Fate of E. coli O157:H7 and metagenomic analysis of bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives

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Complete List of Authors:	Ogunade, Ibukun; University of Florida, 8701581 Kim, D; Gyeongsang National University, Division of Applied Life Science (BK21Plus, Institute of Agriculture and Life Science) Jiang, Y; University of Florida, Department of Animal Sciences, Institute of Food and Agricultural Sciences; Department of Animal Sciences Cervantes, A. A. Pech; University of Florida, Animal Sciences Arriola, Kathy; University of Florida, Animal Sciences Vyas, Diwakar; University of Florida, Animal Sciences; Weinberg, Zwi; The Volcani Center, Food Science; Jeong, Kwang Cheol; University of Florida, Animal Sciences; University of Florida, Emerging Pathogens Institute Adesogan, Adesogan; University of Florida, Animal Sciences
Key Words:	corn silage, Escherichia coli, metagenomics

INTERPRETATIVE SUMMARY

Fate of *E. coli* O157:H7 and metagenomic analysis of bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives. Ogunade et al. To prevent transmission of *Escherichia coli* O157:H7 on dairy farms, we examined if propionic acid or microbial inoculants can inhibit the growth of the pathogen in contaminated corn silage during and after ensiling. The pathogen was eliminated in all silages within 7 d of contamination at ensiling. Propionic acid prevented the growth of the pathogen after contamination at silo opening and *Lactobacillus buchneri* reduced its growth. Metagenomic analysis of the silage revealed that the bacterial community composition in the silages was modified by additive treatment.

Running head: CONTROL OF *ESCHERICHIA COLI* O157:H7 IN CORN SILAGE

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

I. M. Ogunade,* D. H. Kim,† Y. Jiang,* A. A. Pech Cervantes,* K. G. Arriola,* 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, FL 32608

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

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

[1]Corresponding author: Adegbola T. Adesogan, P.O. Box 110910, Department of Animal Sciences, University of Florida, Gainesville, FL 32611 352-3927527 (Phone) 352-392-7962 (Fax) adesogan@ufl.edu

ABSTRACT

The first objective was to examine if addition of microbial inoculants or propionic acid would inhibit the growth of the pathogen in corn silage contaminated with *Escherichia coli* O157:H7 at ensiling, at silo opening, or after aerobic exposure. Another objective was to examine how additive treatment affects the silage bacterial community composition. 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, 10^5 cfu/g of *E. coli* O157:H7 (**EC**); 3, EC and 10^6 cfu/g of *Lactobacillus plantarum* (**ECLP**); 4, EC and 10^6 cfu/g of *Lactobacillus buchneri* (**ECLB**); and 5, EC and 2.2 g/kg of propionic 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 120 were also analyzed for chemical composition, yeasts and molds, bacterial community composition and aerobic stability. The pH of silages from all treatments decreased below 4 within 3 d of ensiling. The pathogen was not detected in any silage after 7 d of ensiling. Treatment with *L. buchneri* and propionic acid resulted in fewer yeasts and greater aerobic stability compared with the Control, EC and ECLP silages. Compared to the Control, metagenomic 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 *Acenitobacter* 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 the pathogen

immediately after silo opening (120 h) had similar low pH values (< 4.0) and no EC were detected. The EC and ECLP silages re-inoculated with the pathogen after 168 h of aerobic exposure had relatively high pH values (> 5.0) and EC counts (5.39 and 5.3 log cfu/g, respectively) 24 h later. Whereas 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: corn silage, *Escherichia coli* O157:H7, metagenomics.

INTRODUCTION

Escherichia coli O157:H7 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 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 the pathogen when untreated manure is used to fertilize corn plants that are subsequently ensiled and fed to cattle and 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; Duniere 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). Whereas, in silages contaminated with the pathogen after aerobic exposure, maintenance of a low pH (below 4.0) due to application of *L. buchneri* at ensiling curtailed the growth of EC (Pedroso et al., 2010). In contrast, Duniere 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 *E. coli.* Therefore, more studies are needed to ascertain the

inhibitory effect of *L. buchneri* or other additives on EC-contaminated aerobically exposed silage additives. Such information is critically needed to reduce or prevent cycling of EC among animals and or people.

Propionic acid is a strong antifungal agent (Woolford et al., 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). 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. Another objective of this study was to understand how the bacterial community of corn silage is affected by EC contamination and additive application.

