Fate of *Escherichia coli* O157:H7 and bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives

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ABSTRACT

Inhibiting the growth of *Escherichia coli* O157:H7 (EC) in feeds may prevent the transmission or cycling of the pathogen on farms. The first objective of this study was to examine if addition of propionic acid or microbial inoculants would inhibit the growth of EC during ensiling, at silo opening, or after aerobic exposure. The second objective was to examine how additives affected the bacterial community composition in corn silage. Corn forage was harvested at approximately 35% dry matter, chopped to a theoretical length of cut of 10 mm, and ensiled after treatment with one of the following: (1) distilled water (control); (2) 1×10^5 cfu/g of EC (ECCH); (3) EC and 1×10^6 cfu/g of *Lactobacillus plantarum* (ECLP); (4) EC and 1×10^6 cfu/g of *Lactobacillus buchneri* (ECLB); and (5) EC and 2.2 g/kg (fresh weight basis) of propionic acid, containing 99.5% of the acid (ECA). Each treatment was ensiled in quadruplicate in laboratory silos for 0, 3, 7, and 120 d and analyzed for EC, pH, and organic acids. Samples from d 0 and 120 were also analyzed for chemical composition. Furthermore, samples from d 120 were analyzed for ammonia N, yeasts and molds, lactic acid bacteria, bacterial community composition, and aerobic stability. The pH of silages from all treatments decreased below 4 within 3 d of ensiling. *Escherichia coli* O157:H7 counts were below the detection limit in all silages after 7 d of ensiling. Treatment with *L. buchneri* and propionic acid resulted in fewer yeasts and greater aerobic stability compared with control, ECCH, and ECLP silages. Compared with the control, the diversity analysis revealed a less diverse bacterial community in the ECLP silage and greater abundance

of *Lactobacillus* in the ECLP and ECA silages. The ECLB silage also contained greater abundance of *Acinetobacter* and *Weissella* than other silages. Subsamples of silages were reinoculated with 5×10^5 cfu/g of EC either immediately after silo opening or after 168 h of aerobic exposure, and EC were enumerated after 6 or 24 h, respectively. All silages reinoculated with EC immediately after silo opening (120 h) had similar low pH values (<4.0) and EC counts were below the detection limit. The ECCH and ECLP silages reinoculated with EC after 168 h of aerobic exposure had relatively high pH values (>5.0) and EC counts (5.39 and 5.30 log cfu/g, respectively) 24 h later. However, those treated with *L. buchneri* or propionic acid had lower pH values (4.24 or 3.96, respectively) and lower EC counts (1.32 log cfu/g or none, respectively). During ensiling, EC was eliminated from all silages at pH below 4.0. During aerobic exposure, the growth of EC was reduced or prevented in silages that had been treated with *L. buchneri* or propionic acid at ensiling, respectively.

Key words: bacterial community, corn silage, *Escherichia coli*

INTRODUCTION

Escherichia coli O157:H7 (EC) is a foodborne pathogen that causes various forms of enteritis in humans ranging in severity from bloody diarrhea to hemolytic uremic syndrome and even death (Kaper et al., 2004). This pathogen is implicated in about 100,000 illness cases and 90 deaths annually in the United States (Mead et al., 1999; Scallan et al., 2011). The estimated annual cost associated with EC illnesses can be as high as \$405 million (Frenzen et al., 2005).

Cattle harbor and shed EC in their manure (Lahti et al., 2003; Mir et al., 2015) and are considered the main reservoirs of the pathogen (Cernicchiaro et al., 2013). Up to 30% of all cattle are asymptomatic carriers of

Received July 15, 2016.

Accepted November 1, 2016.

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EC (Callaway et al., 2006; Reinstein et al., 2007) and livestock feeds are considered to be the vehicle of transmission among livestock (Hancock et al., 2001; Davis et al., 2003). Corn silage forms the bulk of most dairy cattle rations in the United States and it can be contaminated with EC when untreated manure is used to fertilize corn plants that are subsequently ensiled and fed to cattle or after silo opening via the manure and saliva of cattle (Russell et al., 2000; Keen and Elder, 2002; Smith et al., 2005).

The low pH achieved during ensiling of corn eliminates pathogenic EC that is present at ensiling (Pedroso et al., 2010; Dunieri et al., 2011). At silo opening, aerobic conditions cause increases in pH (Weinberg et al., 1995) via stimulation of the growth of lactate-utilizing yeasts. The resulting elevated pH can facilitate growth and spread of EC (Pedroso et al., 2010; Ogunade et al., 2016). In silages contaminated with the pathogen after aerobic exposure, maintenance of a low pH (below 4.0) due to application of *Lactobacillus buchneri* at ensiling curtailed the growth of EC (Pedroso et al., 2010). In contrast, Dunieri et al. (2011) showed that application of *L. buchneri* at the time of ensiling did not prevent the growth of EC when aerobically exposed silages were contaminated with a pathogenic EC. Therefore, more studies are needed to ascertain the inhibitory effect of *L. buchneri* or other additives on EC-contaminated aerobically exposed silage. Such information is critically needed to reduce or prevent cycling of EC among animals and people.

Propionic acid is a strong antifungal agent (Woolford, 1975) that can inhibit the growth of silage yeasts and molds, which are responsible for the rise in pH of silages during aerobic exposure (Kleinschmit et al., 2005). We hypothesized that by maintaining a low pH due to inhibition of the growth of lactate-utilizing yeasts that increase the pH after silo opening, propionic acid would be effective at killing the pathogen during the aerobic phase. To our knowledge, no experiments have examined the use of propionic acid to prevent the growth of EC during or after ensiling. Furthermore, no studies have simultaneously compared the efficacy of inhibiting the growth of EC on silage with propionic acid and obligate or facultative heterofermentative lactic acid bacteria (**LAB**) inoculants. Therefore, this study was aimed at examining the effectiveness of propionic acid and microbial inoculants containing either *L. plantarum* or *L. buchneri* at controlling EC in corn silage during the ensiling and feedout stages.

