $P = 0.07$; Table S4, Table S5). The genus *Orpinomyces* was found to have lower sequence abundance in the low CH_4-emitting heifers (Wilcoxon test $P = 0.01$) and tended to be lower in PP heifers than in either PB or PG heifers (Kruskal–Wallis $P < 0.10$).

Correlation among microbial taxa, physiological, and production traits

A correlation analysis was performed to determine the relationships between bacterial, archaeal and anaerobic fungal taxa, CH_4 emissions, and production variables (Fig. 3, Table S8). The strongest significant correlations between the bacterial community and CH_4 emissions were observed for OTUs classified to the families Bifidobacteriaceae and RF16, as well as for the genera *Acetobacter, Eubacterium*, p-75-a5, and *Shuttleworthia*, with correlations ranging from −0.60 to 0.47 (Spearman's test $P < 0.10$).

OTUs within the family Bifidobacteriaceae and genera *Acetobacter, Clostridium*, *Coprococcus, Fibrobacter,* and *Schwartzia* showed the strongest positive correlations with dDMI and dOMI. Negative correlations with these variables were observed for OTUs in the genera *Eubacterium,* p-75-a5, and SHD-231, with correlations ranging from −0.59 to 0.52 (Spearman's test $P < 0.01$). For average body weight, positive correlations were found to OTUs classified to *Acetobacter*, Bifidobacteriaceae, and *Coprococcus* (r = 0.67 to r = 0.76, Spearman's test $P < 0.01$), while negative correlations to the genera *Eubacterium* and p-75-a5 (r = −0.75 and r = −0.61, Spearman's correlation $P < 0.05$) were determined.

For the archaeal community, *Methanobrevibacter* was weakly correlated with CH_4 emissions when it was corrected for the digestible fractions of feed (dDMI and dOMI). The genus *Methanosphaera* showed strong negative correlations to CH_4 g kg^{-1} of dDMI (r = −0.74, Spearman's test $P = 0.01$) and CH_4 g kg^{-1} of dOMI (r = − 0.79, Spearman's test $P = 0.01$). The candidate genus Vadin CA11 was found to be positively correlated with CH_4 emissions (r = 0.66, Spearman's test $P = 0.01$ and r = 0.65, Spearman's test $P = 0.01$, respectively). Only *Methanosphaera* was found to be significantly correlated with dDMI (r = −0.59, Spearman's test $P = 0.02$) and dOMI (r = −0.60, Spearman's test $P < 0.02$). Body weight was negatively correlated with *Methanosphaera* (r = −0.88, Spearman's test $P = 0.01$) and positively correlated with Vadin CA11 (r = 0.53, Spearman's test $P = 0.05$).

Table 3. Relative abundances of abundant microbial taxa

Amplicon	Taxa	PP	PB	PG	Low	High
Bacteria	Firmicutes	54.88 ± 14.36	52.31 ± 7.62	46.82 ± 4.18	54.71 ± 12.85	48.69 ± 6.41
Bacteria	Bacteroidetes	17.53 ± 8.38*B*	35.21 ± 5.71*A*	28.33 ± 12.47*AB*	20.38 ± 10.24*b*	30.98 ± 10.78*a*
Bacteria	Firmicutes:Bacteroidetes	3.54 ± 1.07*A*	1.55 ± 0.52*B*	1.96 ± 0.91*B*	3.21 ± 1.26*a*	1.810.80 b
Bacteria	*Acetobacter*	0.05 ± 0.06*B*	0.86 ± 0.46*A*	0.81 ± 0.49*A*	0.19 ± 0.34*b*	0.82 ± 0.48*a*
Bacteria	*Eubacterium*	0.03 ± 0.04*A*	0.00 ± 0.00*B*	0.00 ± 0.00*B*	0.03 ± 0.04*a*	0.00 ± 0.00*b*
Bacteria	*p-75-a5*	0.13 ± 0.07	0.08 ± 0.06	0.05 ± 0.03	0.12 ± 0.07	0.06 ± 0.05
Archaea	*Methanobrevibacter*	93.63 ± 1.43*B*	96.14 ± 0.26*A*	95.55 ± 2.37*A*	94.00 ± 1.56*b*	95.81 ± 1.83*a*
Archaea	*Methanosphaera*	6.28 ± 1.43*A*	3.70 ± 0.23*B*	2.86 ± 0.51*C*	5.86 ± 1.63*a*	3.16 ± 0.59*b*
Archaea	*Vadin CA11*	0.10 ± 0.09*B*	0.16 ± 0.15*B*	1.51 ± 1.85*A*	0.14 ± 0.14	0.98 ± 1.58
Fungi	*Orpinomyces*	0.52 ± 1.16*B*	2.94 ± 2.44*A*	2.85 ± 2.10*A*	0.65 ± 1.08*b*	3.09 ± 2.16*a*

