Comparisons of bacterial and archaeal communities in the rumen and a dual-flow continuous culture fermentation system using amplicon sequencing

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ABSTRACT: Dual-flow continuous culture (CC) fermenters are commonly used to study rumen fermentation in vitro. Research using culture-based and oligonucleotide techniques has shown that certain microbial populations within fermenters may be maintained at abundances similar to those observed in vivo. In this study, bacterial and archaeal communities in the rumen of dairy cattle and in a dual-flow CC fermentation system were compared using high-throughput amplicon sequencing targeting the V4 hypervariable region of 16S rRNA. We hypothesized that the in vitro system harbored a comparable bacterial and archaeal community to that observed in the rumen. Members of the *Bacteroidetes* and *Firmicutes* made up the 2 most abundant phyla in the rumen, inoculum,

and fermenters and did not differ among sample types ($P > 0.10$). Similarly, *Prevotellaceae*, the most abundant family in all 3 sample types, did not differ based on source ($P = 0.80$). However, beta diversity analyses revealed that bacterial and archaeal communities differed between fermenters and rumen samples ($P \leq 0.001$), but fermenter bacterial and archaeal communities stabilized by day 4 of each period. While the overall bacterial and archaeal community differs between natural rumens and those detected in in vitro fermenter systems, several prominent taxa were maintained at similar relative abundances suggesting that fermenters may provide a suitable environment in which to study shifts among the predominant members of the microbial community.

Key words: 16S rRNA sequencing, continuous culture, microbiome, rumen

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INTRODUCTION

The study of rumen fermentation can be aided by in vitro continuous culture (CC) systems that simulate the rumen environment. Compared with in vivo models, in vitro methods are less expensive, less time-consuming, and more tightly controlled (Hristov et al. 2012). One of the most commonly used in vitro models is the dual-flow CC fermenter system developed by Hoover et al. (1976b), which maintains fermentation of rumen inocula within

a ~1-liter fermentation vessel by constant addition of feed substrate, bicarbonate buffer, and N₂ or CO₂ to maintain anaerobic conditions, as well as differential removal of solid and liquid effluents. This system has been shown to closely approximate the in vivo effects of fiber digestion and VFA profile (Hoover et al. 1976a, 1976b). However, the ability for this system to maintain certain microbial populations is still unclear.

Experiments characterizing the microbial communities in CC fermenters have been limited in the scope of the microbial community examined, but recent development of next-generation sequencing (NGS) of 16S rRNA amplicons has provided the ability to study microbial environments on a

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community-wide scale. Thus far, a limited number of studies have used 16S rRNA sequencing in communities from CC rumen fermenters. Jin et al. (2016) profiled the microbiome of dual-flow CC fermenters to examine the effects of urea supplementation on the rumen community. The long-term rumen simulation technique (RUSITEC) is another commonly used CC fermentation apparatus in which feed is placed within nylon bags that are agitated inside fermentation vessels containing rumen inocula. Within RUSITEC fermenters, 16S sequencing has been used to study the effects of chitosan and ivy fruit saponins (Belanche et al. 2015) and 2 species of brown seaweed (Belanche et al. 2016) on the rumen microbiome. Despite the emerging use of NGS to monitor changes in rumen microbial communities within in vitro systems, there has been, to our knowledge, no research comparing the microbiomes present in these systems with the true in vivo community.

Understanding how the microbial community in CC fermenters relates to the microbiome of natural rumens is critical for drawing conclusions about treatments applied within these systems. In addition to a lack of comparisons between rumens and CC fermenters, there is little knowledge about changes in the microbiome of CC fermenters over time. Dual-flow CC fermenters traditionally use a 6- or 7-d adaptation period prior to sampling (Windschitl and Stern 1988; Cardozo et al. 2004; Jenkins et al. 2014). This duration was originally chosen based on the results of Hoover et al. (1976b) which demonstrated no differences in in vitro fermentation between days 6 through 9 and 11 through 14 of the experiment. However, more recent evidence confirming this adaptation period length is sparse. Examination of changes in the bacterial and archaeal community of CC fermenters over time may provide insight into the length of incubation time required to achieve a stable microbiome.

To address the paucity of information regarding the microbial communities and community dynamics in CC fermenters, we utilized a NGS amplicon sequencing approach to characterize bacterial and archaeal communities in these systems. The objectives of this experiment were to compare and contrast the bacterial and archaeal communities in the rumen of dairy cows and those of dual-flow CC fermenters, and investigate temporal changes in the community present within fermenters. We expected that some rumen taxa, particularly those associated with fiber degradation and rumen papillae, would decrease in vitro, but the overall

microbial community structure would remain similar to natural rumens.

METHODS

Experimental Diets and Treatments

Cows and fermenters were fed the same basal diet formulated to meet or exceed requirements of a Holstein cow producing 40 kg of milk/d with 2.8% fat and 3.7% protein (NRC 2001). The experimental diet contained approximately 50% corn silage, 14% protein mix, 13% alfalfa haylage, 8% ground corn, 4% corn gluten, 3% molasses, and 2% cottonseed (Table 1). The diet fed to the cows was collected immediately after mixing, and pelleted for use as substrate for CC fermenters. Briefly, diets were mixed and dried in a forced air oven at 55 °C for 48 h. After drying, the diet was ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 2-mm screen, and pelleted in a CL-5 California pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter × 12 mm long. Pellets were dried at room temperature for 48 h. The nutrient composition of the pellets was analyzed and was confirmed to be similar to the nonpelleted diet. DM of the pellets was measured on days 0, 5, and 7 of both experimental periods (AOAC 2005).

Cows and Rumen Contents Collection

Two multiparous, lactating Holstein dairy cows were fitted with rumen cannulas according to the guidelines set by the University of Minnesota Animal Care Committee (Protocol ID: 1304-30557A). Cows were adapted to the basal diet for 21 d prior to rumen sample collection. Fifty milliliters of rumen contents (40 mL liquid and 10 mL solids) were manually collected in conical tubes across 4 time points at 0, 2, 4, and 6 h post-feeding. To ensure a representative community, rumen contents were composited from the cranial and caudal regions of the ruminoreticulum, whereas rumen solids were collected from multiple regions of the rumen mat. Approximately 5 liters of rumen inoculum from each cow were separately collected into prewarmed thermoses, blended at low speed in a Waring blender (Waring Products Inc.), strained through 4 layers of cheesecloth, and homogenized. After filtration and homogenization, 100 mL of inocula were collected from each cow and immediately frozen at −50 °C for DNA extraction and 16S

Table 1. Ingredient and chemical composition of basal experiment diet

Item	Composition ^a
Feed composition	
Corn silage	48.5
Protein mix ^b	13.7
Alfalfa haylage	12.3
Ground corn grain	7.6
Corn gluten feed	4.2
Molasses	3.3
Cottonseed	2.2
Alfalfa hay	1.7
Grass hay	1.6
Energy booster	0.8
Chemical composition	
CP, %	16.1
Undegraded CP (RUP ^c), % of CP	38.8
Degraded CP (RDP ^c), % of CP	61.2
Soluble CP, % of CP	33.0
NDF, %	29.9
ADF, %	19.1
NDFd ^c , %	45.7
Ash	
Starch, %	26.3
Sugar, %	4.4
NFC ^c , %	42.5
Crude fat, %	5.1
TDN, %	74.3
NE _t , Mcal/kg DM	0.78

^aComposition as % of 100 °C DM unless otherwise noted.

