Bacterial diversity and composition of alfalfa silage as analyzed by Illumina MiSeq sequencing: Effects of *Escherichia coli* O157:H7 and silage additives

I. M. Ogunade,* Y. Jiang,* A. A. Pech Cervantes,* D. H. Kim,† A. S. Oliveira,‡ D. Vyas,* Z. G. Weinberg,§ K. C. Jeong,* and A. T. Adesogan*¹

*Department of Animal Sciences, Institute of Food and Agricultural Sciences, University of Florida, Gainesville 32608

†Division of Applied Life Science (BK21Plus, Institute of Agriculture and Life Science), Gyeongsang National University, Jinju 660-701, South Korea

‡Instituto de Ciências Agrárias e Ambientais, Universidade Federal de Mato Grosso, Campus Sinop, Sinop, MT 78557-267, Brazil

§Department of Food Safety and Quality, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel 50250

ABSTRACT

The first objective of this study was to examine effects of adding *Escherichia coli* O157:H7 with or without chemical or microbial additives on the bacterial diversity and composition of alfalfa silage. The second objective was to examine associations between the relative abundance of known and unknown bacterial species and indices of silage fermentation quality. Alfalfa forage was harvested at 54% dry matter, chopped to a theoretical length of cut of 19 mm, and ensiled in quadruplicate in laboratory silos for 100 d after the following treatments were applied: (1) distilled water (control); (2) 1×10^5 cfu/g of *E. coli* O157:H7 (EC); (3) EC and 1×10^6 cfu/g of *Lactobacillus plantarum* (EC+LP); (4) EC and 1×10^6 cfu/g of *Lactobacillus buchneri* (EC+LB); and (5) EC and 0.22% propionic acid (EC+PA). After 100 d of ensiling, the silage samples were analyzed for bacterial diversity and composition via the Illumina MiSeq platform (Illumina Inc., San Diego, CA) and chemically characterized. Overall, *Firmicutes* ($74.1 \pm 4.86\%$) was the most predominant phylum followed by *Proteobacteria* ($20.4 \pm 3.80\%$). Relative to the control, adding *E. coli* O157:H7 alone at ensiling did not affect bacterial diversity or composition but adding EC+LP or EC+LB reduced the Shannon index, a measure of diversity (3.21 vs. 2.63 or 2.80, respectively). The relative abundance of *Firmicutes* (69.2 and 68.8%) was reduced, whereas that of *Proteobacteria* (24.0 and 24.9%) was increased by EC+LP and EC+PA treatments, relative to those of the control (79.5 and 16.5%) and EC+LB (77.4 and 18.5%) silages, respectively. Compared with the control, treatment with EC+LP increased the relative abundance of *Lactobacillus*, *Sphingomonas*, *Pantoea*, *Pseudomonas*, and *Erwinia* by 426, 157, 200,

194, and 163%, respectively, but reduced those of *Pediococcus*, *Weissella*, and *Methylobacterium* by 5,436, 763, and 250%, respectively. Relative abundance of *Weissella* (9.19%) and *Methylobacterium* (0.94%) were also reduced in the EC+LB silage compared with the control (29.7 and 1.50%, respectively). Application of propionic acid did not affect the relative abundance of *Lactobacillus*, *Weissella*, or *Pediococcus*. Lactate concentration correlated positively ($r = 0.56$) with relative abundance of *Lactobacillus* and negatively ($r = -0.41$) with relative abundance of *Pediococcus*. Negative correlations were detected between ammonia-N concentration and relative abundance of *Sphingomonas* ($r = -0.51$), *Pantoea* ($r = -0.46$), *Pseudomonas* ($r = -0.45$), and *Stenotrophomonas* ($r = -0.38$). Silage pH was negatively correlated with relative abundance of *Lactobacillus* ($r = -0.59$), *Sphingomonas* ($r = -0.66$), *Pantoea* ($r = -0.69$), *Pseudomonas* ($r = -0.69$), and *Stenotrophomonas* ($r = -0.50$). Future studies should aim to speciate, culture, and determine the functions of the unknown bacteria detected in this study to elucidate their roles in silage fermentation.

Key words: MiSeq sequencing, silage bacterial diversity, silage additive

INTRODUCTION

Ensiling is a forage preservation method based on lactic acid fermentation that occurs spontaneously under anaerobic conditions because of activities of a complex community of forage epiphytic microbes, which are highly variable in composition and species (Eikmeyer et al., 2013; Muck, 2013). The low pH achieved during ensiling, caused by the production of lactic acid and other organic acids, inhibits or prevents the growth of spoilage and pathogenic organisms (Woolford, 1975). Microbial inoculants, such as homofermentative and heterofermentative lactic acid bacteria, are added to silage to dominate the natural population of bacteria

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¹Corresponding author: adesogan@ufl.edu

in the forage and influence the dynamics of the microbial population during ensiling (Kung, 1998; Wang et al., 2006). Homofermentative lactic acid bacteria are mainly used to improve silage preservation by accelerating lactic acid fermentation and causing a rapid drop in pH (Kristensen et al., 2010; Muck, 2010; Nkosi et al., 2010). Heterofermentative lactic acid bacteria produce high concentrations of acetic or propionic acid, thereby inhibiting the growth of spoilage yeasts and molds and improving the shelf life of silage at feedout (Filya, 2003; Kleinschmit and Kung, 2006; Queiroz et al., 2013). Chemical additives, such as propionic acid, are added mainly as antifungal agents to enhance the shelf life of silages during feedout (Kung et al., 1998, 2000).

