High-grain diets supplemented with phytogetic compounds or autolyzed yeast modulate ruminal bacterial community and fermentation in dry cows

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ABSTRACT

The feeding of concentrate-rich diets may lead to microbial imbalances and dysfermentation in the rumen. The main objective of this study was to determine the effects of supplementing phytogetic compounds (PHY) or autolyzed yeast (AY) on rumen fermentation and microbial abundance in cows intermittently fed concentrate-rich diets. The experiment was carried out as an incomplete 3×4 Latin square design, with 8 nonlactating rumen-fistulated Holstein-Friesian cows. The cows were randomly assigned to a concentrate diet that was either not supplemented (CON), or supplemented with PHY or AY. Each of the 4 consecutive experimental periods was composed of a 1-wk roughage-only diet (RD), 6-d gradual concentrate increase, followed by 1 wk of 65% concentrate (dry matter basis; Conc I), and 1 wk of RD and a final 2-wk 65% concentrate (dry matter basis; Conc II) phase. Digesta samples were collected from the rumen mat for bacterial 16S rRNA gene Illumina MiSeq (Illumina, Balgach, Switzerland) sequencing, and samples of particle-associated rumen liquid were obtained for measuring short-chain fatty acids, lactate, ammonia, and pH during RD (d 6), Conc I (d 19), and Conc II (d 39). The concentrate feeding caused a decrease of overall bacterial diversity indices, especially during Conc I. The genera *Ruminococcus*, *Butyrivibrio*, and *Coprococcus* were decreased, whereas *Prevotella*, *Megasphaera*, *Lachnospira*, and *Bacteroides* were increased in abundance. Supplementation of both feed additives increased the abundance of gram-positive and decreased that of gram-negative bacteria. Supplementation of AY enhanced cellulolytic bacteria such as *Ruminococcus* spp., whereas PHY decreased starch and sugar fermenters including *Bacteroides* spp., *Shuttleworthia* spp., and *Syntrophococcus* spp. Moreover, PHY supplementation increased butyrate percentage in the

rumen in both concentrate phases. In conclusion, intermittent high-concentrate feeding altered the digesta-associated rumen bacterial community and rumen fermentation with more significant alterations found in Conc I than in Conc II. The data also showed that both feed additives had the most significant modulatory effects on the bacterial community, and their subsequent fermentation, during periods of low pH.

Key words: feed additive, subacute rumen acidosis, cow-rumen bacteria, short-chain fatty acid

INTRODUCTION

The highly complex microbial community of the rumen enables multiple metabolic functions in the host. This community is evolutionarily adapted to a fiber-rich herbivore diet. Yet, the feeding of large amounts of concentrates, during periods of high energy needs, may lead to rumen bacterial imbalances (McCann et al., 2016). These imbalances may further result in a decline of fiber degradation (Pourazad et al., 2016), shifts in short-chain fatty acid (SCFA) profile (Kleen et al., 2003), and accumulation of endotoxins (Plaizier et al., 2012) in the rumen, all of which can further impair cow health and productivity.

With the help of next-generation sequencing techniques, it has been possible to gain a deeper understanding of rumen bacterial shifts in cattle in response to concentrates in the diet. However, digesta-associated microbial responses to concentrate-rich diets were typically investigated in challenge studies with short and continuous feeding periods (Hook et al., 2011; Mao et al., 2013b; Petri et al., 2013), and less commonly evaluated using intermittent feeding studies. The application of an intermittent high-concentrate feeding model provides the opportunity to look at changes in the rumen microbiome and fermentation that may more closely resemble variable DMI patterns and dietary energy intake, and subsequent drops in ruminal pH, such as during transition and peak lactation. In a recent study, we observed that cows experiencing 2 intermittent

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concentrate-rich challenges had a more severe drop in ruminal pH and rumination behavior during the first than during the second challenge (Kröger et al., 2017), despite feeding the same diets. These findings suggest a differing response of the ruminal microbiome during the 2 concentrate phases. More interestingly, the same experiment revealed enhancement effects on rumination behavior and ruminal pH in cows supplemented with phytogetic compounds (**PHY**) or autolyzed yeast (**AY**), with effects evolving especially during the first challenge phase. In vitro studies have reported PHY to have a direct bacteriostatic effect and therefore alter fermentation end products (Cardozo et al., 2005; Cal-samiglia et al., 2007), whereas AY has an indirect effect by either acting as ligand for gram-negative bacteria (**GNB**; Nocek et al., 2011; Ganner and Schatzmayr, 2012) or as a substrate for cellulolytic bacteria (Harrison et al., 1988; Mao et al., 2013a) and so increases SCFA load (Kesselring et al., 2017). However, if the effects of intermittent concentrate feeding are related to alterations of ruminal microbiome, and whether PHY and AY supplementation modulate rumen microbiome and fermentation in an in vivo model, have not yet been evaluated.

Therefore, one aim of this study was to determine the changes of the rumen bacterial community and fermentation products, such as SCFA, ammonia, and lactate, when feeding an intermittent concentrate-rich diet to cattle. Another aim was to determine the effect of supplementation of PHY or AY feed additives on rumen bacteria shifts and the fermentation products during that intermittent high-concentrate feeding. Our hypothesis was that the increase of concentrate in the diet will decrease bacterial diversity and mediate rumen bacteria community composition and the end products of its metabolism in the rumen. Further, we hypothesized that PHY and AY will modulate the bacterial community structure in the rumen, likely resulting in a reduction in starch-fermenting bacteria and an increase in cellulolytic bacteria, leading to favorable changes in the fermentation products, such as SCFA profile. Effects are expected to be more pronounced during the first feeding phase because the time cows spent under pH 6.0 was longer during this phase (Kröger et al., 2017).

MATERIALS AND METHODS

Animals, Diets, and Experimental Setup

All procedures involving animal handling and treatment were approved by the institutional ethics com-

mittee of the University of Veterinary Medicine (Vet-meduni) Vienna and the national authority according to §26 of the Law for Animal Experiments, Tierversuchsgesetz 2012- TVG (GZ: BMFWF-68.205/0023-WF/V/3b/2015).