^bProtein mix composition (DM basis): fine-rolled corn, 37%; soybean meal, 15%; canola meal, 12%; treated soybean meal, 10%; distillers dried grains, 8%; blood meal, 5%; calcium carbonate, 4%; sodium bicarbonate, 4%; trace minerals, 2%; protected methionine, 2%; salt, 2%; potassium carbonate, 1%; urea, 1%.

^cRUP, rumen undegradable protein; RDP, rumen degradable protein; NDFd, NDF digestibility; NFC, non-fiber carbohydrates.

rRNA gene sequencing. Inoculum from each cow was randomly assigned to 4 prewarmed fermenters (1048 ± 28 mL per fermenter). Pelleted diet (25 g) was added to the fermenters immediately after inoculation.

CC Operation

Eight CC fermenters, as described by [Hannah et al. \(1986\)](#), were used in 2 consecutive 10-d periods, with 7 d of adaptation and 3 d of sampling. Fermenters were modified with a pH measurement and control system, which maintained fermenter pH between 5.6 and 6.4 by automated addition of 5 N sodium hydroxide or 3 N hydrochloric acid. Fermenter pH was recorded every 15 s using an electronic data acquisition system (DASYLab

v13.0, Measurement Computing, Norton, MA). Pelleted feed was provided to the fermenters at a rate of 75 g of DM/liter of fermenter volume/d by an automated feeding system that delivered feed at eight 90-min intervals across the day. Artificial saliva buffer (pH = 8.29) was prepared according to [Weller and Pilgrim \(1974\)](#) to provide a final concentration (g/liter) of NaHCO₃, 5.0; Na₂HPO₄, 1.76; KHCO₃, 1.6; KCL, 0.6; MgSO₄, 0.05; and urea, 0.4. Liquid dilution rate for each fermenter was set to 10%/h through steady infusion of artificial saliva buffer, while solids dilution rate was set at 5.5%/h by regulating liquid removal. Liquid effluent was removed from fermenters through two 300-μm porosity stainless steel filters using peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL). Anaerobic conditions were maintained within fermenters by the addition of N₂ gas at a rate of 20 mL/min. Fermenter temperature was maintained at 38.5 ± 0.1 °C by indwelling heaters. A magnetic agitator stirred fermenter contents at 350 rpm to maintain homogenous culture conditions.

Sample Collection

Thirty milliliter samples of solid and liquid effluent were collected from fermenters at 0900, 1100, and 1300 h and composited by day. Samples were immediately frozen at -20 °C after collection. Daily effluent samples were stored at -60 °C until DNA extraction. On the final 3 d of each experimental period, composited solid and liquid effluent was collected into separate vessels maintained at 1 °C in a water bath to reduce enzymatic and microbial activity. Solid and liquid effluents were combined, composited by fermenter for all 3 sampling days and analyzed for DM, OM, ash, NDF, ADF, VFA, total nitrogen flow, ammonia-nitrogen (NH₃-N) flow, and total purines. A portion of the effluent sample was lyophilized for analysis of DM, OM, NDF, ADF, ash, and purines. At the end of each experimental period, fermenter contents were filtered through 4 layers of cheesecloth and centrifuged at 1,000 × g to remove feed particles. Supernatant was then centrifuged at 20,000 × g to isolate microbial cells which were collected and lyophilized for analysis of DM, OM, total N, and purines.

DNA Extraction

Daily solid and liquid effluent samples were thawed and 16.5 mL of solid and 13.5 mL of liquid

effluent were combined and mixed. These volumes were chosen to match the 55:45 ratio of solid:liquid effluent leaving the fermenters. Aliquots of rumen contents (7.5 mL) from each of the 4 time points were pooled and mixed. Rumen, inoculum, and fermenter effluent samples were blended in a Waring blender to remove solid-associated bacteria from feed particles, and centrifuged at $1,000 \times g$ to sediment the remaining feed. Supernatant was centrifuged at $4,000 \times g$ to isolate the bacterial pellet. DNA was extracted from the microbial cells using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Quantity and quality of DNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Rumen and inoculum sample nucleic acid concentrations averaged 102 ng/ μ L. Nucleic acid concentration in fermenter samples averaged 71 ng/ μ L. DNA quality was assessed spectrophotometrically by $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ and $A_{260 \text{ nm}}/A_{230 \text{ nm}}$ ratios. Rumen and inoculum samples averaged 1.86 and 1.22 for $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ and $A_{260 \text{ nm}}/A_{230 \text{ nm}}$ ratios, respectively. Fermenter effluent generated an average $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ ratio of 1.86 and an average $A_{260 \text{ nm}}/A_{230 \text{ nm}}$ ratio of 1.44. These values are similar to those previously reported for rumen microbial DNA (Kang et al. 2009; Popova et al. 2010; Henderson et al. 2013).

DNA Sequencing and Sequence Processing

The V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the 515F/806R bar-coded primer set with KAPA HiFidelity Hot Start Polymerase (Gohl et al. 2016). Pooled, size-selected samples were denatured with NaOH, diluted to 8 pmol in Illumina's HT1 buffer, spiked with 15% PhiX, and heat-denatured at 96 °C for 2 min immediately before loading into the sequencer. Replicate sequence data were generated using paired-end sequencing of purified amplicon pools using Illumina MiSeq [300 nucleotide (nt) read length] (Illumina, Inc., San Diego, CA). Library preparation and sequencing were performed by the University of Minnesota Genomics Center. Sequencing data have been deposited into GenBank at the NCBI (<http://www.ncbi.nlm.nih.gov/>) under accession number SRP119665.

Sequence processing was performed using mothur software ver. 1.34.0 (Schloss et al. 2009). Sequences were trimmed to 100 nt, paired-end alignment was performed using fastq-join (Aronesty 2013), and sequences were screened for quality. Sequences were excluded from analysis if they had a quality score less than 35 over a 50 nt

window, a mismatch to a primer or barcode sequence, homopolymers that were greater than 8 nt or at least one ambiguous base. Singleton sequences were removed in mothur and chimeras were removed using UCHIME (Edgar et al. 2011). The number of sequence reads in each sample was normalized by random subsampling to 30,000 sequence reads per sample. Sequences were aligned against the SILVA database ver. 119 (Quast et al. 2012). Operational taxonomic units (OTUs) were clustered using the furthest-neighbor algorithm at 97% similarity and OTUs were classified against the Ribosomal Database Project ver. 14.

