Effects of the dose and viability of *Saccharomyces cerevisiae*.

1. Diversity of ruminal microbes as analyzed by Illumina MiSeq sequencing and quantitative PCR

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ABSTRACT

This study was conducted to examine effects of the dose and viability of supplemental *Saccharomyces cerevisiae* on the ruminal fermentation and bacteria population and the performance of lactating dairy cows. Four ruminally cannulated lactating cows averaging 284 ± 18 d in milk were assigned to 4 treatments arranged in a 4×4 Latin square design with four 21-d periods. Cows were fed a total mixed ration containing 41.7% corn silage, 12.1% brewer's grains, and 46.2% concentrate on a dry matter basis. The diet was supplemented with no yeast (control) or with a low dose of live yeast (5.7×10^7 cfu/cow per day; LLY), a high dose of live yeast (6.0×10^8 cfu/cow per day; HLY), or a high dose of killed yeast (6.0×10^8 cfu/cow per day; HDY). Microbial diversity was examined by high-throughput Illumina MiSeq sequencing (Illumina Inc., San Diego, CA) of the V4 region of the 16S rRNA gene. The relative abundance of select ruminal bacteria was also quantified by quantitative PCR (qPCR). Adding LLY to the diet increased the relative abundance of some ruminal cellulolytic bacteria (*Ruminococcus* and *Fibrobacter succinogenes*) and amylolytic bacteria (*Ruminobacter*, *Bifidobacterium*, and *Selenomonas ruminantium*). Adding live instead of killed yeast increased the relative abundance of *Ruminococcus* and *F. succinogenes*; adding HDY increased the relative abundance of *Ruminobacter*, *Bifidobacterium*, *Streptococcus bovis*, and *Selenomonas ruminantium*. The most dominant ($\geq 1\%$ of total sequences) bacteria that responded to LLY addition whose functions are among the least understood in relation to the mode of action of yeast include *Paraprevotellaceae*, CF231, *Treponema*, and *Lachnospiraceae*. Future studies should aim to spec-

ate, culture, and examine the function of these bacteria to better understand their roles in the mode of action of yeast. A relatively precise relationship was detected between the relative abundance of *F. succinogenes* ($R^2 = 0.67$) from qPCR and MiSeq sequencing, but weak relationships were detected for *Megasphaera elsdenii*, *Ruminococcus flavefaciens*, and *S. ruminantium* ($R^2 \leq 0.19$).

Key words: *Saccharomyces cerevisiae*, dairy cows, rumen bacteria, MiSeq sequencing

INTRODUCTION

Yeasts are microbial feed additives that have been widely used for decades in the dairy industry (Beauchemin et al., 2008). Several studies have examined the effects of yeasts on ruminal fermentation (Chaucheyras-Durand and Fonty, 2001; Lila et al., 2004; Krause and Oetzel, 2006) and the performance of ruminant livestock (Erasmus et al., 2005; Bach et al., 2007; Ferraretto et al., 2012). Some studies have examined the effects of yeast on the abundance of ruminal protozoa (Chaucheyras-Durand and Fonty, 2002), lactate-utilizing bacteria (Chaucheyras et al., 1996; Rossi et al., 2004), and cellulolytic bacteria (Callaway and Martin, 1997). Such studies are important because the beneficial effects of yeast on milk yield and weight gain are attributed largely to their effects on ruminal bacteria (Pinloche et al., 2013). However, most of these studies have focused only on a few specific bacteria due to the limitations of the traditional culture or quantitative PCR (qPCR) techniques used. Several studies have used sequencing of the 16S rRNA gene to provide a more complete understanding of ruminal bacterial diversity (Whitford et al., 1998; Tajima et al., 1999; Kocherginskaya et al., 2001). Relative to traditional methods, next-generation high-throughput sequencing is a newer method that provides a more complete relative quantification of microbial community composition, and it may allow a more precise inference of their functions (Zhang et al.,

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2011) in a much shorter time and at considerably less expense. Very few studies have examined the effects of yeasts on the ruminal microbial community using next-generation high-throughput sequencing systems. Pinloche et al. (2013) used 454 pyrosequencing to demonstrate that addition of live yeast increased the populations of main fibrolytic and lactate-utilizing bacteria in the rumen. Based on 454 pyrosequencing, Sandri et al. (2014) reported that lyophilized live yeast supplementation increased the abundance of *Bacillus* and dry brewer's yeast supplementation increased the abundance of an unassigned genus of family *Erysipelotrichaceae*. However, they examined only the effects on the liquid ruminal fraction.

The Illumina MiSeq sequencing system (Illumina Inc., San Diego, CA) has higher throughput per run, lower error rates, and greater depth and breadth of coverage than the 454 pyrosequencing system (Loman et al., 2012; Frey et al., 2014). Therefore, the MiSeq sequencing system may provide a more detailed characterization of the effects of yeast on ruminal diversity. This is particularly important because the beneficial effects of yeast on milk yield and weight gain have been attributed to only a few of the diverse microorganisms in the rumen. Moreover, little is known about the effects of the dose and viability of live yeast on the ruminal microbiome. Such information is needed to understand the relative effects and modes of action of live yeast and yeast culture to increase their efficacy.

The objective of this study was to examine the effect of the dose and viability of live yeast on the diversity of the ruminal microbial population using high-throughput MiSeq 16S rDNA sequencing and qPCR techniques. A second objective was to examine the relationship between estimates of the relative abundance of certain ruminal bacteria from both methods. Because all yeast treatments increased or tended to increase NDF and ADF digestibility (Jiang et al., 2016) and only the low dose of live yeast increased milk production, we hypothesized that all yeast treatments would increase the relative abundance of cellulolytic ruminal bacteria, but the response would be greatest for the low dose of live yeast. We also hypothesized that similar bacterial diversity responses to yeast treatments would be achieved with qPCR and MiSeq sequencing, and that MiSeq sequencing would reveal several uncultured or unknown ruminal bacteria that are important candidates for future studies because of their response to yeast supplementation.

MATERIALS AND METHODS

All experimental cows were managed according to the guidelines approved by the University of Florida

Institute of Food and Agricultural Sciences Animal Research Committee.

Animals, Housing, and Feeding

The experiment was conducted from December 2013 to April 2014. Four ruminally cannulated lactating primiparous Holstein cows at 284 ± 18 DIM were randomly assigned to 1 of 4 treatment sequences in a 4×4 Latin square experimental design with four 21-d experimental periods.

Cows were housed in a freestall, open-sided barn with sand-bedded stalls ($1.14 \times 2.31 \times 1.22$ m). The experimental pens were fitted with 2 rows of fans (1 fan/6 linear meters) with low-pressure nozzles for cooling the cows; the nozzles were activated by ambient temperatures of 21.1°C . Artificial light was provided by suspended fluorescent bulbs. Water was freely available at all times. Areas between the feed bunks and the freestalls were flushed twice daily to remove manure.

Each cow was randomly assigned to a feeding gate (Calan Broadbent feeding system; American Calan Inc., Northwood, NH) for measurement of individual cow feed intake. All cows were trained to eat from their specific Calan gate during a 5-d period before a 10-d pre-study covariate period, when cows were fed the control diet and DMI and milk production were measured.