MATERIALS AND METHODS

Forage and Treatments

Whole-plant corn forage, harvested 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 *Escherichia coli* O157:H7 EDL933 alone (**EC**); 3) EC + 1×10^6 cfu/g *Lactobacillus plantarum* R2014 (**ECLP**); 4) EC + 1×10^6 cfu/g *Lactobacillus buchneri* R1102 (**ECLB**); 5) EC + 2.2 g/kg propionic acid (**ECA**). The pathogen was supplied by University of Florida Emerging Pathogens Institute, Gainesville, and the silage inoculant bacteria were supplied by Lallemand Animal Nutrition, Milwaukee, WI. Bacterial counts and their viability in the inoculants were

verified before silage inoculation by plating on de Man-Rogosa-Sharpe (**MRS**) agar (Difco, Detroit, MI), at 37°C for 48 h, and appropriate amounts of the inoculants were added to achieve the desired application rate. The *E. coli* O157:H7 was grown on Luria-Bertani (**LB;** Sigma-Aldrish, Saint Louis, MO) broth overnight for 18 h at 35°C. The cells in the suspension were adjusted to an optical density of 0.5 at 600 nm (GENESYS 20 spectrophotometer; Thermo Scientific, Waltham, MA). Bacterial counts in the suspension were confirmed by direct plating on sorbitol-MacConkey agar (**SMAC**; Oxoid, Basingstoke, UK) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) to make **CT-SMAC** after incubation at 35°C for 24 hours. 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. The EC suspension was also mixed with 5 mL of water and applied to the fresh forage. Equal quantities of water were applied to the treated and control forages.

Treatments were applied to each of quadruplicate samples per treatment separately (1.5 kg each). Each treated sample was hand-mixed, manually packed into a 14 × 21cm, thick-walled plastic bag, which was vacuumed and sealed with silo ties. 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 20°C. Subsamples of these silages were analyzed for EC, pH, organic acids, ammonia-N, aerobic stability, and yeast and mold counts. Furthermore, subsamples from day 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, re-inoculated 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, the time (h) for the silage temperature to exceed 2.0°C above the ambient temperature, was subsequently measured. After 168 h of aerobic exposure, silages were re-inoculated 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 minute 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, lactic acid bacteria (LAB), yeasts and molds. Enumeration of EC was done by serial dilution in buffered peptone water (**BPW**) 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 *E. coli* O157:H7 and confirmed by latex agglutination (Remel, Lenexa, KS) according to the manufacturer's instructions. The detection limit for EC counts was 10 cfu/g. An aliquot of the serially diluted extract was also plated on malt extract (Thermo Scientific-Oxoid, OXCM0059B) and MRS agars at 32°C for 72 and 48 h for enumeration of yeast and mold, 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 × *g* for 15 min at 4°C. The supernatant was analyzed for ammonia-N using an adaptation for a 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). 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 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 Luria-Bertany 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 × *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 and tested for antibacterial activity against EC.

Metagenomic 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, metagenomic analysis of the d 120 silage samples was performed using Illumina sequencing (Macrogen, South Korea). 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 minutes using Sonicor SC-150 (Sonicor Instrument Corporation, Copiague, NY) followed by centrifugation at 9000 × *g* for 15 minutes to create a pellet for subsequent DNA extraction.

Total metagenomic DNA was extracted from the resulting pellet by using a PowerLyzer PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). Bead beating (Bullet Blender Storm 24, Averill Park, NY) was used for mechanical disruption of bacterial cells. The purity and concentration of gDNA were measured with a spectrophotometer (Nanodrop® 115 1000, Thermo Scientific, Waltham, MA) and the integrity of DNA was verified by agarose (0.7%) gel electrophoresis.

Each 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, USA) and Nanodrop (Thermo Scientific, Waltham, MA). Ten nanograms of the gDNA 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, San Diego) and qualified using the LabChip GX HT DNA High-Sensitivity Kit (PerkinElmer, Massachusetts, USA). The paired-end (2 × 300 bp) sequencing was

211 performed by Macrogen Inc. (Seoul, Korea) using the Illumina MiSeq™ platform (Illumina, San
212 Diego).

213 ***Data and Statistical Analysis***

214 The Experiment had a completely randomized design with 5 treatments and 4 replicates per
215 treatment. All microbial data were transformed to log units and are presented on a wet weight basis.
216 Data were analyzed using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC) and the
217 model for analyzing data collected over time included effects of treatment, day and treatment × day.
218 Where treatment x day interactions were significant, effects of treatment on each day were
219 examined. The model for analyzing data collected at one-time point only included the treatment
220 effect.

221 For the metagenomics data, CD-HIT-OTU was used for clustering of sequences to identify
222 Operational Taxonomic Units (**OTUs**) (Li et al., 2012). The taxonomic composition of each sample
223 was generated using QIIME UCLUST (Edgar, 2010). Alpha diversity (rarefaction curve for OTUs,
224 Chao1, and Shannon indices) and beta diversity (unweighted principal coordinated analysis (**PCoA**)
225 UniFrac distance) were generated using the QIIME software package with a script
226 core_diversity_analyses.py (Caporaso et al., 2010). A nonparametric Monte Carlo test was used to
227 compare the within-treatment and between-treatment UniFrac distances. Variables, such as relative
228 abundance of bacteria, number of reads, and species richness and diversity, were analyzed using the
229 GLIMMIX procedure of SAS and a model that included the treatment effect. Differences between
230 means were determined using the Tukey test. Significant differences were declared at $P \leq 0.05$.

231 **RESULTS**

232 ***Anaerobic phase***

The chemical composition of the forage used for each treatment before ensiling was similar except that the pH of the ECA corn silage was lower ($P = 0.01$) than those of other silages (Table 1).

