EFFECTS OF MYCOTOXIN ADSORBENTS ON AFLATOXINS AND OF MICROBIAL ADDITIVES ON *Escherichia coli* O157:H7 IN FEEDS FOR DAIRY COWS

By

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To my wife, Adeola, and my daughter, Tomisin
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<td>ADF</td>
<td>Acid-detergent fiber</td>
</tr>
<tr>
<td>AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFM&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Aflatoxin M&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered-peptone water</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>DAEC</td>
<td>Diffusely-adherent <em>E. coli</em></td>
</tr>
<tr>
<td>DFM</td>
<td>Direct-fed microbial</td>
</tr>
<tr>
<td>DIM</td>
<td>Days-in-milk</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DMI</td>
<td>Dry matter intake</td>
</tr>
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<td>DON</td>
<td>Deoxynivalenol</td>
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<tr>
<td>EAEC</td>
<td>Entero-aggregative <em>E. coli</em></td>
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<td>EC</td>
<td><em>Escherichia coli</em> O157:H7</td>
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<td>EIEC</td>
<td>Entero-invasive <em>E. coli</em></td>
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<td>EPEC</td>
<td>Entero-pathogenic <em>E. coli</em></td>
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<td>ETEC</td>
<td>Entero-toxigenic <em>E. coli</em></td>
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<td>FCM</td>
<td>Fat-corrected milk</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>HPLC</td>
<td>High-performance liquid chromatograph</td>
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<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
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<td>LAB</td>
<td>Lactic acid bacteria</td>
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<td>LB</td>
<td><em>Lactobacillus buchneri</em></td>
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<td>Abbreviation</td>
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<tr>
<td>LP</td>
<td><em>Lactobacillus plantarum</em></td>
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<td>MFI</td>
<td>Mean florescence intensity</td>
</tr>
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<td>MRS</td>
<td>Man-Rogosa-Sharpe</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral-detergent fiber</td>
</tr>
<tr>
<td>NE_L</td>
<td>Net energy of lactation</td>
</tr>
<tr>
<td>NH₃-N</td>
<td>Ammonia nitrogen</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PC</td>
<td>Principal coordinate</td>
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<td>PCoA</td>
<td>Principal coordinate analysis</td>
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<td>SCC</td>
<td>Somatic cell counts</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEQ</td>
<td>Sequestering agent</td>
</tr>
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<td>SMAC</td>
<td>Sorbitol-macConkey agar</td>
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<td>STEC</td>
<td>Shiga toxin-producing <em>E. coli</em></td>
</tr>
<tr>
<td>TMR</td>
<td>Total mixed ration</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>ZEA</td>
<td>Zearalenone</td>
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Three experiments were conducted to evaluate the effects of two microbial cultures and a chemical additive on the survival of pathogenic \textit{Escherichia coli} O157:H7 (EC) and bacterial diversity in corn and alfalfa silages. A fourth experiment examined the effects of sequestering agents on the performance and health of dairy cows challenged with dietary aflatoxin B$_1$.

Experiment 1 examined whether adding \textit{Lactobacillus plantarum} (LP), \textit{Lactobacillus buchneri} (LB) or propionic acid to alfalfa silage contaminated with EC would inhibit the growth of the pathogen during or after ensiling. The bacterial inoculants hastened the inhibition of EC during ensiling and, like propionic acid, they also prevented its growth after ensiling during aerobic exposure.

Experiment 2 assessed the effects of silage additives on bacterial diversity of alfalfa silage analyzed via Illumina MiSeq sequencing. Inoculation with LP or LB reduced the silage bacterial diversity by increasing the abundance of the respective bacteria. Relative to the uninoculated forage, inoculation with LP increased the abundance of \textit{Lactobacillus}, \textit{Sphingomonas}, \textit{Pantoea}, \textit{Pseudomonas}, and \textit{Erwinia}, but reduced that of genera \textit{Pediococcus}, \textit{Weissella}, and \textit{Methylobacterium}. Abundance of \textit{Weissella} and \textit{Methylobacterium} also were
reduced in the EC+LB silage compared to uninoculated alfalfa. Application of propionic acid did not affect the abundance of genera *Lactobacillus*, *Weissella*, and *Pediococcus*. Strong correlations between some unknown bacteria and indices of silage fermentation quality, such as lactate, acetate, and ammonia-N, were detected.

Experiment 3 examined the fate of EC and bacterial diversity in corn silage contaminated with the pathogen and treated with a chemical additive or two microbial inoculants prior to ensiling. The pathogen was eliminated in all silages within 7 d of contamination at ensiling because of rapid drops in pH. Propionic acid prevented the growth of the pathogen during the feed-out stage by keeping the pH of the silage below 4.0. The composition of the bacterial community of the silages was modified by additive treatment, notably, the abundance of *Lactobacillus* was greater in silages treated with LP or propionic acid. Silage treated with LB at ensiling contained greater abundance of *Acinetobacter* and *Weissella* than other silages.

Experiment 4 examined if adding three mycotoxin-sequestering agents to diets contaminated with aflatoxin B₁ would reduce milk aflatoxin M₁ concentration and improve the performance and immune status of dairy cows. The sequestering agent mixtures contained varying proportions of *Saccharomyces cerevisiae*, clay, and chlorophyll-based products. The sequestering agents did not prevent the toxin from being transferred to milk but they reduced toxin-induced inflammatory stress in the dairy cows.
CHAPTER 1
INTRODUCTION

The safety of animal feeds has emerged as an important issue because of the recent deaths and illnesses because of feed and food-borne pathogens and toxins in the United States (US) and other parts of the world. *Escherichia coli* is a facultative anaerobic bacterium commonly found in the mammalian intestinal tract (Drasar and Barrow, 1985) and *E. coli* O157:H7 (EC) is a Shiga-toxin-producing bacterium that causes hemorrhagic colitis, bloody diarrhea, and hemolytic uremic syndrome (HUS) in humans (Kaper et al., 2004). This pathogen has been isolated from both dairy and beef cattle and cattle are considered the primary reservoir of EC (Cernicchiaro et al., 2013; Mir et al., 2015). Manure from cattle containing viable EC can contaminate the water supply on farms such that EC subsequently can be consumed directly in drinking water or contaminated irrigation water or manure used as fertilizer used on harvested forages (Weinberg et al., 2004; Mir et al., 2015) that are later fed to cows. Furthermore, pathogens also can be found in improperly fermented silages. Fenlon and Wilson (2000) reported that the number of EC in poorly fermented silage grew from 3 to 6 log cfu/g during the first 7 d of ensiling when the pH stayed above 5. Consequently, livestock feeds have been suggested to be the vehicle of transmission of EC on the farm (Hancock et al., 2001; Davis et al., 2003). Therefore, inhibiting the growth of the pathogen may help reduce its cycling and spread on the farm.

Fungal contamination of animal feeds is a common occurrence on farms and the production of mycotoxins by some fungal species poses a significant threat to the livestock industry because of the detrimental effects of fungal mycotoxins on all classes of farm animals. Aflatoxin is the most studied mycotoxin because of its carcinogenic potential and transmission into milk of dairy cows (Diaz et al., 2004; Liu and Wu, 2010). In fact, up to 160,000 cases of
cancer in the US have been traced to aflatoxin (Liu and Wu, 2010). Clay-based sequestering agents have reduced the transfer of aflatoxins into milk but may interfere with absorption of vitamins and minerals (Chestnut et al., 1992). Yeast-based sequestering agents have reduced the transfer of dietary aflatoxins into milk (Diaz et al., 2004); however, the efficacy of yeast-based binders is greater at lower levels of dietary aflatoxin contamination (Xiong et al., 2015).

Therefore, more research is needed on alternative aflatoxin-sequestering agents that reduce the transfer of dietary aflatoxins to milk and prevent or ameliorate negative effects of the toxin on the performance of livestock. Therefore, the studies presented in this dissertation investigated the protective effects of sequestering agents and silage additives against aflatoxin and EC, respectively, in feeds for dairy cows.

The studies presented in Chapters 3 and 4 examined the efficacy of microbial and chemical additives at inhibiting the growth of EC in alfalfa silage during and after ensiling, and examined if the additives influence bacterial diversity in silage. The study presented in Chapter 5 examined if addition of microbial inoculants or propionic acid would inhibit the growth of the EC in corn silage contaminated with EC at ensiling, at silo opening, or after aerobic exposure, as well as how additive treatment affected the composition of the bacterial community in silage.

The study in Chapter 6 examined if adding a yeast cell wall product, clay, or a chlorophyll-based product to diets contaminated with aflatoxin B₁ (AFB₁) would reduce milk aflatoxin M₁ (AFM₁) concentration and improve the performance and immune status of dairy cows.
Silage Fermentation

Anaerobic fermentation of fresh forage is a preservation method that involves the conversion of plant sugars into organic acids, mainly lactic acid, by epiphytic lactic acid bacteria (Weinberg and Muck, 1996; Kung, 2010). Production of these organic acids lowers the pH of the ensiled forage and inhibits the growth of spoilage organisms (Weinberg and Muck, 1996; Driehuis, 2013). Forages constitute a significant proportion of the feed costs of livestock production, and preserved forages, such as silage, form the bulk of the ration of dairy cows and many beef cattle in the US, therefore, production of high quality silage is paramount (Addah, 2014). The main aim of preserving forages is to make them available at the same nutritive value all-year-round, especially, during the winter period when forages experience little or no growth. In addition to oxygen restriction and moisture content of forages, successful preservation of forages as silage is dependent on the activities of the silage microbial population, especially the bacterial population (Muck, 2013), which often needs to be dominated by inoculated bacteria or restricted by additives to optimize the fermentation. Improvements in ensiling processes by rapid acidification, maintenance of anaerobic conditions during ensiling, and reducing aerobic spoilage after opening the silo, also have helped ensure production of quality silage (Kaiser and Evans, 1997; Muhlbach, 1998; Kung, 2010).

Phases of Silage Fermentation

Forage fermentation involves the following phases (Weinberg and Muck, 1996; Kung, 2010): aerobic, fermentation, stable, and feed-out phases.
Aerobic Phase

This phase occurs for a few hours after sealing the silo. During this phase, respiration of plants and aerobic organisms continues because of the presence of oxygen within and between the forage plants. This phase continues until the oxygen is used up. Aerobic respiration utilizes the soluble carbohydrate mass needed by lactic acid bacteria for fermentation. Furthermore, aerobic respiration produces heat which can reduce protein digestibility when the temperature reaches 32°C (Collins and Owens, 2003; Pahlow et al., 2003). In addition, plant proteases are still active during this phase and they metabolize protein through proteolysis and deamination resulting in ammonia accumulation.

For efficient silage fermentation, the aerobic phase must be shortened as much as possible to reduce the growth of unwanted and spoilage microorganisms, such as yeast, mold and aerobic bacteria which can deplete soluble carbohydrates in the ensiled forage (Kung, 2010). Wilting of the forage to recommended dry matter concentrations, chopping to proper lengths, and proper and quick packing or compaction are some of the practices that help to shorten the duration this phase (McDonald et al., 1991; Kung, 2010).

Fermentation Phase

This phase begins when the oxygen in the silo is used up and anaerobic conditions prevail. During this phase, the lactic acid bacteria convert plant sugars to organic acids, primarily lactic acid. This reduces the pH of the silage to 3.6 to 5.0, depending on the forage type and ensiling conditions. The drop in pH helps to preserve the nutritive value and reduce shrinkage of the silage by preventing the growth of spoilage bacteria, yeasts, and molds (Addah et al., 2014). This phase can last for several days or weeks depending on the forage type and ensiling conditions.
Stable Phase

Once the pH is low and air ingress into the silo is prevented, the silage environment becomes relatively stable. However, some acid-tolerant enzymes and micro-organisms, e.g. L. buchneri, still function at a slow rate during this stage (Schmidt et al., 2008), whereas other organisms such as clostridia and bacilli survive the acidic environment as spores (Muck, 2010).

Feed-out Phase

Immediately after silo opening, aerobic conditions prevail, therefore dormant aerobic organisms such as yeasts, are resuscitated. Aerobic spoilage occurs because of depletion of lactate by lactate-utilizing yeasts and acetic acid bacteria (McDonald et al., 1991). This increases the pH, thereby, creating a conducive environment for the growth of molds and pathogenic organisms (McDonald et al., 1991). Yeasts and molds utilize sugars, nutrients and lactate as substrates, producing heat in the process. This denatures protein and depletes nutrients in the silage (Kung, 1998). In addition, molds can produce mycotoxins which are detrimental to the health of livestock and humans (Campbell et al., 2004; Fung and Clark, 2004).

Silage Additives

Production of high quality silage is dependent on enhancing the fermentation processes during the different phases of forage preservation. Silage additives have been employed for this purpose for many years (Henderson, 1993). Some of the main classes of silage additives are fermentation stimulants (microbial inoculants, enzymes, and fermentable carbohydrate sources), fermentation inhibitors (acids and formaldehyde), aerobic deterioration inhibitors (lactic acid bacteria, acids and acid salts), and nutrients (McDonald et al., 1991; Kaiser, 1999).

Bacterial inoculants have been the subject of considerable research because, relative to chemical additives, they are less expensive, less hazardous, and non-corrosive to farm machinery.
The following are the main bacterial inoculants currently used for silage preservation.

**Homofermentative Bacteria**

These are commonly referred to as first-generation bacterial additives (Muck, 2010; Addah et al., 2014). *Lactobacillus plantarum, L. casei, Pediococcus* species, and *Enterococcus faecium* are the species most commonly used as first generation inoculants to rapidly reduce silage pH by increasing production of lactic acid. These bacteria ferment 1 mole of glucose to 2 moles of lactic acid and 2 moles of adenosine triphosphate (ATP) via the Embden-Meyerhof pathway. *Lactobacillus plantarum* is also capable of fermenting pentoses to lactic acid, CO$_2$, and acetic acid via the heterofermentative pathway in absence of glucose. Consequently, it is now classified as a facultative heterofermenter (Holzer et al., 2003). Homofermentative bacterial strains used as silage inoculants are chosen for their rapid growth and ability to compete against and dominate the silage microbial community. Previous studies have confirmed greater lactic acid concentrations along with reduced pH and acetic acid, ammonia-N and butyric acid concentrations in silage inoculated with homofermentative bacteria (Hu et al., 2009; Queiroz et al., 2013). Similar findings were reported in a review of 230 studies conducted by Muck and Kung (1997). Furthermore, a recent meta-analytic study from our laboratory confirmed that inoculation with these bacteria improved silage fermentation (Oliveira et al., 2017).

The effectiveness of homofermentative bacteria at improving silage fermentation is affected by factors such as population of epiphytic bacteria, plant sugar concentration, and buffering capacity (Muck and Kung, 1997; Filya et al., 2007). The benefits of inoculation are more pronounced in forages with low epiphytic bacterial counts, high sugar concentrations, and/or high buffering capacity (Filya et al., 2007). However, inoculation with homofermentative
bacteria may increase aerobic spoilage because of the poor antifungal effects of lactate (Kung, 1998; Johnson et al., 2002) as well as the utilization of lactate by spoilage yeasts (Kung, 2010).

**Heterofermentative Bacteria**

The problem of aerobic deterioration at feed-out led to the development of heterofermentative or second-generation bacterial additives (Addah et al., 2014). Heterofermentative bacteria produce lactic acid, ethanol, CO\(_2\), and acetic acid during fermentation (Oude Elferink et al., 2001) because they lack the fructose diphosphate aldolase enzyme. Consequently, during the fermentation, heterofermentative bacteria ferment glucose-6-phosphate into 6-phosphogluconate rather than fructose-6-phosphate (Kung et al., 2003). These bacteria use the heterofermentative pathway to generate antifungal compounds, such as acetic and propionic acids (Oude Elferink et al., 2001; Krooneman et al., 2002) which inhibit the growth of spoilage yeasts (Moon, 1983). *Lactobacillus buchneri* is the most commonly used heterofermentative bacterial inoculant; others include *L. brevis*, *Propionibacterium freudenreichii*, and *P. acidipropionici*. *Lactobacillus buchneri* converts lactic acid to acetic acid, 1,2-propanediol, and ethanol (Oude Elferink et al. 2001). Silage inoculation with *L. buchneri* reduced the lactate and increased acetate concentrations (Kleinschmit and Kung, 2006b), subsequently reducing the number of yeasts and improving aerobic stability of corn, barley and sorghum silages (Huisden et al., 2009). However, heterofermentation in silage by *L. buchneri* involves DM losses (1 to 3%; Kleinschmit and Kung, 2006b) because of release of one mole of CO\(_2\) for every mole of acetic acid produced.

*Lactobacillus brevis* is another heterofermentative bacterium often used as a silage additive as it can generate acetic acid from carbohydrate fermentation (Holzer et al., 2003). However, this bacterium is not as effective as *L. buchneri* in producing acetic acid and improving aerobic stability of silages. Danner et al. (2003) reported that on a DM basis, corn
forage treated with *L. brevis* had greater acetate concentration (2.86 vs. 1.65%) and aerobic stability (72 h vs. 40 h) compared to the uninoculated silage. Similarly, silage treated with *L. buchneri* increased acetate concentration (5.53 vs. 1.65%) and aerobic stability (274 h vs. 40 h) compared to the uninoculated silage. The greater efficacy of *L. buchneri* at improving acetate concentration and subsequently aerobic stability, explains why it is used more commonly as a bacterial inoculant than *L. brevis*.

*Propionibacteria* produce propionic acid which is the strongest antifungal agent of all the short chain fatty acids (Moon, 1983); however, because of their inability to compete with and dominate the epiphytic bacterial population at low pH, silage inoculants containing *P. freudenreichii* and *P. acidipropionici* have not affected the propionic acid concentration and aerobic stability of corn silages (Taylor et al., 2002; Pedroso et al., 2010; Arriola et al., 2011).

**Ferulate Esterase-producing Bacteria**

The quest for microbial inoculants that have several synergistic benefits led to the development of third generation inoculants containing strains like *L. buchneri* PTA 6138 and *L. crispatus* NRRL B-30868 that can improve fiber digestibility as well as fermentation and aerobic stability. The improved fiber digestion occurs because of production of ferulate esterase, an enzyme that targets ferulic acid, which limits the digestion of forage fiber in ruminants (Yu et al., 2005; Addah et al., 2014) by inhibiting the attachment of fibrolytic bacteria in the rumen (Varel and Jung 1986). Ferulic acid esters of arabinoxylans impede access of xylanases to xylan (Gorbacheva and Rodinova, 1977). In addition, ferulic acid forms linkages with lignin and proteins (Rawel and Rohn, 2010) which further reduce the nutritional value of forages. Ferulate esterases release ferulic acid from arabinoxylans, thereby increasing susceptibility of the cell wall to enzymatic degradation (Bartolome et al., 1995). Nsereko et al. (2008) evaluated effects of adding eight lactic acid bacteria that produce ferulate esterase (*L. buchneri* PTA 6138, NNRL B-
30866; *L. crispatus* NRRL B-30868, 30869 and 30870; *L. reuteri* NRRL B-30867, *L. brevis* NRRL B-30865; and an unidentified *Lactobacillus* strain NRRL B-30871) at the rate of $1 \times 10^5$ colony forming units (cfu)/g on fermentation and neutral detergent fiber (NDF) digestibility of perennial ryegrass. All treated silage samples had approximately 10% greater NDF digestibility compared with the control. However, only silages treated with *L. buchneri* strain 6138 and NRRL B-30866 had lower pH and greater acetate concentration relative to the control. In the same experiment, inoculation of corn forage with a combination of *L. buchneri* 6138 and *L. paracasei* tolerans 6135 at ensiling improved the aerobic stability by up to 128 h and improved NDF digestibility by 7% relative to the control. In another study, Kang et al. (2009) evaluated the effects of an esterase-producing inoculant containing $1.0 \times 10^4$ cfu/g of *L. casei* strain PTA6135 and $1.0 \times 10^5$ cfu/g of *L. buchneri* strain PTA6138 on two corn silage hybrids (Croplan Genetics 851RR2 and Vigoro 61R36). The authors reported that inoculation of the first hybrid decreased lactate to acetate ratio, increased propionate concentration, improved the aerobic stability, and increased the potentially degradable DM fraction. However, inoculation of the second hybrid did not affect fermentation, but decreased aerobic stability and increased total degradable DM fraction, 24-h and 48-h DM digestibility, and 48-h NDF digestibility. These results suggest that responses to these inoculants may also depend on properties of the forage such as differences in their epiphytic bacterial population, water activity, water soluble carbohydrates, and cell-wall component concentrations (McDonald et al., 1991; Kang et al., 2009).

**Inoculants Containing *Saccharomyces* Strains**

Advancements in silage inoculant science that focus on delivering silage with probiotic properties that enhance the performance and health of animals led to the development of fourth generation of silage inoculants. These inoculants contain *Saccharomyces cerevisiae* yeast strains
that synergistically can improve silage fermentation, digestibility, and aerobic stability, and alter microbial ecology within the gastrointestinal tract of ruminants to benefit health and/or production efficiency. Some *S. cerevisiae* strains have been documented widely as direct-fed microbials (DFM) that are effective at improving feed efficiency, decreasing ruminal acidosis, and mitigating methane emissions (Desnoyers et al., 2009; McAllister et al., 2011). A recent study examined if probiotic yeasts can persist in silage. Duniere et al. (2015) evaluated the viability of three unnamed *S. cerevisiae* strains added at the rate of $1.0 \times 10^3$ cfu/g fresh weight to whole-plant corn forage ensiled for 90 d. They confirmed survival of the inoculated strains during ensiling and noted that they did not affect the nutritional quality or aerobic stability of the silage. This study suggests that silage can be used as a vehicle to supply yeasts to cattle. A recent study from our laboratory examined the effects of the dose ($5.7 \times 10^7$ or $6.0 \times 10^8$ cfu/cow/day) and viability (live or killed) of a *S. cerevisiae* strain isolated from corn silage on the ruminal fermentation and bacterial population of dairy cows (Jiang et al., 2017). Adding the low dose of live yeast to the diet increased the relative abundance of ruminal cellulolytic bacteria, such as *Ruminococcus* and *Fibrobacter*, and amylolytic bacteria, such as *Ruminobacter* and *Bifidobacterium* and increased milk production by dairy cows. The results from the latter two studies suggest that fourth generation inoculants containing *Saccharomyces* could be used to deliver probiotics that enhance the performance and health of dairy cows. However, more studies are needed to validate this theory.

**Silage Microbial Community Analysis by Molecular Techniques**

The microbial ecology of ensiling is complex and a better understanding of the silage microbial community may improve silage fermentation and preservation. The microbial population of silages has been described previously using culture-based methods, which characterize microbial ecology using commercial growth media such as Luria–Bertani medium,
nutrient agar, and tryptic soy agar (Kirk et al. 2004). Culture-based techniques are relatively quick and inexpensive (Kirk et al., 2004) but their main limitation is that they grossly underestimate the bacterial diversity (Ercolini, 2004) because of the inability to cultivate 99% of naturally-occurring species (Temmerman et al., 2003).

In recent years, molecular approaches have increased our understanding of the silage microbial community (Rossi and Dellaglio, 2007; McEniry et al., 2008; Dolci et al., 2011). These techniques have allowed detection and enumeration of strains that cannot grow on agar and identified new lactic acid-producing bacterial species (Muck, 2013). Techniques that have been used to study microbial communities in silage include length heterogeneity polymerase chain reaction (PCR) (Brusetti et al., 2006), terminal restriction fragment length polymorphism (McEniry et al., 2008), denaturing gradient gel electrophoresis (Parvin et al., 2010; Santos et al., 2015), ion torrent sequencing platform (Kraut-Chen et al., 2016), and single molecule, real-time sequencing technology (Bao et al., 2016). Most of these techniques utilize PCR to amplify a portion, especially the 16S rRNA gene, of microbial DNA (Muck, 2013).

**Terminal Restriction Fragment Length Polymorphism**

Terminal restriction fragment length polymorphism (T-RFLP) is based on variations in the DNA sequence present in PCR-amplified 16S rRNA genes (Smit et al. 1997). The PCR products are digested with restriction enzymes and terminal restriction fragments (T-RFs) are separated using a DNA sequencer (Thies, 2007; Rastogi and Sani, 2011). Microbial diversity is estimated by analyzing the size, numbers, and peak heights of resulting T-RFs. Each T-RF represents a single Operational Taxonomic Unit (OTU) (Rastogi and Sani, 2011). The limitation of the T-RFLP method is that it underestimates community diversity because less than 100 bands per gel can be resolved and different species can have the same T-RF length (Rastogi and Sani, 2011). McEnry et al. (2008) studied the temporal diversity of bacteria in grass silage using T-
RFLP technique, which revealed a reduction in the abundance of species with time (0, 2, 6, 14, 35, and 98 d of ensiling). However, identification of species was not possible because multiple species share the same fragment length.

**Length Heterogeneity Polymerase Chain Reaction**

Length heterogeneity polymerase chain reaction (LH-PCR) utilizes the variation in the length of a gene to differentiate microbial species (Mills et al., 2007; Muck, 2013). The technique interrogates the hypervariable regions present in 16S rRNA genes. It utilizes a fluorescent dye-labeled forward primer. A fluorescent internal size standard is run with each sample to measure the amplicon lengths in base pairs (Rastogi and Sani, 2011). The area under the peak in the electropherogram is used to indicate the relative abundance of that amplicon. Unlike T-RFLP, one advantage of using LH-PCR is that it does not require any restriction digestion and therefore PCR products are directly analyzed by a fluorescent detector (Rastogi and Sani, 2011). The main limitation of the LH-PCR technique includes underestimation of diversity as taxa may produce same-length amplicons, which cannot be differentiated by LH-PCR (Mills et al. 2007). Brusetti et al. (2006) studied the development of various lactic acid bacterial species during ensiling of whole-plant corn forage using LH-PCR and up to 58% of the peaks could not be identified. In addition, species of *Weissella* and *Enterobacter* could not be differentiated.

**Denaturing Gradient Gel Electrophoresis**

In this technique, PCR products are obtained from DNA using 16S rRNA primers and separated on a polyacrylamide gel containing a linear gradient of DNA denaturant, such as a mixture of urea and formamide (Muyzer et al. 1993). The movement of each DNA segment is based on the composition of the DNA nucleotides rather than the length of the DNA sequence (Muck, 2013). The main limitation is that Denaturing Gradient Gel Electrophoresis (DGGE)
underestimates the microbial diversity because not all sequence changes in 16S rDNA fragments can be detected by DGGE, and partial 16S ribosomal DNA sequences differing by more than one base pair can migrate to identical positions in DGGE gels (Jackson et al., 2000). Santos et al. (2015) reported variations in the DGGE-based microbial structure of corn silages inoculated with three strains of *L. buchneri* (UFLA SLM11, UFLA SLM103 and UFLA SLM108) by using DGGE technique.