Diets and Treatments

Cows received a typical mid-lactation TMR formulated to meet or exceed the nutrient requirements of dairy cows producing at least 30 kg of milk (NRC, 2001) using CPM-Dairy software (version 3.0.10; www.cpm dairy.net). Diets consisted of corn silage, a concentrate mixture, and a vitamin and mineral premix. All cows were offered 110% of their feed intake on the previous day.

Cows were randomly assigned to the following treatments: (1) A control treatment fed no yeast; (2) a low dose of live yeast (**LLY**; 5.7×10^7 cfu/cow per day); (3) a high dose of live yeast (**HLV**; 6.0×10^8 cfu/cow per day); and (4) a high dose of killed yeast (**HDY**; fed 6.0×10^8 cfu/cow per day). Yeast in the HDY treatment were killed in the liquid form by heating in a water bath for 1.5 h at 80°C . Exactly 5 g of a maltodextrin carrier containing the respective yeast treatment was top-dressed on the relevant TMR at each of the 2 daily feedings. Allotments of yeast for each daily feeding were prepared 4 d before the beginning of each period and freeze-dried. The specific yeast used was a proprietary strain of *Saccharomyces cerevisiae* (Dupont Pioneer, Johnston, IA) that was isolated from corn silage.

Rumen Sampling

Ruminal fluid was collected at 0 h (immediately before feeding) on d −1 of period 1 (covariate) and at 0, 2, 4, 6, 8, and 10 h after the morning feeding on d 21 of each period. Samples were filtered through 4 layers of cheesecloth to separate solid and liquid fractions, which were both frozen (−80°C) and dispatched on dry ice to the Dupont Pioneer Analytical-Genomics Technologies laboratory (Johnston, IA) for further analysis.

DNA Extraction and Preparation

Each ruminal liquid or solid sample (~25 g) was ground coarsely in liquid nitrogen in a precooled (−196°C) mortar, transferred to a precooled (−196°C) grinding vial equipped with a stainless steel impactor and then ground in liquid nitrogen at 6 cycles per second for 6 min and then at 8 cycles per second for 6 min in an enclosed freezer mill (no. 6970EFM; SPEX SamplePrep, Metuchen, NJ). The DNA was then extracted and purified from 0.20 g of ground samples with a PowerSoil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA). The integrity of the DNA was verified by agarose (0.7%) gel electrophoresis, and the DNA was stored at −20°C until further use.

Illumina MiSeq Sequencing (Experiment 1)

The V4 region of the 16S rRNA gene was PCR-amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWCTTAAT-3') (Caporaso et al., 2010) using Accuprime Pfx DNA polymerase (Life Technologies, Carlsbad, CA) with a reaction volume of 20 µL. A 12-bp barcode for sample identification was included in the forward primers. Amplification was achieved with the Applied Biosystems Veriti Thermocycler (Life Technologies) using the following steps: (1) initial denaturation at 95°C for 2 min; (2) 30 cycles of further denaturation at 95°C for 15 s, annealing at 50°C for 30 s, and extension at 68°C for 1 min; (3) concluding extension at 68°C for 7 min. All PCR reactions were performed in triplicate, and PCR products were combined, assessed for integrity by agarose (2%) gel electrophoresis, quantified using the Qubit dsDNA BR Assay Kit in accordance with the manufacturer's instructions (Life Technologies; https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_BR_Assay_UG.pdf) and subsequently pooled in equal proportions based on DNA concentration. Pooled amplicons were then purified using a QIAquick Gel Extraction Kit (Qiagen; Venlo, Limburg, Belgium). Paired-end reads with 250 bp were generated using the MiSeq v. Four sequencing platform (Illumina

Inc., San Diego, CA) from the purified amplicons. Sequences were trimmed, quality-filtered, and de-convoluted based on the 12-bp barcode sequence using an in-house Dupont Pioneer pipeline. All paired ends were joined, and sequences with short reads were extended by merging paired-end reads using FLASH (Magoč and Salzberg, 2011). Any read pairs that could not be assembled and any single reads were discarded.

Sequence Analysis

Sequences were trimmed, quality-filtered (with a minimum per base Q score of 16 and a minimum average Q score of 20) and de-convoluted based on the 12-bp barcode sequence using an in-house Dupont Pioneer pipeline. Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME; v. 1.8.0) according to Caporaso et al. (2010). Chimeras were removed by script `identify_chimeric_seqs.py` with the `usearch61` option (Edgar, 2010). The `Screen.seqs` function in Mothur (Schloss et al., 2009) was used to remove sequences that were longer than 275 bp, with ambiguous bases, or with homopolymers longer than 8 bases. Operational taxonomic units (OTU) were picked at a 97% identity threshold using the `pick_de_novo_otus.py` script. Representative OTU were classified by `Uclust` (Edgar, 2010) against the Greengenes reference database (release 12_10; DeSantis et al., 2006). Singletons were removed with the `filter_otus_from_otu_table.py` script (Bokulich et al., 2013) before further analysis. The number of reads were normalized to 13,787 and within (α)- and between (β)-sample phylogenetic diversity and the relative abundance of OTU were compared with the `core_diversity_analyses.py` script as described by Lozupone and Knight (2005). Unweighted UniFrac distances (Lozupone and Knight, 2005) between sets of taxa in the phylogenetic tree were used to build principal coordinate analysis (PCoA) plots.

Quantitative PCR Analysis (Experiment 2)

Primer sets used for qPCR analysis of select rumen bacteria and protozoa are listed in Table 1. *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii*, and *Prevotella ruminicola* primer sets were based on those from Stevenson and Weimer (2007). A total bacteria primer set (Maeda et al., 2003) that amplifies all bacteria was selected as a reference primer set for the bacteria-specific primer sets. The population of the *Saccharomyces cerevisiae* strain used in this study was quantified by qPCR using primers TM4F and TM4R; Research Disclosure database number 612005). The

protozoa population was quantified by qPCR using the primers 316F and 539R and methods described by Sylvester et al. (2004), except that the platinum Taq DNA polymerase was replaced with a QuantiTect Multiplex PCR Master Mix (Qiagen).

Quantification of the selected ruminal microorganisms was performed with the Applied Biosystems ViiA7 platform (Life Technologies) using fluorescence detection of EVAgreen dye (Biotium, Hayward, CA). Each microtiter plate well contained the following: 4.75 μ L of 2 $\times$ QuantiTect Multiplex PCR Mastermix (Qiagen), which contained DNA polymerase, reaction buffer, deoxynucleotide triphosphates (dNTP), and ROX dye (Life Technologies) as a passive reference; forward and reverse primers at a final concentration of 360 nM; 0.25 μ L of EVAgreen; 0.1 μ L of uracil N-glycosylase, 1.0 μ L of each sample; and 3.83 μ L of distilled water to give a total of approximately 10 μ L per well. The plate was briefly centrifuged and placed in the thermocycler for analysis. Amplification consisted of an initial hold of 95°C for 15 min, followed by 40 cycles of 94°C for 60 s and 60°C for 60 s.