Detailed information about cows, feeding, and experimental setup is given in our companion paper (Kröger et al., 2017). In brief, the experiment used 8 nonlactating rumen-fistulated Holstein-Frisian cows (863 ± 65 kg of BW $\pm$ SD) in an incomplete 3×4 Latin square design, balanced for carry-over effects. Cows were randomly assigned to 1 of the 3 feeding groups: control (**CON**, no feed additive), PHY (Digestarom; phytogetic product, containing herbs, spices, essential oils, and nonvolatile extracts, with major active compounds derived from mint, thyme, rosemary, and clove; BIOMIN Holding GmbH, Getzersdorf, Austria; 3 g per cow per day), or AY (Levabon, Rumen E; containing yeast cell components from autolysis, as mannan oligosaccharides, glucans, chitin, nucleotides, peptides, and AA; BIOMIN Holding GmbH; 15 g per cow per day). Each of the 4 periods included 1 wk of baseline feeding (100% roughage diet, **RD**), 6 d of gradual concentrate adaptation, 1 wk of high-concentrate challenge (65% concentrates, on a DM basis, **Conc I**), 1 wk of recovery with roughage only, followed by a second 65% concentrate challenge lasting 2 wk (**Conc II**). This feeding model was used to reflect the dietary challenge milking cows undergo during the transition period, and by using dry, non-pregnant cows, excluding any lactation-cycle-dependent influences. Feed additives were provided in the concentrate mix except during the recovery phase, where the daily doses of PHY and AY were given via the rumen cannula. Each experimental period was followed by a 3-wk washout phase to avoid carry-over effects.

Exact protocols of feeding and feed sampling, as well as chemical composition of dietary ingredients, were described in the companion paper (Kröger et al., 2017). In brief, the RD consisted of grass silage and hay in 50:50 ratio (DM basis) and the concentrate mixture contained barley grain (33%), wheat (30%), rapeseed meal (16%), corn (15%), beet pulp (3.2%), a mineral-vitamin premix (1%; containing 13.5% Ca, 9% Mg, 5% P, 1.5% Na, 2,100,000 IU of vitamin A/kg, 300,000 IU of vitamin D/kg, and 7,500 mg of vitamin E/kg), beet molasses (1%), calcium carbonate (0.5%), and NaCl (0.3%) on a DM basis. Diets were offered separately via individual feeding troughs equipped with computer-controlled electronic scales. Access was regulated electronically with transponder access gates (Insentec B.V., Marknesse, the Netherlands) and daily feed intake was recorded for each individual cow.

Sampling Procedures

Rumen Microbiota. Sampling of solid digesta-associated microbiota was performed on d 6 (RD), 19 (Conc I), and 39 (Conc II) for the 3 feeding groups ($n = 8$) 8 h after morning feeding (1600 h). Approximately 200 g of solid digesta from the middle (in transversal and sagittal median of the rumen, 15 to 20 cm below the surface) of the rumen mat was collected via the rumen cannula, and put into a sterile plastic tray. To avoid contaminations, subsamples were taken with sterile tweezers from the middle of the sample, placed into two 2-mL Cryotubes (Sarstedt AG, Wiener Neudorf, Austria), shock frozen in liquid nitrogen, and stored at -80°C until further analysis.

Rumen Fermentation Parameters. For each experimental period, sampling was performed on d 6 (RD), 19 (Conc I), and 39 (Conc II) for all feeding groups ($n = 8$) before the morning feeding (0600 h), 4 h after the morning feeding (1200 h), and 8 h after morning feeding (1600 h). Approximately 500 g of digesta from the middle of the rumen mat was collected via the rumen cannula and manually squeezed to obtain about 250 mL of the particle-associated rumen liquid (PARL; Zebeli et al., 2008). The PARL samples were collected in a plastic container and the pH was immediately measured with a portable pH-meter (SG2-SevenGo, Mettler Toledo, Vienna, Austria). After pH measurement, the PARL sample was filtered through 4 layers of cheesecloth. From the filtered liquid, samples for SCFA, lactate, and ammonia were taken and stored at -20°C until analysis.

DNA Extraction and Sequencing

Solid digesta samples were handled according to a modified protocol by (Kong et al., 2010). A 250-mg digesta sample was combined with 750 μL of 0.4 M potassium phosphate buffer in lysis A tubes (Sigma-Aldrich, Vienna, Austria) and homogenized by beat beating for 90 s (3×30 s) at speed 4.5 (Fastprep FP120, Savant Instruments Inc., Holbrook, NY). Samples were then centrifuged 10 min at $9,391 \times g$ at room temperature and the supernatant discarded. Pre-lysis buffer (20 mM Tris/Cl, 2 mM EDTA, 1% Triton-X 100; pH 8.0) was added (500 μL) to the pellet and vortexed until the pellet was resuspended. The sample was then heated at 95°C for 5 min (thermomixer compact, Eppendorf Austria GmbH, Vienna, Austria) with agitation (900 motions per minute). The tubes were centrifuged again at $18,407 \times g$ for 5 min at room temperature and the supernatant separated afterward in clean 2-mL tubes, and stored on ice until DNA extraction. An amount of 1.2 mL of 0.4 M potassium phosphate, 100 μL of

100 mg/mL of lysozyme, and 10 μL of 2.5 U/ μL of mutanolysin (Sigma-Aldrich) were added to the remaining pellet. The samples were then incubated at 37°C for 30 min (Friocell, MMM group, Munich, Germany) while shaking at 125 motions per minute. After incubation, 20 μL of 18.6 mg/mL proteinase K (Sigma Aldrich, Vienna, Austria) was added and then samples were further incubated for 1 h at 37°C , 125 motions per minute. Then the samples underwent a second bead-beating step (3×45 s, speed: 4.5), and were centrifuged at $14,000 \times g$ for 3 min at room temperature, and the supernatant was pooled with the previously separated supernatant.