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) was similar for all silages (Table 2). The pathogen was not detected after 3 d of ensiling in EC, ECLP and ECLB silages suggesting that it had been eliminated. Although it was detected in the ECA silage on d 3, EC was not detected in any of the d 7 silages (Figure 2).

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), the lactate concentration was similar to those of Control, EC 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 (Table 2; Figure 4). Propionate was detected only in the ECA silage (0.41% DM), 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 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, EC and ECLP silages (5.08, 5.30 and 4.91 log cfu/g, respectively).

Aerobic phase

Escherichia coli O157:H7 was not detected in silages within 6 h of re-inoculation 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 re-inoculated with the pathogen on d 127 (Figure 5). In addition, the d 128 EC and ECLP silages had greater pH values (5.67, and 6.13, respectively) 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 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, EC 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 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 ± 2,642 reads per sample after quality filtering. Coverage values were greater than 99% for all the samples. Rarefaction curves plateaud in all samples sequenced, indicating that the number of reads used in analysis was sufficient in identifying OTUs (Figure S1). The unweighted PCoA 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 Proteabacteria) were detected in all samples. *Firmicutes* and *Proteobacteria* accounted for approximately 99.9% of

the bacterial communities. 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).

At the genus level, six genera (*Lactobaccillus*, *Weissella, Serratia, andAcenitobacter, and Stenotrophomonas*) accounted for approximately 98% of the bacterial community. Compared to 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 *Acenitobacter* 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 *L. diolivorans* (96.8%) and *L. plantarum* (0.98%) when compared to 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 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). While the disappearance of EC was hastened by microbial inoculation in 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 confirms that the elimination of EC during ensiling is most probably due to low pH. Previous studies have shown that the antibacterial activity of the extracts of alfalfa and corn silages adjusted to pH 5.5 and 5.0, respectively, was not detected 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) reported that pure cultures of *L. plantarum, Enterococcus faecium,* and *L. buchneri* inhibited the growth of *Micrococus 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 Robury (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 (Gregory 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.

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 to 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).

Aerobic post-ensiling 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 re-inoculation 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 *E. coli* O157:H7 was not detected in barley silage 24 h after re-inoculation 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 re-inoculation at silo opening (Pedroso et al., 2010) because the pH values of the silages were still below.

The inhibitory effects of acetate and propionic acid 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-assimilating 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 three

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).

While no EC or low counts of the pathogen were detected in ECLB and ECA silages re-inoculated with the pathogen after 168 h of aerobic exposure, high counts were detected in EC and ECLP silages probably due to their pH values (pH > 5) compared to 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/or 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 component analysis (PCoA) of diversity based on UniFrac (Unweighted) distances indicates compositional differences based on the presence or absence of OTUs among samples (Park et al., 2016). The fact the bacterial diversity and taxonomic composition of the EC 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 abundances of *Lactobacillus* and *L. plantarum* in ECLP and ECA silages, and greater abundances of *Acenitobacter* and *Weissella* in ECLB silage compared to the

Control silage. Studies that utilized DGGE technique reported little or no change in the bacterial community structure of corn silage treated with *L. buchneri, L. plantarum* or *L. rhamnosus* (Li and Nishino, 2011; Santos et al., 2015).

The high abundance of *L. diolivorans*, phylogenetically belonging to *L. buchneri* group (Krooneman et al., 2002), in ECLB silages, could be due to presence as an epiphytic lactic acid bacterium on the fresh corn forage. The presence of this organism, which converts propanediol to propanol and then propionic acid (Krooneman et al., 2002), may be partly responsible for the relatively greater aerobic stability of the least stable silage in this study versus those in our earlier similar study (Pedroso et al., 2010). Addition of *L. diolivorans* to corn silage resulted in greater acetate concentration and improved aerobic stability (Charley and Kung, 2005).

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 ± 1.63%) of *Lactobacillus* spp. due to the low pH value of corn silage (< 4.0). The more the abundance of a dominant specie, the less diverse the microbial community (Polley et al., 2007; Allen et al., 2009).

In this study, we detected two 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 other antifungal compounds, such as 3-hydroxy fatty acids, in addition to acetate and phenyllactate, by *W. paramesenteroides* isolated from fermented cassava. Thus, the

greater abundance of *Weissella* in the ECLB silage may have been partly responsible for its greater acetate concentration. Other silages contained equal or greater abundance of *L. diolivorans*, which also produces lactate and acetate (Charley and Kung, 2005) and no other bacteria that are known to produce lactate or acetate responded to the inoculant treatments. 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.

Acenitobacter spp. are aerobic, non-fermenting bacteria, which can be found in different environments (Berg et al., 1996) but are supposedly absent in silage (Li and Nishino, 2011). Some *Acenitobacter* species can survive in an anaerobic environment in the presence of acetate as a substrate (Fuhs and Chen, 1975). The uptake of acetate by *Acenitobacter* in an anaerobic condition requires energy from carbohydrate degradation (Satoh 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 micro-organisms at the initial stage of ensiling (Pitt, 1986). Aerobic respiration utilizes readily available carbohydrate and produces heat and the consequent silage DM and energy losses during ensiling (Muck and Holmes, 2000). The increased abundance of this *Acenitobacter* 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). Future studies should examine if *Acenitobacter* 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 discribed above were either too low (< 0.01%) or did not respond to silage treatment.

