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Pyrosequencing reveals shifts in the bacterial epimural community relative to dietary concentrate amount in goats

S. U. Wetzels,*†‡ E. Mann,†‡ B. U. Metzler-Zebeli,‡§ M. Wagner,†‡ F. Klevenhusen,*‡ Q. Zebeli,*‡ and S. Schmitz-Esser†‡[1]
*Institute of Animal Nutrition and Functional Plant Compounds,
†Institute for Milk Hygiene, Milk Technology and Food Science,
‡Research Cluster Animal Gut Health, and
§University Clinic for Swine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, 1210 Vienna, Austria

ABSTRACT

Ecological balance in the rumen is highly sensitive to concentrate-rich diets. Yet the effects of these feeding practices on the caprine bacterial epimural microbiome (CBEM), a microbial community with putative important physiological functions in the rumen, are largely unexplored. This study aimed to investigate the effect of dietary concentrate amount on ruminal CBEM. Seventeen growing goats were fed diets with 0 [n = 5; 6.2 MJ of metabolizable energy (ME)/d], 30 (n = 6; 7.3 MJ of /d), or 60% (n = 6; 10.2 MJ of ME/d) concentrate for 6 wk. Two hours after their last feeding, goats were euthanized and tissue samples of the ventral rumen wall were collected, washed in phosphate-buffered saline to detach loosely attached bacteria, and stored at −20°C for further processing. Genomic DNA was isolated from thawed rumen mucosa samples and used for Roche/454 Life Science (Branford, CT) 16S rRNA gene amplicon pyrosequencing yielding 122,458 reads. Pyrosequencing data were clustered into 1,879 operational taxonomic units (OTU; 0.03 distance level). Pyrosequencing revealed *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Spirochaetes* as the most abundant phyla (97.7%). Compared with the 30% group, both the 60 and 0% concentrate groups harbored significantly more *Firmicutes* and *SR1*, respectively. On an OTU level, a *Bergeriella*-related OTU was most abundant in the CBEM, followed by 2 *Campylobacter* OTU, which responded differently to diets: 1 OTU was significantly increased whereas the other significantly decreased with highest concentrate amount in the diet. At the genus level, the 0% concentrate group harbored increased *Kingella*-like sequences compared with the other feeding groups. Furthermore, the 0% concentrate group tended to have more *Bergeriella* than the 30 and 60% concentrate groups. The genus *Bergeriella* was significantly decreased in the 60% feeding group compared with the other diets. In conclusion, this is the first report of CBEM using deep-sequencing methods on the genus and OTU level, and our study revealed major shifts in the CBEM in response to concentrate-rich diets with potential health relevance in goats.

Key words: rumen epithelium, microbiota, subacute rumen acidosis, goat, feeding

INTRODUCTION

The gastrointestinal tract of ruminants is a highly complex and mostly anaerobic ecosystem. Trillions of microorganisms, including bacteria, archaea, ciliate protozoa, fungi, and viruses interact or compete in a symbiotic, commensal, or potentially pathogenic way with the host (Hungate, 1966; Luckey, 1972; Kamra, 2005). In the rumen, bacteria are the predominant microorganisms, fermenting largely indigestible plant biomass to products such as short-chain fatty acids (**SCFA**), an essential energy source for the host ruminant (Bergman, 1990; Mackie, 2000). The ruminal mucosa-associated (epimural) bacterial fraction performs a multitude of symbiotically relevant functions necessary for ruminants' overall metabolism and health (Sadet et al., 2007; Chen et al., 2011; Li et al., 2012). Early cultivation-dependent and -independent studies revealed a distinct bacterial community pattern between the rumen content and the epimural site (Cheng and Wallace, 1979). Due to the higher absolute number of bacteria, the metabolic activity of luminal bacteria is more substantial compared with epimural bacteria. Nevertheless, the bacterial epimural microbiome is the primary contact line to the mucosal sites of the rumen. Moreover, epimural bacteria competitively exclude with adhesive and putative pathogenic microorganisms and provide protection against harmful microbiota by, for example, forming a protecting biofilm on the ruminal

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[1]Corresponding author: Stephan.Schmitz-Esser@vetmeduni.ac.at

epithelium (Leser and Molbak, 2009; Sadet-Bourgeteau et al., 2010; Chen et al., 2011).

Among the first groups to cultivate bacteria from caprine rumina, Dehority and Grubb (1977) described up to 70% of the cultivated bacteria classified as *Butyrivibrio* affiliating to the class *Clostridia*. Although cultivation-dependent methods underestimate bacterial diversity in the rumen (Hungate, 1966; Cheng and Wallace, 1979; Amann et al., 1995), *Clostridia*, together with *Bacteroidia*, have also been described recently for being predominant in the caprine rumen by cloning and sequencing 16S rRNA genes (Cunha et al., 2011). More recently, Lee et al. (2012) used pyrosequencing for the characterization of the microbiome in the goat rumen content and described it as clearly distinct from those of other ruminants. However, the literature lacks information about the caprine bacterial epimural microbiome (**CBEM**) and little is known about how it adapts to dietary changes (Huo et al., 2014; Han et al., 2015; Liu et al., 2015). This makes it difficult to predict adaptive responses of the CBEM in the rumen. In contrast to cattle and sheep, the goat is a browser ruminant, adapted to thrive under low-quality forage dietary conditions. However, the intensive goat production systems dictate feeding of high-energy diets. Consequently, the roughage-based feeding practices have been consistently replaced by concentrate-rich diets in intensive goat farming. These shifts in feeding practices typically result in a decrease in ruminal pH, increase of osmolarity, change in composition of the SCFA and substrates available for rumen microbes, and changes in fermentation processes (Khafipour et al., 2009). The accumulation of these events could lead to SARA, a severe rumen metabolic disorder in ruminants (Fernando et al., 2010). Indeed, SARA affected the diversity and community structure of rumen liquid and solid associated bacteria in goats (Huo et al., 2014). We recently fed goats diets based on hay (0% concentrate) and 30 or 60% concentrate and observed major changes in electrophysiological properties and permeability (Klevenhusen et al., 2013), greater keratinization, and differences in the abundance of luminal microbial populations and LPS, as well as depletion of cellular protective factors of the rumen epithelium (Hollmann et al., 2013; Metzler-Zebeli et al., 2013a,b). Because of the inherent relationship of CBEM with rumen epithelial lining, we hypothesized that feeding of high-concentrate diets and the resulting acidotic challenge in the rumen would also result in shifts in the community structure of CBEM. Major aims of our study were to define the core CBEM and to investigate shifts in the CBEM in response to 3 different levels of energy supply in the diet using Roche/454 Life Science (Branford, CT) pyrosequencing of 16S rRNA gene amplicons.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

The animal experimental protocol of this research was discussed and approved by the institutional ethics committee of the University of Veterinary Medicine Vienna in accordance with Good Scientific Practice guidelines and national legislation (Protocol no. 15/03/97/2011). The experiment was performed at the experimental station of the Institute for Organic Farming and Biodiversity in Wels, Austria. Experimental animals were the same as in previous studies where rumen digesta microbiota and rumen and colon epithelial host response were examined (Hollmann et al., 2013; Klevenhusen et al., 2013; Metzler-Zebeli et al., 2013a,b). In Supplementary Table S1 (http://dx.doi.org/10.3168/jds.2014-9166), relevant results from previous publications outside the current study are summarized. Seventeen goats, 4 mo of age, were used in our study. Before the experiment started, the goats were fed a high-forage diet containing 1.5 kg of chopped second-cut meadow hay (88% DM, 11.2% CP, 8.4% ash, 55.6% NDF) as forage and 200 g of coarsely ground barley concentrate (88.3% DM, 3.4% ash, 13% CP, 20.5% NDF; Lagerhaus, Wels, Austria) daily per animal. The animals were randomly allocated to 1 of the 3 experimental diets differing in their concentrate amount. The groups were balanced for BW and breed. Diets and feeding procedure have been explained in detail previously (Metzler-Zebeli et al., 2013a). Briefly, the control goats received a diet composed purely of hay (6.2 MJ of ME/d) without any concentrate (0% concentrate diet). The medium (7.3 MJ of ME/d) and high (10.2 MJ of ME/d) concentrate diets consisted of hay plus 30 and 60% concentrate (coarsely ground barley, a rapidly fermentable grain) on a DM basis, respectively. The hay and concentrate fed to the goats originated from 1 batch and the goats were adapted to the 30 and 60% concentrate diets by increasing the concentrate level step-by-step by 2.5 and 5% per day, respectively. Barley grain was selected as concentrate due to its rapid degradation in the rumen combined with a high potential to cause acidotic conditions in the rumen. The experiment started after all goats reached the 30 and 60% concentrate level. The animals were fed separately, twice a day at 0630 and 1330 h, and were weighed weekly for adjusting the feed allowance. In total, the feeding experiment lasted 6 wk.