Chemical Analysis

DM and ash content of the lyophilized effluent and microbial cells, and the experimental diet were determined by drying in an oven at 105 °C for 24 h followed by combustion in a muffle furnace at 550 °C for 24 h (AOAC 2005). Sequential detergent fiber analysis was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F58 fiber bags (ANKOM Corp., Fairport, NY). The digestibility of NDF and ADF was corrected for ash content within samples. $\text{NH}_3\text{-N}$ was determined on the supernatant of centrifuged ($5,000 \times g$) effluent by steam distillation with magnesium oxide using a Kjeltac 2300 Analyzer Unit (Foss Tecator AB, Höganäs, Sweden). Total N of both effluent and diet was determined via the Kjeldahl method (AOAC 1990). Purine concentration of lyophilized effluent and microbial pellets was determined according to the procedure by Zinn and Owens (1986) with spectrometry performed on a Synergy 2 Plate Reader with a Take3 Micro-volume plate (BioTek Instruments, Inc., Winooski, VT). The purine to N ratio in effluents and microbial cells was used to determine flow of bacterial N and OM in the effluent.

Effluent VFA concentrations were determined using gas chromatography. Prior to chromatography, fermenter effluent was clarified by centrifugation at $5,000 \times g$ for 10 min. Supernatant proteins were denatured using 25% meta-phosphoric acid, frozen overnight at -20°C and thawed, followed by an additional centrifugation at $5,000 \times g$ for 10 min to remove denatured proteins. Clarified fluid was filtered through a 0.45- μm polyethersulfone micro-pore filter (Thermo Fisher Scientific Inc., Waltham, MA). VFA concentration was measured using an HP6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) with a 2 m $\times$ 6.35 mm $\times$ 2 mm

Carbopack glass column (Supelco, Bellefonte, PA) as previously described (Ceconi et al. 2015).

Statistical Analysis

OM, DM, fiber, crude protein (CP) digestibility, VFA production, individual VFA concentrations, nitrogen flows, and pH were analyzed as a randomized block design with experimental period serving as a block and inoculum donor equally represented within block using the GLM procedure of SAS 9.2 (SAS Institute, Inc., Cary, NC). Fermentation pH recorded every 15 s during 3 d of sampling was summarized to determine arithmetic mean, minimum, and maximum on an hourly basis. Repeated measures analysis was performed on the hourly averages using the MIXED procedure of SAS with a compound covariance structure. The model treated inoculum donor as a fixed effect and fermenter nested within period as a random effect. Time spent below pH 5.8, between pH 5.8 and 6.2, and above 6.2 were calculated using trapezoidal integration. Data were analyzed using the GLM procedure of SAS using LSMEANS with the PDIFF option.

Alpha diversity, calculated as the Shannon index of diversity, and abundance-based coverage (ACE) estimate of richness were calculated using *mothur*. The effect of sample type (rumen vs. inocula vs. fermenter) on bacterial and archaeal community composition was examined using data from the last 3 days (days 8 to 10) of each period to avoid the confounding effects of fermenter adaptation. ANOVA analyses were performed with Tukey's post hoc test. Comparisons of β -diversity between samples were made using Bray–Curtis dissimilarity matrices (Bray and Curtis 1957). Differences in Bray–Curtis dissimilarity between sample types and days of fermenter operation were assessed using analysis of similarity (ANOSIM), which uses rank order differences in community structure based on Bray–Curtis dissimilarity (Clarke 1993), with a Bonferroni correction for multiple comparisons. Principal coordinate analysis (PCoA) was performed on Bray–Curtis distances in *mothur*, and abundances of families that correlated with ordination position were determined using the *corr.axes* function in *mothur* with Spearman correlations.

Comparisons between relative abundances of OTUs at the domain, phylum, class, order, family, and genus level were made among rumen, inocula, and fermenter samples, as well as across days of incubation within fermenters using the MIXED procedure of SAS 9.2. The effect of sample type at each taxonomic level was analyzed using the

fixed effects of sample type, period, and their interaction, the random effect of inoculum donor, and the repeated effect of day. For comparisons between sample types, fermenter samples prior to day 8 were excluded to prevent the confounding effects of fermenter adaptation. Day of fermenter operation was also analyzed using the fixed effects of day, period, and day by period interaction, and the random effect of inoculum donor. Mean abundances at each taxonomic level were separated by sample type using the LSMEANS function with the PDIFF option and a Tukey adjustment. Statistical significance was determined at $P < 0.05$, while a trend was recognized at $0.10 > P \geq 0.05$.

RESULTS AND DISCUSSION

Fermentation Parameters

Digestion of OM, DM, fiber, and CP, VFA production, individual VFA concentrations, nitrogen flows, and pH were analyzed to confirm that operation of CC fermenters was similar to previous studies. For all fermenters, apparent and true DM digestibility averaged 41.3% and 55%, respectively, while apparent and true OM digestion averaged 31.7% and 44.1%, respectively (Supplementary Table S1). Total $\text{NH}_3\text{-N}$ concentration averaged 8.1 mg/dL, CP degradation averaged 66.7%, and efficiency of microbial protein synthesis (EMPS) averaged 21.8 g of N/kg of truly digested OM. Means for total VFA, A:P ratio, and branched-chain VFA were 99.5, 1.73, and 0.91 mM, respectively (Supplementary Table S2). Acetate concentration within fermenters was 48.1 mol/100 mol of VFA, while propionate was 27.7, and butyrate was 13.4 mol/100 mol VFA. Hourly mean, minimum, and maximum pH were 5.76, 5.70, and 5.83, respectively. Average pH of fermenters was lower than that of natural rumens, which averaged 6.15. This reduction in pH may have been due to decreased buffering capacity of the McDougall's buffer due to vaporization of CO_2 gas (Licitra et al. 1996). Over the 3-d sampling period, time below pH 5.8 was 2,834 min, time between pH 5.8 and 6.2 was 1,439 min, and time above pH 6.2 was 34.3 min. All fermentation parameters were within ranges previously observed in dual-flow CC studies (Hannah et al. 1986; Mansfield et al. 1995; Bach et al. 2008).

