Changes in Bacterial Diversity Associated with Epithelial Tissue in the Beef Cow Rumen during the Transition to a High-Grain Diet

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Our understanding of the ruminal epithelial tissue-associated bacterial (defined as epimural bacteria in this study) community is limited. In this study, we aimed to determine whether diet influences the diversity of the epimural bacterial community in the bovine rumen. Twenty-four beef heifers were randomly assigned to either a rapid grain adaptation (RGA) treatment (n = 18) in which the heifers were allowed to adapt from a diet containing 97% hay to a diet containing 8% hay over 29 days or to the control group (n = 6), which was fed 97% hay. Rumen papillae were collected when the heifers were fed 97%, 25%, and 8% hay diets. PCR-denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR analysis were used to characterize rumen epimural bacterial diversity and to estimate the total epimural bacterial population (copy numbers of the 16S rRNA gene). The epimural bacterial diversity from RGA heifers changed (P = 0.01) in response to the rapid dietary transition, whereas it was not affected in control heifers. A total of 88 PCR-DGGE bands were detected, and 44 were identified from phyla including Firmicutes, Bacteroidetes, and Proteobacteria. The bacteria Treponema sp., Ruminobacter sp., and Lachnospiraceae sp. were detected only when heifers were fed 25% and 8% hay diets, suggesting the presence of these bacteria is the result of adaptation to the high-grain diets. In addition, the total estimated population of rumen epimural bacteria was positively correlated with molar proportions of acetate, isobutyrate, and isovalerate, suggesting that they may play a role in volatile fatty acid metabolism in the rumen.

The ruminal bacteria digest complex and simple carbohydrates in the rumen and produce nutrients, such as volatile fatty acids (VFA), microbial protein, and vitamins, for the host. The rumen bacteria have been classified into three groups based upon their locations of colonization within the rumen: those associated with liquid, attached to solid particles, and attached to the ruminal epithelium (defined as epimural bacteria) (4). To date, most studies have focused on the bacteria attached to solid particles and associated with liquid (13, 16, 23, 35, 42). These studies have revealed that the bacterial diversity in ruminal contents is highly responsive to changes in diet, age, and antibiotic use, with the health status of the host, geographical location, and season further contributing to variation in diversity (42).

While they account for only approximately 1 to 2% of the total bacterial population in the rumen (34), it has been suggested that epimural bacteria play essential roles in oxygen scavenging (6), urea hydrolysis (10, 47), and tissue recycling (25). As epimural bacteria are directly attached to the epithelial lining, they may also be involved in host-microbial interactions, and they may have a role in barrier function for rumen tissue, which is exposed to various fermented products. The epimural bacteria have been reported to be taxonomically distinct from those in the rumen fluid or bacteria attached to solids in dairy cattle (7, 25) and sheep (5), using culture-based techniques. Recent studies using culture-independent techniques have also confirmed that the epimural bacterial community is distinctly different from the liquid- and particle-associated bacteria (7, 27, 35, 36). However, the ecology of the epimural bacteria and whether diet affects the diversity of this population in the rumens of beef cattle have not been studied.

Rapid transition from a high-forage diet to a high-grain diet is common practice in the nutritional management of feedlot cattle. It is known that changing the proportions of forage and concentrate in diets affects ruminal fermentation characteristics, such as VFA and ruminal pH (39), and the known effects of changes in ruminal pH and VFA concentrations on microbial activity have been documented (28, 33). For example, low pH (<6.0) has negative effects on fibrolytic bacteria in the rumen, and the population of amylolytic bacteria decreases as pH continues to decline (24, 28). In the current study, we hypothesized that diet affects the diversity and population of the ruminal epimural bacteria. Therefore, we evaluated the diversity and density of rumen epimural bacteria from beef heifers (n = 18) while they transitioned from a high-forage to a high-grain diet in comparison to heifers (n = 6) fed the high-forage diet throughout the study. The diversity and density of rumen epimural bacteria were investigated using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative real-time PCR (qRT-PCR) analysis. In addition, correlation analysis was used to evaluate the association between the diets and fermentation characteristics, including the molar proportion of VFA and ruminal pH and the epimural bacterial diversity and population.

MATERIALS AND METHODS

Animals and sampling. Twenty-four ruminally cannulated beef heifers (about 8 months old, weighing 244 kg to 369 kg) were cared for in the Laired McElroy Environmental and Metabolic Center at the University of Alberta. Animal care and use followed the guidelines of the Canadian Council on Animal Care (2009).

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The experiment protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock (protocol number OBA077). Heifers were randomly assigned to either the control (CON; n = 6) group or a rapid grain transition treatment (RGA; n = 18) group throughout a 29-day experiment period. Heifers in the RGA group were initially fed a diet containing 97% hay (days 1 to 4) and transitioned to a final diet containing 8% hay using the following intermediate diets: 60% hay (days 5 to 8), 40% hay (days 9 to 12), 25% hay (days 13 to 16), 15% hay (days 17 to 20), and 8% hay (days 21 to 29). Heifers assigned to the CON group were fed the 97% hay diet throughout the experiment. Detailed descriptions of the dietary ingredients, chemical composition, and rapid-transition protocol are presented in Table 1.

Ruminal papillae were biopsied when the heifers were fed 97% hay at 2:00 p.m. on day 3 (the 3rd day of a 4-day feeding period), 25% hay on day 15 (the 3rd day of a 4-day feeding period), and 8% hay on day 26 (the 6th day of a 9-day feeding period). The excised ruminal papillae (approximately 500 mg) were immediately washed with sterile 0.01 M phosphate-buffered saline (PBS) buffer (pH 6.8). The papillae were scraped to remove attached fecal particles and rinsed three times to remove the nonadherent bacteria. The cleaned tissues were then transferred into RNA-later solution (Invitrogen, Carlsbad, CA) and stored at −20°C until further molecular analysis was performed.