**Next-generation Sequencing Methods**

Deoxyribonucleic acid sequencing is a method that determines the order of occurrence of nucleotides in a DNA strand (Raza and Ahmad, 2016). The first-generation sequencing method, called Sanger sequencing, was developed by Edward Sanger in 1975. The basis of Sanger sequencing is rapid DNA synthesis with DNA polymerase and the use of 2', 3'-dideoxy nucleotide (ddNTP) as a specific inhibitor for the process (Sanger et al., 1977). Sanger sequencing was considered the gold standard of DNA sequencing for several years (Grada and Weinbrecht et al., 2013) because of its high quality, long read length and low error rate (Liu et al., 2012). However, because of limitations in throughput and speed, Next-generation sequencing (NGS) methods were developed to cater for high demand for cheaper and faster sequencing technology (Hall, 2007; Raza and Ahmad, 2016). The NGS is a powerful biological tool that provides greater through-put, and it is a cheaper and faster alternative to traditional Sanger sequencing. Some of the existing NGS platforms include Roche 454 pyrosequencing, the Pacific Biosciences real-time sequencing, ion torrent personal genome machine and Illumina MiSeq platform.

**Roche 454 sequencing pyrosequencing**

Roche 454 was the first commercially available NGS that was first developed in 1996 (Ronaghi et al., 1996; Liu et al., 2012). This sequencer uses pyrosequencing technology which
relies on the detection of pyrophosphate released during nucleotide incorporation (Ronaghi et al., 1996). Pyrosequencing is a commonly used DNA sequencing method because of its long read length and fast sequencing speed (Liu et al., 2012). One of the limitations is the relatively high error rate, particularly for poly-bases longer than 6 base pairs (Liu et al., 2012). Currently, no studies have utilized this technique to analyze the microbial diversity of silage.

**Single-molecule real-time (SMRT) sequencing**

The SMRT sequencing platform was developed by Pacific Bioscience (Menlo Park, CA, USA). This is based on detection of a fluorescent pulse in real time as sequencing proceeds (Quail et al., 2012). Advantages of SMRT include a short read time (4 to 6 hours) and long read length (average of 1300 base pairs). Also, it does not need the PCR preparation step, which reduces bias and error caused by PCR (Liu et al., 2012). However, SMRT has lower throughput than other NGS methods (Liu et al., 2012). Bao et al. (2016) applied the SMRT platform to examine the microbial community composition of alfalfa silage because it generates much longer sequence read lengths. The authors found that the relative abundance of bacterial species reduced after fermentation, and *Pediococcus acidilactici* and *L. plantarum* were the most prevalent species.

**Ion torrent personal genome machine platform**

This platform uses semiconductor sequencing technology (Quail et al., 2012). It is based on detection of released protons during nucleotide base incorporation (Rothberg et al., 2011; Quail et al., 2012). It is quick, inexpensive and involves a small-sized instrument (Quail et al., 2012). The sample preparation time is less than 6 hours for 8 samples and it can read 200 base pairs in 2 hours (Liu et al., 2012). A recent study that applied 16S ribosomal RNA gene sequencing using the Ion Torrent Personal Genome Machine revealed that 99% of the sequence
reads detected were *L. reuteri* and *L. pontis* in corn, sorghum, and wheat silages (Kraut-Cohen et al., 2016).

**Illumina MiSeq platform.**

The Illumina sequencing platform allows for deeper sequencing at much lower costs than other sequencers (Degnan and Ochman, 2012) and is currently one of the most popular methods for microbial community profiling. It integrates all steps of sequencing together in the Illumina MiSeq desktop. The process from sample preparation to analyzed data can be completed within 8 hours (Degnan and Ochman, 2012; Liu et al., 2012). Illumina MiSeq has great advantages over other sequencers in terms of the cost to sequence 1 megabase pair (Quail et al., 2012). Though, Illumina Miseq platform generates short read length (150 bases, Quail et al., 2012), low error rate (0.80%, Quail et al., 2012) and high throughput (55.5 megabase pairs/h, Loman et al., 2012) makes it one of the most accurate and widely used sequencers (Degnan and Ochman, 2012). Currently, no studies have utilized this technique to analyze the microbial diversity of silage. Therefore, one of the objectives of this dissertation was to examine the diversity of silage bacteria in corn and alfalfa silages via Illumina Miseq sequencing in order to better characterize the complex microbial community of silages and to suggest roles of unknown silage bacteria by investigating if their abundance correlates with silage quality indices.

**Pathogenic *Escherichia coli***

*Escherichia coli* is a gram-negative, facultative anaerobic bacterium that accounts for approximately 1% of the gut microbial population in mammals (Winfield and Groisman, 2003; Callaway et al. 2009; Tchaptchet and Hansen, 2011). Most strains of *E. coli* are commensal organisms. However, some strains, often referred to as intestinal pathogens, cause diarrhea and associated diseases (Robins-Browne et al., 2016). Under normal conditions, out of 8 - 9 L of fluid presented to the intestine daily from intake and other metabolic processes in the body,
approximately 200 mL are excreted in the stool (Hodges and Ravinder, 2010). These enteric pathogens can alter this balance leading to greater excretion of fluid in the stool (diarrheal disease) (Hodges and Ravinder, 2010). According to Farrokh et al. (2013), the six types of pathogenic *E. coli*, based on their virulence genes and phenotypic characteristics, are STEC (also called verocytotoxin-producing *E. coli*), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). The STEC strains possess a phage-encoded potent cytotoxin, called Shiga toxin, as the main virulence factor (Nataro and Kaper, 1998). In North America, Japan and parts of Europe, the most commonly identified serotype is O157:H7, a strain belonging to the STEC group (Farrokh et al., 2013; Centers for Disease Control and Prevention (CDC), 2015), which is a zoonotic pathogen of significant worldwide public health concern (Chase-Topping et al., 2008). The high virulence of STEC serotype is because of its ability to resist stress conditions, such as low pH, and its low infectious dose (Viazis and Diez-Gonzalez, 2011; Jafari et al., 2013). This pathogen was first discovered in 1977 (Konowalchuk et al., 1977) and was first detected as a food-borne pathogen in 1982 (Riley et al., 1983). It has been implicated in several illness outbreaks in more than 50 countries including the US (Chase-Topping et al., 2008). *Escherichia coli* O157:H7 causes gastrointestinal illness, leading to bloody diarrhea, haemolytic uremic syndrome (HUS), renal failure, and death, especially among children and elderly people (Siegler, 2003; Thorpe, 2004; Razzaq, 2006). *Escherichia coli* O157:H7 is responsible for more than 60 deaths and 70,000 illness cases in the US annually (Mead et al., 1999) and the annual cost to the economy is approximately $1 billion (USDA-ERS, 2001). According to the CDC, two multistate outbreaks of STEC were reported in the US in 2016.
Classification of EPEC is based on the presence of specific virulence genes known as the locus of enterocyte effacement (LEE) pathogenicity island (Croxen et al., 2013). Expression of LEE is associated with attaching and effacing lesions that enable the attachment of bacteria to the intestinal epithelial cells, mediated by a protein called intimin, which is encoded by the eae gene, type 3 protein secretory system and Tir protein (Jafari et al., 2013; Ingle et al., 2016). The exact pathogenesis of these bacteria is not yet fully understood, and may involve factors other than attaching and effacing lesions (Jafari et al., 2013; Ingle et al., 2016).

Enterotoxigenic E. coli strains attach to intestinal epithelial cells via proteinaceous surface structures called colonization factors (Jafari et al., 2013). Following attachment and colonization, ETEC strains cause diarrhea by producing enterotoxins. Strains of ETEC are the leading causes of diarrhea or cholera-like disease in all age groups in areas with poor sanitation and inadequate clean water, especially in developing countries (Jafari et al., 2013).

Enteroaggregative E. coli is a newly discovered pathotype that has been associated with diarrhea in children and adults in developing countries (Robins-Browne et al., 2016). The EAEC strains colonize the intestinal mucosa via aggregative adherence fimbriae. However, the virulence-associated determinants of EAEC are not yet known and therefore, there is no clear definition of EAEC strains (Croxen et al., 2013; Robins-Browne et al., 2016).

Diffusely adherent E. coli is a heterogenous group of strains that originally were identified by their adherence to tissue culture cells (Nataro et al., 1987). They possess an autotransporter protein that generates a diffuse adherence pattern (Jafari et al., 2013). However, Robins-Browne et al. (2016) reported that ingestion of up to $10^{10}$ cfu of two DAEC strains failed to cause diarrhea in humans, thereby casting doubts on the role of DAEC in diarrhea.
Adherent-Invasive *E. coli* strains are not associated with diarrhea and are believed to be associated with the development of Crohn’s disease because they are isolated more commonly from patients with the disease than from healthy people. However, it is not yet known whether AIEC strains contribute to the pathogenesis of Crohn’s disease (Robins-Browne et al., 2016).

**Cattle as a Reservoir of *Escherichia coli* O157:H7**

Cattle are considered to be the main reservoir of EC and the main source from which this pathogen enters the food chain (Callaway et al., 2009). In cattle, *Escherichia coli* represent less than 1% of the ruminal microbial population (Min et al., 2007) because of the competition with other ruminal microbes and the bactericidal effect of the volatile fatty acids produced in the rumen (Wallace et al., 1989). However, the high pH and low volatile fatty acid concentrations of the lower gut, particularly the colon, are favorable for *E. coli*; however, the terminal end of the colon and the lymphoid tissue of the recto-anal junction are the major primary sites of EC colonization (Lim et al., 2007). Cattle are asymptomatic carriers of EC because they lack Globotriaosylceramides (Gb3) and Globotetraosylceramides (Gb4), the vascular receptors for Shiga toxins (Samuel et al., 1990; Obrig et al. 1993).

Reinstein et al. (2007) reported that the fecal prevalence of EC in beef cattle was 7.1% in a study with 933 cows that were delivered to two commercial US abattoirs for slaughter. However, EC was isolated from 13.5% of the fecal samples collected from cows in five commercial dairies (Stanford et al., 2005). In fact, the population of EC in wet feces ranged from $10^1$ to $10^9$ cfu/g (Stephens et al., 2009; Munns et al., 2015). Cattle that shed at least $10^4$ cfu/g of wet feces are termed super-shedders (Matthews et al., 2006a, b). The prevalence of super-shedders is dependent on several factors, such as size and method of sample collection (fecal or recto-anal swab sampling), sampling time (summer versus winter), source of measure (farm, pen, truck, or animal), and method of enumeration (Munns et al., 2015). Up to 7% of a given
population of beef cattle are super-shedders (Cobbold et al., 2007; Stanford et al., 2012) that can be significant contributors to transmission of EC in their environments. Up to 90% of EC shedding within feedlot pens was linked to super-shedders (Omisakin et al., 2003; Chase-Topping et al., 2008; Stephens et al., 2009). Therefore, research towards reducing outbreaks of EC has focused on pre-harvest control of the pathogen by inhibiting the growth and viability of super-shedders (Matthews et al., 2006a).

**Pathogenic *Escherichia coli* in Ensiled Forages**

Dairy and beef producers are more concerned about the nutritive value and occasionally the toxin concentration of feeds and forages than on their pathogen load as the former are more likely to directly affect animal performance (Diaz and Trujillo, 2007). However, feeds and forages also can play a role in disease transmission, or as a source of disease agents (Diaz and Trujillo, 2007). Feeds, especially ensiled forages, can harbor pathogens that can compromise the health and performance of animals and humans and cause fatalities (Driehuis, 2013). The following section summarizes the current scientific knowledge on the occurrence and control of pathogenic *E. coli* in ensiled forages.

Species of Enterobacteriaceae, such as EC, are part of the epiphytic microbial population of most forage crops (Driehuis, 2013) and they compete with lactic acid bacteria for nutrients during the early phase of ensiling. However, as fermentation progresses, the abundance of *E. coli* is reduced because their survival decreases at low silage pH (< 4.0). A rapid drop in pH was shown to eliminate *E. coli* in corn silage (Bach et al., 2002; Byrne et al., 2002). Pedroso et al. (2010) evaluated the effectiveness of 3 commercial bacterial inoculants at controlling EC in corn silages. The pathogen was eliminated within 3 d of ensiling with or without silage inoculation when the pH dropped below 4.0. In a similar study, EC was eliminated from ensiled, artificially contaminated wheat and corn forages when the pH dropped below 5.0 (Chen et al., 2005).
Similar findings were observed in corn silage contaminated with *E. coli* O26, a different strain of STEC (Duniere et al., 2011). The elimination of this pathogen in these studies was caused by the inhibitory low pH and/or the enhanced antimicrobial activities of organic acids at low pH (Bjornsdottir et al., 2006).

Chen et al. (2005) reported that inoculated *E. coli* survived longer in wilted versus unwilted wheat and corn silages because the pH of the former decreased more slowly than the latter. In theory, pathogens like *E. coli* will persist and survive for longer periods in ensiled forages with high buffering capacity, like alfalfa silage, because of slow decline in pH. However, no published studies on the persistence or survival of pathogens in legume or high buffering capacity silages were found. Consequently, one of the objectives of this dissertation was to examine the survival of EC in alfalfa silage and to determine if microbial inoculants can inhibit the survival of EC in alfalfa silage, thereby reducing its cycling on farms.

Laboratory mini silos have a more controlled environment than farm silos which are more prone to air penetration and dirt contamination (Johnson et al., 2005). Studies have shown that the presence of oxygen prolongs the survival of pathogenic *E. coli* such that it can survive the ensiling phase (Duniere et al., 2011; Driehuis, 2013) and grow during the feedout stage when the pH increases (Duniere et al., 2011). For instance, up to $10^7$ cfu/g of *E. coli* were found in the shoulders of commercial wheat and corn silages stored in bunker silos because the shoulders of such silages are the most susceptible sites for air penetration, subsequent less acidic pH values and greater spoilage (Weinberg et al. 2004). Pedroso et al. (2010) monitored the survival of EC in laboratory corn silage samples aerobically exposed for 7 days and then inoculated with the pathogen to mimic its survival during the feedout stage on farms. Up to $6 \log_{10}$ cfu/g of the pathogen were detected after 24 hours of inoculation in all silages except those inoculated with
L. buchneri alone or L. buchneri and P. pentosaceus (1.97 log_{10} \text{cfu/g} or none, respectively) at ensiling, which maintained a lower pH than the control silage (3.91 or 3.66 vs. 4.71).

Other pathogens that occur in silage include Listeria monocytogenes, Clostridium botulinum, Neospora caninum, Bacillus cereus, and Salmonella organisms. These organisms cause listeriosis, botulism, neosporosis, emesis, and salmonellosis, respectively in animals. Just like EC, proper silage management practices to ensure effective fermentation, and minimization of contamination with soil, manure, and animal carcasses will effectively control the occurrence of these agents on farms. The major sources of most of these pathogenic organisms are either dirt, manure and carcass contamination, or improperly managed silage, which create conducive conditions (high pH and aerobic condition) for the development of pathogens (Vissers et al. 2006; Driehuis, 2013). Like E. coli, L. monocytogenes, C. botulinum, N. caninum, B. Cereus, and Salmonella require oxygen and high pH for growth and are therefore likely to persist and grow in aerobically-deteriorating silage (Weinberg et al., 2004; Vissers et al. 2006; Driehuis, 2013).

Control of Fecal Shedding of EC by Ruminants

Research studies have focused mainly on pre-harvest control of the EC because of the severity of infections in humans. The following approaches have been employed to reduce shedding of EC in ruminants:

Diet Change

Changing from a grain to a forage diet has affected fecal shedding of EC (Callaway et al., 2009). Kudva et al. (1995) reported an increase in the number of sheep shedding EC when the diet was suddenly switched from alfalfa pellets to a sagebrush grass (Artemisia tridentate). In another study, cattle fed a corn-soybean-based ration shed more generic E. coli (10^8 vs. 10^5 cfu/g) than those fed medium-quality timothy hay (14% crude protein, 40% NDF) (Diez-Gonzalez et al., 1998). These authors suggested that switching the diet of feedlot cattle from a
grain-based diet to a hay-based diet a few days before slaughter might help reduce carcass contamination in the abattoir. However, this approach reduces the final bodyweight of cattle and thus might not be economical, particularly in feedlot cattle (Keen et al., 1999; Stanton and Schutz, 2000). When cattle were switched from an 85% corn diet to a diet containing 30% millet hay and 62% corn 5 days before slaughter, they lost an average of 1 kg/head/day but had fewer (1 × 10^6 vs. 3.2 × 10^7) total generic E. coli counts in the feces (Stanton and Schutz, 2000). Similarly, Keen et al. (1999) reported a decrease in body weight (0.6 kg/head/day) and a reduction (18 vs. 52%) in the proportion of cattle shedding EC when they were abruptly switched from a diet of corn grain to alfalfa hay. The food safety benefits in these studies should be weighed against potential reduced income from lighter carcasses. However, food safety concerns may be more important because slaughter houses and farms can be shut down if they are found to be the source of EC outbreaks (USDA, 1998).

The effects of changes in diet on EC counts may be attributed to the change in microbial population caused by the altered availability and concentrations of fermentable substrates in the lower intestinal tract (Callaway et al., 2009). The greater abundance of EC in feces with grain-based diets is probably because of higher starch supply from the rumen to the colon. A switch from a grain-based to hay-based diet shifts the site of digestion from the colon to the rumen, which leads to reduced starch availability in the colon (Callaway et al., 2009). It is suggested that this change in nutrient availability causes a shift in the microbial population of the gut. In particular, organisms that are dominant on hay diets, such as Enterococcus, are thought to competitively exclude (Gregory et al., 2000), displace and prevent colonization by EC (Callaway and Martin, 2006). Furthermore, tannins and lignin in forages can reduce fecal shedding of generic E. coli and kill EC in vitro and in manure (Wells et al., 2005; Min et al., 2007).
**Vaccinations**

Vaccination is a recent approach that reduces fecal shedding of EC (Snedeker et al., 2011). *Escherichia coli* O157:H7 vaccines exploit the antigenic components important for bacterial adherence to the intestinal epithelium or virulence of the pathogen, such as type III secreted proteins, intimin protein, and the O157 lipopolysaccharide (Dean-Nystrom et al., 2002; Potter et al., 2004). Siderophore receptor and porin (SRP) protein-based vaccine is a commercially available vaccine that uses SRP proteins as antigens so immunized animals produce anti-SRP antibodies that block iron transport by binding to the outer membrane proteins of bacterial cells (Cull et al., 2012). Blocking iron transport reduces the survival of the gram-negative bacteria, such as EC, in a mixed microbial environment (Cull et al., 2012). The SRP-based vaccine reduced the prevalence of EC, fecal shedding of the pathogen, and the number of high-shedders in feedlot cattle (Fox et al., 2009). In a similar study, vaccinated cattle had less prevalence of EC and less prevalence of high shedders relative to the unvaccinated cattle (Cull et al., 2012). However, despite the proven effectiveness of vaccines, their high cost has hindered the adoption of this approach by producers.

**Direct-Fed Microbials**

Direct-fed microbials (DFM) are utilized primarily to increase feed efficiency in feedlot cattle, enhance milk production in dairy cows, and improve health and performance of young calves (Fuller, 1989; Krehbiel et al., 2003). Some DFM have reduced shedding of EC by ruminants (Lema et al., 2001; Ohya et al., 2001). However, the method of monitoring the EC can affect the results. Bach et al. (2003) reported that a *Saccharomyces cerevisiae* DFM culture reduced counts of EC in a batch culture but not in a continuous flow culture system (Rusitec apparatus). This was because the populations of EC in the continuous culture system declined...
steadily from the onset of inoculation, perhaps because of the high dilution rate of the system (0.32 mL/min).

In recent years, probiotic products that are aimed specifically at reducing fecal shedding of EC have been developed (Callaway et al., 2009). A microbial culture containing \( L. \) \textit{acidophilus} alone or in combination with \( P. \) \textit{freudenreichii} reduced fecal shedding of EC in cattle (Elam et al., 2003; Younts-Dahl et al., 2004). Microbial products containing \( \textit{Streptococcus faecium} \) alone or in combination with \( L. \) \textit{acidophilus}, \( L. \) \textit{casei}, \( L. \) \textit{fermentum}, and \( L. \) \textit{plantarum} were effective, whereas \( L. \) \textit{acidophilus} alone was not effective at reducing fecal shedding of EC in sheep (Lema et al., 2001). Direct-fed microbials might be an effective strategy for reducing the abundance of EC when they improve the performance of ruminants, which can economically justify their inclusion in rations (Cull et al., 2012). However, animal responses to DFM have been inconsistent because of differences in the types, strains, and combinations of organisms that have been used (Kenney et al., 2015), or lack of understanding of the exact modes of action of many DFM.

\textbf{Bacteriophage Therapy}

Some viruses, called bacteriophages, can lyse and kill bacteria commonly found in the gut of food-producing animals and environments (Klieve and Swain, 1993). Lysis of bacterial cells can occur because of phage replication in the host cell. The bacteriophages release a hydrophobic polypeptide that forms pores in the cell membrane, thus, provoking the lysis of the bacterial cell (Andreoeletti et al., 2009). Lysis also can occur in the absence of phage replication. In this case, phages act like DNA or RNA viruses that inject their genetic material into the bacterium, therefore disrupting bacterial expression of genes and proteins, resulting in impaired metabolism (Andreoeletti et al., 2009). Some studies have shown that bacteriophages specific for EC reduced shedding of the pathogen in cattle (Callaway et al., 2008; Niu et al., 2009). However,
regulatory approval is needed for use of phage therapies (LeJeune and Wetzel, 2007), partly because long term exposure to a specific phage preparation can result in bacterial resistance (Jassim and Limoges, 2014; Kateryna and Rai, 2016). Therefore, phage therapy requires clinical testing of every component of the phage preparation (Kateryna and Rai, 2016). There is currently no regulatory approval required for the use of phages to control pathogenic organisms in live food animals. However, the US Food and Drug Administration (FDA) recently approved the use of a *Listeria*-specific bacteriophage preparation on Ready-to-Eat meat and poultry products (FDA, 2014).

**Mycotoxins in Ensiled Forages**

Mycotoxins are a group of secondary metabolites secreted by fungal organisms mostly belonging to genera *Aspergillus*, *Fusarium*, *Claviceps*, and *Penicillium* (Yiannikouris and Jouany, 2002; CAST, 2003; Kabak et al., 2006). Fungi often thrive well in environments with high humidity, high temperature, and oxygen access at all stages of plant production and material storage (Egal et al., 2005). Toxic effects, such as reduced feed intake and milk production, reproductive problems, immunosuppression, and death can occur when animals are fed mycotoxin-contaminated diets (Jouany, 2001; Whitlow and Hagler, 2005).

Approximately 25,000 to 166,000 new cases of liver cancer are annually caused by aflatoxins (Liu and Wu, 2010). The ubiquitous nature of mycotoxins and the severity of their effects on human health make them a major food safety concern. In addition to the severity of their effects on livestock and human health, direct costs of disposal of condemned food and feed ingredients, and indirect costs of regulatory enforcement and quality control measures caused by fungal toxin contamination in the USA were estimated at approximately $1 billion (CAST, 2003; Milicevic et al., 2010).
Prevalence of Mycotoxins in Foods and Feeds

The Food and Agriculture Organization estimates that 25% of all crops worldwide are contaminated with mycotoxins annually (CAST, 1989). In addition, Pittet (1998) reported that 40% of 25,000 food and feed samples collected in 30 countries around the world were contaminated with one or more mycotoxins. A three-year (2009 to 2011) survey on the worldwide occurrence of mycotoxins revealed that 81% of 7,049 livestock feed samples collected from the Americas, Europe and Asia were positive for at least one mycotoxin (Rodrigues and Naehrer, 2012). Mycotoxins are present in concentrates, forages and in preserved feeds such as hay and silage (Biomin, 2015). Most research studies have focused primarily on mycotoxin occurrence in grains and cereals (Binder, 2007; Zinedine et al., 2007; Bhat et al., 2010), perhaps caused by their greater consumption by humans than other parts of forages. Other studies have focused on exposure of cows to mycotoxins via forages (Boysen et al., 2000; O’Brien et al., 2006) because forages account for a high proportion of the ruminant diet (Cheli et al., 2013). A survey by Driehuis et al. (2008) revealed that the contribution of ensiled forage to mycotoxin ingestion in dairy cows is approximately three times more than that of finished feed.

Factors that Predispose to Mold and Mycotoxin Production in Silage

Ensiled forages may contain a mixture of mycotoxins, originating from pre-harvest contamination by *Fusarium* species (Baath et al., 1990), and/or from post-harvest contamination with toxigenic molds that are common in silage, such as *Aspergillus, Monascus, Rhizopus,* and *Geotrichum* (Boysen et al., 2000; Garon et al., 2006; Fink-Gremmels, 2008). Anaerobic conditions and low pH are required to restrict the growth of these molds during ensiling and low pH after ensiling curtails their resuscitation after aerobic exposure. Except for *Penicillium,* typical silage molds, such as *Fusarium, Aspergillus, Monascus, Rhizopus,* and *Geotrichum* species are intolerant of low oxygen and low pH conditions (Pahlow et al., 2003). Therefore,
during ensiling, molds are often found at the top layer or sides of silos that are commonly prone to oxygen infiltration. In agreement, Gonzalez-Pereyra et al. (2008) noted that improperly managed silage promotes aerobic conditions and less extensive fermentation that favors the growth of toxigenic molds that are normally less tolerant of acidic or anaerobic conditions. Once silos are opened, the aerobic conditions allow the growth of lactate-utilizing yeasts that metabolize lactate to CO$_2$ and thereby, increase the pH. The increased pH and exposure to oxygen allow toxigenic fungi to grow aggressively during the feed-out phase (McDonald et al., 1991), particularly in poorly-managed silages.

Although the most notorious negative effect of molds is the secretion of mycotoxins, feeding moldy feeds has been reported to expose animals to respiratory problems, decreased reproductive function, kidney damage, and skin and eye irritation (Di Costanzo et al., 1995; Scudamore and Livesey, 1998; Alm et al., 2002). In fact, Aspergillus fumigatus has been proposed to be associated with mycotic hemorrhagic bowel syndrome, a fatal intestinal disease of adult dairy cows in the US (Puntenney et al., 2003). Many common molds do not produce mycotoxins, and the presence of molds in silage does not indicate the presence of mycotoxins nor does their absence confirm mycotoxins are absent (Zain, 2011).

