Control of *Escherichia coli* O157:H7 in contaminated alfalfa silage: Effects of silage additives

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

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

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

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

ABSTRACT

This study was conducted to examine if adding microbial inoculants or propionic acid to alfalfa silages contaminated with *Escherichia coli* O157:H7 would inhibit the growth of the pathogen during or after ensiling. Alfalfa forage was harvested at the early bloom stage, wilted to a dry matter concentration of 54%, chopped to 19-mm lengths, and ensiled after treatment with one of the following: (1) distilled water (control); (2) 1×10^5 cfu/g of *E. coli* O157:H7 (EC); (3) EC and 1×10^6 cfu/g of *Lactobacillus plantarum* (EC+LP); (4) EC and 1×10^6 cfu/g of *Lactobacillus buchneri* (EC+LB); and (5) EC and 2.2 g/kg of propionic acid (EC+PA). Each treatment was ensiled in quadruplicate in laboratory silos for 0, 3, 7, 16, and 100 d and analyzed for EC counts, pH, and organic acids. In addition, samples from d 100 were analyzed for chemical composition, ammonia-N, counts of yeasts and molds, and aerobic stability. *Escherichia coli* O157:H7 was detected in all silages until d 7, but by d 16 it was not detected in those treated with EC+LB and EC+LP, though it was still detected in EC and EC+PA silages. However, by d 100, the pathogen was not detected in any silage. The rate of pH decrease to 5.0 was fastest for the EC+LP silage (7 d), followed by the EC+LB silage (16 d). Nevertheless, all silages had attained a pH of or less than 5.0 by d 100. The rapid decrease in pH in EC+LP and EC+LB silages was observed due to higher lactate and acetate concentrations, respectively, relative to the other silages during the early fermentation phase (d 3–16). Propionic acid was only detected in the EC+PA silage. Yeast counts were lowest in EC+LB and EC+PA silages. Subsamples of all d-100 silages were reinoculated with 1×10^5 cfu/g of EC immediately after silo opening. When the pathogen was subsequently enumerated after 168 h of aerobic exposure, it was not detected

in silages treated with EC+PA, EC+LB, or EC+LP, which all had pH values less than 5.0. Whereas the EC silage had a pH value of 5.4 and $2.3 \log$ cfu/g of the pathogen. Certain bacterial inoculants can hasten the inhibition of *E. coli* O157:H7 during ensiling, such as propionic acid, and they can also prevent its growth on silage contaminated with the pathogen after ensiling.

Key words: alfalfa silage, microbial inoculants, *Escherichia coli* O157:H7, propionic acid

INTRODUCTION

Escherichia coli O157:H7 is a Shiga toxin-producing food-borne pathogen associated with hemorrhagic colitis and hemolytic uremic syndrome in humans (USDA-APHIS, 2001). This pathogen has been implicated in outbreaks of these illnesses in the United States, Canada, and Europe and accounts for about 95% of hemolytic uremic syndrome cases (Griffin, 1995). *Escherichia coli* O157:H7 is estimated to cause about 75,000 illness cases and 50 deaths yearly in the United States (Mead et al., 1999). Cattle are considered the primary reservoir of *E. coli* O157:H7 (Cernicchiaro et al., 2013), and they shed the pathogen in their manure (Lahti et al., 2003; Mir et al., 2015).

Animal feeds, such as silage, have been suggested to be the vehicle for transmission of the pathogen among livestock (Hancock et al., 2001; Davis et al., 2003). Additive treatments of silages may reduce the prevalence and spread of the pathogen on farms and reduce the risk of human exposure (Matthews et al., 2013). Corn silage and alfalfa hay or silage are the most commonly fed forages in dairy cow diets in the United States and can be contaminated with *E. coli* O157:H7 through the manure and saliva of cows (Russell et al., 2000). The rapid drop in pH during ensiling kills *E. coli* O157:H7 in corn silage contaminated with the pathogen before ensiling. *Lactobacillus plantarum* hastened this decrease in pH (Chen et al., 2005), but it did not hasten or increase inhibition of the pathogen during ensiling (Pedroso et al., 2010). However, *Lactobacillus buchneri*