Means in any row that are followed by different upper case letters are different by the Kruskal–Wallis test ($P < 0.10$). Means + standard deviation in any row followed by different lower case letters are different by the Wilcoxon test ($P < 0.10$). Rows without letters were not significantly different between any groups.
PP, prepubertal heifers; PB, pubertal heifers; PG, pregnant heifers.

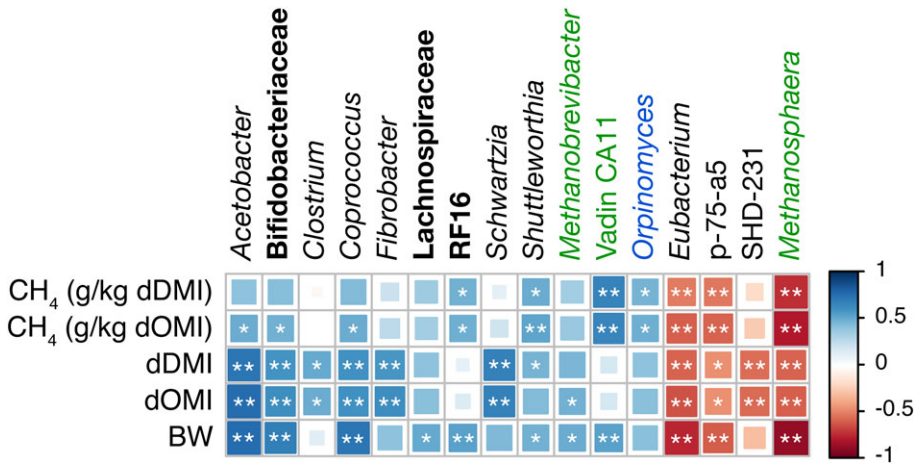

Figure 3. Spearman's rank correlations of CH_4 emissions, production parameters, and microbial taxa. Names in bold indicate families, whereas non-bold names indicate genera. Names in black indicate bacteria, green indicate archaea, and blue indicate fungi. Correlation values are displayed as color and size within blocks. CH_4: methane emissions; dDMI: digestible dry matter intake; dOMI: digestible organic matter intake. *$P \leq 0.05$; **$P \leq 0.01$.

For the fungi, the genus *Orpinomyces* was found to be correlated with energy-corrected CH_4 emissions expressed as CH_4 g kg^{-1} of dDMI (r = 0.46, Spearman's test $P = 0.09$) and CH_4 g kg^{-1} of dOMI (r = 0.48, Spearman's test $P = 0.08$).

None of the bacteria, archaea, and anaerobic fungi found to be correlated with CH_4 emissions showed any significant correlations (Mantel's test $P > 0.1$) with the overall VFA profile or individual concentrations of acetate, propionate, or butyrate (Table 2).

DISCUSSION

Ruminant livestock are responsible for a large proportion of worldwide CH_4 production,[44] with methanogenic archaea as the primary producers of CH_4 in the gastrointestinal tract. As a result, methanogens have been the target of many enteric CH_4 mitigation strategies. However, the relationship between rumen methanogens and CH_4 is unclear, with previous work finding weak[45] to no correlation[17,46] between absolute methanogen abundance and CH_4 production in adult cattle. Moreover, little to no work has considered the methanogenic output of heifers, even though these animals produce more CH_4 per unit feed intake than older, lactating cows.[11,12] Here, we sought to more fully understand the relationship between ruminal microbial communities and enteric CH_4 emissions in dairy heifers at different physiological stages. To accomplish this, rumen microbial communities in prepubertal (PP), pubertal (PB), and pregnant heifers (PG) were assessed along with CH_4 emissions and production traits to identify future targets for mitigation efforts in these young animals.