Temperature and insect activity are the major factors influencing mycotoxin contamination of feedstuffs (Coulombe, 1993). Molds can grow at a temperature range of 10° to 40°C, a pH range of 4 to 8, and above 0.7 aw (Whitlow, 2003). In wet feeds, such as silage, high moisture levels allow mold growth if oxygen is available (Whitlow, 2003). Delayed harvesting, slow or delayed filing of silos, inadequate packing and sealing of silos, slow feedout rates, bridging in silage bags, and damaged plastic wrap, bags, or silo covers can create a conducive microclimate for mold proliferation and mycotoxin production. (Whitlow, 1993). Other factors
that may predispose to mold growth and mycotoxin production in feeds include rodent, rain, hail, drought, and lodging damage.

**Types of Mycotoxins in Ensiled Forages**

More than 400 mycotoxins occur naturally, however, only a few have been studied extensively (Whitlow and Hagler, 2005). Mycotoxins that are frequently present in ensiled forages include deoxynivanenol, fumonisin, aflatoxin, ochratoxin A, zearalenone, T2-toxin, ergot, and roquefortine C (Driehuis et al., 2008; Whitlow, 2009).

**Fusarium Toxins**

*Fusarium* species are field fungi because they proliferate in the field during plant growth (Christensen et al., 1977; Storm et al., 2008). Their activities are favored at high humidity (>70%) and temperatures that fluctuate between hot days and cool nights (CAST, 2003). Ensiling conditions are not favorable for *Fusarium* species, as they do not tolerate low pH and anaerobic conditions. Nevertheless, *Fusarium* toxins can tolerate ensiling (Lepom et al., 1990). Hence, concentrations of *Fusarium* toxins detected in silage usually reflect the contamination levels at the time of harvesting.

**Deoxynivalenol**

Deoxynivalenol (DON) is a toxin produced by *Fusarium* species such as *F. graminearum, F. sporotrichioides, F. culmorum, F. poae, F. roseum,* and *F. tricinctum.* Cold, wet periods followed by a short, dry period favor their secretion (Diekman and Green, 1992). The toxin also is prevalent when wet conditions coincide with warm days and cool nights (De Wolf et al., 2005). Deoxynivalenol also is known as vomitoxin because it is associated with vomiting in swine, as well as feed refusal, diarrhea, reproductive problems, and eventual death (Canady et al., 2001).
Deoxynivalenol is the most commonly detected mycotoxin in preserved forages at very high concentrations (Rotter et al., 1996). A three-year survey of 7,049 livestock feed samples to estimate the worldwide occurrence of mycotoxins revealed that DON represents one of the most frequent threats to livestock with a prevalence rate of 59% and an average contamination level of 1,104 µg/kg (Rodrigues and Naehrer, 2012). In another survey on the presence of mycotoxins in feed ingredients in the Netherlands (Driehuis et al., 2008), corn and wheat silages were found to be the main sources of DON in diets of dairy cattle, with average concentrations of 854 and 621 µg/kg, respectively, and maximum concentrations 3,142 and 1,165 µg/kg, respectively.

Ruminants are relatively resistant to DON (Pestka, 2007), because rumen microorganisms can extensively convert DON to non-toxic, de-epoxy DON (Swanson et al., 1987; Marczuk et al., 2012). Charmley et al. (1993) reported only a numerical loss in milk yield (-1.4 kg/d) in first lactation dairy cows consuming a DON-contaminated diet (2.6 – 6.5 mg/kg).

Likewise, no reduction in milk production was observed when DON was fed to dairy cows at 66 mg/kg or 6.4 mg/kg for 5 days or 6 weeks, respectively (Trenholm et al., 1985; Cote et al., 1986). A similar result was obtained by Korosteleva et al. (2009) who reported no reduction in milk production by dairy cows consuming diets contaminated with 2.6 or 6.5 mg/kg of DON. However, altered rumen fermentation and reduced protein flow to the duodenum (Danicke et al., 2005; Seeling et al., 2005) were observed in dairy cows when 8.21 mg/kg DM of DON and 0.09 mg/kg of ZEA were fed daily for 4 weeks, possibly because of the synergistic toxic effects of ZEA and DON (Grenier and Oswald, 2011).

The guidance level for DON in ruminant feeds in Western Europe is 5 mg/kg DM (European Commission, 2006) and the advisory guidelines stipulated by the US FDA are 5 and 10 mg/kg of diet DM for dairy and beef cattle, respectively (FDA, 2011).
**Fumonisins**

Fumonisin B₁ and B₂ are the most important of a group of at least 28 silage toxins produced by two *Fusarium* species, *F. verticillioides* and *F. proliferatum* and a strain of *F. nygamai* (Marasas, 1996; Whitlow and Hager, 2005). Hot, dry periods followed by humid conditions and insect damage are the major predisposing factors that favor the secretion of fumonisins by *Fusarium* species (DeWolf et al., 2005). Fumonisin represents the most frequent threat to livestock among mycotoxins with a 64% prevalence rate and an average concentration of 1,965 µg/kg in 7,049 livestock feed samples collected from the Americas, Europe, and Asia (Rodrigues and Naehrer, 2012). Gonzalez-Pereyra et al. (2008) reported that fumonisin levels in corn silage varied from 340 to 2,490 µg/kg of DM, with higher concentrations detected in samples taken from the top layer and side walls of the silo, which are normally prone to air infiltration and less extensive fermentation.

The structural formula of fumonisin is similar to sphingosine, which is a component of sphingolipids found at high concentrations in nerve tissues (Soriano et al., 2005). Fumonisin interrupts sphingolipid biosynthesis (Diaz and Boermans, 1994), thereby, causing leucoencephalomalacia, also known as moldy corn poisoning, in horses (Kellerman et al. 1990), pulmonary edema in swine (Colvin and Harrison 1992), and hepatotoxicity in rats, mice and rabbits (Gumprecht et al., 1995; Voss et al., 1995). Because it is rumen degradable (60 to 90%), the toxin tends to be less potent in ruminants (WHO, 2000); however, fumonisin has been nephrotoxic to calves fed 1 mg/kg BW of the toxin (Mathur et al., 2001). Similar results were observed in beef calves supplemented with 148 mg/kg of total fumonisin in the diet for 31 d (Osweiler et al., 1993). Diaz et al. (2000) reported lower milk yield in Holstein and Jersey cows fed diets containing 100 mg/kg of fumonisin from 7 days before calving to 70 d after parturition. Nevertheless, carry-over of the toxin into milk is believed to be negligible (Scott et al., 1994),
and hence the toxin poses no serious food safety concern for people. The US FDA advisory guideline for fumonisnin is 30 mg/kg of diet DM for lactating dairy cows, 30 mg/kg of diet DM for breeding ruminants, and 60 mg/kg of diet DM for calves over 3 months that are intended for slaughter (FDA, 2011).

**Zearalenone**

Zearalenone (ZEA) is an estrogenic metabolite produced by several species of *Fusarium* such as *F. graminearum*, *F. culmorum*, and *F. crookwellense* (Saeger et al., 2003). *Fusarium graminearum* occurs naturally in high-moisture corn and it has also been detected in moldy hay and pelleted feeds (CAST, 2003). It also co-occurs with DON in grain crops, like corn, barley and sorghum at very low concentrations (CAST, 2003). High humidity conditions with alternating low (11-14°C) and moderate (27°C) temperatures favor its production (Vigier et al. 1997; De Wolf et al., 2005).

Several studies have reported the occurrence of ZEA in ensiled forages. Whitlow and Hagler (2005) reported an average ZEA concentration of 525 µg/kg of DM and 30% incidence of ZEA in 461 corn silage samples in the US. Driehuis et al. (2008) reported ZEA contamination in 13 and 50% of grass and corn silage samples in Denmark with average concentrations of 180 and 146 µg/kg, respectively. Reed and Moore (2009) reported that sorghum and alfalfa silages contained 660 µg/kg and 79.8 mg/kg of ZEA, respectively. Zearalenone was the most common mycotoxin detected in a study that assessed the exposure of Danish cattle to mycotoxins from maize silage (Storm et al., 2014). A recent survey reported that approximately 45% of 7,049 livestock feed samples collected from the Americas, Europe, and Asia contained ZEA, with an average concentration of 233 µg/kg (Rodrigues and Naehrer, 2012).

The structural similarity of ZEA to estrogen enables the toxin to mimic the hormone (Saeger et al., 2003). This causes numerous reproductive problems, including hyper-estrogenism,
vaginitis, and mammary gland enlargement, particularly in swine (Diekman and Green, 1992; Marczuk et al., 2012). Ruminants are less susceptible than swine because ZEA is converted to its hydroxyl-metabolites, α and β-zearalenol, by ruminal flora, predominantly by ruminal protozoa (Kennedy et al., 1998). Although α-zearalenol has higher affinity for estrogen receptors than ZEA, its lower absorption rate and or interconversion to β zearalenol in the liver decrease its negative effects (Malekinejad et al., 2006; Fink-Gremmels, 2008). Nevertheless, the capacity of ruminal microbes may be exceeded by high intakes of ZEA. Weaver et al. (1986) reported reduced conception rates in dairy heifers fed 12.5 mg/kg of ZEA. Furthermore, depressed intake and milk production, diarrhea, and reproductive failure were reported in heifers fed diets contaminated with 660 µg/kg ZEA and 440 µg/kg DON (Coppock et al., 1990).

The major concern with ZEA toxicity is its negative effect on animal health and reproduction because, like fumonisins, the extent of transfer into milk is negligible (Seeling et al., 2005). Consequently, there are no FDA action limits, guidance, or advisory levels for ZEA. However, the guidance value for ruminant feeds in Europe is 500 µg/kg (European Commission, 2006).

T-2 Toxin

T-2 is a potent Fusarium-produced mycotoxin (Saito and Okubo, 1974). It is more prevalent in cereal grains than ensiled forages (Whitlow and Hagler, 2005) and is produced during wet and warm conditions (Xu et al., 2014). Approximately 5% of 388 corn silage samples collected from different countries contained 65 µg/kg of T-2 toxin (Biomin, 2015). The T-2 toxin is degraded by ruminal flora to its less toxic metabolites (Whitlow, 1993), nevertheless, T-2 is associated with gastroenteritis and intestinal hemorrhages (Hsu et al., 1972), immunosuppression (Gentry et al., 1984; Black et al., 1992), and reduced performance and reproductive problems in cattle (Kegl and Vanyi, 1991). A study noted that it is toxic to cattle at high intake level of 0.7 to
1.5 mg/kg (Li et al., 2011). Calves consuming 10 to 50 mg/kg of T-2 toxin experienced abomasal ulcers and sloughing of the ruminal papillae. However, no death was recorded (Cheeke, 1998). Guidelines for T-2 toxin have not been established by US FDA or European legislative agencies; nevertheless, feeding levels close to the toxic dose of Li et al. (2011) is not recommended.

**Aspergillus Toxins**

*Aspergillus* molds are considered storage fungi because usually, they do not infect crops before harvest. Some species, such as *Aspergillus flavus* may infect crops and produce aflatoxins in the field (Wicklow, 1983; Storm et al., 2008) when temperatures are high (> 32°C) and during drought stress (Whitlow and Hagler, 2005).

**Aflatoxins**

Aflatoxins are a group of toxic, mutagenic and carcinogenic compounds produced by *Aspergillus flavus, A. parasiticus, and A. nominus* (Deiner et al., 1987; Kurtzman et al., 1987; Creepy, 2002). High humidity (> 80%) and temperature (> 32°C), insect damage and drought stress (DeWolf et al., 2005; Tsitsigiannis et al., 2012) are conducive conditions for aflatoxin production. Natural forms of aflatoxins such as B₁ and G₁, and their dihydroderivatives, B₂ and G₂, are naturally present in food and feed ingredients because of the ability of *Aspergillus* to grow over a broad range of temperatures and humidities (Dutton, 1988; Phillips, 1999; Tulayakul et al., 2005). Aflatoxin B₁ (AFB₁) is the major aflatoxin and the most potent natural carcinogen produced by toxigenic *Aspergillus* (Squire, 1981). The incidence of aflatoxin is relatively low in silages compared to other mycotoxins, probably because *A. flavus* and *A. parasiticus* are less tolerant of acidic and anaerobic silage environments. Aflatoxins were present in 33% of 7049 livestock feed samples at an average concentration of 63 µg/kg (Rodrigues and Naehrer, 2012). In a survey conducted in Denmark by Driehuis et al. (2008), no AFB₁ was detected in 140 corn silage samples. However, high concentrations of aflatoxin can occur in poorly made silages or in
silages made in hot, humid areas or with diseased corn plants (Gonzalez-Pereyra et al. 2008; Queiroz et al., 2012). In addition, aflatoxins are more prevalent in trench silos because *Aspergillus* is one of the main fungal contaminants in such silos (González Pereyra et al., 2011). Up to 51 µg/kg DM of AFB$_1$ was detected in corn silage collected in a trench silo in France (Richard et al., 2009). Furthermore, Gonzalez-Pereyra et al. (2008) reported an AFB$_1$ concentration of 156 µg/kg of DM in corn silage stored in a trench type silo without proper sealing.

Aflatoxins have been the most studied mycotoxin because of the severe public health concern associated with their carcinogenicity (CAST, 2003). Aflatoxins do not have a lactone ring and consequently, are relatively resistant to ruminal microbial degradation; moreover, low concentrations can inhibit ruminal bacterial growth (Yiannikouris and Jouany, 2002). Long exposure to aflatoxins in diets of lactating cows caused adverse effects such as reduced health and performance of cows, impaired liver function, (Garrett et al., 1968), suppressed immune status and increased susceptibility to diseases despite vaccinations (Diekman and Green, 1992). The symptoms associated with acute aflatoxin ingestion (aflatoxicosis) include inappetence, ataxia, rough hair coat, and enlarged liver (Guthrie and Bedell, 1979; Whitlow and Hagler, 2005). Precise diagnosis of aflatoxicosis is difficult because the symptoms are not specific to the condition (Coulombe, 1993). Increased liver weight and reduced performance were observed in beef cattle fed a diet containing 100 µg/kg of aflatoxin (Garret et al., 1968; Patterson and Anderson, 1982). Furthermore, reduced feed efficiency because of compromised ruminal function was reported in steers fed 600 µg/kg (Diekman and Green, 1992). Queiroz et al. (2012) noted decreased milk yield and milk protein concentration, as well as, altered immune response in dairy cows fed 75 µg/kg of aflatoxin daily for 5 d. However, other studies reported no changes
in milk yield when 170 (Kutz et al., 2009) or 112 µg/kg (Stroud, 2006) of AFB1/kg of TMR DM were fed for 7 or 11 d, respectively. This indicates that several factors, other than the amount ingested, may influence the response of cows to the toxin; such factors may include heat or acidic stress, subclinical disease, etc.

After ingestion of contaminated feeds, a portion of the ingested aflatoxin B1 is degraded by the ruminal microbes, resulting in the formation of aflatoxicol (Upadhaya et al., 2010). In humans and susceptible animals, the toxin is metabolized by cytochrome P-450 enzymes into aflatoxin-8,9-epoxide (highly toxic, mutagenic and carcinogenic), which causes chromosome damage by binding with and forming adducts on DNA (Jouany et al., 2009). Other metabolic fates of AFB1 in the liver include O-dealkylation to AFP1, ketoreduction to aflatoxicol, and hydroxylation to AFM1 (highly toxic and carcinogenic), AFQ1, or AFB2α (Kuilman et al., 2000; Wu et al., 2009; Jafarian-Dehkordi and Pourradi, 2013). Aflatoxin B1 is excreted in milk of dairy cows as AFM1 (Diaz et al., 2004; Battacone et al., 2005), which represents a safety risk of milk and milk products because both AFB1 and AFM1 are Group 1 human carcinogens (International Agency for Research on Cancer, IARC, 2002). The rate of transmission of aflatoxin from feed into milk can be as high as 6% (EFSA, 2004). Therefore, aflatoxin is the only mycotoxin that has government legislation for its maximum permissible level in feedstuffs and milk products. The Action Levels established by the US FDA are 0.5 and 20 µg/kg for fluid milk and feed ingredients offered to dairy cattle, respectively (FDA, 2011), while the maximum level permitted in Europe for fluid milk is 0.05 µg/kg (European Commission, 2006).

**Penicillium Toxins**

*Penicillium* molds can grow in typical silage environments with low water activity (0.79 – 0.83 aw), low oxygen concentration (1%) and low pH (3.0 – 6.0) (Boysen et al., 2000; Nevarez
et al., 2009). *Penicillium* molds are more common during storage, but they also can grow on plants under very wet conditions (Christensen et al., 1977).

**Ochratoxin A**

Ochratoxins are structurally-related secondary metabolites produced by *Aspergillus ochraceus*, *A. clavatus* and *Penicillium verrucosum*, particularly when temperatures range between 20 and 25°C. Ochratoxin A, which is carcinogenic, immunotoxic, and which inhibits glucose metabolism, is considered the most toxic of the group (Whitlow, 1993; Yiannikourisa and Jouany, 2002). However, this toxin is not currently classified as being carcinogenic to humans. Veldman et al. (1992) reported 27% incidence of ochratoxin A in 89 randomly selected feed ingredient samples, especially in grains, with an average concentration of 120 µg/kg.

Ochratoxin A is extensively and rapidly degraded in the rumen to a less toxic product, ochratoxin-alpha (Hult et al., 1976; Fink-Gremmels, 2008), which explains tolerance of ruminants to the toxin. However, it also may be metabolized to ochratoxin C, which is similarly toxic (Chu, 1974). Bioavailability of dietary ochratoxin may be increased with high-grain diets, probably because of acidotic conditions (Hohler et al., 1999). Furthermore, the detoxifying capacity of the rumen may be exceeded at high intake, in which case the toxin can reduce milk production and be transferred into milk (Whitlow, 1993; Yiannikourisa and Jouany, 2002). Overall, the occurrence of ochratoxins in feed ingredients, especially in ensiled forages, is low because the toxin is not stable in silos and it poses no significant threat to food safety and supply.

**Mycophenolic acid and Roquefortine C**

These are secondary metabolites of *Penicillium roqueforti* (Miller and Trenholm, 1994; Schneweis et al., 2000). *Penicillium roqueforti* is commonly referred to as the silage mold (Pahlow et al., 2003) because it is acid-tolerant and it can grow at low oxygen concentrations.
Consequently, it is the most frequently isolated *Penicillium* mold in silage (Sumarah et al., 2005).

Roquefortine C and mycophenolic acid have been frequently detected in silage. In Netherlands, Roquefortine C was detected in 19% of 47 corn and grass silage samples and 7% of 29 samples of ensiled by-products. Mycophenolic acid was detected in 13 and 10% of samples of these feed classes, respectively. (Driehuis et al., 2008). A survey of silage samples in Germany showed prevalence estimates of 56 and 32% of roquefortine C and mycophenolic acid, respectively, (Schneweis et al., 2000).

Symptoms, such as reproductive disorders, mastitis, and lack of appetite were detected in cattle herds fed silages containing 0.2 to 1.5 mg/kg of roquefortine C in Northern Germany (Auerbach et al., 1998). Also, paralytic effects were reported in cows fed 4 to 8 mg/kg of Roquefortine C (Haggblom, 1990). However, no clinical signs of intoxication were observed in sheep fed 25 mg/kg of Roquefortin C for 18 days (Tuller et al., 1998), indicating that sheep are more tolerant to Roquefortine C than cattle.

Mycophenolic acid is considered an immunosuppressive agent; it blocks the proliferative response of T and B lymphocytes, and inhibits antibody formation and production of cytotoxic T cells (Eugui et al., 1991). Research data on the effects of mycophenolic acid in cattle are lacking. However, Mohr et al. (2007) found no effects in sheep exposed to 10 to 300 mg/kg daily for 44 days.

**Ruminal Degradation of Mycotoxins**

Ruminants are more protected from toxic effects of mycotoxins than monogastrics because rumen microbes degrade or inactivate some of the toxic molecules (Pestka et al., 2007; Cheli et al., 2013). However, the capacity of rumen detoxification can be saturated and it varies with diet and or metabolic disease infestation (Fink-Gremmels, 2008). In addition, starch-
induced low ruminal pH (5.5 to 5.8) and high ruminal passage rates of high-producing cows may greatly reduce microbial detoxification (Fink-Gremmels, 2008; Pantaya et al., 2016). Therefore, some mycotoxins, such as ochratoxins and aflatoxins, can partially escape ruminal degradation and be transferred to animal products, particularly milk, posing a severe health hazard to humans.

Kiessling et al. (1984) investigated the ruminal degradation of different mycotoxins and reported that whole rumen fluid degraded ZEA, T-2 toxin, and ochratoxin to their less toxic metabolites (zearalenol, HT-2 toxin, ochratoxin alpha, respectively), but had no effect on AFB₁ and DON. The authors also demonstrated that 90 to 100% of the metabolism of ochratoxin A, ZEA, and T-2 toxin were achieved by the rumen protozoa and, therefore, they are considered important agents of ruminal microbial mycotoxin biodegradation (Upadhaya et al., 2010). However, some studies have demonstrated that ruminal bacteria also are involved in mycotoxin degradation (Schatzmayr et al., 2006; Yang, 2010).

**Co-occurrents of Mycotoxins in Livestock Feeds**

Contamination of livestock feeds by multiple mycotoxins is a common occurrence on farms because most fungi can produce several mycotoxins, and several fungi can simultaneously contaminate feed commodities (Bottalico, 1998; Sweeney and Dobson, 1998; Alassane-Kpembi et al., 2016). Therefore, studying the occurrence and effects of a single mycotoxin will more likely give incomplete and/or false information about the associated risk of mycotoxin exposure. An extensive literature review revealed that more than 75% of feed samples in Europe contain one or more mycotoxins (Streit et al., 2012). In addition, almost half of 7049 animal feed samples collected from the Americas, Asia, and Europe simultaneously contained at least two of DON, aflatoxins, ZEA, fumonisins, and ochratoxin A (Rodrigues and Naehrer 2012). Aflatoxin B₁ was found to co-occur with ochratoxin A, ZEA, or the combination of both toxins in 31, 12,
or 27% of 123 Spanish barley samples, respectively (Ibáñez-Vea et al., 2012). In another study in the Netherlands, Driehuis et al. (2008) reported that DON and ZEA co-occurred in 44% of dairy diets, and corn silage was the major source of both mycotoxins in the feeds.

Synergistic negative effects of several toxins in the diet can worsen health and production problems of cattle consuming mycotoxin-infested feeds. A meta-analytic study summarizing 112 publications reported negative synergistic or additive effects of different mycotoxins on animal performance (Grenier and Oswald, 2011), which emphasizes the need to study co-occurrence of mycotoxins in feeds to get a clearer picture of their adverse effects on the performance and health of livestock.

**Dietary Contribution of Corn Silage to Mycotoxin Intake in Cattle**

Whole-plant corn silage is an important component of dairy and beef diets in the US, Europe and some other parts of the world as approximately 120 million tons of corn silage are produced and fed to ruminants annually in the US (USDA, 2016). Corn forage and silage can be contaminated with mycotoxins in the field or during or after ensiling, respectively. The percentage contribution of corn silage mycotoxins to total dietary intake of mycotoxins varies widely among studies (Table 2-1). In the worst case scenario, the contributions may be as high as 81, 134, 107, 68, 23, and 270% of dietary concentrations for AFB₁, ZEA, DON, fumonisin, mycophenolic acid, and roquefortine C, respectively (Table 2-1). However, the actual range varies widely because of factors such as location, sampling technique, method of mycotoxin analysis, prevailing weather conditions, etc. Nevertheless, these data suggest that affordable mycotoxin analysis should be conducted on silages to increase feed safety.
Preventing Mycotoxin Contamination of Feeds

Field or Pre-harvest Phase

Pre-harvest control of mycotoxins is the first step to ensure that feeds are safe. Mycotoxin contamination can be reduced by minimizing environmental stress on the plant through appropriate agronomic practices such as use of varieties or hybrids that are adapted to local conditions and are naturally resistant to fungal infection, control of insect infestation, proper management of crop residues and weeds, application of fungicides and pesticides, use of irrigation, timely crop rotation, and proper fertilization (Edwards, 2004; Whitlow and Hagler, 2005). Insect management is the most important practice for reducing the risk of mycotoxins, particularly fumonisins and aflatoxins, in temperate regions because they act as vectors of fungal spores and insect damage predisposes crop plants, such as maize, to fungal infection (Munkvold, 2014).

The public health concern over the use of pesticides and fungicides as well as development of resistance to fungicides by fungal organisms has led to development of bio-agents to control mycotoxin contamination on the field (Tsitsigiannis et al., 2012). Application of atoxigenic strains of A. flavus or A. parasiticus to crops is a successful approach that has been used to reduce aflatoxin contamination of the field (Tsitsigiannis et al., 2010). Two atoxigenic strains of A. flavus, AF36 and NRRL 21882, are widely used in the US to minimize aflatoxin contamination of crops. An in-depth review on biological control of mycotoxins was published by Tsitsigiannis et al. (2012) and is an excellent supplement to this section.

Harvest Phase

Careful harvest timing is important in controlling the level of mycotoxin contamination. The risk of mycotoxin contamination is reduced significantly with earlier harvests (Jones et al.,
1981). However, harvest should be timed to ensure optimum yield, DM concentration, and nutritive value.

The cutting height of the crop harvesters should be set to minimize soil contamination because of the widespread occurrence of *Fusarium* spores in soil (Jean Pierre, 2007). In addition, harvesting equipment should be properly maintained and calibrated to avoid physical damage to crops at harvest because contact with soil and kernel damage predisposes forage to contamination with fungal spores (Whitlow and Hagler, 2005). Furthermore, immediate storage of harvested feeds is necessary to minimize the risk of mycotoxin contamination, because delayed storage may expose the crop plants to temperature and humidity conditions that favor continued growth of toxigenic fungi and mycotoxin contamination (Munkvold, 2014).

**Ensiling Phase**

Controlling mycotoxin production at the pre-harvest stage via physical, chemical, and chemical methods is best; however, problems about feed safety, potential losses in quality of commodities, limited efficacy and cost implications (Köhl et al., 2011) limit the success at this stage (Köhl et al., 2011). Hence, mycotoxin control during storage is often critical.

Low silage pH and anaerobic conditions during ensiling inhibit the growth of spoilage and toxigenic fungi, and reduce production of mycotoxins in silage (Pahlow et al., 2003). Plants should be harvested at the recommended DM of 65 – 70% for pit silage and 45–50% for baled silage, and silos should be filled rapidly, tightly packed and completely sealed to preserve anaerobic conditions (Jouany, 2007). Cavallarin et al. (2011) reported that using an oxygen-barrier plastic film for ensiling limits aflatoxin production. A similar result was reported by Amaral et al. (2010). To reduce mold infestation and or mycotoxin contamination during feed--out, aerobic stability-enhancing measures should be taken including maintaining a straight firm
silo face, feeding out the silage at a minimum rate of 10 to 16 cm per day, and feeding the silage immediately after removal from the silo (Jouany, 2007).