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¹Corresponding author: adesogan@ufl.edu

prevented the growth of the pathogen on contaminated aerobically exposed corn silage (Pedroso et al., 2010). Despite its widespread usage in dairy cow diets due to its high nutritional value (Albrecht and Beauchemin, 2003), no studies have examined the use of microbial inoculants to inhibit the spread of *E. coli* O157:H7 via alfalfa silage. The high buffering capacity and low water-soluble carbohydrate concentration of alfalfa hinder the rapid pH decline necessary for efficient fermentation. Therefore, pathogens eliminated by the rapid pH decline and sustained acidic conditions in ensiled corn may not be eliminated in ensiled alfalfa. Whether microbial inoculants or acid additives can reduce the transmission of *E. coli* O157:H7 on dairy farms by inhibiting the growth of the pathogen on alfalfa forage during or after ensiling is unknown. The objective of our study was to evaluate the effectiveness of obligate or facultative heterofermentative lactic acid bacteria (**LAB**) inoculants or propionic acid at controlling *E. coli* O157:H7 in alfalfa silage during and after ensiling.

MATERIALS AND METHODS

Harvesting, Ensiling, and Inoculation

Alfalfa forage was harvested at the early bloom stage with a mower (John Deere 946, Moline, IL), wilted to a DM of 54%, and chopped to a theoretical length of cut of 19 mm with a forage harvester (John Deere 7950). The following treatments were applied in quadruplicate with a hand-held garden sprayer to 1.5 kg of the forage: (1) distilled water (control); (2) 1×10^5 cfu/g of *Escherichia coli* O157:H7 EDL933 alone (**EC**); (3) EC + 1×10^6 cfu/g of *L. plantarum* R2014 (**EC+LP**); (4) EC + 1×10^6 cfu/g of *L. buchneri* R1102 (**EC+LB**); and (5) EC + 2.2 g/kg of propionic acid containing 99.9% of the acid (**EC+PA**). The EC was supplied by the University of Florida Emerging Pathogens Institute (Gainesville) and the silage inoculant bacteria were supplied by Lallemand Animal Nutrition (Milwaukee, WI). The number of bacteria in the inoculants was verified by serial dilutions in buffered peptone water (Oxoid, Basingstoke, UK) followed by plating on de Man, Rogosa, Sharpe (**MRS**) agar (Difco, Detroit, MI) and incubating at 37°C for 48 h. The EC was grown in Luria-Bertani broth (Sigma-Aldrich, Saint Louis, MO) overnight at 35°C for 12 h. The cells in the suspension were adjusted to an optical density of 0.5 at 600 nm using a GENESYS-20 spectrophotometer (Thermo Scientific, Waltham, MA). The number of EC in the suspension was verified by spread plating on sorbitol MacConkey agar (**SMAC**; Oxoid) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L; **CT-SMAC**), and incubated at 35°C for

24 h. The pathogen inoculum was diluted in distilled water (1:5 ratio) and applied to the forage to supply 5 log cfu/g at ensiling. The bacterial inoculants and propionic acid were also diluted in distilled water (1:2 and 1:0.3 ratio, respectively) and applied to the forage at 3 mL/kg of fresh forage (Pedroso et al., 2010) to give application rates of 1×10^6 cfu/g for each inoculant and 0.22% for the acid. Control silages were also treated with the 3 mL of distilled water/kg of fresh forage. After proper mixing, the treated forages were packed into 14 × 21 cm, thick-walled plastic bags that were subsequently vacuumed to remove air and sealed with plastic ties. Each treatment was ensiled for 0, 3, 7, and 16 d at 20°C. Samples from these ensiling durations were analyzed for EC counts, pH, organic acids, and ammonia-N. In addition, each treatment was applied to 4.5 kg of forage in quadruplicate, packed into 20-L plastic mini-silos lined with thick-walled plastic bags, and ensiled for 100 d at 20°C. On d 100, samples from these silos were analyzed for EC, pH, organic acids, ammonia-N, aerobic stability, and yeast and mold counts. In addition, samples from d 0 and 100 were analyzed for chemical composition.