Most studies that assessed the effects of silage additives on the microbial community composition of silage have been based on culture-based techniques (Ercolini, 2004), real-time PCR (Stevenson et al., 2006), or char-

acterization of denaturing gradient gel electrophoresis bands, which all underestimate the microbial community composition (Kraut-Cohen et al., 2016). The application of 454 pyrosequencing has increased our understanding of complex microbial communities (Degnán and Ochman, 2012) such as the rumen. Furthermore, the Illumina MiSeq sequencing system, which gives higher throughput per run, lower error rates, and greater depth and breadth of coverage than the 454 pyrosequencing system, at much lower costs (Degnán and Ochman, 2012; Frey et al., 2014), has enhanced our understanding of microbial communities. A recent study applied a newer sequencing technology, called single molecule real-time sequencing technology, to examine the bacterial profile of alfalfa silage because of its power to generate long sequence reads (Bao et al., 2016); however, this platform suffers from high error rate (Quail et al., 2012). To our knowledge, no studies have used 454 pyrosequencing or Illumina MiSeq sequencing system to examine the silage microbiome. In addition, no studies that examined inhibitory effects of silage additives on pathogens have characterized the microbiome of silage. Therefore, the second objective of this study was to understand how propionate and inoculants affected the bacterial community composition in corn silage using the Illumina sequencing platform.

MATERIALS AND METHODS

The Biological Safety Office, Division of Environmental Health and Safety, and the Institutional Animal Care and Use Committee of the University of Florida approved all procedures for this study (protocol no. 201408675).

Forage and Treatments

Whole-plant corn forage (D598R50, Dyna Gro, East Lansing, MI), harvested with a forage chopper (Claas 940, Claas of America Inc., Omaha, NE) at approximately 35% DM and chopped to a theoretical length of cut of 10 mm, was treated with the following: (1) distilled water (control); (2) 1×10^5 cfu/g EC EDL933 alone (**ECCH**); (3) ECCH + 1×10^6 cfu/g *Lactobacillus plantarum* R2014 (**ECLP**); (4) ECCH + 1×10^6 cfu/g *L. buchneri* R1102 (**ECLB**); and (5) ECCH + 2.2 g/kg (fresh weight) of propionic acid, containing 99.5% of the acid (**ECA**). *Escherichia coli* O157:H7 EDL933 was supplied by University of Florida Emerging Pathogens Institute (Gainesville, FL), the silage inoculant bacteria were supplied by Lallemand Animal Nutrition (Milwaukee, WI), and the propionic acid was purchased from Sigma-Aldrich (St. Louis, MO). Bacte-

rial counts and their viability in the inoculants were verified before silage inoculation by plating on de Man, Rogosa, Sharpe agar (Difco Laboratories Inc., 288210), at 37°C for 48 h, and appropriate amounts of the inoculants were added to achieve the desired application rate. The EC was grown on Luria-Bertani (LB; Sigma-Aldrich, L3022) broth overnight for 18 h at 35°C. The next day, the culture was diluted (1:100) in fresh LB broth and again incubated at 37°C for 4 h. The cells in the suspension within 16-mm pathlength tubes were adjusted to an optical density of 0.50 at 600 nm (GENESYS 20 spectrophotometer; Thermo Fisher Scientific-Oxoid Ltd., Basingstoke, UK, 4001-000). Bacterial counts in the suspension were confirmed by direct plating on Sorbitol-MacConkey agar (SMAC; Sigma-Aldrich, 83339) supplemented with cefixime (0.05 mg/L, Sigma-Aldrich, 18588) and potassium tellurite (2.5 mg/L, Sigma-Aldrich, 60539) to make CT-SMAC after incubation at 35°C for 24 h. The bacterial inoculants and propionic acid were diluted in distilled water and applied to the forage at 3 mL/kg of fresh forage, resulting in application of 1×10^6 cfu/g and 2.2 g/kg of bacterial inoculants and propionic acid, respectively. Prior to inoculation, the EC suspension was replated on CT-SMAC to ensure accurate application rates. The EC suspension was mixed with 5 mL of water and applied to the fresh forage at 1×10^5 cfu/g. Equal quantities of water (9.5 mL each) were applied to the treated and control forages. Treatments were applied, using a handheld garden sprayer, to each of quadruplicate samples per treatment separately (1.5 kg each). Each treated sample was hand-mixed, manually packed into a 14 × 21 cm, thick-walled plastic bag (International Plastics Inc., Greenville, SC, PB61430), which was vacuumed (Canister Vacuum 4122, Bissell Inc., Grand Rapids, MI) and sealed with silo ties (Uline Corp., Pleasant Prairie, WI, S12356). The silages in the bags were ensiled for 3 and 7 d at 20°C after which subsamples were analyzed for EC, pH, organic acids, and ammonia N. Additionally, 4.5 kg of the forage was treated with each additive or nothing (control) in quadruplicate and packed into 20-L plastic buckets lined with thick-walled plastic bags and ensiled for 120 d at ambient temperature (25°C). Subsamples of these silages were analyzed for EC, pH, organic acids, ammonia N, LAB, yeast and mold counts, and aerobic stability. Furthermore, subsamples from d 0 and 120 were analyzed for chemical composition.

Survival of *E. coli* O157:H7 in Aerobically Exposed Silages

After the bucket silos were opened on d 120, about 1 kg of silage from each replicate was transferred to an

open-top 20-L plastic bucket, reinoculated with 5×10^5 cfu/g of EC and stored at 20°C for 24 h. Subsamples were taken after 6, 12, 18, and 24 h to assess changes in EC counts. Furthermore, 2 kg of silage from each d 120 replicate was placed in a different open-top 20-L plastic bucket. Wireless sensors (Onset Computer Corporation, Cape Cod, MA) that recorded silage temperatures at 30-min intervals were placed in the geometrical center of the silage mass in each bucket. Aerobic stability was defined as the time (h) for the silage temperature to exceed 2.0°C above the ambient temperature. After 168 h of aerobic exposure, silages were reinoculated with 5×10^5 cfu/g of EC on d 127, and 24 h later (d 128), the population of the pathogen on the silages was counted.