The potential of silage lactic acid bacteria to mitigate aflatoxin contamination of silage has been evaluated in vitro (Gourama and Bullerman, 1995; Ahlberg et al., 2015). Lactic acid bacterial inoculants have been used successfully to enhance silage fermentation, increase aerobic stability and reduce fungal populations in silages (Mari et al., 2009; Pedroso et al., 2010). However, research reports on using additives to reduce forage aflatoxin contamination are limited. Inoculation of harvested corn plant infested with southern rust with a mixture of *Pediococcus pentosaceus* and *L. buchneri* at ensiling increased aerobic stability and prevented production of aflatoxins (5.2 vs. 0 mg/kg) (Queiroz et al., 2012). In another study, *L. buchneri* inoculation reduced aflatoxin concentration but had no effects on concentrations of DON or ZEA in corn silage (Iglesias et al., 2005). Cavallarin et al. (2011) reported that limiting aerobic spoilage by inoculating corn forage with *L. buchneri* at ensiling also reduced aflatoxin production. Teller et al. (2012) studied the effects of additive application and damaging ears of corn in the field before harvest on production of selected mycotoxins. Concentrations of DON and fumonisins were increased in damaged ears but not in undamaged ears and inoculation with *L. buchneri* 40788 (400,000 cfu/g of fresh forage) and *Pediococcus pentosaceus* (100,000 cfu/g), or treatment with potassium sorbate, reduced mold and yeast counts and aerobic spoilage, but had no effect on the concentration of DON, ZEA, and fumonisins in the corn silage. The main deduction from these studies is that proper silage management and use of mold-inhibiting additives or microbial inoculants can decrease aerobic spoilage and reduce the growth of toxigenic fungi in silage (Kung et al., 2003), but these measures may not always reduce concentrations of mycotoxins existing on plants before ensiling. In summary, good silage
management principles and practices at all stages of silage making and feeding are essential to reduce mycotoxin contamination of silages.

**Mycotoxin Detoxification Strategies**

Strategies for detoxification of mycotoxins are aimed at destroying or reducing the concentration of a mycotoxin, or making it non-toxic and or unavailable for absorption in the gut of ruminants (CAST, 2003). Despite several efforts to control mycotoxins at pre and post-harvest stages, frequent instances of feed contamination with mycotoxins occur, emphasizing the need for effective detoxification of feed mycotoxins. Such measures should target detoxification of silage mycotoxins because silage represents the bulk of dairy cattle diets in the US and many other parts of the world, and it is prone to mycotoxin contamination particularly when poorly managed.

Various mycotoxin decontamination strategies, such as manual removal, cleaning, or milling of contaminated grains (Bata and Lastity, 1999; Siwela et al., 2005), thermal irradiation (Rater and Matissek, 2008), ammoniation (Hogeboom et al., 2001), and ozonation (McKenzie, 1997) are considered effective; however, in addition to the high cost and safety issues, these strategies are not suitable for ensiled forages probably because of their high volume. Dilution of contaminated ingredients with others in rations is also an effective strategy for reducing the ingestion of mycotoxins, though this does not involve detoxification of the toxin. Though effective, it is not an ideal strategy for contaminated silage because it reduces the feed-out rate which may promote further mold growth (Whitlow and Hagler, 2005) and mycotoxin production.

A feasible, safe, and relatively cost effective alternative strategy to reduce problems associated with ingestion of mycotoxins is to decrease their bioavailability in the gut. This involves inclusion of sequestering agents in the diets (CAST, 2003). The use of clay minerals as sequestering agents in the feeds of livestock has been well documented (Stroud, 2006). Clay
minerals are fine-grained components of geological materials, occurring as particles with a phyllosilicate structure that are formed usually as products of chemical weathering (Veldt and Meniere, 2008). Clay minerals can hold water and dissolved nutrients because of the presence of unbalanced electrical charges (Veldt and Meniere, 2008). Therefore, they can adsorb organic substances on their external surfaces or within their inter-layer spaces by the interaction or substitution of the exchanged cations within these spaces (Veldt and Meniere, 2008). Aflatoxins can be adsorbed into this porous structure and be trapped by electric charges (Jouany, 2007). Adsorbent clay minerals are effective at binding AFB₁ in liquid media (Moschini et al., 2008).

Kutz et al. (2009) examined the efficacy of two calcium montmorillonite clay-based sequestering agents, soils (Novus International Inc., St. Charles, MO) and overspills (Engelhard Corp., Cleveland, OH), in mid lactating cows fed 100 μg/kg of aflatoxin B₁. Addition of soils and overspills reduced the milk AFM₁ concentration by 45% and 48%, respectively. Queiroz et al. (2012) evaluated the effects of dietary addition of 0.5 and 1% of diet DM of a montmorillonite-based mycotoxin sequestering agent on milk AFM₁ concentrations and reported that only the high dose reduced the concentration of AFM₁ in the milk. Furthermore, a recent study by Maki et al. (2016) revealed that transfer rate of aflatoxin into milk was reduced in cows fed overspills.

Although, clay-based products have been effective at adsorbing aflatoxin in diets, their composition varies significantly by lot and type; therefore, the efficacy and safety of each product should be evaluated before inclusion in animal diets (CAST, 2003). The most extensively studied clay minerals are a range of products known as hydrated sodium calcium aluminosilicate (HSCAS) and montmorillonite is one of those frequently used (Jouany, 2007). This clay mineral binds with aflatoxin by chelating the β-dicarbonyl moiety in aflatoxin with uncoordinated metal ions in the clay materials (Phillips et al., 1991; Whitlow, 2006). Hydrated
sodium calcium aluminosilicate has high affinity for AFB$_1$ (Phillips et al., 1988). In fact, the aflatoxin-HSCAS binding complex formed is stable over a wide pH (2 to 10) and temperature (25 to 37°C) range (Sarr et al., 1990, 1991). Other clay products that have been studied include bentonites, zeolites, clinoptilolites, and others that are not characterized (Whitlow, 2006). Clay minerals differ in terms of ion exchange capacity, surface area, and particle size, shape and distribution (Grim, 1968). Montmorillonite clay is believed to have the greatest cation exchange capacity (Grim, 1968). Due to such compositional differences, it is important for research studies to report the characteristics of clay products being tested. Some of the limitations of clay-based products as mycotoxin sequestering agents are that they accumulate in manure, may be contaminated with toxic metals and dioxins, and they are only effective at binding aflatoxins (Bursian et al., 1992; Phillips et al., 1999). For instance, the inclusion of HSCAS in the DON-contaminated diet of pigs did not mitigate the effects of DON (Patterson and Young, 1993). Also, HSCAS reduced the toxicity of aflatoxin, but did not prevent that of ochratoxin A alone or combined with aflatoxin in poultry (Huff et al., 1992). In addition, clay-based sequestering agents may bind with dietary minerals and vitamins. Chestnut et al. (1992) reported impaired absorption of zinc, magnesium, and manganese in sheep fed HSCAS. Similar findings were observed in poultry (Chung et al., 1990) and swine (Schell et al., 1993). No published reports indicating that clay-based sequestering agent ingestion impaired mineral absorption in dairy and beef cattle were found. However, a recent study reported an increased loss in milk yield as concentration of clay product increased from 0.5 to 2% in dairy cows fed 100 µg/kg of dietary aflatoxins (Sulzberger et al., 2017). The clay product used in this study contained vermiculite, nontronite, and montmorillonite.
To ensure protection against other mycotoxins and reduce the need for clay products in diets, yeast and yeast cell wall products were evaluated for their ability to bind aflatoxins and other mycotoxins (Devegowda et al., 1998). A yeast cell wall product (MTB-100) reduced the AFM$_1$ concentration of milk by 59% when fed at 0.05% of a diet contaminated with 55 µg/kg of AFB$_1$ (Diaz et al., 2004). Xiong et al. (2015) reported that adding a blend of yeast extract and yeast culture reduced transfer of AFB$_1$ into milk from diets contaminated with 20 µg/kg of AFB$_1$ but had no effect when diets were contaminated with 40 µg/kg of AFB$_1$. Furthermore, no effects of yeast products have been found in diets contaminated with at least 60 µg/kg of AFB$_1$ (Waltman et al., 2008; Kutz et al., 2009; Kissell et al., 2013), indicating that the efficacy of yeast products is dependent upon the extent of mycotoxin contamination.

Glucans and mannans are major components of yeast cells (Fruhauf et al., 2012). Sequestration of mycotoxins by yeast is caused by adhesion of the toxin to the cell wall components (mannan and β-glucan) (Pfliegler et al., 2015). Yeast cell wall glucans, besides being nutritional aids and growth promoters, can bind mycotoxins in vitro and in vivo (Fruhauf et al., 2012). Beta-glucans (glucomannan) fed at 0.05 or 0.1% of diet to dairy cow or poultry, respectively, bound aflatoxins, ochratoxin, and T-2 toxin (Raju and Devegowda, 2000; Diaz et al., 2004). Furthermore, Freimund et al. (2003) noted that a glucan polymer can bind both T-2 toxin and ZEA in vitro. An additional benefit of adding yeast or yeast products to diets is that β-glucan can improve the performance of animals by stimulating both nonspecific and specific immunological responses (Keller et al., 2015).

Activated charcoal is a mycotoxin sequestering agent with a large surface area and excellent adsorptive capacity (Whitlow, 2006). However, the effects of activated charcoal have been variable. Galvano et al. (1996) reported that two activated charcoals differing in surface
area and iodine number were fed at 2% of the diet reduced the transfer of toxin into the milk of Friesian cows fed 11 µg/kg of AFB$_1$ by 22 to 45%, but these responses were not better than that with HSCAS. However, when cows were fed 55 µg/kg of AFB$_1$, feeding 45 g/cow daily of activated charcoal did not reduce milk aflatoxin residues, whereas bentonite clay binder (225 g/cow daily) or an esterified glucan (10 g/cow daily) reduced milk aflatoxin (Diaz et al., 2004). This suggests that the efficacy of activated charcoal is dependent on the level of contamination. Studies with broilers (Edrington et al., 1997) and turkey poults (Edrington et al., 1996) also suggest that charcoal is not as effective as clay-based sequestering agents.

A synthetic water-soluble polymer, polyvinlypyrrolidone (PVP), also has been evaluated as a mycotoxin sequestering agent. Alegakis et al. (1999) reported binding of ZEA by PVP ranging from 33.5 to 66.2% per 25 mg of PVP in an in vitro medium. However, no studies on the binding efficacy of PVP in animals were found.

The binding effects of chlorophyll-based products on AFB$_1$ also have been studied in vitro (Hsu et al., 2008). The mechanism of action of chlorophyll-based product involves trapping AFB$_1$ and other carcinogens via binding of their planar ring structures to that of chlorophyll (Arimoto et al., 1993), thereby, preventing the toxin from forming adducts with DNA, and hence facilitating the clearance of the toxin from the body (Hsu et al., 2008). In humans challenged with 30 ng of AFB$_1$, two chlorophyl products fed at 150 mg daily produced a significant 40% to 60% reduction in excretion of urinary aflatoxin equivalents (Jubert et al., 2009).

**Summary.** This review discussed the phases of silage fermentation, the importance of silage additives, the types and modes of action of different silage additives, different techniques for silage microbial evaluation, and the critical need for proper understanding of silage microbial composition and its relationship to the metabolites in silage. In addition, the review highlighted
the role of feeds and ruminant livestock in cycling of pathogenic EC on farms, and emphasized the need for effective strategies to prevent the growth of EC on feeds. Chapters 3, 4, and 5 summarize experiments that examined the efficacy of strategies to inhibit the growth of EC in corn and alfalfa silage. These experiments also characterized the microbial composition of the treated and untreated silages and examined relationships between the abundance of known and unknown silage microbes and indices of silage quality.

This review also described the role, importance and danger of mycotoxins for animals and humans, and their prevalence. It also showed that equivocal results have been achieved when sequestering agents were used to bind mycotoxins and reduce their transfer from the diet to the milk of dairy cows. This emphasizes the need to develop more effective sequestering agents and to continue to evaluate their effects on milk AFM\textsubscript{1} concentration, as well as on milk yield and quality. Chapter 6 describes an experiment aimed at examining whether dietary addition of three novel \textit{Saccharomyces cerevisiae} fermentation products could act as mycotoxin-sequestering agents that would reduce transfer of aflatoxin to milk and improve the performance and health of dairy cattle challenged with dietary aflatoxin.
Table 2-1. Estimated dietary contributions of whole plant corn silage to total dietary mycotoxin in diets.*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mycotoxin</th>
<th>Mean Concentration (µg/kg)</th>
<th>Maximum concentration (µg/kg)</th>
<th>% mean contribution in dairy diet</th>
<th>% maximal contribution in dairy diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richard et al. 2009</td>
<td>AFB₁</td>
<td>28.0</td>
<td>NA</td>
<td>42 (168)*</td>
<td>NA</td>
</tr>
<tr>
<td>Keller et al. 2013</td>
<td>AFB₁</td>
<td>33.0</td>
<td>54</td>
<td>49.5 (198)*</td>
<td>81.0 (324)*</td>
</tr>
<tr>
<td>Driehuis et al. 2008</td>
<td>ZEA</td>
<td>174</td>
<td>943</td>
<td>13.05</td>
<td>70.7</td>
</tr>
<tr>
<td>Storm et al. 2014</td>
<td>ZEA</td>
<td>66.0</td>
<td>311</td>
<td>3.96</td>
<td>23.3</td>
</tr>
<tr>
<td>Schollenberger et al. 2006</td>
<td>ZEA</td>
<td>432</td>
<td>1790</td>
<td>25.92</td>
<td>134.3</td>
</tr>
<tr>
<td>Eckard et al. 2011</td>
<td>ZEA</td>
<td>180</td>
<td>430</td>
<td>10.8</td>
<td>32.3</td>
</tr>
<tr>
<td>Storm et al. 2010</td>
<td>DON</td>
<td>1056</td>
<td>5094</td>
<td>6.336</td>
<td>38.2</td>
</tr>
<tr>
<td>Driehuis et al. 2008</td>
<td>DON</td>
<td>854</td>
<td>3142</td>
<td>5.124</td>
<td>23.6</td>
</tr>
<tr>
<td>Storm et al. 2014</td>
<td>DON</td>
<td>1629</td>
<td>2974</td>
<td>9.774</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>2100</td>
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</tr>
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<tr>
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<td>34,000</td>
<td>NA</td>
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<tr>
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<td>1824</td>
<td>1.23 (0.37)*</td>
<td>3.65 (1.09)*</td>
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<tr>
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<td>30400</td>
<td>6.00 (1.8)*</td>
<td>60.8 (18.2)*</td>
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<td>Roquefortine C</td>
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<td>240</td>
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*Estimated daily corn silage intake of 7.5 kg; Estimated concentrations of mycotoxins in the diets; aflatoxins (AFB₁; 20 µg/kg, FDA, 2010; 5 µg/kg, European commission, 2006), Deoxynivalenol (DON; 5 mg/kg, FDA, 2011 and European Commission, 2006), fumonisín (30 mg/kg, FDA, 2011; 50 mg/kg, European commission, 2006), ZEA (0.5 mg/kg, European commission, 2006), and mycophenolic acid (300 mg/kg, Mohr et al., 2007) and Roquefortine C (4 mg/kg; Haggblom, 1990)

#Values in parenthesis are estimates based on maximum permissible level of the mycotoxin in Europe.

NA = Not given
CHAPTER 3
CONTROL OF Escherichia coli O157:H7 IN CONTAMINATED ALFALFA SILAGE:
EFFECTS OF SILAGE ADDITIVES

Background

Escherichia coli O157:H7 is a Shiga toxin-producing food-borne pathogen associated with hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans (USDA-APHIS, 2001). This pathogen has been implicated in outbreaks of these illnesses in the US, Canada and Europe and accounts for about 95% of HUS cases (Griffin, 1995). Escherichia coli O157:H7 is estimated to cause about 75,000 illness cases and 50 deaths yearly in the US (Mead et al., 1999). Cattle are considered the primary reservoir of EC (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 US and they can be contaminated with EC through the manure and saliva of cows (Russel et al., 2000). The rapid drop in pH during ensiling kills EC 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, L. buchneri 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 EC 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 EC 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 examine the effectiveness
of obligate or facultative heterofermentative lactic acid bacteria (LAB) inoculants or propionic
acid at controlling EC in alfalfa silage during and after ensiling.

**Materials and Methods**

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

**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, Moline, IL). 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) $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 EDL933 alone (EC); (3) EC + $1 \times 10^6$
cfu/g of *Lactobacillus plantarum* R2014 (EC+LP); (4) EC + $1 \times 10^6$ cfu/g of *L. buchneri* R1102
(EC+LB); and (5) EC + 2.2 g/kg (fresh weight) 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 (BPW; 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 (LB) 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, Basingstoke, UK) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) (CT-SMAC), and incubated at 35°C for 24 hours. 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 $1 \times 10^6$ cfu/g for each inoculant and 0.22% for the acid. Control silages were also treated with the 3 mL/kg fresh forage of distilled water. 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 days 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 re-inoculated 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, an 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 minute 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 BPW followed by direct 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 enumerated as EC. 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, Detroit, MI) 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 × 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 LB 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 × g for 15 min at 4°C. The pH of the supernatants was adjusted to 5.5. The MRS broth was also adjusted to a pH of 5.5 and used as the Control. Silage extracts were similarly centrifuged and the cell-free supernatants were adjusted to pH 5.5. Two sterile cloning discs (6.0-mm diameter; cat. no. 231039, Becton Dickinson Co.) 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 for 24 h at 35°C. The diameter of zones of inhibition around the paper discs were 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 × day. When treatment × day interactions were significant, effects of treatment on each day were examined. The model for analyzing the data collected once included the treatment effect. Differences between means were determined using the Tukey 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 3-1). 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 3-1). 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 3-2). 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 \( (0.17 \pm 0.03, 0.24 \pm 0.06 \) 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 3-3), which is the minimal 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 3-4), possibly because of 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 3-2).

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 3-2). This was caused by 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. 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-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 $2.65 \log_{10} \text{cfu/g}$, respectively) than in Control, EC and EC+LP silages ($5.22$, $4.85$ and $4.50 \log_{10} \text{cfu/g}$, respectively).

**Aerobic Phase**

After 168 h of re-inoculation and aerobic exposure, $2.3 \log_{10} \text{cfu/g}$ of EC were detected in the EC silage but no EC was detected in the Control, inoculant or propionic acid- treated silages (Figure 3-5). The pH values of the inoculant and propionic acid-treated silages were below 5.0 (Figure 3-6) 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 3-5). 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 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 US. 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 two 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 of forages and silages because of 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 *E. coli* O157 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 non-pathogenic *E. coli* in well preserved corn and grass silages because of the rapid drop in pH during ensiling is well known (Byrne et al., 2002; Chen et al., 2005; Pedroso et al., 2010, Duniere 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 because of the high buffering capacity of alfalfa which decreases the rate of pH decline during ensiling.

This 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. This suggests that inoculant treatment is needed to eliminate EC in contaminated forage that is ensiled for relatively short periods. This was likely caused by the 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 jenzenii* 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 (Duniere et al., 2011; Pedroso et al., 2010).

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 this 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 3-3).

The inhibitory effect of the inoculants on EC may have been partly because of 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 because of their antibacterial effects on EC. This is evident from the inhibition zones caused by pure cultures of the inoculants on the Kirby bauer disks coated with EC. These inhibitory effects may have been caused by the 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 because of
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 Post-Ensiling 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 this study, inoculation with *L. buchneri* resulted in higher concentration of acetic acid and about 1000-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, 2006a).

Propionic acid has the greatest antimycotic characteristics of all the short chain fatty acids (Woolford, 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 this 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 caused by 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 this 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 this 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, Duniere et al. (2011) showed that E. coli O26 was not detected in corn silages when contamination occurred at silo opening because of 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_{10} 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 Control, 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 because the 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.
Table 3-1. 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.

<table>
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<th>Item</th>
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<td>EC+LP</td>
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<tr>
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<td>6.20&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>56.3</td>
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<td>22.9</td>
<td>23.6</td>
<td>23.1</td>
</tr>
<tr>
<td>NDF</td>
<td>36.4</td>
<td>35.8</td>
<td>37.0</td>
</tr>
<tr>
<td>ADF</td>
<td>29.3</td>
<td>28.3</td>
<td>28.9</td>
</tr>
</tbody>
</table>

<sup>ab</sup>Means within a row with different superscripts differ (*P* < 0.05).

¹Control = distilled water; EC = 1 × 10<sup>5</sup> cfu/g of *E. coli* O157:H7; LP = 1 × 10<sup>6</sup> cfu/g of *L. plantarum*; LB = 1 × 10<sup>6</sup> cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid
Table 3-2. 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.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EC</td>
<td>EC+LP</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>4.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.82&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.57&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.95&lt;sup&gt;cb&lt;/sup&gt;</td>
<td>0.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate: acetate</td>
<td>2.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.83&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yeast</td>
<td>5.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aerobic stability (h)</td>
<td>166</td>
<td>160</td>
<td>&gt;168</td>
</tr>
<tr>
<td>DM (%)</td>
<td>54.5</td>
<td>54.1</td>
<td>54.7</td>
</tr>
<tr>
<td>NDF</td>
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<td>42.1</td>
<td>40.5</td>
</tr>
<tr>
<td>ADF</td>
<td>32.6</td>
<td>34.4</td>
<td>32.2</td>
</tr>
<tr>
<td>CP</td>
<td>24.3</td>
<td>23.1</td>
<td>23.6</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>0.34</td>
<td>0.42</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<sup>a-c</sup>Means within a row with different superscripts differ (*P* < 0.05).

<sup>1</sup>Control = distilled water; EC = 1 × 10⁵ cfu/g of *E. coli* O157:H7; LP = 1 × 10⁶ cfu/g of *L. plantarum*; LB = 1 × 10⁶ cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid.
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 = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; LP = $1 \times 10^6$ cfu/g of *L. plantarum*; LB = $1 \times 10^6$ cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid; Treatment × day $P$-value = 0.01; *Concentrations differed at the day of ensiling specified ($P < 0.05$). Error bars represent SE.
Figure 3-2. 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 = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; LP = $1 \times 10^6$ cfu/g of *L. plantarum*; LB = $1 \times 10^6$ cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid; Treatment × day P-value = 0.01; *Concentrations differed at the day of ensiling specified (P < 0.05). Error bars represent SE.
Figure 3-3. 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 = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; LP = $1 \times 10^6$ cfu/g of *L. plantarum*; LB = $1 \times 10^6$ cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid; Treatment × day *P*-value = 0.01; *Concentrations differed at the day of ensiling specified (*P* < 0.05). Error bars represent SE.
Figure 3-4. *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 *L. plantarum*; LB = 1 × 10^6 cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid; Treatment × day P-value = 0.01; *Concentrations differed at the day of ensiling specified (P < 0.05). Error bars represent SE.
Figure 3-5. Effect of reinoculation with $1 \times 10^5$ 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 = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; LP = $1 \times 10^6$ cfu/g of *L. plantarum*; LB = $1 \times 10^6$ cfu/g of *L. 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.
Figure 3-6. Effect of reinoculation with $1 \times 10^5$ 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 = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; LP = $1 \times 10^6$ cfu/g of *L. plantarum*; LB = $1 \times 10^6$ cfu/g of *L. buchneri*; PA = 2.2 g/kg of propionic acid; Bars with different letters differed ($P < 0.05$); Treatment SE = 0.29
CHAPTER 4
BACTERIAL DIVERSITY AND COMPOSITION OF ALFALFA SILAGE AS ANALYZED BY ILLUMINA MISEQ SEQUENCING: EFFECTS OF E. coli O157:H7 AND SILAGE ADDITIVES

Background

Ensiling is a forage preservation method based on lactic acid fermentation that occurs spontaneously under anaerobic conditions because of activities of a complex community of forage epiphytic microbes, which are highly variable in composition and species (Eikmeyer et al., 2013; Muck, 2013). The low pH achieved during ensiling, caused by the production of lactic acid and other organic acids, inhibits or prevents the growth of spoilage and pathogenic organisms (Woolford, 1975). Microbial inoculants, such as homofermentative and heterofermentative bacteria, are added to silage to dominate the natural population of bacteria in the forage and influence the dynamics of the microbial population during ensiling (Kung, 1998; Wang et al., 2006). Homofermentative bacteria are mainly used to improve silage preservation by accelerating lactic acid fermentation and causing a rapid drop in pH (Kristensen et al., 2010; Muck, 2010; Nkosi et al., 2010). Heterofermentative bacteria produce high concentrations of acetic or propionic acid, thereby inhibiting the growth of spoilage yeasts and molds and thus, improving the shelf life of silage during the feedout stage (Filya, 2003; Kleinschmit and Kung, 2006b; Queiroz et al., 2013). Chemical additives, such as propionic acid, are added mainly as antifungal agents to enhance the shelf life of silages during the feedout stage (Kung et al., 1998, 2000).

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

Forages and silages may be contaminated with various micro-organisms, including pathogenic Escherichia coli when manure and irrigation slurry are applied as fertilizers (Beuchat and Ryu, 1997). Dairy manure can contain up to 8 log cfu/g of E. coli (Walczak and Xu, 2011) and counts of more than 2 log cfu/g have been detected on forages and silages (Chen et al., 2005; Ogunade et al., 2016). Effective control measures are needed to inhibit the growth of the pathogen and prevent its cycling on farms or entry into the food chain. The first objective was to examine effects of adding E. coli O157:H7 with or without chemical or microbial additives on the bacterial diversity and composition of alfalfa silage. The second objective was to examine
associations between the abundance of known and unknown bacterial species and indices of silage fermentation quality.

**Materials and Methods**

**Metagenomic DNA Extraction and Purification**

Approximately 100 g of each silage sample from Chapter 3 was added to 200 mL of PBS + 0.01% Tween-80 in a sterile stomacher bag. The mixture was sonicated for 30 min using Sonicor SC-150 (Sonicor Instrument Corporation, Copiague, NY). The wash was centrifuged at 9000 × g for 1 h to create a pellet from which DNA was extracted using a PowerLyzer PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Bead beating (Bullet Blender Storm 24, Next Advance, Averill Park, NY) was used for homogenizing the suspension and for mechanical disruption of bacterial cells. The purity and concentration of gDNA were measured with a spectrophotometer (Nanodrop 115 1000, Thermo Fisher Scientific-Oxoid) and the Integrity of the DNA was verified by agarose (0.7%) gel electrophoresis.