Bacterial DNA was isolated using PowerSoil DNA Isolation Kit (Mobio Laboratories, Qiagen, Carlsbad, CA) according to the manufacturer's instructions with the following modifications: 250 μL of the prepared sample was used; after adding solution C1, the tubes were vortexed for only 1 min (instead of 10 min) on the vortex adapter (vortex genie2, Scientific Industries Inc., Bohemia, NY); to elute DNA, 2×50 μL of 70°C sterile DNA-Free PCR grade Water (DEPC treated water, Thermo Fisher Scientific Inc., Waltham, MA) was used. After DNA extraction, samples were stored at -20°C . Total DNA quantity after isolation was measured for all samples using a Qubit Fluorometer 2.0 (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions. Amplicon sequencing was performed using Illumina MiSeq paired-ends sequencing technology (Microsynth AG, Balgach, Switzerland). The hypervariable region V3 to V5 of bacterial 16S rRNA genes was amplified by bridge amplification using the primer set 357 forward (5'-CCTACGGGAGGCAGCAG-3'), and 926 reverse (5'-CCGTCAATTCMTTTRAGT-3'; Peterson et al., 2009) to generate an approximate amplicon size of 570 bp. Multiplexed libraries were constructed by ligating sequencing adapters and indexes onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina). Primers were trimmed and corresponding overlapping paired-end reads were stitched by Microsynth (Microsynth AG).

Sequence Processing

The sequencing data were deposited into the European Nucleotide Archive and can be accessed via accession number PRJEB21562 (<http://www.ebi.ac.uk/ena/data/view/PRJEB21562>). Sequence data were analyzed with the open source bioinformatics pipeline QIIME (version 1.9.1. <http://qiime.org/>), based on the recommended workflow of QIIME tutorials (<http://qiime.org/tutorials/index.html>; last accessed November 14, 2016) and Navas-Molina et al. (2013). Briefly,

sequences of low quality were trimmed with a quality score of 20. The chimeric sequences were identified using the gold.fa reference database (Mukherjee et al., 2017), and subsequently filtered. A total of 1,182,865 sequences passed the quality control and chimera check. Sequences were then clustered into operational taxonomic units (OTU) with a 97% 16S rRNA gene similarity cutoff by performing open reference OTU picking. The database of SILVA (<https://www.arb-silva.de>, version 123; last accessed August 25, 2016) was used for taxonomic classification. The OTU with less than 10 sequences in total were excluded for data normalization. A total of 5,400 OTU were found; however, 47 OTU were unassigned at kingdom level and were therefore removed. The remaining 5,353 OTU, with a total number of 1,181,185 sequences, were used for taxonomic-based analyses. The percent relative abundances of all OTU were calculated and OTU were ranked according to their abundance. Secondary taxonomic classification was performed for highly abundant OTU using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; last accessed April 1, 2017). Measures of α diversity were also determined using QIIME, specifically nonparametric species richness estimator Chao 1, as well as the diversity indices Shannon and Simpson. The rarefaction depth was equalized for all samples to 6,245 sequences per sample based on the minimum sequence number achieved. For β -diversity analysis, the weighted UniFrac distance matrix (Lozupone and Knight, 2005) was also calculated with the same normalization of sequences per sample. Venn diagrams were created using Venn Diagram Plotter (version 1.5., PNNL, Richland, WA) and Venny (version 2.1., Oliveros, 2007). Sequencing results were analyzed at the genus level to overview the changes of the whole population. For interpreting changes of the highly dominant taxa, the most abundant 100 OTU with their taxonomic assignment of $\geq 97\%$ sequence similarity to SILVA or NCBI were chosen. The assignment to gram-negative and gram-positive bacterial cell wall was based on literature (Dworkin, 2006a,b; Whitman, 2009).

Rumen Fermentation Variables

Concentrations of SCFA (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, caproate) were determined by GC as previously described by Kumar et al. (2016). Briefly, the samples were centrifuged at $20,000 \times g$ for 25 min at 4°C , 0.2 mL of HCl (1.8 mol/L) was added to the supernatant, followed by addition of 0.2 mL of the internal standard (4-methylvaleric acid, Sigma-Aldrich Co. LLC, St. Louis, MO). The mixture

was centrifuged at $20,000 \times g$ for 25 min at 4°C to remove any precipitated substrates. The clear supernatant was transferred into the GC vial and was analyzed for SCFA concentrations via GC apparatus (GC Model 8060 MS172DPFC, no. 950713, Fisons, Rodena, Italy). Injector and detector were at temperatures of 170 and 190°C , respectively, and helium was used as a carrier gas with a flowrate of 6 mL/min. As reference, an external standard for each targeted acid with a known concentration was used. Generation and evaluation of chromatograms was done with Stratos Software (Stratos Version 4.5.0.0, Polymer Laboratories, Church Stretton, Shropshire, UK).

The ammonia concentration in the PARL samples was determined using the indophenol reaction (Weatherburn, 1967). For this analysis, the samples were thawed at room temperature and then centrifuged at $15,115 \times g$ for 10 min at room temperature. Subsequently, the clear supernatant was diluted with distilled water to obtain a concentration within the standard calibration curve. Both ammonia and phenol were oxidized by sodium hydroxide in the presence of sodium nitroprusside and dichloroisocyanuric acid to form a blue complex. After 90 min of reaction, the absorbance was measured at 655 nm using U3000 Spectrophotometer (INULA GmbH, Vienna, Austria). The blue color intensity was linearly correlated with ammonia concentration of the sample.

Lactic acid contents of the PARL samples were analyzed photometrically using an enzymatic test kits according to the manufacturer's instructions (Megazyme D-, L-lactate assay kit; Megazyme International, Wicklow, Ireland) for 8 h after morning feeding only.