Laboratory Analyses

Silage subsamples (20 g each) taken on d 120 were diluted with 180 mL of distilled water and homogenized for 1 min in a stomacher (UL Lab-Blender 400, Seward Laboratory, London, UK) to obtain silage extracts for further analyses. The suspension was filtered through 2 layers of cheesecloth and immediately analyzed for pH and counts of EC, LAB, yeasts, and molds. Enumeration of EC was done by serial dilution in buffered peptone water [BPW; peptone (10 g/L) and sodium chloride (5 g/L); Thermo Fisher Scientific-Oxoid Ltd., Basingstoke, UK, CM0009] followed by pour plating on duplicate plates of CT-SMAC agar (Zadik et al., 1993). Plates were incubated for 24 h at 35°C and sorbitol-negative colonies were presumptively identified as EC and confirmed by latex agglutination (Thermo Fisher Scientific-Oxoid Ltd., DR0120M; March and Ratnam, 1989). The detection limit for EC counts was 10 cfu/g. An aliquot of the serially diluted extract was also plated on malt extract (Thermo Fisher Scientific-Oxoid, CM0059B) and de Man, Rogosa, Sharpe agars at 32°C for 72 and 48 h for enumeration of yeasts and molds, and LAB, respectively. The pH of silage extracts was measured using a digital pH meter (Accumet AB15, Fisher Scientific). For organic acid and ammonia N analyses, 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 Auto Analyzer (Technicon, Tarrytown, NY) and an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification, and for organic acids using a High Performance Liquid Chromatograph system (Hitachi L2400, Tokyo, Japan) coupled to a UV detector set at 210 nm and an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) with 0.015 M sulfuric acid mobile phase and a flow rate of 0.7 mL/min at 45°C. The DM content of the fresh forages and silages was determined by drying samples in a forced-air oven at 55°C

for 48 h. Dried samples were ground to pass the 1-mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ). The procedures of Van Soest et al. (1991) were used for NDF and ADF analyses. Heat-stable amylase was used in the NDF analysis but no sodium sulfite was added, and the results were expressed on a DM basis inclusive of residual ash. Crude protein was calculated by multiplying N measurements obtained from Kjeldahl digestion by 6.25.

Antibacterial Activity of the Silage Extracts

Antibacterial activity against EC of the silage extracts was determined by the agar disk diffusion method of Bauer et al. (1966). The EC isolates were cultured in LB broth and adjusted to an optical density of 0.5 at 600 nm and the inoculum was prepared on CT-SMAC agar. Silage extracts were centrifuged at $5,000 \times g$ for 30 min at 4°C and the cell-free supernatants were collected. Two sterile cloning discs (6.0-mm diameter; cat. no. 231039, Becton Dickinson Co., Franklin Lakes, NJ) were immersed in the supernatants for 20 s using flame-sterilized forceps and then placed on the surface of CT-SMAC agar plates containing EC. Plates were prepared in duplicate and incubated for 24 h at 35°C. The diameters of zones of inhibition around the paper discs were measured with a ruler. In addition, the pH of the cell-free supernatants of the silage extracts was adjusted to pH 5.0 using 2 *N* NaOH and tested for antibacterial activity against EC.

DNA Extraction and Sequencing

To understand the influence of EC with or without silage additives on the composition and diversity of the silage microbial community, bacterial diversity analysis of the d 120 silage samples was performed using the Illumina MiSeq platform (Illumina, San Diego, CA). Approximately 100 g of each sample was added to 200 mL of PBS + 0.01% Tween-80 in a sterile 1-L Erlenmeyer flask. The mixture was sonicated for 45 min using the Sonicor SC-150 (Sonicor Instrument Corporation, Copiague, NY) followed by centrifugation at $9,000 \times g$ for 15 min to create a pellet for subsequent DNA extraction.

Total metagenomic DNA was extracted from the resulting pellet using a PowerLyzer PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA). Bead beating (0.1 mm, glass beads; Bullet Blender Storm 24, Averill Park, NY) was used for mechanical disruption of bacterial cells. The purity and concentration of genomic DNA were measured with a spectrophotometer (Nanodrop 115 1000, Thermo Fisher Scientific-

Oxoid Ltd., Basingstoke, UK) and the integrity of DNA was verified by agarose (0.7%) gel electrophoresis.

Each replicate sample was prepared for sequencing according to the Illumina 16S metagenomic sequencing library protocols to amplify the V3 and V4 regions (Klindworth et al., 2013). The DNA quality was measured by using PicoGreen (Invitrogen, Carlsbad, CA) and Nanodrop (Thermo Scientific, Waltham, MA). Ten nanograms of the genomic DNA was PCR-amplified with the following barcoded fusion primer sequences: 519F (5'-CCTACGGGNGGCWGCAG-3'), 806R (5'-GACTACHVGGGTATCTAATCC-3'). The purified product was quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina sequencing platforms, Illumina) and qualified using the LabChip GX HT DNA High-Sensitivity Kit (Perkin Elmer, Waltham, MA). The paired-end (2 $\times$ 300 bp) sequencing was performed by Macrogen Inc. (Seoul, Korea) using the Illumina MiSeq platform (Illumina).

Data and Statistical Analysis

The experiment had a completely randomized design with 5 treatments and 4 replicates per treatment. All microbial data were transformed to log units and were presented on a wet weight basis. Data were analyzed using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC) and the model for analyzing data collected over time included effects of treatment, day, and treatment $\times$ day interaction. When treatment $\times$ day interactions were significant, effects of treatment on each day were examined. The model for analyzing data collected at one-time point only included the treatment effect. Means were separated using the Tukey test. Significant differences were declared at $P \leq 0.05$.