CONCLUSIONS

This study shows that EC was undetected in all silages contaminated at ensiling with 5 log cfu/g of the pathogen when the pH dropped to 4.0 within 3 d of ensiling. The exception was the ECA silage, which required 7 d of ensiling for elimination of EC, likely because of habituation of the pathogen 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. Re-inoculation of silages with *E. coli* O157:H7 after 168 h of aerobic exposure resulted in relatively high EC counts of 5.39 and 5.30 cfu/g in EC 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 not detected in the ECA silage, which had a pH 3.96. This suggests that maintenance of pH of approximately 4.0 with additives can curtail or prevent the growth of EC in contaminated aerobically exposed silages, and inhibit cycling of the pathogen 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. Metagenomic analysis of the silage revealed that the bacterial community composition in the silages was modified by additive treatment. Our results revealed that exactly 98.3 ± 0.65% of the bacterial community involved in lactic acid fermentation in corn silage belong to phylum *Firmicutes* and 96.5 ± 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*.

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Table 1: Characteristics (% of DM except as noted) 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	EC	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	8.75	8.77	8.37	8.26	8.55	0.23	0.16
NDF	41.6	41.4	40.6	42.5	42.4	1.40	0.68
ADF	24.9	24.8	25.5	25.4	25.0	1.41	0.98

[ab]Means within a row with different superscripts differ ($P < 0.05$).

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

Table 2: Fermentation indices and chemical composition (% of DM except as noted) and microbial counts (log cfu/g) 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	*P*-value
	Control	EC	ECLP	ECLB	ECA		
pH	3.93	3.91	3.89	3.91	3.87	0.04	0.16
Lactate	2.99^{ab}	3.10^{ab}	3.50^{a}	2.59^{b}	3.06^{ab}	0.20	0.01
Acetate	2.33^{b}	2.43^{b}	1.85^{b}	3.39^{a}	2.26^{b}	0.30	0.01
Propionate	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.41^{a}	0.05	0.01
Yeasts	5.08^{a}	5.30^{a}	4.91^{a}	3.43^{b}	2.92^{b}	0.48	0.01
DM (%)	30.3	30.2	30.6	30.8	31.0	0.44	0.30
NDF	42.5	42.3	43.3	42.5	43.3	1.89	0.97
ADF	28.4	28.0	28.6	28.7	28.6	1.33	0.98

CP	9.58	9.11	9.23	9.02	9.37	0.21	0.11
Ammonia-N	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

682 $^{a–c}$Means within a row with different superscripts differ ($P < 0.05$).
683 [1]Control = distilled water; EC = 1 × 10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1 × 10^6 cfu/g of *L.*
684 *plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1 × 10^6 cfu/g of *L.*
685 *buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic
686 acid.
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696 Table 3: Bacterial community composition and diversity of whole-plant corn silage inoculated with
697 *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid

Item	Treatment[1]					SE	*P*-value
	Control	EC	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 (%)							
Firmicutes	97.8^{bc}	98.4^{ab}	99.0^{a}	97.4^{c}	98.7^{a}	0.29	0.01
Proteobacteria	2.07^{ab}	1.56^{bc}	0.98^{c}	2.63^{a}	1.18^{c}	0.28	0.01
Genus (%)							
Lactobaccillus	94.9^{cd}	96.5^{bc}	98.3^{a}	94.8^{d}	97.9^{ab}	0.54	0.01
Sphingobacterium	0.05	0.03	0.00	0.09	0.02	0.02	0.07

Weissella	1.57[b]	1.42[b]	0.72[c]	2.39[a]	0.79[c]	0.20	0.01
Acenitobacter	0.71[b]	0.63[b]	0.44[b]	1.16[a]	0.51[b]	0.12	0.01
Stenotrophomonas	0.22	0.20	0.17	0.36	0.15	0.07	0.06
Lactobaccillus species (%)							
L. diolivorans	94.3[bc]	95.3[abc]	96.8[a]	93.75[c]	95.5[ab]	0.55	0.01
L. plantarum	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 ($P < 0.05$).
[1]Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1×10^6 cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid.

Figure 1.