Statistical Analyses

Statistical analyses for SCFA, ammonia and lactate content, and genus and OTU taxonomy were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC). Overall, the treatments and blocking variables did not interact, as assumed for the Latin square design. Data were checked for normality and variance homogeneity before further statistical analysis. For variables that did not meet the assumptions required for ANOVA, generalized linear mixed models were applied (GLIMMIX procedure of SAS), whereas the rest of the data were analyzed using the MIXED procedure of SAS (version 9.2, SAS Institute Inc.). All statistical models were performed with the feeding phase (RD, Conc I, and Conc II) and feeding group (CON, PHY, AY) considered as fixed effects. Interactions between feeding phase and feeding group (phase $\times$ group) were also analyzed. For variables of SCFA and ammonia, the fixed effect of sampling hour after the morning feeding

as well as the interaction between sampling hour and feeding phase (hour $\times$ phase) was also accounted in the model. To account for repeated measurements of the same animal within a feeding group over time, a variance-components variance-covariance structure was used according to the Bayesian information criterion. Cow nested within square and the experimental period was considered as a random effect. Treatment effects are only given for feeding phases Conc I and Conc II because feed additives were only administered during those phases. Comparisons among the least squares means were performed with pdiff option and considered as significant with $P < 0.05$ and as a trend with $0.05 \leq P \leq 0.10$. Principal coordinate analysis of the Weighted Unifrac distance matrix data was performed using PROC PRINCOMP of SAS (version 9.2, SAS Institute Inc.). Correlations between the fermentation parameters and the microbiota data of the most abundant 100 OTU were analyzed using Pearson correlation coefficient (PROC CORR of SAS). According to Hinkle et al. (2003), the r was interpreted as follows: 0.00 to 0.30 as negligible; 0.30 to 0.50 as low; 0.50 to 0.70 as moderate; 0.70 to 0.90 as high, and 0.90 to 1.00 as substantial. For our interpretation we considered moderate, high, or substantial correlations from $r > 0.5$.

RESULTS

The influences of concentrate feeding and the feed additives on DMI, minimum reticulorumenal pH, and duration of pH < 6.0 have been previously published in the companion study (Kröger et al., 2017), and are used in this article only for correlating with the current microbiological data. The objectives of the current study were to evaluate how an intermittent high-concentrate feeding model and the supplementation of PHY or AY feed additives alters the rumen bacterial community and rumen fermentation parameters such as SCFA, ammonia, and lactate.

Effect on Bacterial Population Diversity and Phylogeny

Data of α -diversity indices are shown in Figure 1. The Shannon index was decreased with concentrate feeding compared with RD (Figure 1A). Both Chao 1 (Figure 1B) and the Simpson index (Figure 1C) were even further decreased in Conc I compared with Conc II ($P < 0.01$). All 3 parameters had a lower standard deviation for RD than for Conc I and Conc II. In the PHY group (0.908), Simpson index showed a significant ($P = 0.02$) decrease compared with CON (0.947) during Conc I. Principal coordinate analysis of the Weighted Unifrac distance matrix revealed a clear clustering of the RD

samples. Moreover, the RD cluster was located apart from the 2 concentrate groups with a high variation of 52% along the principal component 1 axis (Figures 2A and 2C). The samples of the 2 concentrate phases showed a more widespread clustering within their samples (Figures 2A, 2B, and 2C). UniFrac distance matrix analysis showed no clustering according to the 3 feeding groups CON, PHY, and AY. Between the 3 feeding phases, 2,646 of the total 5,353 OTU were shared (Figure 3). The RD had the highest number of unique OTU and the 2 concentrate phases shared the highest number of OTU.

According to the OTU assignment a total of 35.08% of the OTU could be assigned to 53 genera, 46 of them being affected by the concentrate feeding. The 2 concentrate challenges Conc I and Conc II decreased most

Figure 1. Alpha diversity indices Shannon (A), Chao 1 (B), and Simpson (C) derived from the QIIME (<http://qiime.org/>) command α rarefaction. Different lowercase letters a, b, and c indicate significant ($P < 0.01$) changes between feeding phases roughage diet for 1 wk (RD), 65% concentrates for 1 wk (Conc I), and 65% concentrates for 2 wk (Conc II). OTU = operational taxonomic unit; upper box = 2nd quartile; mid line = median; lower box = 3rd quartile; whiskers = highest and lowest quartile.

Figure 2. (A, B, C) Two-dimensional principal coordinate analysis (PCoA) plots based on weighted UniFrac distance matrices, showing the phylogenetic clustering according to the 3 feeding phases 1 wk roughage diet (RD; triangle), 1 wk 65% concentrates (Conc I; square), and 2 wk 65% concentrates (Conc II; circle). The 3 axes [principal component (PC) 1, 2, 3] indicate the variation between the samples in percent. Color version available online.

of the genera (39 and 41 genera, respectively) and increased only 14 and 12 genera, respectively, in comparison to RD (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13565>). When looking at the differences in relative abundance between the 2 concentrate feeding phases, 8 genera were higher in Conc II than Conc I and 7 genera lower in Conc II than in Conc I. Changes in percent abundance, both positive and negative, were identical for both high-concentrate challenges relative to RD for all genera. The most abundant genus, *Prevotella* (12.7% total relative abundance), was increased by 49% ($P < 0.01$) in both concentrate challenges, as well as *Shuttleworthia*, which increased by 98% in Conc I ($P < 0.01$; 0.9% total relative abundance). Most of the other high abundance genera such as *Ruminococcus* (5.4% total relative abundance), *Butyrivibrio* (4.6%), *Coprococcus* (2.2%), *Blautia* (1.0%), and *Pseudobutyrvibrio* (0.8%) decreased ($P \leq 0.03$) with concentrate feeding by 49, 37, 24, 88, and 79%, respectively. Within the less abundant genera, *Fibrobacter* (0.2%) decreased by 70% ($P < 0.01$) in the 2 concentrate diets, whereas others, such as *Lachnospira* (0.2%), *Megasphaera* (0.2%), *Lactobacillus* (0.08%), *Succinivibrio* (0.06%), and *Bacteroides* (0.04%) significantly increased ($P \leq 0.03$) at least in 1 of the 2 concentrate phases by 63, 100, 100, 88, and

100%, respectively (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13565>). Both feed additives PHY and AY had effects on both high and low abundance genera, most of them occurring during Conc I (Figure 4A and 4B). Autolyzed yeast significantly increased *Ruminococcus* ($P < 0.01$) and *Mogibacterium* ($P < 0.05$), tended to increase *Clostridium* ($P = 0.08$) and *Dorea* ($P = 0.06$), and tended to decrease *Methylobacterium* ($P = 0.07$). Both PHY and AY decreased low abundance genera such as *Treponema* ($P < 0.01$) and *Bacteroides* ($P < 0.01$ for PHY, $P = 0.07$ for AY), and decreased *Anaerovorax* ($P = 0.07$), and *Fusobacterium* ($P < 0.08$) with a trend. Moreover, PHY had a significant decreasing effect on *Roseburia* ($P = 0.03$), YRC22 ($P = 0.02$), and a trend to decrease *Bifidobacterium* ($P < 0.07$), and a significant increasing effect on *Oscillospira* ($P = 0.02$) and *Curtobacterium* ($P = 0.02$) and an increasing trend on *Anaerostipes* ($P = 0.05$; Figures 4A and 4B).