For the bacterial community composition data, CD-HIT was used for clustering of sequences to identify operational taxonomic units (OTU; Li et al., 2012). The taxonomic composition of each sample was generated using QIIME UCLUST (Edgar, 2010) against the Ribosomal Database Project Release 11 (Cole et al., 2014). Alpha diversity (rarefaction curve for OTU, Chao1, and Shannon indices) and β diversity (unweighted principal coordinate analysis UniFrac distance) were generated using the QIIME software package with a script `core_diversity_analyses.py` (Caporaso et al., 2010). A nonparametric Monte Carlo test was used to compare the within-treatment and between-treatment UniFrac distances. Variables, such as relative abundance of bacteria, number of reads, and species richness and diversity, were analyzed using the GLIMMIX procedure of SAS and a model that included the treatment effect.

Table 1. Characteristics of whole-plant corn forage inoculated with *Escherichia coli* O157:H7 (EC) alone or EC and bacterial inoculants or propionic acid before ensiling

Item	Treatment ¹					SE	P-value
	Control	ECCH	ECLP	ECLB	ECA		
pH	5.94 ^a	5.99 ^a	5.93 ^a	5.93 ^a	5.14 ^b	0.04	0.01
DM (%)	34.7	35.7	35.5	35.4	34.8	0.88	0.75
CP (% of DM)	8.75	8.77	8.37	8.26	8.55	0.23	0.16
NDF (% of DM)	41.6	41.4	40.6	42.5	42.4	1.40	0.68
ADF (% of DM)	24.9	24.8	25.5	25.4	25.0	1.41	0.98

^{ab}Means within a row with different superscripts differ ($P < 0.05$).

¹Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO).

Differences between means were determined using the Tukey test. Significant differences were declared at $P \leq 0.05$.

RESULTS

Anaerobic Phase

The DM, CP, NDF, and ADF concentrations of the forage used for each treatment before ensiling were similar and averaged $35.2 \pm 0.44\%$ ($P = 0.75$), 8.54 ± 0.23 (% DM, $P = 0.16$), 41.7 ± 0.78 (% DM, $P = 0.68$),

and 25.1 ± 0.31 (% DM, $P = 0.98$; Table 1). The pH of the ECA corn forage was lower ($P = 0.01$) than those of other silages after the treatments were applied.

After 3 d of ensiling, all silages had pH values below the threshold of 4.0. The pH values remained low for the entire ensiling duration (Figure 1), such that the pH at final silo opening (120 d) counts were similar for all silages (Table 2). *Escherichia coli* O157:H7 was below the detection limit after 3 d of ensiling in ECCH, ECLP, and ECLB silages, suggesting that it had been eliminated. Although it was detected in the ECA silage on d 3, EC was below the detection limit in all d 7

Figure 1. Changes in the pH of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment $\times$ day SE and P -value = 0.02 and 0.001, respectively; an asterisk (*) indicates that values differed at this ensiling duration ($P < 0.05$). Error bars represent SE. Color version available online.

Figure 2. Changes in *Escherichia coli* O157:H7 (EC) counts of whole-plant corn forage inoculated with 1×10^5 cfu/g of EC alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = 1×10^5 cfu/g of EC (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment $\times$ day SE and *P*-value = 0.05 and 0.001, respectively; an asterisk (*) indicates that counts differed at this ensiling duration ($P < 0.05$). Error bars represent SE. Color version available online.

silages (Figure 2), though it is possible that selective enrichment of samples may have led to recovery of EC.

By d 7 of ensiling, the ECLP silage had greater ($P = 0.01$) lactate concentration than other silages (Figure 3); however, at final silo opening (d 120), its lactate concentration was similar to those of control, ECCH, and ECA silages (Table 2). The acetate concentration was similar for all silages at the initial stages of ensiling (on d 3 and 7); however, the concentration was higher

in the ECLB silage than other silages at d 120 (Figure 4; Table 2). Propionate was detected only in the ECA silage (0.41% DM), reflecting the addition at ensiling, whereas butyrate was not detected in any silage. Ammonia N concentration was higher ($P < 0.05$) in ECLB silage than ECLP silage at final silo opening. Yeast and mold counts were lower ($P = 0.01$) in the ECLB and ECA d 120 silages (3.43 and 2.92 log cfu/g, respectively) than in corresponding control, ECCH, and ECLP

Table 2. Fermentation indices and chemical composition and microbial counts of corn silage that was inoculated with *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid after 120 d of ensiling

Item	Treatment ¹					SE	<i>P</i> -value
	Control	ECCH	ECLP	ECLB	ECA		
pH	3.93	3.91	3.89	3.91	3.87	0.04	0.16
Lactate (% of DM)	2.99 ^{ab}	3.10 ^{ab}	3.50 ^a	2.59 ^b	3.06 ^{ab}	0.20	0.01
Acetate (% of DM)	2.33 ^b	2.43 ^b	1.85 ^b	3.39 ^a	2.26 ^b	0.30	0.01
Propionate (% of DM)	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^b	0.41 ^a	0.05	0.01
Yeasts and molds (log cfu/g)	5.08 ^a	5.30 ^a	4.91 ^a	3.43 ^b	2.92 ^b	0.48	0.01
Lactic acid bacteria (log cfu/g)	7.06	7.41	7.79	7.89	7.46	0.51	0.52
DM (%)	30.3	30.2	30.6	30.8	31.0	0.44	0.30
NDF (% of DM)	42.5	42.3	43.3	42.5	43.3	1.89	0.97
ADF (% of DM)	28.4	28.0	28.6	28.7	28.6	1.33	0.98
CP (% of DM)	9.58	9.11	9.23	9.02	9.37	0.21	0.11
Ammonia N (% of DM)	0.10 ^{ab}	0.10 ^{ab}	0.08 ^b	0.12 ^a	0.09 ^{ab}	0.01	0.04
Ammonia N (% of total N)	6.63 ^{ab}	7.14 ^{ab}	5.20 ^b	8.41 ^a	6.23 ^{ab}	0.89	0.03

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

¹Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO).

