EFFECTS OF BACTERIAL INOCULANTS AND MYCOTOXIN ADSORBENTS TO IMPROVE THE QUALITY AND SAFETY OF FEED INGREDIENTS

By

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Ensiling is an important preservation method that allows storage of forages harvested at maturity stages that optimize their productivity and nutritive value for use at times when forages are dormant or unavailable. Consequently, silage is one of the major components of the diet of ruminant livestock in many countries. However, reductions in forage nutritive value and safety often occur during ensiling. To study methods to minimize such problems, three experiments were conducted to evaluate the effects of additives on the fermentation, shelf life, microbiology, pathogenicity, and toxicity of corn silage. Because of the occasional presence of mycotoxins in silage and challenges with existing detoxification methods, a fourth experiment was conducted to evaluate the effect of dietary addition of a mycotoxin-sequestering agent on measures of health, immune response, and performance of dairy cows fed a diet contaminated with a mycotoxin.

The objective of Experiment 1 was to evaluate the effect of applying different bacterial inoculants containing homofermentative, heterofermentative, or both types of bacteria on the survival of pathogenic *E. coli* O157:H7 in corn silage during the anaerobic fermentation and aerobic exposure stages of silage production. *Escherichia*

coli O157:H7 did not survive after the pH dropped below 4.0 during ensiling. Unlike others, only inoculants containing *Lactobacillus buchneri* increased the concentration of acetic acid. Because of the antifungal properties of acetic acid, such inoculants prevented the growth of yeasts and molds, kept the pH below 4 after ensiling, and thereby prevented the growth of the pathogen after ensiling.

Experiment 2 aimed to determine effects of applying an inoculant containing homofermentative and heterofermentative bacteria on the fermentation, nutritive value, aerobic stability, and nutrient losses from corn silage produced in farm-scale silos. Applying the inoculant made the fermentation more heterolactic, by increasing acetic acid concentrations, tended to inhibit the growth of yeasts, and substantially reduced the amount of spoilage and the associated energy and nutrient losses.

The objective of Experiment 3 was to examine the association between increasing severity of Southern rust infestation and fermentation, nutritive value, and safety of corn silage, and to determine if inoculant application could mitigate adverse effects of rust infestation on silage quality and safety. The nutritive value and fermentation of corn silage were reduced drastically as the severity of rust infestation increased. Uninoculated corn silage with the highest level of rust infestation had 200 times more aflatoxin than the Food and Drug Administration Action (FDA) Level. Application of the bacterial inoculant improved the nutritive value, fermentation, and shelf life of rust-infested corn silage and prevented accumulation of aflatoxin in the silage.

The objectives of Experiment 4 were to determine the effect of feeding two doses of a mycotoxin adsorbent on milk aflatoxin M_1 concentrations and performance and immune response of dairy cows fed a diet contaminated with aflatoxin B_1. Feeding the

high dose of the adsorbent kept the aflatoxin concentration of the milk below the FDA action level, whereas feeding low dose of the adsorbent or the toxin-contaminated diet alone did not. Both doses of the adsorbent prevented the heightened inflammatory stress response and decrease in milk production and quality caused by feeding the toxin without the adsorbent.

CHAPTER 1
INTRODUCTION

Silage is defined as the material produced by the controlled fermentation of a crop of high moisture content (McDonald, 1991). The main objective of producing silage is to harvest crops at maturities that optimize their quality and productivity and preserve them to feed animals during winter or dry seasons (Pahlow et al., 2003) or for year-round feeding of housed ruminant livestock. This method of forage conservation has been adopted in many countries and has replaced traditional methods such as haymaking. In the US, silage is fed year-round to most dairy cattle because the variability in quantity and quality of pastures, climatic challenges, difficulties of milking cows on pasture, and large herd sizes make it more challenging to raise dairy cows on pastures. The area of corn planted for silage in the US in 2010 was 13.7 million ha, which is six times greater than the estimated planted area in 1994 (Wilkinson and Bolsen ,1996; USDA, 2011).

Quality silage production is dependent on specific fermentation pathways, without which growth and prevalence of spoilage and pathogenic bacteria and toxigenic fungi occur. Fenlon and Wilson (2000) reported an increase in the population of *Escherichia coli* O157:H7, a Shiga toxin-producing, acid tolerant bacteria, in poorly fermented ryegrass silage. This bacterium is a major food safety concern and contamination of food by this bacterium has led to several health scares, deaths, extensive recalls of various food items, and culminated in closure of major food companies in the US and around the world (Mead and Griffin, 1998; Rangel et al., 2005). Another food safety concern with poorly made silage is the production of mycotoxins. Gonzalez-Pereyra et al. (2008) noted that poorly prepared corn silage samples were contaminated with a high concentration (60 µg/kg DM) of aflatoxin. This toxin is a mutagenic, carcinogenic,

and toxic metabolite produced by Aspergillus species and it can be transferred from the diet of dairy cows to their milk and ultimately to consumers (Creppy, 2002; CAST 2003). Aflatoxin is responsible for causing 25,000 to 155,000 new cases of cancer around the world and is estimated to have cost the US economy approximately $350 million in 2010 (Wu, 2010). Poor fermentation and aerobic deterioration are the main sources of silage spoilage, nutrient losses from silage (McDonald, 1991; Tabacco et al., 2011), and silage pathogenicity. Feeding diets containing increasing levels of spoiled silage has linearly reduced feed intake and animal performance, and predisposed cows to various health complications (Whitlock et al., 2000; Whitlow and Hagler, 2005).

Because of the widespread need for and use of silage in US dairy farms, research in the last few decades has focused on development of technologies to improve silage quality but little attention has been focused on food or feed safety aspects of silage production. Various scientific advances have been made in the use of additives and inoculants to improve silage fermentation and shelf life (Kung et al., 2003; Huisden et al., 2009). Homolactic bacteria inoculants, which synthesize lactic acid as the sole product of hexose fermentation, have been used successfully to rapidly increase the acidity in silages to values ($\leq$ 4) that reduce the growth of fermentation-impairing bacteria such as *Enterobacteria* and *Clostridia* (Pahlow et al., 2003; Filya et al., 2007). Heterolactic bacterial inoculants have been developed to ferment hexose and lactate to antifungal acids like acetic acid, which inhibit the growth of spoilage and toxigenic fungi and hence increase the shelf life of forages (Huisden et al., 2009). However, silage quality improvement efforts have focused almost exclusively on improving the fermentation and shelf life of silage. Consequently, no proven methods of destroying

silage pathogens or detoxifying silage mycotoxins by applying additives at the time of ensiling exist. Rather, the main strategy for preventing adverse effects of ingesting mycotoxin-contaminated silage or feed ingredients involves dietary addition of mycotoxin sequestering agents. The interaction of the toxin and binder results in a macromolecular complex that cannot be absorbed by the animal (Phillips, 1999). Mycotoxin sequestering agents do not detoxify because they do not change the concentration of the toxin in the diet; however, because they prevent absorption of the toxin, they decrease its adverse effects on the animal and its transfer to milk (Diaz et al., 2004). The main objectives of this dissertation were to examine potential effects of different inoculants on the fermentation, aerobic stability, nutrient and dry matter (DM) losses of silage, as well as effects on the pathogenicity and toxicity of silage. An additional objective was to evaluate the efficacy of using different doses of a mycotoxin sequestering agent on the performance, immune response, and milk aflatoxin concentration of dairy cows fed a diet contaminated with aflatoxin. In order to achieve these objectives four experiments were performed. Experiment 1 investigated if different bacterial inoculants containing homofermentative bacteria, heterofermentative bacteria, or both types of bacteria could curtail the growth of pathogenic *E. coli* O157:H7 in corn silage during the anaerobic and aerobic stages of silage production. A second objective of this experiment was to examine whether pH-independent antibacterial activity against *E. coli* O157:H7 existed in the inoculants and persisted in inoculated silages. Experiment 2 examined the effects of applying a dual-purpose inoculant containing homolactic and heterolactic bacteria selected based on the results of Experiment 1, on the fermentation, nutritive value, aerobic stability, and nutrient losses from corn silage

produced in farm-scale silos. Experiment 3 examined the relationship between increasing severity of Southern rust infestation and the quality and safety of corn silage, and whether the dual-purpose inoculant used in Experiment 2 could mitigate adverse effects of Southern rust on the quality and safety of corn silage. Experiment 4 determined the effect of adding two doses of a mycotoxin adsorbent on milk aflatoxin M_1 (AFM_1) concentrations and the performance and immune response of dairy cows fed a diet contaminated with aflatoxin B_1 (AFB_1).

CHAPTER 2
LITERATURE REVIEW

The Importance of Silage

Silage is defined as the material produced by controlled fermentation of a crop of high moisture content McDonald (1991). The main rationale for preserving forages is to be able to harvest and store them at a growth stage that optimizes their productivity and nutritive value and use them at periods when forages experience little or no growth, very often during the winter. Ensilage, the technique of making silage, has been practiced for over 3000 years; however, interest in this process only became widespread in the latter part of nineteenth century (McDonald, 1991). The importance of this practice is evident from its increasing adoption by producers. In western European countries, silage has been the main forage preservation method in the last few decades, far exceeding the production of hay (Wilkinson et al., 1996). The area of corn planted for silage in the US in 2010 was 13.7 million ha, which is six times greater than that in 1994 (Wilkinson and Bolsen, 1996; USDA, 2011). Countries such as Brazil, Argentina, Chile, Australia, Mexico, and New Zealand experienced a rapid increase in silage production in the 1990s (Wilkinson et al., 2003) mainly because of improvements in ensiling technologies (Kaiser and Evans, 1997; Muhlbach, 1998;). These technologies have facilitated adherence to the basic concepts of silage making, which are 1) to obtain the anaerobic conditions necessary to promote rapid fermentation and, consequently, enhance the rate of acidification of silage and thereby impair the growth of undesirable microorganisms; 2) to densely pack the forage into silos immediately after harvest and rapidly achieve and maintain anaerobic conditions for the ensiling duration, and 3) to manage the silage during the aerobic feed-out phase in a way that minimizes spoilage,

nutrient depletion, and mycotoxin production. To understand these concepts and how new technologies can improve the quality of silage, the process of ensiling will be discussed under four main chronological phases: aerobic, active fermentation, stable fermentation, and feed-out phases (Weinberg and Muck, 1996).

Ensiling Phases

Initial Aerobic Phase

This phase is characterized by use of the oxygen trapped within the forage mass and is frequently accompanied by an increase in temperature of the ensiled material. Ongoing respiration by harvested plants and microorganisms results in nutrient oxidation and heat generation during this phase. Obligate and facultative aerobic microorganisms continue to utilize hexose sugars and the presence of oxygen sustains the growth of molds, yeasts and undesirable bacteria that may later produce toxins or predispose the silage to the growth of pathogens at feed out. Plant enzymes, proteases, and carbohydrases initiate the decomposition of proteins and carbohydrates to amino acids and soluble sugars, respectively (Pahlow et al., 2003). Oxygen also delays the decrease in pH necessary for ensiling by reducing the rate of plasmolysis of plant cells (Greenhill, 1964), which releases the intracellular fluid needed by lactic acid bacteria (LAB) for synthesis of lactic acid. All these processes negatively affect silage quality and this initial phase can last for hours in well-compacted silage or days if oxygen is present within the silage matrix. Thus, optimizing silage density at silo filling and immediately sealing silos is necessary to expel residual oxygen (McDonald, 1991) and prevent further oxygen ingress into the silage at ensiling.

Main Active Fermentation Phase

The fermentation phase begins when the oxygen in the silage mass is greatly reduced after the initial aerobic phase and it is characterized by the fermentation of water-soluble carbohydrates (WSC) by bacteria particularly LAB to acids that decrease the pH. Obligate and facultative anaerobic LAB as well as *Clostridia, Enterobacteria, Bacilli* and yeasts compete for fermentation substrates like WSC, but in most cases, LAB prevail and become dominant whereas the population of other microorganisms tends to decrease (Pahlow et al., 2003). However, if the pH decreases slowly, *Enterobacteria* can outcompete LAB (Lin, 1992; Kizilsimsek et al., 2007).

The fermentation phase can last for several days or weeks depending on the crop used (McDonald, 1991). Pedroso et al. (2005) reported active fermentation in sugarcane silage during the first 15 days (d) of fermentation causing the WSC concentration and pH to decrease by 14% (DM basis) and 1.55 units, respectively. Once active fermentation was over on d 15 of ensiling, no further decreases in pH or WSC were detected during the 165-d ensiling period. Common external signs during the active fermentation phase are the production of effluent, formation of gases, and shrinkage of the silage mass (Pahlow et al., 2003) because of increased cell plasmolysis, formation of CO_2, and dry matter losses, respectively.

Stable Fermentation Phase

During this phase, the low pH and fermentation product accumulation reduce the activity of most microorganisms in the silage. Even the population of LAB undergoes a three-fold logarithmic reduction (Pahlow et al., 2003). Nevertheless, acid-tolerant enzymes continue to cause a slow and continuous release of WSC by hydrolyzing polysaccharides as long as anaerobic conditions are maintained. Kleinschmit and Kung

(2006a) noted that the concentration of WSC in corn forage ensiled for an extended period (361 d) fluctuated with time. Silages treated with LAB achieved the lowest concentration of WSC after 28 d of fermentation; however, by d 361, the concentration was greater. This is probably caused by hydrolysis of complex carbohydrates by acid-tolerant enzymes. The authors also reported that treatment with *Lactobacillus buchneri* slightly increased concentrations of acetate (1% DM) and 1, 2-propanediol (0.4% DM) from d 56 to 361 of fermentation. This low rate of synthesis of fermentation products is characteristic of microorganisms surviving the stable phase because the prevailing conditions hinder optimization of their metabolic processes (Oude Elferink et al., 2001). Acid-tolerant yeast species also can become less active and survive this phase as can *Clostridia* and *Bacilli*, which can become dormant endospores (Pahlow et al., 2003).

The Feed-out Phase

The feed-out phase begins when the silo is opened and the silage is exposed to air. Once oxygen penetrates the silage mass, dormant facultative aerobic organisms within the silage grow and multiply, as do airborne microorganisms that penetrate the silage. This phase can be divided into two main stages (Oude Elferink., 2011). The first one is characterized by metabolism of organic acids by yeast or bacteria (McDonald, 1991). Ohyama et al. (1971) isolated acid-tolerant yeasts of the genera *Candida*, *Hansenula* and *Pichia*, which are involved in initiating the aerobic deterioration of corn and grass silages and can use organic acids as a substrate. Oxidation of acids by these fungi increases the pH, making the silage more conducive for the growth of less acid-tolerant aerobic microorganisms that characterize the second stage of aerobic deterioration. The succession of microorganisms in these phases is well represented by

two thermal peaks on aerobic stability curves. The first one is by acid-tolerant yeasts or bacteria and the second by later growth of molds (McDonald, 1991).

In addition to acid-tolerant yeasts, acetic acid bacteria (AAB) can also use lactic and acetic acids as a substrate. The complete oxidation of organic acids by these bacteria was studied by Spoelstra et al. (1988) who reported that AAB also are capable of causing the first stage of aerobic deterioration in corn silage. Aerobic deterioration is a major cause of nutrient loss in silage and is also often responsible for silage potential pathogenicity and toxicity, which can lead to poor animal performance, diseases and death (Whitlow and Hagler, 2005; Gonzalez-Pereyra et al., 2008). Consequently, concerted efforts have been directed at minimizing aerobic deterioration in the last few decades and these have culminated in development of a newer group of silage inoculants.

Silage Microbiology

Fermentation is the process responsible for the preservation of forage during ensiling. To describe the associated microbiology, the process is divided into primary and secondary fermentation (Pahlow et al., 2003).

Primary Fermentation

Primary fermentation is caused by LAB, which acidify the silage mainly by synthesizing lactic acid, and thereby inhibiting the growth of undesirable microorganisms that cause secondary fermentation. Several bacteria are classified as LAB but those from genera *Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Lactococcus,* and *Streptococcus* are found most frequently in silages. The LAB are obligate fermenters and they generally but not always use carbohydrates as the source of energy (Oude Elferink et al., 2001; Pahlow et al., 2003). Their presence in the

epiphytic microbial population of crops is probably because of their defensive role against reactive oxygen species; for instance, their high manganese concentration works as an intracellular oxygen scavenger (Archibald and Fridovich, 1981). Because they are fermenters, LAB perform well under anaerobic conditions and oxygen can be lethal for most of those that do not secrete catalase (Codon, 1987). Most LAB will grow at temperatures between 25 and 40^{o}C; however, some strains can survive more extreme conditions. Mulrooney and Kung (2008) reported that as incubation temperatures increased from 30 to 45^{o}C for 6 h, the viability of several LAB decreased, however *L. plantarum* MTD/1 was thermotolerant and its viability was maintained even at 45^{o}C. Under anaerobic conditions, LAB dominate the microbial population and decrease forage pH from 6 and above to approximately 4 by fermenting a wide variety of substrates and synthesizing lactic acid as well as other products (Pahlow et al., 2003).

