Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants

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ABSTRACT

Three ruminally cannulated Holstein cows were used to characterize the dynamics of bacterial colonization of rice straw and alfalfa hay and to assess the differences in the composition and inferred gene function of the colonized microbiota between these 2 forages. Non-incubated (0 h) rice straw and alfalfa hay samples and residues in nylon bags incubated for 0.5, 2, 6, 16, and 48 h were analyzed for dry matter and were used for DNA extraction and MiSeq (Illumina Inc., San Diego, CA) sequencing of the 16S rRNA gene. The microbial communities that colonized the air-dried and nonincubated (0 h) rice straw and alfalfa hay were both dominated by members of the *Proteobacteria* (contributing toward 70.47% of the 16S RNA reads generated). In situ incubation of the 2 forages revealed major shifts in the community composition: *Proteobacteria* were replaced within 30 min by members belonging to the *Bacteroidetes* and *Firmicutes*, contributing toward 51.9 and 36.6% of the 16S rRNA reads generated, respectively. A second significant shift was observed after 6 h of rumen incubation, when members of the *Spirochaetes* and *Fibrobacteria* phyla became abundant in the forage-adherent community. During the first 30 min of rumen incubation, ~20.7 and 36.1% of the rice straw and alfalfa hay, respectively, were degraded, whereas little biomass degradation occurred between 30 min and 2 h after the rice straw or alfalfa hay was placed in the rumen. Significant differences were noted in attached bacterial community structure between the 2 forage groups, and the abundances of dominant genera *Anaeroplasma*, *Butyrivibrio*, *Fibrobacter*, and *Prevotella* were affected by the forage types. Real-time PCR results showed that the 16S rRNA copies of total bacteria attached to these 2 forages were affected by the forage types and incubation time, and higher numbers of attached bacterial 16S

rRNA were observed in the alfalfa hay samples than in the rice straw from 0.5 to 16 h of incubation. The metagenomes predicted by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) revealed that the forage types significantly affected 21 metabolic pathways identified in the Kyoto Encyclopedia of Genes and Genomes, and 33 were significantly changed over time. Collectively, our results reveal a difference in the dynamics of bacterial colonization and the inferred gene function of microbiota associated with rice straw and alfalfa hay within the rumen. These findings are of great importance for the targeted improvement of forage nutrient use efficiency in ruminants.

Key words: rumen, rice straw, alfalfa hay, microbiome

INTRODUCTION

Ruminant animals and ruminal microorganisms have evolved together for millions of years, resulting in a rumen microbial population consisting of a highly diverse collection of obligately anaerobic microorganisms, including fungi, protozoa, bacteria, and archaea (Russell and Rychlik, 2001). Within this microbiome, bacteria are the dominant domain and make the greatest contribution to digestion and conversion of feeds to volatile fatty acids and microbial proteins (Kim et al., 2011). A previous study showed that bacterial attachment is instrumental in the process of microbial degradation of dietary plant material (McAllister et al., 1994), and particle-associated bacteria account for 70 to 80% of rumen microbial matter (Craig et al., 1987). Therefore, studies on the mode of bacterial attachment and the subsequent digestion of the plant biomass are essential for improving ruminant nutrient use efficiency.

Over the last decade, many experiments have demonstrated that rumen microorganisms rapidly associate with and colonize recently ingested feed particles (Edwards et al., 2007; Huws et al., 2013, 2016; Piao et al., 2014, 2015), and these studies have revealed that the composition of colonizing microbial communities is affected by the incubation time (Bowman and Firkins,

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1993; Huws et al., 2013, 2016; Piao et al., 2014, 2015). Forage type is recognized as an important factor that affects the rumination time, rumen fermentation, and performance of the ruminant (Welch and Smith, 1969; Brask et al., 2013; Ding et al., 2015). One forage type, rice straw, is abundant and inexpensive, but is typically considered low-quality roughage. A second type, alfalfa, is well known for its high quality and is used worldwide as an important dietary forage in ruminant production; nevertheless, rice straw is the predominant forage source for ruminants in the tropical zones of the world. These 2 forages are known to differ in the digestibility of their DM in the rumen (Yang et al., 2011), and these differences could be attributed to differences in the physical structure and chemical characteristics between the 2 types of forage. However, the difference in the composition of degrading and colonizing ruminal bacterial communities of the 2 forages may also be an important factor.

In the current study, we hypothesized that forages with different quality will affect the composition of the microbiota that colonize and degrade the forage within the rumen, which will likely be accompanied by a difference in community function. Therefore, the primary objective of this study was to compare the effects of forage sources (rice straw vs. alfalfa hay) on the composition, structure, and inferred function of ruminal particle-associated microbiota in dairy cows. An additional aim was to investigate the temporal changes in the bacterial communities that colonize and degrade rice straw and alfalfa hay in the rumen.

MATERIALS AND METHODS

Animals and Sample Collection

Three mature, rumen-cannulated, nonlactating Holstein cows (average BW: 502 ± 25 kg) were used in this study. Cows were individually housed in 3 stalls in a barn with good ventilation and were fed 15 kg of DM (75% Chinese wild grass hay, 25% corn-based diet) per animal per day. The cattle were fed at 0700 and 1730 h, with one-half of the allowed daily ration given at each feeding. The cattle had free access to drinking water. All animal care procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Air-dried rice straw and alfalfa hay were ground into 2-mm pieces using a Wiley mill, and the ground samples were weighed into individual in situ nylon bags (10×20 cm; pore size = $50 \mu\text{m}$). Three bags containing 2 g of forage for each of the 2 forage treatments and 1 blank bag (i.e., a total of 7 bags for each of the 5 time periods, for a total of 35 bags per cow) were placed

into the rumen of each cow and removed after 0.5, 2, 6, 16, and 48 h. After removal from the rumen, 4 bags containing forages (2 for rice straw and 2 for alfalfa hay) and the blank bag were rinsed and manipulated in cold water until the water ran clear, then squeezed by hand to remove excess water and used for nutritional ingredient degradation analysis. The remaining 2 bags (one filled with rice straw and one with alfalfa for each cow) were washed with sterile saline to remove loosely attached microbes and then immediately frozen on dry ice and transported to the laboratory for storage at -80°C until DNA extraction.