Figure 3. Changes in lactate concentration of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment $\times$ day SE and *P*-value = 0.08 and 0.001, respectively; an asterisk (*) indicates that concentrations differed at this ensiling duration ($P < 0.05$). Error bars represent SE. Color version available online.

silages (5.08, 5.30, and 4.91 log cfu/g, respectively). Lactic acid bacterial counts were unaffected ($P = 0.52$) by treatment.

Aerobic Phase

Escherichia coli O157:H7 was below the detection limit in silages within 6 h of reinoculation with the pathogen at silo opening (d 120). Except for the ECA

silage, EC was detected on d 128 in all silages that had been reinoculated with the pathogen on d 127 (Figure 5). In addition, the d 128 ECCH and ECLP silages had greater pH values (5.67 and 6.13, respectively; $P < 0.05$) and EC counts (5.39 and 5.30 log cfu/g, respectively; $P = 0.01$) than the ECLB and ECA silages, which had pH values of 4.24 and 3.96, and had approximately 10,000-fold lower and no EC, respectively. These responses suggest that the lower pH of the ECLB

Figure 4. Changes in acetate concentration of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment $\times$ day SE and *P*-value = 0.09 and 0.001, respectively; an asterisk (*) indicates that concentrations differed at this ensiling duration ($P < 0.05$). Error bars represent SE. Color version available online.

Figure 5. *Escherichia coli* O157:H7 (EC) counts and pH of whole-plant corn silage (d 128) reinoculated with 5×10^5 cfu/g of EC 168 h after silo opening. Control = distilled water; ECCH = 1×10^5 cfu/g of EC (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Similarly shaded bars with different letters differed ($P < 0.05$); SE values for pH and EC = 0.17 and 0.44, respectively. Error bars represent SE.

and ECA silages prevented the growth of EC, which was facilitated by pH values that exceeded 5.0. The ECLB and ECA silages also had greater ($P = 0.001$) aerobic stability than control, ECCH, and ECLP silages (Figure 6).

Antibacterial Activity

The cell-free supernatants from all silage extracts that were not pH-corrected produced a similar zone of inhibition (4.15 ± 0.71 mm) against EC, whereas no

Figure 6. The aerobic stability (h) of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 alone or with bacterial inoculants or propionic acid and ensiled for 120 d. Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Bars with different letters differ ($P < 0.05$). Treatment SE and P -value = 4.8 and 0.001, respectively. Error bars represent SE.

Table 3. Bacterial community composition and diversity of whole-plant corn silage inoculated with *Escherichia coli* O157:H7 alone or with bacterial inoculants or propionic acid¹

Item	Treatment ¹					SE	P-value
	Control	ECCH	ECLP	ECLB	ECA		
Species diversity and richness							
Shannon index	0.53 ^a	0.41 ^{ab}	0.30 ^b	0.52 ^a	0.42 ^{ab}	0.06	0.02
Chao index	33.5	26.4	22.5	28.7	32.9	4.68	0.16
Phylum (%)							
<i>Firmicutes</i>	97.8 ^{bc}	98.4 ^{ab}	99.0 ^a	97.4 ^c	98.7 ^a	0.29	0.01
<i>Proteobacteria</i>	2.07 ^{ab}	1.56 ^{bc}	0.98 ^c	2.63 ^a	1.18 ^c	0.28	0.01
Genus (%)							
<i>Lactobacillus</i>	94.9 ^{cd}	96.5 ^{bc}	98.3 ^a	94.8 ^d	97.9 ^{ab}	0.54	0.01
<i>Sphingobacterium</i>	0.05	0.03	0.00	0.09	0.02	0.02	0.07
<i>Weissella</i>	1.57 ^b	1.42 ^b	0.72 ^c	2.39 ^a	0.79 ^c	0.20	0.01
<i>Acinetobacter</i>	0.71 ^b	0.63 ^b	0.44 ^b	1.16 ^a	0.51 ^b	0.12	0.01
<i>Stenotrophomonas</i>	0.22	0.20	0.17	0.36	0.15	0.07	0.06
<i>Serratia</i>	0.69	0.45	0.34	0.47	0.48	0.15	0.06
<i>Lactobacillus</i> species (%)							
<i>L. diolivorans</i>	94.3 ^{bc}	95.3 ^{abc}	96.8 ^a	93.75 ^c	95.5 ^{ab}	0.55	0.01
<i>L. plantarum</i>	0.52 ^{dc}	0.71 ^{bc}	0.98 ^{ab}	0.35 ^d	1.08 ^a	0.11	0.01
Uncultured	0.70	0.40	0.47	0.46	0.78	0.15	0.09

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

¹Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Values are averages of 4 replicates of each treatment.

zone was detected for pH-corrected supernatants of the extracts.

Bacterial Community Composition and Diversity

Sequencing of the V3 to V4 regions of the bacterial 16S rRNA gene resulted in 1,250,072 reads, with an average of $62,504 \pm 2,642$ reads per sample after quality filtering. Coverage values were greater than 99% for all the samples. Rarefaction curves plateaued in all samples sequenced, indicating that the number of reads used in analysis was sufficient for identifying OTU (Supplemental Figure S1; <https://doi.org/10.3168/jds.2016-11745>). The unweighted principal coordinate analysis UniFrac plot showed that compositional differences ($P = 0.05$) existed among the bacterial communities of the different silages (Figure 7), and the Monte Carlo test revealed that the between-treatment variation in the unweighted UniFrac distance was greater ($P < 0.001$) than the within-treatment distance. Relative to the control, the ECLP silage resulted in a lower ($P < 0.05$) Shannon index, which measures diversity based on the number and evenness of species (Table 3). The Chao1 index, a measure of the richness of the bacterial species based on number of rare species, was unaffected by treatment (Table 3).

