UNIVERSITY OF COPENHAGEN

Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows

Zhu, Zhigang; Kristensen, Lise; Difford, Gareth F.; Poulsen, Morten; Noel, Samantha J.; Abu Al-Soud, Waleed; Sørensen, Søren Johannes; Lassen, Jan; Løvendahl, Peter; Højberg, Ole

Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows

Zhigang Zhu,*[1] Lise Kristensen,† Gareth F. Difford,† Morten Poulsen,* Samantha J. Noel,*
Waleed Abu Al-Soud,‡ Søren J. Sørensen,‡ Jan Lassen,† Peter Løvendahl,† and Ole Højberg*[1]
*Department of Animal Science, Aarhus University, DK-8830 Tjele, Denmark
†Center for Quantitative Genetics and Genomics, Department of Molecular Biology and Genetics, Aarhus University, Tjele, Denmark
‡Department of Biology, Faculty of Science, University of Copenhagen, DK-2100 Copenhagen, Denmark

ABSTRACT

In the present study, we hypothesized that the rumen bacterial and archaeal communities would change significantly over the transition period of dairy cows, mainly as an adaptation to the classical use of low-grain prepartum and high-grain postpartum diets. Bacterial 16S rRNA gene amplicon sequencing of rumen samples from 10 primiparous Holstein dairy cows revealed no changes over the transition period in relative abundance of genera such as *Ruminococcus*, *Butyrivibrio*, *Clostridium*, *Coprococcus*, and *Pseudobutyrivibrio*. However, other dominant genus-level taxa, such as *Prevotella*, unclassified *Ruminococcaceae*, and unclassified *Succinivibrionaceae*, showed distinct changes in relative abundance from the prepartum to the postpartum period. Overall, we observed individual fluctuation patterns over the transition period for a range of bacterial taxa that, in some cases, were correlated with observed changes in the rumen short-chain fatty acids profile. Combined results from clone library and terminal-restriction fragment length polymorphism (T-RFLP) analyses, targeting the methyl-coenzyme M reductase α-subunit (*mcrA*) gene, revealed a methanogenic archaeal community dominated by the *Methanobacteriales* and *Methanomassiliicoccales* orders, particularly the genera *Methanobrevibacter*, *Methanosphaera*, and *Methanomassiliicoccus*. As observed for the bacterial community, the T-RFLP patterns showed significant shifts in methanogenic community composition over the transition period. Together, the composition of the rumen bacterial and archaeal communities exhibited changes in response to particularly the dietary changes of dairy cows over the transition period.
Key words: rumen bacteria, archaea, Illumina MiSeq, terminal-RFLP, transition period

INTRODUCTION

The dairy cow transition period, defined as 3 wk before to 3 wk after parturition, is characterized by dramatic physiological changes and imposes severe challenges on the animal for maintaining positive nutrient and energy balances (Bell, 1995; Ingvartsen, 2006). In ruminants, dietary carbohydrates are fermented by rumen microbes into short-chain fatty acids (**SCFA**), which are the major energy source for the host animal (Reynolds et al., 1988). During the prepartum period, feed intake is typically low but still covering the energy needs of the cow. Postpartum, the demands for glucose, AA, and fatty acids are dramatically increased but the slow increase in feed intake cannot satisfy the animal's nutrient and energy needs (Bell, 1995). A period of negative energy balance is therefore encountered by many cows during early lactation (Drackley, 1999), where rates of hepatic gluconeogenesis and adipose fat mobilization are greatly accelerated, potentially leading to ketosis or fatty liver development. To help alleviate the negative energy balance and sustain high milk production, the diet composition (roughage-to-concentrate ratio) and amount is commonly adjusted postpartum.

For the rumen bacterial community, high-grain diets typically induce a decline in diversity as well as an increase in starch-degrading and lactate-utilizing or propionate-producing members of the genera *Prevotella*, *Streptococcus*, *Selenomonas*, and *Megasphaera* and a decrease in fibrolytic members of *Butyrivibrio*, *Ruminococcus*, and *Fibrobacter* (Tajima et al., 2001; Fernando et al., 2010; Petri et al., 2013). Consequently, dietary changes may alter the rumen fermentation characteristics, such as SCFA production rate and profile (relative proportions of mainly acetate, propionate, and butyrate), which may again affect feed efficiency of the cow (Wang et al., 2012).

Compared with the rumen bacterial community, the archaeal community is less diverse and, based on small subunit ribosomal RNA analyses, the domain *Archaea* has been reported to comprise only 0.5 to 2.5% of the

entire rumen microbial community (*Prokarya* and *Eukarya*) in domestic ruminants (Lin et al., 1997; Jeyanathan et al., 2011). Moreover, the rumen *Archaea* is represented almost solely by the *Euryarchaeota* phylum, covering all methanogens, and seems to be dominated by relatively few species within the *Methanobrevibacter* and *Methanosphaera* genera and the *Methanomassiliicoccales* order in ruminants across animal species, diet type, and geographical location (Jeyanathan et al., 2011; Sirohi et al., 2013; Henderson et al., 2015). It should be kept in mind, however, that methanogens, although low in total numbers, are the main hydrogen scavengers of the rumen and, as such, are key players in driving fermentation processes. The dynamics of not only the bacterial, but also the methanogenic archaeal, community of the rumen may therefore be crucial for dairy cows, particular in critical life phases such as the transition period, when the animals are highly dependent on optimal energy metabolism.

Advanced sequencing technologies have enabled in-depth analysis of microbiomes (composition and function). It has been demonstrated that the rumen microbiome of dairy cows is dynamic and changes according to, for example, age (Jami et al., 2013; Rey et al., 2014), parity (Pitta et al., 2014a; Lima et al., 2015), and lactation cycle stage (Jewell et al., 2015), and distinct prepartum to postpartum shifts in the rumen bacterial community composition have been observed (Mohammed et al., 2012; Wang et al., 2012; Pitta et al., 2014a; Lima et al., 2015). Most of the studies investigating the rumen microbiota over the transition period have focused on *Bacteria* (Mohammed et al., 2012; Wang et al., 2012; Pitta et al., 2014a; Lima et al., 2015; Zhu et al., 2017). For the present study, as in only one other study (Dieho et al., 2017), we included not only *Bacteria*, but also *Archaea*, hypothesizing that the composition of both domains changes over the transition period as an adaptation to mainly the dietary changes, thereby supporting optimal host nutrient utilization and energy supply. We used next-generation sequencing technology (Illumina MiSeq, Illumina Inc., San Diego, CA) to characterize the bacterial community, applying a universal prokaryotic primer set to target the V3–V4 region of the 16S rRNA gene (Caporaso et al., 2011). The less-diverse archaeal community was characterized by use of an *mcrA* gene-based clone library and terminal (**T**)-RFLP analyses.

Compared with primiparous cows, multiparous cows may possess a more mature rumen microbiome, better adapted for coping with shifts in substrate availability after several transitions. The present study focuses on primiparous cows, anticipating that an understanding of the rumen microbiome of primiparous cows and its response to dietary changes over the transition period may lead to strategies to, potentially, manipulate the rumen microbiome and improve the health and nutritional status of primiparous cows in their first transition period as well as later in life.

MATERIALS AND METHODS

Animals, Diets, and Rumen Sampling

The animal experimental procedure was performed according to a protocol approved by The Animal Experiments Inspectorate, Danish Veterinary and Food Administration, Ministry of Environment and Food of Denmark (approval number 2016-15-0201-00959). The study included 10 primiparous Holstein cows with close predicted calving dates, housed at a research farm (Danish Cattle Research Centre; www.DKC-Foulum .dk). All cows were fed ad libitum with a low-grain (high-forage) prepartum and a high-grain (low-forage) postpartum diet (Table 1), and had free access to drinking water. Feed intake data of the individual cows were recorded automatically at each visit to the feeders (Insentec B.V., Marknesse, the Netherlands; Supplemental Figure S1; https://doi.org/10.3168/jds.2017 -14366). Gestating cows were grouped in a barn with straw bedding. After calving, the cows were moved individually to a barn for lactating cows, equipped with a voluntary milking station (VMS, DeLaval, Tumba, Sweden). The cows were offered a limited amount of concentrate when visiting the milking robot. Rumen samples (7 per cow, 70 in total) were taken once a week after morning feeding (between 0900 and 1000 h after feeding) for 7 consecutive weeks according to the sampling scheme shown in Figure 1. Because the expected and actual day of parturition differed, the cows were unintentionally sampled unevenly relative to parturition. Consequently, cow 1 contributed with 6 prepartum samples and 1 postpartum sample, whereas cow 10 contributed with 7 postpartum samples. For sampling, a rumen flora scoop was inserted via the mouth and esophagus into the rumen and unfractionated samples of rumen content were withdrawn (Geishauser et al., 2012). Approximately 40 mL of each rumen sample was transferred into a 50-mL polypropylene centrifuge tube and stored on ice for immediate transport to the laboratory. After vigorous mixing of the rumen samples, sub-samples (1.2 mL) were immediately aliquotted into Eppendorf tubes using blunt-ended pipette tips (2.5–3 mm in diameter) to allow particles to enter, snap frozen in liquid nitrogen and stored at −80°C until further analysis. The remaining rumen sample was stored at −20°C.

Short-Chain Fatty Acids

Rumen samples stored at −20°C were thawed and homogenized and 1-mL sub-samples were retrieved for SCFA analysis. The concentrations of SCFA were determined by gas chromatography, as previously described (Jensen et al., 1995; Canibe et al., 2007), using a Hewlett Packard gas chromatograph (model 6890; Hewlett Packard, Agilent Technologies, Naerum, Denmark) equipped with a flame-ionization detector and a 30-m ZB-5 column (Phenomenex, Værløse, Denmark) with an internal diameter of 0.32 mm and coated with 5%-phenyl 95%-dimethylpolysiloxane with a film thickness of 0.25 µm. The internal standard was 2-ethyl-butyric acid (109959 Aldrich, Sigma-Aldrich Denmark A/S, Copenhagen, Denmark).

DNA Extraction

Snap-frozen rumen samples were thawed at room temperature and 200 µL of homogeneously mixed sub-sample was retrieved for DNA extraction. The DNA extraction was performed using QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction, with a bead-beating step (Star Beater, VWR, Søborg, Denmark) added to

Table 1. Ingredients and chemical composition (% of DM) of the TMR fed prepartum and postpartum

Item	Prepartum	Postpartum
Spring barley	—	9.85
NaOH-treated wheat	—	9.85
Rapeseed meal I 4% fat	8.47	—
Rapeseed cake II 10.5% fat	—	11.19
Sugar beet pellets (unmolassified)	—	11.19
Grass silage I[1]	—	22.38
Maize silage[2]	44.03	25.52
Grass silage II[3]	25.40	8.95
Spring barley straw	21.17	—
Urea	—	0.16
Feed salt	—	0.18
Cowmix (Komix) 302–318[4]	—	0.72
Cowmix (Komix) Gold[5]	0.93	—

[1]Chemical analysis: DM, 29.93%; ash, 10.16% of DM; fiber, 22.02% of DM; NDF, 36.08% of DM; soluble nitrogen 1.79% of DM; fat, 4.13% of DM; total sugar, 0.33% of DM; rumen-soluble OM, 81.48% of DM; pH = 4.