Rumen digests were collected at 8:00 a.m., 10:00 a.m., 12:00 noon, and 2:00 p.m. on day 2 (the 2nd day of a 4-day 97% hay feeding period), day 14 (the 2nd day of 4-day 25% hay feeding period), and day 25 (the 5th day of 9-day 8% hay feeding period). Fifty milliliters of digesta sample was directly collected using a 50-ml sterile Falcon tube, placed on dry ice immediately after collection, and then stored at −80°C until further molecular analysis was performed. The digesta samples (approximately 200 ml) were immediately transferred through a perforated screen (Petex; pore size, 355 μm; Sefar Canada Inc., Scarborough, Ontario, Canada). Each 10 ml of strained rumen fluid was acidified with 2 ml of metaphosphoric acid and stored at −20°C until analysis for VFA was performed, including acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total VFA concentration. To determine VFA concentrations, acidified fluid samples were centrifuged at 13,000 × g for 20 min, and the supernatant was transferred into a gas chromatography (GC) apparatus (model 3400; Varian, Walnut Creek, CA) for analysis using a 170°C autosampler (model 8200; Varian) in a Stabilwax-DA column and measured with a 190°C detector. Peak integrations were calculated for each heifer. These response variables have been widely used as indicators for the extent or severity of ruminal acidosis (40). In this study, they were used to study the relationship among bacterial diversity, bacterial density changes, and ruminal pH values during the diet transition. The pH threshold of 5.5 was used in this study because beef cattle were fed highly fermentable diets.

**DNA extraction.** Rumen tissue samples were thawed and washed using fresh sterile 0.01 M PBS buffer (pH 6.8) three times to ensure the removal of nonadherent bacteria and the residual RNA-later solution. In brief, the tissue was transferred onto a sterile plastic petri dish (90 mm by 15 mm) containing ~20 ml PBS buffer and was incubated for 3 min at room temperature with light shaking on the bench every 1 min. The buffer was then removed, and the same amount of buffer was added to repeat the wash step three times. Total DNA was extracted using a bead-beating method (48). Briefly, the tissue sample (100 to 250 mg) was transferred to a 2-ml microcentrifuge tube containing zirconium beads (0.3 g; diameter, 0.1 mm) and washed with 1 ml of TN150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) twice by vortexing and centrifugation at 14,600 × g for 5 min at 4°C. Then, the pellet was resuspended in 1 ml of TN150, followed by physical disruption in a Mini Bead-Beater-8 (BioSpec Products, Bartlesville, OK) at 4,800 rpm for 3 min. The tube was immediately placed on ice and incubated for 2 minutes. The beat-beating process was then repeated to ensure the maximum recovery of bacterial DNA. Phenol and chloroform-isomyl alcohol (24:1) were used to remove protein, and DNA was precipitated with 70% cold ethanol and dissolved in 30 μl of nucleic-free water. The amount and quality of DNA were measured based on absorbance at 260 and 280 nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

**Design of a reference marker for PCR-DGGE analysis.** In this study, an internal reference lane containing 47 amplicons from 16S rRNA full-length sequences with known taxonomic identities was used for all PCR-DGGE analysis. According to the principles of PCR-DGGE, DNA fragments consisting of the same sequences migrate to the same location on the DGGE gel, indicating that if the bands migrate to the same locations as the reference sequences, they are likely to have the same identity with the reference sequences at the genus level (based on 93 to 96% similarity) (2). Based on studies using a reference system with known sequences to confirm the identity of the DGGE bands (14, 19, 52), we decided to use a reference system containing distinct taxonomic identification in the V2-V3 region for DGGE analysis to predict the taxonomy of DGGE bands at the genus level. To generate this reference lane, 1,026 full-length 16S rRNA gene sequences were selected from a previous study using sequencing analysis of an epimural bacterial community (M. Li, M. Zhou, E. Adamowicz, J. A. Basarab, and L. L. Guan, unpublished data) (NCBI accession numbers GU303006 to GU304593). The sequences were aligned from base positions 300 to 600 of the V2-V3 region 16S rRNA gene using the ClustalX program (http://www.molecularevolution.org) (45). Forty-seven unique sequences were determined based on the alignment score (50) and taxonomic identification (Table 2). The plasmid DNA extracted from colonies containing each of these 47 full-length 16S rRNA gene sequences was used as a template to amplify the DNA fragment (~200 bp) using the HDA1-GC and HDA2 primers and the program outlined previously (49). Then, the 47 amplicons were mixed to be used as reference markers and loaded in one lane, while the individual
amplicons were loaded in separate lanes of the same DGGE gel in order to determine the migration position of each band in the reference marker. The DGGE (30 to 55% gradients with 6% acrylamide) was run at 130 V and 60°C for 4 h using the Bio-Rad Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA). After electrophoresis, the gels were stained with 0.1% ethidium bromide for 20 min and destained with Milli-Q water for 30 min. The gels were then photographed with the FluorChem SP imaging system (Alpha Innotech, Hercules, CA). After electrophoresis, the gels were stained with 0.1% ethidium bromide for 20 min and destained with Milli-Q water for 30 min. The gels were then photographed with the FluorChem SP imaging system (Alpha Innotech, Hercules, CA).

**PCRDGGE analysis.** Total DNA (10 ng/µl) extracted from individual ruminal tissue was used as a template to generate the amplicon for PCR-DGGE analysis using nested PCR. The nested PCR was performed by amplifying a 1.5-kb product targeting the full-length 16S rRNA gene with a universal bacterial primer pair, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TAC GGYTACCTTGTAGACTAC-3') (21). The nested-PCR conditions were as follows: initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 90 s; and a final elongation for 7 min at 72°C. The PCR product was then diluted 10 times as a template to amplify an ~200-bp DNA fragment using the HDA1-GC and HDA2 primers (49). The PCR-DGGE analysis was performed under the same conditions described above for the reference marker, which was included for every gel.