Community Alpha Diversity

Among all samples characterized by Illumina sequencing, a mean Good's coverage estimate of

98.3 $\pm$ 0.4% was achieved, indicating nearly complete community characterization. Individual samples were estimated to have between 1,063 and 4,091 OTUs, as determined using ACE, although diversity was significantly lower among fermenter samples compared to the rumen or inocula ($P < 0.0001$; Fig. 1A). Richness also declined throughout fermenter operation and was significantly lower on day 10 than the time of inoculation ($P < 0.0001$; Fig. 1B). Alpha diversity, measured using the Shannon index, was similarly lower among fermenter samples than in communities from the rumen and inocula ($P < 0.0001$; Fig. 1C), and diversity similarly trended toward a significant decrease through the 10 d of operation ($P = 0.051$; Fig. 1D). The decrease in microbial diversity within CC may be due to the relatively homogeneous environment present within fermenters. Microorganisms inhabit various ecological niches within the rumen, including the high-fiber rumen mat, the liquid phase containing partially digested feed, and the rumen wall which possesses its own unique microbial community (Kong et al. 2010). The CC fermenter system

in the current experiment uses constant agitation to mix rumen contents, and therefore does not allow distinct communities to form.

Community Beta Diversity

Bray–Curtis dissimilarity matrices were used to determine β -diversity (differences in community composition) between samples, and the effects of sample type (rumen vs. inocula vs. fermenter) were assessed using ANOSIM. Analysis of similarity revealed that β -diversity varied by sample type ($r = 0.54$, $P < 0.001$), with community differences occurring between fermenters and both rumen ($r = 0.56$, $P = 0.001$) and inocula ($r = 0.60$, $P < 0.001$) samples, but not between inocula and rumens ($r = -0.08$, $P = 0.665$). These results indicate that the bacterial community within fermenters is modified compared to the rumen, and that bacterial community profiling of fermenters should be interpreted with caution. Several characteristics inherent to CC fermenters may be responsible for the observed changes in bacterial and archaeal

Figure 1. Comparisons of alpha diversity among sample types and days of fermenter operation. (A) ACE richness by sample type. (B) ACE richness by day of fermenter operation. (C) Shannon index by sample type. (D) Shannon index by day of fermenter operation. Sample type comparisons were performed using rumen ($n = 4$), inocula ($n = 4$), and fermenter samples collected from days 8 to 10 ($n = 48$). Each sampling day was represented by 16 samples. Error bars represent the SEM for each sample type or day of fermenter operation.

communities. In the Hoover et al. (1976b) system, fermenters are continuously homogenized with a spinning agitator, whereas the rumen mixes contents with pulsatile peristaltic contractions of distinct sacs (Hristov et al. 2012). Pulsatile contraction creates stratification of the rumen environment, allowing survival of slow-growing microbes, such as protozoa (Carter and Grovum 1990). Rumen papillae are also absent from fermenters, resulting in a lack of nutrient absorption, osmotic regulation, and presence of the epimural bacterial community. These inherent differences in the fermentation environment of the rumen and CC fermenters likely contribute to the observed differences in bacterial and archaeal communities. Future work with CC fermenters should focus on modifying the agitation and turnover rates of these systems to maintain a core microbiome more similar to that of the rumen. A promising alteration to the dual-flow CC fermenters has been described by Karnati et al. (2009), which added a multistage filtration system to maintain protozoa at a much higher abundance than unmodified fermenters. It is expected that the bacterial and archaeal communities of the modified system may more closely resemble the rumen microbiome than those observed in the current study.

Interestingly, there was no change in β -diversity between rumen and inocula, suggesting collection, transport, and filtration did not impact the bacterial community. These results were surprising considering that rumen samples consisted of composited rumen contents from 4 sampling times throughout the day, while inoculum was collected from a single time point 4 h post-feeding, and the fact that the pH of the composited rumen samples was 6.15 while inoculum pH was 5.56. Previous research indicates that cows with widely different rumen pH values may have similar bacterial community compositions (Palmonari et al. 2010). Weimer et al. (2010) observed that after near-complete exchange of rumen fluid between 2 cows, the cow's microbial community returns to its original structure in less than 24 h, even though pH was dramatically reduced during fluid transplantation. This observation suggests that microbial populations are resilient to short-term declines in pH. In the current study, inocula maintained the same microbial community as rumens, despite a drastic decline in pH.

Temporal Changes in Fermenter Community

The effect of incubation time within CC fermenters on Bray–Curtis dissimilarity was visualized using PCoA (Fig. 2) and evaluated statistically

using ANOSIM. Principal coordinate analysis revealed that day 0 samples exhibited a unique clustering pattern that slightly overlapped with rumen and inoculum samples. Analysis of similarity confirmed that there was no difference in community composition among rumen, inocula, or day 0 samples ($r = -0.08$ to 0.17 , $P \geq 0.172$). By day 3, fermenters had a significantly different community composition than inocula, which persisted throughout the rest of the study period ($r \geq 0.54$, $P < 0.001$). However, throughout the 10 d study period, communities in fermenters on specific days did not differ from rumen communities at Bonferroni-corrected $\alpha = 0.001$ ($r \leq 0.60$). This result contrasts with the analysis by sample type in which samples from days 8 to 10 were pooled, and may suggest that differences between the rumen and fermenters among pooled samples reflect only very minor and short-term temporal variations in community membership and abundance. Communities in fermenters at day 0 were significantly different from communities at day 3 and onward ($r = 0.29$ to 0.49 , $P < 0.001$), communities between days 3 and 9 differed significantly ($r = 0.20$, $P < 0.001$), and day 10 communities also differed significantly from those at days 2 and 3 ($r = 0.27$ and 0.26 , $P < 0.001$). No other comparisons among fermenter communities differed significantly, suggesting that the communities had normalized after 3 d.

Digestion experiments using dual-flow CC fermenters customarily use adaptation periods of 6 to 8 d prior to sampling (Cardozo et al. 2004; Jenkins et al. 2014; Lascano et al. 2016). This adaptation period length was originally established in the classic paper by Hoover et al. (1976b), which showed no difference in the fermentation parameter results from samples collected either from days 6 to 9 or from days 11 to 14. Results of the current experiment suggest that bacterial and archaeal communities acclimate somewhat faster than previously thought and are adapted by day 4 after inoculation. Lengowski et al. (2016) demonstrated that the relative abundance of protozoa, methanogens, total bacteria, *F. succinogenes*, *R. albus*, *R. amylophilus*, *P. bryantii*, and *S. ruminantium* decreased during the first 48 h after inoculation in a RUSITEC system, but did not change between days 2 and 13, which is more similar to the results observed in the current experiment.