[2]Chemical analysis: DM, 24.59%; ash, 3.28% of DM; fiber, 25.08% of DM; NDF, 49.7% of DM; soluble nitrogen, 0.71% of DM; fat, 2.13% of DM; starch, 19.86% of DM; rumen-soluble OM, 70.72% of DM; pH = 3.8.

[3]Chemical analysis: DM, 23.51%; ash, 9.6% of DM; fiber, 29.44% of DM; NDF, 48.99% of DM; soluble nitrogen, 1.27% of DM; starch, 2.03% of DM; fat, 2.9% of DM; total sugar, 0.22% of DM; rumen-soluble OM, 71.79% of DM; pH = 3.9.

[4]Cowmix 302–318 (mineral; Vitfoss, Gråsten, Denmark) containing (100 g): Ca, 30.3%; Mg, 7.8%; S, 1.3%.

[5]Cowmix Gold (mineral; Vitfoss) containing (1 kg): Ca, 1.65%; Mg, 19.33%; P, 3.05%; K, 0.3%; Na, 3.7%.

enhance cell lysis. Bead-beating was performed twice for 20 s at high speed, placing the tubes on ice for 30 s between the 2 bead-beatings. The concentration of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and the quality of DNA was checked by agarose gel electrophoresis. The DNA samples were stored at −20°C until further analysis.

16S rRNA Gene Amplicon Sequencing

Total genomic DNA was used for sequencing library construction based on a 2-step PCR procedure (Berry et al., 2011; Tian et al., 2016; Dieho et al., 2017). The universal prokaryotic primers modified 341F (5′-CCTAYGGGRBGCASCAG) and modified 806R (5′-GGACTACNNGGGTATCTAAT) were used to amplify the V3–V4 hypervariable region of the 16S rRNA gene (Sundberg et al., 2013). The PCR mix (20 µL) contained: 1× AccuPrime PCR Buffer II (15 mM MgCl$_2$), 0.24 U of AccuPrime Taq DNA Polymerase (Life Technologies), 0.5 µmol of each primer, 2 µL of diluted template, and water to a total of 20 µL (Life Technologies, Carlsbad, CA). The PCR incubation conditions were as follows: an initial activation of hot-start polymerase at 94°C for 2 min, followed by 15 cycles at 94°C for 20s, 56°C for 20s, and 68°C for 30s, and final extension at 68°C for 5 min. The samples were incubated at 70°C for 3 min and then moved directly to ice. A second PCR step was performed to add adapters and indexes to the amplified 16S rRNA fragments by use of the universal primers 515F (5′-GTGBCAGCMGC-CGCGGTAA) and modified 806R. Each primer had a barcode sequence of 8 nucleotides at the 5′ and 3′ ends, which was unique for each sample. Long amplicons were purified by Agencourt AMpure XP system (Beckman Coulter, Brea, CA). Subsequently, the purified PCR products were quantified on a Qubit 2.0 Fluorometer, using the Qubit dsDNA Assay kit (Life Technologies), and then pooled in a single tube with equimolar DNA concentrations of all samples. The pooled samples were concentrated using the DNA clean and concentrator-5 kit (Zymo Research, Orange, CA) and sequenced using 250 base paired-end sequencing with the Illumina MiSeq platform, according to the manufacturer's instructions (Illumina Inc.).

Amplicon Sequence Analysis

Amplicon sequences were analyzed using the QIIME 1.7.0 data analysis pipeline (Caporaso et al., 2010b). Briefly, the raw sequences were de-multiplexed and assigned to corresponding samples based on the specified barcode. Quality filtering was performed using the

criteria of minimum average quality score of 25 and ≤6 homopolymers and chimera were removed by USE-ARCH in the QIIME pipeline (Edgar, 2010). Operational taxonomic units (**OTU**) were picked against the Greengenes database (version: gg_13_5; greengenes. secondgenome.com), using the BLAST OTU picker with the default sequence similarity parameter (97%). Representative sequences from each OTU were aligned using PyNAST (Caporaso et al., 2010a), and taxonomic information for each representative sequence was determined with RDP classifier (Wang et al., 2007). A phylogenetic tree of the representative sequences was constructed using the default FastTree method (Price et al., 2009). α-Diversity analysis was performed with QIIME, and richness (Chao1 index), number of distinct OTU (observed species), and phylogenetic diversity (**PD**) whole tree within the bacterial community were calculated from the OTU table. To estimate β-diversity based on the OTU, we used the weighted UniFrac metric (Hamady et al., 2010). Principal coordinate analysis was implemented in the R software (R version 3.2.3, https://www.r-project.org/) on UniFrac matrices to compare the dynamic shift of bacterial communities across the transition period. Statistical differences in the sample groups were tested on weighted UniFrac distance matrix using the ANOSIM method in QIIME (Fierer et al., 2010).

mcrA Gene Clone Library

Twenty of the 70 rumen samples (3 samples from each sample week, except 1 wk where only 2 samples were included) were selected to cover the entire transition period and from these, a *mcrA* gene clone library was constructed. To target the *mcrA* gene of all methanogens, we used the forward primer 5′-GGTGGTGTMG-GDTTYACHCARTA modified from Steinberg and Regan (2008), and the reverse primer 5′-FAMCGTTCAT-BGCGTAGTTVGGRTAGT, used in the same study. Pooled PCR products for individual samples were purified with QIAquick PCR Purification Kit following the manufacturer's instructions (Qiagen GmbH). Purified PCR products from all the 20 samples were pooled at equimolar concentrations and subsequently ligated to pGEM-T Easy Vector according to the instruction of the manufacturer (Promega, Madison, WI). Plates were incubated overnight at 37°C, and positive clones were selected based on blue-white screening. Finally, white clones were transferred to microtiter-plates containing SOC medium (Sigma-Aldrich, St. Louis, MO) and sent for sequencing at MWG Eurofins (Ebersberg, Germany).

Clone Sequence Analysis and Phylogenetic Tree Construction

The quality of the raw *mcrA* gene sequences was checked using GENEIOUS (Auckland, New Zealand) and any chimeric clone sequences were removed. High quality sequences were subjected to data analysis using Mothur (http://www.mothur.org/wiki/Mothur _manual). The clone sequences were assigned to individual OTU by de novo picking based on 97% similarity; no database was involved for this step. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 (Tamura et al., 2011). The tree was evaluated with the bootstrap test based on 500 resamplings of the data sets. All of the clone sequences
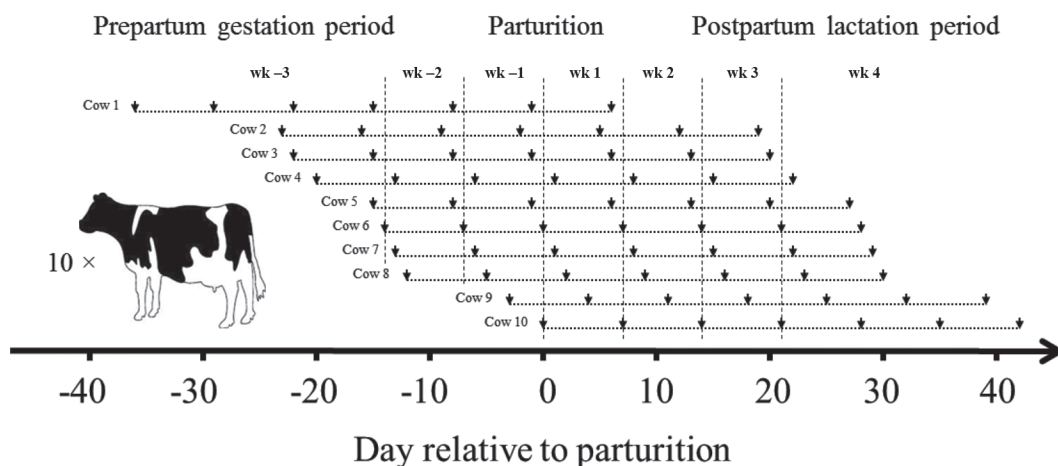

Figure 1. Diagram depicting the 7 sample days relative to parturition (d 0) for each of the 10 cows. For statistical analysis and data presentation, the samples were grouped into week of sampling relative to parturition: wk −3: d 15 or more before parturition (10 samples); wk −2: d 7–14 before parturition (9 samples); wk −1: d 0–6 before parturition (10 samples); wk 1: d 1–7 after parturition (10 samples); wk 2: d 8–14 after parturition (9 samples); wk 3: d 15–21 after parturition (9 samples); wk 4: d 22 or more after parturition (13 samples).

reported here were deposited in GenBank under the accession numbers KM491218 to KM491302.

T-RFLP-Based mcrA Gene Analysis

Terminal-RFLP analysis was performed on the *mcrA* gene. The initial step was the generation of PCR amplicons of the *mcrA* gene, using the primers described in the clone library construction. For each of the 70 samples, 4 replicate PCR products were prepared and pooled into a final volume of 50 µL, followed by purification with QIAquick PCR Purification Kit (Qiagen GmbH), according to the manufacturer's instructions. For each pooled sample, 200 ng of purified PCR amplicon was digested with the *Taq*I restriction endonuclease (New England Biolabs, Ipswich, MA), according to Cheng et al. (2009). Digested fragments were precipitated with 3 *M* sodium acetate and 96% ice-cold ethanol, washed with 70% ice-cold ethanol, and mixed with 10 µL of formamide and 0.2 µL of Megabase ET900-R size standard (GE Healthcare, Amersham, UK). After drying, the samples were run on an ABI 3730XL Capillary Sequencer (Life Technologies).

Statistical Analysis

To investigate the influence of transition period on DMI, individual components of SCFA, T-RFLP data, the relative abundance of bacteria taxa, and α-diversity parameters of the bacterial community, a linear model was constructed using PROC MIXED in SAS (SAS 9.3, SAS Institute Inc., Cary, NC). The model included "week relative to parturition" as a fixed factor, and animals and sample weeks were considered random effects. The 70 samples were grouped into week of sampling relative to parturition, as outlined in the sampling scheme (Figure 1). These 7 groups contained 10, 9, 10, 10, 9, 9, and 13 samples, respectively. The significance level of the fixed factor "week relative to parturition" in the linear mixed model was denoted by the *P*-value, and the relative abundance of bacterial taxa between sample groups was compared by least squares means *t*-test. *P*-values ≤0.05 were considered significant and tendencies were represented by *P*-values between 0.05 and 0.10. Pearson correlation coefficients between the relative abundance of individual bacterial taxa and VFA components were calculated by PROC CORR in SAS (SAS 9.3, SAS Institute Inc.), and only significant correlations ($P \leq 0.05$) were imported into Excel 2010 (Microsoft Corp., Redmond, WA) to create a heatmap. Using R software (R version, 3.2.3; https://www.r-project.org/), principal component analysis (**PCA**) was performed on the relative abundance values of

predominant T-RF from the T-RFLP profiles to assess the shifts of the methanogenic community over the transition period. To visualize the distribution of OTU identified in the bacterial profiles of the prepartum and postpartum rumen samples, Venn diagrams were constructed by use of Venny 2.1.0 (Oliveros, 2007).