Rumen Mucosa Sampling

To investigate shifts in the CBEM, the goats were euthanized 2 to 3 h after the morning feeding on the last day of the feeding experiment (d 42) by an intravenous

injection of Release (50 mg of pentobarbital/kg of BW; WDT, Garbsen, Germany) and Xylosol (0.5 mg of xylazine/kg of BW; Ogris Pharma, Wels, Austria). After opening the abdominal cavity, the rumen was carefully removed and opened from the dorsal side. The rumen was emptied and a 4- × 4-cm piece of the ventral rumen wall tissue was cut and placed into 100 mL of sterile 1 × PBS (room temperature) for transferring to the laboratory. In the laboratory, the rumen wall was rigorously washed in 100 mL of 1× PBS (this procedure was done 3 times) to detach the free and loosely attached bacteria. The samples were cut into 1- to 2-cm^2 pieces and stored in 1× PBS at −20°C for further processing.

DNA Extraction

The rumen mucosa samples were thawed on ice and genomic DNA was extracted from 0.25 g of rumen mucosa (cut in 2- × 2-mm pieces), using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturers protocol (http://www.mobio.com), with 1 additional step (after the first vortexing for 10 min, the sample was ground by rotating the mortar 10 times to the left and to the right, followed by 5 min of vortexing again). The DNA concentration was quantified using the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, Vienna, Austria).

Preparation of 16S rRNA Gene Amplicon Libraries, Pyrosequencing and Processing, and Taxonomic Assignment of Sequence Reads

The DNA samples were sequenced at the Center for Medical Research Medical University in Graz (Austria) using a 454 GS FLX Titanium Sequencing System (Lib-L kit primer A-primer B; Roche/454, Life Science). The 16S rRNA genes were amplified using the universal primers F27 (forward = 5′-AGA GTT TGA TCC TGG CTC AG-3′; Microsynth, Balgach, Switzerland) and R357 (reverse = 5′-CTG CTG CCT YCC GTA-3′; Microsynth) targeting the V1-V2 hypervariable regions of the 16S rRNA gene. The 25-μL PCR reaction, performed in triplicate, contained 1× fast start buffer; 1.25 U of high-fidelity enzyme; 200 μ*M* (each) dATP, dTTP, dGTP, and dCTP; 0.4 μ*M* barcoded primers (Eurofins MWG, Ebersberg, Germany); 2.5 m*M* $MgCl_2$; and PCR-grade water (Roche Diagnostics, Mannheim, Germany). Total genomic DNA (100 ng) was added to the PCR. Amplification in a standard thermocycler after initial denaturation at 95°C for 3 min was performed in 30 cycles at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min with a final extension for 7 min. Amplicons were purified using a WAVE System (Transgenomic Inc., Omaha, NE) and eluted by a linear gradient of acetonitrile in 0.1 *M* trimethyl ammonium acetate; amplicon DNA was purified subsequently on NucleoFast 96 PCR plates (Macherey-Nagel, Düren, Germany). The DNA concentrations were determined using a PicoGreen double-stranded DNA assay kit (Life Technologies, Carlsbad, CA). Barcode-labeled amplicons were pooled equimolar and analyzed using a 2100 Bio Analyzer (Agilent Technologies, Waldbronn, Germany) using a DNA 7500 kit. Emulsion PCR of pooled samples was performed with the GS Titanium MV emPCR kit (Roche/454 Life Science) according to manufacturer's instructions. Sequencing of the pool was performed using the GS FLX Titanium Sequencing Kit XLR70 (Roche/454 Life Science) according to manufacturer instructions.

Analysis of the sequence data (17 samples; 122,458 reads in total) was done with the software package mothur (version 1.33.3, http://www.mothur.org/) according to the procedure described previously (Schloss et al., 2009; Schloss and Westcott, 2011). Primers, barcode sequences, and sequences of short length and low quality were trimmed with a minimum average quality score of 30 and a minimum length of 145 bp. The command "Chimera.uchime" was used to exclude chimeric sequences. A total of 82,047 sequences (67%) passed the quality control. The sequences were clustered into operational taxonomic units (**OTU**) using 97% 16S rRNA gene similarity as a threshold (0.03 distance; Kuczynski et al., 2012). The OTUs containing only 1 sequence were removed (2,363 OTU). For calculating the nonparametric estimators, Chao 1 and ACE, and the diversity indices, Shannon, Simpson, and Bray-Curtis similarity, the "summary.single" command was used after rarefying to the minimum number of sequences per sample. Rarefaction curves were generated and modified after an algorithm described by (Griffen et al., 2012). Heatmaps were created using JcolorGrid (http://jcolorgrid.sourceforge.net/; Joachimiak et al., 2006). To illustrate microbial shifts on community level, linear discriminant analysis was done in JMP Pro (SAS Institute, Cary, NC) with the 45 most-abundant OTU as covariates and diet as the categorical variable. Cluster analysis was done with Cluster 3.0 (Clustering Library version 1.52; de Hoon et al., 2004). The OTUs that were detected in all feeding groups (n = 322) were normalized and k-means clustering was performed. In the heatmap, Euclidean distances (median values) are visualized in Java Treeview (Saldanha, 2004).

Diet-group-specific prediction of the metagenomic potential of the CBEM was performed with PICRUSt, based on 16S rRNA data (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States;

Langille et al., 2013). This tool is based on the assumption that phylogeny can reliably predict gene content. For PICRUSt, another OTU picking was performed with quality controlled sequences by aligning sequences to Greengenes reference OTUs (downloaded from http://greengenes.secondgenome.com/downloads/database/13_5) and OTUs were clustered based on 0.03 distance limit. This OTU table was used for PICRUSt on the online Galaxy interface (http://galaxyproject.org/), with a workflow described by the developers (http://picrust.github.com/picrust/tutorials/quickstart.html#quickstartguide).

Cloning and Sanger-Sequencing for Comparison with Pyrosequencing Data

The PCR amplification of 16S rRNA genes were done with primers 616F (forward = 5′-AGA GTT TGA TYM TGG CTC-3′; Juretschko et al., 1998) and 1492R (reverse = 5′-GGY TAC CTT GTT ACG ACT T-3′; Lane, 1991). Reaction mixtures (total volume = 25 μL) containing each primer at a concentration of 0.2 pmol/μL were prepared by using 1× reaction buffer (Ex Taq Buffer; 20 m*M* MgCl) and 0.025 U of TaKaRa Ex Taq Hot Start polymerase (TaKaRa 5 units/μL), 0.8 m*M* (0.2 m*M* each) of dNTP (TaKaRa, Shiga, Japan), and 0.5 μL of template DNA. Thermal cycling was carried out by using an initial denaturation step of 95°C for 5 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 40 s, and elongation at 72°C for 60 s. Cycling was completed by a final elongation step of 72°C for 5 min. The StrataClone PCR Cloning Kit (Agilent, Santa Clara, CA) was used for cloning following the manufacturer's instructions. In total, 328 clones were sequenced by Sanger sequencing (LGC Genomics, Berlin, Germany) using vector-specific primers. Overlapping sequences were assembled and vector sequences were removed using the DNA Baser software (http://www.dnabaser.com/). Comparison of pyrosequencing OTUs and cloning OTUs was performed with local BLASTn searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Each of the near full-length sequences showing ≥99% similarity to pyrosequencing OTU were considered fitting. Sanger sequencing OTUs were classified against type strains using RDP (Ribosomal Database Project; http://rdp.cme.msu.edu/index.jsp).

Accession Numbers

The pyrosequencing data are available in the European Nucleotide Archive under accession number PRJEB7404. The clone sequences are available in the European Nucleotide Archive under accession numbers LN612774-LN613101.