**PCR Amplification of the V3 and V4 Hypervariable Regions of Bacterial 16S rRNA Genes**

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

**Sequencing and Diversity Analysis**

Overlapping reads were merged using fast length adjustment of short reads (Magoc and Salzberg, 2011). Pre-processing and clustering of sequences to identify OTUs was done using CD-HIT-OTU software (Li et al., 2012). Taxonomic composition of the samples from phylum to species was generated using QIIME UCLUST (Edgar 2010) against the Ribosomal Database Project Release 11. Alpha diversity (rarefaction curve for OTUs, Chao1, and Shannon-Weaver diversity indices) and beta diversity (unweighted principal coordinate analysis (PCoA) 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 with no Bonferroni correction was used to compare the within-treatment and between-treatment UniFrac distances, which represent phylogenetic distances based on the presence/absence of OTUs among samples (Park et al., 2016).

**Statistical Analysis**

The experiment was a completely randomized design with 5 treatments and 4 replicates per treatment. Continuous variables, such as relative abundance of bacteria, number of reads, and species richness and diversity were analyzed using the GLIMMIX procedure of SAS. The model included the treatment effect and means were separated using the PDIF procedure of SAS, which is based on the Fisher’s F-protected least significant difference test. Significant differences were declared at $P \leq 0.05$. Pearson’s correlation coefficients were generated using the R software (http://www.r-project.org). Significant correlations were declared at $P \leq 0.10$. 
Results

Fermentation quality indices, counts of \textit{E. coli} O157:H7, and chemical composition of the silages at silo opening (d 100) were reported previously in Chapter 3. Briefly, the pH was lower ($P < 0.05$) in EC+LP and EC+LB silages (4.57 and 4.64, respectively) than in the Control and EC silages (4.88 and 4.82, respectively), and the EC+PA silage had a similar ($P > 0.10$) pH to inoculated silages. Compared to the Control, silages treated with EC+LP and EC+LB had greater ($P < 0.05$) concentrations of lactate and acetate, respectively. Propionate was only detected in EC+PA silage. \textit{Escherichia coli} O157:H7 was eliminated from the silages once the pH dropped below 5.0. This occurred earlier (16 vs. 100 d of ensiling) in EC+LP and EC+LB silages because they produced more lactate and acetate throughout the fermentation than other silages.

Sequencing Results and Bacterial Diversity

Sequencing of the V3 and V4 regions of the bacterial 16S rRNA gene of the 20 samples yielded 1,702,729 reads with an average of 85,136 ± 4522 reads per sample after quality filtering. Rarefaction curves plateaued in all the samples sequenced (figure not shown), indicating that the number of reads was sufficient for identifying OTU. The average Good’s coverage for all the samples was greater than 99%, indicating that the depth of sequencing was adequate for reliable analysis of the bacterial community. Compared to the others, silages treated with microbial inoculants had lower ($P < 0.05$) Shannon index values (Figure 4-1A), which measures species diversity based on the number and evenness of species. The Chao1 index, a measure of species richness based on the number of rare species was unaffected ($P > 0.10$) by treatment (Figure 4-1B). The unweighted PCoA UniFrac plot revealed compositional differences in the bacterial community of the 20 samples (Figure 4-2). The Control samples seemed to cluster apart from most other samples, except one EC sample, and the Monte Carlo test revealed
that the between-treatment variation (0.12) was greater \( P < 0.01 \) than that (0.07) of within treatment.

**Comparisons at the Phylum Level**

The microbial community structure of alfalfa silage was represented by 5 phyla (Table 4-1). Phylum Firmicutes \( (73.3 \pm 6.05) \) was more abundant than others, followed by Proteobacteria \( (20.4 \pm 3.80) \). Compared to the Control, EC treatment did not affect the abundance and composition of bacteria at the phylum level. The abundance of Firmicutes in the Control and EC+LB silages \( (79.5 \text{ and } 77.4\%, \text{ respectively}) \) was greater \( P < 0.05 \) than that in the EC+LP or EC+PA silages \( (69.2 \text{ or } 68.8\%, \text{ respectively}) \), whereas, that of Proteobacteria was greater in the EC+LP and EC+PA silages \( (24.0 \text{ and } 24.9\%, \text{ respectively}) \) than in the Control, EC and EC+LB \( (16.5, 18.0, \text{ and } 18.5\% \text{ respectively}) \) silages. The abundance of Bacteroidetes, Actinobacteria and Cyanobacteria were similar for all treatments \( P > 0.10 \).

**Comparisons at the Family Level**

Treatment effects on the percentage distribution of the 13 most abundant families that represented 91.7% of the silage bacterial community are shown in Table 4-2. Lactobacillaceae was the most abundant family followed by Leuconostocaceae. Compared to the Control, EC treatment did not affect the abundance of bacteria at the family level, but treatment with EC+LP and EC+LB increased \( P < 0.05 \) the abundance of Lactobacillaceae and reduced \( P < 0.05 \) those of Leuconostocaceae and Enterococccaceae. Compared to the Control, EC+PA did not affect \( P > 0.10 \) the abundance of Lactobacillaceae but decreased \( P = 0.01 \) those of Leuconostocaceae and Enterococccaceae. Among the families belonging to phylum Proteobacteria, the abundance of Methyllobacteriaceae was less \( P = 0.01 \) in EC+LP and EC+LB silages whereas those of Enterobacteriaceae, Pseudomonadaceae and Xanthomonadaceae were greater \( P < 0.05 \) in EC+LP and EC+PA silages, compared with the Control. Also, the
abundance of Sphingomonadaceae was greater ($P = 0.02$) in EC+LP, EC+LB and EC+PA silages relative to the Control.

**Comparisons at the Genus Level**

Treatment effects on the percentage distribution of the 16 most abundant genera, accounting for approximately 90.3% of the silage bacterial community are shown in Table 4-3. *Pediococcus* and *Weissella* were the most abundant genera in the Control silage. Compared to the Control, EC treatment did not affect the abundance of bacteria at the genus level. Treatment with EC+LP resulted in the greatest ($P = 0.01$) abundance of *Lactobacillus* (62.6%), followed by EC+LB (47.8%), whereas Control, EC and EC+PA silages had similar ($P > 0.50$) and smaller ($P < 0.50$) values than EC+LB silage (16.7, 16.8, and 8.98%, respectively). Relative to the Control, EC+LP silage had lower ($P < 0.05$) abundance of *Pediococcus* (29.9 vs. 0.55%), *Weissella* (27.7 vs. 3.89%) and *Methylobacterium* (1.50 vs. 0.60%), and increased ($P < 0.05$) abundance of *Sphingomonas* (1.86 vs. 2.93%), *Pantoea* (4.14 vs. 8.28%), *Pseudomonas* (1.26 vs. 2.44%) and *Erwinia* (0.35 vs. 0.57%); whereas EC+LB silage contained less ($P < 0.05$) abundance of *Weissella* (29.7 vs. 9.19%), and *Methylobacterium* (1.50 vs. 0.94%) relative to the Control. Compared to the Control, treatment with EC+PA resulted in greater ($P < 0.05$) abundance of *Sphingomonas* (1.86 vs. 3.61%), *Pantoea* (4.14 vs. 7.21%), *Pseudomonas* (1.26 vs. 2.75%), and *Erwinia* (0.35 vs. 0.63%) but EC+PA did not affect the abundance of three most abundant genera; *Lactobacillus*, *Weissella* and *Pediococcus*.

**Comparisons at the Species Level**

The relative abundance of bacterial species that accounted for more than 0.1% of the bacterial community is shown in Table 4-4. Treatment effects on the abundance of the bacterial species that were affected by treatments are shown in Table 4-5. Unknown *Pediococcus* and *Weissella* species were the most abundant species in Control silages and the abundance of
bacterial species was similar \( (P > 0.10) \) in Control and EC silages. The abundance of \textit{L. plantarum} and \textit{L. buchneri} were greatest \( (P = 0.01) \) in EC+LP and EC+LB silages, respectively. Relative to the Control, the abundance of \textit{L. acidipiscis}, \textit{Weissella koreensis}, \textit{Methylobacterium gossypiicola}, \textit{M. populi} and unknown \textit{Lactobacillus} and \textit{Weissella} species, were reduced \( (P < 0.05) \), whereas that of unknown \textit{Pseudomonas} species was increased in EC+LP and EC+LB silages. Furthermore, EC+LP silage contained greater \( (P < 0.05) \) abundance of \textit{Pseudomonas fulva} and unknown \textit{Rhodococcus} and \textit{Sphingomonas} species, and less \( (P < 0.05) \) abundance of an unknown \textit{Pediococcus} species compared to the Control. The EC+PA silage contained lower \( (P < 0.05) \) abundance of \textit{L. acidipiscis}, unknown \textit{Lactobacillus} and \textit{Weissella} species, \textit{W. koreensis}, and \textit{M. populi}, and increased abundance of an unknown \textit{Sphingomonas} and \textit{Pseudomonas} species compared to the Control.

**Correlations between Abundance of Bacteria and Fermentation Quality Indices**

The correlations between the abundance of bacterial genera and silage fermentation indices are presented in Table 4-6. Lactate concentration correlated positively with the abundance of \textit{Lactobacillus} \( (r = 0.56, P = 0.01) \), and negatively with the abundance of \textit{Pediococcus} \( (r = -0.41, P = 0.08) \). Abundance of genera \textit{Sphingomonas} \( (r = -0.51) \), \textit{Pantoea} \( (r = -0.46) \), \textit{Pseudomonas} \( (r = -0.45) \), and \textit{Stenotrophomonas} \( (r = -0.38) \) were negatively correlated \( (P < 0.10) \) with ammonia-N concentration. Silage pH was negatively correlated \( (P < 0.10) \) with the abundance of \textit{Lactobacillus} \( (r = -0.59) \), \textit{Sphingomonas} \( (r = -0.66) \), \textit{Pantoea} \( (r = -0.69) \), \textit{Pseudomonas} \( (r = -0.69) \), and \textit{Stenotrophomonas} \( (r = -0.50) \), and positively correlated \( (P < 0.10) \) with the abundance of \textit{Pediococcus} \( (r = 0.44) \), \textit{Methylobacterium} \( (r = 0.53) \), \textit{Weissella} \( (r = 0.60) \), and \textit{Erwinia} \( (r = 0.40) \). Abundance of \textit{Sphingomonas} \( (r = -0.49) \), \textit{Pantoea} \( (r = -0.48) \), \textit{Pseudomonas} \( (r = -0.45) \), and \textit{Stenotrophomonas} \( (r = -0.37) \) were negatively correlated \( (P < 0.10) \) with yeast and mold counts.
Correlations between the abundance of known and unknown bacterial species and silage fermentation quality indices are presented in Tables 4-7 and 4-8, respectively. As expected, the abundance of *L. buchneri* was positively correlated (r = 0.42, P = 0.06) with acetate concentration and negatively (r = -0.41, P = 0.06) correlated with yeast and mold counts. The abundance of *L. plantarum* and *Pseudomonas fulva* were positively correlated with lactate concentration (P ≤ 0.10) and negatively correlated with silage pH (P ≤ 0.10). The abundance of *L. acidipiscis* (r = 0.52), *Methylobacterium gossypiicola* (r = 0.48), *M. populi* (r = 0.50) and *Weissella koreensis* (r = 0.43) were positively correlated (P ≤ 0.10) with silage pH. The abundance of *L. acidipiscis* (r = 0.41), *M. populi* (r = 0.50), and *W. koreensis* (r = 0.43) were positively correlated (P ≤ 0.10) with yeast and mold counts. Acetate concentration was positively correlated (r = 0.51, P = 0.02) with an unknown species and genus in family Xanthomonadaceae. Lactate concentration was negatively correlated (P ≤ 0.10) with abundance of unknown species belonging to genera *Rathayibacter* (r = -0.37) and *Pediococcus* (r = 0.51), and positively correlated (P ≤ 0.10) with those of unknown species belonging to genera *Rhodococcus* (r = 0.43) and *Pseudomonas* (r = 0.53), and an unknown genus in family Xanthomonadaceae (r = 0.39). Ammonia-N concentration was negatively correlated (P ≤ 0.10) with abundance of unknown species belonging to genera *Salana* (r = -0.47), *Frigoribacterium* (r = -0.41), *Pseudorhodobacter* (r = -0.44), *Pantoea* (r = -0.45), *Stenotrophomonas* (r = -0.44), *Rhodococcus* (r = -0.40), *Pseudomonas* (r = -0.40) and *Sphingomonas* (r = -0.51). Abundance of unknown species belonging to genera *Saguibacter* (r = -0.38), *Pseudorhodobacter* (r = -0.44), *Pantoea* (r = -0.79), *Stenotrophomonas* (r = -0.72), *Rhodococcus* (r = -0.39), *Sphingomonas* (r = -0.68) and *Pseudomonas* (r = -0.78) were negatively correlated (P ≤ 0.10) with pH. Whereas, unknown species belonging to *Pediococcus* (r = 0.40), *Erwinia* (r = 0.48), *Weissella* (r = 0.60) and
Lactobacillus (r = 0.53) had positive correlations (P ≤ 0.10) with pH. Abundance of unknown species belonging to family Xanthomonadaceae and genera Stenotrophomonas and Pantoea were negatively correlated (P ≤ 0.10) with yeast and mold counts.

**Discussion**

This experiment was conducted as part of a broader study aimed at understanding the probiotic and food safety-enhancing effects of lactic acid bacterial silage inoculants. Silages were inoculated with E. coli O157:H7 to monitor its fate during ensiling of alfalfa that had been ensiled with or without additives. The pathogen was not detected in any silage at silo opening because of the inhibitory effect of the low pH resulting from silage acidification (Chapter 3).

**Bacterial Diversity and Composition**

This study revealed the abundance and diversity of bacteria in alfalfa silage treated with EC with or without microbial or chemical additives. The Illumina Miseq sequencing platform used for the analysis is a powerful tool for studying phylogeny and taxonomy of complex microbial communities of environmental samples (Gloor et al., 2010; Degnan and Ochman, 2012).

It is well known that majority of the bacterial community involved in lactic acid fermentation in silage belong to phylum Firmicutes and to the genera Lactobacillus, Pedicoccus, Lactococcus, Weissella and Leuconostoc (Pahlow et al., 2003; Pang et al., 2011). Our study revealed that approximately 74.1 ± 3.28% of the alfalfa silage bacterial community belong to phylum Firmicutes, consisting mainly of families Lactobacillaceae and Leuconostocaceae, and genera Lactobacillus, Pedicoccus, and Weissella. Approximately 20.4 ± 1.65% belonged to phylum Proteobacteria, consisting mainly of families Enterobacteriaceae, Xanthomonadaceae, Sphingomonadaceae and Aurantimonadaceae, and genera Sphingomonas, Pantoea, Pseudomonas and Stenotrophomonas. Bao et al. (2016) assessed the bacterial community of
alfalfa silage via single molecule real-time sequencing technology and reported that majority of the species detected belong to genera *Lactobacillus, Weissella, Pediococcus* and *Pantoea*. In a similar study, McGarvey et al. (2013) monitored the bacterial population before and after ensiling of alfalfa forage and found that the abundance of Proteobacteria reduced from 89.6 to 26.9%, while that of Firmicutes increased from 8.1 to 70.6% after 40 days of ensiling, indicating that silage fermentation involves a shift in the bacterial community from Proteobacteria to Firmicutes.

The fact that the bacterial diversity and taxonomic composition of the EC silage did not differ from that of the Control silage suggests that the microbiome shifts in the additive-treated silages reflect the effects of the respective additives alone. Inoculation with microbial inoculants has been employed for several years to enhance fermentation of silage (Stokes 1992; Whiter and Kung, 2001). Microbial inoculants are added to dominate and control microbial events during silage fermentation (Kung, 1998; Wang et al., 2006). Our results revealed that silage inoculation with *L. plantarum* or *L. buchneri* increased the abundance of the respective bacteria, thereby, reducing the diversity of the bacterial community (Polley et al., 2007; Allen et al., 2009). Similar results were obtained by Eikmeyer et al. (2013) and Ogiy et al. (2015), who reported dominance of inoculated *L. buchneri* and *L. plantarum* in grass and wheat silages, respectively. However, in some cases, inoculated strains may be outcompeted by the indigenous microbial population (Stevenson et al., 2006).

Silage inoculation with *L. plantarum* reduced the abundance of *Weissella* and *Pediococcus* after 100 d of ensiling probably because of the faster rate of pH decline during ensiling and lower pH at silo opening (Ogunade et al., 2016). Most species of *Weissella* are obligate heterofermentative bacteria that produce lactate and acetate as major end products of
sugar metabolism (Graf et al., 2016). *Weissella* species are considered early colonizers (Dellaglio and Torriani, 1986) as they are outcompeted by acid-tolerant *Lactobacillus* species because of the pH drop as fermentation progresses (Graf et al., 2016). That the abundance of *L. buchneri* and species of *Weissella*, both of which are obligate heterofermenters, were reduced in EC+LP silage partly explain greater lactate to acetate ratio usually observed in alfalfa silage treated with *L. plantarum* compared with the untreated silage (Whiter and Kung, 2001; Kizilsimsek et al., 2007; Ogunade et al., 2016).

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

*Lactobacillus buchneri* is primarily added to silage to ferment lactate to acetate and 1,2-propanediol (Driehuis et al., 2001). As a potent ant mycotic agent, acetate inhibits the growth of spoilage yeasts, thereby, improving the aerobic stability of silage (Danner et al., 2003; Tabacco et al., 2009). Treatment with *L. buchneri* improved the aerobic stability of corn, sorghum, wheat,
barley, and alfalfa silages (Kung and Ranjit 2001; Weinberg et al. 2002; Kung et al., 2003). However, acetic acid production has often been associated with DM loss in silage (Wilkinson and Davies, 2012). In this study, irrespective of treatment applied, the alfalfa silage was stable for the entire duration of the aerobic phase (168 h; Ogunade et al., 2016), however DM loss was not measured. Like *L. plantarum*, silage treatment with *L. buchneri* increased the abundance of *Lactobacillus* and reduced that of *Weissella*, but had no effect on abundance of *Pediococcus*. In a similar study, Eikmeyer et al. (2013) studied the taxonomic profiles of ensiling microbial communities of grass silage inoculated with *L. buchneri*. The authors observed greater abundance of *Lactobacillus* genus and lower abundance of *Weissella* genus relative to the untreated silage.

Chemical additives, such as formic acid, propionic acid-based products, and formaldehyde are added as antifungal agents to improve the aerobic shelf life of silage by reducing the population of yeasts and molds, which cause aerobic deterioration (Kung et al., 1998, 2000). As expected, propionic acid treatment had no effects on the fermentation. In addition, it did not affect bacterial diversity or abundance of *Lactobacillus, Weissella* and *Pediococcus*, the three most abundant genera responsible for driving silage fermentation in this study. However, treatment with propionic acid increased the abundance of bacteria belonging to genera *Pantoea, Pseudomonas* and *Sphingomonas* and *Erwinia*.

**Correlations between Bacterial Abundance and Fermentation Quality Indices**

In support of previous findings (Whiter and Kung, 2001; Kizilsimsek et al., 2007), abundance of *L. plantarum* was positively (*r* = 0.57) and negatively correlated (*r* = -0.49) with lactate and pH, respectively. Abundance of bacteria belonging to genera *Pantoea, Pseudomonas* and *Sphingomonas* were greater in EC+LP versus Control silages and their abundance correlated negatively with pH (*r* = -0.69, -0.69, and -0.66, respectively) and ammonia-N concentration (*r* = -
0.46, -0.45, and -0.51, respectively). High concentrations of ammonia-N in silage is usually indicative of protein breakdown, and is typically caused by extensive plant protease activity and/or clostridial fermentation (Kung and Shaver, 2001). Tao et al. (2012) showed that plant proteolytic enzymes in alfalfa silage are active at pH 4.0 to 8.0, however, most of them had higher activities at pH 5.0 to 6.0. The rate of pH decline during ensiling of alfalfa is slow because of the high buffering capacity and low water-soluble carbohydrate content in this forage (Kung et al., 2003; Muck, 1998). Consequently, alfalfa is particularly susceptible to extensive proteolysis and clostridial fermentation (Kung et al., 2003). True protein degradation in alfalfa silage has been successfully reduced by *L. plantarum* inoculation because of the rapid drop in pH which inhibits proteolytic bacteria (Weinberg and Muck, 1996; Filya et al., 2007). While *L. plantarum* inoculation may play a major role in true protein preservation in alfalfa, our results suggest that *Pantoea*, *Pseudomonas* and *Sphingomonas* may also contribute to the effect because their abundances were negatively correlated with ammonia-N concentration. Studies on waste water treatment revealed greater abundance of bacterial species belonging to genera *Pseudomonas* and *Sphingomonas* towards the onset of nitrification (Bal-Krishna et al., 2013), a microbiological process whereby ammonia is oxidized to nitrite and then, to nitrate (Bal-Krishna et al., 2013); however, silage studies have not involved these bacteria. Fitzgerald et al. (2015) monitored ammonia-oxidizing microbial communities in reactors under low dissolved oxygen conditions and reported that some species of *Pseudomonas*, *Rhodococcus*, and *Sphingomonas* are involved in nitrification. Therefore, the negative correlations between *Pseudomonas* and *Sphingomonas*, and ammonia-N concentration may be partly due to nitrification. However, previous studies have not shown that nitrification by these bacteria can occur under anaerobic conditions, and in this study, nitrate-N concentrations were well below the detection limit of 300
mg/kg or the recommended safe level in ruminant diets (< 1000 mg/kg nitrate-N, Rasby, 1988), possibly because of the low abundances of the bacteria. Therefore, more research is needed to elucidate the role of these bacteria in reducing silage ammonia-N concentrations.

The negative correlations of these same bacteria (Sphingomonas, Pantoea, and Pseudomonas) with yeast and mold counts may reflect the inhibitory effect of propionic acid on yeast and mold growth (Kung et al., 1998, 2000). Notably, compared to that for Control silage, these bacteria were also more abundant ($P \leq 0.05$) in EC+PA silage which also had the lowest ($P = 0.01$) yeast and mold counts (Ogunade et al., 2016).

* Methylobacterium* and *Erwinia* were among the dominant genera in pre-ensiled alfalfa forage (McGarvey et al., 2013; Ni et al., 2016). However, in agreement with our results, their abundance was low (approximately 1%) after ensiling in the study of Ni et al. (2016). Species of *Methylobacterium* are strictly aerobic and neutrophilic (Doronina et al., 2002; Knief et al., 2012), supporting their positive correlation ($r = 0.53$) with pH and reduced ($P \leq 0.05$) abundance in silages treated with the microbial additives in this study. Certain species of *Erwinia* do not grow below pH 5.0 (Shrestha et al., 2005), supporting their low abundance in the silage and the positive correlation ($r = 0.40$) with pH. Although, the abundance of *Erwinia* was increased ($P \leq 0.05$) by EC+LP and EC+PA treatments, but this difference may not have practical significance because of their low abundance (< 0.6%) and the small differences among the treatments.

Using 16S gene sequences for taxonomic assignment of lactobacilli to the species level may be misleading because of the relatively little sequence diversity among lactobacilli (Berthier and Elrhich, 1998; Felis and Dellaglio, 2007). Therefore, the species-level taxonomic assignment in these and similar studies should be cautiously interpreted.
Bacterial Candidates for Future Research

Silage inoculation with *L. plantarum* increased the abundance of the added *L. plantarum* and that of *Pseudomonas fulva* by approximately 750 and 524%, respectively, relative to the Control. Both *L. plantarum* and *P. fulva* had strong positive correlations with lactate concentration. *Pseudomonas fulva* belongs to the *Pseudomonas putida* group (Anzai et al., 2000), which, like *L. plantarum*, possesses lactate dehydrogenase (Nelson et al., 2002), a key enzyme for homolactic fermentation (Gaspar et al., 2014). While *L. plantarum* has been extensively studied for its role in silage homolactic fermentation, *P. fulva* has not, possibly because of the associated public health and safety concerns. Human infections with *P. fulva* have been documented in previous studies (Almuzara et al., 2010; Seok et al., 2010) and the bacterium has caused meningitis in a young girl (Almuzara et al., 2010) and bacteremia in a 56-year-old man (Seok et al., 2010), but it is susceptible to a wide range of antibiotics (Liu et al., 2014).

Adequate control measures and strict regulations will be required for evaluation or use of this bacterium as a silage inoculant because of the possible health and safety risks.

This study shows perhaps for the first time the associations of some unknown silage bacteria and key silage quality indices. For instance, an unknown bacterial species belonging to family Xanthomonadaceae was positively correlated with lactate and acetate concentrations and negatively correlated with yeast and mold counts. Furthermore, unknown bacterial species belonging to genera *Rhodococcus* and *Pseudomonas* had strong positive correlations with lactate concentration. In addition, unknown species belonging to genera *Salana, Frigoribacterium, Stenotrophomonas, Pantoea* and *Pseudorhodobacter* had strong negative correlations with ammonia-N concentration. These unknown bacteria should be identified, cultured and investigated further to determine their roles in silage fermentation. To our knowledge, no member of the aforementioned genera and/or families has been evaluated as a microbial additive.
Further studies on these bacterial species are needed as at least some of them may be candidates for development into new silage inoculants that can improve the preservation of forages.

**Conclusions**

This study revealed that 74.1 ± 3.28% and 20.4 ± 1.65% of the alfalfa silage bacterial community belong to phylum Firmicutes and Proteobacteria, respectively. *Lactobacillus, Weissella* and *Pediococcus* were the most abundant genera detected. Relative to the Control, adding *E. coli* O157:H7 alone at ensiling did not affect the bacterial community composition of the silage. The abundance of Firmicutes was reduced (115 and 116%) and that of Proteobacteria was increased (145 and 151%) by EC+LP and EC+PA treatments, respectively. Treatment with EC+LP resulted in the greatest abundance of *Lactobacillus*, but reduced those of *Pediococcus, Weissella*, and *Methylobacterium*. Abundance of *Weissella* and *Methylobacterium* were also reduced in the EC+LB silage compared to the Control. Application of propionic acid did not affect the abundance of *Lactobacillus, Weissella* and *Pediococcus*. Unknown bacterial species that were positively correlated with lactate and acetate concentrations, and negatively correlated with ammonia-N concentration were detected. These unknown bacteria should be speciated and examined for their respective potential roles in fermentation and true protein preservation in alfalfa silage.
Table 4-1. Relative abundance (%) of phyla isolated from alfalfa silage treated without (Control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of two bacterial inoculants or propionic acid and ensiled for 100 d.