RESULTS

16S rRNA Gene Amplicon Sequencing

After the quality filtering step, we retrieved a total of 11,771,910 high-quality sequences from the 70 rumen samples (10 cows, 7 sampling weeks) with a mean read count of 167,398 per sample and maximum read count of 1,385,345, represented by 3,566 OTU, with the exclusion of OTU representing <0.001% of all sequences. Although we retrieved several archaeal amplicons, assigned to the *Methanobrevibacter* and *Methanosphaera* genera, in the 16S rRNA gene analysis, they were lost in the filtering step because of their low abundance. Overall, the rumen prokaryote community was dominated by the following 7 bacterial phyla (numbers indicate average relative abundance across the transition period): *Bacteroidetes* (50%), *Proteobacteria* (21%), *Firmicutes* (14%), *Spirochaetes* (5%), *SR1* (3%), *Fibrobacteres* (1%), and *Tenericutes* (0.9%). Unassigned sequences and sequences assigned to less-prevalent phyla accounted for ~5% of all sequences and were grouped as "others" (Figure 2). Within these phyla, 34 genus-level taxa were represented ubiquitously in the microbiome of all rumen samples across the transition period, including CF231, YRC22, *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Ruminobacter*, *Succinivibrio*, *Pseudobutyrivibrio*, *Clostridium*, *Coprococcus*, *Treponema*, *Fibrobacter*, unclassified *Ruminococcaceae*, unclassified *Lachnospiraceae*, and unclassified *Succinivibrionaceae*.

The distribution of OTU identified from the bacterial profiles across the 7 sample groups is illustrated by Venn diagrams (Figure 3). There were 3,373 OTU shared by the 3 prepartum sample groups (Figure 3A) and 3,118 OTU shared by the 4 postpartum sample groups (Figure 3B); the numbers of OTU specific to the 2 periods were 356 and 101, respectively (Figure 3C). The 356 OTU specific to the prepartum microbiome were associated with unclassified *Bacteroidales* (79 OTU), *Ruminococcaceae* (75), *Paraprevotellaceae* (25), RF16 (24) and unclassified *Clostridiales* (20). The 101 OTU specific to the postpartum microbiome were associated with *Succinivibrionaceae* (31), *Prevotella* (19), and *Lachnospiraceae* (11).

The *Bacteroidetes* phylum, represented solely by the *Bacteroidales* order, showed a significant decrease (*P*
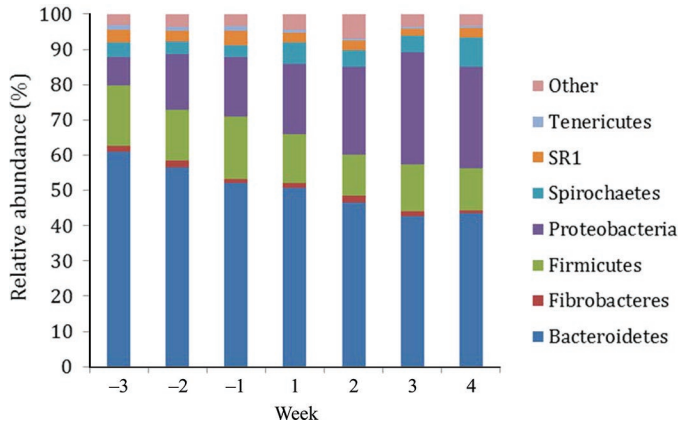
Figure 2. Taxonomic composition of bacterial community at the phylum level, showing relative 16S rRNA amplicon abundance (percent reads out of total reads) of the 7 predominant phyla. The remaining minor bacterial phyla were compiled into "other." Color version available online.

< 0.0001) in relative abundance from 61% prepartum to 43% postpartum (Table 2), but some lower taxa showed different trends. The dominant family-level taxa, RF16 and unclassified *Bacteroidales*, along with the less dominant BS11 and *Bacteroidaceae*, all showed significant decreases (for *P*-values, see Table 2) in relative abundance over the transition period. In contrast, the *Prevotellaceae* family, with *Prevotella* as the only genus, increased significantly ($P = 0.04$) in abundance (from 4.5 to 8.5%).

At the phylum level, we observed no significant changes for *Firmicutes*, dominated by the *Clostridiales* order and 6 families: *Ruminococcaceae* (6.14%), *Lachnospiraceae* (3.68%), unclassified *Clostridiales* (2.92%), *Clostridiaceae* (0.63%), *Erysipelotrichaceae* (0.47%), and *Christensenellaceae* (0.11%). However, we observed significant reductions ($P < 0.01$ and $P < 0.0001$, respectively) for *Ruminococcaceae* (from 8.1 to 4.7%) and *Christensenellaceae* (from 0.2 to 0.05%) over the transition period. At the genus level, we observed a significant decrease ($P < 0.0001$) for unclassified *Ruminococcaceae* (from 5.6 to 2.7%) but no significant changes for *Ruminococcus* and the less-dominant genera *Butyrivibrio*, *Clostridium*, *Coprococcus*, and *Pseudobutyrivibrio*. Albeit with lower relative abundances in the prepartum period, the least dominant genera *Moryella* and *Shuttleworthia* showed significant increases ($P = 0.02$ and $P < 0.01$, respectively) in the postpartum period.

Proteobacteria increased significantly ($P < 0.01$) in relative abundance, from 8 to 29%. The majority of the *Proteobacteria* sequences grouped as unclassified *Succinivibrionaceae* (*Gammaproteobacteria* class, *Succinivibrionaceae* family), a genus-level taxon that in-
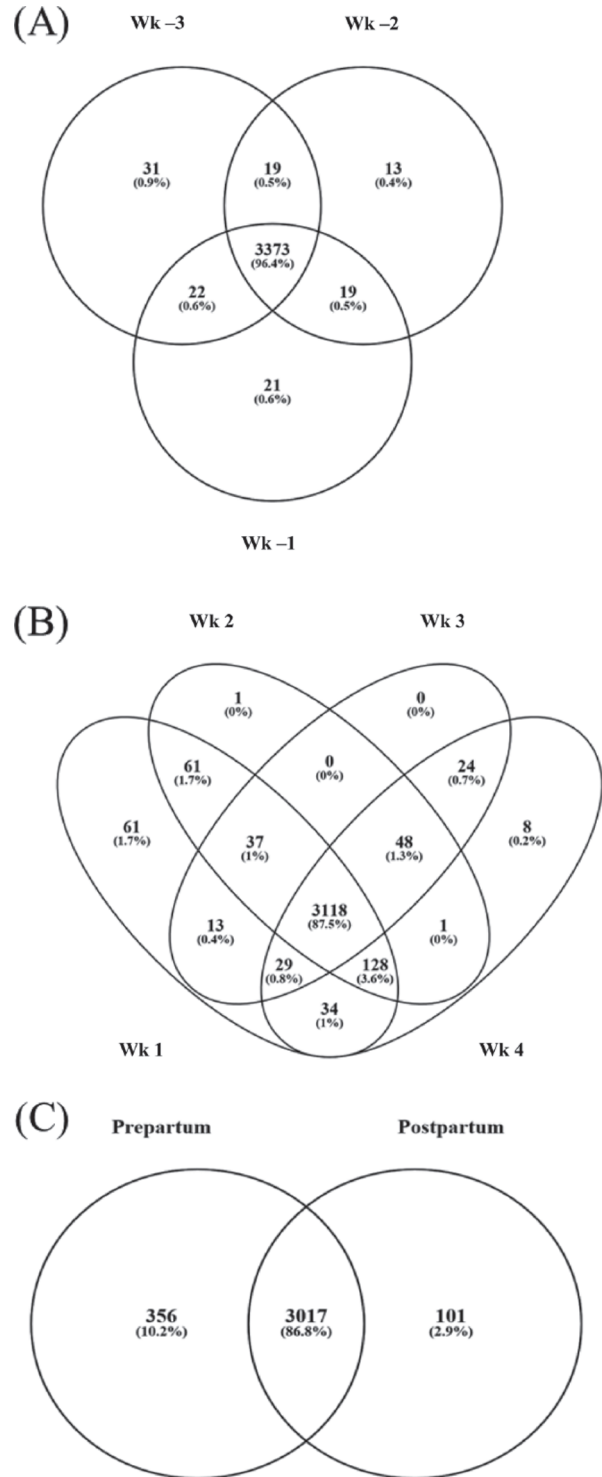
Figure 3. Venn diagram showing the distribution of operational taxonomic units (OTU) in sample groups. A total of 3,566 OTU were identified from Illumina MiSeq 16S rRNA amplicon sequencing (Illumina Inc., San Diego, CA). Three panels indicated the number of OTU shared by (A) 3 groups (wk −3, wk −2, and wk −1) from the prepartum period; (B) 4 groups (wk 1, wk 2, wk 3, and wk 4) from the postpartum period; and (C) from both the prepartum and postpartum period. The percentage of OTU shared by different sample groups is given below the shared OTU number.

creased significantly ($P < 0.0001$) in abundance over the transition period from ~5 to ~27%. The less abundant *Alphaproteobacteria* class decreased significantly ($P < 0.0001$) from 1.8 to 0.7%.

The *Spirochaetes* phylum increased significantly ($P = 0.03$) from ~3 to ~8% over the transition period, whereas the less dominant phyla (*Tenericutes, Fibrobacteres,* and SR1) showed no significant changes.

Table 2. Relative abundance[1] (%) of major bacterial taxa in the rumen of dairy cows across transition period (for those sequences classified from phylum to genus, intermediate taxonomic identifications were omitted)