Statistical Analyses

Data were first analyzed for normality using Shapiro-Wilk test with PROC UNIVARIATE in SAS (version 9.2, SAS Institute Inc.). The parametric analysis of the data was conducted using PROC MIXED of SAS. Data were submitted to ANOVA with a model accounting for the fixed effects of feeding group and random effect of animal. Polynomial contrasts were used to evaluate linear and quadratic effects of concentrate inclusion and to contrast the effects of 0 versus 30 and 60% grain and 0 and 30 versus 60% concentrate. Degrees of freedom were estimated using the method of Kenward-Roger. Significance was defined as $P \leq 0.05$ and trends were defined at a level of $0.05 < P \leq 0.10$. Results are shown as least squares means ± standard error of the mean.

RESULTS

Pyrosequencing Data and OTU Classification

All 122,458 reads deriving from 17 samples were processed together throughout the bioinformatics workflow. In total, 82,047 sequences (67%) with a minimum length of 145 bp passed the quality control (4,357 ± 855 sequences per sample). Sequences were clustered into 4,242 OTU, from which 2,363 OTU were excluded because they consisted of only 1 sequence. The remaining 1,879 OTU were used for all further downstream analyses. Each of the 12 most abundant OTU contained >1,000 sequences (up to 10,364 sequences; Supplementary Table S2; http://dx.doi.org/10.3168/jds.2014-9166).

Assessment of Diversity and Surveys of CBEM Structure

Rarefaction curves (Figure 1A) were calculated for all samples and summarized to diet means to determine the coverage of sequenced diversity per group. Curves revealed sufficient high diversity coverage for all feeding groups. Rank abundance curves (Figure 1B) indicated that the samples contained a low proportion of highly abundant OTUs, whereas most of the present diversity was composed of rare organisms. Diversity indices estimating species richness and evenness are shown in Table 1. Diversity indices were not different among feeding groups ($P > 0.1$). The actual numbers of OTUs found in each feeding group were 851, 1,129, and 1,179 in the 0, 30, and 60% concentrate diets, respectively.

As shown in Figure 1, 323 OTU (17.2%) were shared among the 3 feeding groups, whereas 634 OTU (33.8%) could be detected in 2 out of 3 feeding groups, and 922 OTU (49%) were detected in only 1 of the feeding

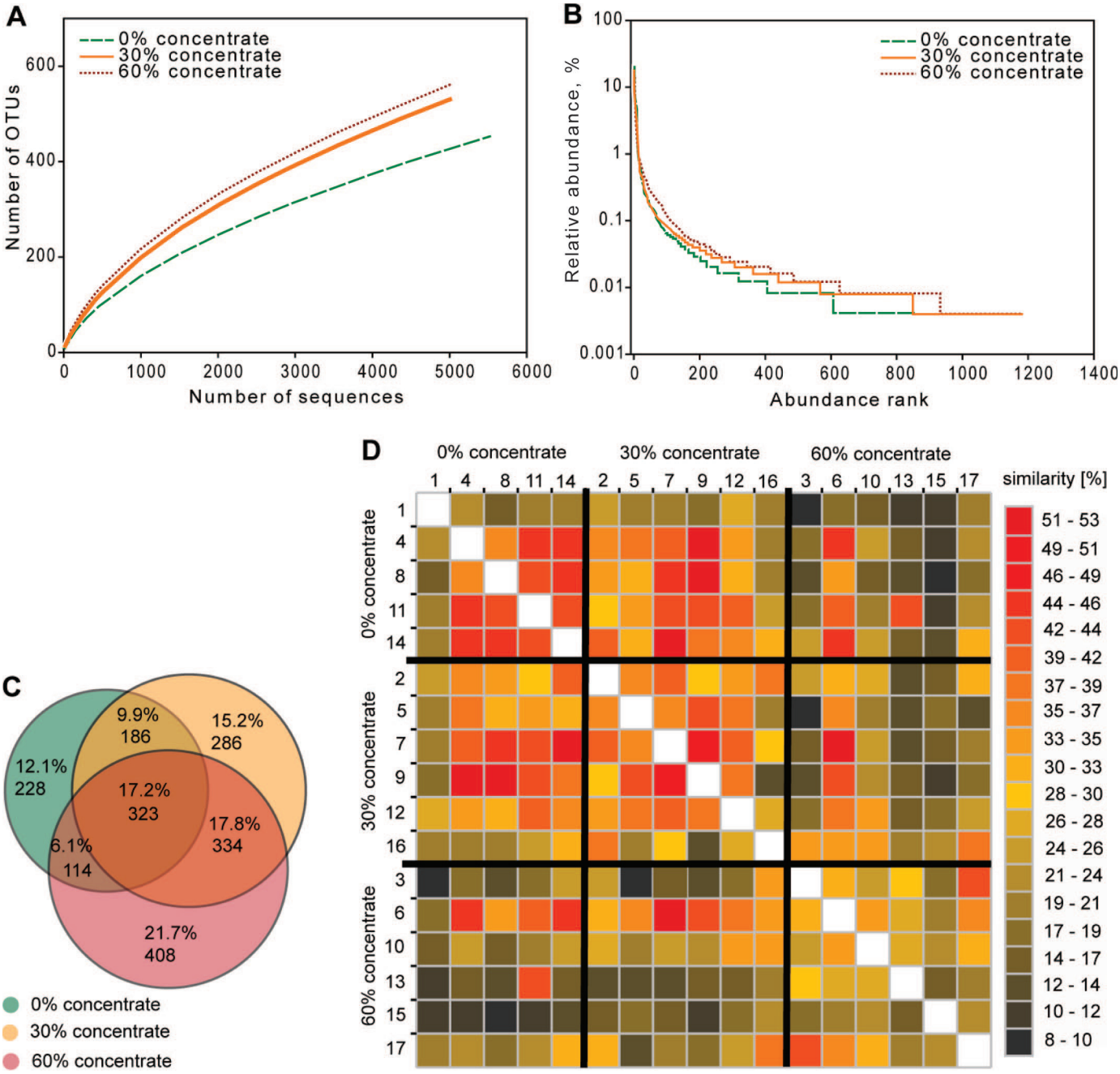

Figure 1. Diversity of bacteria attached to the rumen wall. (A) Rarefaction curves and (B) rank abundance curves based on an operational taxonomic unit (OTU) definition threshold of 0.03 16S rRNA distance are shown. Rarefaction and rank abundance curves were calculated for each sample and depicted as mean values per feeding group. (C) Venn diagram showing the number of shared OTU between feeding groups. The size of the circle is in proportion to the number of OTUs detected in each feeding group. (D) Bray-Curtis analysis displays 16S rRNA gene similarity results between the 3 feeding groups (0, 30, and 60% barley concentrate) and the single goats compared with each other. Color version available online.

groups (Figure 1C). On a community level, shown by Bray-Curtis similarities, the comparison of 0 and 30% concentrate groups showed relatively high similarity scores (between 30 and 53% similarity), except for goat 1 compared with all other goats. In contrast, the 60% concentrate group (with the exception of goat 6) displayed only low similarity to the other 2 feeding groups as well as low similarity within the 60% concentrate group (Figure 1D). However, pairwise comparisons of the microbial communities from different feeding

Table 1. Species richness and diversity indices for bacteria attached to the rumen wall by estimator[1]

Feeding group	Chao 1	ACE[2]	Shannon	Simpson
0% concentrate	713	1,070	3.34	0.11
	(662–1,003)	(1,063–1,360)	(3.69–3.84)	(0.08–0.09)
SEM	103	157	0.38	0.02
30% barley concentrate	816	1,235	3.79	0.08
	(662–1,003)	(1,063–1,360)	(3.69–3.84)	(0.08–0.09)
SEM	94	143	0.082	0.02
60% barley concentrate	855	1,269	4.09	0.06
	(662–1,003)	(1,063–1,360)	(3.69–3.84)	(0.08–0.09)
SEM	94	143	0.28	0.02

[1]Values of richness and diversity indices did not differ significantly among feeding groups. Values in parentheses represent confidence intervals.

[2]ACE = abundance-based coverage estimator.

groups by using libshuff analysis revealed that all feeding groups were statistically distinct from each other (data not shown).