The obtained PCR-DGGE profiles were analyzed using the BioNumerics software package (version 6.0; Applied Maths, Austin, TX). To be able to predict the identities of the PCR-DGGE bands using the reference marker and to compare the PCR-DGGE profiles among different gels, it is critical to define the optimal parameters, such as optimization and tolerance for similarity coefficient settings for all given fingerprint types obtained within an experiment. In this study, optimization of 0.8% and a tolerance position of 0.88% were obtained based on the calculation function of the software for this particular experiment. Similarity matrices were generated using the Dice similarity coefficient (Dc), and a dendrogram was obtained using the unweighted-pair group method with mathematical averages (UPGMA) clustering algorithm (11, 29). The similarity between bacterial PCR-DGGE profiles was calculated as a percentage. Multidimensional scaling (MDS) and principal-components analysis (PCA)

| Clone identifier | Taxonomic identification (similarity [%]) | Accession no. |
|-----------------|-----------------------------------------|---------------|
| 406RT6-G12      | Uncultured Mycoplasma sp. (90)          | AB089057.1    |
| 406RT1-G07      | Treponema refringens (92)               | AF426101.1    |
| 206RT5-D08      | Prevotella (100)                        | AB239482.1    |
| 206RT2-A09      | Clostridiales (97)                      | DQ394637.1    |
| 406RT3-E12      | Treponema bryantii (96)                 | M57737.1      |
| 206RT1-D07      | Treponema bryantii (98)                 | M57737.1      |
| 406RT1-G04      | Succinivibrionaceae (95)                | AY61926.1     |
| 406RT5-D01      | Comamonadaceae bacterium MPsc (93)      | EU831508.1    |
| 406RT2-F02      | Clostridiales (97)                      | EU835464.1    |
| 206RT1-A03      | Proteobacteria (82)                     |              |
| 206RT1-C06      | Ruminobacter amylophilus strain H18 (97)|              |
| 206RT2-B09      | Uncultured rumen bacterium clone TWBRB53 (98)| FJ799156.1 |
| 206RT1-A06      | Incertae sedis XV (98)                  | EU831431.1    |
| 206RT5-G02      | Ruminococcus flavefaciens strain AR72 (90)| AF104841.1  |
| 406RT1-F10      | Ruminococcaceae (100)                   | EU831687.1    |
| 406RT3-A06      | Rumen bacterium (95)                     | AB239489.1    |
| 206RT2-F11      | Rumen bacterium R-9 gene (93)           | AB239482.1    |
| 206RT3-A06      | Uncultured rumen bacterium clone P5_G03 (96) | EU831963.1  |
| 406RT3-F10      | Desulfobulbus sp. (94)                  |AY080536.1    |
| 206RT1-C02      | Uncultured rumen bacterium clone T33H60F43 (88)| AB270115.1  |
| 406RT4-C05      | Anaerovibrio lipolytica (98)            | BAY621303.1   |
| 206RT1-B02      | Butyribivio (94)                        | EU831849.1    |
| 206RT1-D02      | Mitsuokella jalaludinii strain M9 (94)   | NR_028840.1   |
| 206RT2-A08      | Eggerthella sinensis strain HKU14 (93)  | AY321958.1    |
| 206RT4-G01      | Clostridiales (98)                      | AB185741.1    |
| 406RT4-D04      | Lachnospiraceae bacterium DJF VP52 (90)| EU728778.1    |
| 406RT2-C03      | Campylobacter fetus strain 03-427 (96)  | AY297796.1    |
| 406RT3-A04      | Alphaproteobacteria (97)                | AB124367.93   |
| 206RT6-A08      | Desulfosporus sp. AAN04 gene (96)       | AY005036.1    |
| 206RT1-E07      | Desulfobulbus sp. oral clone CH031 (94)| AY349370.1    |
| 206RT1-A02      | Deferrribacteres sp. oral clone JV001 (98)| EF436307.1  |
| 206RT1-D04      | Bacteroidales (100)                     |               |
| 406RT1-F08      | Unidentified rumen bacterium JW16 (95)  | AF108445.1    |
| 406RT4-B05      | Succiniclasticum ruminii strain DSM 9236 (93)| NR_026205.1 |
| 406RT3-F08      | Desulfotobacterium hafniense DCB-2 (96)| CP001336.1    |
| 206RT3-A01      | Desulfobulbus sp. oral clone CH031 (94)| AY005036.1    |
| 206RT2-H02      | Clostridiales (100)                     | AB185814.1    |
| 206RT6-G05      | Eubacterium sp. C2 (90)                 | AF044945.1    |
| 406RT4-A10      | Nettericaceae (100)                     | AY551997.1    |
| 206RT1-A02      | Mogibacterium (96)                      | AB034014.1    |
| 406RT2-D09      | Victivallis (99)                        | FJ028789.1    |
| 206RT1-C01      | Rumen bacterium YS2 (91)                | AF544207.1    |
| 206RT1-A11      | Uncultured bacterium clone CHIMPI_aaj40e05 (94)| EU462343.1  |
| 406RT3-F09      | Porphyromonaceae (94)                   | EF686526.1    |
| 406RT4-C04      | Uncultured rumen bacterium clone YRC60 (93)| EU259436.1  |
| 406RT3-E11      | Atoxobium parvulum DSM (93)             | CP001721.1    |
| 206RT5-A07      | Rumen bacterium YS2 (92)                | AF544207.1    |
were also performed using the MDS and PCA modules supplied with the BioNumerics software package.