Because of the fermentative differences within the group, LAB have been classified by the type of fermentation they promote (McDonald, 1991; Pahlow et al., 2003) into homofermentative or heterofermentative types. Homofermentative LAB ferment hexose sugars to pyruvate almost exclusively by the glycolytic pathway followed by reduction of pyruvate to lactic acid (Figure 2-1, part A). However, they cannot ferment pentoses since they do not have phosphoketolase, which is necessary for the synthesis of acetyl-phosphate and glyceraldehyde-3-phosphate (White, 2007). Facultative heterofermentative LAB utilize the same pathway as homofermentative LAB to ferment hexoses, as they secrete aldolase, which is necessary for the synthesis of 3-phosphoglyceraldehyde and dihydroxy-acetone phosphate. However, they also secrete

phosphoketolase and can therefore ferment pentoses. Obligate heterofermentative LAB are characterized by their fermentation of hexoses to other products in addition to lactic acid (Figure 2-1, part B).

Secondary Fermentation

Enterobacteria

Enterobacteria species are well known for their capacity to decarboxylate and deaminate amino acids during secondary fermentation of silage. Those commonly isolated from silage are gram-negative, rod-shaped, often motile, catalase-expressing bacteria capable of reducing NO_3 (Pahlow et al., 2003). Most *Enterobacteria* are strictly dependent on carbohydrates for growth under anaerobic conditions (Pahlow et al., 2003) and are not pathogenic. However poor fermentation may cause the establishment of *E.coli* O157:H7 (Fenlon and Wilson, 2000) on silage and increase the chances of feed and food contamination with the pathogen.

In general, *Enterobacteria* are very sensitive to climatic changes, which cause fluctuation in the epiphytic population of plants; nevertheless, their minimum population is frequently 100 times higher than that of LAB (Pahlow et al., 2003). At the beginning of the initial aerobic phase, *Enterobacteria* often multiply to numbers (10^8 to 10^{10} cfu/g) that may exceed those required for LAB dominance during the active fermentation phase (Lindgren et al., 1985). This capacity to compete with beneficial LAB for nutrients can cause extensive proteolysis and DM losses (McDonald, 1991); therefore, their presence in silage is highly undesirable.

Clostridia

Clostridia species are important participants in the secondary fermentation in silages and they are as undesirable as *Enterobacteria* species. Clostridial growth is

optimized by conditions, which lead to poor fermentation such as low DM at harvest, limiting WSC concentration, high buffering capacity, and the presence of residual oxygen in the silage mass (McDonald, 1991). Clostridial species are gram-positive obligate anaerobes capable of forming dormant endospores that survive adverse conditions (Madigan et al., 2009). Clostridial growth is possible at pH of 4.6 to 7.0 and from 3.5 to 50^{o}C (Kendall, 2006). Strains frequently isolated from silage include saccharolytic types that usually ferment carbohydrates such as *C. tyrobutyricum* and *C. butyricum* and proteolytic types like *C. sporogenes* (Pahlow et al., 2003). The main negative effects of *Clostridia* on the quality of silage are degradation of amino acids, which increases NH_3 concentration and synthesis of butyric acid, in particular, as well as acetic, propionic, isobutyric, and 2-methyl and 3–methyl butyric acids (McDonald, 1991). *Clostridia* also may synthesize biogenic amines and CO_2 via decarboxylation of amino acids (Hui and Sherkat, 2006). The butyric acid and biogenic amines typically decrease silage intake (Dulphy and Van Os, 1996). Such biogenic amines reduced ruminal function and decreased DM intake by up to 25% in steers fed contaminated alfalfa silage (Phunstok et al., 1998).

Yeasts

Yeasts are eukaryotic microorganisms that mainly grow as a single cell (Brock, 2009). Yeasts belonging to the *Candida* and *Hansenula* genera can initiate aerobic deterioration of silage by using lactate as a substrate during the final aerobic phase, but yeasts also have the capacity to cause secondary fermentation under anaerobic conditions. When present in the epiphytic population of crops with high WSC concentrations such as sugarcane, yeasts ferment hexoses to ethanol primarily via an alcoholic fermentation. Co-existence of yeasts and heterofermentative LAB cause

considerable DM losses and result in silages of poor quality with high ethanol concentrations (Pedroso et al., 2005; Avila et al., 2009). Jonsson and Pahlow (1984) noted that under anaerobic conditions, fermentative, non-lactate assimilating *Saccharomyces cerevisiae* are predominant, however under aerobic conditions, lactate-assimilating yeasts are dominant.

Dominance of the microbial population by LAB can curtail secondary fermentation but the outcome depends on the DM concentration of the silage and the pH or the acidification rate. Silages with low DM concentrations require lower pH to be anaerobically stable (Pahlow et al., 2003). Ensuring a fast acidification of silage is the main mechanism for preventing the growth of the *Clostridia* and *Enterobacteria* that cause secondary fermentation in silage.

Silage Additives

Three of the main silage making goals are preventing secondary fermentation, establishing and maintaining anaerobic conditions during ensiling, and preventing entry of oxygen into the silage mass during the final aerobic phase. Silage additives are often employed to address these issues; however, a wide range of additives are available and they have different effects and modes of action. The ensuing section describes the main additives currently used for silage preservation.

Bacterial Additives

Bacterial additives or inoculants contain selected strains of bacteria that ferment sugars to either lactic acid, which causes a rapid pH decline and, thereby, preserves the forage, or to antifungal acids that inhibit the growth of spoilage-causing fungi. These bacteria are classified as homolactic and heterolactic types, respectively. Both types

can be used to improve silage quality but they have different roles and act on different phases of the ensiling process.

Homolactic bacteria

The first recorded use of homolatic bacteria to preserve plant products occurred at the beginning of the twentieth century when French workers applied cultures of bacteria to preserve sugar beet pulp (Watson and Nash, 1960). Homolactic bacteria ferment glucose into lactic acid via a pathway that is energetically efficient because no energy-containing byproducts are produced. The transformation of 1 mole of glucose to 2 moles of lactic acid and 2 moles of adenosine triphosphate (ATP) via the Embden-Meyerhof pathway yields high recoveries of energy (99.3%) and DM (100%; Kung et al., 2003; White, 2007).

Lactobacillus plantarum: The use of homolactic bacteria was common at the end of 1970s (Kung et al., 2003). At that time, most silage inoculants were developed based on the criteria of Whittenbury (1961) who recommended that inoculant bacteria should grow vigorously, be able to dominate the microbial population during fermentation, and be homofermentative and acid tolerant in order to produce significant amounts of lactic acid and rapidly decrease the pH. The microorganism that fit all these criteria was *Lactobacillus plantarum*, which is still the bacteria most commonly found in commercial silage inoculants. *Lactobacillus plantarum* is a gram positive, rod shaped bacterium commonly isolated from fermented food and silages (Kung et al, 2003). It is tolerant to aerobic conditions due to the high intracellular content of manganese polyphosphate, which works as an oxygen scavenger to lower the amount of reactive oxygen species (Archibald and Fridovich, 1981). The latter survival mechanism of *L. plantarum* and its

physiological and biochemical properties make it a perfect candidate for dominating the fermentation and meeting Whittenbury's other criteria for silage inoculants.

Lactobacillus plantarum was classified previously as an obligate homofermentative bacterium based on its ability to ferment one mole of glucose to 2 moles of lactic acid via the energetically efficient homofermentative Embden-Meyerhof pathway. However, *L. plantarum* is now classified as a facultative heterofermenter because when glucose is lacking, it shifts the fermentation away from that based on exclusive lactic acid production to one based on fermenting pentoses to lactic acid, CO_2, and acetic acid via the less efficient heterofermentative pathway (Holzer et al., 2003). Inadequate glucose supply also reduces the concentration of fructose-1, 6-bisphosphate, an essential activator of lactate dehydrogenase (Pahlow et al., 2003). The new classification method is based on phylogenetic comparison of 16 ribosomal ribonucleic acid (16S rRNA) and is more accurate than the traditional physiological and biochemical system. Nevertheless, *L. plantarum* is still considered a homolactic bacteria when glucose availability does not limit its fermentative activity. Thus, in silages with adequate glucose concentrations, *L. plantarum* usually synthesizes lactate exclusively, and the lactate reoxidizes NADH, thus allowing the Embden-Meyerhof pathway to be continuously repeated during the metabolism of carbohydrates (McDonald, 1991).

Lactobacillus plantarum has been used successfully to decrease the pH of silages particularly those with high buffering capacity. Filya et al. (2007) reported that application of three strains of *L. plantarum* reduced the pH of alfalfa from 5.08 in the Control silage to an average of 4.43 in inoculated silages and increased the lactate to acetate ratio from 2.88 to 6.53. Conaghan et al. (2010) showed that *L. plantarum*

increased lactic acid concentrations in ryegrass silages from 73 (Control) to 95 g/kg of DM and reduced the pH from 4.5 to 4.0.

Pediococcus pentosaceus: *Pediococcus pentosaceus* is a homolatic, gram positive, facultatively anaerobic, non-spore forming bacterium that often is used as a silage inoculant. Like *Lactobacillus plantarum*, it is acid tolerant and is capable of synthesizing L and D forms of lactic acid (Garvie, 1986; Axelsson, 1998). However, it grows more actively than L. plantarum and most other silage bacteria when the pH is 5 to 6.5 and residual oxygen is still present during the early stages of the fermentation (Kung et al., 2003; McDonald, 1991). *Pediococcus* strains also can grow well at high DM concentrations and under low water activity (Tanaka and Ohmomo, 2000). These characteristics allow *Pediococcus* strains to start the acidification of silage during the initial aerobic phase at early stages of fermentation when Lactobacillus strains grow less vigorously due to the high pH. Thus, some inoculants contain both P. pentosaceus and L. plantarum in order to exploit their complementary growth niches and thereby increase the rate of acidification during the early and later stages of the fermentation (Lin et al., 1992). Cocconcelli et al. (1991) used DNA (deoxyribonucleic acid) probes to verify the colonization of corn silage by P. pentosaceus and L. plantarum. The authors verified that the population of *Pediococcus pentosaceus* was maximized after an exponential increase during the first 12 h of ensiling, whereas dominance by Lactobacillus plantarum occurred only after 48 h. Cai et al. (1999) inoculated alfalfa and ryegrass silages with isolates of *Pediococcus acidilactici* or *Pediococcus pentosaceus* at 25 or 48^{o}C. The authors noted that the quality of silage kept at 25^{o}C was improved by both strains as evidenced by decreases in DM and gas losses and products of

secondary fermentation such as NH_3 and butyric acid. Similar but less pronounced trends were evident at 48^{o}C, which suggests that P. pentosaceus may be an unsuitable homolactic bacteria for silage produced in subtropical or tropical areas.

Enterococcus faecium: *Enterococcus faecium* is an important producer of lactic acid during the initial stages of fermentation like *P. pentosaceus*. These bacteria are gram-positive facultatively anaerobic cocci that synthesize L-lactic acid and can grow at pH 4.5 to 9.6 (Cai, 1999). Cai (1999) isolated 48 strains of lactic acid bacteria from forage crops, from which 4 isolated strains of *Enterococcus* and two commercial strains of *Lactobacillus* were applied to alfalfa and grass silage. Silages inoculated with the commercial additives had lower pH, butyric acid, ammonia-N, gas production, and DM losses compared with Control silages, but those inoculated with the *Enterococcus* strains did not. The authors concluded that *Enterococcus* species cannot improve silage fermentation likely because they were unable to grow at pH below 4.5. Nevertheless, like *P. pentosaceus, Enterococcus* spp. are used in homolactic inoculants containing *L. plantarum* to 1) dominate the initial fermentation phase and quickly initiate the pH decrease and thereby prevent the onset of secondary fermentation and 2) to decrease the pH to levels conducive for the growth of *L*. plantarum. Filya et al. (2000) applied *Enterococcus faecium* and *Lactobacillus plantarum* to fresh and wilted wheat silage and reported that a pH of 5 was achieved after 15 and 70 d in Control silages versus 1 and 5 d in inoculated silages. Filya et al. (2007) studied the effect on the fermentation of alfalfa silage of inoculants containing 2 strains of *E. faecium* that were applied separately, and mixtures of *E. faecium* and *L. plantarum,* or *E. faecium, L. plantarum,* and *P. pentosaceus*. Combination of *E. faecium* with *L. plantarum* reduced the pH, increased

lactic acid concentration, and decreased ethanolic fermentation, however addition of *P. pentosaceus* did not increase the effectiveness of the inoculant compared to using *E. faecium* and *L. Plantarum*. One of the two inoculants containing only *E. faecium* improved the fermentation but did not reduce ethanol concentration likely due to secondary fermentation by *Enterobacteria* and or yeasts, whereas the other one did not have any effects when compared to the Control treatment. This study shows the complementary effects of *L. plantarum and E. faecium* on silage fermentation.

The effects of inoculants containing homolactic acid bacteria on silages were reviewed by Kung and Muck (1997). Homolactic bacteria inoculants frequently cause a rapid pH decrease by increasing lactic acid concentration and thereby reducing proteolysis, deamination, and the potential for ethanolic, butyric, or acetic acid – dominated fermentations, such that secondary fermentation is decreased and DM recovery is increased (Kung et al., 2003). Therefore, one of the main benefits of inoculation with homofermentative bacteria is to reduce losses of energy, nutrients, and DM associated with secondary fermentation. However, homolactic bacteria do not reduce the risk of aerobic deterioration of silage. Kung and Muck (1997) reported that homolactic inoculants do not affect and in fact sometimes worsen the aerobic deterioration of inoculated silages. This is largely because lactic acid is not a strong antifungal agent and therefore, it does not usually inhibit the growth of spoilage fungi. In contrast, the propionic and acetic acids produced during heterolactic fermentation (Moon, 1983; Huisden et al., 2009) are strong antimycotic agents that inhibit spoilage fungi and thereby reduce silage deterioration.

The term aerobic stability is used frequently to express how long silage remains without signs of microbial deterioration under aerobic exposure. During the aerobic exposure phase, acid-tolerant yeasts that use lactic acid as a growth substrate indirectly increase the pH to levels that are ideal for molds and other spoilage and pathogenic microbes that worsen silage deterioration (Adesogan and Queiroz, 2009; Queiroz et al., 2011). Respiration by such microbes results in rapid metabolism of nutrients and increased DM loss (Henderson et al.,1979; Cai, 1999). Poor aerobic stability does not occur only on the silo face but also within the silo (Pitt and Muck, 1993). Air can penetrate up to 4 meters into the silage mass, which implies that silage can begin to deteriorate for days before it is exposed on the silo face and fed (Parsons, 1991).

Whittenbury's criteria for the ideal bacterial silage inoculant did not account for the inability of lactic acid to reduce fungal growth and aerobic deterioration, perhaps because aerobic deterioration problems were less extensive and common in the 1960s due to the smaller silo sizes that were prevalent. With the large silos in current use, the need to maintain or increase aerobic stability is very critical. Heterolactic inoculants were developed to address this need.

Heterolactic bacteria

Heterolactic bacteria produce lactic acid and additional products such as ethanol, CO_2, and acetic acid during hexose fermentation (Oude Elferink et al., 2001). This is because these bacteria lack fructose diphosphate aldolase, thus glucose 6-phosphate is fermented to 6- phosphogluconate rather than fructose-6-phosphate (Kung et al., 2003).

Adding heterolactic bacteria instead of homolactic bacteria to silage can increase DM and energy losses. For instance, respective losses of up to 27 and 1.7% were reported for heterolactic fermentation, whereas with homolactic fermentation, no DM

loss occurs and only about 0.7% of energy is lost (McDonald, 1991). Nevertheless, the heterofermentative pathway is attractive because it generates antifungal agents such as acetate or propionate (Oude Elferink et al., 2001; Krooneman, 2002), which are powerful antifungal agents that have increased the aerobic stability of corn, sorghum, and ryegrass silages inoculated with heterolactic inoculants (Kung and Ranjit, 2001; Tabacco et al., 2011; Driehuis et al., 2001; Huisden et al., 2009). The acetate produced by heterofermentative bacteria can also curtail yeast-induced ethanolic fermentation by inhibiting fungal growth in forages with high sugar concentrations.