Supplemental Figure S2 (<https://doi.org/10.3168/jds.2017-13565>) provides a list with the first most abundant 100 OTU, representing those with a total relative abundance of $\geq 0.15\%$, and their changes of abundance along the 3 feeding phases RD, Conc I, and Conc II. Overall, only 9 out of the 100 most abundant OTU were not affected by the concentrate feeding. Statistically significant changes in relative abundance between the 2 Conc phases occurred in 44 OTU. Similar to the genera data, Conc I and Conc II always showed the same abundance changes in comparison to RD, apart

Figure 3. Venn diagram illustrating the number and percentage of individual and shared operational taxonomic units (OTU) of the 3 feeding phases: 100% roughage diet for 1 wk (RD), and 65% concentrates for 1 wk (Conc I) and 2 wk (Conc II).

Figure 4. (A, B) Effect of the additives phytochemical (PHY) and autolyzed yeast (AY) on high (A) and low (B) abundance genera during 65% concentrate for 1 and 2 wk (Conc I and Conc II, respectively). CON = control. Different lowercase letters indicate significant differences between the feed additives ($P < 0.01$), and lowercase letters in parentheses indicate a trend ($0.05 < P < 0.1$). Error bars indicate SEM.

from 1 OTU; the *Butyrivibrio fibrisolvens* OTU_62 was significantly higher in Conc I ($P < 0.01$) and had a trend to be lower in Conc II ($P = 0.08$) in comparison to RD. From the most taxonomically similar OTU found, 20 *Prevotella* spp. associated OTU increased with concentrate feeding, 2 did not differ between the feeding phases and only one single *Prevotella* OTU decreased in the 2 concentrate phases. The *Clostridiales* order (97% similarity) was represented by 7 OTU, including the most abundant OTU_1, and was increased in 5 instances from RD to concentrate feeding. *Ruminococcaceae* (10 OTU) and *Ruminococcus* spp. (12 OTU) were decreased in 16 cases and increased in 4 OTU. *Lachnospiraceae* family was represented by 12 OTU, most of them increasing with concentrate feeding. *Butyrivibrio* spp. decreased in 5 out of 8 OTU with concentrate feeding. Within the 100 most abundant OTU, PHY and AY showed effects only on the OTU in Conc I as compared with CON, except an increasing effect of AY on *Ruminococcus flavefaciens* (OTU_23) in Conc II (Figure 5). The 4 *Ruminococcaceae* family OTU were increased by PHY, whereas *Prevotella* spp. associated OTU were decreased by PHY and AY. Additionally, one *Ruminococcus* spp. (OTU_48) was increased by AY in Conc I (Figure 5).

To elucidate shifts of major bacterial groups of the rumen, we analyzed the data according to known Gram staining of the genera found using taxonomic classification and found 50% to be gram-positive bacteria (GPB) and 31% GNB. The rest were of variable or undefined Gram staining (Supplemental Figures S1 and S2; <https://doi.org/10.3168/jds.2017-13565>).

Effect on Rumen Fermentation Profile

The percentage of acetate, iso-butyrate, caproate, acetate:propionate ratio, and PARL pH were significantly ($P < 0.05$) decreased in both high-concentrate challenges in comparison with the RD (Figure 6; Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13565>). Propionate, butyrate, and valerate increased in both concentrate challenges in comparison to the RD level ($P < 0.01$). Propionate increased even further from Conc I to Conc II, whereas butyrate decreased slightly from Conc I to Conc II but did not return to RD levels ($P < 0.01$). The PHY group had a higher percentage of butyrate in both concentrate challenges (Conc I, $P = 0.01$; Conc II, $P = 0.08$), higher trend for iso-valerate in Conc I, and lower trend for propionate, and significantly lower valerate and caproate in Conc I compared with CON (Table 1). The AY supplementation group showed significantly lower valerate and caproate in Conc I, whereas a higher trend for valerate in Conc II, and significantly lower acetate and

iso-valerate in Conc II compared with CON. Sampling time point affected propionate and butyrate differently depending on the feeding phase (Figure 6). Acetate and the acetate:propionate ratio decreased with time after morning feeding and were significantly different 8 h after feeding ($P < 0.01$) for RD and Conc I. No effect of feed additives on acetate:propionate ratio was seen. Total lactate, as well as the fractions of D-lactate and L-lactate, were increased in both concentrate phases ($P < 0.04$), and the fraction of L-lactate showed an increasing trend in the AY group during Conc I (Table 1). Ammonia concentration was decreased significantly in Conc II in comparison to the RD level ($P = 0.03$; Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13565>), without any effects of feed additives. Total SCFA showed no overall difference between RD and the concentrate phases ($P = 0.69$) or feed additives ($P = 0.47$), but when considering the different sampling time points, total SCFA was higher in RD 4 h after feeding and highest in both concentrate phases 8 h after feeding ($P < 0.01$; Figure 6). Particle-associated pH showed no time point effect in RD, but decreased significantly 8 h after feeding in both concentrate phases ($P < 0.01$).

Correlation analysis was performed for the first 100 OTU and the evaluated fermentation parameters, as well as pH and DMI (Figure 7). For most OTU, a positive correlation was observed with mean reticular and PARL pH, as well as acetate and iso-butyrate. Conversely, a negative correlation was observed with concentrate intake (DM), time pH <6.0, propionate, butyrate, and valerate. Caproate also had positive moderate correlations with *Shuttleworthia* spp., *Syntrophococcus* spp., and *Olsenella* spp. OTU. For total SCFA, iso-valerate, lactate, and NH_3 , only 1 OTU showed a moderate correlation for each parameter, being OTU_29 *Lachnospiraceae* spp., OTU_95 *Moryella* spp., OTU_73 *Prevotella* spp., and OTU_32 *Prevotella bryantii* [best Basic Local Alignment Search Tool nucleotide (BLASTn) hit, 100% similarity], respectively (Figure 7).