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

The objective of this phase of the experiment was to determine whether prior treatment with the silage additives at ensiling would enhance the aerobic stability and prevent the growth of EC in silages contaminated with the pathogen after silos were opened. Approximately 1 kg of d-100 silages from each replicate was transferred to a 20-L plastic mini-silo and reinoculated with 5 log cfu/g of EC. The silages in each silo were covered with 2 layers of cheese cloth to avoid dehydration and stored at 20°C for 7 d. Silage temperature was monitored with wireless temperature sensors (Onset Computer Corporation, Bourne, MA) that were placed in the geometrical center of the silage mass in each silo. The sensors recorded silage temperatures at 30-min intervals. Two additional sensors were placed in the room to record ambient temperature. Aerobic stability was defined as the number of hours the temperature in the silages remained stable before rising more than 2°C above room temperature. On d 107, samples from these silos were analyzed for EC, yeast and mold counts, pH, and aerobic stability.

Laboratory Analyses

Silage extracts for microbial enumeration, VFA, pH, and ammonia-N analyses were prepared by blending 20 g of silage with 180 mL of distilled water in a stomacher

(UL Lab-Blender 400, Seward Laboratory, London, UK) for 1 min and filtering through 2 layers of cheese cloth. The filtrate was immediately analyzed for EC, yeast and mold counts, and pH. For EC enumeration, serial 10-fold dilutions were prepared in buffered peptone water followed by direct plating of 0.1-mL aliquots of the dilutions 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 enumerated as EC. The detection limit for EC enumeration was 10 cfu/g. Colonies were confirmed for the O157 and H7 antigens by latex agglutination (Remel, Lenexa, KS; March and Ratnam, 1989). Yeasts and molds were enumerated after spread-plating 10-fold serial dilutions on malt extract agar (Difco) and incubated at 32°C for 72 h. The pH of silage extracts was measured using a digital pH meter (Accumet AB15, Fisher Scientific, Pittsburgh, PA). An aliquot of the silage extract was centrifuged at $1,800 \times g$ for 15 min at 4°C and the supernatant was analyzed for ammonia-N using an adaptation for the Technicon Auto Analyzer of the method of Noel and Hambleton (1976). Organic acids were quantified using a Merck Hitachi Elite La-Chrome High-Performance Liquid Chromatograph system (Hitachi L2400, Tokyo, Japan) fitted with a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) with a 0.015 M sulfuric acid mobile phase and a flow rate of 0.7 mL/min at 50°C (Muck and Dickerson, 1988). The DM concentrations of the fresh forage and silage were 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 (A. H. Thomas, Philadelphia, PA). Concentrations of NDF and ADF were measured in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY) using the procedures of Van Soest et al. (1991). 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 after Kjeldahl digestion by 6.25.

Antibacterial Activity of the Microbial Inoculants and Silage Extracts

Antibacterial activity against EC of pure cultures of the microbial inoculants and d-100 silage extracts was determined by the agar disk diffusion method of Bauer et al. (1966). The EC isolates were cultured in Luria-Bertani broth and adjusted to an optical density of 0.5 at 600 nm and the inoculum was prepared on CT-SMAC agar. Bacteria in the commercial inoculants were grown in MRS broth at 35°C for 24 h. Cell-free supernatants were prepared by centrifuging the cultures at $3,000 \times g$ for 15 min at 4°C. The pH of the supernatants was ad-

justed to 5.5. The MRS broth was also adjusted to pH of 5.5 and used as control. Silage extracts were similarly centrifuged and the cell-free supernatants were adjusted to pH 5.5. Two sterile cloning discs (6.0 mm in 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 triplicate and incubated at 35°C for 24 h. The diameter of inhibition zones around the paper discs was measured with a ruler.

Statistical Analysis

The experiment had a completely randomized design with 5 treatments and 4 replicates per treatment. All microbial data were transformed to $\log_{10}$. Data were analyzed using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). The model for analyzing data collected repeatedly over time (pH, EC counts, and organic acid concentrations) included effects of treatment, day, and treatment $\times$ day. Where treatment $\times$ day interactions were significant, effects of treatment on each day were examined using the PDIF procedure of SAS, which differentiates means based on Fisher's F-protected least significant difference test. The model for analyzing the data collected once included the treatment effect. Differences between means were determined using the PDIF procedure. Significance was declared at $P < 0.05$.