Lactobacillus buchneri: *Lactobacillus buchneri* is the most commonly used heterofermentative bacteria in silage inoculants. Avila et al. (1999) reported that when added to sugarcane silage, *L. buchneri* increased the acetic acid concentration from 17 g/kg DM in the Control silage to 43 g/kg of DM, reduced ethanol concentration by 33 g/kg of DM, reduced yeast counts by 4-log cfu/g, and increased aerobic stability by 36 hours. *Lactobacillus buchneri* is a gram positive, rod- shaped, non-spore forming and anaerobic bacterium. It is one of the most commonly used LAB in silage inoculants due to its capacity to synthesize acetic acid even under low pH. Oude Elferink et al. (2001) described the pathway used by *L. buchneri* to convert lactic acid to acetic acid, 1,2-propanediol, and traces of ethanol under anoxic conditions. They also reported that the conversion of lactate to these products is dependent on environmental conditions, such as pH and temperature. All the *L. buchneri* strains they evaluated degraded lactate when temperatures were increased 15 to 25^{o}C, however when temperatures were further increased to 30^{o}C, only one strain degraded lactate and at 35^{o}C, none had this effect. The degradation of lactic acid was also dependent upon environmental pH. At pH

5.8, the concentration of lactate was steady for 200 h, while reduction of pH to 4.3 and 3.8 increased the rate of lactate degradation.

Lactobacillus buchneri has been used to increase aerobic stability in corn, barley, alfalfa, sorghum, sugarcane, grass and other silages (Filya, 2003; Huisden et al., 2009; Pedroso et al., 2005). Kleinschmit and Kung (2006b) performed a meta-analysis with 33 studies to understand the effect of *L. buchneri* on corn, grass and small-grain silages. The authors reported that *L. buchneri* increased acetate concentration, decreased lactate concentration, and consequently reduced yeast counts. However, the latter benefit occurred at the expense of a relatively small increase in DM loss caused by the inefficiency intrinsic to heterolactic fermentations. Effects of *L. buchneri* on corn silage were also dose dependent with doses $>10^5$ being more effective than $\leq 10^5$.

The effect of *L. buchneri* on grass and small grain silages in the meta analysis was different from that on corn silages. Treatment with *L. buchneri* increased ethanol and propionic acid concentrations of grass and small-grain silages and yeast not detected in most of such silages; consequently no relationship existed between yeasts and acetate concentration. All the other effects of *L. buchneri* application on corn silage such as improved aerobic stability were also evident in grass and small-grain silages. The fact that application of *L. buchneri* increased aerobic stability in grass and small-grain silages without affecting yeast counts is possibly because the acetic acid produced by the inoculant decreased the growth of other spoilage fungi or bacteria after aerobic exposure.

That *L. buchneri* application increases propionic acid in silages is desirable, because propionic acid has stronger antifungal properties than acetic acid. However, *L.*

buchneri treatment has not increased consistently propionic acid concentration. Combining propionic and acetic acids result in a synergistic antifungal effect, which results in increased aerobic stability. Driehuis et al. (1999) reported that corn silage treated with increasing concentrations of *L. buchneri* had increasing concentrations of acetic, propionic and 1-propanol instead of the 1, 2-propanediol often synthesized in other studies. Corn silage inoculated with $1x10^6$ cfu/g of *L. buchneri* contained 113 mmol of propionic acid/kg of DM, which represents a ten-fold increase over the value for the Control silage. This increase in propionic acid and the a three-fold increase in acetic acid resulted in an aerobic stability of 792 h versus 42 h in Control silages. The authors hypothesized that 1, 2-propanediol was being converted to 1-propanol and propionic acid by *Lactobacillus buchneri* or another microorganism. This hypothesis was later confirmed by Krooneman et al. (2002), who isolated two facultative anaerobic, heterofermentative, novel strains named *Lactobacillus diolivorans*. These strains can co-exist in corn silage inoculated with *L. buchneri* and convert 1,2-propanediol to 1-propanol and the desirable propionic acid. The need for co-existence of these bacteria is probably why effects of *L. buchneri* on propionic acid are inconsistent in the literature. Propionic acid concentrations would only be increased by *L. buchneri* treatment in locations where *L. diolivorans* is part of the natural epiphytic population of crops or if it is added in to the *L. buchneri* inoculant.

Lactobacillus brevis: *Lactobacillus brevis* is a rod shaped, heterofermentative, gram-positive bacterium frequently found in aerobically stable silages (Holzer et al., 2003). It is also known for its ability to synthesize acetic acid from fermentation of carbohydrates (Holzer et al., 2003) and thereby increase aerobic stability of silages

(Wang and Nishino, 2008). Danner et al. (2003) examined effects of different bacterial strains on the fermentation and aerobic stability of corn silage. The authors reported that silage treated with *L. brevis* had 28.6 g/kg DM of acetic acid, 73% more than that in the Control silage and 42 h longer aerobic stability than the Control silage. Nevertheless, *L. buchneri*, was more effective than *L. brevis* at increasing acetic acid concentration (55.3 g/kg of DM) and aerobic stability (274 h). This study and the more frequent and successful use of *L. buchneri* for increasing silage aerobic stability, indicate that *L. brevis* is less effective for this purpose.

Propionibacteria freudenreichii: *Propionibacteria freudenreichii* is a gram positive, non-spore forming, oxygen tolerant bacterium found in dairy products and silage (Woolford, 1975). Propionibacteria can synthesize propionic acid, which is a potent antimycotic agent (Moon, 1983). Merry and Davies (1999) reviewed the effects of *Propionibacteria* on silage and their role in biological control of aerobic spoilage. They concluded that the evidence that inoculation with *P. freudenreichii* increases the propionic acid concentration of silages is equivocal. This may be because *Propionibacteria* are not competitive when the pH decreases quickly in silages with adequate fermentable carbohydrate concentrations and LAB populations such as corn silage. Arriola et al. (2011) reported that adding an inoculant containing *P. freudenreichii* and *P. Pentosaceus* did not affect propionic acid concentration, the fungal population, or aerobic stability of corn silages. Similar results were observed by Kung and Ranjit (2001), Taylor et al. (2002) and Pedroso et al. (2010) when they applied *P. freudenreichii, L. plantarum,* and *P. pentosaceus* together.

Correct use of bacterial inoculants requires an understanding of the specific purposes of different inoculants. As a general rule, homolatic bacteria inoculants are used to improve silage fermentation, whereas heterolactic bacteria inoculants are used to increase aerobic stability. This general concept was illustrated by the work of Tabacco et al. (2011) who compared effects of applying homolactic *L. plantarum* or heterolactic *L. buchneri* to corn and sorghum silages. Silages inoculated with *L. buchneri* had higher pH, lower lactic acid concentration, greater acetic acid concentration and DM loss (0.8%), and greater aerobic stability (690% and 572% greater, respectively) than those in untreated silages. The DM losses during 14 d of aerobic exposure were 15% for *L. buchneri* - treated corn silage versus 44 and 42% for Control and *L. plantarum* –treated silages.

The complementary roles of homolactic and heterolactic bacteria in silage fermentation have led to the development of inoculants containing both types of bacteria in order to improve the fermentation and aerobic stability of the silage. Such 'Dual-purpose' or 'Combo' inoculants have been used successfully to improve the preservation of corn, alfalfa, sorghum, and bermudagrass silage (Filya, 2003; Schmidt et al., 2009; Schmidt and Kung, 2010). Kleinschmit and Kung (2006a) reported that corn silages treated with a mixture of *L. buchneri* (4×10^5 cfu/g) and *P. pentosaceus* (1×10^5 cfu/g) had lower concentration of NH_3, greater concentration of acetate, and greater aerobic stability compared with the untreated silages after 361 d of fermentation. Filya (2003) showed that corn and sorghum silages treated with a dual-purpose inoculant had lower NH_3 from d 2 of ensiling until d 90 (silo opening). Inoculation also increased

acetate concentration in both silages and thereby reduced the yeast population and increased aerobic stability.

Chemical Additives

Chemical additives are applied to silage to totally or partially reduce microbial activity during fermentation or feed out, therefore they can be classified as inhibitors of fermentation or enhancers of aerobic stability.

Fermentation inhibitors

Sulfuric acid: Inorganic acids have been used as silage additives since the late 1800s (Watson & Nash, 1960), however their use is declining due to their hazardous and extremely corrosive potential (Kung et al., 2003). The inorganic acids most frequently used for forage preservation are sulfuric, hydrochloric, and phosphoric acids, which are very strong acids capable of decreasing silage pH immediately after application. These acids do not have any specific antimicrobial activity rather their mode of action is direct acidification (Woolford, 1978). Due to their lack of antimicrobial activity, low to moderate rates of application (<2L/ton of fresh weight) are not effective at eliminating microorganisms tolerant to acidic conditions such as yeasts and coliforms (Chamberlain & Quig, 1987). The latter authors noted that applying 0, 2, 4, and 6 L of sulfuric acid per ton of fresh weight of perennial ryegrass (*Lolium perenne L.*) decreased pH, and lactic, acetic, and butyric acids and also increased residual WSC and ethanol concentrations. The increase in ethanol was due to the presence of yeasts capable of surviving pH lower than 3.5.

The effect of sulfuric acid and other inorganic acids on animal performance is inconsistent. O' Kiely et al. (1996) reported that DM intake (DMI) and live-weight gain increased in beef cattle fed grass silage treated with 2.3 L/t of sulfuric acid instead of

the untreated silage. However, O ‘Kiely (1996) reported that no improvement in weight gain occurred when beef heifers were fed grass silage treated with 2.5 L/t of sulfuric acid. Reasons for such discrepancies are unclear.

Formic acid: Formic acid is an organic acid that inhibits fermentation by promoting acidification of silage and by conferring specific antimicrobial activity. Formic acid has a pronounced inhibitory effect against *Clostridia* and *Enterobacteria*, but yeasts are more tolerant to the acid (McDonald et al., 1991). Formic acid is stronger than other organic acids frequently found in silage such as propionic, lactic, acetic, and butyric acids but it promotes a moderate rate of acidification compared with inorganic acids (Kung et al., 2003). Thus, it is common to use mixtures of formic acid with inorganic acids for forage preservation (Kung et al., 2003). Formic acid application is typically associated with decreased production of fermentation products like lactic, acetic, and butyric acids, increased residual WSC concentration, and reduced ammonia nitrogen concentration (Barry et al., 1978). Waldo et al. (1978) reported that DMI (9.7 vs. 8.6 kg/d) and milk yield (17.7 vs. 17.1 kg/d) increased when dairy cattle were fed formic acid-treated ryegrass silage instead of untreated silage. Keady and Murphy (1996) demonstrated that formic acid-treated ryegrass silages had lower ammonia nitrogen concentration (43 vs. 61 g/kg of total N), greater aerobic stability, and lower DM digestibility than the untreated silage. These authors detected no difference in DMI or milk yield due to inoculation, however milk protein and fat concentrations were greater in cows fed formic acid-treated silage. Formic acid treatment does not frequently increase aerobic stability of silages because yeasts can survive its acidifying and antimicrobial effects (McDonald et al., 1991).

Enhancers of aerobic stability

Propionic acid: Propionic acid is an organic acid that is used as a silage additive primarily because of its strong antimycotic effects. Propionic acid is fungicidal or fungistatic but these properties are only exhibited by the undissociated form, which increases proportionally as pH decreases (Lambert and Stratford, 1999). Inhibition of the growth of yeasts and molds by this acid concomitantly increases aerobic stability because these organisms are responsible for initiating and promoting silage spoilage, respectively. Kung et al. (1998) applied 0.1 or 0.2% of propionic acid to whole-plant corn and reported that the only effect on the fermentation was an increase in the concentration of the acid, whereas the treatment increased aerobic stability by more than 90 hours. Kung et al. (2004) reported that silages treated with 0.1 or 0.2% of a propionic acid-based additive had a 10 to 100 fold decrease in yeast counts and increases in aerobic stability of 39 and 57 h, respectively.

Combining propionic acid with other acids can result in a synergistic inhibition of the growth of yeasts and molds (Moon, 1983). Jung (1972) showed that a mixture of propionic and formic acids resulted in better preservation of corn silage than the use of propionic acid alone. Silage containing both acids had greater aerobic stability and lower protein degradation than silage containing the individual acids. Kung et al. (1998) reported that a combination of propionic acid and other silage additives such as acetic, benzoic and citric acids, ammonia, and sorbate resulted in increased aerobic stability of corn silage by of 278 h.

Benzoic acid: Benzoic acid also enhances aerobic stability by its antifungal effect (Woolford, 1975). Pedroso (2003) applied 0.5 to 2.0 g/kg of sodium benzoate to sugarcane, which is well known for its ethanolic fermentation due to high WSC

concentrations that predispose to the growth of spoilage and ethanol-producing yeasts. Benzoic acid treatment increased in vitro DM digestibility (IVDMD; 45.4 vs. 49.8%DM) and aerobic stability (65 vs. 79h), and decreased ethanol concentration (3.83 vs. 2.52%DM). Pedroso et al. (2006) showed that heifers fed sugarcane silage treated with 0.1% of benzoate had greater feed efficiency than heifers fed untreated silages (7.6 vs. 9.4 kg of DM/kg of live weight). Lattemae and Lingvall (1996) reported that grass silage treated with sodium benzoate had less fungal counts and greater aerobic stability than the untreated silage (Lattemae & Lingvall, 1996).

Urea and ammonia: Urea is frequently classified as a nutrient silage additive because it is a source of nitrogen for ruminal fauna, however the main purpose of treating silage with urea is to obtain ammonia, which enhances aerobic stability via its alkalinity. Urea contains 46% nitrogen and it can be converted to ammonia by urease enzymes in silage. The main reasons why producers use urea instead of ammonia for forage preservation are the equipment needed, expenses involved, and danger associated with application of ammonia. Once the urea is transformed into ammonia, the caustic and antimycotic effect of ammonia curtails fungal growth because in alkaline environments, uncharged NH_3 diffuses through fungal membranes and kills the microorganism by metabolic poisoning (Kung et al., 2003). The same authors reported that in general, ammonia-treated silages have a higher pH, lower lactic to acetic ratio, lower residual water-soluble carbohydrates, and greater nitrogen, aerobic stability and digestibility than untreated silages. The reason why digestibility is increased by ammoniation is that the alkali hydrolyzes linkages between hemicelluloses and lignin in the plant cell wall. However, ammoniation can reduce the rate of pH decrease in silage.

This factor coupled with the alkalinity can favor the growth of *Enterobacteria* and *Clostridia* that cause secondary fermentation. A further problem is that under high temperatures, ammonia reacts with sugars in forages to form 4-methylimidazole, which causes hyperexcitability in cattle (Kerr et al., 1987).

Mode of Action of Bacterial Inoculants

Acid-Induced Inhibition

The main function of bacterial silage inoculants is to produce organic acids, which reduce the pH and or inhibit the growth of spoilage-causing fungi. Homofermentative bacteria are primarily involved in the former and are considered stimulators of fermentation whereas, heterofermentative bacteria are involved in the latter and classified as aerobic stability enhancers. Adding homofermentative LAB considerably increases the lactic acid concentration of silage. Although lactic acid has weak antifungal effects, it is a strong acid that can lead to rapid acidification of the silage because of its low dissociation constant (3.08; Madigan et al., 2009). Acidification to pH of 4 or below is recommended in silages because most undesirable microbes are less active at this pH range (Kung et al., 2003). Lindgren et al. (1985) reported that the following pH values restrict microbial activity: 4.6 for *Enterobacteria*, 4.2 to 5.0 for *Clostridia*, 4.5 to 5.0 for Bacilli, 5.0 for Streptomyces and 2.0 for yeasts and molds. Therefore, acidification of silage to pH of 4 inhibits all undesirable microorganisms except yeasts and molds

Antifungal Inhibition

The ability of yeasts to survive acidic environments allows them to worsen silage fermentation and to initiate aerobic spoilage. This is because these organisms can make the fermentation ethanolic instead of homolactic and they can metabolize lactic

acid, thereby increasing silage pH to the threshold that is conducive for other spoilage and pathogenic microbes (Pedroso et al., 2005; Gonzalez-Pereyra et al., 2008). The inability of lactic acid or even strong inorganic acids to prevent the growth of fungi in silages suggests that mechanisms other than pH reduction should be used to inhibit their growth (Chamberlain and Quig, 1987; Kung et al., 2003). Consequently, heterofermentative bacteria that synthesize antifungal acids like propionic and acetic acid (Moon 1983) are being used increasingly to inhibit spoilage fungi.