Chemical Analysis

Relative biomass degradation during rumen incubation was determined by DM, CP, NDF, and ADF analysis. Dry matter and CP were determined according to AOAC methods (AOAC, 1990), and NDF and ADF were determined using the procedures of Larter (1992).

DNA Extraction and Quantitative Real-Time PCR Analysis

Total microbial genomic DNA was extracted from 200 mg of the nonincubated control sample and from each rumen-incubated rice straw and alfalfa sample. The DNA was extracted by a bead-beating method using a mini-bead beater (Biospec Products, Bartlesville, OK), followed by phenol-chloroform extraction, as described by Mao et al. (2012). The concentration and purity of DNA were determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE), and DNA samples were stored at -80°C until further processing.

Total attached bacterial 16S rDNA was quantified by real-time PCR. Primer pairs for the total bacteria were bacF (5'-CCATTGTAGCACGTGTGTAGCC-3') and total bacR (5'-CGGCAACGAGCGCAACCC-3'), as reported by Hook et al. (2011). The PCR amplifications were performed in triplicate with SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hertfordshire, UK). The total volume of reaction solution (20 μL) contained 0.5 ng of DNA, 10 μL of Fast SYBR Green Master Mix (Applied Biosystems), 0.4 μL of each primer (5 μM), 6.8 μL of nuclease-free water, 0.4 μL of ROX Reference Dye (50 $\times$), and 2 μL of the template, as described previously (Mao et al., 2015). Amplification was carried out using the following program: 95°C for 30 s for the initial denaturation and then 40 cycles of 95°C for 5 s followed by annealing/extension for 30 s at 60°C . A standard

curve was constructed using serial dilutions of plasmid DNA containing the 16S rRNA gene sequence of *Streptococcus bovis*. The real-time PCR efficiency ranged between 90 and 103%; negative controls without the DNA template were run with every assay to assess the overall specificity.

PCR Amplification, MiSeq Sequencing, and Sequencing Data Processing

Amplification and sequencing were performed as described elsewhere (Mao et al., 2015). Briefly, the V3–V4 regions of bacterial 16S rRNA genes were amplified using primers 338F (5'-barcode-ACTCCTRCGGGAG-GCAGCAG)-3' and 806R (5'-GGACTACCVGGG-TATCTAAT-3'), where the barcode is an 8-base sequence unique to each sample. Amplicons were purified and then pooled in equimolar amounts and paired-end sequenced (2×250) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) according to standard protocols. For data analysis, raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using the Quantitative Insights into Microbial Ecology (QIIME, v.1.8.0), as described by Caporaso et al. (2010b) with the following criteria, as described by Mao et al. (2015): operational taxonomic units (OTU) were clustered with a 97% similarity cutoff using UPARSE (Edgar, 2013), and chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). The most abundant sequences within each OTU (representative sequences) were aligned to the Greengenes database using PyNAST (Caporaso et al., 2010a) with the default parameters set by QIIME. Taxonomy was assigned to representative sequences using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) with a confidence value of 0.8 against the Greengenes 16S rRNA gene data set (v.13.8) (DeSantis et al., 2006). A phylogenetic tree was constructed within QIIME using FastTree. Rarefaction curves, α diversity, and β diversity calculations were also performed using QIIME. Principal coordinates analysis (PCoA) was used to compare groups of samples based on unweighted Uni-Frac distance metrics (Lozupone and Knight, 2005), and an unweighted distance-based analysis of molecular variance (AMOVA) was conducted to assess significant differences among samples using the Mothur v.1.3.0 program (Schloss et al., 2009). The similarity between the microbial communities associated with the 2 forages was analyzed through a hierarchical cluster analysis using the average-neighbor method. This analysis was conducted using the function hclust in the R stats package (R Project 3.0.2; <http://cran.r-project.org/>).

Functional Gene Prediction

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) is a bioinformatics tool that uses 16S ribosomal DNA sequences to predict the functional gene content of microorganisms (Langille et al., 2013). In the present study, we used PICRUSt to obtain an overview of the genomic and metabolic features represented by the adherent bacterial communities in our samples. We associated OTUs with known bacterial genomes precalculated in PICRUSt, by first picking closed OTUs against the Greengenes 16S rRNA gene database (13.5) using QIIME 1.7.0 (Caporaso et al., 2010b). The resulting OTU table was then normalized using the script `normalize_by_copy_number.py` and used for metagenome inference of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs using PICRUSt. The difference in the predicted molecular functions of the bacterial communities attached to the rice straw and alfalfa hay for the different incubation times was determined by partial least squares discriminant analysis (PLS-DA) using the SIMCA-P (11.5) software package (Umetrics, Umeå, Sweden).