Phylum Affiliation

In total, 23 phyla were identified and *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Spirochaetes* were the most abundant, with 97.7% of all reads affiliating to these 4 phyla. In Figure 2 and Supplementary Table S3 (http://dx.doi.org/10.3168/jds.2014-9166), relative abundances of the phyla are depicted with respect to the feeding groups. There was a trend of decreasing *Proteobacteria* ($P = 0.053$) in the 60% concentrate group compared with the other feeding groups. A significant increase of *Firmicutes* ($P = 0.041$) in the 60% concentrate group and *SR1* ($P = 0.034$) in the 0% concentrate group could be detected compared with the other feeding groups. A trend was also observed for a decreasing average number of phyla in the 60% concentrate group compared with the other 2 feeding groups ($P < 0.08$).

Diet-Related Shifts on the OTU Level

Linear discriminant analysis with diet as the categorical variable revealed strong differences among the feeding groups in response to concentrate content of the diet. All feeding groups clustered separately, with no overlapping in their 95% confidence intervals (Figure

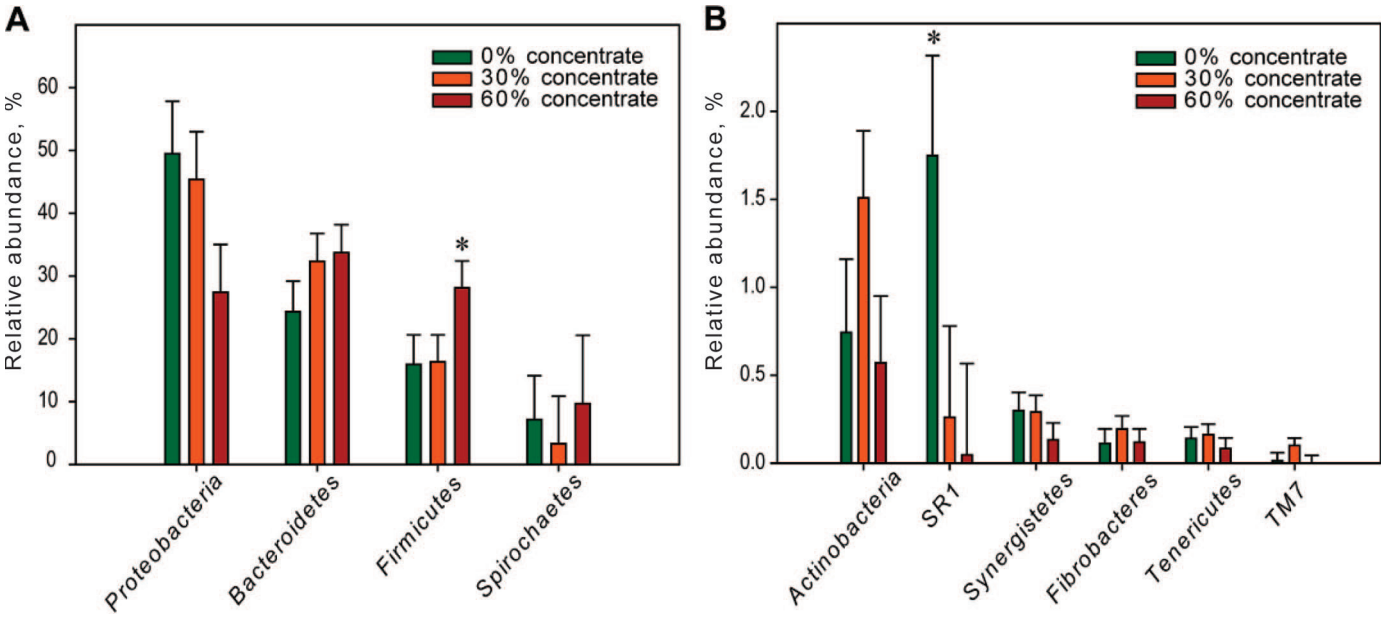

Figure 2. Relative abundances of the 10 most-abundant bacterial phyla attached to the rumen wall. (A) Abundant phyla with more than 2% mean abundance are shown for each feeding group (0, 30, and 60% barley concentrate). (B) Relative abundances of rare phyla for each feeding group. Error bars represent SEM. Significance in one feeding group compared with the other 2 feeding groups are marked with an asterisk (*) and defined as $P \leq 0.05$. Color version available online.

3). Relative abundances of the 45 most abundant OTU are shown in Figure 4 and listed in detail in Supplementary Tables S2 and S4 (http://dx.doi.org/10.3168/jds.2014-9166). In total, the 45 most abundant OTU accounted for 73.8% of all sequences, whereas 28.9% of the 45 most abundant OTU (n = 13) differed significantly between the feeding groups.

In the 0% concentrate group, only 5 OTU were highly abundant (relative abundance >5%), classified as *Bergeriella*, *Campylobacter*, *Prevotella*, *and Meniscus*. *Ottowia*-OTU 14 was significantly decreased (0.2 fold-change, $P < 0.05$) and OTU 20 (unclassified *SR1*) was significantly increased (12.78 fold-change, $P < 0.04$) in the 0% concentrate group compared with the other feeding groups. In the 30% concentrate group, only 3 OTU were highly abundant (relative abundance >5%) and classified as *Bergeriella*, *Campylobacter*, and *Prevotella*, the 3 most abundant genera in our study across all feeding types. In the 60% concentrate group, 4 OTU were highly abundant (relative abundance >5%). Interestingly, *Campylobacter*-OTU 2 was significantly increased (2.89 fold-change, $P < 0.02$) in the 60% concentrate group, whereas *Campylobacter*-OTU 3 is significantly decreased (0.25 fold-change, $P < 0.03$) in the 60% concentrate group compared with the other feeding groups.

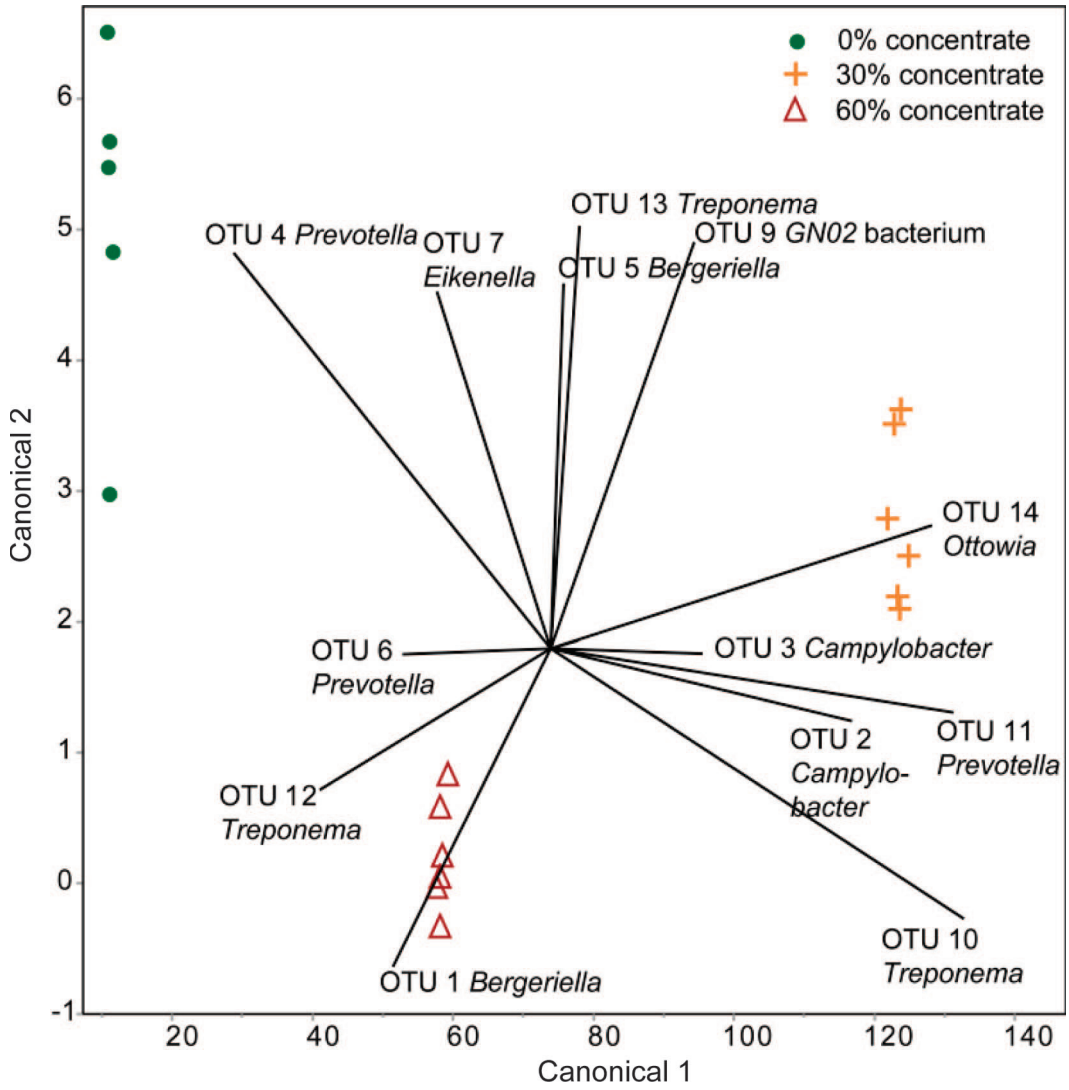

Figure 3. Linear discriminant analysis with the first 2 principal components for the epimural bacteria dependent on 3 different diets. For the discriminant analysis the 45 most-abundant operational taxonomic units (OTU) (representing 73.8% of all reads) were used as covariates and the diet as the categorial variable. Diets were abbreviated as follows: green circles: 0% concentrate group, orange crosses: 30% barley concentrate group, red triangles: 60% barley concentrate group. Color version available online.