To identify which PCR-DGGE bands were affected by diet, a best-fit Gaussian curve for each band from all DGGE patterns was calculated. All the assigned bands were then exported with a normalized relative position. After the band-matching procedure, a binary matrix in which all the bands were allocated to the defined numbers of categories was created for the 24 heifers. Three dietary conditions (97% hay, 25% hay, and 8% hay) were used to define the presence or absence of particular bands on each variable using the PROC CATMOD model developed in house (15). In this model, the effects of all variables on the prevalence of each band were determined based on the transformation of the cell probabilities (response function). Afterwards, the FREQ procedure of SAS (version 9.2; SAS Institute, Cary, NC) was used to estimate the frequencies of the bands in all animals, and the results were plotted.

Estimation of the total rumen microbial population using qRT-PCR. qRT-PCR analysis was performed to estimate the total rumen bacterial population by measuring the copy numbers of the 16S rRNA gene using the primer pair U2 (forward, 5′-ACTCTACTGGGAGGAGCAG-3′; reverse, 5′-GAC TACCGGGTATCTAATCC-3′) (41) with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR green chemistry. The total volume of each reaction solution contained 10 μl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 μl of each primer (20 pmol μl⁻¹), 8 μl of nuclease-free water, and 1 μl of DNA template (10 ng μl⁻¹). The standard curve was constructed using plasmid DNA containing the 16S rRNA sequence of Butyrivibrio hungatei with serial dilution of the initial concentration of 9.1 × 10⁵ molecules μl⁻¹. The range of the copy numbers in the standard curve was from 9.1 × 10¹ to 9.1 × 10⁶ molecules μl⁻¹. Each standard dilution and sample was assayed in triplicate. Amplification was carried out using the following program: 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 20 s, followed by annealing/extension for 1 min at 62°C.

Standard curves were plotted in StepOnePlus software version 2.0. The copy numbers of total 16S rRNA genes in the samples were determined by relating the threshold cycle (Cₚ) values to standard curves. The calculation of the copy number for the 16S rRNA gene in 0.5 g of tissue was performed using the formula from the study of Li et al. (23). The corresponding RT-PCR efficiency ranged between 86 and 100% in this study.

Statistical analysis. The experiment was analyzed as a randomized complete block design. The PROC MIXED procedure of SAS (version 9.2; SAS Institute, Cary, NC) was used to analyze the VFA concentration and total rumen bacterial population. In the statistical model, period (diet), and treatment were analyzed as fixed effects, with heifer as a random effect and with all potential 2- and 3-way interactions. Interactions having P values of >0.05 were removed from the model, and the data were reanalyzed using reduced models. Least-squares means were compared using the Bonferroni mean separation method, and significance was declared at a P value of 0.05.

PCA was performed using the composite data set of the normalized location and intensity of each DGGE band, ruminal pH variables (mean, minimum, and maximum pH or the duration or area under the pH threshold of 5.5), and the molar proportion of VFA. All these data were also used to evaluate the relationships and to investigate the contributions of these variables to the variation of the data using the PRINCOMP procedure in SAS. For each dietary treatment (period), the mean value of each measured molar proportion of VFA or total VFA concentration from four time points was used for analysis. The rumen variables included all the ruminal pH characteristics (mean, minimum, and maximum pH or the duration or area under the pH threshold of 5.5) and VFA molar proportions (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total VFA) as PCA ordinations. This procedure standardizes the variables to a mean of 0 and a standard deviation of 1. The correlation matrix was used to generate principal-component eigenvalues and associated loadings (38).

Correlation among total ruminal bacterial population, ruminal pH, and the molar proportion of VFA were analyzed using the PROC CORR and REG procedures of SAS.

RESULTS

Rumen fermentation parameters. Interactions between period and treatment were significant for all data except for the area under the pH threshold of 5.5 (pH × min/day), the molar proportion of isobutyrate, and the concentration of total VFA. There were no differences for any measured variables, including ruminal pH, and the concentration of total and individual VFA over the 3 periods for the CON group and for the RGA group when fed the 97% hay diet (period 1 [P1] in Table 3). When RGA heifers were fed the 25% hay diet, the mean ruminal pH decreased from 6.75 to 6.12 (P = 0.001), the duration below pH 5.5 increased from 0 to 356 min/day, and the area below pH 5.5 (pH × min/day) increased from 0 to 156 relative to 97% hay-fed heifers. For VFA profiles, the molar proportions of propionate, butyrate, valerate, and isovalerate increased, while no differences were detected for the total VFA concentration for heifers fed the 25% hay diet compared to those fed the 97% hay diet (Table 3). When heifers were fed the 8% hay diet, the ruminal pH and the VFA profile did not differ, except for the molar proportion of valerate (P = 0.03), compared with those fed the 25% hay diet. However, when RGA heifers were fed the 8% hay diet, they had lower mean ruminal pH and molar proportion of acetate but higher molar proportions of propionate, butyrate, valerate, and isovalerate than those fed the 97% hay diet (Table 3). Among individual heifers, the mean ruminal pH change was strongly dependent on the host. The mean ruminal pH changes of 18 RGA cattle were found to be follow three patterns: the mean ruminal pH continually decreased as the proportion of hay decreased (6 heifers), the ruminal pH decreased only when the diet transitioned from 97% hay to 25% hay and then increased during the transition to the diet containing 8% hay (7 heifers), and the ruminal pH decreased when the diet transitioned from 97% hay to 25% hay with little change in the ruminal pH thereafter (5 heifers) (data not shown).