For bacteria standards, 16S rRNA genes of the species of interest were retrieved from GenBank and the portion of DNA fragments that contained the primer binding sites were synthesized at Integrated DNA Technologies Inc. (Coralville, IA). The strains of the individual species used were *B. fibrisolvens* strain H17c, *F. succinogenes* strain S85, *M. elsdenii* strain T81, *P. ruminicola* 23, *R. albus* strain 7, *R. flavefaciens* strain FD1, *S. ruminantium* ssp. *lactilytica* TAM6421, and *Strept. bovis* strain JB1. The protozoa standard was generated by PCR using P.SSU-54f/P.SSU-1747r primers (Sylvester et al., 2004) with pooled rumen DNA samples as the template. Copy numbers of protozoa 18S rRNA gene were calculated based on the molecular weight as reported by Sylvester et al. (2004; 9.4×10^5 g/mol or 6.4×10^8 copies/ng of PCR product). The number of bacterial and protozoal DNA molecules synthesized were calculated, serially diluted [10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 1 molecule(s) per PCR reaction], and used as the standards for quantifying the species of interest by qPCR.

Gene copy numbers (for bacteria) of each sample were calculated based on cycle threshold values from standard curves. The relative abundance of protozoa and specific bacteria was calculated by dividing the population of protozoa and the bacteria of interest by that of the total bacteria reference.

Statistical Analysis

The experimental design was a single 4×4 Latin square with 4 experimental units per treatment. The

within-sample phylogenetic distance metric was compared at the highest rarefaction depth using the GLM procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). A nonparametric Monte Carlo test with no Bonferroni correction was used to compare the within-treatment and between-treatment UniFrac distances.

The relative abundance of OTU was analyzed using the GLIMMIX procedure of SAS. The model for analyzing the data is expressed in the form:

$$Y = \mu + T_i + P_j + C_k + CV_1 + TH_m + (T \times TH)_{im} + \varepsilon_{ijklm},$$

where Y is the dependent variable, μ is the overall mean, T_i is the treatment effect, P_j is the period effect, C_k is the cow effect (random), CV_1 is the covariate effect, TH_m is the effect of sampling time, $(T \times TH)_{im}$ is the interaction between treatment and time, and ε_{ijklm} is the residual error.

Normality was tested by examining the distribution of residuals. Denominator degrees of freedom were estimated using the Kenward-Roger option in the MODEL statement. Time was used in the repeated-measures statement with autoregressive order 1 repeated measure covariance structure, which was chosen because it gave the smallest Akaike information criterion values, and because measurements taken close together were more correlated than those that were farther apart (SAS Institute, 2016). Preplanned nonorthogonal contrasts that were examined to compare means included the following: control vs. LLY (effect of supplementation with LLY), LLY versus HLY (effect of the dose of live yeast), average of LLY and HLY versus HDY (effect of supplementing with live vs. killed yeast), and control versus HDY (effect of supplementing with killed yeast). Treatment effects and their interactions were declared significant at $P \leq 0.05$, and tendencies to significance were declared at $0.05 \leq P \leq 0.15$. To compare the quantification of key ruminal bacteria by qPCR and MiSeq sequencing, the relationship between the relative percentage of *F. succinogenes*, *R. flavefaciens*, and *S. ruminantium* measured by both methods was examined using the REGRESSION procedure of SAS.

RESULTS

Sequencing Depth and Coverage and Relative Abundance of Taxa

The quality-filtered reads for 16S rRNA sequences were demultiplexed and assembled, yielding 13,374,890 sequences in total with a median sequence length of 253 bases and an average coverage of 55,728 sequences per

Table 1. Species-specific primers for the quantification of selected ruminal microbial populations using a real-time quantitative PCR assay

Target species	Primer	Primer sequence (5'-3')	Reference	qPCR efficiency ¹ (%)
Bacteria general	BacF	GTGSTGCAYGGYTGTCGTCA	Maeda et al. (2003)	109.06
	BacR	ACGTCRTCCMCACCTTCCTC		
<i>Fibrobacter succinogenes</i>	FibSuc3F	GCGGGTAGCAACACAGGATTAGA	Stevenson and Weimer (2007)	101.83
	FibSuc3R	CCCCGGACACCCAGTAT		
<i>Ruminococcus albus</i>	RumAlb3F	TGTTAACAGAGGGAAGCAAAAGCA	Stevenson and Weimer (2007)	104.90
	RumAlb3R	TGCAGCCTACAATCCGAAGTAA		
<i>Ruminococcus flavefaciens</i>	RumFla3F	TGGCGGACGGGTGAGTAA	Stevenson and Weimer (2007)	105.15
	RumFla3R	TTACCATCCGTTTCCAGAAAGCT		
<i>Selenomonas ruminantium</i>	SelRum2F	CAATAAGCATTCGGCCTGGG	Stevenson and Weimer (2007)	106.18
	SelRum2R	TTCACTCAATGTCAAGCCCTGG		
<i>Streptococcus bovis</i>	StrBov2F	TTCTAGAGATAGGAAGTTTCTTCGG	Stevenson and Weimer (2007)	107.31
	StrBov2R	ATGATGGCAACTAACAAATAGGGGT		
<i>Butyrivibrio fibrisolvens</i>	ButFib2F	ACGGCATAAGCGCAGGGA	Stevenson and Weimer (2007)	108.08
	ButFib2R	CGGGTCCATCTTGTACCGATAAAT		
<i>Megasphaera elsdenii</i>	MegEls2F	AGATGGGGACACACAGCTGGA	Stevenson and Weimer (2007)	100.89
	MegEls2R	CGAAAAGTCCGAAAGAGCCT		
<i>Prevotella ruminicola</i>	PreRum1F	GAAAGTCGGATTAAATGCTCTATGTTG	Stevenson and Weimer (2007)	100.74
	PreRum1R	CATCTATAGCGGTAAACCTTTGG		
<i>Saccharomyces cerevisiae</i> YE1496	TM4 F	CCAGCTACCACTTCCAACT		95.22
	TM4 R	GGAGCGCTAGGGTAAGCATA		
Protozoa	316F	CAYGTCTAAGTATAAATAACTAC		
	539R	CTTGCCCTCYAATCGTWCT		
	P.SSU-54f	CAYGTCTAAGTATAAATAACTAC	Sylvester et al. (2004)	93.16 (316F/539R)
	P.SSU-1747r	CTCTAGGTGATWWGRTTAC		

¹Measured efficiencies of the primers in the qPCR reactions. F = forward; R = reverse.

sample. The overall number of OTU detected by the analysis was 75,355, based on 97% nucleotide sequence identity between reads.