Taxonomic level (phylum, class, order, family, genus)	Week relative to parturition[2]							SEM[3]	P-value[3]
	−3	−2	−1	1	2	3	4		
Bacteroidetes	60.93ᵃ	56.58ᵃᵇ	52.10ᵃᵇᶜ	50.66ᵇᶜᵈ	46.70ᶜᵈ	42.78ᵈ	43.49ᵈ	0.021	<0.0001
Bacteroidia	60.93ᵃ	56.58ᵃᵇ	52.10ᵃᵇᶜ	50.66ᵇᶜᵈ	46.70ᶜᵈ	42.78ᵈ	43.49ᵈ	0.021	<0.0001
Bacteroidales	60.93ᵃ	56.58ᵃᵇ	52.10ᵃᵇᶜ	50.66ᵇᶜᵈ	46.70ᶜᵈ	42.78ᵈ	43.49ᵈ	0.021	<0.0001
Paraprevotellaceae	7.44ᵃᵇ	6.99ᵃᵇ	6.18ᵇ	8.95ᵃ	8.21ᵃᵇ	6.10ᵇ	7.41ᵃᵇ	0.006	<0.01
CF231	3.25ᵃᵇ	3.25ᵃᵇ	2.86ᵃᵇ	4.07ᵃ	3.11ᵃᵇ	2.34ᵇ	2.70ᵇ	0.004	0.02
YRC22	3.14ᵃᵇ	2.92ᵃᵇ	2.39ᵇ	3.59ᵃ	4.13ᵃ	2.46ᵇ	3.46ᵃᵇ	0.004	0.02
Prevotellaceae	4.47ᵇ	5.79ᵃᵇ	4.76ᵇ	7.30ᵃᵇ	7.97ᵃᵇ	11.35ᵃ	8.47ᵃᵇ	0.016	0.04
Prevotella	4.47ᵇ	5.79ᵃᵇ	4.76ᵇ	7.29ᵃᵇ	7.96ᵃᵇ	11.34ᵃ	8.46ᵃᵇ	0.016	0.04
RF16	18.98ᵃ	18.99ᵃ	18.78ᵃ	12.25ᵃᵇ	13.06ᵃᵇ	9.88ᵇ	11.58ᵃᵇ	0.021	<0.01
BS11	0.87ᵃ	0.65ᵃᵇ	0.33ᵇᶜ	0.11ᶜ	0.01ᶜ	0.03ᶜ	0.03ᶜ	0.001	<0.0001
Bacteroidaceae	0.24ᵃ	0.17ᵃ	0.11ᵃᵇ	0.05ᵇ	0.03ᵇ	0.04ᵇ	0.02ᵇ	0.000	<0.0001
BF311	0.21ᵃ	0.16ᵃᵇ	0.11ᵇᶜ	0.04ᶜᵈ	0.02ᶜᵈ	0.03ᶜᵈ	0.02ᵈ	0.000	<0.0001
S24–7	0.05	0.05	0.04	0.06	0.09	0.09	0.08	0.000	0.64
Unclassified *Bacteroidales*	28.88ᵃ	23.92ᵃᵇ	21.90ᵇᶜ	21.94ᵇᶜ	17.33ᵇᶜ	15.29ᶜ	15.91ᶜ	0.015	<0.0001
Firmicutes	17.04	14.49	17.53	13.80	11.65	13.15	11.87	0.017	0.08
Clostridia	16.54	13.93	17.00	13.35	11.20	12.69	11.51	0.017	0.07
Clostridiales	16.54	13.93	17.00	13.35	11.20	12.69	11.51	0.017	0.07
Lachnospiraceae	3.40	3.00	3.98	3.48	3.36	4.83	3.70	0.006	0.41
Coprococcus	0.63	0.59	0.57	0.68	0.66	0.85	0.76	0.001	0.43
Butyrivibrio	0.76	0.65	0.83	0.81	0.59	0.68	0.64	0.001	0.82
Moryella	0.10ᵇ	0.10ᵇ	0.11ᵃᵇ	0.14ᵃᵇ	0.12ᵃᵇ	0.13ᵃᵇ	0.18ᵃ	0.000	0.02
Pseudobutyrivibrio	0.28	0.26	0.27	0.31	0.21	0.28	0.22	0.001	0.90
Shuttleworthia	0.02ᵇ	0.02ᵇ	0.02ᵇ	0.06ᵇ	0.17ᵃᵇ	0.38ᵃ	0.17ᵃᵇ	0.001	<0.01
Unclassified *Lachnospiraceae*	1.55	1.30	2.07	1.37	1.48	2.12	1.55	0.004	0.36
Ruminococcaceae	8.10ᵃ	6.62ᵃᵇ	8.51ᵃ	6.38ᵃᵇ	4.85ᵇ	3.98ᵇ	4.66ᵇ	0.008	<0.01
Ruminococcus	2.42	2.05	2.78	2.56	1.93	1.26	1.94	0.004	0.18
Unclassified *Ruminococcaceae*	5.57ᵃ	4.47ᵃᵇ	5.62ᵃ	3.74ᵃᵇ	2.87ᵇ	2.64ᵇ	2.66ᵇ	0.005	<0.0001
Clostridiaceae	0.78	0.66	0.62	0.61	0.51	0.72	0.54	0.001	0.40
Clostridium	0.77	0.66	0.61	0.60	0.50	0.67	0.52	0.001	0.30
Christensenellaceae	0.18ᵃ	0.15ᵃ	0.16ᵃ	0.10ᵃᵇ	0.06ᵇ	0.07ᵇ	0.05ᵇ	0.000	<0.0001
Erysipelotrichaceae	0.50	0.55	0.53	0.45	0.45	0.46	0.36	0.001	0.77
Unclassified *Clostridiales*	3.85	3.27	3.52	2.57	2.22	2.74	2.26	0.005	0.08
Proteobacteria	8.22ᵈ	15.72ᶜᵈ	16.94ᵇᶜᵈ	19.84ᵃᵇᶜᵈ	24.95ᵃᵇᶜ	31.73ᵃ	28.95ᵃᵇ	0.033	<0.01
Gammaproteobacteria	6.13ᵈ	14.07ᶜᵈ	14.79ᵇᶜᵈ	18.48ᵃᵇᶜᵈ	24.08ᵃᵇᶜ	30.89ᵃ	28.01ᵃᵇ	0.033	<0.01
Aeromonadales	6.10ᵈ	14.04ᶜᵈ	14.76ᵇᶜᵈ	18.41ᵃᵇᶜᵈ	24.03ᵃᵇᶜ	30.88ᵃ	27.98ᵃᵇ	0.033	<0.01
Succinivibrionaceae	6.10ᶜ	14.04ᵇᶜ	14.76ᵇᶜ	18.41ᵃᵇ	24.03ᵃᵇ	30.88ᵃ	27.98ᵃ	0.033	<0.01
Ruminobacter	0.80ᵃᵇᶜ	2.41ᵃ	1.88ᵃᵇ	1.38ᵃᵇᶜ	0.25ᶜ	0.42ᵇᶜ	0.32ᶜ	0.004	<0.01
Succinivibrio	0.45	0.79	0.61	0.90	1.17	0.82	0.95	0.002	0.71
Unclassified *Succinivibrionaceae*	4.83ᵈ	10.84ᵈ	12.28ᶜᵈ	16.13ᵇᶜᵈ	22.62ᵃᵇᶜ	29.63ᵃ	26.72ᵃᵇ	0.032	<0.0001
Alphaproteobacteria	1.76ᵃ	1.41ᵃᵇ	1.90ᵃ	1.11ᵇ	0.65ᵇ	0.71ᵇ	0.70ᵇ	0.002	<0.0001
RF32	0.51ᵃᵇ	0.52ᵃᵇ	0.72ᵃ	0.62ᵃᵇ	0.40ᵇ	0.36ᵇ	0.42ᵇ	0.001	<0.01
Unclassified *Alphaproteobacteria*	0.70ᵃ	0.45ᵃᵇ	0.70ᵃ	0.13ᵇ	0.09ᵇ	0.22ᵇ	0.08ᵇ	0.001	<0.0001
Spirochaetes	4.00ᵃᵇ	3.55ᵃᵇ	3.20ᵇ	6.27ᵃᵇ	4.54ᵃᵇ	4.80ᵃᵇ	8.37ᵃ	0.012	0.03
Spirochaetes	3.80ᵃᵇ	3.33ᵃᵇ	2.98ᵇ	6.20ᵃᵇ	4.52ᵃᵇ	4.79ᵃᵇ	8.36ᵃ	0.012	0.03
Treponema	3.78ᵃᵇ	3.32ᵃᵇ	2.96ᵇ	6.19ᵃᵇ	4.52ᵃᵇ	4.79ᵃᵇ	8.35ᵃ	0.012	0.03
Fibrobacteres	1.71	1.89	1.24	1.45	1.85	1.39	0.78	0.003	0.16
Fibrobacter	1.71	1.89	1.24	1.45	1.85	1.39	0.78	0.003	0.16
SR1[4]	3.78	3.01	4.16	2.72	2.72	2.03	2.68	0.005	0.29
Tenericutes	1.26	1.25	1.41	0.88	0.72	0.59	0.40	0.002	0.17
Unassigned	2.35ᵇ	2.91ᵇ	2.65ᵇ	4.00ᵇ	6.69ᵃ	3.30ᵇ	3.28ᵇ	0.007	<0.0001

ᵃ⁻ᵈLeast squares means within a row with different superscripts differ significantly ($P < 0.05$).

[1]Number of sequences allocated to the individual taxa relative to total number of sequences (wk −3: 157,644; wk −2: 164,646; wk −1: 141,524; wk 1: 173,283; wk 2: 155,134; wk 3: 114,874; wk 4: 237,035) obtained in the analysis.

[2]Sample numbers for each study week were wk −1: 10; wk −2: 9; wk −3: 10; wk 1: 10; wk 2: 9; wk 3: 9; wk 4: 13.

[3]SEM represents mean standard error and *P*-value indicates the significance level of fixed effect "week relative to parturition."

[4]Sulfur River 1 (Harris et al., 2004).

Diversity of the Bacterial Community

We calculated 3 estimates of the bacterial community α-diversity (Chao 1, observed species, and PD whole tree) on individual samples, indicating mean values for the week groups (Table 3). The Chao1 richness index showed a significant decrease from the prepartum to the postpartum period. In agreement with this, the observed species number and PD whole tree of the bacterial community showed significant decreases over the transition period (Table 3). Principal coordinate analysis (**PCoA**) of the weighted UniFrac metric revealed a shift of community diversity from 3 wk prepartum and 4 wk postpartum along principal coordinates (**PC**)1 and PC2 (Figure 4A). Taken together, the first 2 PC explained more than 50% of the variation in microbial community. The sample variation was less in the prepartum period than in the postpartum period. There was a significant difference on the weighted UniFrac distance between wk −3 (prepartum) and wk 4 (postpartum; ANOSIM $P = 0.001$, $R^2 = 0.32$). The results may have been biased by the fact that the cows were unintentionally sampled unevenly relative to parturition, because the expected and actual day of parturition differed. Thus, cows 1 and 10 were mainly sampled prepartum and postpartum, respectively, and variation between cows may have affected the observed shifts of the bacterial community. However, we did observe community shifts in individual cows sampled over the entire transition period (e.g., cows 3, 5, 6, and 7) as illustrated by the PCoA plot colored by cow individuals (Figure 4B). This plot also shows that the samples of cows 1 and 10 clustered with samples of other cows from the same sample weeks.

SCFA and Correlations to the Relative Abundance of Bacterial Taxa

The concentration of total SCFA ranged between 80 and 100 mmol/kg of wet rumen sample and did not change significantly over the transition period (Figure 5). The relative proportions of the 3 major SCFA (ac-etate, propionate, and butyrate) on the other hand, changed significantly, such that the proportion of acetate decreased compared with that of propionate and butyrate. Consequently, the acetate-to-propionate (**A:P**) ratio decreased significantly over the transition period (Figure 5). Correlations between the SCFA components and bacterial taxa with relative abundance >1% (family level) or >0.5% (genus level) are illustrated in a heatmap (Figure 6). The *Bacteroidetes* and *Firmicutes* phyla showed positive correlations with acetate proportion and the A:P ratio, as did the lower taxa *Bacteroidia*, *Bacteroidales*, *Clostridia*, and *Clostridiales.* Further, the lower taxa *Ruminococcaceae*, *Ruminococcus*, and unclassified *Ruminococcaceae* of the *Clostridiales* order showed positive correlations with the acetate proportion and A:P ratio as well. The *Prevotellaceae* family and *Prevotella* genus of the *Bacteroidetes* phylum were exclusively positively correlated with propionate concentration and proportion. The *Ruminococcus* genus was the only taxon showing positive correlation with acetate concentration. Except for *Ruminobacter* and *Alphaproteobacteria*, the lower taxa of the *Proteobacteria* phylum showed positive correlations with the propionate concentration and proportion, and thus correlated negatively with A:P. Ten bacterial taxa, including the genus *Prevotella*, the genus-level taxa YRC22 and CF231 of the *Paraprevotellaceae*, and unclassified *Succinivibrionaceae* showed positive correlations with either butyrate concentration or proportion.

mcrA Gene Clone Library Analysis

We identified 85 clones from the pooled PCR products, representing 36 different OTU (phylotypes), based on a 3% dissimilarity cut-off (Figure 7). Phylogenetic analysis demonstrated that all the clones were covered by 2 orders: *Methanobacteriales* (51 clones) and *Methanomassiliicoccales* (34 clones). Within the *Methanobacteriales* order, 25 of the 51 clones were distributed among 17 OTU belonging to *Methanobrevibacter*, and 23 clones were distributed among 6 OTU belonging to

Table 3. α-Diversity analysis of the bacterial community in dairy cows over the transition period

Item[1]	Week relative to parturition							SEM[2]	P-value[2]
	−3	−2	−1	1	2	3	4		
Chao1	2,936.22[a]	2,893.71[a]	2,912.56[a]	2,784.98[a]	2,435.37[b]	2,303.06[b]	2,345.90[b]	74.35	<0.0001
Observed species	2,607.00[a]	2,549.75[a]	2,557.67[a]	2,412.22[a]	2,029.78[b]	1,936.63[b]	1,942.18[b]	82.47	<0.0001
PD whole tree	231.90[a]	228.76[a]	230.30[a]	222.17[a]	198.28[b]	190.61[b]	191.39[b]	5.16	<0.0001

[a,b]Least squares means within a row with different superscripts differ significantly ($P < 0.05$).