Within the rumen archaeal community, *Methanosphaera* exhibited strong negative correlations with CH_4 emissions, and its sequence abundance was higher in PP and low CH_4-emitting heifers. This finding is consistent with previous reports in sheep,[3] but contrasts with studies on adult beef cattle, where a greater abundance of *Methanosphaera* was identified in high CH_4-emitting animals.[47] These results may point to differences in rumen CH_4 production as it relates to body weight, since both sheep and young dairy heifers have lower body weights than adult beef cattle. This is supported by our finding in this study that body weight was negatively correlated with *Methanosphaera* abundance across all heifer physiological stages. This highlights the potential need for different mitigation strategies across the life of the cow.

In contrast, *Methanobrevibacter* did not significantly correlate with CH_4 in this study, likely due to the difficulty in resolving the sequences generate in this study to the species and strain level. Importantly, *Methanobrevibacter* species belonging to the *smithii–gottschalkii–millerae–thaurei* (SGMT) clade are associated with higher CH_4 emissions than those in the *ruminantium–olleyae* (RO) clade,[17] which may partially explain this finding. Additionally, candidate genus Vadin CA11 showed significant positive correlations with CH_4 emissions and, in general, its relative abundance was greater in groups with greater CH_4 emission levels. However, as a candidate genus with no cultured isolates, little is known about this group, and further studies are required to determine its role in rumen methanogenesis.

Taken together, and given the use of relative abundances in this study, these archaeal microbiota results support the previous hypothesis that reductions in CH_4 production by ruminants may be achieved by shifting the archaeal community from predominately *Methanobrevibacter* to *Methanosphaera* [3]. In particular, members of the *Methanobrevibacter* produce 1 mol of CH_4 for each mole of CO_2 reduced,[48] while members of the *Methanosphaera* produce 3 mol of CH_4 per 4 mol of methanol consumed.[49] Thus, higher abundances of *Methanosphaera* may translate to less CH_4 production per unit of carbon input. However, interventions directly targeting the archaeal community often do not result in measurable reductions in CH_4 emissions, and those that have some success usually have negative consequences for animal health or production.[50] Since archaea rely on the degradation products of other microorganisms in the rumen, particularly bacteria and fungi,[2] alterations to these other communities may provide alternative solutions to reducing methanogenesis in livestock.

Our study shows that bacteria from genera *Eubacterium* and *Acetobacter* are negatively and positively correlated with CH_4 emissions, respectively. Species in the genus *Eubacterium* are non-saccharolytic, ammonia-hyperproducing bacteria that are thought to play an important role in amino acid fermentation.[51] In contrast, members of the *Acetobacter* are characterized by their ability to produce acetate[52] and, though usually obligate aerobes, some species have been found to persist with trace oxygen[53] at levels that can transiently exist in the rumen.[54] Importantly, ammonia production in the rumen consumes hydrogen gas,[55] while acetate production generates hydrogen gas.[4,10] Thus, *Eubacterium* may contribute to CH_4 reduction by depriving methanogens of hydrogen for methanogenesis, while *Acetobacter* may positively contribute to CH_4 production by increasing hydrogen availability. This is further supported by our findings of higher concentrations of acetate in high CH_4-producing animals. We note that the bacterial taxa identified in this study contrast with those found in previous work that identified *Prevotella* and *Succinivibrio* from a comparison of PP heifers (9–10 months of age, similar in age to the PP heifers used in this study) to older cows (45+ months of age).[16]

Finally, our analysis of the ruminal fungal communities did not identify any putative mitigation targets. This is likely a result of the sampling method, which predominantly represents the liquid portion of the rumen community. Since anaerobic fungi are primarily found attached to solid particles within the rumen,[56] this method was likely insufficient to fully evaluate this specific community.

In summary, this study presents a comprehensive view of the rumen microbiota of heifers as it relates to CH_4 production, and identifies microbes in the genera *Methanobrevibacter* and *Acetobacter* as potential targets for reduction as a means of lowering CH_4 production. In addition, our findings also suggest that increasing the abundances of microbes in the genera *Methanosphaera* and *Eubacterium* may also lead to reductions in CH_4 production due to the metabolic features of members within these genera. While some of these taxa were previously identified as potential targets in older ruminants, our work describes their association with CH_4 emissions in heifers, and further identifies other novel ruminal microbes that may serve as potential candidates for CH_4 mitigation strategies.