The microbial population of silages has previously been described using culture-based methods, which are notorious for underestimating the bacterial diversity (Ercolini, 2004) because of their inability to cultivate most naturally occurring species (Temmerman et al., 2003). In recent years, a better understanding of the microbial populations in silages has been achieved through molecular approaches, such as denaturing gradient gel electrophoresis (Dolci et al., 2011), random amplified polymorphic DNA PCR (Rossi and Dellaglio, 2007), and terminal restriction fragment length polymorphism (McEniry et al., 2008), that do not rely on culturing. However, these methods can identify only a few of the predominant operational taxonomic units (OTU) (Temmerman et al., 2003). The application of 454 pyrosequencing to simultaneously sequence thousands of 16S rDNA sequences has increased our understanding of complex microbial communities (Degnan and Ochman, 2012). Furthermore, the Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA), which generates higher throughput per run with lower error rates and greater sequence coverage than the 454 pyrosequencing system at much lower costs (Loman et al., 2012; Frey et al., 2014), has enhanced our understanding of complex microbial communities of the rumen and silage. Recent studies have applied Ion Torrent (DNA Electronics Inc., Carlsbad, CA) and single molecule real-time sequencing technologies to examine the microbial communities of corn, alfalfa, and sorghum silages (Bao et al., 2016; Kraut-Cohen et al., 2016); however, these platforms have lower throughput (Loman et al., 2012) and higher error rate (Quail et al., 2012), respectively, than Illumina MiSeq sequencing.

Forages and silages may be contaminated with various microorganisms, including pathogenic *Escherichia coli*, when manure and irrigation slurry are applied as fertilizers (Beuchat and Ryu, 1997). Dairy manure can contain up to 8 log cfu/g of *E. coli* (Walczak and Xu, 2011), and counts of more than 2 log cfu/g have

been detected on forages and silages (Chen et al., 2005; Ogunade et al., 2016). Although the ensiling process has been shown to kill *E. coli* O157:H7 in cereal and corn silages due to acidification (Dunière et al., 2011; Ogunade et al., 2017), the pathogen may persist and alter microbial populations in high-DM alfalfa silage with high pH values due to the high buffering capacity of alfalfa. The first objective of this study was to examine effects of adding *E. coli* O157:H7 with or without chemical or microbial additives on the bacterial diversity and composition of alfalfa silage. The second objective was to examine associations between the relative abundance of silage bacteria and indices of silage fermentation quality. This study complements the results of a companion study (Ogunade et al., 2016) that examined the fate of *E. coli* O157:H7 in contaminated alfalfa silage treated with the same chemical or microbial additives used in this study.

MATERIALS AND METHODS

Harvesting, Ensiling, and Inoculation

Whole-plant alfalfa forage was harvested at the early bloom stage with a mower (John Deere 946, Moline, IL), wilted to a DM of 54%, and chopped to a 19-mm theoretical length of cut with a forage harvester (John Deere 7950). Approximately 4.5 kg per replicate of the chopped forage was treated with the following in quadruplicate: (1) distilled water (control); (2) 1×10^5 cfu/g of *E. coli* O157:H7 (**EC**); (3) EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (**EC+LP**); (4) EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (**EC+LB**); or (5) EC + 0.22% (fresh weight basis) of propionic acid containing 99.9% of the acid (**EC+PA**). The *E. coli* O157:H7 was supplied by the University of Florida Emerging Pathogens Institute (Gainesville). A single colony of *E. coli* O157:H7 was grown in 5 mL of Luria-Bertani broth (Sigma-Aldrich, St. Louis, MO) at 35°C with shaking at 200 rpm overnight. The next day, the culture was diluted 1:100 in fresh Luria-Bertani broth and again incubated at 35°C until the early log stage (optical density at 600 nm = 0.5). The number of *E. coli* O157:H7 cells in the suspension was verified by spread plating on sorbitol MacConkey agar (Oxoid, Basingstoke, UK) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L). The propionic acid was purchased from Sigma-Aldrich. The silage inoculant bacteria were supplied by Lallemand Animal Nutrition (Milwaukee, WI) and their counts and viability were confirmed by plating on de Man, Rogosa, and Sharpe agar (Difco Laboratories Inc., Detroit, MI) at 37°C for 48 h. Appropriate amounts of the

inoculant bacteria or propionic acid were mixed with distilled water and applied to the forage at 3 mL/kg of fresh forage. After proper mixing, the treated forages were packed into 14 × 21 cm, thick-walled plastic bags (PB61430, International Plastics Inc., Greenville, SC) that were subsequently vacuumed (Canister Vacuum 4122, Bissell Inc., Grand Rapids, MI) to remove air, sealed with plastic ties (S12356, Uline Corp., Pleasant Prairie, WI), and ensiled at 25°C for 100 d.

Analysis of Fermentation Products

After 100 d of ensiling, silage subsamples (20 g each) were diluted with 180 mL of distilled water, homogenized for 1 min in a stomacher (UL Lab-Blender 400, Seward Laboratory, London, UK) to obtain silage extracts, filtered through 2 layers of cheesecloth, and immediately analyzed for pH using a digital pH meter (Accumet AB15, Thermo Fisher Scientific/Oxoid Ltd., Basingstoke, UK). For analyses of organic acids (lactate, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate) and ammonia-N, an aliquot of the silage extract was centrifuged at $1,800 \times g$ for 15 min at 4°C. The supernatant was analyzed for ammonia-N using an adaptation for the Technicon Auto Analyzer of the Noel and Hambleton (1976) method and for organic acids using a Merck Hitachi Elite La-Chrome High Performance Liquid Chromatograph system (Hitachi L2400, Tokyo, Japan) coupled to a UV detector set at 210 nm. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) with a 0.015 M sulfuric acid mobile phase and a flow rate of 0.7 mL/min at 45°C was used.