PCR-DGGE profiling of ruminal epimural bacteria. The PCR-DGGE profiles of epimural bacteria from 24 heifers fed different diets were generated and compared. The PCR-DGGE profiles of the 6 heifers in the CON group did not change among three periods (P = 0.58) when the similarities were compared using Dₛₑ (data not shown). For heifers on the RGA treatment, the bacterial PCR-DGGE profiles were clustered by diet (period) (Fig. 1A). Similarity analysis showed that the bacterial profiles were more similar (average Dₛₑ = 81.22%) within the same diet than among different diets (average Dₛₑ = 69.32%).

To further verify the bacterial PCR-DGGE profile changes in response to diet, MDS and PCA, two alternative grouping methods, were used to produce two- or three-dimensional plots for relatedness of the bacterial diversity among all animals. The MDS analysis showed that PCR-DGGE profiles from heifers fed 97% hay (Fig. 1B, green symbols) or 25% hay (Fig. 1B, dark-blue symbols) diets were grouped closely based on the diet, while the PCR-DGGE profiles from heifers fed an 8% hay diet were scattered into several groups (Fig. 1B, purple, red, and yellow symbols) and mixed with the outliers from the other two diets. PCA of PCR-DGGE profiles across diets also revealed a trend toward PCR-DGGE profiles being grouped by diet (data not shown).

Assessment of PCR-DGGE bands. In total, 88 bands were detected from all PCR-DGGE profiles. When the PCR-DGGE bands were subjected to multivariate statistical analysis, the frequency of presence for most of the PCR-DGGE bands changed in response to diet (Table 4). For instance, when the concentration of hay decreased, the frequency of presence of bands 23, 39, 42, 44, 51, 52, 53, 57, 59, 60, 64, 78, and 84 was reduced, while it increased for bands 4, 9, 15, 20, 30,
TABLE 3. Analysis of ruminal fermentation parameters and total epimural bacterial population between and within treatments

| Parameter | CON (0%) | P1 (97% hay) | P2 (85% hay) | P3 (60% hay) | P4 (60% hay) | P5 (6% hay) |
|-----------|----------|--------------|--------------|--------------|--------------|-------------|
|          | T        | T × P        | T            | T × P        | T × P        | T × P       |
| Rumen pH | Mean     | 6.48 ± 0.02a | 5.29 ± 0.02a | 6.76 ± 0.04b | 6.73 ± 0.02a | 6.71 ± 0.003a |
|           | Minimum  | 6.14 ± 0.03a | 5.03 ± 0.02a | 6.34 ± 0.02b | 5.05 ± 0.02a | 6.30 ± 0.002a |
|           | Maximum  | 6.83 ± 0.06a | 6.46 ± 0.07a | 7.01 ± 0.03a | 6.02 ± 0.04a | 6.97 ± 0.005a |
| VFAs     | Mean     | 71.6 ± 4.2a  | 54.6 ± 4.4a  | 54.6 ± 4.4a  | 54.6 ± 4.4a  | 54.6 ± 4.4a  |
|           | Minimum  | 47.8 ± 2.8a  | 34.0 ± 2.5a  | 24.0 ± 2.5a  | 24.0 ± 2.5a  | 24.0 ± 2.5a  |
|           | Maximum  | 79.1 ± 6.6a  | 64.2 ± 5.6a  | 60.3 ± 5.6a  | 60.3 ± 5.6a  | 60.3 ± 5.6a  |
| Isobutyrate (%) | Mean   | 0.95 ± 0.06a  | 1.80 ± 0.10b | 1.80 ± 0.10b | 1.80 ± 0.10b | 1.80 ± 0.10b |
| Butyrate (%)  | Mean   | 9.11 ± 0.44b | 7.66 ± 0.88b | 7.66 ± 0.88b | 7.66 ± 0.88b | 7.66 ± 0.88b |
| Isovalerate (%) | Mean | 1.11 ± 0.10b | 0.11 ± 0.10b | 0.11 ± 0.10b | 0.11 ± 0.10b | 0.11 ± 0.10b |
| Total VFAs (mM) | Mean | 84.3 ± 9.05b | 78.3 ± 7.09b | 78.3 ± 7.09b | 78.3 ± 7.09b | 78.3 ± 7.09b |

Comparison of the total population of rumen epimural bacteria. The total epimural bacterial population was estimated using the total copy numbers of bacterial 16S rRNA genes. It did not change over time ($P = 0.78$) for CON heifers but differed between CON and RGA cattle ($P = 0.05$) (Table 3). For RGA heifers, the total epimural bacterial numbers dramatically increased when the heifers were fed the 25% hay diet compared to those fed the 97% hay diet ($P = 0.01$) and significantly decreased when the dietary hay content decreased from 25 to 8% ($P < 0.01$). When comparing the 97% hay diet (period 1) and the 8% hay diet (period 3), the total epimural bacterial populations were not different ($P = 0.36$). When the individual heifers in the RGA group were compared, the changes in the total epimural bacterial population under the three diets did not follow a consistent pattern (Fig. 3).

Correlations among total population of rumen epimural bacteria, rumen pH, and VFA profiles. To identify whether epimural bacteria are associated with rumen fermentation parameters, the relationships among the total epimural bacterial population, ruminal pH, and proportion of individual VFA were evaluated. Significant positive correlations ($P < 0.05$) were detected only between the total population of epimural bacteria and duration (min/day) under the pH threshold of 5.5 (Table 5). There was no correlation between the total epimural bacterial population and the molar proportion of VFA.