ACKNOWLEDGEMENTS

This work was supported by Brazilian governmental agencies CNPq, CAPES, FAPEMIG, INCT-CA, Embrapa, and PECUS-RumenGases. We thank all members of the Veloso, Marcondes, Mantovani, and Suen labs for their support and helpful discussions. We would like to acknowledge the CAPES Doctorate Sandwich Program that supported C.S.C. This work was also supported, in part, by the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) Agriculture and Food Research Initiative (AFRI) HATCH grant #WIS01729 and foundational program grant 2015-67015-23246 to G.S.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Roehe R, Dewhurst RJ, Duthie C-A, Rooke JA, McKain N, Ross DW *et al.*, Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. Leeb T, editor. *PLoS Genet* **12**:e1005846 (2016) [Internet]. Available: http://dx.plos.org/10.1371/journal.pgen.1005846 [1 December 2017].

2 Flint HJ, Bayer EA, Rincon MT, Lamed R and White BA, Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Micro* [Internet]. **6**:121–131 (2008).

3 Kittelmann S, Pinares-Patiño CS, Seedorf H, Kirk MR, Ganesh S, McEwan JC *et al.*, Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS ONE* **9**:e103171 (2014) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/25078564 [1 December 2017].

4 Boadi D, Benchaar C, Chiquette J and Massé D, Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can J Anim Sci* **84**:319–335 (2004) [Internet]. Available: http://www.nrcresearchpress.com/doi/10.4141/A03-109 [3 December 2017].

5 Stewart CS, Flynt HJ and Bryant MP, The rumen bacteria, in *The Rumen Microbial Ecosystem*, ed. by Hobson P and Stewart C. Blackie Academic and Professional, New York, NY, pp. 10–72 (1997).

6 IPCC, in *Climate Change 2014: Synthesis Report*, ed. by Pachauri RK and Meyer LA. Intergovernmental Panel on Climate Change, Geneva, p. 151 (2014).

7 Johnson KA and Johnson DE, Methane emissions from cattle. *J Anim Sci* **73**:2483 (1995) [Internet]. Available: https://www.animalsciencepublications.org/publications/jas/abstracts/73/8/2483 [1 December 2017].

8 de Menezes AB, Lewis E, O'Donovan M, O'Neill BF, Clipson N and Doyle EM, Microbiome analysis of dairy cows fed pasture or total mixed ration diets. *FEMS Microbiol Ecol* **78**:256–265 (2011) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21671962 [1 December 2017].

9 Reis WLS, Detmann E, Batista ED, Rufino LMA, Gomes DI, Bento CBP *et al.*, Effects of ruminal and post-ruminal protein supplementation in cattle fed tropical forages on insoluble fiber degradation, activity of fibrolytic enzymes, and the ruminal microbial community profile. *Anim Feed Sci Technol* **218**:1–16 (2016) [Internet] Available: https://