Metagenomic DNA Extraction and Purification

Approximately 100 g of each silage sample was added to 200 mL of PBS + 0.01% Tween-80 in a sterile stomacher bag. The mixture was sonicated for 10 min using Sonicator SC-150 (Sonicator Instrument Corp., Copiague, NY). The wash (liquid fraction) was centrifuged at $9,000 \times g$ at 4°C for 10 min to create a pellet from which DNA was extracted using a PowerLyzer PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. Bead beating (Bullet Blender Storm 24, Next Advance, Averill Park, NY) at a speed of 4,000 rpm for 30 s was used to homogenize the suspension and to mechanically disrupt the bacterial cells. The purity and concentration of genomic DNA were measured with a spectrophotometer (Nanodrop 115 1000, Thermo Fisher Scientific-Oxoid) and the integrity of the DNA was verified by agarose (0.7%) gel electrophoresis.

PCR Amplification of the V3–V4 Hypervariable Region of Bacterial 16S rRNA Genes

Samples for sequencing were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3–V4 region. The DNA quality was measured by PicoGreen and Nanodrop (Thermo Fisher Scientific-Oxoid), and 10 ng of genomic DNA was amplified using the following PCR conditions: 94°C for 3 min followed by 35 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min, followed by a final elongation step of 8 min at 72°C. The barcoded fusion primer sequences used for amplifications were 519F: 5'-CCTACGGGNGGCWGCAG-3' and 806R: 5'-GACTACHVGGGTATCTAATCC-3'. The final purified product was quantified using real-time quantitative (q)PCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Waltham, MA). The paired-end (2 × 300 bp) sequencing was done by Macrogen (Seoul, Korea) on the Illumina MiSeq platform.

Sequencing and Diversity Analysis

Overlapping reads were merged using fast length adjustment of short reads (Magoc and Salzberg, 2011). Sequences were quality-filtered with a Q score of 20. Pre-processing and clustering of sequences to identify OTU was done using CD-HIT-OTU software (Li et al., 2012). Taxonomic composition of the samples from phylum to genus was generated using QIIME UCLUST (Edgar, 2010) against the Ribosomal Database Project Release 11 (<https://rdp.cme.msu.edu/>). α -Diversity (rarefaction curve for OTU, Chao1, and Shannon-Weaver diversity indices) and β -diversity (principal coordinate analysis, **PCoA**, of unweighted UniFrac distances) were generated using the QIIME software package with a script `core_diversity_analyses.py` (Caporaso et al., 2010). A nonparametric Monte Carlo test with no Bonferroni correction was used to compare the within-treatment and between-treatment UniFrac distances, which represent phylogenetic distances based on the presence or absence of OTU among samples (Park et al., 2016).

Statistical Analysis

The experiment was a completely randomized design with 5 treatments and 4 replicates per treatment. Continuous variables, such as relative abundance of bacteria, number of reads, and species richness and diversity,

were analyzed using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). The model included the treatment effect, and means were separated using the PDIF procedure of SAS, which is based on the Fisher's *F*-protected least significant difference test. Significant differences were declared at $P \leq 0.05$. Pearson correlation coefficients were generated using the R software (<http://www.r-project.org>). Significant correlations were declared at $P \leq 0.10$.

RESULTS

Fermentation quality indices, counts of *E. coli* O157:H7, and chemical composition of the alfalfa silages at silo opening (d 100) were reported previously by Ogunade et al. (2016). Briefly, the pH was lower ($P < 0.05$) in EC+LP and EC+LB silages (4.57 and 4.64, respectively) than in the control and EC silages (4.88 and 4.82, respectively), and the EC+PA silage had a similar ($P > 0.10$) pH to inoculated silages. Compared with the control, silages treated with EC+LP and EC+LB had greater ($P < 0.05$) concentrations of lactate and acetate, respectively. Propionate was only detected in EC+PA silage. Butyrate, isobutyrate, valerate, and isovalerate were not detected in any silage. *Escherichia coli* O157:H7 was eliminated from the silages once the pH dropped below 5.0. This occurred earlier (16 vs. 100 d of ensiling) in EC+LP and EC+LB silages because they produced more lactate and acetate throughout the fermentation than other silages.

Sequencing Results and Bacterial Diversity

Sequencing of the V3–V4 region of the bacterial 16S rRNA gene of the 20 samples yielded 1,702,729 reads with an average of $85,136 \pm 4,522$ reads per sample after quality filtering. Rarefaction curves plateaued in all samples sequenced (data not shown), indicating that the number of reads was sufficient for identifying OTU. The average Good's coverage for all the samples was greater than 99%, indicating that the depth of sequencing was adequate for reliable analysis of the bacterial community. Compared with the others, silages treated with microbial inoculants had lower ($P < 0.05$) Shannon index values (Figure 1A), which measures species diversity based on the number and evenness of species. The Chao1, an estimate of species richness based on the number of rare species, was unaffected ($P > 0.10$) by treatment (Figure 1B). The unweighted PCoA UniFrac plot revealed compositional differences in the bacterial community of the 20 samples (Figure 2). The control samples seemed to cluster apart from most other samples except one EC sample, and the Monte Carlo

test revealed that the between-treatment variation was greater ($P < 0.01$) than the within-treatment variation.