Correlations among epimural bacterial PCR-DGGE profiles, ruminal pH, and molar proportion of VFA. To identify the correlations between rumen epimural bacterial diversity (PCR-DGGE bands) and fermentation characteristics (pH and VFA), the associations among all variables were investigated using PCA analysis. Three significant principal components were extracted, describing 60% of the total variance. Two significant principal components were extracted describing 39.1% (PC1) and 16.9% (PC2) of the variation. In the first principal component (PC1), isovalerate, isobutyrate, acetate, and bands 1, 13 (Treponema sp.), 19, 21 (Proteobacteria), 27, 28, 34 (rumen bacterium), 38 (uncultured rumen bacterium), and 40 had the highest contributions, and they were orthogonal to VFA. Rumen pH, minimum pH, and bands 3 (uncultured Mycoplasma sp.), 10 (Clostridiales), 22, 24, 28, 35, 37 (rumen bacterium), and 61 (Desulfovibulbus sp.) were described in the second principal component (PC2).
DISCUSSION

Diet is one of the major factors influencing the structure and function of the microbial community in the rumen contents (18, 44, 52). The nature of feed materials and the physicochemical changes induced by their fermentation are known to favor the development of certain microbial ecotypes in the ruminal solid and liquid phases (24). Meanwhile, the diversity of the ruminal epimural microflora has been reported to be stable relative to that in the ruminal contents through 8 weeks (35) and under different dietary regimens (35, 36). In this study, we hypothesized that transition from a high-forage diet to a high-grain diet can affect the diversity of epimural bacteria in beef cattle. The PCR-DGGE profiles and total bacterial population of CON animals throughout this study indicate that the epimural bacterial composition was not altered over time at both the structural and density levels, which confirms the findings of a previous study with a static diet (23). The observed changes in the PCR-DGGE profiles and total epimural bacterial population from the rumens of RGA animals in response to the decreased forage proportion of the diet show that dietary changes can induce marked changes in the diversity and density of the epimural bacteria.

At the phylum level for the bacteria detected in this study, the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* and unknown bacteria were predominant in the epimural bacterial community (Fig. 2). This confirmed the finding from previous
TABLE 4. Identification of PCR-DGGE bands and associations with different rumen parameters

| DGGE band | Predicted taxonomic identification (% similarity) | Presence (+) or absence (−) in the rumen and/or probable associations with different parameters |
|-----------|-----------------------------------------------|--------------------------------------------------------------------------------------|
|           | Genus level | Phylum level | 97% hay diet | 25% hay diet | 8% hay diet |
| 1 UD      | UD          | —           | +           | —           | —           |
| 2 UD      | UD          | —           | +           | —           | —           |
| 3 Uncultured Mycoplasma sp. (90) | Tenericutes (81) | +; pH, minimum, butyrate | +           | —           | —           |
| 4 Treponema sp. (92) | Spirochaetes (100) | +           | +           | —           | —           |
| 5 Prevotella (100) | Bacteroidetes (100) | +           | +           | +           | +           |
| 10 Clostridales (97) | Firmicutes (98) | +; valerate | +           | +           | +           |
| 11 Treponema sp. (96) | Spirochaetes (100) | +; total VFAs | +           | +           | +           |
| 12 UD      | UD          | —           | —           | —           | —           |
| 13 Treponema sp. (98) | Spirochaetes (100) | +; propionate | +           | +           | +           |
| 14 UD      | UD          | —           | —           | —           | —           |
| 15 Succinivibrionaceae (95) | Proteobacteria (100) | +           | +           | —           | —           |
| 20 Clostridiales (97) | Firmicutes (99) | +           | +           | +           | +           |
| 21 Proteobacteria (82) | Proteobacteria (82) | +; total VFAs, isobutyrate | +           | +           | +           |
| 25 Ruminococcus sp. (97) | Ruminococcus (100) | +; butyrate, valerate | +           | +           | +           |
| 29 Uncultured rumen bacterium clone TWBRB53 (98) | Bacteroidetes (100) | +; acetate, butyrate, TEBP, pH, min, time, area | +           | +           | +           |
| 30 Incertae sedis XV (98) | Firmicutes (99) | +           | +           | +           | +           |
| 32 Ruminococcus sp. (90) | Firmicutes (100) | +; isobutyrate, isovalerate | +           | +           | +           |
| 33 Ruminococaceae (100) | Firmicutes (100) | +           | +           | +           | —           |
| 34 Rumen bacterium (95) | Firmicutes (100) | +           | —           | —           | —           |
| 38 Uncultured rumen bacterium clone P5_G03 (96) | Bacteroidetes (100) | +; propionate | +           | +           | +           |
| 39 Desulfobulbus sp. (94) | Proteobacteria (100) | +           | +           | —           | —           |
| 42 Anaerovibrio sp. (98) | Firmicutes (100) | +           | —           | —           | —           |
| 43 Butyrivibrio (94) | Firmicutes (99) | +           | +           | +           | —           |
| 44 Ruminococcus sp. (93) | Actinobacteria (100) | +           | —           | —           | —           |
| 46 Clostridiales (98) | Firmicutes (99) | +           | —           | —           | —           |
| 48 Lachnospiraceae sp. (90) | Firmicutes (100) | +           | —           | —           | —           |
| 50 Campylobacter sp. (96) | Proteobacteria (100) | +           | —           | —           | —           |
| 51 Alphaproteobacteria (97) | Proteobacteria (97) | +; min, propionate | +           | +           | +           |
| 52 Desulfomarinus sp. (96) | Firmicutes (99) | +           | +           | +           | —           |
| 53 Desulfobulbus sp. (94) | Proteobacteria (100) | +; isovalerate | +           | +           | —           |
| 54 Defterribacteres sp. (98) | Firmicutes (98) | +           | —           | —           | —           |
| 55 Ruminococcus sp. (93) | Firmicutes (100) | +           | —           | —           | —           |
| 56 Bacteroidales (100) | Bacteroidetes (100) | +; TEBP | +           | +           | —           |
| 58 Unidentified rumen bacterium JW16 (95) | Bacteroidetes (97) | +           | +           | +           | +           |
| 59 Succinivibrionaceae (93) | Firmicutes (100) | +; TEBP, isobutyrate | +           | +           | +           |
| 60 Desulfobacterium sp. (96) | Firmicutes (100) | +           | +           | +           | —           |
| 61 Desulfobulbus sp. (94) | Proteobacteria (100) | +           | +           | +           | —           |
| 62 Clostridiales (100) | Firmicutes (100) | +           | +           | +           | +           |
| 65 Eubacterium sp. C2 (90) | Firmicutes (100) | +           | —           | —           | —           |
| 69 Mogibacterium (96) | Firmicutes (100) | +           | —           | —           | —           |
| 70 Verrucomicrobiaceae (98) | Firmicutes (100) | +           | +           | +           | —           |
| 72 Rumen bacterium YS2 (91) | Proteobacteria (68) | +; butyrate, propionate | +           | +           | +           |
| 74 Uncultured bacterium clone CHIMPI_saj40b05 (94) | Firmicutes (100) | +; total VFAs | +           | +           | +           |
| 75 Porphyromonadaceae (94) | Firmicutes (100) | +           | +           | +           | +           |
| 78 Uncultured rumen bacterium clone YRC61 (93) | Bacteroidetes (100) | +; isovalerate | +           | +           | +           |
| 79 Rumen bacterium YS2 (92) | Proteobacteria (52) | +; TEBP | +           | +           | —           |
| 80 UD      | UD          | —           | —           | —           | —           |
| 81 UD      | UD          | —           | —           | —           | —           |
| 85 UD      | UD          | —           | +           | +           | +           |
| 86 UD      | UD          | +           | —           | —           | —           |
| 87 UD      | UD          | +           | —           | —           | —           |
| 88 UD      | UD          | +           | —           | —           | —           |