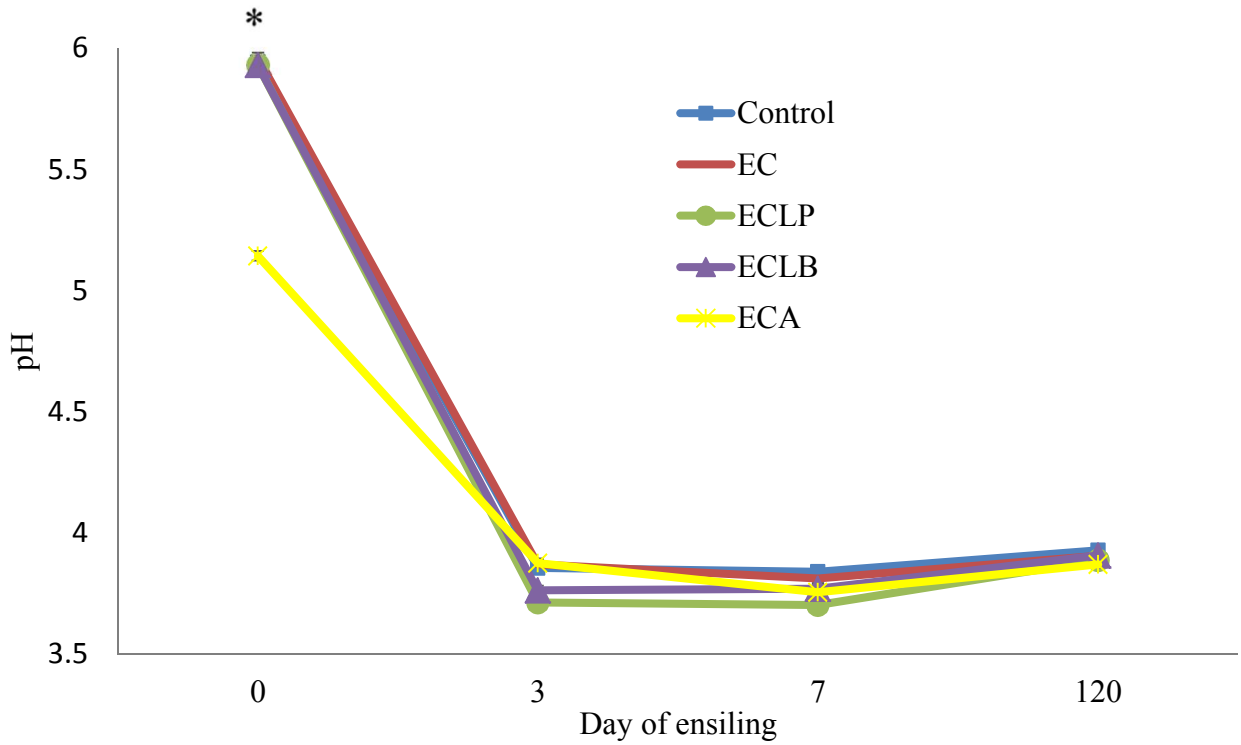

Figure 1. Changes in the pH of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations

Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1×10^6 cfu/g of *L.*

720 *buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic
721 acid. Treatment × day SE and *P*-value = 0.02 and 0.001, respectively; an asterisk (*) indicates that
722 values differed at this ensiling duration ($P < 0.05$). Error bars represent SE.
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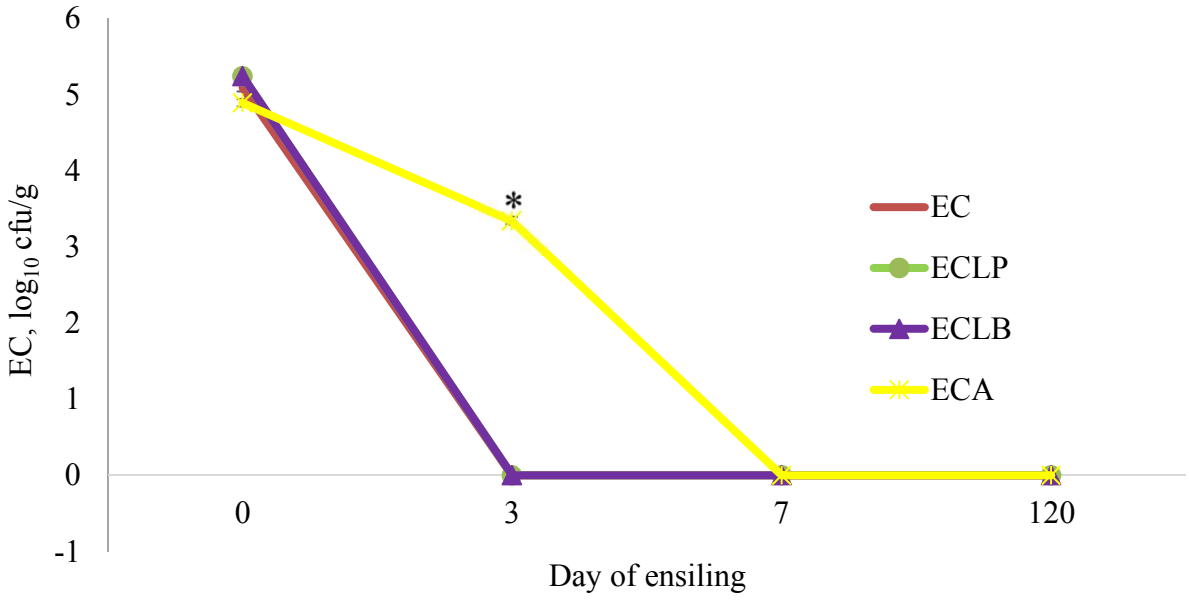
738 Figure 2.