[1]Chao1 index, observed species number, and phylogenetic diversity (PD) whole tree were used to measure the bacterial diversity of individual samples.

[2]SEM represents mean standard error and *P*-value indicates the significance level of the fixed effect "week relative to parturition."
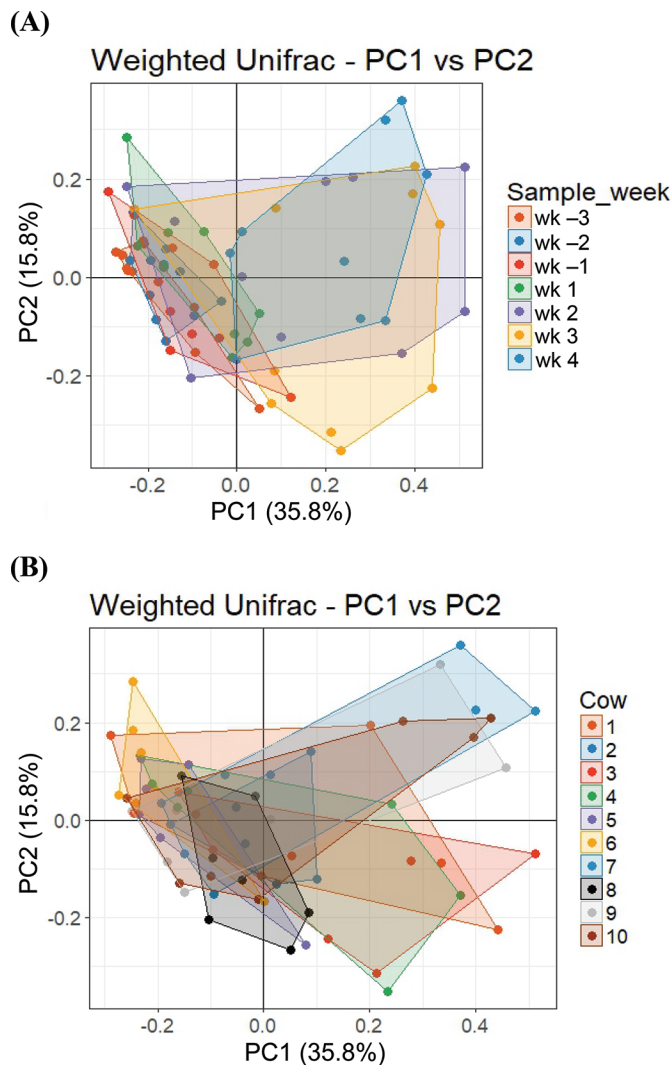
(A)

(B)

Figure 4. Principal coordinates (PC) analysis (PCoA), showing the similarities of the bacterial communities based on weighted UniFrac distances are shown in 2-dimensional PCoA plots. (A) Weekly sample groups (wk −3, wk −2, wk −1, wk 1, wk 2, wk 3, wk 4) are indicated by different colors and cow individuals allocated to the same group are represented by the same color; (B) individual cows numbered from 1 to 10 are indicated by different colors. Color version available online.

Methanosphaera. The remaining 3 *Methanobacteriales* clones were covered by a single OTU, MTH6, with an unclassified methanogen as closest relative. The 34 *Methanomassiliicoccales* clones were distributed among 12 OTU, all belonging to *Methanomassiliicoccus*. We included 2 *mcrA* gene sequences of uncultured methanogenic archaeon clones (GenBank accession numbers JN229252 and JN229233), obtained from rumen samples in a previous study and reported to have more than 95% similarity to rumen cluster C, RCC, (*Methanomassiliicoccales*) archaea (Poulsen et al., 2013) in the phylogenetic tree, and they clustered closely with the *Methanomassiliicoccales* clones of the present study.
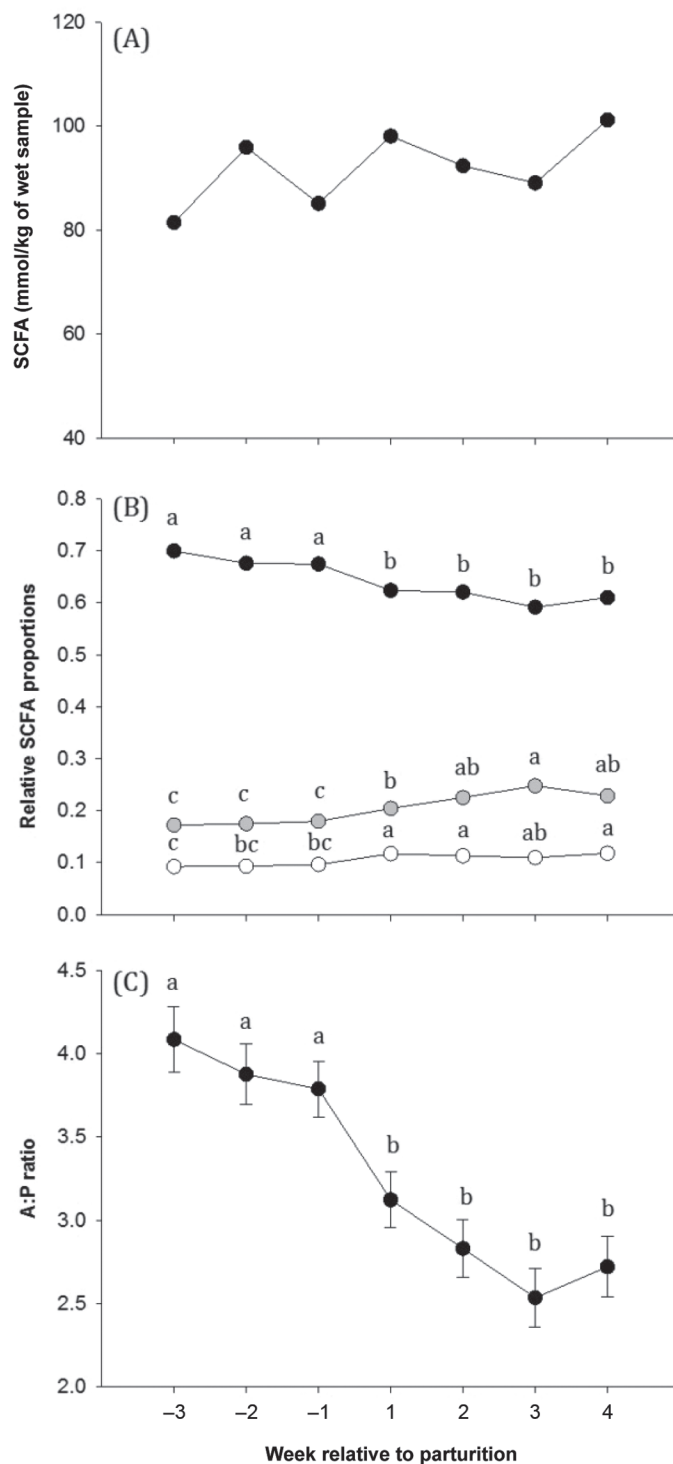

Figure 5. Ruminal short-chain fatty acids (SCFA) over the transition period: (A) accumulated total SCFA; (B) relative proportions of the 3 major SCFA: acetate (black), propionate (gray), and butyrate (white); (C) acetate-to-propionate ratio (A:P). Error bars in panel C represent SEM. Different letters (a–c) denote significant difference ($P < 0.05$) between weeks.

mcrA Gene T-RFLP Analysis

Based on in silico digestion of the clone library sequences (http://www.restrictionmapper.org/), we obtained a tentative identification of most individual T-RF (Figure 7), and we depicted their contribution to the methanogen community composition over the transition period by their relative abundance, as illustrated for the T-RFs 210 and 267 bp (Supplemental Figure S2; https://doi.org/10.3168/jds.2017-14366). The majority of clone sequences, clustering within *Methanobrevibacter*, generated a T-RF of 39 bp (Figure 6). This was also the most abundant fragment in the electropherograms of the T-RFLP analysis, with no significant changes over the transition period (Table 4).

All *Methanosphaera* clones generated a T-RF of 267 bp (Figure 7), the abundance of which increased significantly over the transition period (Table 4). Several T-RF (105, 154, 202, 297, and 393 bp) were generated by the clone library sequences of the *Methanomassiliicoccales* order (Figure 7). The most dominating of these, T-RF 105 and 297 bp, showed opposite patterns over the transition period with significant increases and decreases, respectively (Table 4).

PCA of T-RF Based on Their Relative Abundances

The first 2 principal components explained 55% (32.3 and 22.7% for PC1 and PC2, respectively) of the total variance (Figure 8). A clear separation was observed
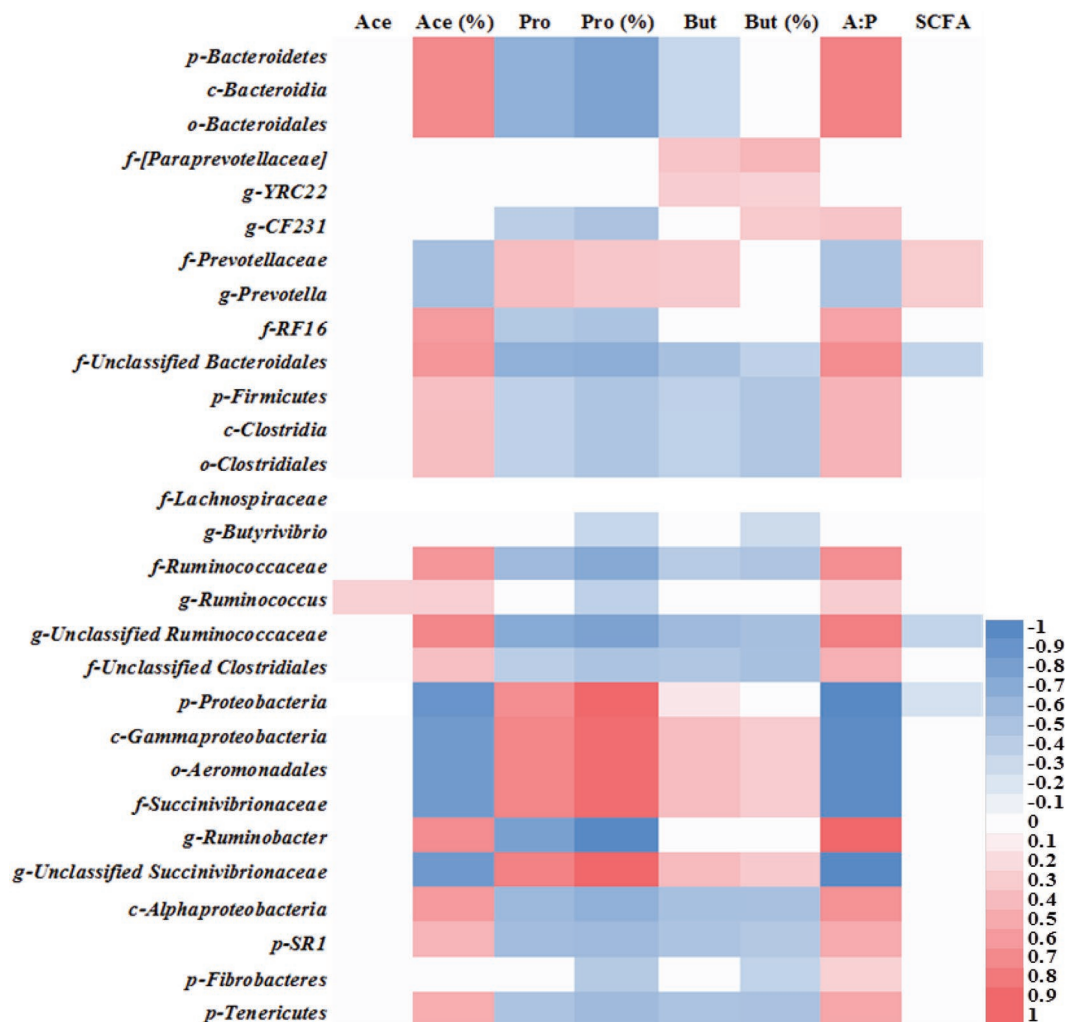