DISCUSSION

Effect of Feeding 2 Intermittent High-Concentrate Diets

The main aim of this study was to determine rumen bacterial community changes and fermentative effects of feeding an intermittent concentrate-rich diet in cattle. The growth, activity, and homeostatic regulation of rumen microbes depends mainly on the substrate availability and specificity of rumen microbes (Henderson et al., 2015). We found that the change from forage-only diet to a high-grain diet clearly de-

OTU Number	Taxonomic classification according to SILVA and NCBI best BLASTn hit ≥ 97% identity	Identity %	Feeding phase						
			Conc I			Conc II			
			CON	PHY	AY	CON	PHY	AY	
OTU_6	<i>Ruminococcaceae</i> (f)	12		**					<div>Relative abundance %</div> <div><div></div> ≥ 2.0</div> <div><div></div> 1.0 - 2.0</div> <div><div></div> 0.5 - 1.0</div> <div><div></div> 0.2 - 0.5</div> <div><div></div> < 0.2</div> <div><div>**</div> $P < 0.01$</div> <div><div>*</div> $0.01 < P < 0.05$</div>
OTU_14	<i>Syntrophococcus</i> (g)	100		**					
OTU_17	<i>Ruminococcaceae</i> (f)	97		**					
OTU_21	<i>Prevotella ruminicola</i> (s)	100		**	*				
OTU_23	<i>Ruminococcus flavefaciens</i> (s)	99						*	
OTU_26	<i>Saccharofermentans</i> (g)	100			**				
OTU_31	<i>Ruminococcaceae</i> (f)	97		**					
OTU_48	<i>Ruminococcus</i> (g)	97			**				
OTU_50	<i>Prevotella ruminicola</i> (s)	99		**	**				
OTU_52	<i>Roseburia intestinalis</i> (s)	97			*				
OTU_59	<i>Shuttleworthia</i> (g)	97		**					
OTU_60	<i>Ruminococcaceae</i> (f)	97		**					
OTU_76	<i>Prevotella</i> (g)	99		**					
OTU_95	<i>Moryella indoligenes</i> (s)	96		**					
OTU_99	<i>Olsenella</i> (g)	99		**	**				
OTU_100	<i>Eubacterium pyruvativorans</i> (s)	99		**					

Figure 5. Heatmap showing the influence of the feed additives phytogetic (PHY) and autolyzed yeast (AY) compared with control (CON) onto the first most abundant 100 operational taxonomic units (OTU). Only feeding-phase $\times$ feed-additive interactions that had $P < 0.1$ for the feed additive contrast or fixed effects feed-additive were considered in this table. Based on the higher sequence identity to the reference database, either results from OTU picking against SILVA (<https://www.arb-silva.de>, version 123; last accessed August 25, 2016) or the best Basic Local Alignment Search Tool nucleotide (BLASTn) hit against the National Center for Biotechnology Information was chosen as the taxonomic reference. The letter in parentheses is indicative for the taxonomic level reached with at least 97% sequence identity: family (f), genus (g), and species (s). Conc I = 65% concentrate (DM) for 1 wk; Conc II = 65% concentrate (DM) for 2 wk. Color version available online.

creased rumen bacteria diversity (Mao et al., 2013b). Interestingly, the second concentrate challenge had a higher Simpson index, higher number of unique OTU, and a separated phylogenetic clustering in comparison to Conc I. This indicates that Conc I had a stronger effect, with a clearly disrupted bacterial community, than Conc II, despite the same diet was fed. During 1 wk of roughage-only break, the bacterial community seemed to more closely resemble a high-grain diet microbiome than a roughage microbiome (Petri et al., 2013), which would underline the adaptation of the rumen microbiome to the concentrate diet in the second challenge. Principal coordinate analysis showed that RD samples clearly form a separate cluster against concentrate diet samples, which suggests that the bacterial community was able to return to the initial RD composition within 3 wk of washout and 1 wk of RD (Hook et al., 2011).

The findings of distribution of GPB (50%) and GNB (31%) in the digesta samples corresponded with other studies (53% GPB and 41% GNB) using 40% concentrate as control and 70% concentrate as challenge (Mao et al., 2013b). Mao et al. (2013b) further found that the relative abundance of GPB was higher in the 70%

concentrate group than in the control group. In the current study, the GNB increased with concentrate feeding and had at genus level numerically a higher mean relative abundance (2.45%) in the concentrate phases than GPB (1.79%). These results are mainly driven by the increase of the most prevalent genus in our study, *Prevotella*, a gram-negative rod, which is known to be found in high abundance (42–60%), and high genetic variability in the rumen (Stevenson and Weimer, 2007). Previous research has shown variable levels of *Prevotella* with relation to dietary change (Mao et al., 2013b). However, with the use of sequence data, it must be considered that taxonomic databases are based predominantly on cultured and fully sequenced species of *Prevotella* such as *P. bryantii*, *P. ruminicola*, and *P. brevis*, which represent only 2 to 4% of all *Prevotella* found in the rumen (Stevenson and Weimer, 2007). Therefore, it is highly probable that other uncultured fastidious *Prevotella* species have variable substrate specificities and are capable of growing under low pH conditions (Purushe et al., 2010). A similar contradiction was seen with another highly abundant GNB OTU, *Syntrophococcus* spp., which increased in

Figure 6. Effect of sampling time point on total short-chain fatty acid (SCFA) amount acetate, propionate, and butyrate proportion; acetate:propionate ratio, and particle-associated rumen liquid (PARL) pH during 100% roughage for 1 wk (RD), 65% concentrates for 1 wk (Conc I), and 65% concentrates for 2 wk (Conc II). The lowercase letters a, b, and c indicate significant ($P < 0.01$) changes between the 3 sampling time points 0 (before feeding), 4 (4 h after morning feeding), and 8 (8 h after feeding). The lowercase letters x and y indicate a significant ($P < 0.01$) difference between RD and the 2 Conc diets (x) or between Conc I and Conc II (y) during the sampling time points.

our study with concentrate feeding. *Syntrophococcus* is identified as a genus that preferentially grows in a pH range of 6.0 to 7.6 (Whitman, 2009). Because the pH in our study was found to be below 6.0 for extended duration of time under high-concentrate feeding (Kröger et al., 2017), these results suggest that the pH sensitivity of certain GNB and GPB species needs to be reevaluated (Nagaraja and Titgemeyer, 2007). This research question is of particular interest due to the release of possible toxic LPS from GNB, as it is known for the

GNB *Escherichia coli*, causing both local and systemic inflammation (Khafipour et al., 2009).