The diversity of bacteria in the solid and liquid fraction differed (Figures 1 and 2). *Prevotella* dominated (24%) the solid fraction of the ruminal contents, followed by *Fibrobacter* (10%), and then *Treponema* (6%; Figure 1). In the liquid fraction, *Prevotella* accounted for 50% of ruminal bacteria, followed by *Succinivibrionaceae* (7%), and then by several bacteria that each had a relative abundance of approximately 2%, including *Butyrivibrio*, *Lachnospiraceae*, *Ruminococcus*, and *Treponema* (Figure 2). Because OTU were not classified to the species level, we further compared representative sequences from OTU from the *Prevotella* genus against the 16S sequences from *P. ruminicola* strain 23 and *P. bryantii* B14 using BLAST to suggest what percentage of the *Prevotella*, the most abundant genus, corresponded to well-known species such as *P. ruminicola* or *P. bryantii*. Sequences that had expectation values less than 1×10^{-100} and over 99% identity to reference sequences were classified as the respective species. Based

on these classifications, *P. bryantii* accounted for 0.83 and 0.72% of *Prevotella* in the solid and liquid fraction, respectively, whereas *P. ruminicola* accounted for 13.11% and 16.56% of *Prevotella* in the solid and liquid fraction, respectively. However, these results should be carefully interpreted, because the analysis was based solely on the V4 region of 16S and may or may not reflect the classification by the full-length 16S gene.

Within-Sample (α) and Between-Sample (β) Diversity

The average Good's coverage of all the samples was $0.91 \pm \text{SEM } 0.001$ (Supplemental Material; <https://doi.org/10.3168/jds.2016-11263>), indicating that the sequencing depth was adequate for reliable analysis of the microbial community of all the samples. Treatments did not affect within-sample (α) phylogenetic diversity (Chao1 estimates; Figure 3), and this was confirmed statistically ($P > 0.15$). The PCoA plots of between-sample (β) phylogenetic diversity (Figure 4) show that bacteria in the liquid and solid samples clustered separately, and this was also confirmed by

Figure 1. Relative abundance (%) of the 20 most dominant bacteria genera or families in the ruminal solid fraction of dairy cows as analyzed by MiSeq (Illumina Inc., San Diego, CA) 16S rDNA sequencing (experiment 1). *L. Ruminococcus* = *Lachnospiraceae Ruminococcus*; *Veillonellaceae*, *Porphyromonadaceae*, *Clostridiaceae*, *Paraprevotellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*, and *Lachnospiraceae* represent unknown genera in the respective families. Color version available online.

Figure 2. Relative abundance (%) of the 20 most dominant bacteria genera or families in the ruminal liquid fraction of dairy cows as analyzed by MiSeq (Illumina Inc., San Diego, CA) 16S rDNA sequencing (experiment 1). YS2 = uncultured or unidentified taxa; *Porphyromonadaceae*, *Veillonellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Paraprevotellaceae*, and *Succinivibrionaceae* represent unknown genera in the respective families. Color version available online.

statistical analysis ($P = 0.001$). These findings indicate that compositional differences existed among the bacterial communities of the liquid and solid fractions, which has been observed by Tajima et al. (1999) and Michalet-Doreau et al. (2001). The Monte Carlo test revealed that the within-treatment variation in the unweighted UniFrac distances used to estimate β diversity was smaller ($P = 0.001$) than the between-treatment variation (Figure 4). This suggests that treatments had different effects on ruminal diversity. The time of taking ruminal samples did not affect β diversity (Figure 5; $P > 0.15$).

Treatment Effects on Dominant Bacteria Groups Analyzed by MiSeq Sequencing

Solid Fraction. Although 65.8% of sequences could be assigned to the genus level using GreenGene as the reference, only 7.8% of the sequences could be assigned to the species level; 87.8, 98.4, 98.5, and 98.7% of the sequences could be assigned to family, order, class, and phylum, respectively.

The relative percentage in the solid fraction of *Prevotella*, *Succinivibrionaceae*, and *Fibrobacter*, which were among the most dominant bacteria, were unaf-

fected by treatment ($P > 0.15$, Table 2), but it is unclear if changes in the relative abundance of the species occurred. Adding LLY to the diet decreased the relative abundance of *Treponema* ($P = 0.01$), *Lachnospiraceae* ($P = 0.07$), and *Coprococcus* ($P = 0.04$), but increased that of CF231 (an uncultured/unidentified taxon in family *Paraprevotellaceae*; $P = 0.06$), *Ruminobacter* ($P = 0.10$), *Porphyromonadaceae* ($P = 0.13$), and *Bifidobacterium* ($P = 0.01$). Increasing the live yeast dose (HLY vs. LLY) increased the relative abundance of *Treponema* ($P = 0.03$) but decreased that of *Paraprevotellaceae* ($P = 0.11$), CF231 ($P = 0.11$), and *Bifidobacterium* ($P = 0.12$). Adding live yeast instead of killed yeast to the diet increased the relative abundance of *Ruminococcus* ($P = 0.07$) and decreased that of S24-7 (uncultured/unidentified taxon in order *Bacteroidales*; $P = 0.01$) and *Clostridiaceae* ($P = 0.04$). Adding HDY decreased the relative abundance of *Lachnospiraceae* ($P = 0.06$) and *Coprococcus* ($P = 0.01$) and increased that of *Ruminobacter* ($P = 0.02$), *Porphyromonadaceae* ($P = 0.12$), and *Bifidobacterium* ($P = 0.03$).

Liquid Fraction. Table 3 shows treatment effects on dominant bacteria in the ruminal liquid fraction. Adding LLY to the diet increased the relative abundance of *Butyrivibrio* ($P = 0.11$), *Ruminococcus* ($P =$

Figure 3. Phylogenetic diversity for bacterial communities in ruminal fluid of cows fed diets supplemented without or with 2 doses of live yeast or 1 dose of killed yeast (experiment 1). Values are mean $\pm$ SEM. CON (control) = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496. The phylogenetic diversity metric was compared across treatments at highest rarefaction depth. No treatment effects were evident ($P > 0.15$). Color version available online.

Figure 4. Principal coordinate (PC) analysis plot of β diversity of rumen samples from cows fed diets supplemented without or with 2 doses of live yeast or 1 dose of killed yeast (experiment 1). CON (control) = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496. Color version available online.

0.06), and *Bifidobacterium* ($P = 0.02$) and decreased that of *Treponema* ($P = 0.14$), RFP12 (uncultured/unidentified taxon in order *WCHB1-41*; $P = 0.03$), and *Paludibacter* ($P = 0.02$). Increasing the live yeast dose tended to decrease the relative abundance of *Bifidobac-*

Figure 5. Principal coordinate (PC) analysis plot of β diversity of rumen samples taken at different times after the morning feeding of dairy cows fed different doses of live yeast and 1 dose of killed yeast (experiment 1). Color version available online.