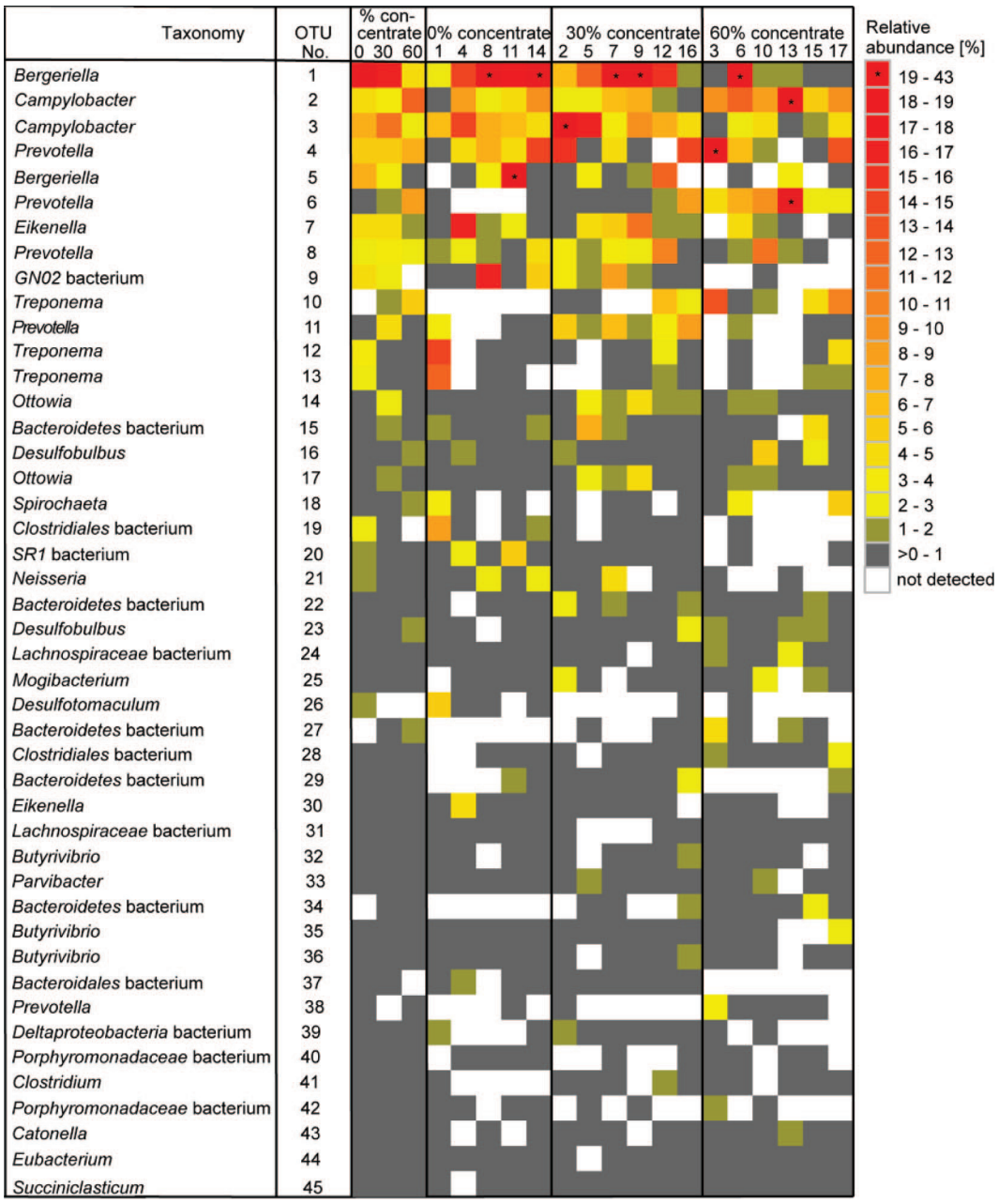

Figure 4. Relative abundance of operational taxonomic units (OTU) per feeding group (0, 30, and 60% barley concentrate), single goat, and diet-related shifts. Heatmap showing the relative abundances of the 45 most-abundant OTU sorted per feeding group and per individual goat. Diets were abbreviated as follows: A: 0% concentrate group, B: 30% concentrate group, and C: 60% concentrate group. For better resolution, relative abundance values higher than 19% (ranging from 19 to 43%) are shown as 19% and are denoted with an asterisk (*). Color version available online.

The k-means clustering was performed with OTUs that were detected in all 3 feeding groups (n = 322, Figure 5). Cluster I consisted of 12 OTU that were highly abundant in the 0% concentrate feeding group, moderately abundant in the 30% concentrate feeding group, and scarcely abundant in the 60% feeding group. These OTUs were classified as *Meniscus*, *Treponema*, *Lutispora*, unclassified *SR1*-phylum, *Neisseria*, *Bergeriella*, *Kingella*, *Mahella*, *Butyrivibrio*, *Paraprevotella*, and *Sediminitomix*. Cluster IV consisted of 62 OTU and contained OTUs that were highly abundant in the 60% concentrate group and low to moderately abundant in the 2 other feeding groups. These OTUs were classified as *Prevotella*, *Desulfobulbus*, *Spirochaeta*, *Guggenhei-*