Statistical Analysis

We related changes in total microbial composition to the chemistry of forage using redundancy analysis (RDA), as implemented in the Canoco 5.0 software package (Microcomputer Power, Ithaca, NY; Ter Braak and Smilauer, 2012). Different chemical composition was introduced as environmental (explanatory) variables. The relative contributions of the top 30 genus-level phylogenetic groups were used as response variables. Redundancy analysis was performed focusing on intersample correlation, and the Monte Carlo Permutation test was applied to decide whether chemical composition had any statistically significant influence on the microbial composition. Chemical ingredients were considered to have significant effects on microbial composition for P values < 0.05 .

The DM loss, real-time PCR data, bacterial prevalence, and relative abundance values of the KEGG pathways were analyzed using general linear model (GLM) of SPSS (SPSS v.16, SPSS Inc., Chicago, IL) according to the following equation: $Y_{ijk} = \mu + G_i + T_j + GT_{ij} + e_{ijk}$, where Y_{ijk} was the observation, μ was the overall mean, G_i was the forage effect ($i = 2$), T_j was the incubation time effect ($j = 5$), GT_{ij} was the interaction between the forage types and incubation time, and e_{ijk} was the residual error. All P -values from the GLM tests of relative abundance of bacterial taxa

were corrected for a false discovery rate (**FDR**) of 0.05 using the Benjamini–Hochberg method (Benjamini and Hochberg, 2000), and FDR-corrected *P*-values below 0.05 ($\text{FDR} < 0.05$) were considered significant. If GLM tests indicated a significant difference between means, subsequent Tukey's multiple range tests was done to determine which of the means differed from each other. Main effect and differences were considered significant when $P < 0.05$.

RESULTS

Chemical Composition and Biomass Degradation of Rice Straw and Alfalfa Hay

The chemical composition of the individual forages is listed in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2016-11398>). Compared with rice straw, alfalfa hay had greater amounts of CP and ether extract, and lower amounts of NDF, ADF, and ash. During the rumen incubation period, alfalfa hay had higher DM loss ($P < 0.001$), NDF degradation ($P = 0.001$), and CP degradation ($P < 0.001$) than rice straw (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2016-11398>). Dry matter loss, NDF degradation, and CP degradation were significantly affected by incubation time ($P < 0.001$, Supplemental Table S2).

Data Acquisition and Analysis

In this study, a 16S rRNA gene sequence analysis of samples generated 1,865,166 sequences with a length greater than 400 bp (Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2016-11398>) and an average of $51,810 \pm 6,115$ sequences per sample. Quality filtering by QIIME generated 1,230,669 high-quality reads, accounting for 66% of the raw reads, and an average of $34,185 \pm 8,724$ sequences per sample. The average sequence length was 437 bp.

Dominant Taxa Colonizing the Rice Straw and Alfalfa Hay at 0 h and Their Fate During Rumen Incubation

The microbial communities colonizing the nonincubated (0 h) rice straw and alfalfa were both dominated by the members of *Proteobacteria* (70.47%), *Actinobacteria* (18.69%), *Bacteroidetes* (6.30%), and *Firmicutes* (4.26%) (Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2016-11398>). The 16S rRNA data showed that the relative abundances of the communities colonizing the rice straw and alfalfa both decreased substantially to very low levels within the 0.5 h of rumen incubation, with some taxa decreasing

to below detection limits (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2016-11398>).

Diversity of the Bacterial Microbiota Attached to Rice Straw and Alfalfa After 0.5 h of Rumen Incubation

We assessed whether sampling provided sufficient OTU coverage to describe the bacterial composition of each region accurately by generating individually based rarefaction curves for each sample (Supplemental Figure S2; <http://dx.doi.org/10.3168/jds.2016-11398>). The Good's coverage was greater than 0.98, implying that sampling was sufficient for the samples collected at all incubation times (Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2016-11398>). At the 0.03 dissimilarity level, the OTU numbers and the abundance-based coverage estimator (**ACE**), Chao 1, and Shannon indices were significantly affected ($P < 0.001$ to $P = 0.005$) by the forage types and incubation time. The ACE, Chao 1, and Shannon indices were lower in both the rice straw and alfalfa samples collected at 48 h of incubation compared with 30 min of incubation (Table 1).

Principal coordinates analysis plots based on unweighted UniFrac distance metrics revealed that the alfalfa-colonizing microbiota exhibited clearly noticeable changes that paralleled the changes in incubation time (AMOVA: *F*-statistic = 1.91, $P = 0.01$) (Figure 1). By contrast, the microbiota attached to the rice straw were not markedly different from each other (AMOVA: *F*-statistic = 0.97, $P = 0.513$). Analysis of molecular variance also showed that the ruminal bacterial communities attached to the rice straw (AMOVA: *F*-statistic = 2.85, $P = 0.006$) were significantly different from the colonizing microbiota of the alfalfa.

Hierarchical cluster analysis of the dendrogram showed that 2 clusters were formed, and it also revealed differences in the composition of microbiota between rumen incubated samples (cluster 1) and nonincubated samples (cluster 2) (Supplemental Figure S3; <http://dx.doi.org/10.3168/jds.2016-11398>). Cluster 2 included 2 distinct subclusters. Most samples from 16 and 48 h of incubation fell within subcluster 1. Conversely, subcluster 2 primarily comprised samples from 0.5, 2, and 6 h of incubation.