Figure 6. Heatmap of correlations between short-chain fatty acid (SCFA) components and bacterial taxa with a relative abundance >1% (family level) or >0.5% (genus level). Pearson correlation coefficients were calculated and the values between −1 and 1 in the color key indicate significantly negative (blue) or positive (red) correlations. Only taxa with a significant correlation ($P \leq 0.05$) to at least one of the SCFA components are shown in the graph. The taxonomic levels are indicated by letters (p = phylum; c = class; o = order; f = family; g = genus). The SCFA components (SCFA = total SCFA; Ace = acetate; Pro = propionate; But = butyrate) represent actual concentrations or relative proportions (%). A:P indicates the acetate-to-propionate ratio. Color version available online.
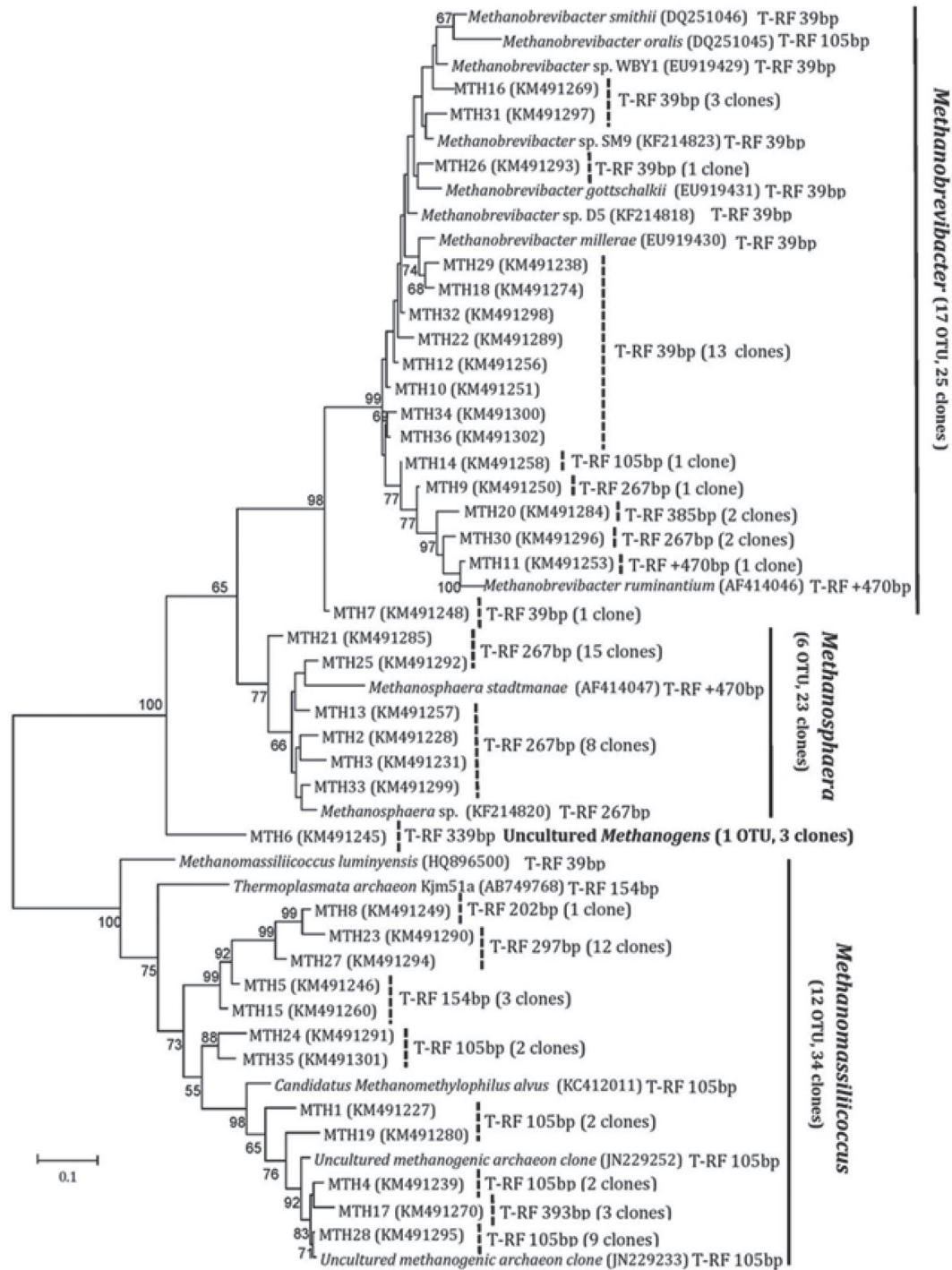
Figure 7. Phylogenetic tree based on the *mcr*A gene clone library, constructed by using the maximum likelihood method based on the Kimura 2-parameter model. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011). The terminal restriction fragment (T-RF) lengths for each representative sequence, as indicated in the figure, were obtained by in silico digestion to identify *Taq*I restriction sites. The number of clones obtained for operational taxonomic units (OTU) with the same T-RF length is denoted in parentheses.

Table 4. Relative abundance[1] of predominant terminal-restriction fragments (T-RF) from *mcrA* gene terminal-RFLP profiles across the transition period and potential T-RF identifications based on clone library *mcrA* gene sequences

| T-RF (bp) | Week relative to parturition | | | | | | | SEM[2] | *P*-value[2] | Clones[3] | Potential identification[4] |
	−3	−2	−1	1	2	3	4				
39	51.6	51.7	49.3	49.8	42.5	39.4	43.5	3.284	0.11	19	*Methanobrevibacter* sp.
105	9.5[bc]	7.9[bc]	8.2[bc]	7.5[c]	13.1[abc]	21.1[a]	16.4[ab]	2.391	<0.01	15	*Methanomassiliicoccus* sp.
										1	*Methanobrevibacter* sp.
154	4.6[a]	4.6[a]	4.6[a]	2.4[b]	1.4[bc]	0.9[c]	0.5[c]	0.396	<0.0001	3	*Methanomassiliicoccus* sp.
202	1.5[a]	1.5[a]	1.4[a]	0.7[ab]	0.4[b]	0.6[ab]	0.6[ab]	0.294	<0.01	1	*Methanobrevibacter* sp.
										1	*Methanomassiliicoccus* sp.
210	0.2[a]	0.2[ab]	0.2[ab]	0.2[ab]	0.1[b]	0.1[b]	0.1[ab]	0.018	<0.01	0[5]	Not identified
267	9.3[cd]	8.1[d]	10.2[cd]	16.0[bc]	16.6[ab]	18.8[ab]	22.3[a]	1.708	<0.0001	22	*Methanosphaera* sp.
										3	*Methanobrevibacter* sp.
297	15.6[a]	16.3[a]	15.9[a]	9.2[ab]	9.3[ab]	9.6[ab]	6.2[b]	1.833	<0.01	12	*Methanomassiliicoccus* sp.
339	ND[6]	ND	ND	ND	ND	ND	ND	ND	ND	3	Uncultured methanogens
342	0.3[bc]	0.5[bc]	0.4[bc]	0.3[c]	0.7[abc]	1.0[a]	0.8[ab]	0.129	<0.01	0	Not identified
385	1.6	1.4	1.6	1.5	1.3	1.3	1.4	0.241	0.88	2	*Methanobrevibacter* sp.
393	ND	ND	ND	ND	ND	ND	ND	ND	ND	3	*Methanomassiliicoccus* sp.
475	1.1	1.2	1.5	1.7	2.1	1.7	2.5	0.396	0.07	0	Not identified

[a–d]Least squares means within a row with different superscripts differ significantly ($P < 0.05$).

[1]Relative abundance was calculated as individual peak (T-RF) height relative to total peak heights for each sample.

[2]SEM represents mean standard error and *P*-value indicates the significance level of fixed effect "week relative to parturition."

[3]Number of clone sequences detected in the *mcrA* gene clone library (85 sequences in total) with the respective T-RF lengths (see Figure 7).

[4]Potential identification of the T-RF based on BLAST search of the detected clone library sequences. See Figure 7.

[5]No *mcrA* gene sequences were detected in the clone library with the given T-RF length.

[6]No T-RF of this length were detected in the electropherograms.

for the methanogenic archaeal community from wk −3 to wk 4. There was greater variation in the postpartum samples, as displayed by the distinct pattern compared with the prepartum samples.

DISCUSSION

Changes in the Rumen Bacterial Community

Over the transition period, 34 bacterial genera were detected in all samples from all animals and thereby met the suggested criteria for constituting a "core microbiome" (Weimer, 2015). Additionally, our results indicate the existence of distinct prepartum and postpartum microbiomes, suggesting that factors that changed over the transition period—such as diet composition and feed intake—shaped the rumen bacterial community.

In agreement with other rumen studies (Pitta et al., 2014a; Lima et al., 2015; Dieho et al., 2017), we observed *Bacteroidetes*, *Proteobacteria*, and *Firmicutes*, to be the dominant bacterial phyla. However, these dominating phyla differ in their relative abundance from study to study and, in our case, over the transition period. This may be explained by differences in the forage-to-concentrate ratio of the respective diets (Petri et al., 2013; Pitta et al., 2014c). It has thus been reported that *Ruminococcaceae* together with unclassified *Bacteroidales* were more abundant in the rumen of animals fed forage diets (Henderson et al., 2015),

indicating that the dietary shift over the transition period may have been the factor driving the observed reduction in relative abundance of *Ruminococcaceae* and *Christensenellaceae*. On the other hand, we observed no significant changes in cellulolytic genera, such as *Ruminococcus*, *Fibrobacter*, and *Butyrivibrio*, over the transition period, which is in contrast to the decrease reported by Lima et al. (2015). The discrepancy may be explained by considerable proportions of NDF from grass and maize silage (36–49%) in the postpartum diet of the present study, providing sufficient substrate to maintain the population of cellulolytic bacteria.