Five phyla (*Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria*) were detected in all samples. *Firmicutes* and *Proteobacteria* accounted for approximately 99.9% of the bacterial communities

(Table 3). Relative to the control (97.8% *Firmicutes* and 2.07% *Proteobacteria*), ECLP and ECA silages had greater ($P < 0.05$) OTU abundance of *Firmicutes* (99.0 and 98.7%, respectively) and lower abundance of *Proteobacteria* (0.98 and 1.18%, respectively). It is important to note that sequences that seemed to represent *Cyanobacteria* may represent chloroplasts from the ensiled corn forage, whose 16S genes have much homology to those of *Cyanobacteria* (Hanshew et al., 2013).

At the genus level, 6 genera (*Lactobacillus*, *Sphingobacterium*, *Weissella*, *Acinetobacter*, *Stenotrophomonas*, *Serratia*) accounted for approximately 99% of the bacterial community (Table 3). Compared with the control (94.9%), ECLP and ECA silages had greater ($P < 0.05$) abundance of *Lactobacillus* (98.3 and 97.8%, respectively). The ECLB silage contained greater ($P < 0.01$) abundance of *Acinetobacter* and *Weissella* (1.16 and 2.39%, respectively) than other silages.

Among the *Lactobacillus* species, the ECLP silage contained greater ($P < 0.05$) abundance of *Lactobacillus diolivorans* (96.8%) and *L. plantarum* (0.98%) compared with the ECLB silage (93.8 and 0.35%, respectively).

DISCUSSION

Anaerobic Phase

The elimination of EC from the corn silage during the early part of the ensiling phase is largely attributable

Figure 7. Unweighted UniFrac principal coordinate analysis (PCoA) plot of individual samples in each treatment. Individual samples were represented with dark circles (control), squares (ECCH), medium circles (ECLP), open circles (ECLB), and light circles (ECA). Control = distilled water; ECCH = 1×10^5 cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1×10^6 cfu/g of *Lactobacillus plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1×10^6 cfu/g of *Lactobacillus buchneri* R1102 (Lallemand Animal Nutrition); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Percentage variation explained by each PCoA is indicated on the axis. Color version available online.

to the low pH achieved due to rapid accumulation of organic acids. Previous studies have shown that EC was eliminated in corn and barley silages, and alfalfa silage when the pH was below 4.0 and 5.0, respectively (Bach et al., 2002; Chen et al., 2005; Ogunade et al., 2016). Whereas the disappearance of EC was hastened by microbial inoculation of alfalfa silage because of the slow pH decline, EC was rapidly eliminated during ensiling of corn, with or without inoculation, because of a rapid drop in pH (Pedroso et al., 2010; Duniere et al., 2011). A microbial inoculant containing *Pediococcus pentosaceus* and *Propionibacterium jensenii* also hastened (7 vs. 15 d) the elimination of EC in barley silages due to a relatively slow pH decline (Bach et al., 2002).

Although antibacterial activity against EC was evident in the low pH silage extracts, it was not observed in the pH-corrected extracts. This supports the notion that the elimination of EC during ensiling is most probably due to inhibitory effect of the low pH. Nevertheless, the pK_a (acid dissociation constant) may also be implicated because at low pH, organic acids are mostly in protonated states, and the protons can diffuse into bacterial cells and cause cell death (Bjornsdottir et al., 2006). Previous studies have shown that antibacterial activity was not detected in extracts of inoculant-treated alfalfa and corn silages adjusted to pH 5.5 and 5.0, respectively, whereas the pH-corrected cell-free supernatants from pure cultures of inoculant bacteria showed activity against EC (Pedroso et al., 2010; Ogunade et al., 2016). Gollop et al. (2005) re-

ported that pure cultures of *L. plantarum*, *Enterococcus faecium*, and *L. buchneri* inhibited the growth of *Micrococcus luteus* and *Pseudomonas aeruginosa*, but very few of the extracts of silages treated with these bacteria showed activity independent of pH.

More time (7 d) was required for elimination of EC in ECA silages than in others probably because of the lower initial pH of the ECA silage before ensiling. Goodson and Rowbury (1989) reported that *E. coli* strains that were previously habituated in a medium with a pH of 5.0 survived longer after short periods of exposure to pH 3.5, whereas those that were previously habituated in a medium with a pH of 7 failed to grow. Likewise, strains of EC habituated in a sub-lethal medium with a pH of 5.0 had greater resistance than those habituated in a medium with higher pH values when they were subsequently exposed to a medium with an acidic pH of 3.85 (Leyer et al., 1995). Similarly, in this study, EC persisted longer in the ECA silage than in other silages and the respective pH values were 5.14 and 5.95 ± 0.03 at ensiling (Table 1).

Addition of *L. buchneri* to silage enhances anaerobic degradation of lactate to acetate (Oude Elferink et al., 2001; Kleinschmit and Kung, 2006; Queiroz et al., 2013). In accordance, in this study, silage inoculation with *L. buchneri* increased the concentration of acetate. The antimycotic properties of acetate (Danner et al., 2003; Schmidt and Kung, 2010) and propionate (Woolford, 1975) resulted in the lower yeast counts of ECLB and ECA silages, respectively, compared with those

in the other silages. *Lactobacillus buchneri*-inoculated silage had higher ammonia N concentration compared with ECLP silage. This agrees with previous studies on corn and sorghum silages (Driehuis et al., 2001; Filya, 2003). A high concentration of ammonia N (>10% of total N) in corn silage is a marker of excessive protein breakdown (Ward, 2008), typically caused by a slow drop in pH and/or clostridial fermentation (Kung and Shaver, 2001). However, the concentrations observed in these silages are within the range for well-preserved corn silage (Ward, 2008). Also, the fact that no butyrate was detected supports the deduction that spoilage due to clostridial fermentation was not evident.