The antifungal effect of relatively weak acids like propionic and acetic acid is caused mainly by their undissociated form, which can diffuse through the plasma membrane of fungi into the cytoplasm (Lambert and Stratford, 1999). Once inside the cell, the neutral intra-cytoplasmic pH causes the acid to dissociate into charged ions. To avoid accumulation of hydrogen ions and cytoplasmic acidification, the cell utilizes a H^+ATPase pump to remove the excess ions. Expenditure of energy for this process reduces the energy available for growth and multiplication of the fungi (Lambert and Stratford, 1999). The proportion of free undissociated acid is dependent on the extracellular pH and on the acid dissociation constant (pKa). The pKa of acetic and propionic acid is 4.8, which implies that in well-made silages with a pH of about 4, more than 50% of ions should return to the undissociated form and allow the cycle described by Lambert and Stratford (1999) to continue. The percentage of undissociated acids increases as the pH drops, indicating that combining strong acids with acetic and propionic acid would intensify their antifungal action. Inoculants containing combinations of heterolactic and homolactic bacteria exploit this concept (Kung et al., 2003) as do mixtures of strong and weak acids.

Bacteriocin-Mediated Inhibition

In addition to the modes of action described above, silage inoculants may also contain bacteriocins, which are small proteins that inhibit or kill closely related bacteria species or even different strains of the same species (Yildirim, 2001). Bacteriocins synthesized by one bacterial strain can bind to specific receptors on the membrane of susceptible cells. The receptors are proteins whose normal function is to transport substances, growth factors, or micronutrients from the outer membrane (Madigan et al., 2009). Bacteriocins also can have deleterious effects on DNA and RNA of target cells. *Lactobacillus buchneri* for example produces buchnericin, a bacteriocin that inhibits the growth of select species of *Listeria, Bacillus, Micrococcus, Enterococcus, Streptococcus, Lactobacillus, Leuconostoc*, and *Pediococcus* genera (Yildirim, 2001). Buchnericin is bactericidal because it makes susceptible cells lose high amounts of intracellular potassium ions and become more permeable to o-nitrophenol-β-D-galactopyranoside (Yildirim et al., 2002). Sparo et al. (2006) characterized enterocin MR99, a bacteriocin produced by *Enterococcus* and isolated from corn silage, which has bactericidal effects on strains of *Listeria monocytogenes*, *Staphilococcus aureus*, and bovine mastitis agents and it had bacteriostatic effects on *E. coli*. Marcinakova et al. (2008) inoculated grass silage with a bacteriocin-producing strain of *Enterococcus faecium* and reported that the population of *Listeria* species was reduced after 105 days of ensiling. However, it was not clear whether the latter was caused by the pH decline during ensiling or by the bacteriocin itself.

In general, little is known about effects of bacteriocins on silage but the limited data available indicates that bacteriocins from silage inoculant bacteria can inhibit

pathogenic bacteria (Kung et al., 2003). However, the effects of bacteriocins on silage fermentation and aerobic stability have not been properly characterized.

Other inhibitors of undesirable microorganisms in silage produced by inoculant bacteria include benzoic acid and mevalonolactone, which is produced by *Lactobacillus plantarum* (Niku-Paavola et al., 1998). The authors observed that the combination of these compounds produced by *L. plantarum* and lactic acid had synergistic effect to reduce *Fusarium* species. Sorbic, formic and nitric acids or their salts also have inhibited undesirable organisms in other studies (Kung et al., 2003).

Pathogenic Agents in Silage

Pathogenic microorganisms can be found in silage and some produce toxic compounds. The presence of such organisms in silage is due to their tolerance of acidic conditions and inadequate management during the fermentation or aerobic exposure phases. Poor management of silage during the latter phase causes aerobic spoilage, which can predispose to the growth of opportunistic pathogenic microorganisms such as *Listeria and Bacillus* species. Pathogenic organisms such as *Clostridia* and *Enterobacteria* may also thrive if silage is poorly managed during the initial aerobic phase and the subsequent anaerobic phase of ensiling.

Bacillus

Bacillus are gram positive, facultatively anaerobic, sporulating bacteria. Their growth can be decreased by environmental factors such as low water activity (<0.935) and pH (<4.8) and high temperature (>30^{o}C) (Quintavalla and Parolari, 1993) but their endospores tolerate harsh environmental temperatures. *Bacillus cereus* is particularly notorious because its spores can pass through the digestive tract intact, contaminate the milk of dairy cows, survive pasteurization temperatures, and decrease the shelf life

of milk and cream (Christiansson et al., 1999; Pahlow et al., 2003). Furthermore, enterotoxins produced by this bacteria cause foodborne illnesses, notably emesis and diarrhea (Ankolekar et al., 2009). Diarrheal types, also cause abdominal pain and are more commonly associated with milk from contaminated silage. Bacillus can dominate other silage microorganisms in certain instances such as under high temperatures (Lindgren et al., 1985), in big bale silages, or after treatment with formaldehyde (Barry et al., 1978) or antibiotics (Woolford et al.,1982). They are also more common in corn and small-grain cereal silages (McDonald et al., 1991; Adesogan and Quieroz, 2009). Bacillus spp. are unable to initiate aerobic deterioration of silages, however they can be present at later stages after yeasts indirectly increase the pH by metabolizing lactate (Holden, 1989). However, *Bacillus* spp. also can produce bacteriocin – like substances that inhibit the growth of yeasts and thereby increase aerobic stability of silages (Pahlow et al., 2003). Goodman et al. (1995) demonstrated that *B. subtilis* and *B. licheniformis* are capable of produce zymocin, a bacteriocin that could impair the growth of yeasts and molds. However, factors that determine whether Bacillus spp. decrease or enhance aerobic stability are unclear.

Listeria

Listeria are opportunistic gram-positive bacteria that cause high mortality rates and a wide range of diseases in immunocompromized animals and humans including meningitis, encephalitis, septicemia, gastroenteritis, mastitis and abortions (McDonald et al., 1991; Adesogan and Queiroz, 2009). Listeria monocytogenes (formerly called Bacterium monocytogenes) is the main causative agent and the main source of contamination of ruminants in spoiled silage (Wiedman, 2003) although some others strains such as *L.* ivanovii can infect ruminants (Sleator et al., 2009). This facultatively

anaerobic bacterium is ubiquitous in nature because it can tolerate refrigeration temperatures, low water activity, and a wide range of pH above 5.0 (Tienungoon et al., 2000). *Listeria* normally resides in decaying plant matter in the soil but is also associated with the gastrointestinal tract in a number of animals. On farms it is commonly found in baled silages because of their relatively low density, high pH, and high surface area to mass ratio (McDonald et al., 1991). In well-prepared bunker silages, *L.* monocytogenes only thrives in areas exposed to a prolonged, low rate of oxygen infiltration because they can be dominated by more aerobic organisms like yeasts and molds and they don't survive the low pH conditions in well-made silage (McDonald et al., 1991). Using rybotyping techniques, Ryser et al. (1997) demonstrated that 77% of haylages with pH of 5 to 6 had some ribotype of *L.* monocytogenes or *L.* innocua. They also verified that 18% of all 210 silage samples were contaminated by the pathogen. In Britain, outbreaks of listeriosis are associated more commonly with sheep particularly around parturition because sheep are fed more commonly baled silages and cattle are more resistant though they are often asymptomatic carriers (McDonald et al., 1991; Villar et al., 2007). Fecal shedding increases the prevalence of *Listeria* infection among small ruminants but is a less effective method of recontamination in cattle. The pathogen can be transmitted from contaminated silages into milk and according to Sanaa and Ménard (1994), the presence of *L. monocytogenes* in silage increases the risk of its presence in milk by a factor of 20. Fortunately, the pathogen is destroyed by adequate pasteurization of milk but it may survive in soft cheeses and dairy products, which are not subjected to such treatments (Adesogan and Quieroz, 2009). Zoonotic transmission is also possible by the

consumption of meat and raw foodstuffs infected with the pathogen (Swaminathan & Gener-Smidt, 2007)**.**

Clostridia

Clostrida are gram positive, mostly obligately anaerobic, sporulating bacteria that thrive in silages with low WSC concentrations particularly when plant moisture (>70%) and buffering capacity and the prevailing pH (>4.6) and temperature (>30^{o}C) are high. Consequently, they often dominate the fermentation of unwilted legumes ensiled without additives (McDonald et al., 1991). Those commonly found in silage are saccharolytic types that ferment sugars and organic acids (e.g. *C. butyricum* and *C. tyrobutyricum*) and others that ferment both sugars and protein (e.g. *C. sporogenes* and *C. perfringens*), but those that ferment amino acids exclusively are uncommon in silages (Pahlow et al., 2003). Clostridial presence in silage is mainly from soil contamination or slurry application. When slurry is applied to forages before ensiling, it is common to find high Clostridial spore numbers even when the environment is not ideal for the multiplication of *Clostridia* in silages (Weissbach, 1993). Clostridium butyricum is the most frequently isolated species in silage. This bacterium and others in the same genus can considerably worsen silage fermentation (McDonald et al., 1991) because such saccharolytic *Clostridia* derive energy by fermenting sugars and lactate into butyric acid, CO_2 and H_2. Although the antifungal properties of butyric acid can enhance aerobic stability, its pungent acrid odor typically depresses intake and therefore reduces performance of ruminant livestock (Adesogan and Queiroz, 2009) .

The depletion of lactate by saccharolytic *Clostridia* increases the pH and provides a more conducive environment for growth of proteolytic *Clostridia* that deaminate and catabolize amino acids into fatty acids. Consequent increases in the ammonia

concentration and protein solubility of silages make them less suited for high producing cattle and enhance environmental pollution from livestock operations (Adesogan and Queiroz, 2009). Furthermore, biogenic amines such as cadaverine, glucosamine, histamine, putrescine, and tyramine can be produced during Clostridial proteolysis in silages (Adesogan and Queiroz, 2009). Many of these putrefaction-associated compounds are malodorous and unpalatable, therefore they reduce feed intake by livestock (Neumark, 1967; Neumark and Tadmor, 1968), but some are potentially toxic. For instance histamine is lethal at high doses and when injected intravenously at low doses, it stopped ruminal motility and eructation in sheep (Dain et al., 1955).

An added complication with *Clostridia* is that their spores can be transmitted from silage into milk or dairy products. In cheese, spores can develop unsightly outgrowths and continued butyric fermentation can lead to the formation of gas pockets that can double the size or cause cracks to appear in cheese, a phenomenon called late blowing of cheese (Cocolin et al., 2004). The large quantities of butyric acid produced result in a rancid odor and tainted flavor. *Clostridium perfringens* also causes enteric syndromes characterized by abdominal pain and diarrhea resembling *B. cereus* diarrhea (Adesogan and Queiroz, 2009).

One of the main reasons why inoculation with homolactic LAB has been successful is that it curtails Clostridial secondary fermentation by rapidly reducing the pH and increasing the concentration of bacteriocin-like substances (Pahlow et al., 2003; Pedroso et al., 2010). Arriola et al. (2011) demonstrated that a homolactic inoculant containing a mixture of *E. faecium*, *P. pentosaceus,* and *L. plantarum* was more effective at inhibiting the growth of *Clostridia* than the Control silage or silages treated

with a heterolactic inoculant containing *L. buchneri* alone, dual-purpose inoculants containing *P. pentosaceus* and either *L. buchneri* or *Propionibacteria freudenreichii* or *a homolactic inoculant* containing *L. plantarum* and *P. pentosaceus.* Yet the treatment with the greatest inhibitory effect on *Clostridia* had one of the slowest rates of pH decline, indicating that the mode of action was not based on direct acidification. Thuault et al. (1991) demonstrated that *C. tyrobutyricum* could be inhibited by substances other than hydrogen peroxide and lactic acid from LAB. Other treatment methods to curtail the growth of *Clostridia* include using reduced oxygen permeability film to cover bunker silos (Borreani and Tabacco, 2008), wilting forages, and using chemical additives such as nitrite (Pahlow et al., 2003).

Enterobacteria

Enterobacteria are gram negative facultatively anaerobic bacteria. Epiphytic *Enterobacteria* including *Erwinia herbicola* and *Rahnella aquitilis* often dominate fresh crops, but these are superseded by others like *Escherichia coli, Hafnia alvei*, and *Serratia fonticola* during ensiling (Driehuis and Elferink, 2000). Although *Enterobacteria* actively compete with LAB in the early stages of ensiling, they are inhibited once the pH drops below 4.5 (Pahlow et al., 2003). Those that survive ensiling can start growing actively when the pH increases after aerobic deterioration (Driehuis and Elferink, 2000). Like *Clostridia, Enterobacteria* deaminate and decarboxylate amino acids in silages, thereby enhancing ammonia and biogenic amine production and increasing the risk of depressed intake and ineffiecient N utilization by livestock.

Escherichia coli O157:H7, a shigatoxin producing gram-negative bacteria is the most notorious of the *Enterobacteria*. It has emerged as an important cause of food borne disease. In children and the elderly, it initially causes acute bloody diarrhea but

this may evolve into hemolytic uremic syndrome, a severe illness characterized by anemia and kidney failure. Cattle are the main reservoir of *E. coli* O157:H7 and the pathogen may be present in feces, milk, and feed of dairy cows (Armstrong et al., 1996; Mechie et al., 1997; Chapman et al., 1997; Lynn et al., 1998). Silage can be contaminated with *E. coli* O157:H7 via manure or irrigation water (Weinberg et al., 2004) but the pathogen disappears from contaminated silages when the pH drops below 4 - 5 (Bach et al., 2002; Chen et al., 2005; Pedroso et al., 2010). However, the pathogen has been found in decaying commercial silages with relatively high pH values and it survived for three weeks in grass silages (pH 4 to 4.6) contaminated with the pathogen (Reinders et al., 1999). Therefore, it is critical that silage pH is kept below 4 during and after ensiling to prevent the growth of the pathogen.

Importance of Mycotoxins in Silage

Mycotoxins are secondary metabolites secreted by molds mostly belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genera (Yiannikouris and Jouany, 2002). The ubiquitous nature of mycotoxins and the severity of their effects on human health make them a major food safety concern. The Food and Agriculture Organization (FAO) estimates that 25% of all crops are contaminated with mycotoxins (CAST, 1989). 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 $1.4 billion (CAST, 2003). The social importance of mycotoxins is evident from the 25,000 to 166,000 new cases of liver cancer annually caused by aflatoxins (Liu and Wu, 2010).

Mycotoxins also negatively affect domestic animals causing suppression of the immune system, imbalance of hormonal function, and reduction of nutrient utilization,

which result in decreased performance and eventually death (Whitlow and Hagler, 2005). For the farm system, the costs associated with feeding animals contaminated diets occur in the form of poor animal performance and milk disposal due to high concentration of toxins (Applebaum et al., 1982; Masoero et al., 2007; Kutz et al., 2009). Milk with aflatoxin concentrations above 0.5 µg/kg are considered illegal to be marketed in USA, Brazil, Argentina, and countries belonging to the southern common market, whereas in the European Union, the maximum permissible concentration is 0.05 µg/kg (FDA, 2000; ANVISA, 2002; EFSA, 2004).

Anaerobiosis and low pH inhibit the growth of most fungi, thus ensiling is an effective strategy to prevent the growth of molds and mycotoxin production from many field and storage fungi. However, these conditions provide a more conducive environment for the growth of acid- tolerant and low oxygen-tolerant species, such as *Penicillium roqueforti*, *Aspergillus fumigatus*, *Byssochlamys nivea*, and various *Fusarium* species (Pahlow et al., 2003). Gonzalez-Pereyra et al. (2008) noted that inadequate management of silage can impair fermentation, promote aerobic conditions within the silage mass, and favor the growth of fungi that are normally less tolerant of acidic or anaerobic conditions such as *Aspergillus flavus*. Furthermore, once silos are opened, the ensuing aerobic conditions often allow the growth of spoilage-causing, yeasts, which metabolize lactate to CO_2 and thereby increase the silage pH. This elevated pH predisposes toxigenic fungi to grow actively during the feed-out phase, particularly in poorly managed silages.

Mycotoxin contamination is a ubiquitous problem due to the high adaptive capacity of toxigenic fungi, which allows them to proliferate in all stages of feed production such

as on the field and at harvest, storage, and feed out. Nevertheless, various techniques can be used to prevent or at least minimize contamination of feeds with mycotoxins and effective detoxification methods also exist.