Table 2. Effect of supplementing 2 levels of live yeast or 1 level of killed yeast on the bacterial composition (relative %) of the ruminal solid fraction of dairy cows as analyzed by MiSeq (Illumina Inc., San Diego, CA) sequencing (experiment 1)

Genus	Treatment ¹					Contrast <i>P</i> -value			
	Control	LLY	HLY	HDY	SEM	Control vs. LLY	LLY vs. HLY	LLY + HLY vs. HDY	Control vs. HDY
<i>Prevotella</i>	23.4	25.1	23.8	24.5	1.03	0.26	0.38	0.96	0.45
<i>Succinivibrionaceae</i> ²	4.51	4.61	4.26	5.04	0.77	0.91	0.71	0.43	0.57
<i>Fibrobacter</i>	10.7	10.2	12.4	9.98	1.18	0.75	0.17	0.34	0.66
<i>Treponema</i>	11.1	7.51	10.4	9.40	1.09	0.01	0.03	0.67	0.17
<i>Butyrivibrio</i>	6.77	6.52	6.74	7.01	0.58	0.76	0.78	0.59	0.77
<i>Lachnospiraceae</i> ²	6.09	5.22	5.81	5.17	0.33	0.07	0.20	0.37	0.06
<i>Ruminococcus</i>	4.30	4.26	4.63	3.80	0.30	0.92	0.33	0.07	0.20
<i>Ruminococcaceae</i> ²	3.24	3.20	3.31	3.11	0.12	0.82	0.51	0.33	0.45
<i>Paraprevotellaceae</i> ²	2.18	2.44	2.03	2.25	0.22	0.28	0.11	0.92	0.74
CF231	0.87	1.13	0.91	1.06	0.11	0.06	0.11	0.73	0.16
YRC22	1.21	1.20	1.05	1.09	0.09	0.91	0.21	0.74	0.31
<i>Veillonellaceae</i> ²	0.46	0.41	0.44	0.43	0.03	0.32	0.55	0.92	0.54
<i>Ruminobacter</i>	0.87	1.31	1.28	1.62	0.19	0.10	0.90	0.16	0.02
<i>Porphyromonadaceae</i> ²	0.48	0.66	0.53	0.66	0.08	0.13	0.25	0.47	0.12
<i>Coprococcus</i>	1.48	1.21	1.26	1.12	0.09	0.04	0.64	0.30	0.01
S24-7 ²	0.82	0.69	0.58	0.93	0.11	0.24	0.30	0.01	0.31
<i>Bifidobacterium</i>	-0.03	1.13	0.53	0.87	0.31	0.01	0.12	0.90	0.03
<i>Clostridium</i>	1.04	0.93	1.02	0.99	0.06	0.20	0.30	0.86	0.51
<i>Clostridiaceae</i> ²	0.82	0.73	0.75	0.89	0.05	0.26	0.86	0.04	0.38
<i>Lachnospiraceae Ruminococcus</i>	0.42	0.47	0.52	0.51	0.06	0.54	0.47	0.84	0.26

¹Control = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496.

²Uncultured or unidentified genus in the corresponding family.

terium ($P = 0.12$) but increased that of YS2 ($P = 0.12$). Adding live yeast to the diet instead of killed yeast increased or tended to increase the relative abundance of *Ruminococcaceae* ($P = 0.08$), *Ruminococcus* ($P = 0.01$), *Paraprevotellaceae* ($P = 0.05$), and *Coprococcus* ($P = 0.09$), and decreased those of *Succinivibrionaceae* ($P = 0.13$), *Ruminobacter* ($P = 0.01$), *Veillonellaceae* ($P = 0.05$), *Porphyromonadaceae* ($P = 0.04$) and S24-7 ($P = 0.10$). Adding HDY to the diet decreased or tended to decrease the relative abundance of *Lachnospiraceae* ($P = 0.14$), *Paraprevotellaceae* ($P = 0.01$), *Coprococcus* ($P = 0.07$), and *Paludibacter* ($P = 0.14$) and increased those of *Fibrobacter* ($P = 0.13$), *Ruminobacter* ($P = 0.01$), *Porphyromonadaceae* ($P = 0.12$), and *Bifidobacterium* ($P = 0.05$).

Treatment Effects on Select Microbes Analyzed by qPCR

We detected no treatment effects on total bacteria copy numbers in the solid (Table 4) or liquid fractions (Table 5). In the ruminal solid fraction, the relative abundance of *R. albus*, *R. flavefaciens*, *Strep. bovis*, *M. elsdenii*, and *P. ruminicola* were unaffected by dietary supplement (Table 4). However, adding LLY to the diet tended ($P = 0.14$) to increase the relative abundance of *F. succinogenes*. Increasing the dose of live yeast decreased ($P = 0.02$) the relative abundance of *B.*

fibrosolvens. Adding live yeast instead of killed yeast increased ($P = 0.04$) the relative abundance of *F. succinogenes* and decreased ($P = 0.05$) that of *S. ruminantium*. Adding HDY to the diet tended ($P = 0.15$) to decrease the relative abundance of *B. fibrosolvens* and protozoa ($P = 0.14$ and 0.05 , respectively).

In the liquid fraction, the relative abundance of *F. succinogenes*, *R. flavefaciens*, *B. fibrosolvens*, *M. elsdenii*, *P. ruminicola*, and protozoa were not affected by diet (Table 5). Adding LLY to the diet tended to decrease the relative abundance of *R. albus* ($P = 0.12$) and increase that of *S. ruminantium* ($P = 0.14$). Increasing the dose of live yeast or adding live yeast instead of killed yeast had no effect on the relative abundance of bacteria in the liquid fraction. Adding HDY to the diet tended to increase the relative abundance of *S. ruminantium* ($P = 0.11$) and *Strep. bovis* ($P = 0.06$).

The quantity of *Saccharomyces cerevisiae* strain YE1496 used in the qPCR analysis was below the detection limit (5×10^3 cells/g of sample) in all the samples.

Comparison of Relative Abundance of Specific Bacteria Analyzed by Sequencing and qPCR

We compared the abundance of OTU assigned taxonomically as the defined species of interest (see below) with qPCR results. The relationship between MiSeq

and qPCR estimates of the relative abundance of *F. succinogenes* ($R^2 = 0.60$, $P < 0.001$) in the ruminal solid fraction was more precise than those of *R. flavefaciens* ($R^2 = 0.22$, $P < 0.001$), *S. ruminantium* ($R^2 = 0.05$, $P < 0.001$), and *M. elsdenii* ($R^2 = 0.14$, $P < 0.001$; Figure 6). In the ruminal liquid fraction, the relationships between MiSeq and qPCR estimates of the relative abundance of *F. succinogenes* ($R^2 = 0.16$, $P < 0.01$), *R. flavefaciens* ($R^2 = 0.11$, $P < 0.01$), and *M. elsdenii* ($R^2 = 0.11$, $P < 0.01$) were not as precise as those in the solid fraction, but that of *S. ruminantium* was similar ($R^2 = 0.08$, $P < 0.01$; Figure 7). These data indicate that the relationship was more precise when the relative abundance of the species was higher.

DISCUSSION

Relative Abundance of Taxa

As in the present study, others have noted that *Prevotella* was the most abundant genus in ruminal samples (Stevenson and Weimer, 2007; Wu et al., 2012). Based on qPCR analysis, Stevenson and Weimer (2007) reported that *Prevotella* accounted for 42 to 60% of the bacterial rRNA gene copies in rumen samples. Also, the relative abundance of *Fibrobacter* in the ruminal

solid fraction in the present study agrees with Lee et al. (2012), who reported that *Fibrobacter* was the second most dominant genus in particle-associated bacteria in ruminal fluid samples (4.3 to 10% of the total bacterial sequences). In addition, the high relative abundance of an unknown genus in the family *Succinivibrionaceae* in the ruminal liquid versus solid fraction agrees with the findings of Castro-Carrera et al. (2014).