Comparisons at the Phylum Level

The microbial community structure of alfalfa silage was represented by 5 phyla (Table 1). *Firmicutes* (73.3 ± 6.05) were more predominant than others, followed by *Proteobacteria* (20.4 ± 3.80). Compared with the control, the EC treatment did not affect the relative abundance or composition of bacteria at the phylum level. The relative abundance of *Firmicutes* in the control and EC+LB silages (79.5 and 77.4%, respectively) was greater ($P < 0.05$) than that in the EC+LP or EC+PA silages (69.2 or 68.8%, respectively), whereas that of *Proteobacteria* was greater in the EC+LP and EC+PA silages (24.0 and 24.9%, respectively) than in the control, EC, and EC+LB (16.5, 18.0, and 18.5% respectively) silages. The relative abundances of *Bacte-*

Figure 1. (A) Estimate of α -diversity; treatment P -value = 0.01. (B) Species richness calculated as the Chao1 index; treatment P -value = 0.51. Bars with different letters (a, b) differ ($P \leq 0.05$). Error bars represent SE. Control = distilled water; EC = 1×10^9 cfu/g of *Escherichia coli* O157:H7; EC+LP = EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); EC+PA = EC + 2.2 g/kg of propionic acid.

Table 1. Relative abundance (%) of phyla isolated from alfalfa silage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid and ensiled for 100 d

Phylum	Treatment ¹					SE	P-value
	Control	EC	EC+LP	EC+LB	EC+PA		
<i>Actinobacteria</i>	1.52	2.27	2.03	1.18	2.00	0.58	0.21
<i>Bacteroidetes</i>	1.76	2.81	3.67	2.33	2.91	1.20	0.63
<i>Cyanobacteria</i>	0.79	1.33	1.12	0.59	1.38	0.32	0.12
<i>Firmicutes</i>	79.5 ^a	75.6 ^{ab}	69.2 ^b	77.4 ^a	68.8 ^b	3.28	0.02
<i>Proteobacteria</i>	16.5 ^b	18.0 ^b	24.0 ^a	18.5 ^b	24.9 ^a	1.65	0.01

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; EC+LP = EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); EC+PA = EC + 2.2 g/kg of propionic acid.

roidetes, *Actinobacteria*, and *Cyanobacteria* were similar for all treatments ($P > 0.10$).

Comparisons at the Family Level

Treatment effects on the percentage distribution of the 13 predominant families that represented 91.7% of the silage bacterial community are shown in Table 2. *Lactobacillaceae* was the most predominant, followed by *Leuconostocaceae*. Compared with the control, EC treatment did not affect the relative abundance of bacteria at the family level, but treatment with EC+LP and EC+LB increased ($P < 0.05$) the relative abun-

dance of *Lactobacillaceae* but reduced ($P < 0.05$) those of *Leuconostocaceae* and *Enterococcaceae*. Compared with the control, EC+PA did not affect ($P > 0.10$) the relative abundance of *Lactobacillaceae* but decreased ($P = 0.01$) those of *Leuconostocaceae* and *Enterococcaceae*. The relative abundance of *Methylobacteriaceae* was lower ($P = 0.01$) in EC+LP and EC+LB silages, whereas those of *Enterobacteriaceae*, *Pseudomonadaceae*, and *Xanthomonadaceae* were greater in EC+LP and EC+PA silages ($P < 0.05$), compared with the control. Also, the relative abundance of *Sphingomonadaceae* was greater ($P = 0.02$) in EC+LP, EC+LB, and EC+PA silages relative to the control.

Figure 2. Unweighted UniFrac principal coordinate analysis (PCoA) plot of individual samples in each treatment. The PCoA plot indicates the phylogenetic distance (variation) between samples using 2 principal coordinates (PC1 and PC2). The percentage variation explained by each PC is indicated on each axis. Control = distilled water; EC = 1×10^5 cfu/g of *Escherichia coli* O157:H7; EC+LP = EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); EC+PA = EC + 2.2 g/kg of propionic acid. Color version available online.

Comparisons at the Genus Level

Treatment effects on the percentage distribution of the 16 most predominant genera, accounting for approximately 90.3% of the silage bacterial community are shown in Table 3. *Pediococcus* and *Weissella* were most predominant in the control silage. Compared with the control, the EC treatment did not affect the relative abundance of bacteria at the genus level. Treatment with EC+LP resulted in the greatest ($P = 0.01$) relative abundance of *Lactobacillus* (62.6%), followed by EC+LB (47.8%), whereas control, EC, and EC+PA silages were similar ($P > 0.50$). Relative to the control, EC+LP silage had lower ($P < 0.05$) relative abundance of *Pediococcus* (29.9 vs. 0.55%), *Weissella* (27.7 vs. 3.89%), and *Methylobacterium* (1.50 vs. 0.60%), and greater ($P < 0.05$) relative abundance of *Sphingomonas* (1.86 vs. 2.93%), *Pantoea* (4.14 vs. 8.28%), *Pseudomonas* (1.26 vs. 2.44%), and *Erwinia* (0.35 vs. 0.57%), whereas EC+LB silage had a lower ($P < 0.05$) relative abundance of *Weissella* (29.7 vs. 9.19%) and *Methylobacterium* (1.50 vs. 0.94%) relative to the control. Compared with the control, treatment with EC+PA resulted in greater ($P < 0.05$) relative abundance of *Sphingomonas* (1.86 vs. 3.61%), *Pantoea* (4.14 vs. 7.21%), *Pseudomonas* (1.26 vs. 2.75%), and *Erwinia* (0.35 vs. 0.63%) but EC+PA did not affect the relative abundance of the 3 most predominant genera: *Lactobacillus*, *Weissella*, and *Pediococcus*.