Characterization and Comparison of the Bacterial Communities Attached to Rice Straw and Alfalfa After 0.5 h of Rumen Incubation

A total of 15 bacterial phyla were identified in all samples (Supplemental Figure S4; <http://dx.doi.org/10.3168/jds.2016-11398>). The majority of the se-

Figure 1. Principal coordinate analysis (PCoA) of microbial diversity across all samples using a unweighted UniFrac metric. The percentage of variation explained by PC1 and PC2 are indicated in the axis. S = rice straw samples; A = alfalfa samples. Color version available online.

quences obtained from the samples of rumen-incubated rice straw and alfalfa hay belonged to *Firmicutes* (rice straw, 34.19% vs. alfalfa, 39.05%), *Bacteroidetes* (rice straw, 52.84% vs. alfalfa, 50.91%), *Spirochaetae* (rice straw, 2.47% vs. alfalfa, 1.83%), *Tenericutes* (rice straw, 2.17% vs. alfalfa, 2.81%), *Proteobacteria* (rice straw, 2.08% vs. alfalfa, 1.56%), and *Fibrobacteres* (rice straw, 2.01% vs. alfalfa, 0.42%). The other 9 phyla were relatively minor (<2% of total sequences) in abundance in comparison.

At the genus level, 159 and 163 taxa were observed across all rumen-incubated rice straw and alfalfa samples. For clarity and visualization purposes, the top 50 most abundant taxa detected in the present study are presented in Supplemental Figure S5 (<http://dx.doi.org/10.3168/jds.2016-11398>). The most abundant taxa (those with a relative abundance of $\geq 3\%$ for at least 1 incubation time) attached to the rumen-incubated rice straw and alfalfa hay samples included *Fibrobacter*, *Prevotella*, *Ruminococcus*, *Treponema*, *Butyrivibrio*, and *Anaeroplasma*, as well as the unclassified taxa derived from *Bacteroidales* (family), *Christensenellaceae* (family), *Lachnospiraceae* (family), *Prevotellaceae* (family), *Rikenellaceae* (order), and *Ruminococcaceae* (family). On a genus level, the abundance of the dominant taxa *Anaeroplasma*, *Butyrivibrio*, *Fibrobacter*, *Prevotella*, unclassified *Bacteroidales*, unclassified *Rikenellaceae*, and unclassified *Ruminococcaceae* were affected ($FDR < 0.001$ to $FDR = 0.028$) by the forage diet (Table 2), and the percentage of *Anaeroplasma*, *Butyrivibrio*, *Fibrobacter*, *Prevotella*, *Ruminococcus*, *Treponema*, unclassified *Bacteroidales*, unclassified *Christensenellaceae*, unclassified *Lachnospiraceae*, unclassified *Rikenellaceae*, and unclassified *Ruminococcaceae* changed ($FDR < 0.001$ to $FDR = 0.048$) over time.

The samples of rumen-incubated rice straw showed that most of the dominant taxa *Butyrivibrio*, *Prevotella*, and unclassified *Christensenellaceae* decreased after 0.5 h of incubation ($P < 0.05$) (Table 2), while *Treponema*, unclassified *Bacteroidales*, and unclassified *Rikenellaceae* increased in abundance at 48 h of incubation compared with the levels at 0.5 h, with the highest amounts occurring in the samples at 48 h of incubation. A higher abundance of *Fibrobacteria* was observed

Table 1. The α diversity of bacterial community attached to rice straw and alfalfa hay at different incubation times

Item	OTU ¹ numbers		ACE ²		Chao 1 value		Shannon index	
	Rice straw	Alfalfa	Rice straw	Alfalfa	Rice straw	Alfalfa	Rice straw	Alfalfa
0.5 h	1,546 ^a	1,705 ^a	1,780 ^a	1,839 ^a	1,804 ^a	1,868 ^a	6.05 ^a	6.25 ^a
2 h	1,578 ^a	1,649 ^b	1,761 ^{ab}	1,802 ^a	1,770 ^{ab}	1,820 ^a	6.07 ^a	6.12 ^{ab}
6 h	1,402 ^a	1,580 ^b	1,638 ^{ab}	1,740 ^a	1,658 ^{ab}	1,759 ^a	5.89 ^{ab}	6.05 ^{ab}
16 h	1,513 ^a	1,588 ^b	1,695 ^{ab}	1,763 ^a	1,721 ^{ab}	1,786 ^a	6.04 ^a	6.10 ^{ab}
48 h	1,377 ^b	1,388 ^b	1,576 ^b	1,602 ^b	1,602 ^b	1,628 ^b	5.50 ^b	5.63 ^b
SEM ³	84		91		93		0.26	
P-value ⁴								
F	<0.001		0.01		0.005		<0.001	
T	<0.001		<0.001		<0.001		<0.001	
F $\times$ T	0.219		0.916		0.915		<0.001	

^{a,b}Values within the same column with different superscripts were significantly different ($P < 0.05$).

¹OTU = operational taxonomic units.

²ACE = abundance-based coverage estimator.

³SEM = standard error of the difference of the means.

⁴F = effect of forage types; T = effect of incubation time; F $\times$ T = interaction effect of forage types and incubation time.

Table 2. Comparison of the dominant taxa (average relative abundance $\geq 3\%$ for at least one incubation time) attached to rice straw and alfalfa over time within the rumen