In the present study, only a small proportion of sequences could be assigned to the species level due to the limited number of cultured species and the limited power of 16S-V4 region for species identification. Consequently, sequences were not assigned beyond the genus level for the examination of treatment effects. The level of sequence assignment to the genus level (65.8%) in the present study was greater than that (53%) reported by Pinloche et al. (2013) with 454 pyrosequencing, which might be because a different region of the 16S rRNA gene was amplified, and different database and methods were used to assign taxa. Henderson et al. (2013) cautioned that it is almost impossible to be sure that current DNA isolation methods truly represent the entire ruminal microbial community. Also, the apparent microbial communities detected in this study may not be directly comparable with those from other studies that used a different DNA isolation method.

Table 3. Effect of supplementing 2 levels of live yeast or 1 level of killed yeast on the bacterial composition (relative %) of the ruminal liquid fraction of dairy cows as analyzed by MiSeq (Illumina Inc., San Diego, CA) sequencing (experiment 1)

Genus	Treatment ¹				SEM	Contrast <i>P</i> -value			
	Control	LLY	HLY	HDY		Control vs. LLY	LLY vs. HLY	LLY + HLY vs. HDY	Control vs. HDY
<i>Prevotella</i>	51.7	49.8	49.0	49.0	2.10	0.48	0.76	0.88	0.33
<i>Succinivibrionaceae</i> ²	7.31	6.27	6.44	8.36	1.32	0.48	0.91	0.13	0.47
<i>Fibrobacter</i>	1.13	1.08	1.42	1.52	0.20	0.81	0.16	0.21	0.13
<i>Treponema</i>	1.71	1.27	1.53	1.71	0.20	0.14	0.36	0.22	1.00
<i>Butyrivibrio</i>	2.12	2.77	2.65	2.35	0.27	0.11	0.75	0.28	0.55
<i>Lachnospiraceae</i> ²	2.46	2.23	2.26	2.00	0.22	0.44	0.93	0.34	0.14
<i>Ruminococcus</i>	1.72	2.72	2.81	1.44	0.42	0.06	0.85	0.01	0.56
<i>Ruminococcaceae</i> ²	2.38	2.59	2.44	2.12	0.17	0.37	0.52	0.08	0.28
<i>Paraprevotellaceae</i> ²	2.76	2.59	2.58	2.29	0.11	0.29	0.95	0.05	0.01
CF231	2.33	2.63	2.41	2.48	0.16	0.22	0.37	0.85	0.52
YRC22	1.50	1.55	1.45	1.36	0.12	0.67	0.42	0.21	0.28
<i>Veillonellaceae</i> ²	1.66	1.79	1.68	1.45	0.10	0.42	0.49	0.05	0.18
<i>Ruminobacter</i>	0.85	0.88	1.16	1.84	0.26	0.92	0.38	0.01	0.01
<i>Porphyromonadaceae</i> ²	1.51	1.28	1.55	2.05	0.23	0.48	0.41	0.04	0.12
<i>Coprococcus</i>	0.55	0.55	0.51	0.44	0.04	0.96	0.51	0.09	0.07
S24-7 ²	0.84	0.76	0.73	0.96	0.10	0.60	0.80	0.10	0.38
RFP12 ²	1.54	0.85	1.14	1.34	0.23	0.03	0.29	0.16	0.47
<i>Bifidobacterium</i>	0.00	1.10	0.42	0.88	0.32	0.02	0.12	0.72	0.05
YS2 ³	0.59	0.51	0.64	0.48	0.06	0.30	0.11	0.19	0.18
<i>Paludibacter</i>	0.89	0.42	0.58	0.62	0.18	0.02	0.36	0.44	0.14

¹Control = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496.

²Uncultured or unidentified genus in the corresponding family.

³Uncultured or unidentified genus in order YS2.

Table 4. Effect of supplementing 2 levels of live yeast or 1 level of killed yeast on the bacterial composition (relative %) of the ruminal solid fraction of dairy cows as analyzed by quantitative PCR (experiment 2)

Target	Treatment ¹				Contrast <i>P</i> -value			
	Control	LLY	HLY	HDY	SEM	Control vs. LLY	LLY vs. HLY	LLY + HLY vs. HDY
<i>Fibrobacter succinogenes</i>	3.83	4.44	4.66	3.78	0.27	0.14	0.57	0.04
<i>Ruminococcus albus</i>	0.02	0.02	0.02	0.02	0.002	0.37	0.69	0.75
<i>Ruminococcus flavefaciens</i>	2.45	2.44	2.44	2.41	0.27	0.99	0.98	0.91
<i>Selenomonas ruminantium</i>	0.55	0.49	0.54	0.59	0.03	0.17	0.22	0.05
<i>Streptococcus bovis</i>	0.001	0.001	0.001	0.002	0.0002	0.53	0.69	0.63
<i>Butyrivibrio fibrisolvens</i>	0.044	0.042	0.029	0.037	0.003	0.68	0.02	0.76
<i>Megasphaera elsdenii</i>	0.0001	0.0001	0.0001	0.0001	0.00002	0.75	0.59	0.35
<i>Prevotella ruminicola</i>	0.51	0.54	0.54	0.59	0.05	0.67	0.97	0.38
Total bacteria copy number (per g)	5.90×10^{10}	6.21×10^{10}	5.24×10^{10}	4.42×10^{10}	5.4×10^9	0.76	0.43	0.30
Protozoa	6.09	4.60	5.23	3.75	0.74	0.17	0.55	0.22

¹Control = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496.

Table 5. Effect of supplementing 2 levels of live yeast or 1 level of killed yeast on the microbial composition (relative %) of the ruminal liquid fraction of dairy cows as analyzed by quantitative PCR (experiment 2)

Target	Treatment ¹				Contrast <i>P</i> -value			
	Control	LLY	HLY	HDY	SEM	Control vs. LLY	LLY vs. HLY	LLY + HLY vs. HDY
<i>Fibrobacter succinogenes</i>	1.69	1.66	1.95	1.94	0.27	0.94	0.46	0.68
<i>Ruminococcus albus</i>	0.02	0.01	0.01	0.01	0.004	0.12	0.79	0.69
<i>Ruminococcus flavefaciens</i>	1.65	1.68	1.62	1.94	0.44	0.96	0.91	0.56
<i>Selenomonas ruminantium</i>	0.71	0.80	0.72	0.81	0.04	0.14	0.19	0.34
<i>Streptococcus bovis</i>	0.0022	0.0028	0.0028	0.0035	0.0004	0.37	0.99	0.19
<i>Butyrivibrio fibrisolvens</i>	0.029	0.035	0.036	0.033	0.007	0.55	0.94	0.75
<i>Megasphaera elsdenii</i>	0.0005	0.0004	0.0003	0.0006	0.0001	0.51	0.55	0.19
<i>Prevotella ruminicola</i>	5.63	3.52	3.89	4.98	1.06	0.18	0.81	0.34
Total bacteria copy number (per mL)	1.41×10^{10}	1.63×10^{10}	1.46×10^{10}	1.31×10^{10}	1.50×10^9	0.33	0.44	0.25
Protozoa	22.6	18.3	17.4	19.7	3.63	0.41	0.87	0.68

¹Control = no yeast treatment; LLY and HLY = low (5.7×10^7 cfu/cow per day) and high (6.0×10^8 cfu/cow per day) doses of live *Saccharomyces cerevisiae* YE1496 (DuPont Pioneer, Johnston, IA); HDY = high dose (6.0×10^8 cfu/cow per day) of killed *Saccharomyces cerevisiae* YE1496.