<table>
<thead>
<tr>
<th></th>
<th>Treatment†</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EC</td>
<td>EC+LP</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>1.52</td>
<td>2.27</td>
<td>2.03</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>1.76</td>
<td>2.81</td>
<td>3.67</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.79</td>
<td>1.33</td>
<td>1.12</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>79.5a</td>
<td>75.6ab</td>
<td>69.2b</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>16.5b</td>
<td>18.0b</td>
<td>24.0a</td>
</tr>
</tbody>
</table>

†Means within a row with different superscripts differ (P < 0.05).

Control = distilled water; EC = 1 × 10⁵ cfu/g of *E. coli* O157:H7; EC+LP = EC + 1 × 10⁶ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1 × 10⁶ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); EC+PA = EC + 2.2 g/kg of propionic acid.
Table 4-2. Relative abundance (%) of the 13 most abundant families isolated from alfalfa silage treated without (Control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of two bacterial inoculants or propionic acid and ensiled for 100 d.

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

<sup>a</sup>-<sup>d</sup>Means within a row with different superscripts differ (*P* < 0.05).

<sup>1</sup>Control = distilled water; EC = 1 × 10<sup>5</sup> cfu/g of *E. coli* O157:H7; EC+LP = EC + 1 × 10<sup>6</sup> cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1 × 10<sup>6</sup> cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); EC+PA = EC + 2.2 g/kg of propionic acid.
Table 4-3. Relative abundance (%) of 13 most abundant genera isolated from alfalfa silage treated without (Control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of two bacterial inoculants or propionic acid and ensiled for 100 d.

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

<sup>a-d</sup>Means within a row with different superscripts differ (*P* < 0.05).

<sup>1</sup>Control = distilled water; EC = 1 × 10<sup>5</sup> cfu/g of *E. coli* O157:H7; EC+LP = EC + 1 × 10<sup>6</sup> cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + 1 × 10<sup>6</sup> cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid.
Table 4-4. Relative abundance of bacterial species isolated from alfalfa silage treated without (Control) or with *Escherichia coli* O157:H7 (EC) alone, or EC and either of two bacterial inoculants or propionic acid and ensiled for 100 d.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Average abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salana</em></td>
<td>Unknown</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td><em>Frigoribacterium</em></td>
<td>Unknown</td>
<td>0.36 ± 0.13</td>
</tr>
<tr>
<td><em>Rathayibacter</em></td>
<td>Unknown</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td><em>Sanguibacter</em></td>
<td>Unknown</td>
<td>0.38 ± 0.17</td>
</tr>
<tr>
<td><em>Sphingobacterium</em></td>
<td>Unknown</td>
<td>2.43 ± 1.65</td>
</tr>
<tr>
<td><em>Exiguobacterium</em> acetylicum</td>
<td>Unknown</td>
<td>0.31 ± 0.21</td>
</tr>
<tr>
<td><em>Kroppenstedtia</em></td>
<td>Unknown</td>
<td>0.36 ± 0.70</td>
</tr>
<tr>
<td><em>Pediococcus</em></td>
<td>Unknown</td>
<td>23.0 ± 14.2</td>
</tr>
<tr>
<td><em>Brevundimonas</em></td>
<td>Unknown</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td><em>Aureimonas</em></td>
<td>Unknown</td>
<td>2.04 ± 0.49</td>
</tr>
<tr>
<td><em>Devosia</em></td>
<td>Unknown</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>Unknown</td>
<td>1.66 ± 0.31</td>
</tr>
<tr>
<td><em>Rhodobacteteraceae</em></td>
<td>Unknown</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td><em>Pseudorhodobacter</em></td>
<td>Unknown</td>
<td>0.36 ± 0.10</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>Unknown</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td><em>Erwinia</em></td>
<td>Unknown</td>
<td>0.47 ± 0.16</td>
</tr>
<tr>
<td><em>Pantoea</em></td>
<td>Unknown</td>
<td>5.81 ± 2.02</td>
</tr>
<tr>
<td><em>Xanthomonadaceae</em></td>
<td>Unknown</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td><em>Pseudoxanthomonas</em></td>
<td>Unknown</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td><em>Stenotrophomonas</em></td>
<td>Unknown</td>
<td>2.91 ± 0.75</td>
</tr>
<tr>
<td><em>Lactobacillus</em> diolivorans</td>
<td>Unknown</td>
<td>10.8 ± 18.9</td>
</tr>
<tr>
<td><em>Lactobacillus</em> plantarum</td>
<td>Unknown</td>
<td>16.0 ± 24.1</td>
</tr>
<tr>
<td><em>Lactobacillus</em> acidipiscis</td>
<td>Unknown</td>
<td>0.31 ± 0.53</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>Unknown</td>
<td>1.73 ± 2.06</td>
</tr>
<tr>
<td><em>Rhodococcus</em></td>
<td>Unknown</td>
<td>0.34 ± 0.20</td>
</tr>
</tbody>
</table>
Table 4-4. Continued.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Average abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylobacterium</em></td>
<td>gossipiicola</td>
<td>0.92 ± 0.35</td>
</tr>
<tr>
<td><em>Weissella</em></td>
<td>Unknown</td>
<td>17.4 ± 9.96</td>
</tr>
<tr>
<td><em>Weissella</em></td>
<td>Koreensis</td>
<td>0.81 ± 0.87</td>
</tr>
<tr>
<td><em>Methylobacterium</em></td>
<td>Populi</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>Unknown</td>
<td>2.52 ± 0.80</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Unknown</td>
<td>3.26 ± 1.67</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Fulva</td>
<td>0.39 ± 0.36</td>
</tr>
</tbody>
</table>

*Unknown genus in the indicated family*
Table 4-5. Relative abundance (%) of bacterial species isolated from alfalfa silage treated without (Control) or with Escherichia coli O157:H7 (EC) alone, or EC and either of two bacterial inoculants or propionic acid and ensiled for 100 d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>EC</th>
<th>EC+LP</th>
<th>EC+LB</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus buchneri</td>
<td>1.84(^{b})</td>
<td>2.24(^{b})</td>
<td>0.19(^{b})</td>
<td>46.8(^{a})</td>
<td>3.19(^{b})</td>
<td>2.27</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>8.03(^{b})</td>
<td>10.8(^{b})</td>
<td>61.8(^{a})</td>
<td>0.47(^{c})</td>
<td>5.14(^{b})</td>
<td>3.09</td>
</tr>
<tr>
<td>Lactobacillus acidipiscis</td>
<td>1.13(^{a})</td>
<td>1.21(^{a})</td>
<td>0.00(^{b})</td>
<td>0.01(^{b})</td>
<td>0.22(^{b})</td>
<td>0.26</td>
</tr>
<tr>
<td>Lactobacillus*</td>
<td>4.59(^{a})</td>
<td>2.58(^{ab})</td>
<td>0.63(^{bc})</td>
<td>0.46(^{c})</td>
<td>0.42(^{c})</td>
<td>0.95</td>
</tr>
<tr>
<td>Rhodococcus*</td>
<td>0.23(^{b})</td>
<td>0.40(^{ab})</td>
<td>0.55(^{a})</td>
<td>0.26(^{b})</td>
<td>0.31(^{b})</td>
<td>0.09</td>
</tr>
<tr>
<td>Methylobacterium gossypiicola</td>
<td>1.27(^{a})</td>
<td>1.44(^{a})</td>
<td>0.51(^{c})</td>
<td>0.75(^{bc})</td>
<td>1.11(^{ab})</td>
<td>0.17</td>
</tr>
<tr>
<td>Weissella*</td>
<td>27.8(^{a})</td>
<td>23.4(^{ab})</td>
<td>3.77(^{d})</td>
<td>8.89(^{c})</td>
<td>23.1(^{b})</td>
<td>2.19</td>
</tr>
<tr>
<td>Weissella koreensis</td>
<td>1.89(^{a})</td>
<td>1.41(^{a})</td>
<td>0.12(^{b})</td>
<td>0.30(^{b})</td>
<td>0.32(^{b})</td>
<td>0.39</td>
</tr>
<tr>
<td>Methylobacterium populi</td>
<td>0.35(^{a})</td>
<td>0.35(^{a})</td>
<td>0.16(^{b})</td>
<td>0.21(^{b})</td>
<td>0.21(^{b})</td>
<td>0.03</td>
</tr>
<tr>
<td>Sphingomonas*</td>
<td>1.71(^{c})</td>
<td>2.08(^{bc})</td>
<td>2.78(^{ab})</td>
<td>2.58(^{abc})</td>
<td>3.43(^{a})</td>
<td>0.42</td>
</tr>
<tr>
<td>Pseudomonas*</td>
<td>0.06(^{c})</td>
<td>0.09(^{c})</td>
<td>0.41(^{ab})</td>
<td>0.23(^{b})</td>
<td>0.16(^{bc})</td>
<td>0.06</td>
</tr>
<tr>
<td>Pseudomonas fulva</td>
<td>0.17(^{b})</td>
<td>0.27(^{b})</td>
<td>0.89(^{a})</td>
<td>0.37(^{b})</td>
<td>0.31(^{b})</td>
<td>0.20</td>
</tr>
<tr>
<td>Pseudomonas*</td>
<td>1.04(^{b})</td>
<td>0.93(^{b})</td>
<td>1.14(^{b})</td>
<td>1.40(^{b})</td>
<td>2.28(^{a})</td>
<td>0.33</td>
</tr>
</tbody>
</table>

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

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

*Unknown species in the indicated genus
Table 4-6. Pearson’s correlation between abundance of bacterial genus and fermentation indices of alfalfa forage ensiled for 100 d.

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

*Significant (P ≤ 0.10) Pearson’s correlation coefficient
<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Acetate (% DM)</th>
<th>Lactate (% DM)</th>
<th>Ammonia-N (% DM)</th>
<th>pH</th>
<th>Yeast and mold (log cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus buchneri</em></td>
<td>0.42*</td>
<td>-0.02</td>
<td>-0.03</td>
<td>-0.18</td>
<td>-0.41*</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>-0.24</td>
<td>0.57*</td>
<td>-0.23</td>
<td>-0.49*</td>
<td>0.19</td>
</tr>
<tr>
<td><em>Lactobacillus acidipiscis</em></td>
<td>0.06</td>
<td>-0.06</td>
<td>0.01</td>
<td>0.52*</td>
<td>0.41*</td>
</tr>
<tr>
<td><em>Methylobacterium gossypiicola</em></td>
<td>0.07</td>
<td>-0.28</td>
<td>-0.07</td>
<td>0.48*</td>
<td>0.29</td>
</tr>
<tr>
<td><em>Methylobacterium populi</em></td>
<td>0.01</td>
<td>-0.14</td>
<td>0.15</td>
<td>0.63*</td>
<td>0.50*</td>
</tr>
<tr>
<td><em>Weissella koreensis</em></td>
<td>-0.05</td>
<td>-0.04</td>
<td>-0.19</td>
<td>0.46*</td>
<td>0.43*</td>
</tr>
<tr>
<td><em>Pseudomonas fulva</em></td>
<td>-0.05</td>
<td>0.54*</td>
<td>-0.32</td>
<td>-0.58*</td>
<td>-0.14*</td>
</tr>
<tr>
<td><em>Exiguobacterium acetylicum</em></td>
<td>-0.16</td>
<td>-0.11</td>
<td>-0.18</td>
<td>0.02</td>
<td>-0.21</td>
</tr>
</tbody>
</table>

*Significant (P ≤ 0.10) Pearson’s correlation coefficient
Table 4-8. Pearson’s correlation between abundance of unknown bacterial species and fermentation indices of alfalfa forage ensiled for 100 d.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Acetate (% DM)</th>
<th>Lactate (% DM)</th>
<th>Ammonia-N (% DM)</th>
<th>pH</th>
<th>Yeast and mold (log cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salana</em></td>
<td>-0.14</td>
<td>0.03</td>
<td>-0.47*</td>
<td>-0.15</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Frigoribacterium</em></td>
<td>-0.23</td>
<td>-0.06</td>
<td>-0.41*</td>
<td>-0.11</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Rathayibacter</em></td>
<td>-0.2</td>
<td>-0.37*</td>
<td>-0.26</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td><em>Sanguibacter</em></td>
<td>-0.31</td>
<td>0.23</td>
<td>-0.12</td>
<td>-0.38*</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Sphingobacterium</em></td>
<td>-0.02</td>
<td>0.12</td>
<td>-0.29</td>
<td>-0.32</td>
<td>-0.27</td>
</tr>
<tr>
<td><em>Kroppenstedtia</em></td>
<td>-0.18</td>
<td>-0.05</td>
<td>0.61*</td>
<td>0.33</td>
<td>0.09</td>
</tr>
<tr>
<td><em>Pediococcus</em></td>
<td>-0.01</td>
<td>-0.51*</td>
<td>0.35</td>
<td>0.40*</td>
<td>-0.22</td>
</tr>
<tr>
<td><em>Brevundimonas</em></td>
<td>-0.01</td>
<td>-0.33</td>
<td>-0.16</td>
<td>0.11</td>
<td>-0.16</td>
</tr>
<tr>
<td><em>Aureimonas</em></td>
<td>-0.12</td>
<td>-0.15</td>
<td>-0.16</td>
<td>-0.03</td>
<td>0.15</td>
</tr>
<tr>
<td><em>Devosia</em></td>
<td>-0.33</td>
<td>-0.25</td>
<td>0.14</td>
<td>0.34</td>
<td>0.28</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>-0.20</td>
<td>-0.25</td>
<td>0.05</td>
<td>0.11</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Rhodobacteteraceae</em></td>
<td>-0.04</td>
<td>-0.09</td>
<td>-0.09</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td><em>Pseudorhodobacter</em></td>
<td>-0.17</td>
<td>0.01</td>
<td>-0.44*</td>
<td>-0.38*</td>
<td>-0.09</td>
</tr>
<tr>
<td><em>Erwinia</em></td>
<td>0.08</td>
<td>-0.05</td>
<td>-0.03</td>
<td>0.48*</td>
<td>-0.14</td>
</tr>
<tr>
<td><em>Pantoaea</em></td>
<td>0.03</td>
<td>0.20</td>
<td>-0.45*</td>
<td>-0.79*</td>
<td>-0.48*</td>
</tr>
<tr>
<td><em>Xanthomonadaceae</em></td>
<td>0.51*</td>
<td>0.39*</td>
<td>-0.50*</td>
<td>-0.59</td>
<td>-0.46*</td>
</tr>
<tr>
<td><em>Pseudoxanthomonas</em></td>
<td>0.03</td>
<td>-0.20</td>
<td>-0.17</td>
<td>0.09</td>
<td>-0.17</td>
</tr>
<tr>
<td><em>Stenotrophomonas</em></td>
<td>0.28</td>
<td>0.25</td>
<td>-0.44*</td>
<td>-0.72*</td>
<td>-0.68*</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>-0.01</td>
<td>0.01</td>
<td>-0.15</td>
<td>0.53*</td>
<td>0.22</td>
</tr>
<tr>
<td><em>Rhodococcus</em></td>
<td>-0.18</td>
<td>0.43*</td>
<td>-0.40*</td>
<td>-0.39*</td>
<td>0.29</td>
</tr>
<tr>
<td><em>Weissella</em></td>
<td>0.05</td>
<td>-0.31</td>
<td>0.07</td>
<td>0.60*</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>0.30</td>
<td>0.23</td>
<td>-0.51*</td>
<td>-0.68*</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>0.15</td>
<td>0.53*</td>
<td>-0.40*</td>
<td>-0.78*</td>
<td>-0.11</td>
</tr>
</tbody>
</table>

1Unknown species in this genus
*Unknown genus in the indicated family
*Significant ($P \leq 0.10$) Pearson’s correlation coefficient
Figure 4-1. A. Estimate of alpha diversity. Treatment $P$-value = 0.01. B. Species richness calculated as the Chao1 index, Treatment $P$-value = 0.51. Bars with different letters differed ($P < 0.05$); Control = distilled water; EC = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; EC+LP = EC + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid. Error bars represent SE.
Figure 4-2. Unweighted UniFrac Principal Coordinate Analysis (PCoA) plot of individual samples in each treatment. This PCoA plot indicates the phylogenetic distance (variation) between samples using two principal coordinates (PC1 and PC2). The percentage variation explained by each PC is indicated on each axis. Control = distilled water; EC = $1 \times 10^5$ cfu/g of *E. coli* O157:H7; EC+LP = EC + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); EC+LB = EC + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = EC + 2.2 g/kg of propionic acid.
CHAPTER 5
FATE OF *Escherichia coli* O157:H7 AND BACTERIAL DIVERSITY IN CORN SILAGE CONTAMINATED WITH THE PATHOGEN AND TREATED WITH MICROBIAL AND CHEMICAL ADDITIVES

**Background**

*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 US (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 US and it can be contaminated with EC 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). Whereas, in silages contaminated with the pathogen after aerobic exposure, maintenance of a low pH (below 4.0) because of 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 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 or 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 because of 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 either been based on culture-based techniques (Ercolini, 2004), real time PCR (Stevenson et al., 2006), or characterization of denaturing gradient gel electrophoresis (DGGE) 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 (Degnan 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 (Degnan 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 (IACUC) 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) $1 \times 10^5$ cfu/g *Escherichia coli* O157:H7 EDL933 alone (ECCH); (3) ECCH + $1 \times 10^6$ cfu/g *Lactobacillus plantarum* R2014 (ECLP); (4) ECCH + $1 \times 10^6$ cfu/g *Lactobacillus buchneri* R1102 (ECLB); (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, Florida), the silage inoculant bacteria were...
supplied by Lallemand Animal Nutrition (Milwaukee, WI), and the propionic acid was purchased from Sigma-Aldrich (St. Louis, MO). Bacterial counts and their viability in the inoculants were verified before silage inoculation by plating on de Man-Rogosa-Sharp (MRS) agar (Difco Laboratories, Inc., Detroit, MI, 288210), 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-Aldrich, St. Louis, MO, 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, St. Louis, MO, 83339) supplemented with cefixime (0.05 mg/L, Sigma-Aldrich, St. Louis, MO, 18588) and potassium tellurite (2.5 mg/L, Sigma-Aldrich, St. Louis, MO, 60539) 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. Before inoculation, the EC suspension was re-plated 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 hand-held 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 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 × 10⁵ 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 re-inoculated with 5 × 10⁵ 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, 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 *E. coli* O157:H7 and confirmed by latex agglutination (Thermo Fisher Scientific-Oxoid Ltd., Basingstoke, UK, 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 × 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 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 × 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). 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 the 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 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 gDNA were measured with a spectrophotometer.
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, 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 performed by Macrogen Inc. (Seoul, Korea) using the Illumina MiSeq platform (Illumina, San Diego).

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 × day interaction. When treatment × 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 (OTUs) (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 OTUs, Chao1, and Shannon indices) and beta diversity (unweighted principal coordinate analysis (PCoA) 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. 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 $35.2 \pm 0.44$ (%, $P = 0.75$), $8.54 \pm 0.23$ (% DM, $P = 0.16$), $41.7 \pm 0.78$ (% DM, $P = 0.68$) and $25.1 \pm 0.31$ (% DM, $P = 0.98$) (Table 5-1). 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 5-1), such that the pH at final silo opening (120 d) was similar for all silages (Table 5-2). *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 silages (Figure 5-2), 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 5-3), however, at final silo opening (d 120), its lactate concentration was similar to those of Control, ECCH and ECA silages (Table 5-2). 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 5-4; Table 5-2). 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 (Table 5-2). 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 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 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 5-5). 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 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 5-6).
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 plateaued in all samples sequenced, indicating that the number of reads used in analysis was sufficient in identifying OTU (figure not shown). The unweighted PCoA UniFrac plot showed that compositional differences (P = 0.05) existed among the bacterial communities of the different silages (Figure 5-7), and the Monte Carlo test revealed that the between-treatment variation (0.19) in the unweighted UniFrac distance was greater (P < 0.001) than the within-treatment distance (0.09).

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 5-3). The Chao1 index, a measure of the richness of the bacterial species based on number of rare species, was unaffected by treatment (Table 5-3).

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 5-3). 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, whose 16S genes have much homology to those of Cyanobacteria (Hanshew et al., 2013).

At the genus level, six genera (*Lactobacillus*, *Sphingobacterium*, *Weissella*, *Acinetobacter*, *Stenotrophomonas*, *Serratia*) accounted for approximately 99% of the bacterial community (Table 5-3). 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 *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 *L. diolivorans* (96.8%) and *L. plantarum* (0.98%) when compared to the ECLB (93.8 and 0.35%, respectively) and control silages (94.3 and 0.52%, 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 because of 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 (Chapter 3; Bach et al., 2002; Chen et al., 2005). While 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 because of 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 because of inhibitory effect of the low pH. Nevertheless, the pKa (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). Gollop et al. (2005) reported 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 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 (Table 5-1).

Addition of *L. buchneri* to silage enhances anaerobic degradation of lactate to acetate (Oude Elferink et al., 2001; Kleinschmit and Kung, 2006a; 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). Also, the fact that no butyrate was detected supports the deduction that spoilage because of clostridial fermentation was not evident.

**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 EC 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 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 because of
degradation of lactic acid by lactate-utilizing yeasts in the presence of air (Pahlow et al., 2003; Kung, 2010). A meta-analysis showed that \textit{L. buchneri} inoculation decreased yeast counts and improved aerobic stability of corn silages (Kleinschmit and Kung, 2006a). 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).

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 because of their higher 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 (Chapter 3).

**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 OTUs 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 to the Control silage. Studies that utilized denaturing gradient gel electrophoresis (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 taxonomic classification of lactobacilli to the species level with 16S gene sequences may sometimes misrepresent the microbial population because of 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 *W. confusa* as the dominant bacterial species in corn silage, *L. plantarum* as the dominant species in sorghum and rice silages, and *L. pseudomesenteroides, L. paraplanatarum, 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 *L. reuteri* and *L. 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. Both 1,2-propanediol and 1-propanol were not measured in this study and propionate was only detected in ECA silage because of 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 (PacBio) single molecule, real-time sequencing technology (SMRT) 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 ± 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 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 acetate as well as other antifungal compounds, such as 3-hydroxy fatty acids and phenyllactate, by *W. paramesenteroides* isolated from fermented cassava (*Manihot esculenta*). 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. 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. 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, non-fermenting 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 (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 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, 2006a). 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 shows 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 $1 \times 10^5$ 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. Re-inoculation 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 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*. 
Table 5-1. Characteristics of whole-plant corn forage inoculated with *Escherichia coli* O157:H7 (EC) alone or EC and bacterial inoculants or propionic acid before ensiling.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Treatment(^1)</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.94(^a)</td>
<td>5.99(^a)</td>
<td>5.93(^a)</td>
<td>5.93(^a)</td>
</tr>
<tr>
<td>DM (%)</td>
<td>34.7</td>
<td>35.7</td>
<td>35.5</td>
<td>35.4</td>
</tr>
<tr>
<td>CP (% of DM)</td>
<td>8.75</td>
<td>8.77</td>
<td>8.37</td>
<td>8.26</td>
</tr>
<tr>
<td>NDF (% of DM)</td>
<td>41.6</td>
<td>41.4</td>
<td>40.6</td>
<td>42.5</td>
</tr>
<tr>
<td>ADF (% of DM)</td>
<td>24.9</td>
<td>24.8</td>
<td>25.5</td>
<td>25.4</td>
</tr>
</tbody>
</table>

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

\(^1\)Control = distilled water; ECCH = \(1 \times 10^5\) cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainseville, FL); ECLP = ECCH + \(1 \times 10^6\) cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + \(1 \times 10^6\) cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO).
Table 5-2. Fermentation indices and chemical composition 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.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment 1</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ECCH</td>
<td>ECLP</td>
</tr>
<tr>
<td>pH</td>
<td>3.93</td>
<td>3.91</td>
<td>3.89</td>
</tr>
<tr>
<td>Lactate (% of DM)</td>
<td>2.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate (% of DM)</td>
<td>2.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionate (% of DM)</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yeasts and molds</td>
<td>5.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAB&lt;sup&gt;2&lt;/sup&gt; (log cfu/g)</td>
<td>7.06</td>
<td>7.41</td>
<td>7.79</td>
</tr>
<tr>
<td>DM (%)</td>
<td>30.3</td>
<td>30.2</td>
<td>30.6</td>
</tr>
<tr>
<td>NDF (% of DM)</td>
<td>42.5</td>
<td>42.3</td>
<td>43.3</td>
</tr>
<tr>
<td>ADF (% of DM)</td>
<td>28.4</td>
<td>28.0</td>
<td>28.6</td>
</tr>
<tr>
<td>CP (% of DM)</td>
<td>9.58</td>
<td>9.11</td>
<td>9.23</td>
</tr>
<tr>
<td>Ammonia-N (% of DM)</td>
<td>0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ammonia-N (% of total N)</td>
<td>6.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup>Means within a row with different superscripts differ (*P* < 0.05).

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

<sup>2</sup>Lactic acid bacteria
Table 5-3. Bacterial community composition and diversity of whole-plant corn silage inoculated with *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid.

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

<sup>a</sup>-<sup>d</sup>Means within a row with different superscripts differ (*P* < 0.05).