The shift from acetate toward more propionate production and lower pH conditions in the rumen underlies the changing nutrient supply and subsequent metabolism of rumen bacteria during the transition from a forage to a high-concentrate diet (Kleen et al., 2003; Dijkstra et al., 2012). According to our findings, *Ruminococcus* spp., as well as other acetate producing genera including *Saccharofermentans*, *Blautia*, and

Dorea (Liu et al., 2008; Whitman, 2009; Chen et al., 2010), correlated ($r > 0.5$) with rumen acetate levels in the RD, and correspondingly decreased with concentrate feeding. Additionally, the increased abundance of known propionate producers, including *Prevotella* spp., *Bacteroides* spp., *Olsenella* spp., *Succinivibrio* spp., and *Megasphaera* spp. (Dewhirst et al., 2001; Whitman, 2009; Purushe et al., 2010), correlated with the increased propionate levels during concentrate feeding. Furthermore, the acetate:propionate ratio continued to decrease from Conc I to Conc II, which is supported by similar changes in the microbial population, either significantly or numerically, despite rumen pH being higher in Conc II than in Conc I. The continued decrease in acetate:propionate ratio without a decrease in pH indicates that pH was able to recover to a certain point in Conc II due to higher salivary buffers (Kröger et al., 2017), or higher epithelial absorption and buffering (Aschenbach et al., 2011), whereas microbial groups and their metabolism were even more in favor of propionate production in Conc II. These results suggest that dietary substrates and ruminal pH are not solely responsible for metabolic alterations of the microbiome (van Houtert, 1993; Dijkstra et al., 2012), but other influencing mechanisms took place during Conc II feeding.

The butyrate percentage was increased with concentrate feeding, with the greatest increase during Conc I and an intermediate increase in Conc II, suggesting a negative association with the pH trend (Kröger et al., 2017). According to previous research, there is no clear increasing or decreasing trend for butyrate during concentrate feeding and lowered pH conditions (Kleen et al., 2003; Wang et al., 2013; Danscher et al., 2015).

Bacteria traditionally known to be butyrate producers, including *Butyrivibrio* spp., *Pseudobutyrvibrio* spp., and *Coprococcus* spp. (van Gylswyk et al., 1996), were decreased with concentrate feeding. However, several other bacteria that are also able to produce butyrate, increased during concentrate feeding, such as *Roseburia* spp. (van Gylswyk et al., 1996), *Megasphaera* spp. (Puniya et al., 2015), and *Pseudoramibacter* spp. (Whitman, 2009). The literature also shows no consistent trend for rumen butyrate producers, such as *Butyrivibrio* spp. and *Pseudobutyrvibrio* spp., with concentrate feeding (Hook et al., 2011; Petri et al., 2013; McCann et al., 2016). This inconsistent response of butyrate producers to concentrate feeding was also reflected in our finding that the only taxon that showed differing trends for Conc I and Conc II in comparison to RD was a *Butyrivibrio fibrisolvens* strain (OTU_62). Further research is required to look at the functional overlap of various bacterial taxa, as well as their interaction with protozoa, fungi, archaea, and the host, to determine not only their metabolic capabilities but also their metabolic contributions to fermentation in the rumen (Firkins and Yu, 2015).

Effects of Feed Additive Supplementation

The second aim of our study was to determine the effects of PHY or AY supplementation on rumen fermentation and bacterial changes when cows are challenged intermittently with high-concentrate diets. Overall, most of the effects of the feed additives on the rumen bacterial community occurred during the first concentrate challenge, which was expected based on decreased pH in Conc I (Kröger et al., 2017), and

Table 1. Influence of phytogenic (PHY) and autolyzed yeast (AY) on short-chain fatty acid (SCFA) profile, lactate, and ammonia in the particle-associated liquid during 2 high-concentrate feeding phases¹

Parameter	Conc I			SEM	P-value	Conc II			SEM	P-value
	CON	PHY	AY			CON	PHY	AY		
Total SCFA (mM)	118.8	124.4	115.5	7.70	0.36	117.3	123.5	115.5	7.73	0.47
% of total SCFA										
Acetate	54.7	55.9	55.3	1.10	0.35	53.8 ^a	53.8 ^a	50.8 ^b	1.11	0.03
Propionate	23.8 ^(a)	21.0 ^(b)	23.3 ^(a)	1.16	0.09	27.5	25.1	29.9	1.18	0.14
Butyrate	13.0 ^b	16.0 ^a	14.0 ^{ab}	0.89	0.01	11.8 ^(b)	13.9 ^(a)	11.9 ^(ab)	0.89	0.08
Valerate	5.4 ^a	4.1 ^b	4.3 ^b	0.49	0.04	4.4 ^(b)	4.1 ^(b)	5.4 ^(a)	0.49	0.08
Caproate	1.7 ^a	1.2 ^b	1.2 ^b	0.18	0.02	0.8	0.9	0.7	0.18	0.61
Iso-valerate	0.8 ^(b)	1.2 ^(a)	1.0 ^(ab)	0.16	0.08	1.1 ^a	1.3 ^a	0.6 ^b	0.16	0.01
Iso-butyrate	0.8	0.8	0.7	0.05	0.62	0.7	0.8	0.6	0.05	0.12
Acetate:propionate	2.7	2.8	2.6	0.22	0.65	2.0	2.4	1.7	0.22	0.23
Total lactate (mM)	1.01 ^(b)	0.74 ^(b)	2.89 ^(a)	0.85	0.09	1.99	2.07	1.87	0.852	0.91
D-lactate (mM)	0.40	0.34	1.19	0.42	0.16	0.89	1.09	0.78	0.42	0.72
L-lactate (mM)	0.61 ^(b)	0.40 ^(b)	1.70 ^(a)	0.44	0.06	1.10	0.98	1.09	0.44	0.83
Ammonia (mM)	2.37	1.77	1.86	0.54	0.34	1.24	2.11	1.30	0.542	0.17

^{a,b}Different superscript letters indicate significant ($P < 0.05$) changes or a trend (a,b in parentheses; $0.05 < P < 0.1$) between the feed additive groups (PHY, AY) compared with the control group (CON).