*a* UD, unidentified.

*b* Min, minimum of mean rumen pH.

*c* Max, maximum of mean rumen pH.

*d* TEBP, total epimural bacterial population.

*e* The minimum per day that the ruminal pH was <5.5.

*f* The area (pH × minimum/day) that the ruminal pH was <5.5.
studies (5, 27, 43) that the Gram-positive bacteria (*Firmicutes*) are relatively common in tissue-adherent populations. When our study was compared to a study of sheep by Sadet-Bourgetteau et al. (36), the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Spirochaetes* were detected from both sheep and heifers, suggesting they are the predominant bacteria in the epimural bacterial community in ruminants. The lower proportion of the phyla *Firmicutes* and *Bacteroidetes* (55% in heifers versus 86% in sheep) and the higher proportion of unknown bacteria in our study could be the result of the low resolution of the PCR-DGGE analysis and the numbers of bands identified. Further study is necessary to sequence and identify all PCR-DGGE bands, which may improve the taxonomic identification of the bovine epimural bacterial community. Despite

FIG. 2. Comparison of distributions of epimural bacteria at the phylum level from 18 RGA heifers fed 97%, 25%, and 8% hay diets based on the sequence information from the reference marker. The error bars indicate standard deviations of the mean. Different letters indicate significant differences ($P < 0.05$).

FIG. 3. Changes in estimated total populations of epimural bacteria measured by total copy numbers of 16S rRNA genes from each individual of 18 RGA heifers in response to 97%, 25%, and 8% hay diets.
Firmicutes, the phyla Bacteroidetes, Proteobacteria, and Verrucomicrobia are commonly associated with the microbiome. The divergence in diversity and density among different ruminant species is very limited. Varel and Delhorly reported population differences of ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids, and cattle (46). A recent study by Fuente et al. compared populations of ciliated protozoa in the rumens of different domestic ruminant species, including cattle, sheep, goats, and reindeer, using a culture-based method, showing the divergence of the diversity and populations among these species (12). Our study showed that the total bacterial population of epimural bacteria ranged from $7.3 \times 10^9$ to $2.0 \times 10^{10}$ per g of wet tissue for hay-fed heifers, which was higher than that of hay-fed sheep, ranging from $4.4 \times 10^7$ to $2.2 \times 10^8$ per g of wet tissue weight, as reported by Wallace et al. (47). All these results suggest that we cannot ignore the host effect when we compare the studies on epimural bacterial communities. Future studies to investigate how host genetics can control ruminal epimural bacterial changes are warranted to better explain the different findings in sheep and cattle studies.

This is the first study to report, using culture-independent methods, that the density of epimural bacteria can be impacted by diet. McCowan et al. (25, 26) used classic microscopy techniques to enumerate the epimural bacterial population in the rumens of Hereford bulls, showing a high density of the bacteria attached to the ruminal wall. Previous studies of young lambs (32) showed that the epimural bacterial population was up to $10^7$ to $10^8$ cells cm$^{-2}$ in the rumen of a 2-day-old lamb, and this value increased very rapidly with age, reaching $10^6$ to $10^7$ cells cm$^{-2}$ at day 21 after birth. DNA-based qRT-PCR analysis from our study further confirmed that such populations can be as high as $7.3 \times 10^9$ to $2.0 \times 10^{10}$ cells per g of wet tissue. Culture-independent techniques are more sensitive in detecting species at low abundance, as well as unculturable bacteria. However, it is important to be aware of the limitations for estimating the total bacterial population based on the copy numbers of the 16S rRNA gene. It has been known that microbial genomes can display multiple numbers of operons of this gene, with the bacterial genome having up to 15 operons (1). Acinas et al. suggested a correction factor of $2.5$ for a single operon for estimation of the population of bacteria and archaea (1). A recent study by Case et al. (3) evaluated the copy numbers of the 16S rRNA gene in 111 bacterial genomes, and 460 copies of the gene were recovered, giving an average of 4.2 copies per genome. In addition, other studies have shown that the copy numbers of the 16S rRNA gene vary significantly depending on the growth of the bacteria: fast colony formers ($\sim 5.5$ copies per genome) and slow colony formers ($\sim 1.4$ copies per genome) (17). Therefore, the detected population based on 16S rRNA gene copy numbers may be overestimated due to the multiple heterogeneous copies within a genome (3, 8, 9). To date, there are no data to show the average copy number of this gene per genome in the rumen. To address this limitation, recent studies have been conducted indicating that other housekeeping genes, such as ropB or gyrB, and/or some functional genes can be used instead of the 16S rRNA gene (3, 20, 31, 37, 51). However, our attempt to estimate the bacterial population by measuring copy numbers of the ropB gene