Bacterial and Archaeal Community Composition

Domain. Comparisons of relative abundances of OTUs among rumens, inocula, and fermenters

Figure 2. Principal coordinate analysis plot of Bray–Curtis dissimilarities among rumen, inocula, and fermenter samples ($r^2 = 0.66$). Families shown were among the most abundant that correlated significantly with ordination position (Spearman correlations, $P < 0.05$).

were performed at 6 taxonomic levels: domain, phylum, class, order, family, and genus. Fermenter samples from days 0 through 7 were excluded from analysis to avoid the potentially confounding effect of fermenter adaptation. As expected, bacteria greatly outnumbered archaea within rumens, inocula, and fermenters, representing more than 98% of the total OTUs in all sample types, compared with less than 0.3% for archaea (Fig. 3; Supplementary Table S3). Numerically, there was little change in the relative abundance of bacteria, which was observed at 98.7% in rumens, and 99.1% in both inoculum and fermenters. However, among rumen samples, there tended to be a greater percentage of OTUs that could not be classified to either bacteria or archaea ($P = 0.07$; Supplementary Table S3). Archaeal abundances did not differ between sample types ($P = 0.97$).

Previous reports suggest an increase in bacterial abundance occurs within CC fermenters. Mansfield et al. (1995) observed an increase in the viable count of bacteria in fermenters, compared to in vivo abundances. Similarly, Ziemer and colleagues (2000) observed increases in the absolute and relative abundances of bacterial small subunit rRNA after 240 h in CC. In particular, bacteria increased 11%, relative to the abundance of archaea. The increased bacterial abundance in both studies was associated with a decrease in in vitro protozoal numbers, and likely resulted from lower predation of bacteria by protozoa. The protozoal community was not examined in the current experiment due to the unavailability of 18S primers validated for the phylogenetic characterization of protozoa at the time of the study. Recently,

primers have been developed for sequencing ciliated protozoa, but to our knowledge they have not been used to study the rumen microbial community, and their ability to capture important rumen protozoa is unknown (Ishaq and Wright 2014). Furthermore, results from previous experiments suggest that the contribution of protozoa to the overall community within CC fermenters is minimal (Mansfield et al. 1995). A consistent reduction, and often complete depletion of protozoa, is observed in nearly all CC fermenter systems as a result of slow protozoal growth rates and high turnover rates within fermenters (Hristov et al. 2012).

Phylum. Consistent with previous characterizations of the rumen, *Bacteroidetes* and *Firmicutes* were the most abundant phyla present in all 3 sample types (Kim et al. 2011; Jami et al. 2013).

Figure 3. Relative abundance of bacterial and archaeal domains within rumen, inoculum, and fermenter samples. ¹Domains denoted with a dagger (†) tend to differ by sample type ($0.05 < P < 0.10$). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14.

Neither the abundance of *Bacteroidetes* ($P = 0.18$) nor *Firmicutes* ($P = 0.39$) differed among rumen, inocula, or fermenters (Fig. 4; Supplementary Table S4). Similarly, Mao et al. (2013) observed no difference in relative abundances of *Bacteroidetes* or *Firmicutes* in dairy cows experiencing subacute rumen acidosis. In the current study, the pH of rumen samples averaged 6.15, whereas inocula averaged 5.56 and fermenters averaged 5.76. The ability of these 2 phyla to be maintained within wide ranges of rumen pH may have allowed for their preservation within inocula and fermenters. Abundances of members of *Proteobacteria*, the third most abundant phylum, were dissimilar between sample types ($P < 0.001$), occurring at a greater abundance in fermenter samples, compared with inocula ($P = 0.01$). *Proteobacteria* are highly enriched within the epimural microbial community, and many members of this phylum are thought to be microaerophiles or facultative anaerobes (Sadet-Bourgeteau et al. 2010; Chen et al. 2011). Enrichment of *Proteobacteria* in CC may be due to an inability to generate a truly anaerobic environment within fermenters, encouraging growth of oxygen-utilizing species. The relative abundances of members of the *Tenericutes* ($P = 0.01$), *Spirochaetes* ($P < 0.001$), and *Verrucomicrobia* ($P < 0.001$) also differed between sample types, with fermenters having decreased abundances of *Tenericutes* and *Verrucomicrobia* and greater abundances of members of the *Spirochaetes* phylum, compared with rumens and inocula.

Class. At the class level, *Bacteroidia* and *Clostridia* were the first and second most abundant groups present in all sample types, respectively (Fig. 5; Supplementary Table S5). These results agree with previous observations of dairy cow rumens (Lazuka et al. 2015). Rumen, inocula, and fermenter samples did not differ in relative abundances of members of the *Bacteroidia* ($P = 0.17$) or *Clostridia* ($P = 0.33$) classes. *Bacteroidia* and *Clostridia* were each the most abundant phyla within the *Bacteroidetes* and *Firmicutes* classes, respectively, with *Bacteroidia* making up between 97% and 100% of total *Bacteroidetes* and *Clostridia* comprising between 42% and 50% of total *Firmicutes*. Results observed in the current study differed from those detected by Lazuka et al. (2015). In their experiment, *Clostridia* was the most abundant class present in rumen inocula at 43% of total bacteria, but decreased 2-fold in anaerobic batch culture fermenters. In the study by Lazuka and colleagues, donor cows were nonlactating, and were fed greater concentrations of forage than in the current experiment (Lazuka et al. 2015).

Figure 4. Relative abundance of bacterial and archaeal phyla within rumen, inoculum, and fermenter samples. ¹Phyla denoted with an asterisk (*) differ by sample type ($P < 0.05$). Means with divergent superscripts are different at $P < 0.05$ (Tukey-adjusted). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14. ³Phyla present at less than 1.0% relative abundance.

Clostridia contain several well-described fiber-degrading species including *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Eubacterium cellulosolvens* (Krause et al. 2003). Furthermore, Petri et al. (2013) detected a substantial decrease in *Clostridia*, and a concomitant increase in *Bacteroidia* when cows were switched from a high-forage to a high-grain diet. Together, these results suggest that the differences observed between the current and previous studies are likely related to differences in dietary fiber.

Gammaproteobacteria ($P = 0.01$) and *Mollicutes* ($P = 0.01$), the third and fourth most abundant classes, showed significantly different abundances among sample types, with *Gammaproteobacteria* having greater abundance in fermenters compared to inocula ($P = 0.01$), and *Mollicutes* having lower abundance in fermenters than in rumens ($P = 0.04$) or inocula ($P = 0.04$). *Spirochaetes* ($P < 0.001$), the Verruco-5 clade ($P < 0.001$), *Erysipelotrichi* ($P < 0.001$), and *Betaproteobacteria* ($P = 0.001$) were also dissimilar among sample types. Both *Spirochaetes* and Verruco-5 had greater abundances in vitro compared to natural rumens and inocula, whereas *Erysipelotrichi* abundances were lower in inoculum and fermenters compared to rumens, and *Betaproteobacteria* was lower in fermenters than in both rumens and inoculum.