Figure 6. Relationship between estimates of the relative abundance of (a) *Fibrobacter succinogenes*, (b) *Ruminococcus flavefaciens*, (c) *Selenomonas ruminantium*, and (d) *Megasphaera elsdenii* in the ruminal solid fraction quantified by MiSeq (Illumina Inc., San Diego, CA) sequencing (experiment 1) and quantitative PCR (experiment 2). Color version available online.

Figure 7. Relationship between estimates of the relative abundance of (a) *Fibrobacter succinogenes*, (b) *Ruminococcus flavefaciens*, (c) *Selenomonas ruminantium*, and (d) *Megasphaera elsdenii* in the ruminal liquid fraction quantified by MiSeq (Illumina Inc., San Diego, CA) sequencing (experiment 1) and quantitative PCR (experiment 2). Color version available online.

Treatment Effects on Dominant Bacteria Based on MiSeq Sequencing

The ensuing descriptions use published information about substrate utilization by cultured ruminal bacteria genera or species to explain the treatment effects in this study. These explanations are not definitive because of the low number of ruminal bacteria that have been cultivated, the low number of sequences that were assigned to the species level in this study, and potential differences among strains and species in substrate utilization.

Adding LLY to the diet increased the relative abundance of some genera known for fermentation of cellulose (*Ruminococcus*, Russell, 2002; Suen et al., 2011, liquid fraction), starch, and sugars (*Bifidobacterium*, solid and liquid fractions) and decreased that of *Treponema* (solid and liquid fractions), a genus known for pectin utilization (Stewart et al., 1997; Russell, 2002; Vos et al., 2011). The increase in the relative abundance of *Ruminococcus* partly explains how adding LLY to the diet increased the in vivo digestibility of DM and NDF and the performance of the cows in the corresponding animal trial (Jiang et al., 2016). However, the relative abundance of *Ruminococcus* was increased only in the liquid fraction by LLY. Yet particle-associated *Ruminococcus* plays a more important role in NDF digestion than *Ruminococcus* in the liquid fraction, because the former is attached to feed particles and is more abundant, because the solid fraction contains 92% of total bacteria (Mullins et al., 2013). In addition, genus *Ruminococcus* also includes amylolytic bacteria such as *Ruminococcus bromii* (Larue et al., 2005), which is often present in the rumen of grain-fed cattle (Tajima et al., 2000). Therefore, LLY-induced increases in DM and NDF digestibility and milk production by cows in the performance study may be due to factors other than the increase in abundance of *Ruminococcus* in the liquid fraction.

Adding LLY to the diet also increased the relative abundance in the liquid fraction of *Butyrivibrio*, which uses a variety of substrates, such as cellulose, starch, sugar, and protein (Weimer, 1996; Russell, 2002). In the solid fraction, adding LLY to the diet decreased the relative abundance of *Coprococcus* and an unknown genus that belongs to the family *Lachnospiraceae*. The implications of these changes are unclear because *Butyrivibrio* can use a large variety of substrates and there is limited knowledge available about the function of *Coprococcus* and the unidentified genus in family *Lachnospiraceae*.

Increasing the dose of live yeast increased the relative abundance of *Treponema* in the solid fraction, decreased that of CF231 and an unknown genus in family

Paraprevotellaceae, increased the relative abundance of YS2 in the liquid fraction, and decreased that of *Bifidobacterium* in the solid and liquid fractions. The increase in the relative abundance of *Treponema* by increasing the live yeast dose contradicts the decrease in relative abundance of *Treponema* in both the liquid and solid fractions by dietary addition of LLY. This suggests that a lower yeast dose was associated with decreased *Treponema* relative abundance and greater milk production (Jiang et al., 2016). However, because *Treponema* is known for pectin fermentation in the rumen (Stewart et al., 1997), other ruminal bacteria likely played a greater role in the milk response.

The stimulation of *Ruminococcus*, a cellulolytic bacterium, by live yeast but not by killed yeast is consistent with the increases in NDF and ADF digestibility by these treatments in the animal experiment (Jiang et al., 2016). Some of the major ruminal bacteria in the genus *Ruminobacter*, such as *Ruminobacter amylophilus*, ferment starch (Cotta, 1988; Anderson, 1995); hence, adding live yeast instead of killed yeast to the diet may have decreased the relative abundance of starch fermenters. In addition, live yeast but not killed yeast changed the abundance of S24–7, and genera within *Clostridiaceae*, *Succinivibrionaceae*, and *Porphyromonadaceae*. However, the implication of these changes is unclear because of the unknown functions of these bacteria.

Adding killed yeast to the diet increased the relative abundance of starch and sugar digesters, *Ruminobacter* and *Bifidobacterium*, in ruminal liquid and solid fractions, and increased that of some fibrolytic bacteria (e.g., *Fibrobacter*) in the liquid fraction. Live yeast may have been more effective than killed yeast at increasing NDF and DM digestibility in the animal study (Jiang et al., 2016), because they stimulated *Ruminococcus* in the solid and liquid fractions, whereas HDY stimulated only *Fibrobacter* in the liquid fraction, where it was less prevalent than *Ruminococcus* (Figure 2). Adding killed yeast also altered the relative abundance of unknown genera in *Lachnospiraceae* and *Porphyromonadaceae*. However, due to limited knowledge about these families, the implication of these changes is unknown.

Bacteria Candidates for Future Study

In the present study, the most dominant bacteria that responded to addition of LLY to the diet, and whose functions are among the least understood in relation to the mode of action of yeast, include an unidentified genus in the family *Paraprevotellaceae* in the liquid fraction, and CF231, *Treponema*, and an unidentified genus in the family *Lachnospiraceae* in the solid fraction. The relative abundance of the unidentified genus

in the family *Paraprevotellaceae* was increased by 12% in the liquid fraction, and that of CF231, an unclassified taxon, was increased by 30% in the solid fraction. Adding LLY decreased the relative abundance in the solid fraction of *Treponema* by 32% and decreased that of an unknown genus in the family *Lachnospiraceae* by 14%. Due to the relative abundance of these bacteria in the liquid or solid ruminal fractions and the magnitude of their respective responses to LLY addition, which also increased diet digestibility and milk production by dairy cows (Jiang et al., 2016), future studies should aim to speciate, culture, and examine the function of these bacteria to better understand their roles in the mode of action of yeast.

Treatment Effects on Select Microbes as Analyzed by qPCR

The qPCR results suggest that adding LLY to the diet or adding live yeast instead of killed yeast may have increased DM and NDF digestibility in the animal trial (Jiang et al., 2016) by stimulating *F. succinogenes* in the solid fraction. Although LLY supplementation also decreased the relative abundance of *R. albus* in the liquid fraction, this may have not reduced the digestibility of NDF or DM (Jiang et al., 2016) because of the low relative abundance of *R. albus* in the liquid fraction ($\leq 0.02\%$ vs. 3.8–4.7% for *F. succinogenes*). The qPCR and sequencing results on the main fiber digesters differed. The sequencing results suggested that the relative abundance of *Ruminococcus* (but not *Fibrobacter*) was greater when LLY was added to the diet, whereas the qPCR results suggested the opposite. These differences are discussed further in the section below on differences between the results of both assays.