<sup>1</sup>Control = distilled water; ECCH = 1 × 10<sup>5</sup> cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + 1 × 10<sup>6</sup> cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + 1 × 10<sup>6</sup> cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Values are averages of 4 replicates of each treatment.
Figure 5-1. Changes in the pH of whole-plant corn forage inoculated with $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment × 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.
Figure 5-2. Changes in *Escherichia coli* O157:H7 (EC) counts of whole-plant corn forage inoculated with $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment × 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.
Figure 5-3. Changes in lactate concentration of whole-plant corn forage inoculated with $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). Treatment x 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 5-4. Changes in acetate concentration of whole-plant corn forage inoculated with $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for different durations. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). 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 5-5. *Escherichia coli* O157:H7 (EC) counts (log$_{10}$ cfu/g) and pH of whole-plant corn silage (d 128) re-inoculated with $5 \times 10^5$ cfu/g of EC 168 h after silo opening. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). 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 5-6. The aerobic stability (h) of whole-plant corn forage inoculated with $1 \times 10^5$ cfu/g of *Escherichia coli* O157:H7 (EC) alone or with bacterial inoculants or propionic acid and ensiled for 120 d. Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). 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 5-7. Unweighted UniFrac principal coordinate analysis (PCoA) plot of individual samples in each treatment. Individual samples were represented with blue (Control), red (ECCH), orange (ECLP), purple (ECLB), and yellow (ECA). Control = distilled water; ECCH = $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (Emerging Pathogen Institute, Gainesville, FL); ECLP = ECCH + $1 \times 10^6$ cfu/g of *L. plantarum* R2014 (Lallemand Animal Nutrition, Milwaukee, WI); ECLB = ECCH + $1 \times 10^6$ cfu/g of *L. buchneri* R1102 (Lallemand Animal Nutrition, Milwaukee, WI); ECA = ECCH + 2.2 g/kg of propionic acid (Sigma-Aldrich, St. Louis, MO). The PCoA plot indicates the phylogenetic distance (variation) between samples using two principal coordinates (PC1 and PC2). The percentage variation explained by each PC is indicated on each axis.
CHAPTER 6
EFFECTS OF THREE SEQUESTERING AGENTS ON MILK AFLATOXIN M₁
CONCENTRATION AND THE PERFORMANCE AND IMMUNE STATUS OF DAIRY
COWS FED DIETS ARTIFICIALLY CONTAMINATED WITH AFLATOXIN B₁

Background

Mycotoxins are a group of toxic compounds produced by fungi on growing forages or stored feeds that can reduce the feed intake, growth, health, and reproductive performance of livestock and humans. Aflatoxin B₁ has been the most widely studied and problematic mycotoxin in dairy cows because its metabolite, AFM₁, can be transferred to milk (Diaz et al., 2004). Up to 166,000 cases of hepatocellular carcinoma per year in humans have been attributed to aflatoxins (Liu and Wu, 2010). The United States FDA regulates the maximum permissible amount of AFM₁ in milk at 0.5 μg/kg and AFB₁ in dairy feeds at 20 μg/kg because of the appreciable consumption of milk by humans, particularly infants (FDA, 2000). The maximum milk AFM₁ concentration allowed by the European Commission is 0.05 μg/kg (EFSA, 2004).

Various approaches have been employed to reduce the risk of aflatoxin ingestion by livestock and the resultant aflatoxicoses. Ozonization and ammoniation can minimize the production and effects of the toxins in corn and cottonseed meal (CAST, 2003), but these approaches are costly and time-consuming (Kutz et al., 2009), so they are not typically used on dairy farms. One of the more recent approaches is to use adsorbents that bind aflatoxin in the gastrointestinal tract and reduce its bioavailability (Huwig et al., 2001, Phillips et al., 2002). Clay-based aflatoxin-sequestering agents have shown the potential to bind aflatoxins and impair their gastrointestinal absorption (Kabak et al., 2006), but high doses may reduce utilization of vitamins and minerals (Chestnut et al., 1992). The outcomes of studies testing the effectiveness of adsorbents at reducing the transfer of the toxins to milk have been inconsistent (Kabak et al., 2006). Diaz et al. (2004) reported that 3 sodium bentonite products reduced the concentration of
AFM$_1$ residues in milk by 50 to 65%. Another study (Stroud, 2006) compared 8 binding agents, including 3 combinations of yeast and clay products and 5 clay products, and revealed that 1 of the former and 3 of the latter reduced the transfer, secretion, or concentration of AFM$_1$ in milk. A recent study by Xiong et al. (2015) showed that a product based on yeast and sodium montmorillonite reduced the transfer of aflatoxin to milk at a low dietary dose (20 μg/kg) but had no effect at a high dose (40 μg/kg). We have shown that a high dose (1% of diet DM), but not a low dose (0.2% of diet DM), of montmorillonite clay reduced the transmission of dietary AFM$_1$ to the milk of dairy cows (Queiroz et al., 2012). There is clearly a need to continue to evaluate the effects of aflatoxin-sequestering agents on milk AFM$_1$ concentration, as well as on milk yield and quality. The objective of this study was to examine whether dietary addition of 3 *Saccharomyces cerevisiae* fermentation product–based mycotoxin-sequestering agents would reduce milk AFM$_1$ concentration and improve the performance and health of dairy cattle that were fed diets artificially contaminated with AFB$_1$.

**Materials and Methods**

**Cows, Treatments, and Design**

Cows in this study were cared for according to the University of Florida Animal Research Committee protocols, which were developed from guidelines outlined in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). Fifteen lactating multiparous Holstein cows in mid lactation (150 to 200 DIM) were stratified by parity, DIM, and milk production, and were randomly assigned to 1 of 5 treatments arranged in an incomplete crossover design with four 28-d periods. At the end of each period, cows were switched to a different treatment in a way that ensured no cow was on the same treatment in more than 1 period. Cows were housed in a freestall open-sided barn bedded with sand and equipped with Calan gates (American Calan Inc., Northwood, NH) for individual feeding and with misters and
fans to minimize heat stress. The following dietary treatments were investigated: (1) a control diet with no sequestering agent or mycotoxin added (C); (2) diet C fed with 1,725 μg of AFB1 (T); (3) diet T fed with 20 g/d of sequestering agent 1 (SEQ1); (4) diet T fed with 20 g/d of sequestering agent 2 (SEQ2); (5) diet T fed with 20 g/d of sequestering agent 3 (SEQ3). The sequestering agents were supplied by Diamond V Inc. (Cedar Rapids, IA). They were proprietary formulations of an *S. cerevisiae* fermentation product containing a low (SEQ1) or high (SEQ2) dose of a proprietary chlorophyll-based additive or a low dose of the additive combined with sodium bentonite clay (SEQ3).

Diets were formulated to meet or exceed the nutrient requirements of cows producing at least 30 kg of milk (NRC, 2001) using CPM-Dairy software version 3.0.10 (www.cpmdairy.net). The ingredients and chemical composition of the basal diet are shown in Table 6-1. Cows were fed 1,725 μg/head per day of AFB1 based on an average projected DMI of 23 kg/d, to supply a daily dose of 75 μg/kg of AFB1 (in treatments T, SEQ1, SEQ2, SEQ3). Dietary AFB1 was obtained from an *Aspergillus parasiticus* (NRRL-2999) culture at the University of Missouri Diagnostic Laboratory (Columbia, MO). The AFB1 was mixed with ground corn and molasses, weighed into gelatin capsules and dosed orally to cows once daily before the TMR was fed in the morning; the sequestering agents (SEQ1, SEQ2, SEQ3) were top-dressed on the relevant TMR from d 1 to 28 of each period. The first 20 d of each period were used to ensure adaptation to a new diet and clearance of AFM1 residues from the milk from the preceding period. Mycotoxin-contaminated corn was fed only during d 21 to 25 of each period in the relevant treatments, and clearance of the toxin from the milk was monitored during d 26 to 28. Queiroz et al. (2012) confirmed the suitability of the 5-d toxin-dosing and 3-d toxin-clearance periods for modeling the effects of sequestering agents on ingested AFB1.
Experimental Measurements and Analytical Procedures

Cows were milked twice daily. Milk yield was recorded daily by the Afikim milking system (Afilab mini laboratory, Afimilk Ltd., Israel); milk samples were collected from both 1000 and 2200 h milkings on d 20 to 28 in each period and analyzed separately for milk components. Concentrations of milk fat, milk protein, and SCC were analyzed by the Southeast Milk dairy laboratory (Belleview, FL) using a Bentley 2000 near-infrared reflectance spectrophotometer (Bentley Instruments Inc., Chaska, MN). Values for 3.5% FCM yield were calculated based on the following equation: 
\[(0.4324 \times \text{milk yield}) + (16.218 \times \text{milk fat yield})\] (NRC, 2001). Milk AFM\textsubscript{1} concentrations were measured on composited am and pm milk samples on d 20 to 28 in each period and were quantified by the Southeast Milk dairy laboratory using the radioimmunoassay test (CHARM II, Charm Sciences Inc., Malden, MA) described by Diaz et al. (2004). Aflatoxin M\textsubscript{1} excretion was calculated as the product of milk AFM\textsubscript{1} concentration and milk yield. Transfer of the toxin to milk during the dosing period was calculated as daily AFM\textsubscript{1} excretion/ daily AFB\textsubscript{1} intake. Transfer of the toxin during the withdrawal period was calculated as daily AFM\textsubscript{1} excretion/AFB\textsubscript{1} intake on d 25. Aflatoxin M\textsubscript{1} clearance rates were calculated by dividing the difference between the AFM\textsubscript{1} concentration on d 26, 27, and 28 and that on d 25 by 24, 48, and 72 h, respectively.

The amount of feed offered at each feeding was recorded for each cow. Refused feed was collected and weighed each morning before feeding and used to calculate intakes for the next day. Diets were fed ad libitum (110% of the previous day’s DM intake). In each period, dietary ingredients (corn silage, ryegrass triticale silage mixture, alfalfa orchardgrass hay mixture, and concentrate mix) and orts were sampled (100 g) daily into labeled bags from d 20 to 28 and stored in a refrigerator (4°C) for further analysis. Dietary samples were dried at 55°C for 48 h in a forced-air oven, ground to pass the 1 mm screen of a Wiley mill (A. H. Thomas, Philadelphia,
PA), and composited across days. Feed samples were sent to the Dairy One forage testing laboratory (Ithaca, NY) for DM, N, ash, NDF, ADF, and mineral analyses. Nitrogen was determined by rapid combustion using a Macro Elemental N analyzer (AOAC International, 2000; Vario MAX CN, model ID 25.00–5003; Elementar, Hanau, Germany) and CP was calculated by multiplying the N value by 6.25. Concentrations of NDF and ADF were measured using the non-sequential method of Van Soest et al. (1991) in an Ankom A200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α-amylase was used in the NDF procedure, and the results are expressed exclusive of residual ash. Minerals (Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn) were analyzed using an inductively coupled plasma radical spectrometer (ICAP, Thermo Scientific Inc., Marietta, OH) after microwave digestion. The concentration of aflatoxins, deoxynivalenol, zearalenone, and T-2 toxin in the feed ingredients was analyzed by HPLC at the University of Missouri Veterinary Diagnostic Laboratory using the HPLC method described by Kutz et al. (2009). On d 20 and 25 of each period, blood was collected before the morning feeding from the coccygeal vessels into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin anticoagulant, centrifuged at 2,500 × g for 20 min at 4°C and stored at −20°C for further analysis of acute-phase proteins. Plasma haptoglobin concentrations were determined by measuring haptoglobin/hemoglobin complexing based on differences in peroxidase activity (Makimura and Suzuki, 1982). Plasma ceruloplasmin oxidase activity was measured using the colorimetric procedure described by Demetriou et al. (1974). Acid-soluble protein concentration was determined using the bicinchoninic acid kit for protein determination (Sigma-Aldrich, St. Louis, MO) following plasma acid-soluble protein extraction (Nakajima et al., 1982). Whole blood was analyzed for total and differential cell counts using an automated ProCyte Dx hematology analyzer (Idexx Laboratories Inc., Westbrook, ME). The percentage of
neutrophils exhibiting phagocytosis and oxidative burst activities after *Escherichia coli* challenge was measured according to procedures described by Martinez et al. (2012). Briefly, neutrophils were loaded with dihydrorhodamine 123 (Sigma-Aldrich Co., St. Louis, MO) and then cocultured with propidium iodide-labeled *E. coli* isolated from the milk of a cow with mastitis at a bacteria:neutrophil ratio of 40:1. The samples were run within 2 h of preparation using a Becton Dickinson Accuri C6 digital analyzer flow cytometer (Becton Dickinson). Neutrophils were identified according to their size and granularity using forward and side scatter detectors, and data were analyzed using Flowjo software (version 7.6.5., Tree Star Inc., Ashland, OR). The parameters quantified were mean fluorescence intensity (MFI) for propidium iodide staining as a proxy for the number of bacteria phagocytized per neutrophil, and MFI for dihydrorhodamine 123 staining as an indicator of the amount of reactive oxygen species generated per neutrophil. Adhesion molecules that regulate the migration of leukocytes between blood vessel and lymphoid organs, L-selectin (CD62L) and β2-integrin (CD18), were analyzed as described by Silvestre et al. (2011). Interferon-γ, the proinflammatory cytokine produced by various activated leukocyte populations, was measured as described by Garcia Orellana (2012) using the bovine IFN-γ DuoSet ELISA Development Kit (R&D Systems, Inc., Minneapolis, MN).

**Statistical Analysis**

The experiment had an incomplete crossover design with 12 experimental units per treatment. Data were analyzed using the GLIMMIX procedure of SAS Institute Inc. (2012). The model for analyzing outcomes measured repeatedly over time on the same experimental unit (milk production and components; DMI; and aflatoxin concentration, transfer, and excretion) included the effects of treatment, period, and day; interactions of treatment and day and day and period; and the random effect of cow. Time was used in the repeated measures statement with autoregressive order 1 [AR(1)] as the time-series covariance structure. We used pre-experimental
milk yields as covariates for milk yield data obtained during the predosing (d 16 to 20), toxin-dosing (d 21 to 25), and withdrawal (d 26 to 28) periods. The model for analyzing measures that were not collected serially on the same experimental unit (blood cell counts and immune response data) included the effects of treatment and period and the random effect of cow. Blood sample data from d 20 were used as covariates for analysis of data from samples collected on the last day of toxin-dosing (d 25). We estimated denominator degrees of freedom using the Kenward-Roger option in the MODEL statement for all data, and we corrected for potential carryover effects of treatments between periods by including carryover coefficients in the model (Littell et al., 2006; Jones and Kenward, 2015). We examined data for normality by examining the distribution of residuals and transforming them as needed before statistical analysis. Post hoc mean comparisons were performed using the Tukey-Kramer procedure for pairwise multiple comparisons. Significance was declared at $P \leq 0.05$, and trends toward significance were declared at $0.05 < P \leq 0.10$.

Results and Discussion

Milk AFM$_1$ Concentration

The amount of AFB$_1$ dosed (75 µg/kg) exceeded the FDA action level of 20 µg/kg for dairy cattle feeds and it was transferred into milk in sufficient quantities to raise the AFM$_1$ concentration beyond the FDA action level of 0.5 µg/kg for milk. However, the 75 µg/kg dosed represents a moderate natural level of AFB$_1$ contamination in corn samples for livestock feeds, which can be up to 158 µg/kg (Pietri et al., 2004), even though in other studies (Pietri et al., 2007), most of the samples had concentrations less than 20 µg/kg. Doses used in mycotoxin challenge studies usually range from 20 to 112 µg/kg (Diaz et al., 2004; Firmin et al., 2011; Xiong et al., 2015).
The sequestering agent mixtures used in this study were tested to explore if combining the aflatoxin-binding and immunomodulatory effects of *Saccharomyces cerevisiae*, and the aflatoxin-binding effects of clay and chlorophyll-based products would result in synergistic improvements in binding of aflatoxin, stimulation of the immune response, and enhancement of the performance of dairy cows. The protective effect of *Saccharomyces cerevisiae* against mycotoxins is due to adhesion of the toxin to the cell wall components (mannan and β-glucan) of the yeast (Pfliegler et al., 2015). In addition, yeast-based products act as immune modulators because β-glucan can stimulate non-specific and specific immunological responses, and thus improve the performance of animals (Keller et al., 2015). When fed at levels above 1% of the diet DM to dairy cows (Diaz et al., 2004; Queiroz et al., 2012), clays bind aflatoxins by chemisorption via cation exchange (Phillips et al., 1995). Chlorophyll-based compounds sequester aflatoxin and stimulate the immune response by trapping the toxin via the planar ring structure of the chlorophyll (Arimoto et al., 1993) and stimulating increased activity of glutathione S-transferase (GST) enzyme, which prevents aflatoxin epoxide from forming an adduct with DNA, thereby, facilitating the clearance of the toxin from the body (Hsu et al., 2008). None of the treatments reduced the milk aflatoxin concentration, suggesting that the ingredient amounts in the sequestering agent mixtures were inadequate for considerable toxin binding. Nevertheless, amounts were sufficient to modulate the immune response. SEQ1 had the greatest proportion of the *S. cerevisiae* fermentation product; its greater beneficial effects on indices of the immune response highlights the immunomodulatory effects of the *S. cerevisiae* fermentation product. The presence of clay in SEQ3 and not in SEQ2 probably reduced adverse effects of the toxin on the cows, such that they were able to mount a more pronounced immune response. That SEQ2, which had the greatest proportion of the chlorophyll-based
additive, had the least beneficial effect on the immune response, suggests that the additive may have been less effective at binding the aflatoxin or modulating the immune response than the *S. cerevisiae* fermentation product or the clay.

We detected no AFM$_1$ in the milk of the cows before they were dosed with AFB$_1$ (data not shown). During the toxin-dosing period, all cows except for those fed diet C, received 1725 µg of AFB$_1$ daily and their DMI averaged 23.6 kg/d. Therefore, the average daily ingestion of AFB$_1$ was 73 µg/kg of DMI. No AFM$_1$ was detected in the milk of cows fed diet C, whereas cows fed diets T, SEQ1, SEQ2 and SEQ3 had mean milk AFM$_1$ concentrations of 0.73, 0.74, 0.73 and 0.74 µg/kg, respectively (Table 6-2). Adding sequestering agents to diets containing the toxin did not affect milk AFM$_1$ concentration, excretion or transfer during the Toxin-dosing period. Therefore, none of the sequestering agent treatments prevented milk AFM$_1$ concentrations from rising beyond the FDA action level (0.50 µg/kg). This is consistent with other studies on the AFB$_1$-sequestering ability of yeast cell wall products that contain high levels of AFB$_1$ contamination. Milk AFM$_1$ concentration was unaffected by adding 10 g/day of an undisclosed yeast cell wall product or 125 g/d of a yeast cell wall-based product to dairy cow diets containing 100 (Waltman et al., 2008) or 112 (Kutz et al., 2009) µg/kg of AFB$_1$ respectively. Likewise, Kissell et al. (2013) showed that yeast oligosaccharide-based additives did not affect the milk AFM$_1$ concentration of cows fed diets containing 80 µg/kg AFB$_1$. In addition, Firmin et al. (2011) reported that adding an undisclosed yeast cell wall product at 2 g/kg of the diet did not affect the concentration of AFM$_1$ in the milk of dairy ewes fed a diet containing 60 µg of AFB$_1$/kg. However, yeast cell wall sequestering agents have effectively sequestered AFB$_1$ when the toxin concentration in the diet was lower (Diaz et al., 2004; Xiong et al., 2015). Diaz et al. (2004) reported that adding a yeast cell wall-based product at 10 g/d to a
dairy cow diet contaminated with 55 µg/kg of AFB\(_1\) reduced the milk AFM\(_1\) concentration. Certain combinations of yeast and clay-based products have also been less effective at high contamination levels. Xiong et al. (2015) reported that adding a blend of yeast extract and yeast culture, hydrated calcium sodium aluminosilicates, and other metabolites at 42.5 g/kg of DMI reduced milk AFM\(_1\) concentration, secretion and transfer in diets contaminated with 20 µg/kg of AFB\(_1\) but had no effect in diets contaminated with 40 µg/kg of AFB\(_1\). In general, the efficacy of clay or yeast cell wall products alone or combinations of both depend on the dose of the sequestering agent and the level of mycotoxin contamination in the diet.

Once AFB\(_1\) was withdrawn from the diet, milk AFM\(_1\) concentrations in cows fed the sequestering agents decreased rapidly, such that they fell below the FDA action level within 24 h (Table 6-2), whereas it took 48 h for milk AFM\(_1\) concentrations to decrease below the FDA action level in cows fed diet T. In particular, milk AFM\(_1\) concentrations in cows fed SEQ1 and SEQ3 were lower (\(P < 0.05\)) than those of cows fed T 24 h after withdrawing the toxin (Figure 6-1). This was because the SEQ1 and SEQ3 diets tended to reduce (\(P \leq 0.10\)) the transfer and excretion of the toxin from d 25 into the milk on d 26. The SEQ2 diet tended to reduce (\(P = 0.06\)) the milk AFM\(_1\) concentration compared to that for cows fed diet T but had no effect on the transfer and excretion of the toxin into the milk. Our previous study (Queiroz et al., 2012) showed that during the Toxin-dosing period, a diet containing a montmorillonite clay adsorbent (1% of the diet DM) reduced the milk AFM\(_1\) concentration below the FDA action level when cows were fed a diet contaminated with 75 µg/kg DMI of AFB\(_1\). However, the adsorbent had no effect when it was fed at 0.2% of the diet DM. The dose of clay fed in this current study (0.04% of DMI) was lower than in our previous study, but when combined with the \textit{S. cerevisiae}
fermentation product, it reduced the time required for a reduction in the milk AFM\textsubscript{1} concentration to a safe level by 24 h after dietary withdrawal of the toxin.

By 48 h after withdrawing AFB\textsubscript{1} from the diet, only trace concentrations of AFM\textsubscript{1} were detected in the milk in all treatments and no treatment effects ($P > 0.10$) on the concentration, transfer, or excretion of the toxin were evident. This is consistent with other studies, which reported that AFM\textsubscript{1} concentrations in milk were reduced drastically within 2 to 3 d of withdrawing AFB\textsubscript{1} from the diet (Kutz et al., 2009; Queiroz et al., 2012; Xiong et al., 2015). Clearance rates of milk AFM\textsubscript{1} did not differ among cows fed the toxin during any of the days of the Withdrawal period.

**Animal Performance**

During the predosing period, milk yield and DMI did not differ across treatments ($26.7 \pm 1.20$, $P = 0.64$ and $22.6 \pm 0.81$, $P = 0.30$ kg/d, respectively; Table 6-3). Therefore, feeding the sequestering agents during the adaptation or predosing periods did not affect the performance of the cows. During the toxin-dosing period, DMI was not affected by dietary treatment ($23.7 \pm 0.85$ kg/d, $P = 0.16$). Also, dietary treatments did not affect the change in milk yield from the predosing to the Toxin-dosing periods. However, feeding diet T instead of C during the toxin-dosing period tended to reduce milk yield (26.0 vs. 28.5 kg/d, $P = 0.08$) and 3.5% FCM yield (27.1 vs. 29.8 kg/d, $P = 0.09$). This is consistent with the study of Queiroz et al. (2012) who reported that feeding AFB\textsubscript{1} at the rate used in this study tended to reduce the 3.5% FCM yield of cows. A similar numerical trend was reported when 112 µg/kg of AFB\textsubscript{1} was fed to dairy cows by Kutz et al. (2009). However, studies have also reported no changes in milk yield due to feeding 117 µg/kg of DMI (Maki et al., 2016) or 80 µg of AFB\textsubscript{1}/kg of DMI (Kissell et al., 2013).

Feeding the SEQ3 diet instead of C reduced milk yield (24.8 vs. 28.5 kg/d, $P = 0.01$) and 3.5% FCM yield (25.9 vs. 29.8 kg/d, $P = 0.01$). Also, feeding the SEQ2 diet reduced 3.5% FCM
yield relative to feeding diet C (26.9 vs. 29.8 kg/d, \( P = 0.04 \)). The reasons for these reductions are not clear. In contrast, milk and FCM yields of cows fed the SEQ1 and C diets did not differ \((P > 0.10)\). Therefore, unlike the other sequestering agents, feeding the SEQ1 diet probably prevented the adverse effect of the toxin on milk production by the cows. This may be due to the numerically higher DMI for cows fed the SEQ1 diet relative to those fed SEQ2 and SEQ3. Feed efficiency was unaffected \((P > 0.10)\) by dietary treatments.

Compared with diet C, diet T tended to reduce the milk fat yield \((P = 0.10)\) and the SEQ3 diet reduced milk fat yield \((P = 0.01)\) and milk protein yield \((P = 0.01; \text{Table 6-3})\). These results reflect the reduction in milk yields caused by feeding the T and SEQ3 diets because milk fat and protein concentrations were not influenced by dietary treatment. The latter agrees with Smith et al. (1994), who did not detect changes in milk protein and fat percentages due to feeding 200 \( \mu \)g of AFB\(_1\)/kg of diet DM to goats. In addition, Kutz et al. (2009) reported that milk protein and fat percentages were unaffected when dairy cows were fed 112 \( \mu \)g of AFB\(_1\)/kg of diet DM.

No dietary treatment effects on performance measures were evident during the withdrawal period, except that the milk protein concentration of cows fed the SEQ1 diet was greater \((P = 0.04)\) than those of cows fed the SEQ2 diet, but none of the values differed from those of cows fed diet C (Table 6-3).

**Blood Parameters**

Compared to diet C, feeding diet T reduced the red blood cell count \((P = 0.05)\) and hemoglobin concentration \((P = 0.02; \text{Table 6-4})\). However, the blood cell counts were within the normal range for healthy adult dairy Holstein cows in mid-lactation (George et al., 2010). We found no previous studies on the effect of dietary AFB\(_1\) on the blood cell counts of dairy cows, but studies in poultry (Huff et al., 1986; Mohiuuddin et al., 1986; Basmacioglu et al., 2005) and rams (Dönmez et al., 2012) have shown that aflatoxin suppressed blood cell counts.
Expression of CD62L and CD18 tended to be the highest in cows fed the SEQ1 ($P = 0.10$) and SEQ3 ($P = 0.09$) diets (Table 6-5). Blood neutrophils act as the first line of defense against many pathogens once they have access to skin and mucosal surfaces, and migration of neutrophils involves adhesion mediated by selectins (Smolen et al., 2000). After this initial phase, the neutrophil is activated for adhesion of its integrins to the vascular endothelium (Silvestre et al., 2011). The greater MFI of staining for CD62L and CD18 on neutrophils of cows fed the SEQ1 and SEQ3 diets suggests a more prompt evasion of neutrophil cells from the vasculature to the tissue. Dietary treatments did not affect the production of Interferon-γ and percentage of cells exhibiting phagocytosis and oxidative burst activities (Table 6-5).

The production of proinflammatory cytokines is promoted by inflammasomes, which are a specialized intracellular multiprotein complex (Schroder and Tschopp, 2010). These are innate immune system receptors that trigger inflammation in response to infectious microbes and/or toxins (Guo et al., 2015). Inflammasomes are activated by the lethal anthrax toxin (Boyden and Dietrich, 2006), bacterial toxins such as Shiga toxin (Lee et al., 2015), and bacterial and viral pathogens (Guo et al., 2015). To our knowledge, the role of inflammasomes in mycotoxin-mediated inflammation is unknown. Future studies should examine the involvement of inflammasomes in mycotoxin-mediated responses in dairy cows. Acute-phase proteins are blood proteins that play major roles in systemic reaction to inflammation (Ceciliani et al., 2012), and their increased secretion is an indication of inflammatory stress (Bertoni et al., 2008; Gonzalez et al., 2008; Ceciliani et al., 2012). Haptoglobin and ceruloplasmin concentrations were not affected by dietary treatments (Table 6-5). However, acid-soluble protein concentrations were greater ($P = 0.01$) with diet T than with SEQ2, and tended to be greater with T than with SEQ1 ($P = 0.08$) and SEQ3 ($P = 0.10$). Increases of acid-soluble protein concentration in response to
inflammatory stress have been reported in goats (Gonzalez et al., 2008), sheep (Eckersall et al., 2007), pigs (Eckersall et al., 1996), and cows (Nagahata et al., 1989). The result in this study suggest that feeding the SEQ2 diet reduced the inflammatory response caused by diet T, and the SEQ1 and SEQ3 diets tended to have a similar effect. Queiroz et al. (2012) fed the same dose (75 μg/kg) of AFB1 as that in the current study to dairy cows and reported that haptoglobin and β-integrin concentrations were increased by the toxin. However, Xiong et al. (2015) reported that adding 20 or 40 μg/kg of AFB1 to the diet of dairy cows had no effect on the plasma concentrations of antibodies, IgG, IgM and IgA. The latter study may have been less successful at detecting the effects of AFB1 on measures of the immune response because they fed lower doses of AFB1 and examined different immune response indices. This study showed that the 3 sequestering agents altered the immune status of cows challenged with the toxin and reduced toxin-induced inflammatory stress.