Order. *Bacteroidales* ($P = 0.17$) and *Clostridiales* ($P = 0.33$), the 2 most prominent orders (Fig. 6; Supplementary Table S6), did not differ among rumen, inocula, or fermenters. These orders made up 100% of *Bacteroidia* and *Clostridia*, respectively. The third most abundant order, *Aeromonadales*,

Figure 5. Relative abundance of bacterial and archaeal classes within rumen, inoculum, and fermenter samples. ¹Classes denoted with an asterisk (*) differ by sample type ($P < 0.05$). Means with divergent superscripts are different at $P < 0.05$ (Tukey-adjusted). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14. ³Classes present at less than 1.0% relative abundance.

differed by sample type ($P = 0.01$), with a greater relative abundance in fermenters than inocula ($P = 0.01$). *Burkholderiales* were present at 0.02% and 0.01% of total bacteria in rumens and inocula, respectively, but increased to 0.71% of the bacterial population in fermenters ($P = 0.01$). Increases in *Burkholderiales* were demonstrated in cows fed isoflavone (Kasparovska et al. 2016). The enrichment of *Burkholderiales* within CC fermenters suggests that it may be a poor model for studying the effects of isoflavones on the rumen microbial community. *Erysipelotrichales* abundances also differed by sample type ($P < 0.001$), and was lower in fermenters than rumens ($P = 0.01$).

Family. *Prevotellaceae* was the most abundant bacterial family in all sample types, and their relative abundance was not different between rumen, inocula, or fermenters ($P = 0.80$; Fig. 7; Supplementary Table S7). A large number of microbial families were unable to be classified at the family level, with 13%, 20%, and 22% unclassifiable in fermenter, inocula, and rumen samples, respectively. *Ruminococcaceae* remained unchanged between rumens, inocula, and fermenters ($P = 0.68$), making up 7.2%, 8.2%, and 6.1% of OTUs, respectively, and was correlated with ordination position among these samples and fermenters at days 0 and 1 (Fig. 2). The third most abundant family was *Succinivibrionaceae* which exhibited a 13-fold greater relative abundance in the fermenters compared to inoculum ($P = 0.02$; Fig. 2), and tended to differ between fermenters and rumens ($P = 0.09$). *Succinivibrionaceae* are primarily amylolytic bacteria, and the increase observed in the current study is consistent with

Figure 6. Relative abundance of bacterial and archaeal orders within rumen, inoculum, and fermenter samples. ¹Orders denoted with an asterisk (*) differ by sample type ($P < 0.05$). Means with divergent superscripts are different at $P < 0.05$ (Tukey-adjusted). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14. ³Classes present at less than 0.5% relative abundance.

previous research demonstrating greater digestion of total nonstructural carbohydrates in CC compared with in vivo measurements (Mansfield et al. 1995). *Veillonellaceae* constituted between 5.03% and 8.33% of total OTU abundance and did not differ between sample types ($P = 0.34$), although abundances were correlated with fermenter communities near the end of the study period (Fig. 2). The relative abundance of *Paraprevotellaceae* decreased in fermenters compared to rumens ($P = 0.003$), and tended to be greater in inocula than fermenters ($P = 0.06$). *Lachnospiraceae* were also affected by sample type ($P = 0.01$), with a greater abundance occurring in fermenters than rumens ($P = 0.03$) or inocula ($P = 0.01$). Abundances of families within the *Bacteroidales* order were also significantly correlated with ordination position, and showed a strong association, based on position, with rumen, inocula, and early fermenter communities (Fig. 2). **Genus.** Classification of OTUs at the genus level was poor, with an average of 41% of rumen sequences, 31.4% of inocula sequences, and 38.4% of fermenter sequences unclassifiable (Fig. 8; Supplementary Table S8). This high number of unclassified genera illustrates the need for improved characterization of rumen microbial taxonomy. Of the identified genera, *Prevotella* was the most abundant, comprising 38.9%, 52.4%, and 43.8% of rumen, inocula, and fermenter samples, respectively. Despite the numerical differences in *Prevotella* abundances among sample types, these changes were not significant ($P = 0.77$). Results of the current experiment are consistent with observations by Stevenson and Weimer (2007), who used qPCR to

Figure 7. Relative abundance of bacterial and archaeal families within rumen, inoculum, and fermenter samples. ¹Families denoted with an asterisk (*) differ by sample type ($P < 0.05$). Means with divergent superscripts are different at $P < 0.05$ (Tukey-adjusted). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14. ³Classes present at less than 1.0% relative abundance.

demonstrate that *Prevotella* was the dominant genus within the rumen. Many species of *Prevotella* have well-described, metabolically important functions within the rumen. For example, *P. ruminicola* and *P. bryantii* possess a diverse host of glycoside hydrolases, allowing them to digest noncellulosic polysaccharides (Purushe et al. 2010). Furthermore, many members of the *Prevotella* genus have been noted for their resistance to ionophore antibiotics (Newbold et al. 1993; Callaway and Russell 1999). Results from the current experiment imply little change in abundance of *Prevotella* between natural rumens and dual-flow CC fermenters, proposing that they may be accurately modeled in this in vitro system.

Members of the *Ruminococcus* genus also remained unchanged between rumens, inocula, and fermenters ($P = 0.95$), with no numerical difference in abundance between rumens and fermenters. The two most prominent members of the *Ruminococcus* genus, *R. albus* and *R. flavefaciens*, have important roles in the ruminal fiber degradation (Russell 2002). However, the presence of amylolytic species such as *R. bromii* emphasizes the diverse functionality of this genus (Ze et al. 2012). The maintenance of *Ruminococcus* within CC fermenters at a relative abundance similar to in vivo suggests that differences observed in vitro may correspond to similar changes in the cow. *Succinivibrionaceae* exhibited a dramatic

change among sample types ($P = 0.03$), making up 3.5% of total OTUs in rumens, but only 0.4% in fermenters ($P = 0.01$). The genus CF231 in the *Paraprevotellaceae* family differed considerably by sample type ($P < 0.001$), with variation from 2.53% in rumens to 1.25% in inocula ($P < 0.001$) and 0.34% in fermenters ($P < 0.001$). *Treponema* exhibited a similar change in relative abundance

Figure 8. Relative abundance of bacterial and archaeal genera within rumen, inoculum, and fermenter samples. ¹Genera denoted with an asterisk (*) differ by sample type ($P < 0.05$), while those denoted with a dagger (†) tend to differ ($0.05 < P < 0.10$). Means with divergent superscripts are different at $P < 0.05$ (Tukey-adjusted). ²OTUs unable to be classified within the Ribosomal Database Project ver. 14. ³Classes present at less than 1.0% relative abundance.

in vitro, from 1.84% in rumens to 0.41% in fermenters ($P < 0.001$). *Megasphaera* tended to differ between sample types ($P = 0.06$), and exhibited a greater relative abundance within fermenters. *Dialister* also displayed a substantially greater abundance within fermenters, but the change was not significant ($P = 0.10$).