Effects of Specific Mycotoxins

More than 400 mycotoxins are known to occur naturally, however, only a few of them have been extensively studied (Whitlow and Hagler, 2005). Mycotoxins that are frequently present in silages and feed ingredients include deoxynivalenol (DON), zearalenone (ZEA), fumonisin, and roquefortine C (Driehuis et al., 2008). Aflatoxin can also be present in silages made in hot and humid environments.

Deoxynivalenol

Deoxynivalenol is a toxin produced by *Fusarium* species such as *F. graminearum, F. sporotrichioides, F. culmorum, F. poae, F. roseum,* and *F. tricinctum*. Deoxynivalenol is also known as vomitoxin because it tends to cause emesis in swine. Other than vomiting, DON causes feed refusal, diarrhea, reproductive problems, and eventually death. The effect of DON in dairy cattle is not well established but decreases in animal performance in dairy herds have been associated with the toxin (Whitlow et al., 1994). Deoxynivalenol also has been related to altered rumen fermentation (Seeling et al., 2005) and reduced flow of protein to the duodenum (Danicke et al., 2005). In a survey on the presence of mycotoxins in feed ingredients, Driehuis et al. (2008) reported that corn silage was the main source of DON and ZEA in diets of dairy cattle. These authors reported an average concentration of DON in corn and grass silage samples of 550 µg/kg and a maximum concentration of 1,250 µg/kg. They estimated that these high DON concentrations could result in intakes of 8.4 µg of DON/kg of BW or 5 mg per cow/day. After reviewing several studies, DiCostanzo et al. (1995) concluded that beef

cattle are able to tolerate up to 21 mg/kg of DON. Charmley et al. (1993) demonstrated that a contamination level of 6 mg of DON /kg of diet DM did not adversely affect milk yield or cause carry-over of the toxin into milk. Data available on adverse effects of DON on dairy cattle is limited, and insufficient to allow the establishment of a maximum tolerance level, however the guidance value for DON is 5 mg/kg in Western Europe (European Commission, 2006; Driehuis et al., 2008) and the advisory guidelines stipulated by the Food and Drug Administration (FDA) in the US are 10 and 5 mg/kg DM of diet for beef and dairy cattle, respectively (FDA, 2010). The concern about the intake of DON by dairy cattle is related to its potential negative effect on animal health and production. However, due to the low transfer of this toxin from the diet to milk, DON is not considered a contaminant that reduces the safety of dairy products (EFSA, 2004).

Fumonisin

Fumonisins are mainly produced by two species of *Fusarium*, *F. verticilloides* (formerly *F. moniliforme*) and *F. proliferatum* (Whitlow and Hagler, 2005). The structural formula of fumonisin is similar to sphingosine, which is a component of sphingolipids that abound in nerve tissue. The toxicity of this mycotoxin results from interruption of sphingolipid biosynthesis, therefore it causes paralysis, nervousness, and ataxia in horses, and pulmonary edema in swine (Marasas et al., 1988; Ross et al., 1990; Diaz and Boermans, 1994). The toxin tends to be less aggressive in ruminants than monogastrics, however fumonisin has been shown to be hepatoxic and nephrotoxic to calves fed 1 mg/kg of body weight of the toxin (Mathur et al., 2001). The later authors reported hepatocellular apoptosis and renal tubular necrosis within 7 d of dosing the toxin. Similar results were found in a study with beef calves supplemented with 148 mg/kg of total fumonisin in the diet for 31 d (Osweiler et al., 1993). Diaz et al. (2000)

demonstrated that dosing of 100 mg/kg of fumonisin reduced feed intake and milk yield in dairy cattle. Nevertheless, carry-over of the toxin into milk is minimal (Scott et al., 1994).

Gonzalez-Pereyra et al. (2008) reported that fumonisin levels in corn silage varied from 340 to 2490 µg/kg of DM. These authors also reported that samples from different locations in the silo had different concentrations of the toxin and those from the top layer and sidewalls had high pH values, which could favor development of the toxin. *Fusarium* species tolerate low winter temperatures and colonize crop residues such as maize stalks and rice and wheat stubble, and these become major sources of inocula as temperatures increase in early spring (Binder, 2007). Fungal spores also become airborne during the rainy season and can travel long distances causing mycotoxin contamination epidemics (Binder, 2007; Lanier et al., 2010). The FDA advisory guideline for fumonisin is 15 mg/kg of DM of diet for lactating dairy cows (FDA, 2010).

Zearalenone

Zearalenone is an estrogenic metabolite produced by several species of *Fusarium* such as *F. graminearum, F. culmorum,* and *F. crookwellense* (Saeger et al., 2003). Zearalenone can cause numerous reproductive problems including hyperestrogenism, mammary gland enlargement, and vaginitis (Diekman and Green, 1992). Ruminants are less susceptible than pigs or chickens due to conversion of ZEA to its hydroxyl-metabolites, α and β zearalenol, by ruminal flora (Kiessling et al., 1984; Kennedy et al., 1998). Although α-zearalenol is three to four times more estrogenic than ZEA, its lower rate of absorption and interconversion to β zearalenol in the liver helps to decrease negative effects of ZEA (Fink-Gremmels, 2008). Despite their lower susceptibility to this toxin, studies report that high intakes of the toxin may negatively affect dairy cattle. For

instance, conception rates were decreased by 25% in dairy heifers receiving 12.5 mg/kg of ZEA (Weaver et al., 1986).

Whitlow and Hagler (2005) reported a 30% incidence of ZEA contamination in 461 corn silage samples in the US and the average contamination concentration was 525 µg/kg of DM. 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 silage contained 660 µg/kg of ZEA and alfalfa contained up to 79.80 mg/kg. The main concerns with this toxin focus on its negative effect on animal health and reproduction because like fumonisin the degree of transfer into milk is considered negligible (Seeling et al., 2005). There are no zearalenone action limits, guidance, or advisory levels established by FDA at this time. The guidance value for zearalenone in Europe is 500 µg/kg (European Commission, 2006).

Aflatoxin

Aflatoxin is a mutagenic, carcinogenic, and toxic secondary metabolite produced *Aspergillus flavus*, *A. parasiticus and A. nomius* (Creppy, 2002). Due to the ability of *Aspergillus* to grow over a broad range of temperatures and humidities, aflatoxin is a ubiquitous contaminant of food and feed ingredients worldwide (Phillips, 1999). Feeding aflatoxin-contaminated diets to lactating cows reduces their health and performance and also causes transfer of the toxin to milk and dairy products (Diaz et al., 2004).

The symptoms associated with aflatoxin ingestion or inhalation include inappetence, lethargy, ataxia, enlargement of the liver, liver cancer decreased rumen motility, and reduced feed efficiency and milk production (Mathur et al., 1975; Guthrie and Bedell, 1979; Whitlow and Hagler, 2005). Aflatoxin also reduces the immune

response to opportunistic diseases and interferes with the efficiency of vaccines (Diekman and Green, 1992; Marin et al., 2002). However, these symptoms are not specific to aflatoxicosis, which makes precise diagnosis of the condition difficult (Coulombe, 1993). Garret et al. (1968) demonstrated increased liver weight in beef cattle fed a diet containing 100 µg/kg of aflatoxin. Similar concentrations also decreased animal production and compromised animal health (Patterson and Anderson, 1982). Applebaum et al. (1982) reported that high-producing animals were more sensitive to diets contaminated with impure aflatoxin than those contaminated with pure aflatoxin B_1 (AFB_1).

The incidence of aflatoxin is relatively low in silages compared to other mycotoxins, probably due to low tolerance of *Aspergillus flavus* and *A. parasiticus* to the acidic and anaerobic silage environment. However, high concentrations of aflatoxin can occur in poorly made or managed silages or in silages made with diseased corn plants (Queiroz et al., 2009). Gonzalez-Pereyra et al. (2008) reported an aflatoxin B_1 (AFB_1) concentration of 156 µg/kg of DM in corn silage stored in a trench type silo without proper sealing. Richard et al. (2009) observed aflatoxin concentrations of up to 60 µg/kg of DM in corn silage. Aflatoxin also tends to be more common in silages made in hot, humid areas, which facilitate the growth of the fungal source of the toxin.

The rate of aflatoxin transfer to the milk varies from 1 to 6% (EFSA, 2004). Because of the severity of aflatoxin effects on human health, it is the only one of the 400 known mycotoxins that has an Action Level regulated by governmental agencies. The aflatoxin Action Level established by the FDA for fluid milk is 0.5 µg/kg and it is 20 µg/kg for feed ingredients offered to dairy cattle.

Mycotoxin Detoxification Methods

Despite the existence of several notable advances in pre and post-harvest fungal inhibition technologies, feed contamination with mycotoxins is still very common. Silage traditionally represents a significant proportion of the diet of dairy cows and its year-round, widespread use in some countries and seasonal use at times of low pasture availability in others, emphasize the importance of ensuring that mycotoxin contamination of silage is minimized. Yet no method effectively or completely detoxifies silages contaminated with mycotoxins. However, numerous products that improve aerobic stability via their antimycotic action may indirectly decrease the risk or level of mycotoxin contamination of silage.

Dilution of contaminated ingredients is an effective strategy for detoxifying contaminated grains. However, it is less appropriate for contaminated silage because it would probably reduce the feed-out rate and cause slower utilization of the silage on the silo face. These factors could lead to further mold growth and intensification of the contamination problem (Whitlow and Hagler, 2005). Contaminated grains can also be detoxified through biological, physical, or chemical post-harvest methods but for silage, the most practical and effective detoxification strategy is perhaps by using enterosorbents.

Enterosorbents

Enterosorbents are substances that can bind to toxins in the gastrointestinal tract of animals, reducing their bioavailability and associated toxicities (Philips, 1999). They represent an alternative method to avoid the inaccessibility or high cost of physical detoxification methods and the hazards of chemical methods.

Hydrated sodium-calcium aluminosilicates (HSCAS) are clay-based products that form a stable complex with AFB_1, which cannot cross the luminal membrane in the gastrointestinal tract (Spotti et al., 2005). In vitro evaluation of the binding capacity of HSCAS demonstrated that 80 to 99% of aflatoxin present in various solutions (buffer, water, or rumen fluid) was bound to the clay (Moschini et al., 2008). In vivo studies with HSCAS have shown less complete, but nevertheless useful levels of detoxification. Diaz et al. (2004) evaluated different sequestering agents and reported a wide range of efficacy at reducing AFM_1 in milk (31 to 65%) when a diet containing 100 µg/kg of AFB_1 was fed to lactating dairy cows. Kutz et al. (2009) reported a 44 to 48% aflatoxin reduction in milk when mycotoxin binders were used in the diet. These results emphasize the importance of carefully selecting and screening potential sequestering agents before evaluating them in animals under practical farming conditions. Satisfactory decontamination has been achieved with the use of inorganic sequestering agents such as HSCAS but these have discriminating affinity to aflatoxin, having no effect on other toxins. For instance, the inclusion of HSCAS in diets has not changed the estrogenic effect of zearalenone (Bursian et al., 1992). When used at 0.5 or 1% of the diet, HSCAS did not mitigate the negative effects of DON on the daily gain of nonruminant animals (Patterson and Young, 1993).

Other types of enterosorbents such as activated carbon, glucomannan, and peptidoglycans also have been evaluated. Diaz et al. (2004) studied the effect of bentonites, esterified glucomannan (yeast cell wall), and activated carbon as sequestering agents to reduce AFB_1 absorption and transfer to milk. The authors reported that esterified glucomannan fed at 0.05% of diet DM was similarly effective as

sodium bentonites fed at 1.2%. Both products decreased milk aflatoxin concentration by 58.5% and 64.6%, respectively. In the same experiment, activated carbon showed no significant reduction in aflatoxin M1. The results of using these binders are fairly dependent on the extent of aflatoxin contamination in the diet. For instance, the same esterified glucomannan product that reduced milk AFM_1 concentration when dietary AFB_1 concentration was 55 µg/kg of diet DM (Diaz et al., 2004) had no effect on milk aflatoxin when dietary concentration of AFB_1 was 100 µg/kg of diet (Kutz et al., 2009). Cholestyramine, an insoluble quaternary ammonium anion exchange resin was used (2.5g/kg) to decrease the toxic effect of 6 mg/kg of zearalenone in mice (Underhill et al. 1995)**.** Avantaggiato et al. (2005) stated that cholestyramine effectively binds to zearalenone and fumonisins. The authors also reported that activated carbon was the only absorbent, out of 21 products, capable of binding deoxynivalenol and nivalenol.

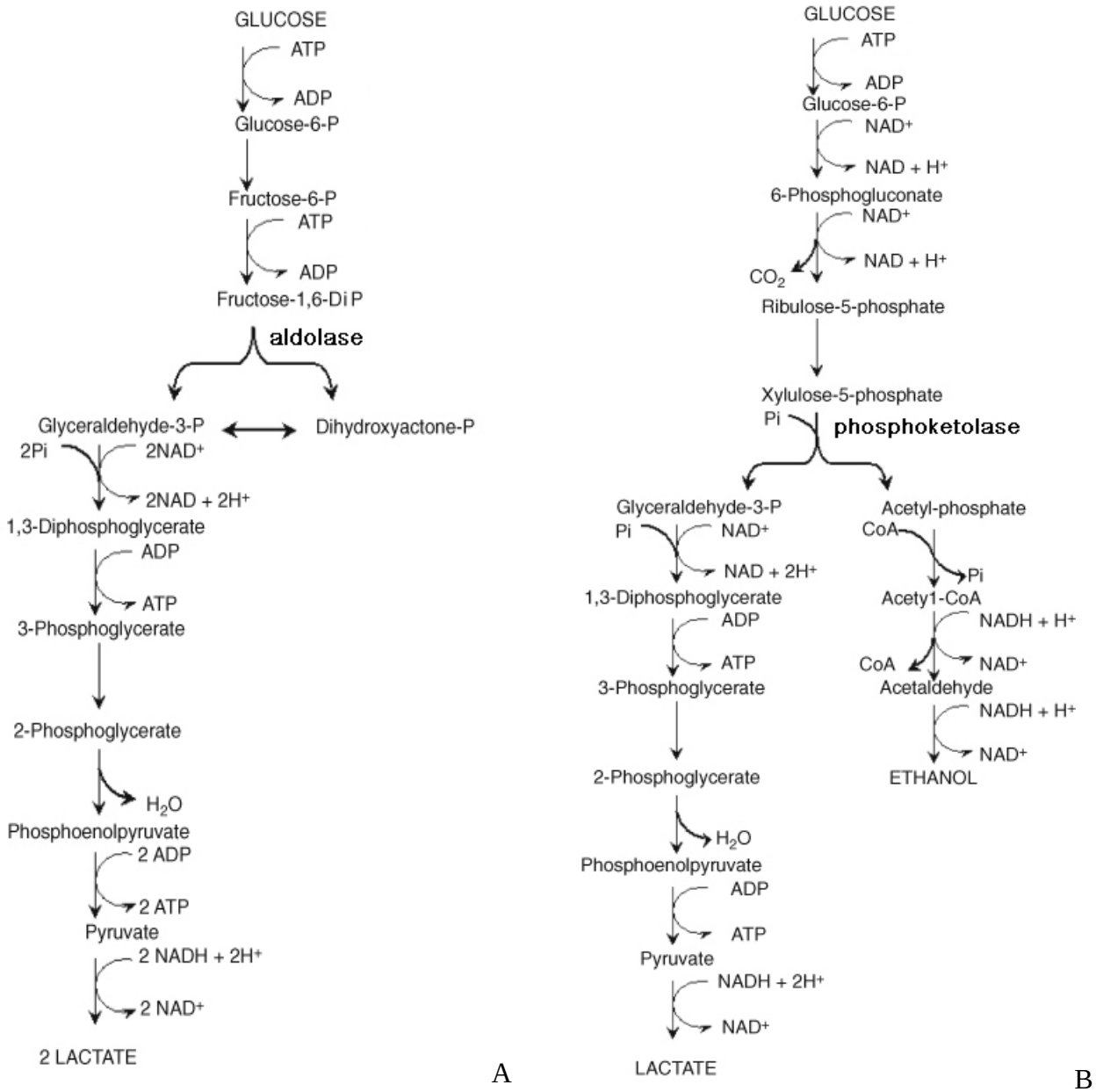

Figure 2-1. Homofermentative (A) and heterofermentative (B) pathways for fermentation of glucose to lactate in silage (Reprinted with permission from Dr. Kenneth Todar; Todar, 2011).