With the shift to a high-grain postpartum diet, we observed a reduction in the relative abundance of potential starch degraders such as *Ruminobacter* over the transition period. Likewise, Wang et al. (2012) reported a reduction in the *Ruminobacter amylophilus* abundance over the transition period, together with an increase in the concentrate proportion of the postpartum diet. On the other hand, we observed higher abundances of *Prevotella* and unclassified *Succinivibrionaceae* in the postpartum period, which is in agreement with other observations that indicate members of these genera to be more abundant in animals fed high-concentrate diets (Henderson et al., 2015). These taxa may include starch-degrading producers of propionate or its precursor, succinate (Bryant and Small, 1956; Strobel, 1992), and their increase could contribute to the observed decrease in A:P. This suggests that as a dominant and
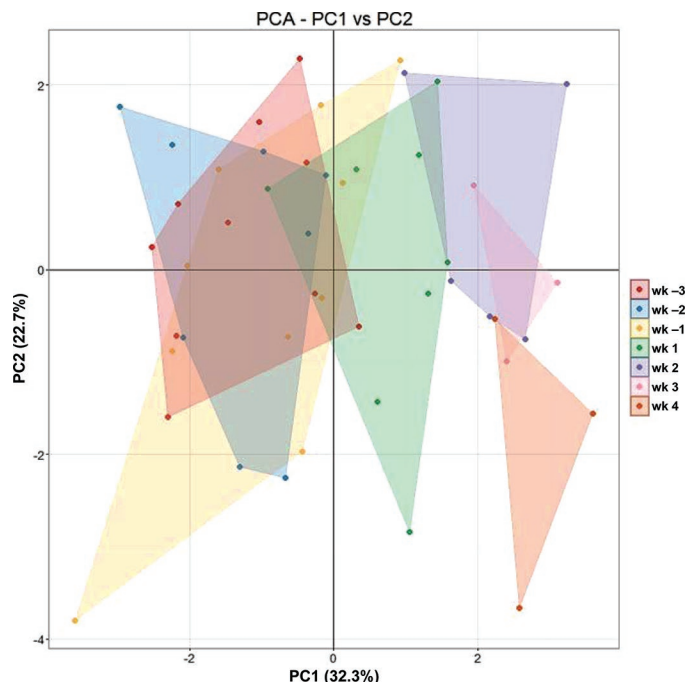
Figure 8. Principal component analysis (PCA), showing the similarities of the methanogenic archaeal communities based on the relative abundance of the terminal restriction fragment (T-RF) identified in the T-RFLP profile used in this analysis. Weekly-based sample groups spanning the transition period (wk −3, wk −2, wk −1, wk 1, wk 2, wk 3, wk 4) are indicated by different colors, and shifts of the methanogen community are depicted. Color version available online.

functionally diverse group, *Prevotella*-related species may more readily adapt to changes in diet composition and take over the role of *Ruminobacter* for starch degradation. However, we also observed a large group of unclassified *Succinivibrionaceae*, which are more closely related to *Ruminobacter* than *Prevotella*. This genus-level taxon increased in abundance postpartum and may also have harbored starch degraders. Overall, we observed a lower *Prevotella* abundance (5–11%) than in other studies (Pitta et al., 2014a, 40–60%; Lima et al., 2015, 20%); however, we observed a large related group of unclassified *Bacteroidales* (15–30%) and these could be fulfilling a similar function to the *Prevotella* in our animals. Moreover, several minor bacterial genera with unknown function were identified in the present as well as in other studies (Jewell et al., 2015; Lima et al., 2015), including CF231 and YRC22 (both in the *Paraprevotellaceae*), BF311 (in the *Bacteroidaceae*), S24–7, *Coprococcus*, *Moryella*, *Pseudobutyrivibrio*, and *Shuttleworthia*, some of which increased or decreased over the transition period.

The rumen bacterial richness decreased significantly from the prepartum to the postpartum period, as also reported by Lima et al. (2015) and Dieho et al. (2017), and the entire bacterial community showed a prepartum to postpartum shift, as illustrated by the PCoA plot (Figure 4A and 4B). This is in line with observations where a shift from a high-forage to a high-starch diet affected the rumen microbial community structure (Tajima et al., 2001; Pitta et al., 2014b,c) and, more specifically, where a high-starch diet (a SARA induction diet) significantly reduced the diversity of the rumen microbial community (Mao et al., 2013).

Although dietary changes over the transition period may be the main driver of changes in the rumen microbial community, the impact of the dietary effects is confounded by host physiological changes over the transition period, which should be taken into consideration. For transition cows, the growth and development of the fetus is energetically a very costly process, with the amount of metabolizable energy required at term being about 175% of that of nonpregnant cow of equal BW (Moe and Tyrrell, 1972). It has also been reported that the total-tract starch digestibility of dairy cows decreased linearly postpartum due to increased DMI, as we also observed in the present study, and increased rumen passage rate (Park et al., 2011). Additionally, the time spent on rumination may be shortened in the post-calving period and the mean retention time of rumen digesta reduced over the transition period in dairy cows (Aikman et al., 2008). Therefore, increased DMI with potentially facilitated ruminal passage rate in the postpartum period may be involved—directly or indirectly—in the modulation of rumen microbiota, with slow-growing microorganisms being washed out of the rumen more easily.

Correlations Between Bacterial Taxa and SCFA

The SCFA are absorbed over the rumen epithelium and utilized in host metabolic processes, where acetate is a key component in milk fat synthesis, propionate is the most important precursor for gluconeogenesis, and butyrate is converted into ketone bodies for energy supply. Ruminal SCFA production and absorption rates have been observed to change with increased concentrate allowance over the transition period (Dieho et al., 2016). This may explain the clear shift in the SCFA profile that we observed in the present study. On the other hand, the abovementioned changes in the relative abundances of major bacteria taxa may be directly related to changes in the rumen microbial fermentation pattern, as reflected by multiple correlations between specific bacterial taxa and individual SCFA components, such as *Ruminococcus* and acetate levels, and *Prevotella* and propionate levels.

Changes in the Rumen Methanogen Community

The general composition of the rumen methanogen community, dominated by members of the orders *Methanobacteriales*, in particular the genera *Methanobrevibacter* and *Methanosphaera*, and the *Methanomassiliicoccales*, is in accordance with other studies (Danielsson et al., 2012; Sirohi et al., 2013; Seedorf et al., 2015).

The *Methanobrevibacter* genus, harboring typical formate-, H_2-, and CO_2-utilizing hydrogenotrophs, dominated the archaeal community and had a high and constant abundance in our study, in accordance with previous observations in primiparous dairy cows (Cersosimo et al., 2016; Dieho et al., 2017). However, as in Dieho et al. (2017), we observed an increased abundance of methylotrophic *Methanosphaera* over the transition period. Members of the genus *Methanosphaera*, like *Methanosphaera stadtmanae*, are hydrogen-dependent methylotrophs, producing methane by reducing methanol with hydrogen as the electron donor. Methanol can be produced via pectin degradation by protozoa and bacteria (Hungate, 1975). For the present study, the inclusion of 11.19% sugar beet pellets in the postpartum diet was a source of pectin, which may have facilitated methanol production and hence the significant increase in *Methanosphaera*; *Methanosphaera* have previously been reported to be more abundant in the rumen of dairy cows fed low-forage diets (Carberry et al., 2014). *Methanomassiliicoccus luminyensis* also produce methane from methanol in the presence of hydrogen (Dridi et al., 2012). *Methanomassiliicoccales* is a less characterized methanogen order, so far solely comprising obligate hydrogen-dependent methylotrophs, but may potentially cover several distinct and physiologically diverse genera and species (Janssen and Kirs, 2008). Rumen *Methanomassiliicoccales* have been observed to decrease in abundance when cows are switched from forage to high-grain diets (Tymensen et al., 2012), and dietary supplementation of rapeseed oil has been reported to inhibit rumen cluster C (*Methanomassiliicoccales*) methanogens specifically (Poulsen et al., 2013). Therefore, the observed reduction in abundance of some *Methanomassiliicoccales* over the transition period may have been associated with increase in dietary concentrate or rapeseed oil content; however, the pattern was not the same for all members of the order.

As further illustrated by the PCA, we observed significant shifts in the composition (relative abundances of the individual *mcrA* gene T-RF) of the rumen methanogen community over the transition period. In accordance with this, shifting cows from a hay diet to a high-concentrate diet has been shown to alter rumen methanogen diversity and community structure but not methanogen abundance (Hook et al., 2011). Moreover, a recent study demonstrated that feed consumption could affect the composition of the methanogen community in the rumen of beef cattle (McGovern et al., 2017). These observations suggest overall that increased DMI over the transition period may affect the methanogen community composition of dairy cows.

CONCLUSIONS

Using a combination of 16S rRNA gene amplicon sequencing and *mcrA*-gene based terminal-RFLP and clone library analyses, we observed significant changes in the relative abundance of individual rumen microbial taxa (within *Bacteria* and *Archaea*) in response to the physiological and dietary perturbations encountered by dairy cows over the transition period. Moreover, we identified core structures of the prepartum and postpartum bacterial and methanogenic archaeal communities. In accordance with the observed shifts in bacterial community composition, we observed a clear shift in the rumen fermentation pattern (SCFA profile). We revealed correlations between the relative abundance of specific bacterial taxa and SCFA components, and the significant decrease in A:P postpartum may again have been a driving factor for the observed changes in the archaeal community, due to increased competition for hydrogen. For future investigations, further application of RNA approaches (e.g., metatranscriptomics) focusing on microbial function may provide a more in-depth understanding of the dynamics of the rumen microbiome in the transition period. However, the present study clearly demonstrated the transition period as being highly dynamic and characterized by distinct perturbations in the rumen microbiome.

ACKNOWLEDGMENTS

REFERENCES

Aikman, P. C., C. K. Reynolds, and D. E. Beever. 2008. Diet digestibility, rate of passage, and eating and rumination behavior of Jersey and Holstein cows. J. Dairy Sci. 91:1103–1114. https://doi.org/10.3168/jds.2007-0724.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73:2804–2819. https://doi.org/10.2527/1995.7392804x.

Berry, D., K. Ben Mahfoudh, M. Wagner, and A. Loy. 2011. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl. Environ. Microbiol. 77:7846–7849. https://doi.org/10.1128/AEM.05220-11.

Bryant, M. P., and N. Small. 1956. Characteristics of two new genera of anaerobic curved rods isolated from the rumen of cattle. J. Bacteriol. 72:22–26.

Canibe, N., O. Højberg, J. H. Badsberg, and B. B. Jensen. 2007. Effect of feeding fermented liquid feed and fermented grain on gastrointestinal ecology and growth performance in piglets. J. Anim. Sci. 85:2959–2971. https://doi.org/10.2527/jas.2006-744.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. Desantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: A flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266–267. https://doi.org/10.1093/bioinformatics/btp636.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. USA 108:4516–4522. https://doi.org/10.1073/pnas.1000080107.

Carberry, C. A., S. M. Waters, D. A. Kenny, and C. J. Creevey. 2014. Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. Appl. Environ. Microbiol. 80:586–594. https://doi.org/10.1128/AEM.03131-13.

Cersosimo, L. M., M. L. Bainbridge, J. Kraft, and A.-D. G. Wright. 2016. Influence of periparturient and postpartum diets on rumen methanogen communities in three breeds of primiparous dairy cows. BMC Microbiol. 16:78. https://doi.org/10.1186/s12866-016-0694-7.