Correlations Between Relative Abundance of Bacteria and Fermentation Quality Indices

The correlations between the relative abundance of bacterial genera and silage fermentation indices are presented in Table 4. Lactate concentration correlated positively with the relative abundance of *Lactobacillus* ($r = 0.56$, $P = 0.01$), and negatively with the relative abundance of *Pediococcus* ($r = -0.41$, $P = 0.08$). Relative abundance of *Sphingomonas* ($r = -0.51$), *Pantoea* ($r = -0.46$), *Pseudomonas* ($r = -0.45$), and *Stenotrophomonas* ($r = -0.38$) were negatively correlated ($P < 0.10$) with ammonia-N concentration. Silage pH was negatively correlated ($P < 0.10$) with the relative abundance of *Lactobacillus* ($r = -0.59$), *Sphingomonas* ($r = -0.66$), *Pantoea* ($r = -0.69$), *Pseudomonas* ($r = -0.69$), and *Stenotrophomonas* ($r = -0.50$), and positively correlated ($P < 0.10$) with the relative abundance of *Pediococcus* ($r = 0.44$), *Methylobacterium* ($r = 0.53$), *Weissella* ($r = 0.60$), and *Erwinia* ($r = 0.40$). Relative abundance of *Sphingomonas* ($r = -0.49$), *Pantoea* ($r = -0.48$), *Pseudomonas* ($r = -0.45$), and *Stenotrophomonas* ($r = -0.37$) were negatively correlated ($P < 0.10$) with yeast and mold counts.

DISCUSSION

This experiment was conducted as part of a broader study aimed at understanding the probiotic and food

Table 2. Relative abundance (%) of the 13 most predominant families isolated from alfalfa silage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid and ensiled for 100 d

Family	Treatment ¹					SE	P-value
	Control	EC	EC+LP	EC+LB	EC+PA		
<i>Firmicutes</i>							
<i>Lactobacillaceae</i>	38.5 ^b	45.5 ^b	63.2 ^a	67.9 ^a	44.7 ^b	4.03	0.01
<i>Leuconostocaceae</i>	29.7 ^a	24.8 ^{ab}	3.89 ^d	9.19 ^c	23.4 ^b	2.31	0.01
<i>Bacillaceae</i>	0.69	3.21	1.20	0.11	0.30	1.57	0.27
<i>Enterococcaceae</i>	0.48 ^a	0.64 ^a	0.04 ^b	0.07 ^b	0.01 ^b	0.12	0.01
<i>Proteobacteria</i>							
<i>Caulobacteraceae</i>	0.12	0.18	0.08	0.09	0.19	0.07	0.13
<i>Aurantimonadaceae</i>	1.94	2.55	1.87	1.60	2.23	0.49	0.24
<i>Hyphomicrobiaceae</i>	0.14	0.19	0.10	0.10	0.12	0.05	0.16
<i>Methylobacteriaceae</i>	1.53 ^{ab}	1.61 ^a	0.61 ^d	0.96 ^c	1.26 ^{bc}	0.16	0.01
<i>Rhizobiaceae</i>	1.80	1.80	1.55	1.33	1.84	0.29	0.27
<i>Sphingomonadaceae</i>	1.93 ^c	2.35 ^{bc}	3.00 ^{ab}	2.78 ^{ab}	3.68 ^a	0.33	0.02
<i>Enterobacteriaceae</i>	4.55 ^c	4.60 ^c	9.70 ^a	5.94 ^c	8.02 ^b	0.76	0.01
<i>Pseudomonadaceae</i>	1.26 ^b	1.29 ^b	2.44 ^a	1.97 ^{ab}	2.75 ^a	0.50	0.03
<i>Xanthomonadaceae</i>	2.65 ^c	2.75 ^{bc}	3.78 ^{ab}	3.21 ^{abc}	4.00 ^a	0.49	0.05

^{a-d}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; EC+LP = EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); EC+PA = EC + 2.2 g/kg of propionic acid.

Table 3. Relative abundance (%) of the 13 most predominant genera isolated from alfalfa silage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid and ensiled for 100 d

Genus	Treatment ¹					SE	P-value
	Control	EC	EC+LP	EC+LB	EC+PA		
<i>Firmicutes</i>							
<i>Lactobacillus</i>	14.7 ^c	16.8 ^c	62.6 ^a	47.8 ^b	8.98 ^c	4.43	0.01
<i>Pediococcus</i>	29.9 ^{ab}	28.7 ^{ab}	0.55 ^c	20.1 ^b	35.8 ^a	5.17	0.01
<i>Weissella</i>	29.7 ^a	24.8 ^a	3.89 ^c	9.19 ^b	23.4 ^a	4.32	0.01
<i>Exigobacterium</i>	0.29	0.48	0.30	0.12	0.37	0.14	0.17
<i>Bacillus</i>	0.14	0.41	0.20	0.11	0.26	0.14	0.25
<i>Proteobacteria</i>							
<i>Aureimonas</i>	1.94	2.15	1.87	1.60	2.22	0.29	0.19
<i>Methylobacterium</i>	1.50 ^{ab}	1.60 ^a	0.60 ^d	0.94 ^{cd}	1.24 ^{bc}	0.16	0.01
<i>Rhizobium</i>	1.80	1.79	1.55	1.33	1.84	0.29	0.27
<i>Sphingomonas</i>	1.86 ^c	2.26 ^{bc}	2.93 ^{ab}	2.71 ^{abc}	3.61 ^a	0.45	0.02
<i>Pantoea</i>	4.14 ^b	4.04 ^b	8.28 ^a	5.40 ^b	7.21 ^a	0.59	0.01
<i>Pseudomonas</i>	1.26 ^b	1.29 ^b	2.44 ^a	1.97 ^{ab}	2.75 ^a	0.50	0.03
<i>Stenotrophomonas</i>	2.43	3.50	2.95	3.56	3.32	0.63	0.27
<i>Erwinia</i>	0.35 ^c	0.35 ^c	0.57 ^{ab}	0.44 ^{bc}	0.63 ^a	0.09	0.02