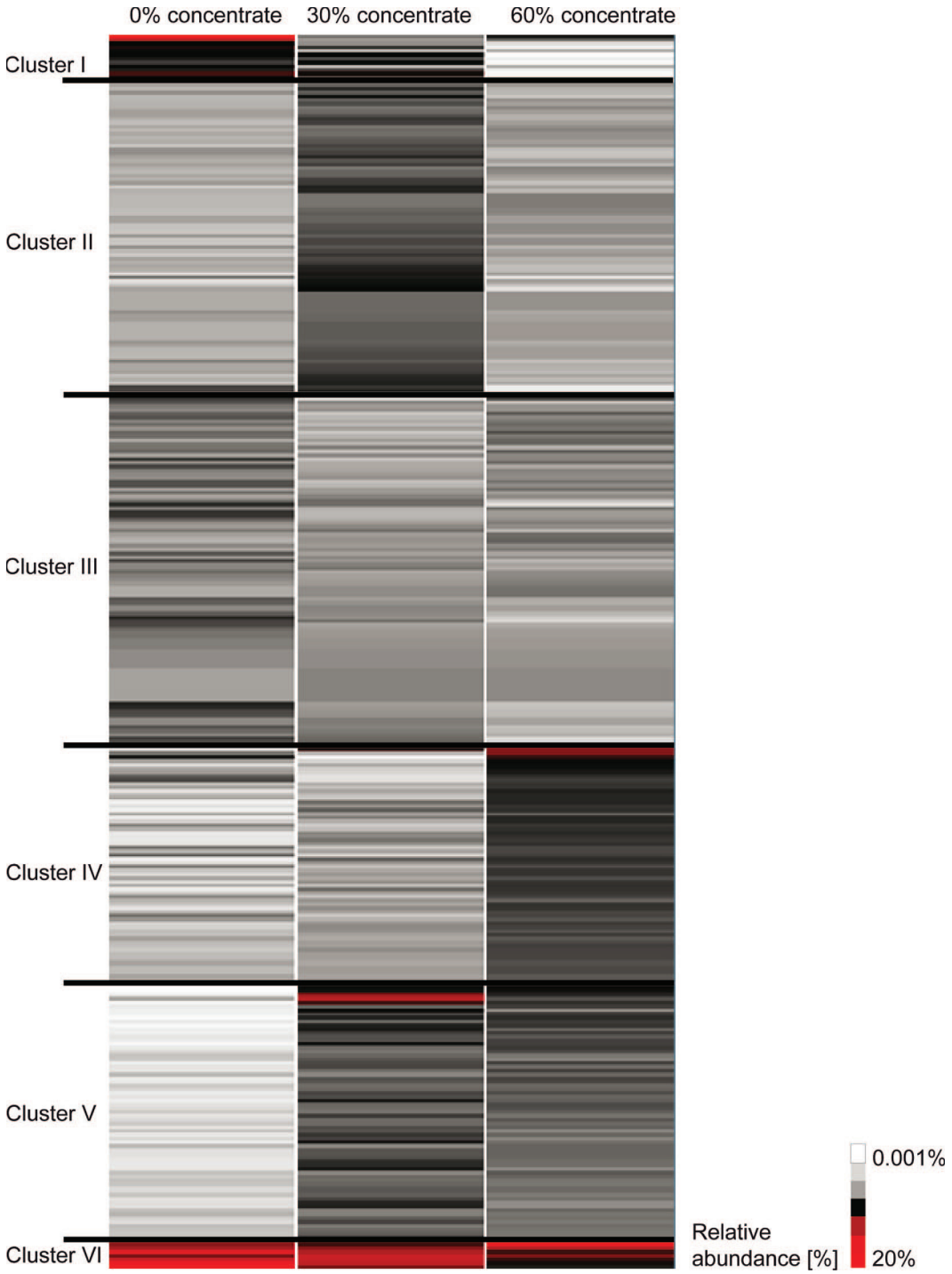

Figure 5. The k-means cluster analysis with operational taxonomic units (OTU) detected in all feeding groups (0, 30, and 60% barley concentrate; n = 322). Sequences were normalized and the Euclidian distances with mean values were calculated and clustered. Color version available online.

mella, *Mogibacterium*, *Phocaeicola*, *Butyrivibrio*, *Howardella*, and *Butyricimonas*. Cluster VI consisted of 8 OTU that were highly abundant in all 3 feeding groups. These OTUs were classified as *Bergeriella*, *Campylobacter*, *Prevotella*, and *Paraprevotella*.

Comparison of the Roche/454 Pyrosequencing Data Set with Near Full-Length Sequences

We sequenced 328 16S rRNA gene clones deriving from 3 goats (1 goat from each feeding group) with

Sanger sequencing and compared them with the pyrosequencing OTUs to obtain better taxonomic resolution of the most abundant OTUs. The OTUs with ≥99% sequence similarity were considered as the same species. In total, 179 OTU of the pyrosequencing data set showed ≥99% sequence similarity with OTUs of the Sanger sequencing data set. In Supplementary Table S2 (http://dx.doi.org/10.3168/jds.2014-9166), the 45 most abundant OTU from the Roche/454 data set and the corresponding near full-length Sanger sequencing matches, if available, are listed.

Metagenome Prediction

To estimate whether diet might also modify the overall metabolic potential of the CBEM, we applied the recently published bioinformatics tool PICRUSt to the 16S rRNA pyrosequencing data (Langille et al., 2013). The predicted metagenomic structure of CBEM is mostly consistent for animals within one dietary group. The results of this analysis (Supplementary Figure S1; http://dx.doi.org/10.3168/jds.2014-9166) suggested consistency for most metabolic functions, particularly for genes encoding for cellular and information processing, such as the ribosomal machinery or translation and transcription, which do not considerably vary among diets fed. However, clusters of orthologs groups category G (genes for carbohydrate transport and metabolism) and T (signal transduction mechanisms) were overrepresented in the 60% concentrate group compared with the other groups (1.3 and 1.2 fold-change, respectively).

Analysis on the Genus Level and Definition of a CBEM Core Microbiome

In addition to the overview on the phylum and OTU level, we combined all OTUs of the pyrosequencing data set in their respective genus to get insights into cumulated shifts on the genus level. In Figure 6, the potential CBEM core microbiome consisting of the 35 most abundant genera out of 220 genera (genera >0.003% relative abundance) is depicted. Relative genera abundances separated per diet are shown in Supplementary Table S5 (http://dx.doi.org/10.3168/jds.2014-9166). The genera *Bergeriella*, *Campylobacter*, and *Prevotella* were the 3 most abundant genera in all feeding groups and accounted for over 49% of all sequences. Independent of the feeding group, *Bergeriella* was the most abundant genus but it represented only the fourth-most abundant genus in the 60% concentrate group. Goats fed with the 0% concentrate diet tended to harbor less *Ottowia* (0.21-fold change, $P < 0.07$) and less *Mogibacterium* (0.24-fold change, $P < 0.03$), whereas *Kingella* increased (5.96-fold change, $P < 0.04$) compared with goats of the other feeding groups. Furthermore, goats fed the 0% concentrate diet tended to have more *Bergeriella* than goats fed the 30% concentrate diet (2.1 fold-change, $P < 0.08$). The genus *Bergeriella* was significantly decreased (0.22-fold change, $P < 0.02$) in the 60% feeding group compared with the 0 and 30% concentrate diets. Feeding of 60% concentrate diet also tended to increase *Prevotella* compared with goats fed the 0 and 30% concentrate diets (1.66 fold-change, $P = 0.07$).

DISCUSSION

In general, environments such as the rumen house fastidious bacteria that are difficult to cultivate in vitro. This has led to limited reference databases, which, in turn, hinder the classification of microbes from such complex ecosystems. Molecular techniques such as those employed in our study have demonstrated a far more complex epimural bacterial community in the rumen than expected based on traditional cultivation-based techniques. The advantage of pyrosequencing is the broad coverage of phylotypes, but the phylogenetic resolution for a classification at species level may not be sufficient. For this reason, we additionally sequenced 16S rRNA gene clones. Unfortunately, not all of the highly abundant OTUs from pyrosequencing were classified as the same species as one of our near full-length sequences. In near future, the "Hungate1000" project, aiming to sequence reference microbial genomes from the rumen, should greatly help in the characterization of rumen microbes (http://www.hungate1000.org.nz/; Creevey et al., 2014). Until this database is fully developed, it may remain difficult to classify certain rumen bacteria due to the absence of closely related characterized reference strains. The present study revealed striking changes in the CBEM in relation to increasing dietary concentrate levels.

The Bacterial Epimural Microbiome

One major finding of our study was the high diversity of the CBEM. We can largely exclude a contamination of the rumen mucosa with bacteria originating from the rumen content, because the mucosa was rigorously washed and rinsed during sampling to remove free and loosely attached bacteria. Furthermore, previous studies revealed distinct clustering of the bacterial communities from different rumen fractions, providing evidence for distinct microbial community structures at the 3 rumen fractions (rumen fluid, attached to solid digesta, attached to rumen mucosa; Cunha et al., 2011; Lee et al., 2012; Huo et al., 2014).