www.sciencedirect.com/science/article/pii/S0377840116301717 [1 December 2017].
10 Van Soest PJ, *Nutritional Ecology of the Ruminant*. Cornell University Press, Ithaca, NY (1994).
11 Jiao H, Yan T, Wills DA, Carson AF and McDowell DA, Development of prediction models for quantification of total methane emission from enteric fermentation of young Holstein cattle at various ages. *Agric Ecosyst Environ* **183**:160–166 (2014) [Internet]. Available: http://www.sciencedirect.com/science/article/pii/S0167880913003885 [1 December 2017].
12 Grandl F, Zeitz JO, Clauss M, Furger M, Kreuzer M and Schwarm A, Evidence for increasing digestive and metabolic efficiency of energy utilization with age of dairy cattle as determined in two feeding regimes. *Animal* **12**:515–527 (2018). [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/28735600 [1 December 2017].
13 Dill-McFarland KA, Breaker JD and Suen G, Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci Rep* **7**:40864 (2017).
14 Cunha IS, Barreto CC, Costa OYA, Bomfim MA, Castro AP, Kruger RH *et al.*, Bacteria and Archaea community structure in the rumen microbiome of goats (Capra hircus) from the semiarid region of Brazil. *Anaerobe* **17**:118–124 (2011).
15 Jami E and Mizrahi I, Composition and similarity of bovine rumen microbiota across individual animals. *PLoS ONE* **7**:1–8 (2012) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22432013 [1 December 2017].
16 Liu C, Meng Q, Chen Y, Xu M, Shen M, Gao R *et al.*, Role of age-related shifts in rumen bacteria and methanogens in methane production in cattle. *Front Microbiol* **8**:1563 (2017) [Internet]. Available: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01563/full [1 December 2017].
17 Danielsson R, Schnürer A, Arthurson V and Bertilsson J, Methanogenic population and CH_4 production in swedish dairy cows fed different levels of forage. *Appl Environ Microbiol* **78**:6172–6179 (2012) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22752163 [1 December 2012].
18 Kumar S, Indugu N, Vecchiarelli B and Pitta DW, Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. *Front Microbiol* **6**:781 (2015) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4521595/ [1 December 2017].
19 Detmann E, Souza MA, Valadares Filho SC, Queiroz AC, Berchielli TT, Saliba EOS *et al.*, *Métodos para análise de alimentos*, 1st edn. Suprema, Visconde do Rio Branco (2012).
20 *Official Methods of Analyses of the AOAC*, 15th edn. Association of Official Analytical Chemists (AOAC) Assoc OFF Agric Chemp, Washington D.C., p. 1-1230 (1990).
21 Lana RP, *Nutrição e Alimentação animal: mitos e realidades*. Universidade Federal de Viçosa, Viçosa, p. 344 (2005).
22 Berchielli T, Pires AV and Oliveira SG, *Nutrição de Ruminantes*, Vol. **583**. FUNEP, Jaboticabal (2006).
23 Johnson K, Huyler M, Westberg H, Lamb B and Zimmerman P, Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. *Environ Sci Technol* **28**:359–362 (1994) [Internet]. Available: http://pubs.acs.org/doi/abs/10.1021/es00051a025 [1 December 2017].
24 Oss DB, Marcondes MI, Machado FS, LGR P, Tomich TR, Ribeiro GO *et al.*, An evaluation of the face mask system based on short-term measurements compared with the sulfur hexafluoride (SF6) tracer, and respiration chamber techniques for measuring CH_4 emissions. *Anim Feed Sci Technol* **216**:49–57 (2016) [Internet]. Available: https://www.sciencedirect.com/science/article/pii/S0377840116300979 [3 December 2013].
25 Johnson KA, Westberg HH, Michal JJ and Cossalman MW, The SF6 tracer technique: methane measurement from ruminants, in *Measuring Methane Production from Ruminants*, ed. by Makkar HS and Vercoe P. Springer, Dordrecht, the Netherlands, pp. 33–67 (2007).
26 Lodge-Ivey SL, Browne-Silva J and Horvath MB, Technical note: Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. *J Anim Sci* **87**:2333 (2009) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/19329475 [1 December 2017].
27 Henderson G, Cox F, Kittelmann S, Miri VH, Zethof M, Noel SJ *et al.*, Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS ONE* **8**:e74787 (2013) [Internet]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24040342 [1 December 2017].
28 Siegfried VR, Ruckermann H, Stumpf G, Siegfried BD, Ruckemann H, Siegfried R. Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirt Forsch (1984). [Internet]. Available: https://www.scienceopen.com/document?vid=12545918-f45c-4b26-ad06-72577b416f09 [1 December 2017].
29 Stevenson D and Weimer P, Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl Microbiol Biotechnol [Internet]* **75**:165–174 (2007).
30 Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI *et al.*, Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One [Internet]* **8**:e47879 (2013).
31 Cunha CS, Veloso CM, Marcondes MI, Mantovani HC, Tomich TR, Pereira LGR *et al.*, Assessing the impact of rumen microbial communities on methane emissions and production traits in Holstein cows in a tropical climate. *Syst Appl Microbiol* **40**:492 (2017, 9) [Internet]. Available: http://www.sciencedirect.com/science/article/pii/S0723202017300966?via%3Dihub [1 December 2017].
32 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB *et al.*, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**:7537–7541 (2009).
33 Kozich JJ, Westcott SL, Baxter NT, Highlander SK and Schloss PD, Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* **79**:5112–5120 (2013) [Internet]. Available: http://aem.asm.org/content/early/2013/06/17/AEM.01043-13.abstract [1 December 2017].
34 Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J *et al.*, SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**:7188–7196 (2007).
35 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K *et al.*, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**:5069–5072 (2006) [Internet]. Available: http://aem.asm.org/content/72/7/5069.abstract [1 December 2017].
36 Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M *et al.*, Towards a unified paradigm for sequence-based identification of fungi. *Mol Ecol* **22**:5271–5277 (2013) [Internet]. Available: https://doi.org/10.1111/mec.12481 [1 December 2017].
37 R Core Team, *R: A Language and Environment for Statistical Computing*. [Internet]. R Foundation for Statistical Computing, Vienna (2017) Available: http://www.r-project.org/ [1 December 2017].
38 Charrad M, Ghazzali N, Boiteau V and Niknafs A, NbClust: an R package for determining the relevant number of clusters in a data set. *J Stat Softw* **61**:1–36 (2014).
39 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. *Vegan: Community Ecology Package* cran.rproject.org 2015, 2.2-1. [Internet]. Available: http://cran.r-project.org/package=vegan [1 December 2017].
40 Clarke KR, Non-parametric multivariate analysis of changes in community structure. *Aust J Ecol* **18**:117–143 (1993).
41 de Mendiburu F. *agricolae: statistical procedures for agricultural research*, cran.rporject.org; 2016 Available: https://CRAN.R-project.org/package=agricolae [1 December 2017].
42 Mantel N, The detection of disease clustering and a generalized regression approach. *Cancer Res* **27**:209–220 (1967) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/6018555 [1 December 2017].
43 Wei T. *Package "Corrplot" – Visualization of a Correlation Matrix* [Internet]. cran.rproject.org; 2012. Available: http://cran.r-project.org/web/packages/corrplot/corrplot.pdf [1 December 2017].
44 Lassey KR, Livestock methane emission: From the individual grazing animal through national inventories to the global methane cycle. *Agric For Meteorol* **142**:120–132 (2007) [Internet]. Available: http://www.sciencedirect.com/science/article/pii/S0168192306002978 [1 December 2017].
45 Wallace RJ, Rooke JA, Duthie C-A, Hyslop JJ, Ross DW, McKain N *et al.*, Archaeal abundance in post-mortem ruminal digesta may