**Conclusions**

Feeding 75 μg/kg of AFB1 increased AFM1 concentration in the milk of lactating dairy cows beyond the FDA action level of 0.5 μg/kg. Feeding the sequestering agents with the toxin did not prevent the milk AFM1 concentration from exceeding the FDA action level. However, within 24 h of withdrawing the toxin, diets that included the sequestering agents had reduced the milk AFM1 concentration below the FDA action level and diet T had not. Feeding AFB1 tended to reduce the yields of milk, 3.5% FCM, milk fat, and milk protein and reduced red blood cell count and hemoglobin concentration. Feeding the SEQ1 diet prevented the reduction in milk and 3.5% FCM yield caused by the toxin and increased the MFI of CD62L and CD18 relative to those for diets T and C. Feeding SEQ2 or SEQ3 did not prevent the adverse effect of the toxin on milk yield or 3.5% FCM yield. However, the SEQ2 diet reduced the acid-soluble protein concentration relative to that for cows fed diet T, and the SEQ3 diet increased the MFI of CD62L
and CD18 relative to those for cows fed diets SEQ2, T, or C. These results imply that the sequestering agents were not effective at preventing the toxin from being transferred to the milk, but they did reduce toxin-induced inflammatory stress in cows. In addition, SEQ1 reduced the adverse effects of the toxin on milk production. Future studies should validate the results of the current study and elucidate the relative efficacy and synergistic effects of bentonite clays, *S. cerevisiae* fermentation products, cell wall oligosaccharides, and chlorophyll-based additives at alleviating the toxic effects of aflatoxin on dairy cows.
<table>
<thead>
<tr>
<th>Ingredient composition (% of DM)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>23.5</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>6.9</td>
</tr>
<tr>
<td>Ryegrass/triticale mixture</td>
<td>8.9</td>
</tr>
<tr>
<td>Wet brewers grain</td>
<td>7.8</td>
</tr>
<tr>
<td>Corn grain</td>
<td>23.5</td>
</tr>
<tr>
<td>Dried citrus pulp</td>
<td>6.5</td>
</tr>
<tr>
<td>Soy hulls</td>
<td>5.9</td>
</tr>
<tr>
<td>Whole cottonseed</td>
<td>4.9</td>
</tr>
<tr>
<td>Soybean meal (47% CP)</td>
<td>5.9</td>
</tr>
<tr>
<td>Amino plus(^1)</td>
<td>2.9</td>
</tr>
<tr>
<td>Mineral and vitamin mix(^2)</td>
<td>3.3</td>
</tr>
</tbody>
</table>

### Chemical composition

- **Crude protein, %**: 16.0
- **ADF, %**: 20.9
- **NDF, %**: 34.2
- **NE\(_L\), Mcal/kg**: 1.66
- **Calcium, %**: 0.86
- **Phosphorous, %**: 0.50
- **Magnesium, %**: 0.70
- **Potassium, %**: 1.31
- **Sodium, %**: 0.38
- **Iron, mg/kg**: 406
- **Zinc, mg/kg**: 110
- **Copper, mg/kg**: 29
- **Manganese, mg/kg**: 98
- **Aflatoxins, µg/kg**: ND\(^3\)
- **Deoxynivalenol, mg/kg**: ND
- **T-2 toxin, mg/kg**: ND
- **Zearalenone, mg/kg**: ND

\(^1\)Aminoplus (Ag Processing Inc., Omaha, NE) composition (% DM): Lysine 3.5, Methionine 0.75, Arginine 3.85, Tryptophan 0.70, Histidine 1.47, Leucine 4.0, Isoleucine 2.43, Phenylalanine 2.64, Threonine 2.01, Valine 2.37, Tyrosine 1.69, Choline 0.29. \(^2\)Vitamin mineral mixture (% as is): ProVAAL-Lys+Met 30 (Perdue Agribusiness, Salisbury, MD), Sodium bicarbonate 28.5, DCAD Plus 13.0 (Soychlor), Calcium carbonate 9.0, Dicalcium phosphate 7.0, Magnesium oxide 7.0, Salt (NaCl) 3.5, Zinpro Availa-4 1.2 (Zinpro Corporation Eden Prairie, Mn), Sel-Plex1000 0.6 (Alltech, Nicholasville, KY), Rumensin90 0.22 (Elanco, Eli Lilly, Indianapolis, IN). 

\(^3\)Concentrations of respective toxins were below lower detection limits (5 µg/kg for aflatoxins, and 0.5 mg/kg for deoxynivalenol, T-2 toxin, and zearalenone).
Table 6-2. Effect of dosing aflatoxin B$_1$ (AFB$_1$) with or without sequestering agents$^1$ on the concentration, transfer and excretion of aflatoxin M$_1$ (AFM$_1$) in the milk of dairy cows during toxin-dosing and withdrawal periods.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment$^2$</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin-dosing period (d 21-25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg)</td>
<td>0.73</td>
<td>0.74</td>
<td>0.73</td>
</tr>
<tr>
<td>Excretion (µg/d)</td>
<td>19.45</td>
<td>19.62</td>
<td>19.15</td>
</tr>
<tr>
<td>Transfer (%)</td>
<td>1.13</td>
<td>1.14</td>
<td>1.11</td>
</tr>
<tr>
<td>Withdrawal period: first day (d 26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg)</td>
<td>0.63$^{a,x}$</td>
<td>0.43$^{b,y}$</td>
<td>0.46$^{ab,y}$</td>
</tr>
<tr>
<td>Excretion (µg/d)</td>
<td>15.87$^x$</td>
<td>11.14$^y$</td>
<td>12.78$^{xy}$</td>
</tr>
<tr>
<td>Transfer (%)</td>
<td>0.92$^x$</td>
<td>0.65$^y$</td>
<td>0.74$^{xy}$</td>
</tr>
<tr>
<td>Clearance (µg/h)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Withdrawal period: second day (d 27)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg)</td>
<td>0.06</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Excretion (µg/d)</td>
<td>1.25</td>
<td>1.02</td>
<td>1.75</td>
</tr>
<tr>
<td>Transfer (%)</td>
<td>0.07</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Clearance (µg/h)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Withdrawal period: third day (d 28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/kg)</td>
<td>ND$^3$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clearance (µg/h)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

$^{a-c}$Means within a row with no common superscript differ ($P \leq 0.05$).

$^{x,y}$Means within a row with no common superscript tend to differ ($P \leq 0.10$).

$^1$Proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc. Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

$^2$T = Control diet + AFB$_1$; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

$^3$ND = Not detected
Table 6-3. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents¹ on the performance of cows during Predosing (d 16 to 20), Toxin-dosing (d 21 to 25) and Withdrawal (d 26 to 28) periods.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment²</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>SEQ1</td>
</tr>
<tr>
<td>Predosing period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>23.4</td>
<td>22.7</td>
<td>22.4</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>27.6</td>
<td>26.6</td>
<td>26.5</td>
</tr>
<tr>
<td>Toxin-dosing period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>24.4</td>
<td>24.2</td>
<td>23.7</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>28.5a,x</td>
<td>26.0ab,y</td>
<td>26.7ab,xy</td>
</tr>
<tr>
<td>Milk yield change³ (kg/d)</td>
<td>0.8</td>
<td>-0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>3.5% FCM (kg/d)</td>
<td>29.8a,x</td>
<td>27.1ab,y</td>
<td>27.7ab,xy</td>
</tr>
<tr>
<td>Efficiency (3.5% FCM/kg DMI)</td>
<td>1.21</td>
<td>1.12</td>
<td>1.19</td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td>3.79</td>
<td>3.83</td>
<td>3.80</td>
</tr>
<tr>
<td>Milk fat (kg/d)</td>
<td>1.07a,x</td>
<td>0.99ab,y</td>
<td>1.00ab,xy</td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td>3.23</td>
<td>3.24</td>
<td>3.24</td>
</tr>
<tr>
<td>Milk protein (kg/d)</td>
<td>0.92a</td>
<td>0.84ab</td>
<td>0.85ab</td>
</tr>
<tr>
<td>SCC (×1000/mL)</td>
<td>221</td>
<td>169</td>
<td>209</td>
</tr>
<tr>
<td>Withdrawal period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>24.4</td>
<td>23.5</td>
<td>24.4</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>26.2</td>
<td>23.5</td>
<td>25.5</td>
</tr>
<tr>
<td>3.5% FCM (kg)</td>
<td>27.3</td>
<td>24.5</td>
<td>26.4</td>
</tr>
<tr>
<td>Efficiency (3.5% FCM/kg DMI)</td>
<td>1.03</td>
<td>1.00</td>
<td>1.02</td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td>3.88</td>
<td>3.84</td>
<td>3.77</td>
</tr>
<tr>
<td>Milk fat (kg/d)</td>
<td>1.05</td>
<td>0.95</td>
<td>0.99</td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td>3.23ab</td>
<td>3.26ab</td>
<td>3.30a</td>
</tr>
<tr>
<td>Milk protein (kg/d)</td>
<td>0.88</td>
<td>0.81</td>
<td>0.86</td>
</tr>
</tbody>
</table>

¹Means within a row with no common superscript differ (P ≤ 0.05).
²Means within a row with no common superscript tend to differ (P ≤ 0.10).
³Proprietary Saccharomyces cerevisiae fermentation product-based sequestering agents (Diamond V Inc. Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

T = Control diet + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

Calculated as mean milk yield during the toxin-dosing period– mean milk yield during the predosing period.
Table 6-4. Effect of dosing aflatoxin B\(_1\) (AFB\(_1\)) with or without sequestering agents\(^1\) on blood cell counts of dairy cows.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^2)</th>
<th>SEM</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>SEQ1</td>
</tr>
<tr>
<td>White blood cells (10(^3)/(\mu)L)</td>
<td>15.22</td>
<td>14.99</td>
<td>14.85</td>
</tr>
<tr>
<td>Red blood cells (10(^6)/(\mu)L)</td>
<td>6.08(^a)</td>
<td>5.80(^b)</td>
<td>5.87(^ab)</td>
</tr>
<tr>
<td>Neutrophils (10(^3)/(\mu)L)</td>
<td>3.26</td>
<td>3.43</td>
<td>3.28</td>
</tr>
<tr>
<td>Monocytes (10(^3)/(\mu)L)</td>
<td>1.65</td>
<td>2.11</td>
<td>1.49</td>
</tr>
<tr>
<td>Eosinophils (10(^3)/(\mu)L)</td>
<td>0.37</td>
<td>0.38</td>
<td>0.40</td>
</tr>
<tr>
<td>Lymphocytes (10(^3)/(\mu)L)</td>
<td>9.51</td>
<td>8.93</td>
<td>10.55</td>
</tr>
<tr>
<td>Basophils (10(^3)/(\mu)L)</td>
<td>0.11</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.97(^a)</td>
<td>9.48(^b)</td>
<td>9.65(^ab)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>29.85</td>
<td>28.59</td>
<td>28.85</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means within a row with no common superscript differ \((P \leq 0.05)\).

\(^1\)Proprietary \textit{Saccharomyces cerevisiae} fermentation product-based sequestering agents (Diamond V Inc. Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

\(^2\)T = Control diet + AFB\(_1\); SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.
Table 6-5. Effect of dosing aflatoxin B$_1$ (AFB$_1$) with or without sequestering agents$^1$ on immune response indices of dairy cows.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment$^2$</th>
<th>SEM</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>SEQ1</td>
</tr>
<tr>
<td>Granulocyte adhesion molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_2$-integrin (CD18; MFI$^3$)</td>
<td>918$^y$</td>
<td>908$^y$</td>
<td>1278$^x$</td>
</tr>
<tr>
<td>L-Selectin (CD62L; MFI)</td>
<td>2188$^y$</td>
<td>2353$^y$</td>
<td>3284$^x$</td>
</tr>
<tr>
<td>Neutrophil function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil phagocytosis (% of cells)</td>
<td>27.72</td>
<td>26.55</td>
<td>28.27</td>
</tr>
<tr>
<td>Oxidative burst (% of cells)</td>
<td>2.94</td>
<td>2.08</td>
<td>3.76</td>
</tr>
<tr>
<td>Cytokine$^4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interferon-$\gamma$ (MFI)</td>
<td>4.79</td>
<td>6.56</td>
<td>6.75</td>
</tr>
<tr>
<td>Interferon-$\gamma$ (% of cells)</td>
<td>3.27</td>
<td>3.08</td>
<td>3.48</td>
</tr>
<tr>
<td>Acute-phase proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haptoglobin$^5$</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/100mL)</td>
<td>19.50</td>
<td>20.59</td>
<td>20.79</td>
</tr>
<tr>
<td>Acid-soluble protein (mg/100 mL)</td>
<td>4.39$^{ab,xy}$</td>
<td>5.10$^{a,x}$</td>
<td>4.25$^{ab,xy}$</td>
</tr>
</tbody>
</table>

$^{a,b}$Means within a row with no common superscript differ ($P \leq 0.05$).

$^x,y$Means within a row with no common superscript tend to differ ($P \leq 0.10$).

$^1$Proprietary Saccharomyces cerevisiae fermentation product-based sequestering agents (Diamond V Inc. Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3).

$^2$T = Control diet + AFB$_1$; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.

$^3$MFI = mean fluorescence intensity.

$^4$Fold increase of ionomycin and phytohemagglutinin-stimulated cells over the non-stimulated cells.

$^5$Arbitrary unit based on absorbance at 450 nm.
Figure 6-1. Effect of dosing aflatoxin B₁ (AFB₁) with or without sequestering agents on the concentration of aflatoxin M₁ (AFM₁) in the milk of dairy cows. *Toxin-dosing period = d 21 to 25, Withdrawal period = d 26 to 28. *AFM₁ concentrations on the day specified differ (P = 0.01). Error bars indicate standard errors. SEQ1, 2 and 3 are proprietary *Saccharomyces cerevisiae* fermentation product-based sequestering agents (Diamond V Inc., Cedar Rapids, IA) containing low (SEQ1) or high (SEQ2) doses of a chlorophyll-based additive or a low dose of the additive and sodium bentonite clay (SEQ3); T = Control diet + AFB₁; SEQ1 diet = T + SEQ1; SEQ2 diet = T + SEQ2; SEQ3 diet = T + SEQ3.
CHAPTER 7
GENERAL SUMMARY

The experiments presented in this dissertation had three main objectives. The first objective was to evaluate the effects of treatment with either of two bacterial cultures or propionic acid on the survival of EC during and after ensiling of corn and alfalfa. The second objective was to determine the effects of inoculation with EC alone or either of two bacterial cultures or propionic acid on the bacterial community composition of corn and alfalfa silages and to examine if the abundance of known and unknown bacteria correlates with key silage fermentation indices. The third objective was to examine if addition of *Saccharomyces cerevisiae* to other aflatoxin-sequestering agents (clay or chlorophyll-based products), would synergistically increase binding of aflatoxin in dairy cows, reduce adverse immune responses to the toxin, and enhance the performance of dairy cows. In order to achieve these objectives, four studies were performed.

The first study examined if adding *L. plantarum* or *L. buchneri* or propionic acid to alfalfa silages contaminated with EC would inhibit the growth of the pathogen during or after ensiling. Alfalfa forage was harvested at the early bloom stage, wilted to a DM concentration of 54%, chopped to 19-mm lengths and ensiled after treatment with one of the following: 1, distilled water (Control); 2, $1 \times 10^5$ cfu/g of *E. coli* O157:H7 (EC); 3, EC and $1 \times 10^6$ cfu/g of *Lactobacillus plantarum* (EC+LP); 4, EC and $1 \times 10^6$ 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. The pathogen 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 pH dropped to 5.0 within 7 d for the EC+LP silage, followed by 16 d for the EC+LB silage and beyond 16 d for the others. 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 because they had higher lactate and acetate concentrations, respectively, relative to the other silages during the early fermentation phase (d 3 to 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 re-inoculated with $1 \times 10^5$ 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 EC during ensiling and like propionic acid, they can also prevent its growth on silage contaminated with the pathogen after ensiling.

The second study applied the Illumina-based metagenomic sequencing technique to examine the effects of silage additives on bacterial diversity and taxonomy of alfalfa silage. The d 100 silage samples from Chapter 3 were analyzed for bacterial diversity and taxonomic composition by Illumina MiSeq Sequencing. Compared to the Control, a less diverse bacterial community was detected in the EC+LP and EC+LB silages. Overall, most sequences were associated with phylum Firmicutes followed by Proteobacteria. Treatment with EC+LP and EC+PA reduced the abundance of Firmicutes, whereas that of Proteobacteria increased compared with the Control and EC+LB silages. Treatment with EC+LP resulted in the greatest abundance of Lactobacillus (62.6%), increased abundance of Sphingomonas, Pantoea, Pseudomonas and Erwinia and reduced abundance of Pediococcus, Weissella and Methylobacterium, relative to the
Control. Abundance of *Weissella* and *Methylobacterium* were also reduced in EC+LB silage relative to the Control. Application of propionic acid did not affect the abundance of *Lactobacillus, Weissella* or *Pediococcus*. At the species level, EC+LB and EC+LP silages contained the greatest abundance of *L. buchneri* and *L. plantarum*, respectively. Lactate concentration correlated positively with abundance of *Lactobacillus* and negatively with abundance of *Pediococcus*. Negative correlations were observed between ammonia-N concentration and abundance of genera *Sphingomonas, Pantoaea, Pseudomonas* and *Stenotrophomonas*. Silage pH was negatively correlated with abundance of *Lactobacillus, Sphingomonas, Pantoaea, Pseudomonas, Erwinia and Stenotrophomonas*. The abundance of bacterial species belonging to *Rhodococcus, Pseudomonas*, and *Xanthomonadaceae*, had positive correlations with lactate and/or acetate, while unknown species belonging to *Salana, Frigoribacterium, Pseudorhodobacter, Pantoaea, Stenotrophomonas*, and *Rhodococcus* had/negative correlations with ammonia-N. Future studies should aim to speciate, culture and validate the functions of these unknown bacteria in order to better understand their roles in silage fermentation.

The third study examined if addition of propionic acid or *L. plantarum* or *L. buchneri* would inhibit the growth of EC on corn silage during ensiling, at silo opening, or after aerobic exposure. The second objective was to examine how additives affect 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), $1 \times 10^5 \text{ cfu/g}$ of EC (ECCH); 3), EC and $1 \times 10^6 \text{ cfu/g}$ of *Lactobacillus plantarum* (ECLP); 4), EC and $1 \times 10^6 \text{ cfu/g}$ 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 (LAB), 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 was below the detection limit in all silages after 7 d of ensiling. Treatment with L. buchneri or propionic acid resulted in fewer yeasts and greater aerobic stability compared with the Control, ECCH, and ECLP silages. Compared to the Control, bacterial diversity was less in the ECLP silage partly because it contained more Lactobacillus. The ECLB silage contained greater abundance of Acinetobacter and Weissella than other silages. Subsamples of silages were reinoculated with $5 \times 10^5$ 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 no EC was detected in such silages. The ECCH and ECLP silages re-inoculated 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. 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).

This study indicates that during ensiling, EC was eliminated from all silages once the pH dropped below 4.0, whereas 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.

The fourth study examined if adding three mycotoxin-sequestering agents to diets contaminated with aflatoxin B$_1$ (AFB$_1$) would reduce milk aflatoxin M$_1$ (AFM$_1$) concentration.
and enhance the performance and immune status of dairy cows. Fifteen lactating dairy cows were used in an experiment with an incomplete crossover design with four 28-d periods. Treatments included a Control diet (C), a toxin (T) diet (1725 µg of AFB\textsubscript{1}/hd/d; 75 µg/kg) and diets containing the toxin and 20 g/hd/d of a proprietary mixture of \textit{Saccharomyces cerevisiae} fermentation product containing a low (SEQ1) or high (SEQ2) dose of a chlorophyll-based additive, or a low dose of the chlorophyll-based additive with sodium bentonite clay (SEQ3). Sequestering agents were top-dressed on the TMR daily in each period and the AFB\textsubscript{1} was orally dosed in gelatin capsules to cows before the TMR was fed on d 21 to 25. Milk was sampled twice daily on d 20 to 28 and plasma was sampled on d 20 and 25. The AFM\textsubscript{1} concentration of the milk was not affected by the sequestering agents during the toxin-dosing period. However, after withdrawal of AFB\textsubscript{1} from the diets, the sequestering agents reduced the time required (24 vs. 48 h) for reduction of the milk AFM\textsubscript{1} concentration below the Food and Drug Administration (FDA) Action level of 0.5 µg/kg. Feeding T instead of C tended to reduce the milk and FCM yields but feeding SEQ1 prevented these effects. Red blood cell count and hemoglobin concentration were reduced by feeding diet T instead of C, but not by feeding diets containing SEQ1, SEQ2 or SEQ3. Mean fluorescence intensity of antibody staining for two leukocyte adhesion molecules, L-selectin (CD62L) and β-integrin (CD18), tended to be greatest when SEQ1 and SEQ3 were fed. Plasma acid-soluble protein concentration was decreased when the SEQ1, SEQ2 and SEQ3 diets were fed instead of diet T. Sequestering agents had no effect on milk AFM\textsubscript{1} concentration but they reduced the time required for reduction of milk AFM\textsubscript{1} concentration to a safe level after withdrawal of AFB\textsubscript{1}. Only SEQ1 prevented adverse effects of AFB\textsubscript{1} on milk and FCM yields.
In addition to their well-established roles in improving silage fermentation, nutrient and DM recovery, and aerobic stability of silage at feed-out, the series of studies demonstrates conclusively that silage additives can be used to inhibit the growth of pathogens in feeds. The results from these studies (Chapters 3 and 4) confirm that pathogenic *E. coli* can be found on forages and show that *L. plantarum* or *L. buchneri* can increase the acidification of corn and alfalfa silages and hasten inhibition of the pathogen during ensiling. In addition to improving the aerobic stability of corn silage, *L. buchneri* and propionic acid also inhibited or prevented the growth of EC when contamination occurred after silo opening and aerobic exposure. Therefore, these silage additives can play a vital role in reducing cycling of EC on farms. This is vital for reducing the frequency of EC outbreaks and the associated food-borne diseases that cost the US approximately $271.4 million annually (USDA, 2014). Future research should examine the effect of controlling the growth of EC in silage on the overall prevalence of EC on farms. If this approach is successful, it would eliminate the high cost of vaccination to reduce EC shedding by ruminants. Another aspect worthy of research is to examine if silage inoculation with *L. plantarum* or *L. buchneri* can reduce fecal shedding of EC by ruminants. Future research should also investigate the prevalence and risk factors associated with EC shedding in dairy cattle. Better knowledge of these factors might guide effective mitigation strategies on farms to reduce EC contamination and cycling.

Studies presented in Chapters 4 and 5 highlight the fact that several silage microorganisms are unknown or uncultured and consequently their roles are unknown; however, some unknown or yet to be cultured silage bacteria are strongly associated with fermentation quality indices, such as lactate, acetate, and ammonia-N. Research is needed on functional characterization of the complex microbial communities in silage and how they are influenced by
external factors like additives. Future research should apply function-based metagenomics to identify and validate the roles of the unknown bacteria in silage fermentation. This may lead to development of new microbial additives that can improve silage preservation and allow more efficient utilization of forage protein by ruminants, thereby, reducing nitrogen excretion to the environment. Future research should also characterize the silage metabolome to provide more comprehensive information on chemical changes during ensiling and on how additives affect silage quality. Future research should integrate the results of multi-omics silage characterization approaches (metagenomics, metatranscriptomics, and metabolomics) to provide definitive insights on the types and roles of micro-organisms present and to show how their metabolites are involved in key signaling and metabolic pathways in silage.

Due to the ubiquitous occurrence of mycotoxins in grains and forages, frequent contamination of livestock feeds with multiple mycotoxins, inconsistent effects of available sequestering agents, and the global threat of mycotoxins to food and feed security, future research should aim to develop more effective mycotoxin sequestering agents that can counteract the additive and or synergistic effects of multiple mycotoxins. Studies on the ecology of specific gut microbes that degrade mycotoxins, particularly aflatoxins, are also needed to reduce their toxic effects and transfer to dairy products. To replace expensive chromatography methods, hazardous radio immunoassay methods and laborious ELISA methods, future research also should develop simple, rapid and accurate mycotoxin detection techniques to foster on-site or on-farm detection of mycotoxins in feeds.
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BIOGRAPHICAL SKETCH

Ibukun Ogunade was born in Lagos, Nigeria. He attended the University of Agriculture, Abeokuta, Nigeria where he earned his Bachelor of Science and Master of Science degrees in 2009 and 2011, respectively. His master’s thesis focused on reducing fecal shedding of *Salmonella* in laying hens with the use of probiotics and prebiotics. Immediately after obtaining his Master of Science degree in Animal Nutrition, he was employed as a Lecturer at the same university from 2011 to 2013 where he taught several undergraduate animal nutrition classes. In June 2013, Ibukun became a Doctor of Philosophy candidate in the Department of Animal Sciences at the University of Florida under Dr. Adebola Adesogan’s supervision, and he received his Ph.D in the spring of 2017. During his doctoral research program, Ibukun performed several applied dairy nutrition research studies investigating forage quality and conservation, feed microbiology and safety, and dietary mycotoxin sequestration strategies. He also collaborated with other researchers within and outside the University on several studies that focused on using fibrolytic enzymes and novel proteins to improve forage utilization, evaluating the use of alternative feed ingredients, and developing alternatives to antibiotics for mitigating greenhouse gas production by ruminant livestock, and improving their productive efficiency. Ibukun’s long-term plan is to become a faculty member at a university in the US.