CHAPTER 3
CONTROL OF E. COLI O157:H7 IN CORN SILAGE WITH OR WITHOUT VARIOUS INOCULANTS: EFFICACY AND MODE OF ACTION

Introduction

Escherichia coli O157:H7, a shigatoxin-producing gram-negative bacteria, has emerged as an important cause of food-borne diseases since it was first isolated in 1982 (Riley et al., 1983). Cattle are the main reservoir of *E. coli* O157:H7 and the pathogen may be present in feces, milk, and feed of dairy cows (Mechie et al., 1997; Chapman et al., 1997; Lynn et al., 1998). Corn silage is one of the most widely used components of dairy cow rations and it can be contaminated with *E. coli* O157:H7 via application of manure or irrigation water (Weinberg et al., 2004). *Escherichia coli* O157:H7 was eliminated from ensiled, contaminated wheat and corn forage when the pH decreased below 5.0 (Chen et al., 2005). Elimination of the pathogen has been hastened by treatment with a bacterial inoculant that accelerated the pH decline (Bach et al., 2002). Weinberg et al. (2004) also confirmed that ensiling is effective in eliminating *E. coli* sp. in contaminated forages but noted that these bacteria may develop in spoiled silages, which often have high pH. To date, no studies have examined if bacterial inoculants that increase aerobic stability and maintain low pH after aerobic exposure such as *L. buchneri* (Filya et al., 2003; Adesogan et al., 2004; Huisden et al, 2009) can also prevent the growth of *E. coli* on aerobically exposed silages.

Many lactic acid and propionic acid-producing bacteria produce substances with antibacterial activity such as peroxides and bacteriocins (Jack et al., 1995; Meile et al., 1999). Gollop et al. (2005) reported that antibacterial activity independent of pH was present in many LAB-based inoculants but the antibacterial activity was not imparted to

some of the silages treated with the bacteria. Little is known about the antibacterial activity of bacterial silage inoculants against *E. coli* O157:H7.

The first objective of this study was to evaluate the effectiveness of bacterial inoculants containing homofermentative or heterofermentative bacteria, and both types of bacteria, at controlling *E. coli* O157:H7 in corn silage during the anaerobic and aerobic stages of silage production. A second objective was to examine if pH-independent antibacterial activity against *E. coli* O157:H7 existed in the inoculants and persisted in inoculated silages.

Materials and Methods

Harvesting, Inoculation and Ensiling

The experiment was conducted at the Department of Animal Sciences, University of Florida from October 2007 to July 2008. Corn forage was harvested at the 50% milk line stage (30% DM) with a forage harvester (Claas Jaguar 900, Claas of America LLC., Columbus, IN) adjusted to achieve a 19-mm theoretical length of cut. The following treatments were applied in triplicate to the forage: 1) distilled water (Control); 2) 5×10^5 cfu/g of *E. coli* O157:H7 (ATCC 43894 - EC); 3) EC and 1×10^6 cfu/g of *Pediococcus pentosaceus* 12455 and *Propionibacterium freudenreichii* (EC+BII); 4) EC and 1×10^6 cfu/g of *Lactobacillus buchneri* 40788 (EC+LB); 5) EC and 1×10^6 cfu/g of *L. buchneri* 40788 and *P. pentosaceus* 12455 (EC+B500). The *E. coli* O157:H7 was supplied by ABC Research Corporation (Gainesville, FL), whereas bacteria in treatments 3, 4, and 5 were commercial inoculants, Biotal Plus II® (BII), *Buchneri* 40788 (LB), and *Buchneri* 500 (B500), respectively (Lallemand Animal Nutrition, Milwaukee, WI). The *E. coli* O157:H7 was grown in trypticase soy broth (Becton, Dickinson and Co., Sparks, MD, USA) for 24 h at 37°C. Bacteria were sedimented by centrifugation (3000 × g for 10

min), washed three times, and resuspended in buffered peptone water (BPW; pH 7.2; Oxoid, Basingstoke, UK). Cells were adjusted to an optical density of 0.5 at 640 nm (Jasco V-530 Spectrophotometer, Jasco Inc. Easton, MD) to achieve 1×10^8 cfu/mL in the suspension (Bach et al., 2002). The population of *E. coli* in the suspension was verified by spread plating on sorbitol MacConkey agar (SMAC, Oxoid, Basingstoke, UK) supplemented with 0.05 mg/L of cefixime and 2.5 mg/L of potassium tellurite (CT, Oxoid, Basingstoke, UK) to yield CT-SMAC. Suspensions of the pathogen were diluted in distilled water and applied to achieve 5×10^5 cfu/g at ensiling. The commercial inoculants were suspended in distilled water and applied at the rate of 3 mL/kg of fresh forage to achieve 1×10^6 cfu/g in the forage and the same amount of distilled water was applied to Control. The number of bacteria in the commercial inoculants was determined previously by serial dilutions in BPW, spread plating in de Man Rogosa Sharpe (MRS) agar and incubating for 24 h at 35^{o}C (Difco; Becton, Dickinson and Company, Sparks, MD).

The forage for each treatment (25 kg) was spread onto a plastic sheet and the treatment was applied using a manual sprayer. Afterwards, the four ends of the plastic sheet were drawn together and the forage was tumbled for 3 min to ensure even distribution of the inoculum. Approximately 1.5 kg of forage from each treatment was manually compacted into a 14 × 21 cm, thick-walled plastic bag. Each treatment was prepared in triplicate and ensiled for 3, 7 or 31 days at 20^{o}C to give a total of 45 bag silos. Samples from these bags were used to determine changes in *E. coli* O157:H7 counts and pH over time. In addition, 4.5 kg of forage from each treatment were manually compacted in triplicate into 20-L mini silos lined with thick-walled plastic bags

and ensiled for 82 d at 20^{o}C at an approximate density of 500 kg/m^{3}. On d 82, mini silos were opened and contents were mixed thoroughly and representatively subsampled for analysis of pH, lactate, VFA, aerobic stability, and *E. coli* O157:H7, yeast, and mold enumeration. Additional samples also were taken for reinoculation with *E. coli*.

Aerobic Stability

Approximately 1.3 kg of d-82 silage from each treatment replicate was transferred to a plastic bag within an open-top styrofoam container. Silage boxes were covered with two layers of cheese cloth to avoid dehydration and stored at 20^{o}C for 144 h. Silage temperature was monitored with thermocouples (Campbell Scientific, Inc., Utah) placed in the geometrical center of the silage mass in each box. Thermocouples were connected to a data logger (CR 1000 data logger; Campbell Scientific, Inc, Utah) that recorded silage temperatures at 30-min intervals. 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.

Survival of E. coli 0157:H7 in Silages Exposed to Aerobic Conditions

This aspect of the experiment determined if treatment with the commercial inoculants at ensiling would prevent the growth of *E. coli* O157:H7 in silages contaminated with the pathogen after silos were opened. Approximately 1 kg of silages sampled at silo opening and those aerated for 144 h in the aerobic stability assay were transferred into separate plastic bags and reinoculated with 1 × 10^{5} cfu/g of *E. coli* O157:H7. Silage pH and *E. coli* counts were determined 24 h after reinoculation.

Laboratory Analyses

Preparation of silage extracts

Silage extracts for enumeration of *E. coli*, analysis of VFA, pH and determination of anti-bacterial activity in the silages were prepared by blending 15 g of silage and 135 mL of distilled water in a stomacher (UL® Lab-Blender 400 - Seward Laboratory, London, UK) for 2 min. For VFA determination, the extracts were filtered through two layers of cheese cloth and frozen (-10^{o}C) after addition of 3 mL of 50% sulfuric acid per 100 mL of extract. The extracts for enumeration of yeasts and molds were prepared by blending 25 g of silage with 225 mL of distilled water for 2 min in the stomacher.

Microbial enumeration

For enumeration of *E. coli* O157:H7 in the suspensions prepared to inoculate silages and in silage extracts, serial 10-fold dilutions were prepared in BPW followed by spread plating on CT-SMAC agar. Plates were incubated for 24 h at 35^{o}C. Colonies on CT-SMAC were screened by API-20E® System (BioMerieux, Inc, Hazelwood, MO) and confirmed as *E. coli* O157:H7 by latex agglutination using O157 and H7 antisera (Difco, Detroit, MI). The number of bacteria in suspensions of commercial inoculants was determined by serial dilutions in BPW and spread plating on MRS agar (Difco; Becton, Dickinson and Company, Sparks, MD). Plates were incubated for 24 h at 35^{o}C. For yeast and mold enumeration, silage extracts were serial diluted in BPW and spread plated on Rose Bengal agar (Oxoid, Basingstoke, UK) supplemented with 1.0 mL/L of dichloran 0.2% in ethanol (Ultra Scientific, Kingstown, RI) and 0.1 g/L of chloramphenicol (Fisher Biotech, Fair Lawn, NJ). Plates were incubated for 5 days at 27^{o}C. For all microbial counts, a range of dilutions was prepared in duplicate and plates yielding 30 to 300 colonies were counted.

Chemical analysis

Dry matter content in the initial forage and silages were determined by drying samples in a forced air oven at 55^{o}C for 48 h. The pH of silage extracts was measured using a digital pH meter (Accumet AB15 - Fisher Scientific). Lactate and VFA concentrations were analyzed by High Performance Liquid Chromatography as described Muck and Dickinson (1988).

Antibacterial activity

Antibacterial activity against *E. coli* O157:H7 in pure cultures of the inoculants and d-82 silage extracts was determined by the Kirby-Bauer disc diffusion test. Petri-plates containing 1% of the *E. coli* O157:H7 inoculum (10^{8} cfu/mL) were prepared on CT-SMAC agar. Bacteria in the commercial inoculants were grown in MRS broth at 35^{o}C for 24 h. Cell-free supernatants were prepared by centrifuging the cultures at 3000 x *g* for 10 min at 5^{o}C. The pH of the supernatants was adjusted to 5.0 with 2 N NaOH. Similary silage extracts were centrifuged and the cell-free supernatants adjusted to pH 5. Two paper discs (6 mm diameter; Cat No. 231039, Becton & Dickinson, Sparks, MD) were immersed into the supernatants for 15 s using flame-sterilized forceps, and placed on the surface of CT-SMAC agar plates containing the indicator microorganism. Plates were prepared in duplicate and incubated for 24 h at 35^{o}C. Zones of inhibition around the paper discs were measured with a ruler.

Statistical Analysis

The experiment had a completely randomized design with five treatments and three replicates per treatment. Data were analyzed with the General Linear Model procedure of SAS (SAS Institute Inc., Cary, NC) and a model including the treatment effect. In addition, the model used to analyze changes in pH and *E. coli* during ensiling

included time and treatment × time. Differences between means were determined using the Tukey test. Significant differences were declared if $P < 0.05$.

Results

Anaerobic Phase

The pH of all silages decreased to below 4 within 3 d of ensiling and remained low at all subsequent ensiling durations (Figure 3-1). The pH at final silo opening (d 82) was greater ($P < 0.05$) in silages treated with commercial inoculants (3.72) than in Control and EC silages (3.52). *Escherichia coli* was not detected in silages after any of the ensiling durations.

Silages treated with inoculants containing *L. buchneri* (EC+LB and EC+B500) had lower lactate and greater acetate concentrations than other treatments (Table 3-1). The EC+LB silage had less ($P < 0.05$) DM than Control, EC, and EC+BII silages. Propionate and butyrate concentrations were low or undetectable in all silages. Applying the inoculant containing propionic bacteria (EC+BII) did not result in greater propionic acid concentration versus other treatments.

Aerobic Phase

Yeasts and molds were not detected in silages treated with *L. buchneri* inoculants but they were present in other silages. Treatment with *L. buchneri* inoculants improved aerobic stability by at least 115% compared with the Control, but treatment with EC or EC+BII did not affect aerobic stability (Figure 3-2).

Escherichia coli O157:H7 was not detected in silages reinoculated with the pathogen at final silo opening (d-82) and exposed to the air for 24 h, probably because pH remained below 4 in all silages during that period (mean =3.78 $\pm$ 0.13). However, the pathogen was present in all silages reinoculated 144 h after silo opening except in

that treated with EC+B500 (Figure 3-3). One day after reinoculation, Control, EC, and EC+BII silages had relatively high pH values (4.71, 5.67, and 6.09) and *E. coli* counts (2.87, 6.73, and 6.87 log cfu/g, $P < 0.05$), whereas those treated with *L. buchneri* inoculants had low pH values (< 4) and undetectable (EC+B500) or low *E. coli* counts (1.97, log cfu/g; EC+LB). Counts of the pathogen were at least 10-fold less in silages treated with *L. buchneri* inoculants versus other treatments.

Antibacterial Activity

The pH-corrected cell-free supernatants from pure cultures of each commercial inoculant produced a 2.5-mm zone of inhibition against *E. coli* O157:H7, whereas no zone was evident from that of the Control. In addition, no inhibition zone against the pathogen was produced by cell-free supernatants from extracts of d-82 silages treated with these inoculants.

Discussion

The fact that *E. coli* O157:H7 was undetected in silages during the anaerobic phase is most probably attributable to the inhibitory low pH resulting from formation of fermentation acids during ensilage. A bacterial inoculant that accelerated the rate of lactate accumulation and the resulting pH decline reduced (7 vs. 15 d) the period required for elimination of *E. coli* O157:H7 from barley silage contaminated with the pathogen (Bach et al., 2002). In this study as in others using corn and grass silage (Byrne et al., 2002; Chen et al., 2005), *E. coli* was eliminated during ensiling even in the absence of inoculation because a low pH was achieved rapidly.

Inoculants containing *L. buchneri* have improved aerobic stability of different silages (Driehuis et al., 2001; Weinberg et al., 2002, Kleinschmit et al., 2005). In this study, inoculation with *L. buchneri* inoculants increased the aerobic stability of corn

silage by at least 115%. This effect is attributable to the inhibitory effect of acetate produced by *L. buchneri* on spoilage-causing fungi (Driehuis et al., 2001; Ranjit et al., 2002). Yeasts were controlled by acetic acid concentrations above 5.6 g/L of culture medium (Woolford, 1975). As in this study, inoculation with *L. buchneri* typically results in acetate concentrations ranging from 36 to 50 g/kg of DM (Driehuis et al., 2001; Taylor et al., 2002).

Propionic acid is the most effective antimycotic agent among short chain fatty acids at pH 5 or lower (Woolford, 1975); therefore, inoculants containing propionic acid bacteria have been tested for their ability to improve aerobic stability. Merry and Davies (1999) indicated that such bacteria have not inhibited consistently the growth of yeasts and molds or improved aerobic stability because they do not grow well when ensiling conditions are conducive to a rapid decline in pH. Previous studies indicated that inoculation with *P. freudenreichii* (1×10^4 cfu/g) with or without homolactic bacteria did not affect propionic acid concentration, yeast and mold counts, or aerobic stability of corn and barley silages (Ranjit et al., 2002; Taylor et al., 2002). Likewise, treatment with the inoculant containing *P. pentosaceus* and *P. freudenreichii* did not affect these measures in this study.

Although antibacterial activity against *E. coli* was evident in cultures of the commercial inoculants, it was not detected in silages treated with the inoculants. Gollop et al. (2005) also reported that pure cultures of different strains of *L. plantarum, Enterococcus faecium* and *L. buchneri* inhibited the growth of *Micrococus luteus* and *Pseudomonas aeruginosa,* but only some of the extracts of silages treated with these bacteria had antibacterial activity independent of pH. Antibacterial activity is dependent

on several factors including water activity, pH, temperature, and VFA concentration, and it varied with crop species and crop moisture concentration in the study of Gollop et al. (2005). The antibacterial activity of inoculant bacteria can be exploited to enhance forage preservation and food safety; therefore future work should determine the source of such activity in silage inoculants, and devise methods to ensure its persistence against spoilage-causing or pathogenic microorganisms in silages.

Fenlon and Wilson (2000) reported that *E. coli* O157:H7 numbers in poorly fermented, artificially inoculated silages grew from 10^3 to 10^6 log cfu/g during the first 7 d of ensilage when the pH stayed above 5. Similarly, in this study, *E. coli* counts in samples reinoculated with the pathogen after 144 h of aerobic exposure increased from 10^5 to over 10^6 cfu/g when the pH was above 5. Weinberg et al. (2004) showed that though low pH achieved during ensilage eliminates *E. coli* from contaminated forages, the pathogen can be found in decaying parts of commercial silages with high pH. In agreement, in this study, *E. coli* was undetected in silages when pH was less than 4 during ensiling. Furthermore, growth of *E. coli* in silages reinoculated with the pathogen after 144 h of aerobic exposure depended on pH. High populations were present in Control, EC and EC+BII silages, which had high pH (> 4), whereas they were at least about 10-fold lower in silages treated with *L. buchneri* inoculants which had pH < 4. These results indicate that preventing aerobic deterioration and the attendant pH increase can prevent or minimize the growth of *E. coli* O157:H7 on silages contaminated with the pathogen during the feedout phase.