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755 **Figure 2**. Changes in *Escherichia coli* O157:H7 (EC) counts of whole-plant corn forage inoculated
756 with 1 × 10^5 cfu/g of EC alone or with bacterial inoculants or propionic acid and ensiled for
757 different durations.

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

Figure 3.

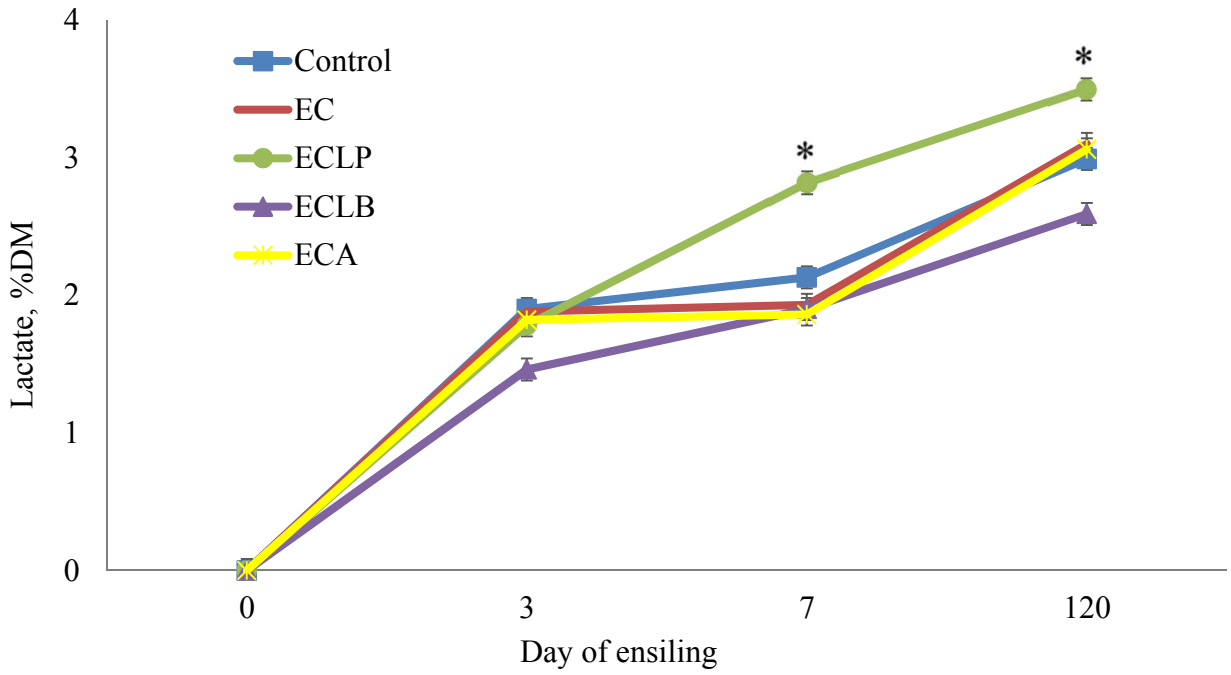

Figure 3. Changes in lactate concentration of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations

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

Figure 4.