^{a-d}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; EC+LP = EC + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); EC+PA = EC + 2.2 g/kg of propionic acid.

safety-enhancing effects of lactic acid bacterial silage inoculants. Silages were inoculated with *E. coli* O157:H7 to monitor its fate during ensiling of alfalfa that had been ensiled with or without additives. The pathogen was not detected in any silage at silo opening due to the inhibitory effect of the low pH resulting from silage acidification (Chen et al., 2005; Dunière et al., 2011; Ogunade et al., 2016).

Bacterial Diversity and Composition

This study revealed the relative abundance and diversity of bacteria in alfalfa silage treated with EC with or without microbial or chemical additives. The Illumina Miseq sequencing platform used for the analysis is a

powerful tool for studying diversity and composition of complex microbial communities of environmental samples (Gloor et al., 2010; Degnan and Ochman, 2012).

It is well known that the majority of the bacterial community involved in lactic acid fermentation in silage belong to the phylum *Firmicutes* and to the genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Weissella*, and *Leuconostoc* (Pahlow et al., 2003; Pang et al., 2011). Our study revealed that approximately $74.1 \pm 3.28\%$ of the alfalfa silage bacterial community belong to phylum *Firmicutes*, consisting mainly of families *Lactobacillaceae* and *Leuconostocaceae* and genera *Lactobacillus*, *Pediococcus*, and *Weissella*. Approximately $20.4 \pm 1.65\%$ belonged to phylum *Proteobacteria*, consisting mainly of families *Enterobacteriaceae*, *Xanthomonada-*

Table 4. Pearson correlation coefficients between abundance of bacterial genera and fermentation indices of alfalfa forage ensiled for 100 d

Genus	Acetate (% of DM)	Lactate (% of DM)	Ammonia-N (% of DM)	pH	Yeast and mold (log cfu/g)
<i>Lactobacillus</i>	0.09	0.56*	-0.27	-0.59*	-0.01
<i>Pediococcus</i>	0.07	-0.41*	0.22	0.44*	-0.10
<i>Weissella</i>	0.04	-0.29	0.04	0.60*	0.12
<i>Aureimonas</i>	-0.01	-0.03	-0.24	0.03	0.24
<i>Methylobacterium</i>	0.06	-0.26	-0.03	0.53*	0.34
<i>Rhizobium</i>	-0.26	-0.04	-0.12	0.16	0.16
<i>Sphingomonas</i>	0.31	0.22	-0.51*	-0.66*	-0.49*
<i>Pantoea</i>	0.07	0.32	-0.46*	-0.69*	-0.48*
<i>Pseudomonas</i>	0.25	0.20	-0.45*	-0.69*	-0.45*
<i>Exigobacterium</i>	-0.09	-0.06	-0.18	0.08	0.14
<i>Bacillus</i>	-0.22	-0.04	-0.06	0.02	0.24
<i>Erwinia</i>	0.13	0.05	-0.06	0.40*	-0.12
<i>Stenotrophomonas</i>	0.26	0.35	-0.38*	-0.50*	-0.37*

*Significant ($P \leq 0.10$) Pearson correlation coefficient.

ceae, *Sphingomonadaceae*, and *Aurantimonadaceae*, and genera *Sphingomonas*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas*. Bao et al. (2016) assessed the bacterial community of alfalfa silage via single molecule real-time sequencing technology and reported that majority of genera detected are *Lactobacillus*, *Weissella*, *Pediococcus*, and *Pantoea*. In a similar study, McGarvey et al. (2013) monitored the bacterial population before and after ensiling alfalfa forage and found that the relative abundance of *Proteobacteria* reduced from 89.6 to 26.9%, whereas that of *Firmicutes* increased from 8.1 to 70.6% after 40 d of ensiling, indicating that alfalfa silage fermentation involves a shift in the bacterial community from *Proteobacteria* to *Firmicutes*.

The fact that the bacterial diversity and taxonomic composition of the EC silage did not differ from that of the control silage suggests that the shifts in microbiome in the additive-treated silages reflect the effects of the respective additives alone. Inoculation with microbial inoculants has been used for several years to enhance fermentation of silage (Whiter and Kung, 2001). Microbial inoculants are added to dominate and control microbial events during silage fermentation (Kung, 1998; Wang et al., 2006). Our results revealed that silage inoculation with *L. plantarum* or *L. buchneri* increased the relative abundance of the predominant genus, *Lactobacillus*, thereby reducing the diversity of the bacterial community. The greater the abundance of dominant bacteria, the less diverse the microbial community (Polley et al., 2007; Allen et al., 2009). The reduction in diversity may have been because *L. plantarum* and *L. buchneri* outcompeted the other bacteria or reduced the pH by increasing lactate and acetate concentrations, respectively (Ogunade et al., 2016), which may have inhibited the growth of the other bacteria. Similar results were obtained by Eikmeyer et al. (2013) and Ogiy et al. (2015), who reported dominance of inoculated *L. buchneri* and *L. plantarum* in grass and wheat silages, respectively. However, in some cases, inoculated strains may be outcompeted by the indigenous microbial population (Stevenson et al., 2006).