RESULTS

Anaerobic Phase

The chemical composition of the fresh alfalfa forage before ensiling was similar for all treatments, except that, as expected, the pH of EC+PA silage was lower ($P = 0.01$) than those of the other treatments (Table 1). The EC+LP silage had the fastest ($P = 0.01$) increase in lactate concentration and it had greater concentrations than other treatments for the entire ensiling duration (Figure 1). The EC+LB silage consistently had greater lactate concentrations than EC, EC+PA, or control silages after 3 d of ensiling. None of the silages contained acetate until d 7, when it was only detected in the EC+LP silage (Figure 2). Yet the EC+LB silage had the greatest acetate concentrations after 16 and 100 d of ensiling and the EC+LP silage had intermediate concentrations. Propionic acid was detected in only the EC+PA silage on d 3, 7, and 16 (0.17 ± 0.03 , 0.24 ± 0.06 , and $0.18 \pm 0.01\%$ DM, respectively).

Within 7 d of ensiling, the pH of the EC+LP silage had decreased below 5.0 (Figure 3), which is the mini-

Table 1. Chemical composition (% of DM, except as noted) and pH of alfalfa forage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid before ensiling

Item	Treatment ¹					SE	P-value
	Control	EC	EC + LP	EC + LB	EC + PA		
pH	6.23 ^a	6.20 ^a	6.21 ^a	6.20 ^a	5.85 ^b	0.04	0.01
DM, %	56.8	56.3	54.8	56.2	56.7	0.87	0.17
CP	22.9	23.6	23.1	23.5	23.8	0.43	0.25
NDF	36.4	35.8	37.0	35.5	37.5	1.27	0.58
ADF	29.3	28.3	28.9	28.3	30.5	1.00	0.30

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

¹Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid.

mal pH value in well-preserved, high-DM alfalfa silage (Kung et al., 1987). In contrast, it took 16 d for the pH of the EC+LB silage to decrease below 5.0 and a longer period for a similar decrease in the other silages.

The fresh, unensiled control forage contained 2.3 log cfu/g of EC (Figure 4), possibly due to contamination from the liquid dairy manure applied to the forage approximately 50 d before it was harvested. Nevertheless, the EC counts of the control silage were about 10,000-fold lower than those of the silages spiked with the pathogen. The pathogen was detected in all silages on d 3 and 7, but by d 16 it was no longer detected in inoculant-treated silages, whereas it persisted for longer periods in the other silages. Nevertheless, no EC was detected in any of the silages by d 100 when all

their pH values were below 5.0 (Table 2). However, it is important to note that selective enrichment of the samples that showed no EC count might have led to recovery of the pathogen.

The pH was lower ($P < 0.05$) in d-100 EC+LP and EC+LB silages (4.57 and 4.64, respectively) than the corresponding control and EC silages (4.88 and 4.82, respectively; Table 2). This was due to the greater ($P < 0.05$) concentrations of lactate and acetate in EC+LP and EC+LB silages, respectively, than in control and EC silages. The EC+PA silage had a similar pH value to silages treated with the microbial inoculants partly because it had a higher propionic acid concentration (0.22% DM) than the other silages, as it had less lactate and acetate than EC+LP and EC+LB, respectively.

Figure 1. Changes in lactate concentration of alfalfa forage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid during ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid. Treatment $\times$ day P -value = 0.01; an asterisk (*) indicates that concentrations differed at the day of ensiling specified ($P < 0.05$). Error bars represent SE. Color version available online.

Figure 2. Changes in acetate concentration of alfalfa forage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid during ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid. Treatment $\times$ day P -value = 0.001; an asterisk (*) indicates that concentrations differed at the day of ensiling specified ($P < 0.05$). Error bars represent SE. Color version available online.

None of other silages contained propionic acid and no butyrate was detected in the silages. The EC+LB and EC+PA silages had lower lactate-to-acetate ratios than the EC+LP silage. Yeast counts were lower ($P < 0.01$) in the EC+LB and EC+PA silages (3.06 and 2.65 log cfu/g, respectively) than in control, EC, and EC+LP silages (5.22, 4.85, and 4.50 log cfu/g, respectively).

Aerobic Phase

After 168 h of reinoculation and aerobic exposure, 2.3 log cfu/g of EC were detected in the EC silage, but no EC was detected in the control, inoculant, or propionic acid-treated silages (Figure 5). The pH values of the inoculant and propionic acid-treated silages

Figure 3. Changes in pH of alfalfa forage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid during ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid. Treatment $\times$ day P -value = 0.02; an asterisk (*) indicates that concentrations differed at the day of ensiling specified ($P < 0.05$). Error bars represent SE. Color version available online.