Maintenance of the low pH achieved at silo opening for 144 h by inoculants containing *L. buchneri* is probably attributable to the inhibitory effect of the acetate

produced by *L. buchneri* on yeasts that metabolize lactate and thereby increase the pH in aerobically exposed silages. Our data suggest that *L. buchneri* inoculants can be used to maintain low pH (< 4) in silages during the feedout phase and thereby curtail the growth of *E. coli* O157:H7, from extraneous sources that might contaminate the forage.

The *E. coli* strain used in this experiment (ATCC 43894) was reported to have low acid tolerance (Benjamin and Datta, 1995), indicating its susceptibility to inhibition during acidic silage fermentation. However, *E. coli* O157:H7 may acquire acid tolerance and acid tolerant strains could conceivably survive the low pH achieved in silages. Nevertheless, all studies we reviewed on the subject indicate that the ensiling process in well-managed silages is efficient at eliminating *E. coli* O157:H7 (Bach et al., 2002; Byrne et al., 2002; Chen et al., 2002). Future work should examine if other factors such as bacteriocins, competition with other microorganisms, and high organic acid concentrations contribute to elimination of the pathogen when forages are ensiled.

Conclusions

This study shows that *E. coli* O157:H7 was eliminated from forages contaminated with 5 log cfu/g of the pathogen when the pH dropped to 4 within 3 d of ensiling. Inoculant treatment did not affect *E. coli* O157:H7 elimination during ensiling but unlike other treatments, application of *L. buchneri* inoculants eliminated yeast and mold populations, increased aerobic stability, and kept the pH below 4 for the duration of the 144-h aerobic exposure period. Reinoculation of silages with *E. coli* O157:H7 after 144 h of aerobic exposure resulted in relatively high ($P > 0.05$) *E. coli* O157:H7 counts of 2.87, 6.73, and 6.87 log cfu/g for the Control, EC and EC+BII silages, respectively. However, counts in EC+LB silages (1.96 log cfu/g) were low and the pathogen was not detected in EC+B500 silages. This suggests that *L. buchneri* inoculants can be used to

curtail the growth of *E. coli* in silages contaminated with the pathogen at the feedout stage. All pure cultures of commercial bacterial inoculants exhibited antibacterial activity independent of pH against *E. coli* O157:H7 but the activity did not persist in the treated silages, suggesting that *E. coli* O157:H7 elimination from the silages was mediated by pH reduction.

Table 3-1. Dry matter, organic acids, and yeast and mold values of corn forage inoculated with Escherichia coli O157:H7 (EC) alone or EC and commercial bacterial inoculants and ensiled for 82 days

Attributes	Control	EC[2]	EC+BII[3]	EC+LB[4]	EC+B500[5]	SE[6]
DM[1],%	26.3[ab]	26.6[ab]	27.1[a]	25.0[c]	25.8[bc]	0.34
Lactate,% DM	2.69[a]	2.10[a]	2.12[a]	0.48[b]	1.10[b]	0.31
Acetate,% DM	2.42[c]	1.73[c]	1.98[c]	4.77[a]	3.81[b]	0.35
Propionate,% DM	0.26	0.00	0.00	0.00	0.01	0.2
Butyrate,% DM	0.21	0.00	0.00	0.00	0.00	0.11
Yeasts and molds, log cfu/g	2.13[ab]	5.67[a]	6.30[a]	0.00[b]	0.00[b]	1.7

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).
[1]DM = Dry matter; cfu/g = colony forming units per gram.
[2]EC = 5×10^5 cfu/g *Escherichia coli* O157:H7.
[3]BII = 1×10^6 cfu/g of *P. pentosaceus* and *Propionibacterium freudenreichii*.
[4]LB = 1×10^6 cfu/g of *L. buchneri*
[5]B500 = 1×10^6 cfu/g of *Pediococcus pentosaceus* and *Lactobacillus buchneri*.
[6]SE = standard error.

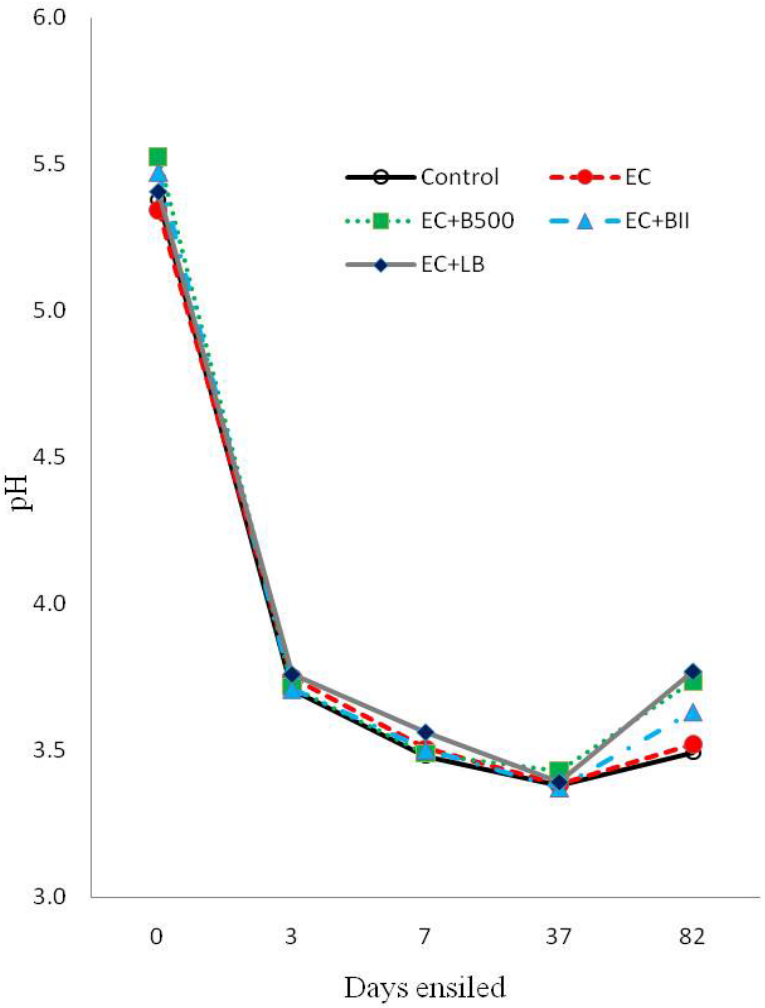

Figure 3-1. Changes in pH in corn forage inoculated with 5×10^5 log cfu/g of *E. coli* O157:H7 (EC) or EC plus bacterial inoculants[1] and ensiled for different durations. [1]BII = *P. pentosaceus* + *P. freudenreichii*; LB = *L. buchneri*; B500 = *P. pentosaceus* + *L. buchneri*. Treatment × day S.E. and *P value* for pH = 0.33 and 1.00, respectively; *. Treatment × day S.E. and *P* value for *E. coli* counts = 0.001 and 0.00, respectively.

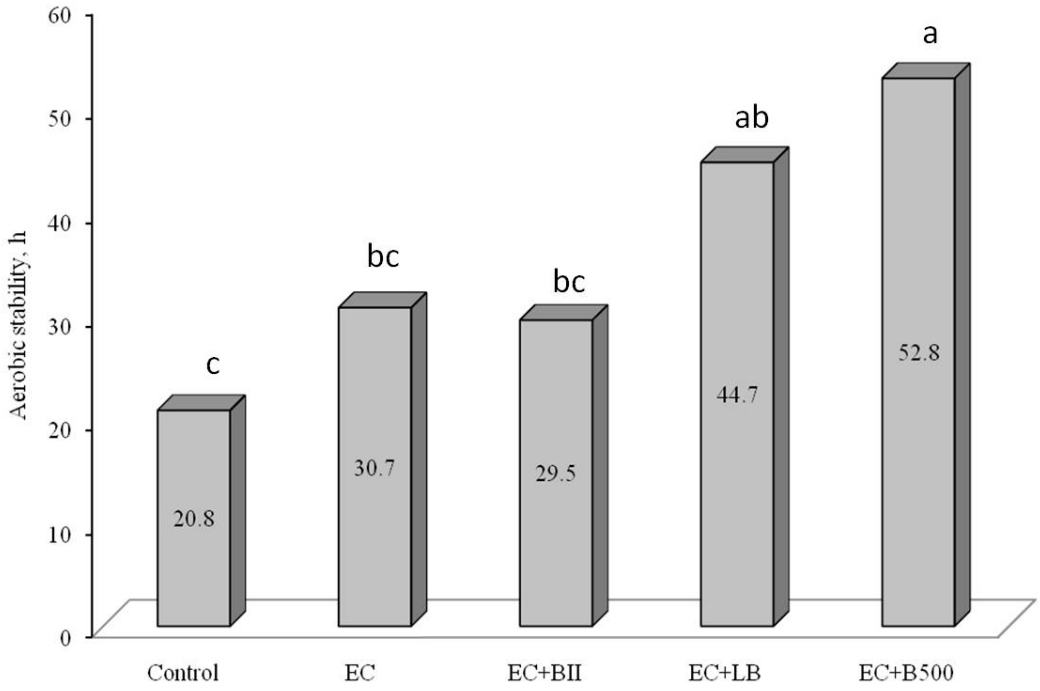

Figure 3-2. Effect of inoculation with 5×10^5 cfu/g of *E. coli* O157:H7 (EC) or EC and bacterial inoculants[1] at ensiling on aerobic stability of corn silages ensiled for 82 d. [1]BII = *P. pentosaceus* + *P. freudenreichii*; LB = *L. buchneri*; B500 = *P. pentosaceus* + *L. buchneri*. S.E. = 4.11. Bars with different letters differed ($P < 0.05$).

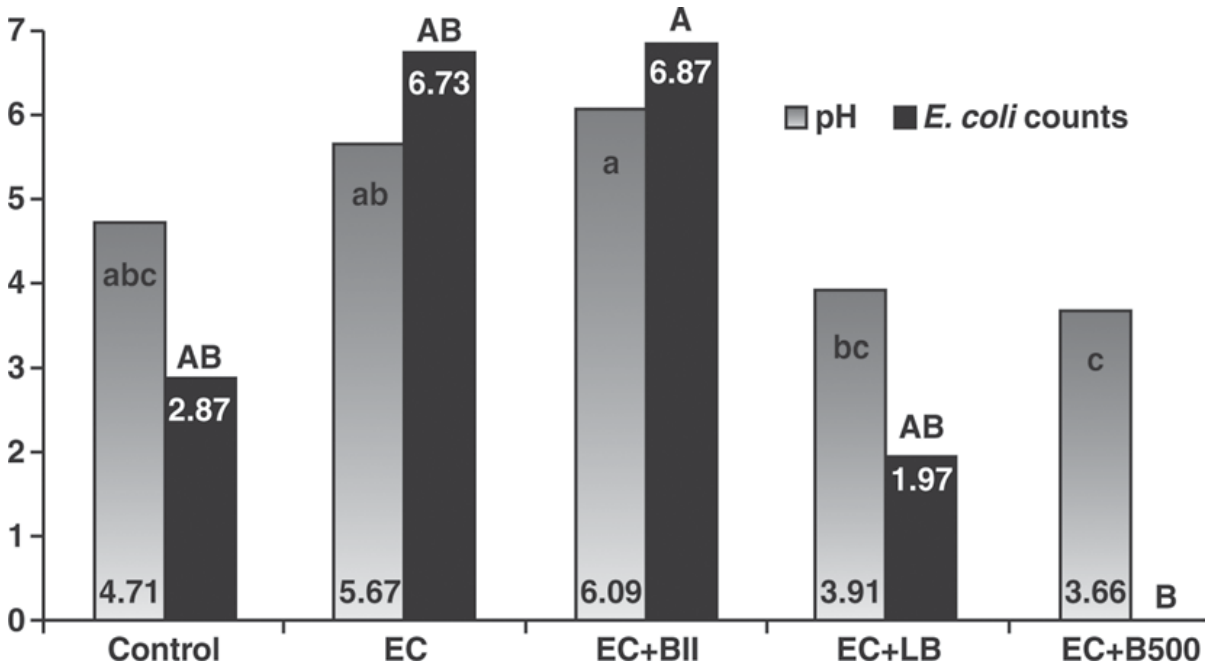

Figure 3-3. Effect of reinoculation of corn silages with 1 × 10^6 cfu/g of *E. coli* O157:H7 (EC) 144 h after silo opening (d 82) on pH and *E. coli* counts (EC; log cfu/g) of silages treated with EC or EC and bacterial inoculants[1] at ensiling. [1]BII = *P. pentosaceus* + *P. freudenreichii*; LB = *L. buchneri*; B500 = *P. pentosaceus* + *L. buchneri*. S.E. values for pH and *E. coli* data were 0.41 and 1.04, respectively. Similarly shaded bars with different letters differed ($P < 0.05$).

CHAPTER 4
EFFECT OF A DUAL PURPOSE INOCULANT ON THE QUALITY AND NUTRIENT LOSSES FROM CORN SILAGE PRODUCED IN FARM-SCALE SILOS

Introduction

Bacterial inoculants have been used to dominate the epiphytic microbial population and thereby improve the fermentation, shelf life and quality of silages (Filya et al., 2007; Pedroso et al., 2010). During ensiling, fermentation of sugars into lactic acid by homofermentative lactic acid bacteria, such as *Pediococcus pentosaceus* causes a rapid pH drop which inhibits growth of undesirable microorganisms (Filya et al., 2006). Heterofermentative lactic acid bacteria ferment sugars into lactic acid as well as other VFA (McDonald et al., 1991). *Lactobacillus buchneri* is a heterofermentative bacterium capable of converting lactate to acetate, which is a potent antimycotic agent and is metabolized to a lesser extent than lactate by aerobic microorganisms (Oude Elferink et al., 2001; Kung, Jr. and Ranjit, 2001). The increase in acetate due to inoculation with *L. buchneri* decreases the growth of spoilage-causing yeasts and molds, thereby enhancing silage aerobic stability (Kleinschmit and Kung, Jr., 2006b; Kristensen et al., 2010; Arriola et al., 2011).

Recently, dual-purpose inoculants containing homofermentative and heterofermentative bacteria have been marketed as ‘Combo’ inoculants that improve the fermentation and aerobic stability of silage. The beneficial effects of dual-purpose inoculants have been proven in numerous studies (Driehuis et al., 2001; Weinberg et al., 2002; Huisden et al., 2009). However, such studies examined silage prepared in laboratory silos. Small scale silos offer ideal conditions for the fermentation process. They are essential tools to model the effect of inoculants on silage fermentation and aerobic stability because this approach is less costly and labor intensive compared with

using farm-scale silos. Nevertheless, it is important to validate inoculant efficacy results obtained with small scale silos in farm-scale silos because the imperfect conditions of the latter are more challenging for proper fermentation (Mari et al., 2009). Also, the relatively small amount of silage used in mini silos prevents accurate estimation of inoculant effects on nutrient and other losses during the aerobic feedout phase, which can last for several months on farms. Therefore, studies demonstrating inoculant efficacy using farm-scale silos are indispensable to validate the effectiveness of inoculants in mini-silo studies. Little is known about effects of dual-purpose inoculants on the fermentation of corn silage prepared using farm-scale silos, and less is known about their effects on silage quality and preservation during the feedout phase in such silos.

The objective of this study was to examine the effects of applying a dual-purpose inoculant on the fermentation, nutritive value, aerobic stability, and nutrient losses from corn silage produced using farm-scale silos. We hypothesized that inoculation would improve the fermentation and aerobic stability of the silages and reduce associated nutrient and energy losses during the feedout phase.

Materials and Methods

Silage Production

A corn hybrid (Dekalb 69-70, Monsanto, St. Louis, MO) was grown at the Dairy Unit, University of Florida, Hague, FL, harvested at 34% DM (½ milk line stage) and chopped to achieve a 19-mm theoretical length of cut using a forage harvester fitted with an 8-row corn head (Claas Jaguar 980, Claas of America LLC., Columbus, IN). Corn plants were treated without (Control) or with a dual-purpose inoculant (LB500) that supplied 1 x 10^5 cfu of *Pediococcus pentosaceous* 12455 and 4 x 10^5 cfu of

Lactobacillus buchneri 40788 per gram of fresh forage (Lallemand Animal Nutrition, Milwaukee, WI). In order to ensure that similar forage was used for both treatments, the inoculant was sprayed on alternate 8-row-wide swaths of forage with a sprayer mounted on the harvester and the respective swaths were alternately packed into Ag-bag silos (Ag-Bag, A Miller-St. Nazianz, Inc. Company, St Nazianz, WI) with a Versa 1012 bagger (Versa Corporation, Astoria, OR). Therefore, each bag containing the Control silage was filled immediately after filling the preceding one with the inoculated silage. This may have allowed minor inoculation of the forage initially packed into Control bags, therefore, silage in the first 5 m from the front of Control and inoculated bags were not used for the experiment. Inoculation and packing was completed on the same day and 4 replicate 45-metric ton bags of forage were prepared for each treatment. Wireless sensors programmed to record temperature data hourly were placed at approximately the same location in each bag during packing.