Taxa	Rice straw (%)					Alfalfa (%)					False discovery rate corrected P -value ²			
	0.5 h	2 h	6 h	16 h	48 h	0.5 h	2 h	6 h	16 h	48 h	SEM ¹	F	T	F $\times$ T
<i>Firmicutes</i>														
<i>Butyrivibrio</i>	1.82 ^a	1.11 ^b	1.22 ^{ab}	0.87 ^b	0.83 ^b	4.43 ^{ab*}	4.47 ^{ab*}	4.19 ^{ab*}	3.09 ^{ab}	1.13 ^b	0.30	<0.001	0.007	0.159
<i>Ruminococcus</i>	2.18 ^{bc}	2.91 ^{ab}	2.31 ^{ab}	3.13 ^a	1.34 ^c	3.05 ^a	2.12 ^{ab}	3.24 ^a	2.87 ^{ab}	0.93 ^b	0.17	0.948	0.001	0.227
Unclassified <i>Christensenellaceae</i>	8.68 ^a	8.52 ^{ab}	7.13 ^{abc}	5.41 ^c	5.98 ^{bc}	12.37 ^a	9.82 ^{ab}	5.60 ^c	4.66 ^c	8.41 ^b	0.44	0.062	<0.001	0.013
Unclassified <i>Lachnospiraceae</i>	7.91	5.57	7.33	8.84	7.91	10.79 ^{ab}	8.76 ^{ab}	11.63 ^a	10.23 ^{ab}	4.68 ^b	0.48	0.101	0.048	0.119
Unclassified <i>Ruminococcaceae</i>	7.47 ^b	11.90 ^a	8.68 ^{ab}	6.55 ^b	6.60 ^b	8.42 ^{ab}	6.68 ^{abc*}	4.51 ^c	4.98 ^c	10.08 ^a	0.43	0.025	0.001	<0.001
<i>Bacteroidetes</i>														
<i>Prevotella</i>	21.60 ^a	10.54 ^b	11.80 ^b	13.98 ^{ab}	8.99 ^b	20.06 ^b	20.11 ^{b*}	31.92 ^{ab*}	17.58 ^b	4.97 ^c	1.47	0.004	<0.001	0.001
Unclassified <i>Bacteroidales</i>	10.03 ^c	20.84 ^a	16.21 ^b	14.14 ^b	19.59 ^a	7.37 ^b	10.58 ^{b*}	7.35 ^{b*}	12.53 ^b	26.91 ^{a*}	1.16	0.004	<0.001	<0.001
Unclassified <i>Prevotellaceae</i>	9.44	5.93	9.27	7.33	5.95	4.75	8.40	7.96	9.05	5.06	0.49	0.808	0.473	0.404
Unclassified <i>Rikenellaceae</i>	9.33 ^b	14.87 ^{ab}	15.66 ^{ab}	12.27 ^b	19.41 ^a	8.32 ^{ab}	9.62 ^{b*}	5.35 ^{c*}	9.63 ^{b*}	22.80 ^a	1.01	0.006	<0.001	0.007
<i>Fibrobacteres</i>														
<i>Fibrobacter</i>	0.69 ^b	0.17 ^b	0.73 ^b	5.29 ^a	3.21 ^{ab}	0.08 [*]	0.18	0.41	1.06 [*]	0.39 [*]	0.34	0.004	0.001	0.045
<i>Spirochaetes</i>														
<i>Treponema</i>	1.61 ^{bc}	0.37 ^c	0.92 ^c	3.83 ^{ab}	4.15 ^a	0.46 ^b	0.56 ^b	1.88 ^b	4.72 ^a	0.69 ^b	0.33	0.381	<0.001	0.021
<i>Tenericutes</i>														
<i>Anaerotrasma</i>	0.74	0.48	0.59	0.80	0.16	0.43 ^b	0.39 ^b	2.01 ^{ab}	3.15 ^{ab*}	0.30 ^b	0.19	0.028	0.002	0.038

^{a-c}Within the same row, mean values with a different superscripted lowercase letter differ significantly.¹SEM = standard error of the difference of the means.²F = effect of incubation time; F $\times$ T = interaction effect of forage types and incubation time.*Within the same row, taxa are significantly different ($P < 0.05$) in relative abundance between rice straw and alfalfa hay at the same incubation time point.

at 16 h of incubation compared with 0.5, 2, and 6 h of incubation. The proportion of genus *Ruminococcus* was higher at 2, 6, and 16 h of incubation compared with 48 h ($P < 0.05$) (Table 2). The samples of rumen-incubated alfalfa showed no change in the proportion of *Butyrivibrio* ($P > 0.05$) from 0.5 to 16 h of incubation and a significant decrease ($P < 0.05$) at 48 h of incubation. The proportion of *Prevotella* was higher at 6 h of incubation compared with those at 0.5, 2, 16, and 48 h. Higher percentages of *Treponema* and *Anaeroplasm*a were observed at 16 h of incubation compared with those at 0.5, 2, and 48 h. The unclassified *Bacteroidales* and unclassified *Rikenellaceae* increased in abundance after 0.5 h of incubation, with the greatest abundance at 48 h. The abundances of *Ruminococcus* and unclassified *Lachnospiraceae* were unchanged from 2 to 16 h of incubation. The percentage of unclassified *Ruminococcaceae* was lower at 6 and 16 h of incubation than at 0.5 and 48 h.

At the genus level, the proportion of *Butyrivibrio* was significantly higher in alfalfa samples than in rice straw samples at 0.5, 2, and 6 h of incubation, and the rice straw presented a higher ($P < 0.05$) percentage of unclassified *Ruminococcaceae* at 2 h of incubation (Table 2). At 2 and 6 h of incubation, a higher percentage of *Prevotella* was observed ($P < 0.05$) in alfalfa samples compared with the rice straw, while unclassified *Rikenellaceae* and unclassified *Bacteroidales* were more abundant ($P < 0.05$) in the rice straw samples. The abundance of *Fibrobacter* was significantly higher in rice straw samples than in alfalfa samples at 16 and 48 h of incubation, and rice straw samples also presented a higher ($P < 0.05$) percentage of *Treponema* at 0.5 h of incubation. *Anaeroplasm*a were more abundant ($P < 0.05$) in the alfalfa samples compared with the rice straw samples at 16 h of incubation. Alfalfa samples presented a higher abundance of unclassified *Bacteroidales* compared with rice straw samples at 48 h of incubation.