Table 2. Fermentation indices, chemical composition (% of DM except as noted) and yeast counts (log cfu/g) of alfalfa silage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid and ensiled for 100 d

Item	Treatment ¹					SE	P-value
	Control	EC	EC + LP	EC + LB	EC + PA		
pH	4.88 ^a	4.82 ^{ab}	4.57 ^c	4.64 ^c	4.67 ^{bc}	0.05	0.01
Lactate	2.14 ^b	1.77 ^b	4.47 ^a	2.59 ^{ab}	1.14 ^b	0.93	0.03
Acetate	0.95 ^{cb}	0.67 ^c	1.27 ^b	2.20 ^a	0.98 ^{cb}	0.23	0.01
Lactate: acetate	2.27 ^{ab}	2.83 ^{ab}	3.93 ^a	1.15 ^b	1.28 ^b	0.92	0.05
Propionate	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^b	0.22 ^a	0.01	0.01
Yeast	5.22 ^a	4.85 ^a	4.50 ^a	3.06 ^b	2.65 ^b	0.53	0.01
Aerobic stability, h	166	160	>168	>168	>168	5.25	0.39
DM, %	54.5	54.1	54.7	54.6	55.6	0.53	0.12
NDF	40.4	42.1	40.5	42.5	42.5	1.38	0.41
ADF	32.6	34.4	32.2	34.3	33.0	1.47	0.52
CP	24.3	23.1	23.6	24.5	24.0	0.82	0.47
Ammonia-N	0.34	0.42	0.30	0.36	0.36	0.10	0.76

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

¹Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid.

were below 5.0 (Figure 6) after the aerobic exposure period whereas those of the control and EC silages were greater ($P < 0.05$), as they had increased to 5.4 and 5.6, respectively. The EC+PA silage had the lowest ($P < 0.05$) yeast counts, followed by the silages treated with the inoculants (Figure 5). All silages were stable for the entire duration of aerobic exposure (168 h).

Antibacterial Activity of Microbial Inoculants and Silage Extracts

The pH-corrected supernatants from pure cultures of *L. plantarum* and *L. buchneri* produced 3.20 ± 0.30 and 3.07 ± 0.25 mm zones of inhibition against EC, respectively, but no inhibition zone was produced by cell-free

Figure 4. *Escherichia coli* O157:H7 (EC) of alfalfa forage treated without (control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of 2 bacterial inoculants or propionic acid during ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid; Treatment $\times$ day P -value = 0.001; an asterisk (*) indicates that concentrations differed at the day of ensiling specified ($P < 0.05$). Error bars represent SE. Color version available online.

Figure 5. Effect of reinoculation with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) at silo opening on counts of EC and yeasts (log cfu/g) on aerobically exposed alfalfa silages that were treated without (control) or with EC alone, or EC and either of 2 bacterial inoculants or propionic acid at ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid. Similarly shaded bars with different letters differed ($P < 0.05$). Treatment SE values for EC and yeasts were 0.23 and 0.50, respectively.

supernatants from extracts of d-100 silages that had been treated with the inoculants at ensiling.

DISCUSSION

Silages form the bulk of the ration of dairy cows and many beef cattle in the United States. Therefore, they are integral initial components of the human food chain. Consequently, the hygienic quality or feed safety

of silage is of paramount importance. In this context, the current experiments studied the effects of 2 LAB inoculants or propionic acid on the survival of EC in alfalfa silage during and after the ensiling periods as part of a broader effort to understand the probiotic and food-safety enhancing effects of such inoculants.

The presence of EC in the fresh control alfalfa forage confirmed the results of various studies and surveys on the potential risk of pathogenic *E. coli* contamination

Figure 6. Effect of reinoculation with 1×10^5 cfu/g of *Escherichia coli* O157:H7 (EC) at silo opening on pH of aerobically exposed alfalfa silages that were treated without (Control) or with EC alone, or EC and either of 2 bacterial inoculants or propionic acid at ensiling. Control = distilled water; EC = 1×10^5 cfu/g of *E. coli* O157:H7; LP = 1×10^6 cfu/g of *Lactobacillus plantarum*; LB = 1×10^6 cfu/g of *Lactobacillus buchneri*; PA = 2.2 g/kg of propionic acid. Bars with different letters (a,b) differed ($P < 0.05$); treatment SE = 0.29. Color version available online.

of forages and silages due to fertilization with manure slurry or irrigation water (Beuchat and Ryu, 1997). Dunn et al. (2004) reported that 38.5% of herds of dairy cows and 6.5% of dairy cows per herd had EC in the manure. The population of *E. coli* in dairy manure varies between 5 to 8 log cfu/g (Zhao et al. 1995; Walczak and Xu, 2011), and the viability of the bacteria in such organic wastes after land application can last for as long as 60 d (Avery et al., 2005).