Silage inoculation with *L. plantarum* reduced the relative abundance of *Weissella* and *Pediococcus* after 100 d of ensiling. This was probably because of the faster rate of pH decline during ensiling and lower pH at silo opening (Ogunade et al., 2016), although competitive inhibition or antagonism by *L. plantarum* may have also been involved. Most species of *Weissella* are obligate heterofermentative bacteria that produce lactate and acetate as major end products of sugar metabolism (Graf et al., 2016). *Weissella* species are considered early colonizers (Dellaglio and Torriani, 1986) as they are outcompeted by acid-tolerant *Lactobacillus* species due

to the pH drop as fermentation progresses (Graf et al., 2016). That the relative abundance of *Weissella*, which are obligate heterofermenters, was reduced in EC+LP silage partly explains the greater lactate-to-acetate ratio usually observed in alfalfa silage treated with *L. plantarum* compared with untreated silage (Whiter and Kung, 2001; Kizilsimsek et al., 2007; Ogunade et al., 2016).

Species of *Pediococcus*, such as *Pediococcus pentosaceus*, are used as silage inoculants because they are acid-tolerant and are capable of synthesizing lactate (Garvie, 1986; Axelsson, 1998). *Pediococcus* species grow rapidly during the early stages of fermentation when the pH is between 5 and 6.5 (McDonald et al., 1991; Kung et al., 2003). These characteristics allow *Pediococcus* species to dominate the early stages of ensiling and promote a pH decrease, thereby stimulating the dominance of *Lactobacillus* species, which become more active and grow vigorously as the pH drops. Lin et al. (1992) studied the succession of lactic acid bacteria during ensiling periods of alfalfa and corn and reported that *P. pentosaceus* was dominant at early stages of fermentation; however, after 7 d of ensiling, *Lactobacillus homohiochii*, *Lactobacillus brevis*, and *Lactobacillus gasseri* dominated the community. This probably explains the negative correlation between *Pediococcus* and lactate concentration, as *Pediococcus* are outcompeted by *Lactobacillus* at low pH due to lactate accumulation. Therefore, some inoculants are formulated to contain both *P. pentosaceus* and *L. plantarum* to increase the rate of acidification during the early and later stages of silage fermentation, respectively (Lin et al., 1992).

Lactobacillus buchneri is primarily added to silage to ferment lactate to acetate and 1,2-propanediol (Driehuis et al., 2001). As a potent antimycotic agent, acetate inhibits the growth of spoilage yeasts, thereby improving the aerobic stability of silage (Danner et al., 2003; Tabacco et al., 2009). Treatment with *L. buchneri* improved the aerobic stability of corn, sorghum, wheat, barley, and alfalfa silages (Kung and Ranjit, 2001; Weinberg et al., 2002; Kung et al., 2003). However, acetic acid production has often been associated with DM loss in silage (Wilkinson and Davies, 2013). In this study, irrespective of treatment applied, the alfalfa silage was stable for the entire duration of the aerobic phase (168 h; Ogunade et al., 2016); however, DM loss was not measured. As seen with *L. plantarum*, silage treatment with *L. buchneri* increased the relative abundance of *Lactobacillus* and reduced that of *Weissella*, but had no effect on relative abundance of *Pediococcus*. In a similar study, Eikmeyer et al. (2013) studied the taxonomic profiles of ensiling microbial communities of grass silage inoculated with *L. buchneri*. The authors

observed greater relative abundance of *Lactobacillus* and lower relative abundance of *Weissella* relative to the untreated silage.

Chemical additives such as formic acid, propionic acid-based products, and formaldehyde are added as antifungal agents to improve aerobic shelf life of silage by reducing the population of yeasts and molds, which cause aerobic deterioration (Kung et al., 1998, 2000). As expected, propionic acid did not affect bacterial diversity or relative abundance of *Lactobacillus*, *Weissella*, and *Pediococcus*, the 3 most predominant genera responsible for driving silage fermentation in this study. The reason for this may be that although the low application rate of propionic acid in this study (0.22%) was sufficient to acidify the silage (Ogunade et al., 2016), it was not sufficient to reduce counts of predominant lactic acid bacteria (Kung, 2010; Zhang et al., 2015), presumably because it did not sufficiently change the concentration of organic acids that modulate the populations of lactic acid bacteria in silage. However, treatment with propionic acid increased the relative abundance of bacteria belonging to genera *Pantoea*, *Pseudomonas*, *Sphingomonas*, and *Erwinia*.