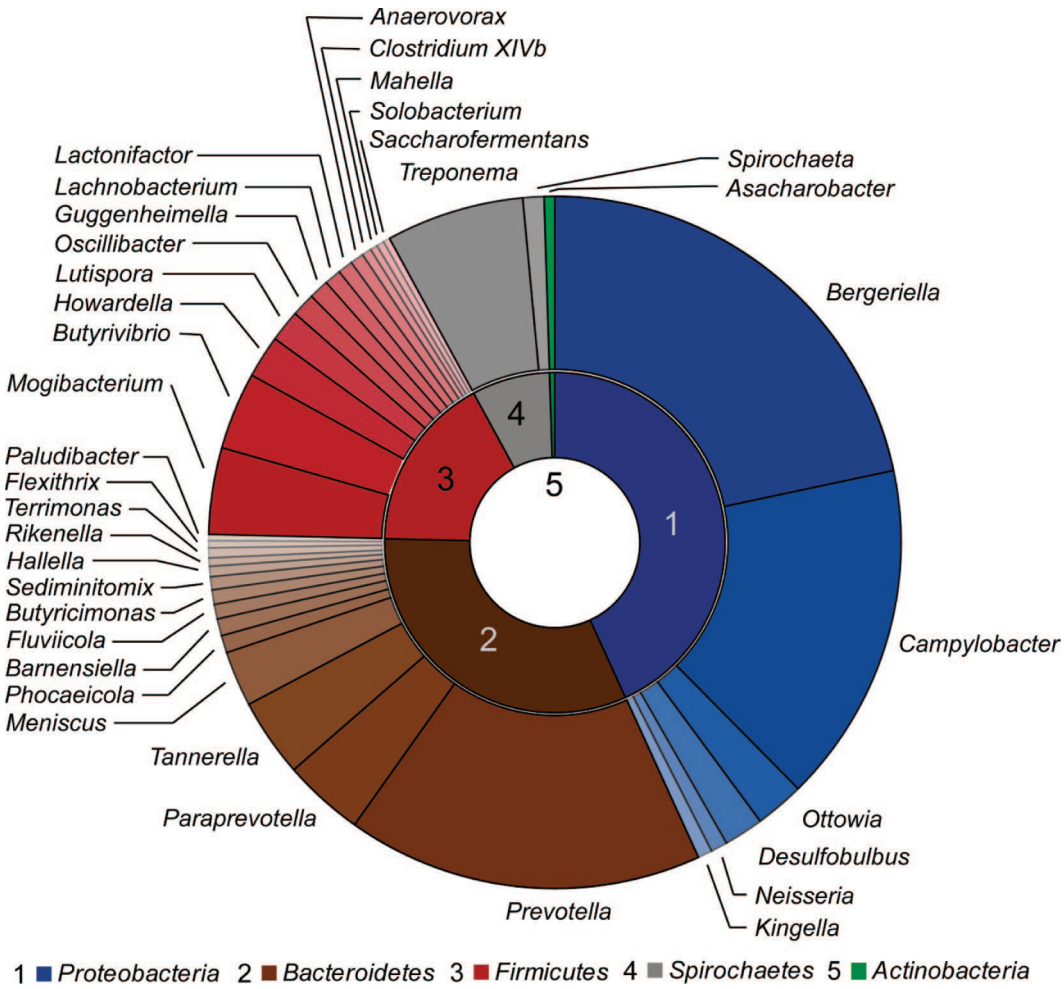

Figure 6. Visualization of the caprine bacterial epimural microbiome core on genus and phylum level, independent of the goat diet. The most abundant genera over all feeding groups (0, 30, and 60% barley concentrate) are shown. The size of the circle fragments is proportional to the number of sequences. Color version available online.

A feeding experiment with different energy supplies revealed *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Lentisphaerae*, *Proteobacteria*, *Spirochaetes*, and *Tenericutes* to be the most abundant phyla in the liquid and solid fraction of the rumen of goats using Roche/454 pyrosequencing (Huo et al., 2014). Also, the bacterial community in the liquid- and solid-associated fractions of the rumen digesta of goats was described using 16S rRNA gene libraries (Cunha et al., 2011). The phyla *Bacteroidetes* and *Firmicutes* were predominant, whereas *Proteobacteria* were only observed in the liquid fraction (0.5%) and *Spirochaetes* were not found. The results of these 2 studies are in contrast to our study, in which we found a high abundance of *Proteobacteria*. Despite all of these studies being conducted on goats, the different results obtained may indicate differences between the luminal core microbiome and CBEM in the rumen. Recently, Liu et al. (2015) also found the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* to be most abundant in the CBEM and increasing *Firmicutes* with high-concentrate diet, which is consistent with our results. *Mogibacterium* increased with increasing concentrate amount in both studies. *Mogibacterium* are known to be nonfermentative, are not nitrate-reducers, and were isolated from human periodontal pockets (Nakazawa et al., 2000); thus, it is difficult to predict their metabolic role in the CBEM. However, due to the different sampling methods, DNA isolation methods, primers, data processing methods, and because different breeds were used, it is hard to compare their study with our study.

In our study, we expected *Proteobacteria* to decrease and *Firmicutes* to increase with increasing concentrate amount because of the susceptibility of many *Proteobacteria* toward low pH (Rousk et al., 2010) and the ability of many *Firmicutes* to degrade easily fermentable carbohydrates, even under low pH. Previous studies al-

ready noted such shifts in the microbiome of the rumen epithelium in other ruminant species fed with different energy levels (Chen et al., 2011; Li et al., 2012; Petri et al., 2013a). This leads to the hypothesis that some parts of the core microbiome may be shared among different ruminants and may behave in a similar way to different feeding strategies. Interestingly, many of the OTUs obtained in our study showed highest similarity (>99%) to sequences originating from cattle and sheep rumen samples (Supplementary Table S6; http://dx.doi.org/10.3168/jds.2014-9166; Sadet-Bourgeteau et al., 2010; Li et al., 2012), which also supports the existence of a potential epimural ruminant core microbiome.

However, *Proteobacteria* could also be triggered by the presence of trace amounts of oxygen, as many organisms belonging to this phylum are microaerophilic or facultative anaerobes (Sadet-Bourgeteau et al., 2010). Bacteria belonging to *Firmicutes* often carry more than 4 rRNA operons, even up to 15 operons, which may have led to an overestimation of the *Firmicutes* abundance (Lee et al., 2009).

At the genus level, our study reveals the most abundant genus to be classified as *Bergeriella*. *Bergeriella* is a nonflagellated commensal, which is known to effectively ferment carbohydrates from its host. *Bergeriella* have rarely been described in rumen samples before (Pallara et al., 2014), but it is well known as a nonpathogen in oral cavities of different mammals (Xie and Yokota, 2005). Unlike *Neisseria* species, it can produce fermentation acids from mannose, glucose, fructose, and sucrose, which may also lead to a decrease of rumen pH. The nitrite-reducing properties of *Neisseria* species might be helpful for the host ruminant to avoid nitrate poisoning (Lewis, 1951; Kahn, 2005). Pathogenic relatives of *Bergeriella*, such as *Neisseria gonorrhoeae*, *Neisseria meningitides*, or *Neisseria ovis*, are associated with gonorrhea, meningitis, and conjunctivitis in ruminants, respectively (Bankemper et al., 1990). Considering the high abundance of *Neisseriaceae* in our study with goats being clinically healthy, we do not expect them to have pathogenic effect on the rumen, but until now it is unclear whether it may have zoonosis relevance. Due to the low sequence similarity (92% for OTU1 and 91% for OTU5), we cannot exclude that these OTUs may belong to a different genus than *Bergeriella*. Pyrosequencing OTUs classified as *Bergeriella* showed highest similarity to *Snodgrassella alvi* (94% similarity, BlastN against GenBank nr), a carboxylic acid-oxidizing dominant member of the honey bee and bumble bee digestive tract (Kwong et al., 2014). Kwong et al. (2014) stated that *Snodgrassella alvi* may form a syntrophic network for sharing metabolic resources with other species. Therefore, we hypothesized that OTUs classified as *Bergeriella* may have a similar function in the goat rumen epithelium.

Campylobacter, the second-most prevalent genus detected, showed no significant difference in response to different concentrate supply. Known pathogens in this genus are *Campylobacter jejuni* and *Campylobacter coli*, which cause enteritis and are common in the intestines of mammals and birds. Further studies are needed to reliably classify *Campylobacter* on species level and to identify whether they represent commensals or potentially pathogens in the caprine rumen (Garrity, 2005; Anderson et al., 2009; Lin et al., 2013).

Prevotella was the third-most abundant genus in our study and is commonly known to be an abundant member of the rumen microbiome (Malmuthuge et al., 2014; Omoniyi et al., 2014; Rosewarne et al., 2014). They have highly diverse functions and are one of the most abundant genera in the rumen content of goats, in particular after high-grain feeding (Khafipour et al., 2009; Metzler-Zebeli et al., 2013b). Correlation analysis showed positive relationships between *Prevotella*, ruminal LPS concentration (Tamura et al., 1992), colonic LPS concentration, and ruminal SCFA concentration (Metzler-Zebeli et al., 2013b). In our study, some *Prevotella*-OTU increased whereas others decreased between different concentrate feeding groups, finally causing no statistically significant shifts on genus level. Due to the high diversity of *Prevotella* species and their metabolic functions, we cannot predict their exact metabolic function. Some *Prevotella* might shed high amounts of LPS, which can float into the blood circuit of the goat and cause systemic inflammation reactions (Khafipour et al., 2009). Second, *Prevotella* species producing SCFA as end products could possibly play an important role in the rumen of goats fed high-energy supply diets, as SCFA are important substrates in the rumen metabolism (Kristensen et al., 1998). Under acidic conditions, high concentrations of SCFA lead to a decrease of ruminal pH, causing epithelial lesions in the rumen wall (Zebeli and Metzler-Zebeli, 2012).