Anaerobic Ensiling Phase

The elimination of pathogenic and nonpathogenic *E. coli* in well-preserved corn and grass silages due to the rapid drop in pH during ensiling is well known (Byrne et al., 2002; Chen et al., 2005; Pedroso et al., 2010; Dunière et al., 2011). To our knowledge, only our previous study (Pedroso et al. 2010) and that of Bach et al. (2002) have investigated the survival of EC in corn and barley silages, respectively, during aerobic exposure. No study seems to have examined the survival of this serotype during the ensiling and aerobic phases of alfalfa silage production. Yet, as shown in this current study, the pathogen is more persistent in untreated ensiled alfalfa silage than in untreated ensiled corn, grass, or barley silages (Bach et al., 2002; Pedroso et al. 2010); this is due to the high buffering capacity of alfalfa, which decreases the rate of pH decline during ensiling.

Our study shows that adding *L. plantarum* or *L. buchneri* to alfalfa at ensiling reduced the time (16 vs. 100 d) required for elimination of EC in alfalfa silages contaminated with the pathogen at ensiling (Figure 4). This suggests that inoculant treatment is needed to eliminate EC in contaminated forage that is ensiled for relatively short periods. This was likely due to faster and greater production of organic acids by the LAB, which rapidly reduced the pH of the silages to values below 5.0. Chen et al. (2005) reported elimination of a recombinant *Escherichia coli* strain in wheat and corn silages when the pH decreased below 5.0, but noted that it persisted longer in wilted silages with a slow pH decline. A microbial inoculant containing *Pediococcus pentosaceus* and *Propionibacterium jensenii* accelerated the accumulation of lactate and reduction in pH of barley silage and thereby hastened (7 vs. 15 d) the elimination of EC that had been added to the barley forage at ensiling (Bach et al., 2002). However, EC is rapidly eliminated during ensiling of corn and grass silages, even in the absence of microbial inoculants, because of the inherent rapid accumulation of organic acids and the subsequent rapid decrease in pH in these forages (Pedroso et al., 2010; Dunière et al., 2011).

The rate and extent of silage fermentation is reduced with increasing DM content (Kung et al., 1984; Muck,

1990; Jones et al., 1992). The high DM at ensiling and high buffering capacity of the alfalfa silages in our study caused their pH to decrease relatively slowly. Application of the inoculants resulted in a faster decrease in pH despite the high DM content of the unensiled forages, and also decreased the pH at d 100 relative to the value for the control silage (Figure 3).

The inhibitory effect of the inoculants on EC may have been partly due to the organic acids they produced. Such acids are generally considered to have strong antimicrobial activity (Buchanan et al., 2004). Within the pH range of 4.0 to 5.5, lactate, the main acid produced during homofermentative ensiling pathways, had the greatest activity in vitro against enterohemorrhagic *E. coli* (Buchanan and Edelson, 1999). Whereas Abdul-Raouf et al. (1993) reported that relative inhibitory activity against EC was higher for acetate than lactate. In addition, Breidt et al. (2004) demonstrated that acetic acid reduced the survival of *E. coli* strains when compared with effects attributed to pH alone.

The inhibitory effect of the inoculants may have also been partly due to their antibacterial effects on EC. This is evident from the inhibition zones caused by pure cultures of the inoculants on the Kirby Bauer disks against EC. These inhibitory effects may have been due to production of bacteriocins (Jack et al., 1995) or other inhibitory compounds. However, the antibacterial effect was reduced or lost during ensiling because extracts of the d-100 inoculant-treated silages produced no inhibition zones around the EC, probably due to loss of metabolic activity of the LAB after 100 d of ensiling. These results are similar to those reported by Pedroso et al. (2010).