help predict methane emissions from beef cattle. *Sci Rep* **4**:5892 (2014) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/25081098 [1 December 2017].
46 Zhou M, Chung Y-H, Beauchemin KA, Holtshausen L, Oba M, McAllister TA *et al.*, Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. *J Appl Microbiol* **111**:1148–1158 (2011) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21848695 [1 December 2017].
47 Wallace RJ, Rooke JA, McKain N, Duthie C-A, Hyslop JJ, Ross DW *et al.*, The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* **16**:839 (2015) [Internet]. Available: http://www.biomedcentral.com/1471-2164/16/839 [1 December 2017].
48 Deppenmeier U, The unique biochemistry of methanogenesis. *Prog Nucleic Acid Res Mol Biol* **71**:223–283 (2002) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/12102556 [3 December 2017].
49 Fricke WF, Seedorf H, Henne A, Krüer M, Liesegang H, Hedderich R *et al.*, The genome sequence of Methanosphaera stadtmanae reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. *J Bacteriol* **188**:642–658 (2006) [Internet]. Available: http://jb.asm.org/content/188/2/642.abstract.
50 Hook SE, Wright A-DG and McBride BW, Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* **2010**: 945785 (2010) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21253540 [1 December 2017].
51 Wallace RJ, McKain N, McEwan NR, Miyagawa E, Chaudhary LC, King TP *et al.*, Eubacterium pyruvativorans sp. nov., a novel non-saccharolytic anaerobe from the rumen that ferments pyruvate and amino acids, forms caproate and utilizes acetate and propionate. *Int J Syst Evol Microbiol* **53**(Pt 4):965–970 (2003) [Internet]. Available: http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.02110-0 [1 December 2017].
52 Andrés-Barrao C, Saad MM, Chappuis M-L, Boffa M, Perret X, Ortega Pérez R *et al.*, Proteome analysis of Acetobacter pasteurianus during acetic acid fermentation. *J Proteomics* **75**:1701–1717 (2012) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22155126 [1 December 2017].
53 Millet V, Vivas N and Lonvaud-Funel A, The development of the bacterial microflora in red wine during ageing in barrels. *J des Sci Tech la Tonnellerie* **1**:137–150 (1995).
54 Scott RI, Yarlett N, Hillman K, Williams AG, Lloyd D, Williams T, The presence of oxygen in rumen liquor and its effects on methanogenesis. *J Appl Bacteriol* **55**:143–149 (1983).
55 van Zijderveld SM, Gerrits WJJ, Apajalahti JA, Newbold JR, Dijkstra J, Leng RA *et al.*, Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J Dairy Sci* **93**:5856–5866 (2010) [Internet]. Available: http://www.sciencedirect.com/science/article/pii/S0022030210006375 [1 December 2017].
56 McAllister TA, Bae HD, Jones GA and Cheng KJ, Microbial attachment and feed digestion in the rumen. *J Anim Sci* **72**:3004–3018 (1994) [Internet]. Available: http://www.ncbi.nlm.nih.gov/pubmed/7730196 [1 December 2017].