| Parameter | TEBP* | \(R\) value | \(P\) value |
|-----------|-------|-------------|-------------|
| Mean      | -0.20 | 0.09        |             |
| Minimum   | -0.15 | 0.10        |             |
| Maximum   | -0.25 | 0.05        |             |
| Durationb | 0.35  | 0.02        |             |
| Area²     | 0.43  | 0.001       |             |
| Acetate   | 0.05  | 0.73        |             |
| Propionate| -0.07 | 0.03        |             |
| Butyrate  | -0.05 | 0.71        |             |
| Isobutyrate| -0.007| 0.96    |             |
| Isovalerate| 0.25 | 0.07        |             |
| Valerate  | -0.12 | 0.40        |             |
| Total VFAs| 0.08  | 0.56        |             |

* TEBP: total epimural bacterial population.

b The minimum per day that ruminal pH was <5.5.
² The area (pH × minimum/day) that ruminal pH was <5.5.
showed that the gene was not suitable for rumen bacteria, since the detected populations were 1,000-fold lower than the known population (data not shown). Further studies are needed to identify more suitable genes to replace the 16S rRNA gene to detect the total bacteria in the rumen. Regardless, the observed significant increase in the total epimural bacterial population and positive correlations ($P < 0.05$) between the total population of epimural bacteria and the duration (min/day) under the pH threshold of 5.5 supply strong evidence that the diet altered the epimural bacterial density, which may be linked to changes in the rumen environment and functions.

Multivariate statistical analysis of individual PCR-DGGE bands further confirmed that diet can impact the presence and/or absence of particular bacterial phylotypes. For example, bands 2, 14, and 25 (Ruminobacter sp.) were present only in the ruminal epithelial samples collected from heifers fed the 25% and 8% hay diets, suggesting that members of this genus attach to the epithelial tissue and that their detectable population can be impacted by diet. Another example is band 79 (rumen bacterium YS2-like). It was not detected under the 8% hay diet, and it was associated with the total bacterial population under the 97% hay diet, suggesting that the diet not only can change the presence or absence of particular species, but also may impact their interactions within the community. Bands 29 and 78, the phylotypes identified with uncultured rumen bacterium clones, were associated with the molar proportions of acetate, butyrate (band 29), valerate (25% hay, band 29 and band 78), isobutyrate (97% hay, band 78), rumen pH (8% hay, band 19), and duration (minutes at a pH of <5.5) (8% hay, band 78), suggesting that diet may impact not only the epimural bacterial diversity and population, but also the association between particular bacterial species, including unknown bacteria with rumen fermentation parameters.

Through the data analysis of the whole study, the variation among individuals in all measured parameters was noticeable. For example, bacterial phylotypes belonging to Tenericutes and Lentisphaerae were detected in only 4 of 18 heifers. This suggests that ruminal epimural bacterial diversity and its response to diet can vary depending on the host animals. Taking heifers 170, 172, and 360 as examples (Fig. 4), the percentage of bacterial phylotypes that belonged to Firmicutes was highest in heifers 170 and 172 fed with the 97% hay diet, while it was highest under 25% hay conditions for heifer 360. Moreover, heifer 360 had a significant pH increase from period 2 (25% hay diet) to period 3 (8% hay diet); such a pH change may be directly associated with the detected difference in bacterial diversity at the phylum level compared to other animals. Although a significant increase in the estimated total epimural bacterial population was detected in the rumens of most animals when they were fed with the 25% hay diet, the pattern of bacterial population changes in each animal was different (Fig. 4). These differences can also be associated with variation in the pH response among individuals. For example, cattle 178 and 360 had the highest total bacterial population numbers, while they had the lowest mean pH under a 25% hay diet. The observation of different pH change patterns in response to the high-grain diet from each animal suggests that the variation in the individual bacterial diversity may have a role in different types of responses at the population level to diet or pH. This strongly suggests that individual variation needs to be taken into account when studying the association between ruminal microbial diversity and host phenotypes.

![Variation in the distributions of epimural bacteria at the phylum level in heifers 170, 172, and 360 in response to 97%, 25%, and 8% hay diets, based on the sequence information from the reference marker.](http://aem.asm.org/)

FIG. 4. Variation in the distributions of epimural bacteria at the phylum level in heifers 170, 172, and 360 in response to 97%, 25%, and 8% hay diets, based on the sequence information from the reference marker.
In conclusion, this study showed that the dietary transition from a forage- to a high-grain-based diet significantly altered the diversity of epimural bacteria in the rumens of beef heifers. These changes may be explained by their association with the concentration of VFA and ruminal pH characteristics in the rumen. In addition, the ruminal epimural bacterial diversity and its response to diet can vary depending on the host animals. Furthermore, the different correlations between particular species of epimural bacteria and fermentation characteristics might indicate a variety of different types of relationships with the host. It provides some preliminary knowledge of the potential roles of epimural bacteria.

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