Conclusions

Results of this study indicate that diversity and richness of the bacterial and archaeal community were lower in fermenters compared with rumens and inocula. The bacterial and archaeal communities differed between rumens and fermenters, but the relative abundances of the most abundant groups within each taxonomic level remained unchanged. At the phyla level, *Bacteroidetes* and *Firmicutes* did not vary between samples, and both *Prevotella* and *Ruminococcus*, the most abundant classified genera, also were maintained in fermenters at levels similar to in vivo. The observed changes in the bacterial and archaeal community appear to be influenced by a small number of lower abundance taxa, within *Proteobacteria* at the phyla level, and both *Succinivibrionaceae* and *Paraprevotellaceae* at the family level. Additionally, several metabolically important genera such as *Succinivibrionaceae* and *Megasphaera* were present at different relative abundances in vitro. The current study maintains that fermenters may be an appropriate model for investigating differences in relative abundance of certain bacterial taxa such as *Prevotella* and *Ruminococcus*, but its efficacy for studying whole-community dynamics may be limited. This experiment also suggests that the bacterial and archaeal community of CC fermenters does not show significant variation after 3 d of operation, and proposes that a 4-d adaptation period is appropriate for stabilization of the bacterial and archaeal community. This study was limited in that no examination of protozoal or fungal communities was made, and future research should consider their contributions to the fermenter microbial community. Furthermore, the relationship between rumen and fermenter communities may be affected by nutrient composition of the diet, and studying the effects of substrate, pH levels, and turnover rates on the microbial populations of CC would be valuable. The present study provides baseline OTU relative abundances for future rumen microbial ecology experiments using CC fermenters with comparable fermentation conditions.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. All authors declare no conflict of interest.

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LITERATURE CITED

- AOAC. 1990. Official Methods of Analysis. 15th ed. Association of Official Analytical Chemists, Arlington, VA.
- AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists, Arlington, VA.
- Aronesty, E. 2013. Comparison of sequencing utility programs. *Open Bioinform. J.* 7:1–8. doi:10.2174/1875036201307010001
- Bach, A., M. Ruiz Moreno, M. Thrune, and M. D. Stern. 2008. Evaluation of the fermentation dynamics of soluble crude protein from three protein sources in continuous culture fermenters. *J. Anim. Sci.* 86:1364–1371. doi:10.2527/jas.2007-0736
- Belanche, A., E. Jones, I. Parveen, and C. J. Newbold. 2016a. A metagenomics approach to evaluate the impact of dietary supplementation with *Ascomyces nodosus* or *Laminaria digitata* on rumen function in rusitec fermenters. *Front. Microbiol.* 7:1–14. doi:10.3389/fmicb.2016.00299
- Belanche, A., E. Pinloche, D. Preskett, and C. J. Newbold. 2016b. Effects and mode of action of chitosan and ivy fruit saponins on the microbiome, fermentation and methanogenesis in the rumen simulation technique. *FEMS Microbiol. Ecol.* 92:1–13. doi:10.1093/femsec/fiv160
- Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27:325–349. doi:10.2307/1942268
- Callaway, T. R., and J. B. Russell. 1999. Selection of a highly monensin-resistant *Prevotella bryantii* subpopulation with altered outer membrane characteristics. *Appl. Environ. Microbiol.* 65:4753–4759.
- Cardozo, P. W., S. Calsamiglia, A. Ferret, and C. Kamel. 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. *J. Anim. Sci.* 82:3230–3236. doi:10.2527/2004.82113230x
- Carter, R. R., and W. L. Grovum. 1990. A review of the physiological significance of hypertonic body fluids on feed intake and ruminal function: salivation, motility and microbes. *J. Anim. Sci.* 68:2811–2832. doi:10.2527/1990.6892811x
- Ceconi, I., M. J. Ruiz-Moreno, N. Dilorenzo, A. Dicostanzo, and G. I. Crawford. 2015. Effect of urea inclusion in diets containing corn dried distillers grains on feedlot cattle performance, carcass characteristics, ruminal fermentation, total tract digestibility, and purine derivatives-to-creatinine index. *J. Anim. Sci.* 93:357–369. doi:10.2527/jas2014-8214