Correlations Between Bacterial Abundance and Fermentation Quality Indices

Relative abundance of *Pantoea*, *Pseudomonas*, and *Sphingomonas* were greater in EC+LP than in control silages and correlated negatively with pH ($r = -0.69$, -0.69 , and -0.66 , respectively) and ammonia-N concentration ($r = -0.46$, -0.45 , and -0.51 , respectively). High concentrations of ammonia-N in silage are usually indicative of protein breakdown and are typically caused by extensive plant protease activity or clostridial fermentation (Kung and Shaver, 2001). No clostridia were detected in this study; therefore, the negative correlation between *Pantoea*, *Pseudomonas*, and *Sphingomonas* and ammonia-N concentration may be due to inhibition of other proteolytic bacteria. Tao et al. (2012) showed that plant proteolytic enzymes in alfalfa silage are active at pH 4.0 to 8.0; however, most had higher activities at pH 5.0 to 6.0. Because of alfalfa's high buffering capacity and low water-soluble carbohydrate content, the rate of pH decline during ensiling of alfalfa is slow (Kung et al., 2003; Muck, 1998). Consequently, alfalfa is particularly susceptible to extensive proteolysis and clostridial fermentation (Kung et al., 2003). True protein degradation in alfalfa silage has been successfully reduced by *L. plantarum* inoculation because of the rapid drop in pH that inhibits proteolytic bacteria (Weinberg and Muck, 1996; Filya et al., 2007). Although *L. plantarum* inoculation may play a major role in true protein preservation in alfalfa, our results

suggest that *Pantoea*, *Pseudomonas*, and *Sphingomonas* may also contribute to protein preservation in alfalfa silage because their relative abundances were negatively correlated with ammonia-N concentration. Studies on wastewater treatment revealed a greater abundance of bacterial species belonging to *Pseudomonas* and *Sphingomonas* toward the onset of nitrification (Bal Krishna et al., 2013), a microbiological process whereby ammonia is oxidized to nitrite and then to nitrate (Bal Krishna et al., 2013); however, alfalfa silage studies have not involved these bacteria. Fitzgerald et al. (2015) monitored ammonia-oxidizing microbial communities in reactors under low dissolved oxygen conditions and reported that some species of *Pseudomonas*, *Rhodococcus*, and *Sphingomonas* are involved in nitrification. Therefore, the negative correlations of *Pseudomonas* and *Sphingomonas* with ammonia-N concentration may be partly due to nitrification. However, previous studies have not shown that nitrification by these bacteria can occur under anaerobic conditions, and in this study, nitrate-N concentrations were well below the detection limit of 300 mg/kg or the recommended safe level in ruminant diets (<1,000 mg of nitrate-N/kg, Rasby et al., 1988), possibly due to the low relative abundances of the bacteria. Therefore, more research is needed to elucidate the role of these bacteria in reducing silage ammonia-N concentrations.

The negative correlations of these same bacteria (*Sphingomonas*, *Pantoea*, and *Pseudomonas*) with yeast and mold counts may reflect the inhibitory effect of the propionic acid applied at ensiling on yeast and mold growth (Kung et al., 1998, 2000). This is because, compared with the control, the EC+PA treatment increased ($P \leq 0.05$) the relative abundance of these bacteria and resulted in the lowest ($P = 0.01$) yeast and mold counts (Ogunade et al., 2016). Whether the inhibition of yeast and molds was due to the direct effect of the propionic acid or its indirect effect via modulation of the bacterial population is unknown.

The use of *L. buchneri* (Oude Elferink et al., 1999; Driehuis et al., 2001) has been shown to inhibit the aerobic deterioration of silages through the fermentation of lactic acid to acetic acid and inhibition of yeast (Driehuis et al., 1999; Tabacco et al., 2009) and clostridial growth (Tabacco et al., 2009).

Methylobacterium and *Erwinia* were among the dominant genera in pre-ensiled alfalfa forage (McGarvey et al., 2013; Ni et al., 2017). However, in agreement with our results, in the study of Ni et al. (2017), their abundance was low (approximately 1%) after ensiling. Species of *Methylobacterium* are strictly aerobic and neutrophilic (Doronina et al., 2002; Knief et al., 2012), supporting their positive correlation ($r = 0.53$) with pH and reduced ($P \leq 0.05$) relative abundance in si-

lages treated with the microbial additives in this study. Certain species of *Erwinia* do not grow below pH 5.0 (Shrestha et al., 2005), supporting their low relative abundance in the silage and the positive correlation ($r = 0.40$) with pH. Although the relative abundance of *Erwinia* was increased ($P \leq 0.05$) by EC+LP and EC+PA treatments, this difference may not have practical significance because of their low relative abundance ($<0.6\%$) and small differences among the treatments.

Bacterial Candidates for Future Research

This study shows, perhaps for the first time, the associations of key silage quality indices and some alfalfa silage bacteria whose roles in alfalfa silage fermentation are among the least understood. For instance, *Sphingomonas*, *Pantoea*, and *Stenotrophomonas* had negative correlations with silage pH, ammonia-N concentration, and yeast and mold counts. These bacteria should be speciated, cultured, and investigated further to determine their roles in silage fermentation. To our knowledge, no member of the aforementioned genera has been evaluated as a microbial additive. Further studies on these bacteria are needed, because at least some of them may be candidates for development into new silage inoculants that can improve the preservation of alfalfa forages.

CONCLUSIONS

This study revealed that $74.1 \pm 3.28\%$ and $20.4 \pm 1.65\%$ of the alfalfa silage bacterial community belong to phyla *Firmicutes* and *Proteobacteria*, respectively. *Lactobacillus*, *Weissella*, and *Pediococcus* were the predominant genera detected. Relative to the control, adding *E. coli* O157:H7 alone at ensiling did not affect the bacterial community composition of the silage. Treatment with *L. plantarum* and *L. buchneri* altered the bacterial composition of the alfalfa silage by increasing the relative abundance of *Lactobacillus*, whereas application of propionic acid did not affect the relative abundance of the predominant bacteria. Negative correlations were found between the relative abundance of *Sphingomonas*, *Pantoea*, and *Stenotrophomonas* and silage pH, ammonia-N concentration, and yeast and mold counts. These bacteria should be speciated and examined for their potential roles in fermentation and true protein preservation in alfalfa silage.

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