Aerobic Postensiling Phase

This aspect of the experiment was conducted to determine whether treatment with the silage additives at ensiling would enhance the aerobic stability of the silages and inhibit the growth of EC in silages contaminated at silo opening (d 120) and after 168 h of aerobic exposure (d 127). The elimination of EC within 6 h of reinoculation and aerobic exposure in all silages on d 120 is probably because the low pH value (3.93 ± 0.04) prevented their survival. Bach et al. (2002) reported that EC was not detected in barley silage 24 h after reinoculation of the pathogen at silo opening because the pH value of the silage was below 4.0. Likewise, EC was not detected in corn silage after 24 h of reinoculation at silo opening (Pedroso et al., 2010) because the pH values of the silages were still below 4.0.

The inhibitory effects of acetate and propionate on spoilage-causing fungi (Ranjit et al., 2002; Kleinschmit et al., 2005) in silages treated with *L. buchneri* and propionic acid are responsible for the greater aerobic stability of such silages. Aerobic spoilage occurs due to degradation of lactic acid by lactate-utilizing yeasts in the presence of air (Pahlow et al., 2003; Kung, 2010). A meta-analysis showed that *L. buchneri* inoculation decreased yeast counts and improved aerobic stability of corn silages (Kleinschmit and Kung, 2006). Similarly, in 3 separate experiments, addition of propionic acid-based products to corn silages at 0.2% of fresh weight improved the aerobic stability of the silages (Kung et al., 1998).

Whereas no or low counts of EC were detected in ECA and ECLB silages reinoculated with the pathogen after 168 h of aerobic exposure, high counts were detected in ECCH and ECLP silages probably due to their higher pH values (pH >5) compared with those of the other silages. Previous studies have shown that EC grows well in corn silage with pH above 4.0 (Fenlon and Wilson, 2000; Pedroso et al., 2010; Duniere et al.,

2011), whereas its growth is curtailed by additives that keep the silage pH below 4.0 after silo opening (Pedroso et al., 2010; Duniere et al., 2011). Likewise, our results showed that keeping the silage pH below 4 after aerobic exposure improved the silage shelf-life and also helped to curtail or prevent the growth and spread of EC. This indicates that keeping the corn silage pH below 4.0 during the feedout stage with appropriate silage additives may help reduce the risk of survival and prevalence of EC or other low pH-sensitive pathogens in aerobically exposed silages. Nevertheless, aerobically stable silage may permit survival of EC that contaminates the silage during the aerobic exposure phase, as shown in this study and our previous study on alfalfa silage (Ogunade et al., 2016).

Bacterial Community Composition and Diversity

Principal coordinate analysis of diversity based on UniFrac (unweighted) distances indicates compositional differences based on the presence or absence of OTU among samples (Park et al., 2016). The fact the bacterial diversity and taxonomic composition of the ECCH silage did not differ from that of the control silage suggests that the microbiome shift observed in the ECLP, ECLB, and ECA silages reflect the effects of the respective additives alone. Notable features of such microbiomes were greater abundance of *Lactobacillus* in ECLP and ECA silages, and greater abundances of *Acinetobacter* and *Weissella* in ECLB silage compared with the control silage. Studies that used the denaturing gradient gel electrophoresis technique reported little or no change in the bacterial community structure of corn silage treated with *L. buchneri*, *L. plantarum*, or *Lactobacillus rhamnosus* (Li and Nishino, 2011; Santos et al., 2016).

The taxonomic classification of lactobacilli to the species level with 16S gene sequences may sometimes misrepresent the microbial population due to the relatively little sequence diversity among lactobacilli (Berthier and Elrhich, 1998; Felis and Dellaglio, 2007). This may partly explain the unexpected low abundance of *L. plantarum* in ECLP silage, absence of *L. buchneri* in ECLB silage, and high abundance of *L. diolivorans* in all silages. Pang et al. (2011) identified *Weissella cibaria* and *Weissella confusa* as the dominant bacterial species in corn silage, *L. plantarum* as the dominant species in sorghum and rice silages, and *Leuconostoc pseudomesenteroides*, *Lactobacillus paraplantarum*, and *L. plantarum* as the dominant species in alfalfa silage. A recent study that applied 16S ribosomal RNA gene amplicon sequencing revealed that 99% of the sequence reads detected were *Lactobacillus reuteri*

and *Lactobacillus pontis* in corn, sorghum, and wheat silages (Kraut-Cohen et al., 2016). *Lactobacillus reuteri* and *L. pontis* have previously been detected in silages, but had never been identified as dominant bacteria (Li and Nishino, 2011; Wu et al., 2014). These dominance reports are also likely to be affected by the ensiling duration or time of sampling, which is known to influence the bacterial community of silage (Beck, 1972; Graf et al., 2016).

Lactobacillus diolivorans, which phylogenetically belongs to *L. buchneri* group, has previously been isolated from corn silage (Krooneman et al., 2002), but we found no study that attempted to quantify the abundance of the bacterium in silage. This bacterium is not regarded as a dominant *Lactobacillus* species in silage (Krooneman et al., 2002), but it can degrade 1,2-propanediol, a fermentation product of *L. buchneri*, to 1-propanol and propionic acid (Krooneman et al., 2002). In corn silage treated with *L. diolivorans* alone or with *L. buchneri*, no 1-propanol or propionate was detected, and the authors suggested that *L. diolivorans* converted 1,2-propanediol that was present into an unknown intermediate compound (Charley and Kung, 2005). In contrast, Zhang et al. (2010) reported that a higher concentration of propionate was formed when *L. diolivorans* was grown with 1,2-propanediol as the sole carbon source than when it was grown with 1,2-propanediol, fructose, and maltose in sourdough. Neither 1,2-propanediol nor 1-propanol was measured in this study and propionate was only detected in ECA silage due to treatment with propionic acid at ensiling. Therefore, no conclusive deductions can be drawn about the presence or role of *L. diolivorans* in the silages. More research is needed to confirm the dominance of *L. diolivorans* in corn silage and to elucidate its role. Such studies should consider other approaches for characterizing the microbial community. For instance, previous studies showed that recA gene sequences are more precise than 16S rRNA for differentiation of LAB species in mixed culture (Felis et al., 2001; Torriani et al., 2001). Furthermore, recent studies claimed that the Pacific Biosciences single molecule, real-time sequencing technology can predict the bacterial profile of target samples to species level because of its power to generate long sequence reads (Zhang et al., 2015; Bao et al., 2016).