- Chen, Y., G. B. Penner, M. Li, M. Oba, and L. L. Guan. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Appl. Environ. Microbiol.* 77:5770–5781. doi:10.1128/AEM.00375-11
- Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* 18:117–143. doi:10.1111/j.1442-9993.1993.tb00438.x
- Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 27:2194–2200. doi:10.1093/bioinformatics/btr381
- Gohl, D. M., P. Vangay, J. Garbe, A. MacLean, A. Hauge, A. Becker, T. J. Gould, J. B. Clayton, T. J. Johnson, R. Hunter, et al. 2016. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat. Biotechnol.* 34:942–949. doi:10.1038/nbt.3601
- Hannah, S. M., M. D. Stern, and F. R. Ehle. 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of mixed diets containing various soya bean products. *Anim. Feed Sci. Technol.* 16:51–62. doi:10.1016/0377-8401(86)90049-0
- Henderson, G., F. Cox, S. Kittelmann, V. H. Miri, M. Zethof, S. J. Noel, G. C. Waghorn, and P. H. Janssen. 2013. Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One.* 8:e74787. doi:10.1371/journal.pone.0074787
- Hoover, W. H., B. A. Crooker, and C. J. Sniffen. 1976a. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* 43:528–534. doi:10.2527/jas1976.432528x
- Hoover, W. H., P. H. Knowlton, M. D. Stern, and C. J. Sniffen. 1976b. Effects of differential solid-liquid removal rates on fermentation parameters in continuous cultures of rumen contents. *J. Anim. Sci.* 43:535–542. doi:10.2527/jas1976.432535x
- Hristov, A. N., C. Lee, R. Hristova, P. Huhtanen, and J. L. Firkins. 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. *J. Dairy Sci.* 95:5299–5307. doi:10.3168/jds.2012-5533
- Ishaq, S. L., and A.-D. G. Wright. 2014. Design and validation of four new primers for next-generation sequencing to target the 18S rRNA genes of gastrointestinal ciliate protozoa. *Appl. Environ. Microbiol.* 80:5515–5521. doi:10.1128/AEM.01644-14
- Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7:1069–1079. doi:10.1038/ismej.2013.2
- Jenkins, T. C., W. C. Bridges, J. H. Harrison, and K. M. Young. 2014. Addition of potassium carbonate to continuous cultures of mixed ruminal bacteria shifts volatile fatty acids and daily production of biohydrogenation intermediates. *J. Dairy Sci.* 97:975–984. doi:10.3168/jds.2013-7164
- Jin, D., S. Zhao, P. Wang, N. Zheng, D. Bu, Y. Beckers, and J. Wang. 2016. Insights into abundant rumen ureolytic bacterial community using rumen simulation system. *Front. Microbiol.* 7:1006. doi:10.3389/fmicb.2016.01006
- Kang, S., S. E. Denman, M. Morrison, Z. Yu, and C. S. McSweeney. 2009. An efficient RNA extraction method for estimating gut microbial diversity by polymerase chain reaction. *Curr. Microbiol.* 58:464–471. doi:10.1007/s00284-008-9345-z
- Karnati, S. K. R., J. T. Sylvester, C. V. D. M. Ribeiro, L. E. Gilligan, and J. L. Firkins. 2009. Investigating unsaturated fat, monensin, or bromoethanesulfonate in continuous cultures retaining ruminal protozoa. I. Fermentation, biohydrogenation, and microbial protein synthesis. *J. Dairy Sci.* 92:3849–3860. doi:10.3168/jds.2008-1436
- Kasparovska, J., M. Pecinkova, K. Dadakova, L. Krizova, S. Hadrova, M. Lexa, J. Lochman, and T. Kasparovsky. 2016. Effects of isoflavone-enriched feed on the rumen microbiota in dairy cows. *PLoS One.* 11:e0154642. doi:10.1371/journal.pone.0154642
- Kim, M., M. Morrison, and Z. Yu. 2011. Phylogenetic diversity of bacterial communities in bovine rumen as affected by diets and microenvironments. *Folia Microbiol. (Praha).* 56:453–458. doi:10.1007/s12223-011-0066-5
- Kong, Y., R. Teather, and R. Forster. 2010. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol. Ecol.* 74:612–622. doi:10.1111/j.1574-6941.2010.00977.x
- Krause, D. O., S. E. Denman, R. I. Mackie, M. Morrison, A. L. Rae, G. T. Attwood, and C. S. McSweeney. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27:663–693. doi:10.1016/S0168-6445(03)00072-X
- Lascano, G. J., M. Alende, L. E. Koch, and T. C. Jenkins. 2016. Changes in fermentation and biohydrogenation intermediates in continuous cultures fed low and high levels of fat with increasing rates of starch degradability. *J. Dairy Sci.* 99:6334–6341. doi:10.3168/jds.2016-11032
- Lazuka, A., L. Auer, S. Bozonnet, D. P. Morgavi, M. O'Donohue, and G. Hernandez-Raquet. 2015. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. *Bioresour. Technol.* 196:241–249. doi:10.1016/J.BIORTECH.2015.07.084
- Lengowski, M. B., K. H. R. Zuber, M. Witzig, J. Mhring, J. Boguhn, and M. Rodehutschord. 2016. Changes in rumen microbial community composition during adaption to an in vitro system and the impact of different forages. *PLoS One.* 11:1–22. doi:10.1371/journal.pone.0150115
- Licitra, G., T. M. Hernandez, and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. *Anim. Feed Sci. Technol.* 57:347–358. doi:10.1016/0377-8401(95)00837-3
- Mansfield, H. R., M. I. Endres, and M. D. Stern. 1995. Comparison of microbial fermentation in the rumen of dairy cows and dual flow continuous culture. *Anim. Feed Sci. Technol.* 55:47–66. doi:10.1016/0377-8401(95)98202-8
- Mao, S. Y., R. Y. Zhang, D. S. Wang, and W. Zhu. 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. *Anaerobe.* 24:12–19. doi:10.1016/J.ANAEROBE.2013.08.003
- Newbold, C. J., R. J. Wallace, and N. D. Walker. 1993. The effect of tetracycline and monensin on fermentation, microbial numbers and the development of ionophore-resistant bacteria in the rumen. *J. Appl. Bacteriol.* 75:129–134. doi:10.1111/j.1365-2672.1993.tb02757.x
- NRC. 2001. Nutrient Requirement of Dairy Cattle, seventh ed. National Research Council, National Academy Press, Washington, DC.
- Palmonari, A., D. M. Stevenson, D. R. Mertens, C. W. Cruywagen, and P. J. Weimer. 2010. pH dynamics and bacterial community composition in the rumen of

- lactating dairy cows. *J. Dairy Sci.* 93:279–287. doi:10.3168/JDS.2009-2207
- Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Environ. Microbiol.* 79:3744–3755. doi:10.1128/AEM.03983-12
- Popova, M., C. Martin, and D. P. Morgavi. 2010. Improved protocol for high-quality co-extraction of DNA and RNA from rumen digesta. *Folia Microbiol. (Praha)*. 55:368–372. doi:10.1007/s12223-010-0060-3
- Purushe, J., D. E. Fouts, M. Morrison, B. A. White, R. I. Mackie, P. M. Coutinho, B. Henrissat, and K. E. Nelson. 2010. Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bryantii*: insights into their environmental niche. *Microb. Ecol.* 60:721–729. doi:10.1007/s00248-010-9692-8
- Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41:D590–D596. doi:10.1093/nar/gks1219
- Russell, J. B. 2002. *Rumen Microbiology and its Role in Ruminant Nutrition*. 1st ed. Cornell University Press, Ithaca, NY.
- Sadet-Bourgeteau, S., C. Martin, and D. P. Morgavi. 2010. Bacterial diversity dynamics in rumen epithelium of wethers fed forage and mixed concentrate forage diets. *Vet. Microbiol.* 146:98–104. doi:10.1016/J.VETMIC.2010.04.029
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75:7537–7541. doi:10.1128/AEM.01541-09
- Stevenson, D. M., and P. J. Weimer. 2007. 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.* 75:165–174. doi:10.1007/s00253-006-0802-y
- Weimer, P. J., D. M. Stevenson, H. C. Mantovani, and S. L. C. Man. 2010. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. *J. Dairy Sci.* 93:5902–5912. doi:10.3168/jds.2010-3500
- Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. *Br. J. Nutr.* 32:341–351. doi:10.1079/BJN19740087
- Windschitl, P. M., and M. D. Stern. 1988. Evaluation of calcium lignosulfonate-treated soybean meal as a source of rumen protected protein for dairy cattle. *J. Dairy Sci.* 71:3310–3322. doi:10.3168/jds.S0022-0302(88)79936-1
- Ze, X., S. H. Duncan, P. Louis, and H. J. Flint. 2012. *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *ISME J.* 6:1535–1543. doi:10.1038/ismej.2012.4
- Ziemer, C. J., R. Sharp, M. D. Stern, M. A. Cotta, T. R. Whitehead, and D. A. Stahl. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ. Microbiol.* 2:632–643. doi:10.1046/j.1462-2920.2000.00146.x
- Zinn, R. A., and F. N. Owens. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Can. J. Anim. Sci.* 66:157–166. doi:10.4141/cjas86-017