Increasing the live yeast dose decreased the relative abundance in the solid fraction of *B. fibrisolvens*, which uses a variety of substrates such as cellulose, hemicellulose, starch, sugar, and protein (Weimer, 1996; Russell, 2002). This may be one of the reasons why HLY did not increase milk yield and LLY did in the companion study.

The effect of the decreased protozoa abundance in the solid fraction by HDY is unclear, because protozoa use many different substrates and bacteria. The stimulation of amylolytic *Strep. bovis* (Asanuma and Hino, 2002) and *S. ruminantium* (Stewart et al., 1997; Russell, 2002) in the liquid fraction by dietary addition of HDY partly supports the sequencing data, which showed that different genera that contain amylolytic bacteria (*Ruminobacter* and *Bifidobacterium*) were stimulated by this treatment. The increase in the relative abundance of *Strep. bovis* by HDY supplementation as determined by qPCR agrees with the trend ($P = 0.12$) for ruminal

lactate concentration to be increased by HDY in the animal experiment (Jiang et al., 2016).

The fate of the supplemented *Saccharomyces cerevisiae* YE1496 in the rumen remains unknown due to the fact that the quantities found in the rumen were below the detection limit for qPCR in this study. More studies are needed to explore the survival of supplemental live yeast in the rumen.

Comparison of MiSeq and qPCR Estimates of the Relative Abundance of Bacteria

Quantitative PCR is used to measure the copy numbers of a gene of interest, such as the 16S rRNA gene. We attempted to validate the MiSeq sequencing results with qPCR using selected species-specific primers commonly used in rumen microbial ecology research. The relationship between estimates of the relative abundance of *F. succinogenes* from qPCR and MiSeq sequencing was moderately precise, but those for *M. elsdenii*, *R. flavefaciens*, and *S. ruminantium* were weak. There are several potential reasons for the weak relationship.

First, differences in treatment effects on specific taxa from the qPCR and sequencing methods were partly due to differences between the techniques, such as the choice and specificity of the primers, particularly those used for qPCR, which are relatively older. Although both methods involve a PCR step, the MiSeq and qPCR primers target different regions. On the 16S rRNA genes, highly conserved and hypervariable sequences are organized alternatively (Chakravorty et al., 2007). For MiSeq sequencing, the hypervariable region 4 (V4) of 16S was amplified by primers that anneal to the flanking of conserved regions. For qPCR, to differentiate species, most species-specific primers were designed based on the regions containing adequate variation. In addition, degenerate primers were used to generate the V4 amplicons (Caporaso et al., 2010) to account for the sequence variability of different bacteria group. These could cause differences in the annealing kinetics of the primers and affect the efficiency of the PCR. In addition, the qPCR primers used also displayed identity to a few strains of closely related species and to some taxonomically unidentified sequences (Stevenson and Weimer 2007), which may yield higher abundance for these taxa.

Second, the V4 amplicon sequence analysis relies on a classification algorithm and a reference database to assign taxonomies to OTU. We used the Greengenes database because, to our knowledge, it is the only publicly available database that provides species-level resolution for some species. However, different classifiers can cause differences in recapturing the taxonomy

information of 16S rRNA sequences, even when the same reference database is used (Liu et al., 2008). In addition, for some species, the short 16S-V4 region may not provide the same classification as the full-length region or other segments of 16S (Jeong et al., 2011). Furthermore, some bacteria are known to have different copies of 16S operons with different sequences. Consequently, sequence-based analyses may classify the different copies as different species (Case et al., 2007).

These factors may contribute the lack of a correlation between the MiSeq and qPCR results. More systematic studies will be needed to identify a standard operating procedure for rumen microbial ecology studies.

Because *F. succinogenes* was more abundant than the other bacteria, the data suggest that the accuracy of the relationship between MiSeq and qPCR estimates of bacterial relative abundance increases with the abundance of the bacteria. Jami and Mizrahi (2012) reported that bacteria with low abundance when measured by qPCR were not detected using pyrosequencing; otherwise, similar abundance estimates were obtained with both methods. Differences in treatment effects on specific taxa from the qPCR and sequencing methods are partly due to differences between the techniques, particularly the choice of the primer. In addition, qPCR is quantitative, but sequencing is semiquantitative because of potential errors of reproducible homopolymer length (Kunin et al., 2010) and bias during sequencing (Firkins and Yu, 2015). Isolation of DNA during qPCR also introduces bias in composition measurements. Nevertheless, both methods indicated that the relative abundance of different cellulolytic organisms was increased by adding LLY or by adding live yeast instead of killed yeast. Both methods also showed that adding killed yeast to the diet increased the relative abundance of different amylolytic organisms. Pinloche et al. (2013) used 454 pyrosequencing and serial analysis of V1 ribosomal sequence tags to examine the effects of adding live yeast to dairy cow diets on ruminal bacterial diversity. As in this present study, they concluded that each method showed that adding live yeast increased the relative abundance of a different fibrolytic bacterium. Both of the methods compared in this study revealed that yeast supplementation caused similar shifts in the population of bacteria that ferment specific nutrients, consistent with the findings of Pinloche et al. (2013).

The results of the present study partly support our first hypothesis that feeding live yeast alone or live yeast instead of killed yeast would increase the relative abundance of cellulolytic bacteria in the rumen. In addition, the results partly support our second hypothesis that similar bacterial diversity responses to yeast treatments will be found with qPCR and MiSeq sequencing.

CONCLUSIONS

The MiSeq sequencing and qPCR methods revealed different treatment effects on the relative abundance of specific ruminal bacteria taxa. Nevertheless, both methods revealed that yeast supplementation caused some similar shifts in the relative abundance of bacteria that ferment specific nutrients. The MiSeq sequencing and qPCR methods revealed that adding LLY to the diet or adding live yeast instead of killed yeast increased the relative abundance of different ruminal cellulolytic bacteria (*Ruminococcus* and *F. succinogenes*, respectively). The respective methods also showed that adding HDY to the diet increased the relative abundance of different amylolytic bacteria (*Ruminobacter* and *Bifidobacteria* vs. *Strep. bovis* and *S. ruminantium*, respectively). The relationship between estimates of the relative abundance of *F. succinogenes* from qPCR and MiSeq sequencing methods was moderately precise, but those for *M. elsdenii*, *R. flavefaciens*, and *S. ruminantium* were weak. The present study confirmed the low relative abundance of several culturable, well-known ruminal bacteria and indicated that the relative abundance and diversity of bacteria in the ruminal solid and liquid fractions differed. The most dominant ($\geq 1\%$ of total sequences) bacteria that responded to LLY addition whose functions are among the least understood in relation to the mode of action of yeast included *Paraprevotellaceae*, CF231, *Treponema*, and *Lachnospiraceae*. Future studies should aim to speciate, culture, and examine the function of these bacteria to better understand their roles in the mode of action of yeast. More systematic studies are needed to develop a standard operation procedure for rumen microbial ecology study.

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