Aerobic Postensiling Phase

Lactobacillus buchneri increases the concentration of acetic acid in silages via anaerobic degradation of lactic acid to acetic acid (Oude Elferink et al., 2001), and thereby prevents accumulation of yeasts and improves the aerobic stability of silages (Weinberg et al., 2002; Kleinschmit et al., 2005; Pedroso et al., 2010; Queiroz et al., 2013). In our study, inoculation with *L. buchneri* resulted in higher concentration of acetic acid and about a 1,000-fold reduction in the number of yeasts compared with the control silage. The antifungal characteristics of acetic acid are well known (Danner et al., 2003; Schmidt and Kung, 2010). A meta-analysis on the effect of *L. buchneri* in corn silages showed that it caused a 10- to 100-fold decrease in yeast counts in corn silage (Kleinschmit and Kung, 2006).

Propionic acid has the greatest antimycotic characteristics of all the short-chain fatty acids (Woelford, 1975) and its undissociated form is effective at reducing

the growth of yeasts and molds, which are responsible for causing and maintaining or enhancing aerobic deterioration in silages, respectively (Kleinschmit et al., 2005). The fraction of propionic acid that is undissociated increases as the pH decreases (Lambert and Stratford, 1999). In our study, treatment with propionic acid predictably increased the concentration of the acid and prevented the growth of yeasts. Kleinschmit et al. (2005) reported that buffered propionic acid (0.1% of fresh forage) did not reduce counts of yeasts in corn silage. This may be due to the relatively low application rate and the concentration of the acid in the product. Rates of application of buffered propionic acid-based products can be as high as 0.25% of fresh forage (Kung et al., 1998), and higher application rates are more effective (Kung et al., 1998, 2000).

Aerobic instability is not a common problem in alfalfa silages with good silo management (Muck, 2008). In our study, the alfalfa silages were stable for the entire aerobic exposure duration. This agrees with the results of Pahlow et al. (2001), who showed that about 90% of legume silages surveyed were stable on exposure to air for the same duration used in our study (168 h).

When the silages contaminated with EC on d 100 were exposed to air for 168 h, 2.3 log cfu/g of the pathogen were detected in the EC silage, yet the pathogen was not detected in silages that had been treated with the inoculants or propionic acid at ensiling. This is probably because the additive-treated silages seem to have kept the pH below 5.0 during the aerobic exposure period. Pedroso et al. (2010) reported that *L. buchneri* prevented the growth of EC on aerobically exposed silages because it maintained an acidic pH (4.0) for 144 h after silo opening and exposure to air. In a similar study, Dunière et al. (2011) showed that *E. coli* O26 was not detected in corn silages when contamination occurred at silo opening due to the low pH in those silages. However, when EC contamination occurred after 144 h when the pH had increased above 6.0, about 2 to 6 log cfu/g of the pathogen were recovered from the silages. This indicates that the risk of survival of pathogenic *E. coli* can be reduced markedly or curtailed with silage additives that maintain acidic pH conditions in aerobically exposed silages. In addition, the more rapid elimination of the pathogen by obligate or facultative LAB may help to curtail the spread of pathogen by preventing it from cycling on dairy farms.

CONCLUSIONS

Escherichia coli O157:H7 was eliminated from alfalfa silages treated with *L. plantarum* or *L. buchneri* within a shorter time (16 vs. 100 d of ensiling) than that required for elimination of the pathogen from con-

trol, EC, and EC+PA silages. Therefore, application of certain inoculants is needed to prevent survival of the pathogen in alfalfa ensiled for short periods. The elimination of EC during ensiling was partly due to the fact inoculants produced more lactate and acetate, caused a faster reduction in silage pH than the other treatments, and exhibited antibacterial effects on EC. Application of *L. buchneri* and propionic acid curtailed the accumulation of yeasts and molds during ensiling and after aerobic exposure. The bacterial inoculants and propionic acid kept the pH below 5.0 for the entire duration of the 168-h aerobic exposure period. Consequently, the pathogen did not survive in aerobically exposed additive-treated silages contaminated with the pathogen at silo opening, but it did survive and persist in silages treated with EC alone, which had a pH of 5.4. This suggests that keeping silage pH below 5.0 during the feedout stage with an appropriate silage additive will help curtail the growth of EC in alfalfa silages contaminated with the pathogen after silo opening.

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