Predicted Functions of Bacteria Attached to Rice Straw and Alfalfa Hay After 0.5 h of Rumen Incubation

Using PICRUSt as a predictive exploratory tool, we found that a total of 40 gene families occurred in all samples (Figure S6; <http://dx.doi.org/10.3168/jds.2016-11398>). We performed PLS-DA on the relative abundance values of KEGG pathways represented from the bacteria associated with rice straw and alfalfa and found a clear distinction between the clustering of rice straw and alfalfa hay samples (Figure 2-A). Of the 40 gene families, the majority of the genes that were ob-

tained belonged to replication and repair (10.76% of total genes inferred by PICRUSt), amino acid metabolism (10.25%), membrane transport (9.87%), carbohydrate metabolism (10.22%), energy metabolism (6.11%), and translation (6.30%). Of the 40 gene families, 21 were significantly affected (FDR < 0.05) by the forage types (Table S5; <http://dx.doi.org/10.3168/jds.2016-11398>), and 33 were significantly changed (FDR < 0.05) over time. Of the 6 predominant gene families mentioned earlier, in the rumen-incubated rice straw samples, the relative abundances of the genes involved in carbohydrate metabolism decreased significantly from 0.5 to 48 h of incubation, while the abundance of genes involved in amino acid metabolism increased significantly ($P < 0.05$) after incubation (Figure 2B). In the rumen-incubated alfalfa samples, the proportion of the gene families related to energy metabolism, replication and repair, amino acid metabolism, and translation increased significantly ($P < 0.05$) from 0.5 to 48 h of incubation, while the abundance of genes involved in membrane transport decreased significantly after incubation, and no significant difference ($P > 0.05$) was observed in the proportion of the gene families related to carbohydrate metabolism within 48 h of incubation.

Attached Bacterial Density

Total bacterial populations in the rice straw and alfalfa hay samples were estimated with a real-time PCR analysis by measuring the total copy number of bacterial 16S rRNA genes. The forage quality and incubation time both had a significant effect on the copy number of bacterial 16S rRNA genes determined on rice straw and alfalfa hay (Table 3). The highest bacterial number in the samples of rumen-incubated rice straw was observed at 48 h of incubation. The alfalfa-associated bacterial densities increased after incubation, and the greatest bacterial numbers were also observed at 48 h of incubation. Higher amounts of attached bacterial 16S rRNA ($P < 0.05$) were observed in the alfalfa hay samples than in the rice straw from 0.5 to 16 h of incubation.

Biomass Degradation Coordinated with Microbial Colonization

Dry matter and CP degradation of rice straw and alfalfa hay was rapidly initiated within 0.5 h of rumen incubation (Figure 3 and Table S2; <http://dx.doi.org/10.3168/jds.2016-11398>). Corresponding to this period, we observed noticeable increases in *Prevotella*, *Ruminococcus*, unclassified *Bacteroidales*, unclassified *Rikenellaceae*, unclassified *Lachnospiraceae*, unclassified

A

B

Figure 2. Metagenomic functional predictions for the samples. (A) Partial least squares (PLS) discriminant analysis of microbial functional diversity across all incubated samples using the relative abundances of functional pathways. (B) Comparisons of the 5 predominant gene pathways of the bacterial microbiota detected in the incubated sample. S = rice straw samples; A = alfalfa samples; KEGG = Kyoto Encyclopedia of Genes and Genomes. Color version available online.

Table 3. Total bacterial numbers ($\log_{10}$ gene copies/g of undigested forage) attached to rice straw and alfalfa hay following incubation for up to 48 h

Forage	Incubation time					SEM ¹	<i>P</i> -value ²		
	0.5 h	2 h	6 h	16 h	48 h		F	T	F × T
Rice straw	10.40 ^d	8.12 ^e	10.77 ^c	11.02 ^b	11.18 ^a	0.12	<0.001	<0.001	<0.001
Alfalfa	10.60 ^b	10.86 ^{b*}	11.15 ^{a*}	11.22 ^{a*}	11.12 ^a				

^{a-c}Mean values with different superscripted lowercase letters within the same row differ significantly ($P < 0.05$).

¹SEM = standard error of the difference of the means.

²Probability of a significant effect due to forage types (F), incubation time (T), and their interaction (F × T).

*Within the same column, means differ significantly ($P < 0.05$).

fied *Christensenellaceae*, unclassified *Prevotellaceae*, and unclassified *Ruminococcaceae* from 0 to 0.5 h of incubation. From 0.5 to 48 h of incubation, DM degradation of rice straw proceeded somewhat linearly, at a rate of ~0.60% biomass per hour. The total biomass degradation of alfalfa hay from 0.5 to 16 h of incubation occurred at a rate of 1.87% biomass per hour of incubation, and it decreased to 0.23% biomass per hour of incubation following 16 h of incubation. We observed noticeable decreases in the abundance of *Fibrobacter*, *Treponema*, and *Butyrivibrio* that corresponded to this period. Compared with the rice straw, the degradation of CP and NDF in alfalfa hay following rumen incubation appeared to be triphasic, and the degradation of CP and NDF degradation mainly occurred between 6 and 16 h, corresponding to degradation of 20 and 40% of the total biomass and an increased abundance of taxa assigned to unclassified *Rikenellaceae* and *Treponema* species in the microbial communities.