¹Conc I = 65% concentrate for 1 wk; Conc II = 65% concentrate for 2 wk.

Figure 7. Pearson correlation analyses of the most abundant 100 operational taxonomic units (OTU) with rumen fermentation relevant parameters. Only OTU that showed at least a moderate correlation to one of the parameters are shown in this figure. Taxonomic assignments based on the higher sequence identity to the reference database, and either the result from OTU picking against SILVA (<https://www.arb-silva.de>, version 123; last accessed August 25, 2016) or the best Basic Local Alignment Search Tool nucleotide (BLASTn) hit against National Center for Biotechnology Information was chosen as the taxonomic assignment. The letter in parentheses is indicative of the taxonomic level reached with at least 97% identity: order (o), family (f), genus (g), and species (s). Pearson correlation coefficient r values $|r| < 0.3$ are considered as negligible, $|0.3 \leq r < 0.5|$ as low, $|0.5 \leq r < 0.7|$ as moderate, $|0.7 \leq r < 0.9|$ as high; n.s. = not significant ($P > 0.1$). SCFA = short-chain fatty acid; PARL = particle-associated rumen liquid. Color version available online.

especially because PHY has been previously described as effective under low pH conditions (Cardozo et al., 2005). Almost all of the bacteria that were decreased by PHY are known starch utilizers, including the genera *Shuttleworthia*, *Olsenella*, *Bacteroides*, *Bifidobacterium*, *Roseburia*, and *Syntrophococcus* (Calsamiglia et al., 2007; Patra, 2011), whereas no fiber fermenting taxa were reduced by PHY. Previous research has indicated that PHY has a bacteriostatic effect affecting cell wall electrolyte exchange, which diverts the cell from metabolic processes (Calsamiglia et al., 2007; Cobellis et al., 2016). In a high-concentrate diet, the increase in starch provided would normally supply amylolytic bacteria with sufficient substrate to promote growth. The decrease of amylolytic bacteria, seen with supplementation of PHY in this experiment, supports the mode of action stated by Calsamiglia et al. (2007) and Cobellis et al. (2016). A reduction in starch utilizers would potentially delay the onset of SCFA fermentation, reduce SCFA accumulation, and thereby prevent rapid and extended duration of low pH after feeding. Moreover, with a decrease in starch utilizers, cellulolytic bacteria might have a chance to evolve due to less competition (Patra and Yu, 2015). This is supported by the higher reticulo-rumen pH previously reported for PHY (Kröger et al., 2017). A distinct effect on certain bacterial groups according to Gram staining was seen, where all GNB decreased and almost all GPB increased for both feed additives. These effects of the feed additive should be further investigated, as some GNB species harbor potential LPS producers and GPB, among others, some major cellulolytic bacteria. However, in vitro studies have suggested that GNB show a higher resistance than GPB against different plant-derived substances because their outer membrane is hydrophilic and does not allow the entrance of lipophilic substances (Cardozo et al., 2005; Calsamiglia et al., 2007; Hart et al., 2008). Thus, our in vivo data produced in cattle fed concentrate-rich diets suggests another potential mode of action for plant-derived substances, which requires more research to be elucidated. For the AY group, the effects on both GNB and GPB are in agreement with literature. As effective ingredient, certain yeast-cell wall components such as mannan-oligosaccharides, β -glucans, chitin, peptides, AA, and nucleotides can act as high-affinity ligands for GNB and remove them from the digestive system without attachment and colonization (Nocek et al., 2011; Ganner and Schatzmayr, 2012). Accordingly, AY decreased *Bacteroides*, a major gram-negative starch fermenting and ammonia utilizing genus (Dworkin, 2006b), during Conc I. Moreover, cell wall components found in AY have also been found to act as substrate for cellulolytic bacteria, promoting growth in the challenged rumen (Harrison et al.,

1988). This suggested prebiotic effect was reflected in our finding that AY increased both *Ruminococcus* spp. significantly and *Clostridium* spp. by trend, throughout the experiment, both of which are known to represent a large number of cellulose degrading rumen species (Whitman, 2009). The increase in cellulolytic bacteria, despite high-concentrate feeding and the reduction of starch fermenting GNB by AY, would contribute to the maintenance of a physiological ruminal pH, which was shown by our accompanying study, where AY tended to stabilize reticulo-ruminal pH despite higher DMI (Kröger et al., 2017).

We reported that with lower pH, butyrate increased in this feeding model; however, PHY increased both pH and butyrate in Conc I. Therefore, it remains to be clarified if PHY has a direct effect on microbial butyrate production and metabolism. Nevertheless, an increased butyrate level with PHY could have favored epithelial uptake of other metabolites and so positively affected ruminal pH because it was described that butyrate is a trigger for enhanced papillae growth and absorption capacity (Steele et al., 2011; Baldwin et al., 2012). In line with other studies (Mao et al., 2013a; Li et al., 2016), the application of AY corresponded with decreased acetate in Conc II, suggesting that AY can also influence the metabolism of cellulolytic bacteria.

CONCLUSIONS

High-concentrate feeding clearly decreased rumen bacterial diversity, with alleviated effects in the second concentrate challenge, revealing a possible adaptation mechanism of rumen bacteria. Interestingly, some GNB were increased with concentrate feeding and low pH, which suggests reevaluation of the pH sensitivity and substrate usage of their species. Our findings suggest that both the PHY and AY feed additive evolve their effects on the rumen microbiome predominantly during low ruminal pH conditions. An interesting finding was that both PHY and AY decreased GNB and increased GPB, which provides background for further research on the mode of action of those 2 feed additives. Autolyzed yeast provided the opportunity to increase cellulolytic bacteria and decrease certain starch fermenters, whereas PHY showed a decreasing effect on overall bacterial diversity and starch fermenting bacteria and increased butyrate levels, which could have contributed to the higher and stabilized reticulo-ruminal pH levels during this experiment.

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