The low bacterial diversity observed in this study, as shown by the low Shannon index (0.44 ± 0.09), is as a result of the high abundance ($96.5 \pm 1.63\%$) of *Lactobacillus* species due to the low pH value of corn silage (<4.0). The more the abundance of a dominant bacterium, the less diverse the microbial community (Polley et al., 2007; Allen et al., 2009).

In this study, we detected 2 genera, *Weissella* and *Acinetobacter*, whose roles in corn silage have not been extensively studied. Bacteria assigned to genus *Weissella* are strictly heterofermentative, producing a mixture of lactate and acetate as the major end products of sugar metabolism (Fusco et al., 2015; Graf et al., 2016). Inoculation of alfalfa silage with heterofermentative LAB strain, *Weissella paramesenteroides*, resulted in increased acetate concentration (Cai et al., 1998). In addition, lactate and acetate are the major acids produced by *W. confusa* during fermentation of rice cake (Baek et al., 2012). Furthermore, Ndagano et al. (2011) reported the production of acetate as well as other antifungal compounds, such as 3-hydroxy fatty acids and phenyllactate, by *W. paramesenteroides* isolated from fermented cassava (*Manihot esculenta*). Other silages contained equal or greater abundance of *Lactobacillus*, which also produce lactate and acetate (Charley and Kung, 2005), and no other bacteria that are known to produce lactate or acetate responded to the inoculant treatments. Thus, the greater abundance of *Weissella* in the ECLB silage may partly explain its greater acetate concentration and greater aerobic stability relative to other silages except ECA. Nevertheless, it is noteworthy that the abundance of *Weissella* was relatively small ($\geq 2.39\%$). Future studies should characterize the antifungal compounds produced by lactic acid bacteria belonging to genus *Weissella* and examine their effects on the fermentation and aerobic stability of corn silage.

Acinetobacter species are aerobic, nonfermenting bacteria, which can be found in different environments (Kämpfer and Glaeser, 2012) but are supposedly absent in silage (Li and Nishino, 2011). Some *Acinetobacter* species can survive in an anaerobic environment in the presence of acetate as a substrate (Fuhs and Chen, 1975). The uptake of acetate by *Acinetobacter* in an anaerobic condition requires energy from carbohydrate degradation (Sato et al., 1996). Silage DM loss during ensiling is typically caused by silage decay as a result of depletion of forage carbohydrate mass by activities of aerobic microorganisms at the initial stage of ensiling (Pitt, 1986). Aerobic respiration uses readily available carbohydrates and produces heat and the consequent silage DM and energy losses during ensiling (Muck and Holmes, 2000). The increased abundance of *Acinetobacter* in ECLB silage may have resulted from the increased acetate concentration, and this may partly explain the small, though important, DM losses sometimes observed in silages that had been treated with *L. buchneri* at ensiling (Filya, 2003; Kleinschmit and Kung, 2006). The practical significance of the seemingly small statistical differences (0.71 vs. 1.16%) in

the abundance of *Acinetobacter* is unknown. Future studies should examine if *Acinetobacter* is responsible for some or all of the DM losses that occur in silages that were treated with *L. buchneri* at ensiling. The abundances of unknown or unculturable bacteria that were not described were either too low (<0.01%) or did not respond to silage treatment.

CONCLUSIONS

This study showed that when the pH dropped below 4.0 within 3 d of ensiling, EC counts were below the detection limit of 10 cfu/g in all silages contaminated at ensiling with 1×10^5 cfu/g of the pathogen. The exception was the ECA silage, which required 7 d of ensiling for elimination of EC, likely because of habituation of EC to acidic conditions when the forage was treated with propionic acid. Application of *L. buchneri* and propionic acid reduced yeast counts, improved aerobic stability, and maintained a low pH for the entire duration of aerobic exposure. Reinoculation of silages with EC after 168 h of aerobic exposure resulted in relatively high EC counts of 5.39 and 5.30 cfu/g in ECCH and ECLP silages, respectively, whereas the pathogen was 10,000-fold lower in ECLB silage, which had a pH of 4.24, and it was below the detection limit in the ECA silage, which had a pH 3.96. This suggests that maintenance of pH of approximately 4.0 or lower with additives can curtail or prevent the growth of EC in contaminated aerobically exposed silages, and inhibit cycling of EC on farms or limit its introduction into the food chain. The fact that extracts of d 120 silages exhibited activity against EC before but not after they were pH adjusted, confirms that EC elimination from the silages was mediated by low silage pH. Bacterial diversity analysis of the silage revealed that the bacterial community composition in the silages was modified by additive treatment. Our results revealed that $98.3 \pm 0.65\%$ of the bacterial community involved in lactic acid fermentation in corn silage belong to phylum *Firmicutes* and $96.5 \pm 1.63\%$ belong to genus *Lactobacillus*. Furthermore, the potential respective roles in corn silage fermentation and DM losses of *Weissella* and *Acinetobacter* should be determined because their abundances increased with inoculation with *L. buchneri*.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding for this project from BARD (United States-284 Israel Binational Agricultural Research and Development Fund),

Project IS-4704-14. We are grateful to Lallemand Animal Nutrition for donation of microbial additives.

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