Cheng, Y. F., J. E. Edwards, G. G. Allison, W. Y. Zhu, and M. K. Theodorou. 2009. Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. Bioresour. Technol. 100:4821–4828. https://doi.org/10.1016/j.biortech.2009.04.031.

Danielsson, R., A. Schnürer, V. Arthurson, and J. Bertilsson. 2012. Methanogenic population and CH4 production in Swedish dairy cows fed different levels of forage. Appl. Environ. Microbiol. 78:6172–6179. https://doi.org/10.1128/AEM.00675-12.

Dieho, K., J. Dijkstra, J. T. Schonewille, and A. Bannink. 2016. Changes in ruminal volatile fatty acid production and absorption rate during the dry period and early lactation as affected by rate of increase of concentrate allowance. J. Dairy Sci. 99:5370–5384. https://doi.org/10.3168/jds.2015-10819.

Dieho, K., B. van den Bogert, G. Henderson, A. Bannink, J. Ramiro-Garcia, H. Smidt, and J. Dijkstra. 2017. Changes in rumen microbiota composition and in situ degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance. J. Dairy Sci. 100:2695–2710. https://doi.org/10.3168/jds.2016-11982.

Drackley, J. K. 1999. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci. 82:2259–2273. https://doi.org/10.3168/jds.S0022-0302(99)75474-3.

Dridi, B., M. L. Fardeau, B. Ollivier, D. Raoult, and M. Drancourt. 2012. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int. J. Syst. Evol. Microbiol. 62:1902–1907. https://doi.org/10.1099/ijs.0.033712-0.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461.

Fernando, S. C., H. T. Purvis, F. Z. Najar, L. O. Sukharnikov, C. R. Krehbiel, T. G. Nagaraja, B. A. Roe, and U. De Silva. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76:7482–7490. https://doi.org/10.1128/AEM.00388-10.

Fierer, N., C. C. L. Lauber, N. Zhou, D. McDonald, E. K. Costello, and R. Knight. 2010. Forensic identification using skin bacterial communities. Proc. Natl. Acad. Sci. USA 107:6477–6481. https://doi.org/10.1073/pnas.1000162107.

Geishauser, T., N. Linhart, A. Neidl, and A. Reimann. 2012. Factors associated with ruminal pH at herd level. J. Dairy Sci. 95:4556–4567. https://doi.org/10.3168/jds.2012-5380.

Hamady, M., C. Lozupone, and R. Knight. 2010. Fast UniFrac: Facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J. 4:17–27. https://doi.org/10.1038/ismej.2009.97.

Harris, J. K., S. T. Kelley, and N. R. Pace. 2004. New perspective on uncultured bacterial phylogenetic division OP11. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.70.2.845-849.2004.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, and P. H. Janssen. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5:14567. https://doi.org/10.1038/srep14567.

Hook, S. E., M. A. Steele, K. S. Northwood, A. D. G. Wright, and B. W. McBride. 2011. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. Microb. Ecol. 62:94–105. https://doi.org/10.1007/s00248-011-9881-0.

Hungate, R. E. 1975. The rumen microbial ecosystem. Annu. Rev. Ecol. Syst. 6:39–66. https://doi.org/10.1146/annurev.es.06.110175.000351.

Ingvartsen, K. L. 2006. Feeding- and management-related diseases in the transition cow: Physiological adaptations around calving and strategies to reduce feeding-related diseases. Anim. Feed Sci. Technol. 126:175–213. https://doi.org/10.1016/j.anifeedsci.2005.08.003.

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. https://doi.org/10.1038/ismej.2013.2.

Janssen, P. H., and M. Kirs. 2008. Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74:3619–3625. https://doi.org/10.1128/AEM.02812-07.

Jensen, M. T., R. P. Cox, and B. B. Jensen. 1995. Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. Anim. Sci. 61:293–304. https://doi.org/10.1017/S1357729800013837.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710. https://doi.org/10.1128/AEM.00720-15.

Jeyanathan, J., M. Kirs, R. S. Ronimus, S. O. Hoskin, and P. H. Janssen. 2011. Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. FEMS Microbiol. Ecol. 76:311–326. https://doi.org/10.1111/j.1574-6941.2011.01056.x.

Lima, F. S., G. Oikonomou, S. F. Lima, M. L. S. Bicalho, E. K. Ganda, J. C. de Oliveira Filho, G. Lorenzo, P. Trojacanec, and R. C. Bicalho. 2015. Prepartum and postpartum rumen fluid microbiomes: Characterization and correlation with production traits in dairy cows. Appl. Environ. Microbiol. 81:1327–1337. https://doi.org/10.1128/AEM.03138-14.

Lin, C., L. Raskin, and D. A. Stahl. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: Comparative analyses using rRNA-targeted oligonucleotide probes. FEMS Microbiol. Ecol. 22:281–294. https://doi.org/10.1016/S0168-6496(97)00002-0.

Mao, S. Y., R. Y. Zhang, D. S. Wang, and W. Y. Zhu. 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. Anaerobe 24:12–19. https://doi.org/10.1016/j.anaerobe.2013.08.003.

McGovern, E., M. S. McCabe, P. Cormican, M. Popova, K. Keogh, A. K. Kelly, D. A. Kenny, and S. M. Waters. 2017. Plane of nutrition affects the phylogenetic diversity and relative abundance of transcriptionally active methanogens in the bovine rumen. Sci. Rep. 7:13047. https://doi.org/10.1038/s41598-017-13013-y.

Moe, P. W., and H. F. Tyrrell. 1972. Metabolizable energy requirements of pregnant dairy cows. J. Dairy Sci. 55:480–483. https://doi.org/10.3168/jds.S0022-0302(72)85519-X.

Mohammed, R., D. M. Stevenson, P. J. Weimer, G. B. Penner, and K. A. Beauchemin. 2012. Individual animal variability in ruminal bacterial communities and ruminal acidosis in primiparous Holstein cows during the periparturient period. J. Dairy Sci. 95:6716–6730. https://doi.org/10.3168/jds.2012-5772.

Oliveros, J. C. 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. BioinfoGP of CNB-CSIC. http://bioinfogp.cnnb.csic.es/tools/venny/index.ht.

Park, A. F., J. E. Shirley, E. C. Titgemeyer, J. M. DeFrain, R. C. Cochran, E. E. Wickersham, T. G. Nagaraja, and D. E. Johnson. 2011. Characterization of ruminal dynamics in Holstein dairy cows during the periparturient period. J. Anim. Physiol. Anim. Nutr. (Berl.) 95:571–582. https://doi.org/10.1111/j.1439-0396.2010.01085.x.

Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS One 8. https://doi.org/10.1371/journal.pone.0083424.

Pitta, D. W., S. Kumar, B. Vecchiarelli, L. D. Baker, J. D. Ferguson, N. Thomsen, D. J. Shirley, and K. Bittinger. 2014a. Temporal dynamics in the ruminal microbiome of dairy cows during the transition period. J. Anim. Sci. 92:4014–4022. https://doi.org/10.2527/jas.2014-7621.

Pitta, D. W., S. Kumar, B. Veiccharelli, N. Parmar, B. Reddy, and C. G. Joshi. 2014b. Bacterial diversity associated with feeding dry forage at different dietary concentrations in the rumen contents of Mehshana buffalo (Bubalus bubalis) using 16S pyrotags. Anaerobe 25:31–41. https://doi.org/10.1016/j.anaerobe.2013.11.008.

Pitta, D. W., N. Parmar, A. K. Patel, N. Indugu, S. Kumar, K. B. Prajapathi, A. B. Patel, B. Reddy, and C. Joshi. 2014c. Bacterial diversity dynamics associated with different diets and different primer pairs in the rumen of Kankrej cattle. PLoS One 9. https://doi.org/10.1371/journal.pone.0111710.

Poulsen, M., C. Schwab, B. Borg Jensen, R. M. Engberg, A. Spang, N. Canibe, O. Højberg, G. Milinovich, L. Fragner, C. Schleper, W. Weckwerth, P. Lund, A. Schramm, and T. Urich. 2013. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nat. Commun. 4:1428. https://doi.org/10.1038/ncomms2432.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26:1641–1650. https://doi.org/10.1093/molbev/msp077.

Rey, M., F. Enjalbert, S. Combes, L. Cauquil, O. Bouchez, and V. Monteils. 2014. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116:245–257. https://doi.org/10.1111/jam.12405.

Reynolds, C. K., G. B. Huntington, H. F. Tyrrell, and P. J. Reynolds. 1988. Net metabolism of volatile fatty acids, D-beta-hydroxybutyrate, nonesterifield fatty acids, and blood gasses by portal-drained

viscera and liver of lactating Holstein cows. J. Dairy Sci. 71:2395–2405.

Seedorf, H., S. Kittelmann, and P. H. Janssen. 2015. Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. Appl. Environ. Microbiol. 81:986–995. https://doi.org/10.1128/AEM.03018-14.

Sirohi, S. K., P. P. Chaudhary, N. Singh, D. Singh, and A. K. Puniya. 2013. The 16S rRNA and mcrA gene based comparative diversity of methanogens in cattle fed on high fibre based diet. Gene 523:161–166. https://doi.org/10.1016/j.gene.2013.04.002.

Steinberg, L. M., and J. M. Regan. 2008. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. Appl. Environ. Microbiol. 74:6663–6671. https://doi.org/10.1128/AEM.00553-08.

Strobel, H. J. 1992. Vitamin B_{12}-dependent propionate production by the ruminal bacterium Prevotella ruminicola 23. Appl. Environ. Microbiol. 58:2331–2333.

Sundberg, C., W. A. Al-Soud, M. Larsson, E. Alm, S. S. Yekta, B. H. Svensson, S. J. Sørensen, and A. Karlsson. 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Ecol. 85:612–626. https://doi.org/10.1111/1574-6941.12148.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Appl. Environ. Microbiol. 67:2766–2774. https://doi.org/10.1128/AEM.67.6.2766-2774.2001.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739. https://doi.org/10.1093/molbev/msr121.

Tian, L., J. Scholte, K. Borewicz, B. van den Bogert, H. Smidt, A. J. W. Scheurink, H. Gruppen, and H. A. Schols. 2016. Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats. Mol. Nutr. Food Res. 60:2256–2266. https://doi.org/10.1002/mnfr.201600149.

Tymensen, L. D., K. A. Beauchemin, and T. A. McAllister. 2012. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. Microbiology 158:1808–1817. https://doi.org/10.1099/mic.0.057984-0.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73:5261–5267. https://doi.org/10.1128/AEM.00062-07.

Wang, X., X. Li, C. Zhao, P. Hu, H. Chen, Z. Liu, G. Liu, and Z. Wang. 2012. Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. Appl. Environ. Microbiol. 78:2386–2392. https://doi.org/10.1128/AEM.07545-11.

Weimer, P. J. 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. Front. Microbiol. 6:296. https://doi.org/10.3389/fmicb.2015.00296.

Zhu, Z., S. J. Noel, G. F. Difford, W. A. Al-Soud, A. Brejnrod, S. J. Sørensen, J. Lassen, P. Løvendahl, and O. Højberg. 2017. Community structure of the metabolically active rumen bacterial and archaeal communities of dairy cows over the transition period. PLoS One 12. https://doi.org/10.1371/journal.pone.0187858.