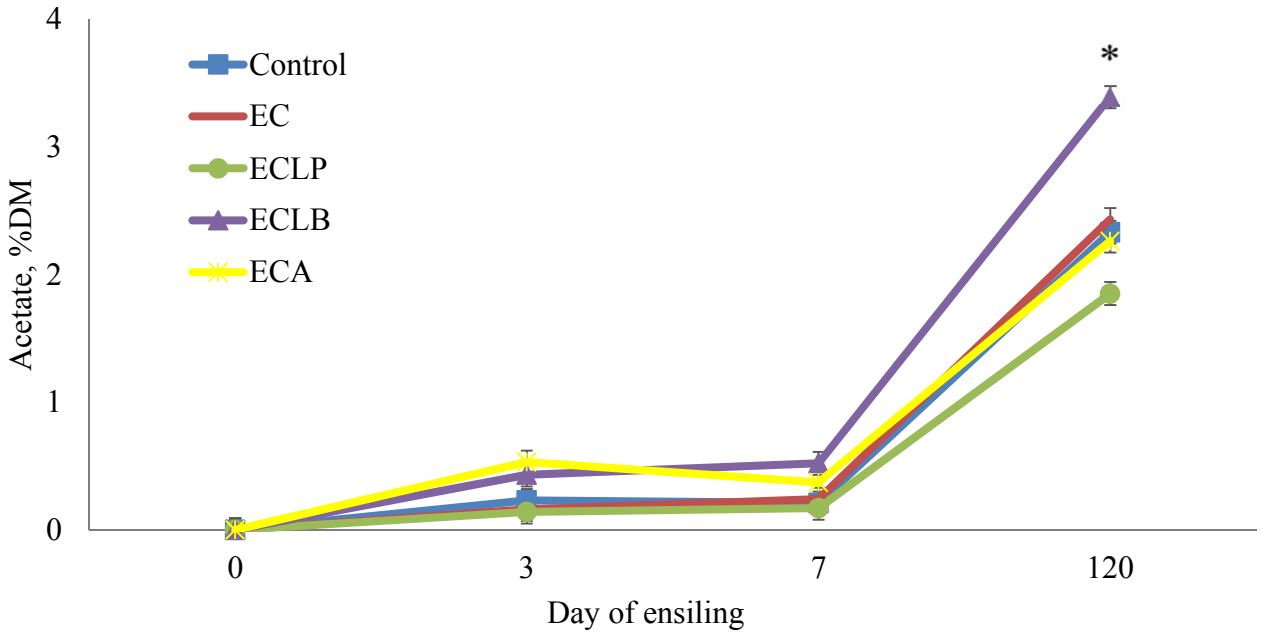

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Figure 4. Changes in acetate concentration of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations

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

925 Figure 5.

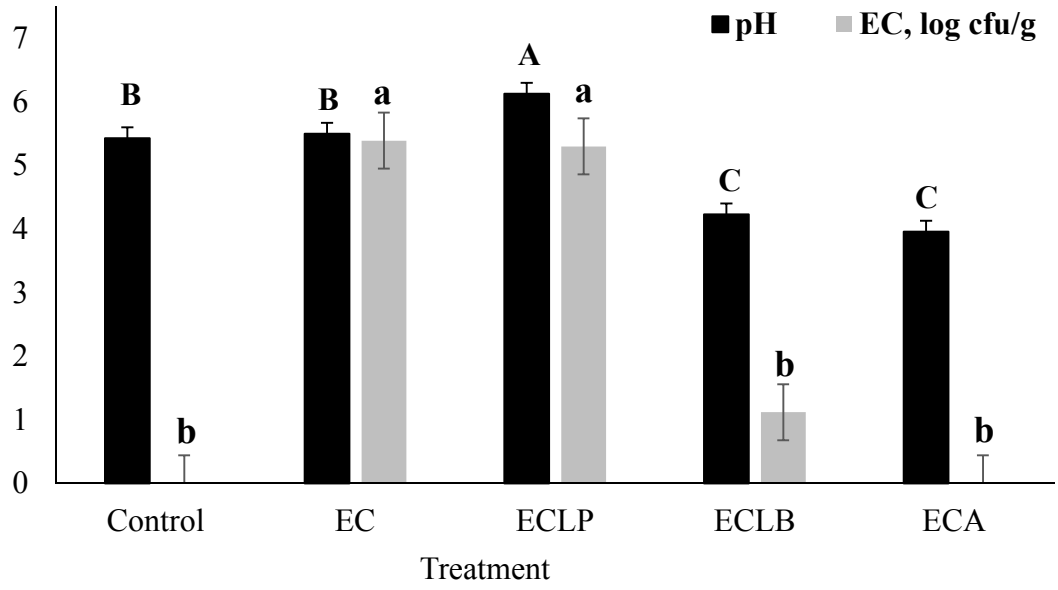

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Figure 5. *Escherichia coli* O157:H7 (EC) counts and pH of whole-plant corn silage (d 128) re-inoculated with 5×10^5 cfu/g of EC 168 h after silo opening
Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1×10^6 cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid. 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.

973 Figure 6

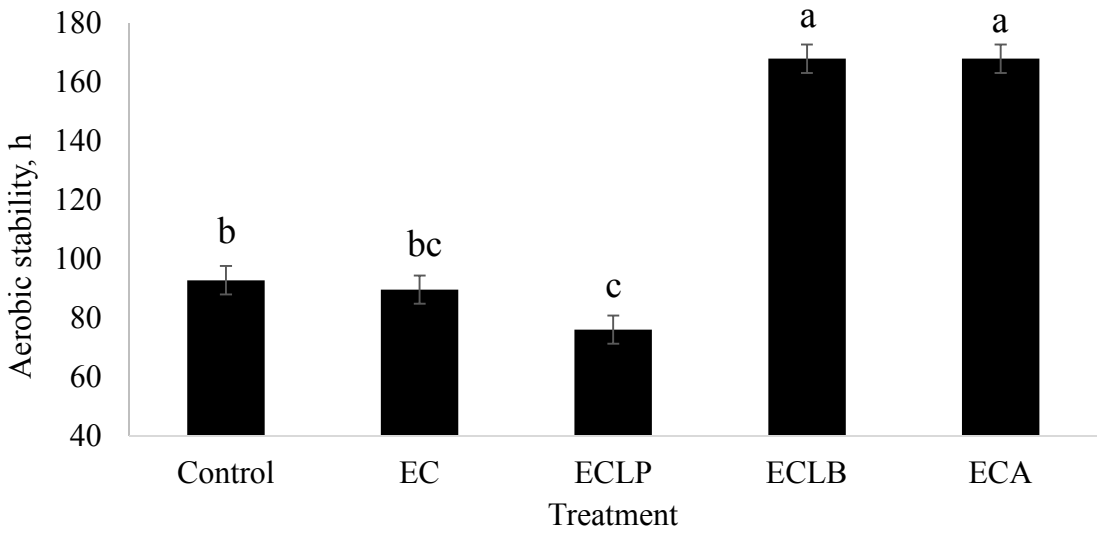

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Figure 6. The aerobic stability (h) of whole-plant corn forage inoculated with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for 120 d

Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1×10^6 cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid. Bars with different letters differ ($P < 0.05$). Treatment SE and *P*-value = 4.8 and 0.001, respectively. Error bars represent SE.