We evaluated the effect of the content of the feed ingredients on the composition of microbiota colonizing the rice straw and alfalfa by performing a redundancy analysis using NDF, CP, and neutral detergent-soluble non-crude protein (DM – NDF – CP) as constrained explanatory variables and the relative abundances of dominant genera (those with a relative abundance of $\geq 2\%$ for at least 1 incubation time) as response variables (Figure 4). Redundancy analysis of the composition of the microbial communities identified the NDF (pseudo- $F = 10.2$, $P = 0.002$), CP (pseudo- $F = 5.2$, $P = 0.002$), and neutral detergent-soluble non-crude protein (pseudo- $F = 2.6$, $P = 0.036$) as significant factors, explaining 27.3 (Axis 1) and 11.1% (Axis 2), respectively, of the variation in microbial composition (Figure 4). On the redundancy analysis ordination plot, 10 genera, including *Butyrivibrio*, *Anaeroplasma*, *Pseudobutyrvibrio*, *Succiniclasticum*, *Prevotella*, *Ruminococcus*, *Saccharofermentans*, unclassified *Lachnospiraceae*, unclassified *Christensenellaceae*, and unclassified *Lentisphaerae*, were correlated with the CP content, while *Bacteroi-*

des, *Saccharofermentans*, *Ruminococcus*, *Papillibacter*, *Succiniclasticum*, *Prevotella*, *Fibrobacter*, unclassified *Ruminococcaceae*, unclassified *Lentisphaerae*, unclassified *Prevotellaceae*, unclassified *Mollicutes*, unclassified *Bacteria*, and unclassified *Christensenellaceae* were related to the CP.

DISCUSSION

We evaluated the temporal changes in the ruminal bacterial community colonizing and degrading rice straw and alfalfa hay in dairy cattle. In the present study, our MiSeq sequencing of 16S rRNA revealed substantial temporal changes occurring within the first 30 min and after 6 h of rumen incubation. The major shift in the community composition during the first 30 min was phylum *Proteobacteria* being replaced by members belonging to the *Bacteroidetes* and *Firmicutes*. The change to a secondary phase was mainly associated with decreases in abundance of the taxa *Butyrivibrio*, *Prevotella*, *Ruminococcus*, and unclassified *Christensenellaceae* and increases in *Fibrobacter*, *Treponema*, unclassified *Bacteroidales*, and unclassified *Rikenellaceae*. These observations are consistent with analogous trials on rumen-incubated air-dried switchgrass, for which pyrosequencing-based analyses identified discrete microbial profiles within the first 30 min and after 4 h of rumen incubation (Piao et al., 2015). Our data also revealed that the temporal changes in the adherent microbiota are associated with the biomass loss of rice straw and alfalfa hay; indeed, the rates of rice straw and alfalfa degradation were greatest within the first 30 min of rumen incubation during which 20.6 and 30.1% of the total biomass was lost, respectively, and this may reflect a rapid utilization of soluble sugars and other easily fermentable nutrients. On the other hand, the bacteria detected from 16 to 48 h play a more focused role in plant fiber degradation, which is reflected in the results showing that more fiber-degrading bacteria, such as *Fibrobacter* and *Treponema*, were observed in

the samples of rice straw and that a higher proportion of unclassified *Bacteroidales* was present in alfalfa hay samples from 16 to 48 h of incubation than from 0.5 to 6 h.

Limited information is available on the effects of different forage types on the structure, diversity, and density of the attached microbiota. In the present study, our results suggest that forage types strongly

Figure 3. Microbial succession and biomass degradation during rumen incubation. Heat maps show succession of genera recruiting >2% of the generated sequences. Line graphs show the relative DM change during rumen incubation. S = rice straw samples; A = alfalfa samples. Color version available online.

affect the composition and structure of the attached microbiota of particles in the rumen of dairy cattle, as reflected by the clustering of the samples using PCoA and confirmed by AMOVA. Our findings also revealed that the 16S rRNA gene copy number of ruminal bacteria attached to the rice straw and alfalfa hay differed markedly from 0.5 to 16 h of incubation. We also found that more bacteria colonized the alfalfa hay than the rice straw, which may be partly explained by the differing physiochemical conditions, including the content of chemical ingredients and availability, between the 2 forages types. Indeed, our results showed that alfalfa hay had a greater content of neutral detergent solubles and a lower NDF content compared with rice straw. Thus, the difference in the composition and density of attached bacterial microbiota between the 2 forage types is likely due to the differing chemical composition in the 2 forages, as mentioned earlier.

The present study revealed that the genera *Butyrivibrio*, *Prevotella*, *Ruminococcus*, *Treponema*, and *Fibrobacter* spp. dominated in both forages, and this

finding is partly consistent with the report of Huws et al. (2016), who reported that *Butyrivibrio*, *Fibrobacter*, *Olsenella*, and *Prevotella* spp. abundances were high in the community of rumen bacteria colonizing fresh perennial ryegrass, indicating that these taxa play an important role in forage degradation in the rumen of dairy cattle. Of the dominant colonizing microbes detected in the present study, the abundances of *Butyrivibrio*, *Fibrobacter*, and *Prevotella* were significantly affected by the forage types. *Butyrivibrio* spp. are known to have proteolytic and polysaccharide-degrading activity (Fulghum and Moore, 1963; Kelly et al., 2010). In the present study, the RDA profile revealed that *Butyrivibrio* abundance was positively associated with the CP content. Thus, the fact that the abundance of *Butyrivibrio* was higher in alfalfa-associated microbiota than in the corresponding rice straw sample from 0.5 to 6 h of incubation might be partly explained by the higher content of CP in alfalfa hay compared with the rice straw. *Prevotella* has been reported as the most abundant genus in the rumen of adult dairy cattle, and it is