Diet-Related Effects and Metabolic Prediction

Increasing the concentrate level of the diet had a substantial effect on the bacterial community attached to the mucosa, as indicated by the trend for an increased species richness and *Firmicutes* abundance in the 60% concentrate group compared with the 2 other groups. Distinct clustering per feeding group in the Bray-Curtis similarity matrix provided strong evidence for distinct microbial communities due to different concentrate amounts confirming our hypothesis. Although all goats grew up in the same barn, distinct community patterns were found within feeding groups (shown by

Bray-Curtis analysis), suggesting an individual CBEM with a shared core microbiome of abundant community members. In a recent study, shifts in the potential luminal goat rumen microbiome due to different energy supply were detected (Huo et al., 2014). The difference in bacterial communities between the 60% concentrate group and the other 2 feeding groups could be due to the strong effect of a decrease in rumen pH, which could lead to rising fermentation and alterations in rumen mucosal functioning. An increase of osmolarity with high concentrate levels fed (Hollmann et al., 2013; Metzler-Zebeli et al., 2013a) may cause increased water influx into the rumen and decrease barrier function of the epithelium (Penner, 2009). Increased water influx into the rumen may be one reason for feed intake depression during SARA in ruminants, which has a negative effect on animal health as well as on dairy production. With high-concentrate diets, rumen conditions are comparable to conditions observed during SARA. For example, ruminal pH was 5.5 and 6.0 in the 60 and 30% concentrate groups, respectively. In cattle, ruminal pH values below 5.8 are typical SARA thresholds (Zebeli and Metzler-Zebeli, 2012), whereas in goats no such ruminal pH thresholds have yet been characterized. Subacute ruminal acidosis is known to provoke greater metabolic stress of microbiota and lower overall fiber digestion. A high number of lactate-producers and disturbed mucosal absorption of SCFA often precede SARA (Zebeli and Metzler-Zebeli, 2012). The cluster analysis revealed nearly half of all OTU abundances were hardly shifted in the 0% concentrate feeding group compared with the 60% concentrate feeding group, which indicates the fraction of relatively stable OTU when a high-concentrate diet is fed. Klevenhusen et al. (2013) analyzed the same goats with respect to altered electrophysiological properties and permeability of the rumen wall. The pH of the rumen was measured directly after euthanasia (2 – 3 h after last morning feeding). Although the values of the ruminal pH during the feeding experiment are not known, we can assume that at least the 60% concentrate feeding group (pH 5.5) have been at high risk of SARA (Klevenhusen et al., 2013).

The OTUs belonging to cluster I (k-means cluster analysis), highly abundant in the 0% concentrate feeding group and low to moderately abundant in the other 2 feeding groups, are expected to mostly ferment cellulose and be involved in fiber digestion. These OTUs, or their close relatives, are common in the gastrointestinal tract of different mammals and ferment different cell-wall carbohydrates to acetic acid and interact with cellulolytic bacteria or are cellulolytic themselves (Stanton and Canale-Parola, 1980; Rumney et al., 1995). *Lutispora* ferments peptone, tryptone, caseine, pyruvate, and some AA and produces acetate, isobutyrate, propionate, and isovalerate (Shiratori et al., 2008). *Mahella* can ferment various plant disaccharides and simple sugars such as arabinose, cellobiose, fructose, glucose, sucrose, xylose, and pyruvate to lactate and formate (Salinas et al., 2004). The accumulation of these OTUs in the 0% concentrate group underlines the importance of these bacteria for cellulose fermenting processes. The most abundant OTUs in cluster IV, highly abundant OTUs in the 60% concentrate group, are mostly responsible for different fermentation processes (e.g., cellulolytic, hemicellulolytic, oligosaccharolytic, proteolytic, amylolytic, and xylanolytic functions) or are not involved in fermentation processes at all (Nakazawa et al., 2000). *Prevotella* is also known to increase with decreasing rumen pH (Petri et al., 2013b). *Desulfobulbus* is a sulfate-reducer that also decomposes FA (Widdel and Pfennig, 1982). *Butyrivibrio* ferments glucose to butyrate, hydrogen, ethanol, carbon dioxide, formate, and lactate (Kopecný et al., 2003). According to the literature, most of these OTUs are involved in the fermentation of easily fermentable carbohydrates or SCFA, as their concentration in the rumen increases with increasing energy supply (Petri et al., 2013a; Huo et al., 2014). The OTUs in cluster VI, highly abundant OTUs in all 3 feeding groups, ferment different carbohydrates (such as cellulose, hemicellulose, saccharose, and others) to acetic acid and other metabolites, interact with cellulolytic bacteria and are cellulolytic themselves (Petri et al., 2013b), or reduce nitrate (Vedros et al., 1983). *Campylobacter* is a nitrate- and sulfite-reducing (except *C. jejuni*), fumarate to succinate reducing, asaccharolytic bacterium relying on fermentation of AA or tricarboxylic acids (Garrity, 2005). All of these phylotypes may also additionally be involved in cross-feeding processes, which might explain their high abundance although their favorite substrate is not known to be produced in high amounts. It is, however, hard to predict which metabolic influence these bacteria have on the host because some of the OTUs showed only low similarity (<90%) to their closest related type strains.

To obtain insight into the putative functional capacity of the CBEM, PICRUSt (Langille et al., 2013) was applied. This tool was used recently for the prediction of gene families and was shown to reliably outline metagenomic approaches from the Human Microbiome Project (Langille et al., 2013). The PICRUSt analysis generated 2 hypotheses: (1) goats have a stable functional metagenome of the CBEM that is hardly influenced by the concentrate percentage in the diet and (2) signal transduction, defense, and carbohydrate metabolism are increased in the 60% concentrate group. This increased carbohydrate metabolism can be

explained by the fact that the 60% concentrate group is associated with a higher amount of easily fermentable carbohydrates in the rumen which serve as easily available energy substrates for rumen CBEM, leading to an adaptation of the carbohydrate metabolism in the rumen epithelium too. The high concentration of easily fermentable carbohydrates and metabolic end products may cause increased osmolarity at the mucosal site. The increased water influx into the rumen can cause damage to the epithelial barrier function and thus foster translocation of pathogens into the blood stream. Although we observed downregulation of several protective proteins in the rumen epithelium due to 60% grain feeding (Hollmann et al., 2013), and also an increase in the permeability of rumen wall (Klevenhusen et al., 2013), the concentration of serum amyloid A did not differ in our study (Klevenhusen et al., 2013). An increase in serum amyloid A would have been a hint for activation of acute-phase response due to endotoxin translocation, which is observed during episodes of SARA (Zebeli and Metzler-Zebeli, 2012). We assume that goats of our study did experience a severe form of SARA with systemic inflammation, as observed in cattle fed similar grain-rich diets (Khafipour et al., 2009). Additionally these goats also showed increased papillae lesion score, increased keratinization score, decreased fecal score, and increased pulse and respiration rates (Klevenhusen et al., 2013; Metzler-Zebeli et al., 2013b).

CONCLUSIONS

Interestingly, many of the OTUs obtained in our study showed the highest similarity to sequences originating from cattle and sheep rumen samples, which supports the hypothesis of some analogies between microbiomes of different ruminants. The results demonstrate dynamics in the CBEM, triggered by high-concentrate feeding, and revealed distinct microbial communities for all 3 diets tested. This confirms the general hypothesis that diet has a big effect on microbial communities in the gastrointestinal tract. Fiber-digesting bacteria decreased whereas starch-fermenting bacteria were enhanced in the high-concentrate feeding group. These shifts underline the severe risk of SARA in ruminants, as it is not only relevant in cattle, but also in goats. Goat dairy and meat production can be negatively affected by inadequate feeding strategies. Collectively, the observed microbial findings may have important relevance in goat-production systems, whose feeding practices are increasingly changing from traditional roughage-based to concentrate-rich diets to support higher production levels. Future work will be important for the characterization of the CBEM to unravel the function of microbes under defined ruminal conditions and for defining indicator species for the prediction of rumen disorders.

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