Figure 7

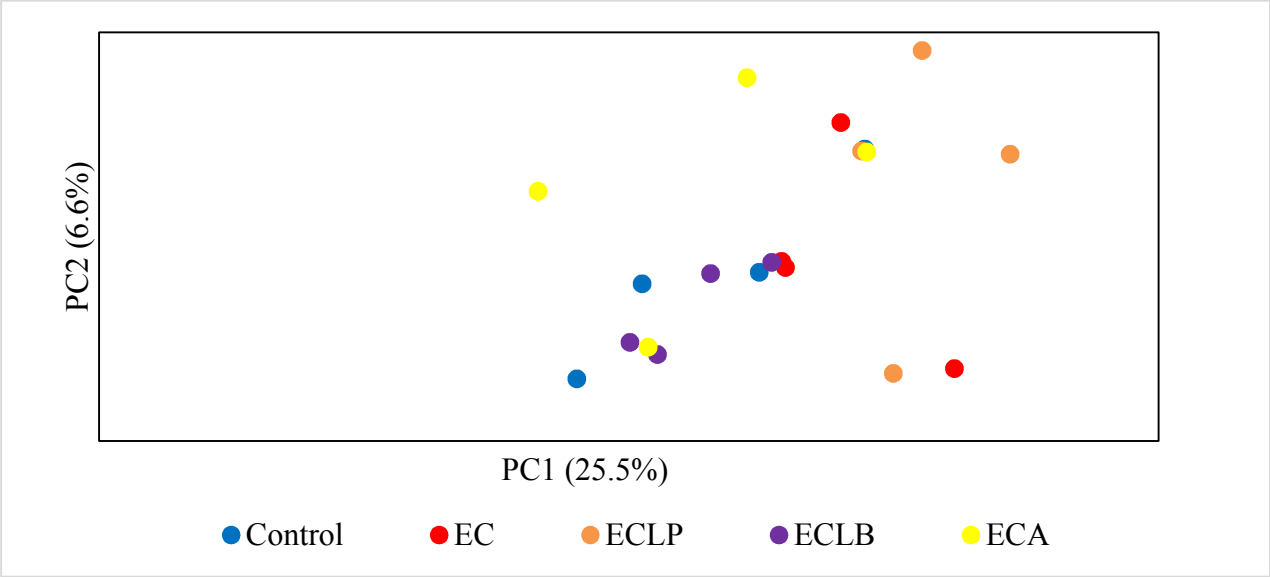

Figure 7. Unweighted UniFrac Principal Component Analysis (PCoA) plot of individual samples in each treatment. Individual sample was represented with blue (Control), red (EC), orange (ECLP), purple (ECLB), and yellow (ECA)
Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; ECLP = EC + 1×10^6 cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = EC + 1×10^6 cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid. Percentage variation explained by each PCoA is indicated on the axis

Figure S1

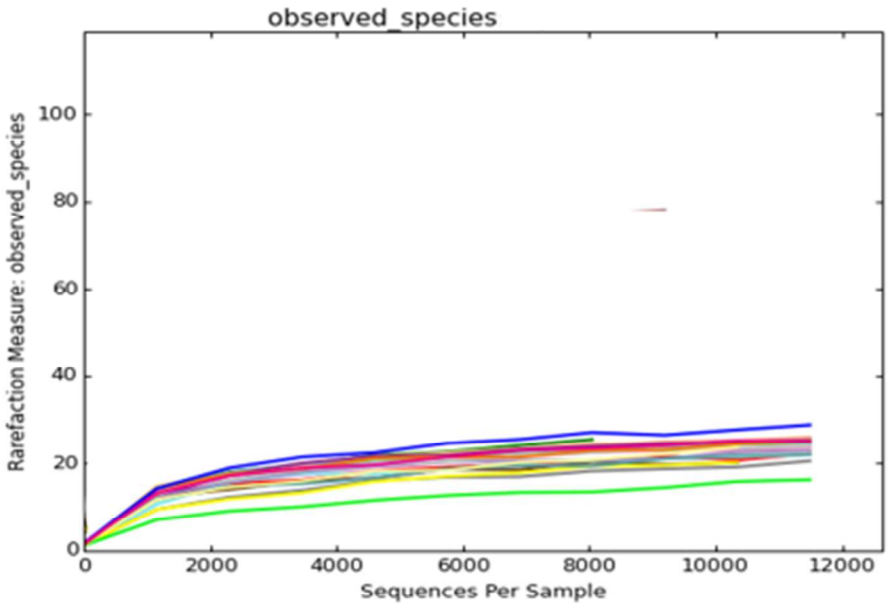

Figure S1. Rarefaction curves of observed OTUs at 97% similarity of the 20 silage samples