Figure 4. Biplot of the redundancy analysis (RDA) based on the relative abundance of dominant genera (>1% of total sequence). Constrained explanatory variables are indicated by open triangle arrows. Neutral detergent-soluble non-crude protein (NDSNCP) is estimated as $\text{NDSNCP} = \text{DM} - \text{NDF} - \text{CP}$. Upper left shows P -value of Monte Carlo permutation test. Color version available online.

also associated with ruminal carbohydrate and protein fermentation (Wallace et al., 1999; Chiquette et al., 2008). The present study showed that *Prevotella* spp. dominated in the associated microbiomes of both rice straw and alfalfa, and this finding is consistent with a previous report (Stevenson and Weimer, 2007). In addition, the present study also revealed that the *Prevotella* abundance was positively associated with the content of CP, and the content of CP is greater in the alfalfa hay compared with rice straw, providing a reasonable explanation for the higher abundance of *Prevotella* observed in the alfalfa samples than in the rice straw samples from 0.5 to 6 h of incubation. *Fibrobacteria* is one of the main cellulose-degrading bacterial genera in the rumen, and it may have an important role in the degradation of low-quality forages such as rice straw (Tajima et al., 2001). In the present study, correlation analysis revealed that *Fibrobacteria* abundance was positively associated with the content of NDF; thus, a higher abundance of *Fibrobacteria* was accordingly observed in the rice straw incubated for 16 and 48 h in the rumen than in the corresponding alfalfa hay samples.

The present study revealed that higher abundances of some unclassified groups, including unclassified *Bacteroidales*, unclassified *Christensenellaceae*, unclassified *Lachnospiraceae*, unclassified *Prevotellaceae*, unclassified *Rikenellaceae*, and unclassified *Ruminococcaceae*, were noted in particles of both forages. Among these organisms of unknown function, the *Lachnospiraceae* and *Ruminococcaceae* were the 2 largest groups of unclassified bacteria in the rumen. A meta-analysis revealed that these 2 unclassified bacterial groups are probably predominant ruminal bacteria and might have an important role in ruminal fiber digestion (Kim et al., 2011). However, little information is available regarding the possible function of the other unclassified groups, such as the *Christensenellaceae* and *Prevotellaceae*, in the rumen. In the present study, the reasons are not clear for why large abundances of unclassified groups were observed in the samples of rice straw and alfalfa hay and why some of these were affected by the incubation time and forage types. The explanations might be related to the potential roles of these organisms in the degradation of fiber and the dynamic changes in chemical composition of forage during incubation. Indeed, our previous study revealed that the unclassified *Rikenellaceae* and unclassified *Ruminococcaceae* dominated in the ruminal bacterial microbiota of dairy cattle fed a corn stover-based diet compared with an alfalfa-based diet (Zhang et al., 2014). In the present study, the RDA profiles showed that unclassified *Rikenellaceae*, unclassified *Ruminococcaceae*, and unclassified *Bacteroidales* were positively correlated with NDF content. Thus, the

results indicate that these 3 unclassified populations may have an important role in the digestion of low-quality forages, such as rice straw.

We determined the potential functions of the attached bacteria in the samples by using PICRUSt to infer putative metagenomes from the 16S rRNA gene profiles. The most abundant functional categories were those corresponding to the functions of amino acid metabolism, carbohydrate metabolism, membrane transport, and replication and repair. These findings are in agreement with general metabolic functions (such as carbohydrate, protein, and amino acid metabolism) being essential for microbial survival, and they are also consistent with the observations of other metagenomic studies in dairy cows (Parmar et al., 2014; Pitta et al., 2016). In addition, findings of the present study revealed significant differences in bacterial function depending on the incubation time. For example, genes relating to carbohydrate metabolism were more abundant in the rice straw-associated microbiota at 0.5, 2, and 6 h of incubation than at 16 and 24 h, whereas the genes associated with amino acid metabolism were more enriched in the colonizing microbiota of both rice straw and alfalfa collected at 48 h of incubation than at 0.5 h. In addition, the genes associated with carbohydrate metabolism were more enriched in the associated microbiota of alfalfa incubated from 6 to 48 h than in the corresponding rice straw digests. One possibility is that alfalfa provides a greater supply of easily fermentable carbohydrates during 6 to 48 h of incubation.

In summary, this study demonstrated that a highly diverse rumen bacterial community colonized rice straw and alfalfa hay rapidly and continuously changed during a 48-h incubation period, revealing high abundances of *Butyrivibrio*, *Prevotella*, *Ruminococcus*, *Treponema*, *Fibrobacter* and some unclassified microbes in the colonizing microbiome. The forage types and incubation times have a significant effect on the diversity, composition, structure, and potential function of colonizing microbiota, and RDA revealed that the content of CP and NDF have a significant effect on the composition of the attached microbiota. A greater abundance of *Butyrivibrio*, *Prevotella*, and unclassified *Lachnospiraceae* was observed in alfalfa hay samples, while the rice straw samples contained a higher proportion of *Fibrobacter*, *Treponema*, and unclassified *Bacteroidales*, indicating that the abundance of dominant taxa in colonizing microbiome is associated with the forage types. Generally, the results obtained here provide a comprehensive overview of the effects of 2 differing quality forages on the diversity, composition, structure, and temporal changes of colonizing microbiota, and they indicate that the rumen microbial colonization events are rapid within the

rumen and that the differences in ecological community are dependent on the differing chemical composition, especially the contents of CP and NDF.

CONCLUSIONS

Collectively, our results reveal differing dynamics of bacterial colonization and inferred gene function of associated microbiota between the alfalfa hay and rice straw within the rumen. These findings are of great importance for the targeted improvement of forage nutrient use efficiency in ruminants.

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