After 166 d of ensiling, the bags were opened and silage was removed from the face at the rate of 500 kg/d, separated into good and spoiled (visibly moldy, darkened, slimy or heating) portions daily by the same three observers, and weighed for 35 d. Temperature sensors were retrieved during unloading of the silage and the data was used to calculate average, minimum, and maximum temperatures, and time to achieve maximum temperature during ensiling.

Laboratory Analysis

Daily samples of good and spoiled silage were analyzed for DM by drying in a forced-air oven at 60^{o}C for 48 h. Additional samples were collected on d 0, 7, 14, 21, 28, and 35 after opening of silage bags and immediatelly analyzed for yeast and mold counts and aerobic stability or stored at -20^{o}C for subsequent determination of chemical

composition, fermentation product profile, and gross energy. Nutrient and energy losses were quantified as the product of the dry weight of the good or spoiled silage removed and the concentration of the nutrient in the silage.

Frozen weekly silage samples were thawed, dried at 60ºC 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 analyzed for DM (105^{o}C for 16 h) and ash (512^{o}C for 8 h). Concentrations of NDF and ADF were measured using the method of Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α-amylase and sodium sulfite were used in the NDF assay. Nitrogen was determined by rapid combustion using a Macro elemental N analyzer (Vario MAX CN, model ID 25.00-5003; Elementar, Hanau, Germany) and CP was calculated as N x 6.25. Water-soluble carbohydrate concentration was determined by the anthrone reaction assay (Ministry of Agriculture, Fisheries and Food, 1986), and ammonia-N was measured by distillation (AOAC, 1985**).** An adiabatic bomb calorimeter (1261 Isoperibol, Parr Instrument Company, Moline, IL) was used to calculate the gross energy concentration of spoiled samples from both treatments.

Aerobic stability was measured on weekly samples by placing 2 kg of good silage in an open-top polystyrene box. Temperature sensors (HOBO temperature data logger 64 k, Onset computer corporation, Cape Cod, MA) were place at the geometric center of each silage sample and data was recorded every 30 min for 7 d. Four additional sensors were placed in the room to record ambient temperature. Silages were covered with 2 layers of cheesecloth to prevent drying. Aerobic stability was denoted as the length of time that elapsed before silage and ambient temperatures differed by more

than 2ºC (Huisden et al., 2009). Also, temperature data was plotted against ‘hours of aerobic exposure’ to calculate the area under the aerobic stability curve.

Silage extract was prepared by mixing 25 g of corn silage with 225 mL of 0.1% peptone water in a stomacher for 3 min. The solution was filtered through 2 layers of cheesecloth and an aliquot was immediately used for yeast and mold counts as described by Schmidt and Kung Jr. (2010). The pH of silage was measured using a pH meter (Corning Model 12, Corning Scientific Instruments, Medfield, MA). An aliquot of 2 mL of silage extract was centrifuged at 2000 x g for 15 min and the supernatant was filtered with a 0.22 µm syringe filter and used for quantification of lactic acid and VFA with a high performance liquid chromatograph (Merck Hitachi®, Elite Lachrom HTA, Tokyo, Japan) coupled to a UV Detector (Merck Hitachi L-2400) set at 210 nm. The column was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) with 0.015 M sulfuric acid mobile phase and a flow rate of 0.7 mL/min at 45°C (Arriola et al., 2011).

Statistical Analysis

The experiment had a completely randomized design with two treatments and repeated measurements over time for each four replicates per treatment. Data were analyzed with the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) and a model including treatment, time, and treatment x time effects. The model included a repeated statement and the ar (1) covariance structure was used to account for sampling from the same bag over time. Differences between means were determined using the PDIFF procedure of SAS, which differentiates means based on the F-protected least significant difference test. Significant differences were declared at $P < 0.05$ and tendencies at $P>0.05<0.10$.

Results and Discussion

The effects of inoculation on chemical composition and quantity of silage classified as good is shown in Table 4-1. The quantity of good silage removed from Control and inoculated silos was similar (156 vs. 159 kg of DM/d, $P = 0.23$), however the proportion of good inoculated silage was greater than that of the good Control silage (96.6 vs. 92.2%, $P = 0.004$). Inoculant application did not change ($P > 0.05$) concentrations of DM (34.8 vs. 34.1%), CP (9.55 vs. 9.47% DM), NDF (41.7 vs. 41.3% of DM), NFC (40.7 vs. 41.1% DM) or WSC (3.46 vs. 3.30% of DM) in good silage. Huisden et al. (2009) reported that except for slightly decreasing WSC concentration, the inoculant used in this study did not affect the chemical composition of silage made from the same corn hybrid used in this study. Mari et al. (2009) also reported that no difference existed between the chemical composition of silage made using farm–scale silos that had been inoculated without or with *L. buchneri* alone.

The quantity (12.88 vs. 5.69 kg/d, $P = 0.002$) and proportion (7.83 vs. 3.39%, $P = 0.004$) of spoiled silage was greater in Control versus inoculated silages (Table 4-2). Therefore, the lower proportion of good silage in Control versus inoculated silages was due to the greater proportion of spoiled silage in the respective silages. That inoculation reduced the quantity of spoiled silage by about 56% is important because feeding spoiled silage can predispose cows to reduced performance and increase ingestion of pathogenic organisms. Whitlock et al. (2000) reported that ingestion of increasing quantities of spoiled silage linearly reduced DMI and NDF digestibility in cattle, and Bolsen and Bolsen (2006) suggested that these reductions could reduce daily milk production from 1.3 to 2.3 kg/d. Spoiled silage also can harbor molds that directly cause diseases such as aspergillosis, farmers lung, and hemorrhagic bowel syndrome and

indirectly cause diseases and reduced performance via mycotoxin production (Adesogan and Queiroz, 2009). In addition, spoiled silage can contain pathogenic bacteria like *Listeria monocytogenes*, *Bacillus cereus,* and *E.coli* O157:H7, which can cause diseases like meningitis, encephalitis, septicemia, gastroenteritis, mastitis, and abortions and reduce the safety, flavor, and shelf life of dairy products (McDonald et al., 1991; Adesogan and Queiroz, 2009; Pedroso et al., 2010).

Inoculated silages had a lower DM concentration than Control silages 35 d after silos were opened for unknown reasons (inoculation x time interaction, $P = 0.07$; Table 4-2; Figure 4-1). Inoculant application did not affect ($P > 0.1$) the mean gross energy value (20.5 vs. 20.5 KJ/g) or mean concentrations of DM (29.8 vs. 31.6% DM), CP (10.1 vs. 10.4% of DM), NDF (47.5 vs. 47.1% of DM), ADF (27.9 vs. 28.7% of DM) or ash (5.01 vs. 5.05% of DM) in spoiled silage. Therefore, inoculation did not affect the composition of good or spoiled silages but it reduced the amount of silage that became spoiled. Consequently, inoculation reduced losses of CP (0. 23 vs. 0. 92 kg/d, $P = 0.03$), NDF (1.34 vs. 4.12 kg/d, $P = 0.04$), ADF (0.80 vs. 2.53 kg/d, $P = 0.04$), ash (0.03 vs. 0.13 kg/d, $P = 0.03$), and gross energy (1.80 vs. 7.69 kJ/g, $P = 0.02$).

Inoculation decreased the pH of silages (3.91 vs. 3.99; $P = 0.01$) but all values were below the threshold of four, which indicates adequate fermentation (Table 4-3). Mean lactic acid concentration did not differ between Control and inoculated silages (7.63 vs. 7.86% of DM; $P = 0.63$), although that of the Control silage tended to be greater 28 d after silo opening (inoculation x time interaction, $P = 0.09$; Figure 4-2) but not before or after. Mean acetic acid concentration tended to be greater in inoculated silages (5.11 vs. 3.56% of DM, $P = 0.07$), therefore the lactate to acetate ratio tended to

be lower in such silages (1.58 vs. 2.53, *P* = 0.08). These results were due to a rapid increase in acetic acid concentration in inoculated silages during the first 7 d of the feedout phase (Figure 4-3) as well as a tendency for greater concentrations of the acid in inoculated versus Control silages at 14 and 21d (inoculant x treatment interaction, *P* = 0.09; Table 4-3). That inoculation increased acetate contribution is attributable at least partly to the presence of *L. buchneri*, which ferments sugars and lactate to acetate (Oude Elferink, 2001). Application of *Lactobacillus buchneri* alone to corn silages often increased acetate concentration at the expense of lactate concentration (Kleinschmit and Kung, 2006b) but this difference is not always evident or statistically significant as when *L. buchneri* was used in combination with homofermentative bacteria (Kleinschmit and Kung, 2006a; Huisden et al., 2009) as in this study. It is interesting to note that acetic acid concentrations of Control and inoculated silages did not differ statistically until 7 d after opening. This delay suggests that aerobic organisms may have contributed to the greater acetate production in inoculated silages because effects of *L. buchneri* on acetic acid concentrations typically occur during ensiling (Driehuis et al., 1999; Oude Elferink, 2001). Acetic acid bacteria can oxidize ethanol and lactate to acetate and they have been implicated in initiating spoilage in corn silage in some studies (Klienschmit and Kung, 2006b).

In addition to increasing the acetic acid concentration of silages, *L. buchneri* may also increase indirectly the propionic acid concentration because it can convert lactic acid into 1, 2-propanediol, which is converted to propionic acid when *L. diolivorans* is present (Krooneman et al., 2002). That inoculation did not affect propionic acid concentration in this study may reflect the absence of *L. diolivorans* in the silages. The

concentrations of butyric acid were similar in Control and inoculated silages (0.19 vs. 0.25% of DM, P = 0.52) and the values were slightly above the threshold of 0.04% which may indicate that Clostridial fermentation occurred (Pahlow et al., 2003; Arriola et al., 2011).

Temperatures recorded during fermentation were typical of those in appropriately packed and sealed silages (Kung Jr., 2008). Maximum ensiling temperature did not differ between treatments (30.1 vs. 28.9^{o}C, *P* = 0.51) and these temperatures were achieved within the first 3 d of ensiling irrespective of treatment (53.8 vs. 49.7 h, *P* = 0.86). The first three ensiling days represent the period when residual oxygen in the silage mass is used up by obligate and facultative microorganisms during the initial part of the aerobic phase (Pahlow et al., 2003). The minimum temperature (20.1 vs. 20.6^{o}C, *P* = 0.57) and temperature range (10.1 vs. 9.3^{o}C, *P* = 0.76) did not differ between treatments. However, mean ensiling temperature tended to be greater in Control versus inoculated silages (22.7 vs. 22.1^{o}C, *P* = 0.08), suggesting that inoculation reduced wasteful heat production from microbial activity during ensiling.

Inoculation reduced yeast and mold counts (2.59 vs. 4.62 cfu/g, *P* = 0.01; Table 4-4) likely reflecting the antifungal activity of the acetic acid, which tended to increase with inoculation. Aerobic stability was numerically, but not statistically increased (14.7 vs. 9.5 h, *P* = 0.71) by inoculation. Aerobic stability is typically increased when *L. buchneri* alone is applied to corn silage. Based on 23 published experiments, Kleinschmit and Kung, Jr. (2006b) used a meta-analysis to demonstrate that application of *L. buchneri* alone increased acetic acid concentration in corn and small grain silages, reduced the yeast and mold population, and increased aerobic stability. Several subsequent studies

have also confirmed the efficacy of using *L. buchneri* inoculants to increase silage aerobic stability (Huisden et al., 2009; Mari et al., 2009). Nevertheless, a few contradictory studies exist. For instance, numerical non-statistical increases ($P > 0.1$) in aerobic stability were detected when *L. buchneri* alone or *L. buchneri* and *P. pediococcus* were applied to corn silage in a mini-silo study (Arriola et al., 2011). Similarly, inoculation with *L. buchneri* alone did not improve the aerobic stability of bermudagrass silage largely because Control silages had undergone a secondary Clostridial fermentation, which made them stable by increasing the concentration of butyric acid (Adesogan et al., 2004). Schmidt and Kung, Jr. (2010) reported that inoculation with *L. buchneri* alone improved the fermentation and aerobic stability of silages made at 3 of 5 locations, further indicating that factors other than the prevailing fungal population can influence the efficacy of *L. buchneri* inoculants. Such factors include the duration of ensiling and initiation of spoilage by acetic acid bacteria rather than yeasts (Pahlow et al., 2003; Kleinschmit and Kung, Jr. 2006b), high epiphytic counts of *L. buchneri* (Arriola et al., 2011), and differences among corn hybrids (Kang et al., 2009).

No treatment difference in aerobic stability was detected when defined as the time that elapsed before silage and ambient temperatures differed by more than $2^{o}C$. However, other important but less commonly used measures of aerobic stability warrant evaluation. For instance, spoilage bacteria and fungi produce CO_2 as a fermentation byproduct (Spoelstra et al., 1988), therefore, CO_2 production has been used as a measure of aerobic stability in some studies (Ashbell et al., 2002; Weinberg et al., 2011). Other studies have simply graphically illustrated changes in temperature over

time for inoculated and Control silages (Salawu et al., 2001; Adesogan et al., 2004). In the current study, total heat accumulation over time during the aerobic feedout phase was reduced by about 16% by inoculation (1916 vs. 2212 ^{o}C × h, *P* = 0.02). This aerobic stability index is particularly useful for detecting effects of inoculation in studies where heat accumulation over time is substantially different between Control and inoculated silages that start heating at approximately the same time. Maximum post ensiling temperature (43.1 vs. 43.4 ^{o}C, *P* = 0.85), silo face temperature (36.18 vs. 34.86^{o}C, *P* = 0.42), and temperature range (17.6 vs. 19.1^{o}C, *P* = 0.32) were not affected by treatment but minimum post ensiling temperatures tended to be reduced by inoculation (25.5 vs. 24.3^{o}C, *P* = 0.09). The short aerobic stability and discrepancy among measures of heat production and aerobic stability in the silages suggest that the inoculant was not as effective at increasing the bunk life of the silage as in other studies. This is attributable to factors such as a relatively low packing density (approximately 168 kg/m^{3}) in the bags, which made it challenging to maintain a straight silo face and to achieve a feedout rate of at least 15 to 30 cm of silage per day (Pitt and Muck, 1993). Furthermore, the prevailing sub-tropical conditions, which are ideal for the growth of spoilage-causing yeasts and molds (Oude Elferink et al., 2001; Adesogan, 2009) may have contributed to poor aerobic stability response.

Conclusions

This study showed that application of a dual-purpose inoculant did not affect the nutritive value of good or spoiled corn silage made using farm-scale silos but reduced the amount and proportion of spoiled silage by over 50%, and thereby reduced the associated energy and nutrient losses from the silage. Inoculation reduced the yeast and mold population and consequently reduced the total heat accumulation during the

aerobic feedout phase. Most beneficial effects of the inoculant were attributed to its tendency to make the fermentation more heterolactic by increasing the concentration of acetate.

Table 4-1. Effect of inoculant treatment[1] on the quantity and chemical composition of 'good'[2] silage removed daily from silos

	Control	Inoculant	SEM[3]	P value treatment	P value time	P value treatment x time
Quantity, kg of good silage DM/d	156	159	2.12	0.23	0.20	0.65
Percentage of good silage, %	92.2	96.6	1.08	0.004	0.76	0.71
Chemical composition of good silage						
DM, %	34.1	34.8	0.96	0.60	0.11	0.51
CP, %	9.47	9.55	0.21	0.79	0.01	0.58
ADF, %	24.9	24.0	0.64	0.29	0.21	0.85
NDF, %	41.3	41.7	1.20	0.81	0.12	0.49
NFC[4], %	41.1	40.7	1.16	0.83	0.06	0.55
WSC, %	3.30	3.46	0.27	0.63	0.61	0.40
Ash, %	3.45	3.29	0.12	0.36	0.60	0.86

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu/g of *P. pentosaceus* and 4 x 10^5 cfu/g of *L. buchneri.*
[2]Silage that was not visibly moldy, dark, heating or slimy.
[3]SEM = standard error of mean.
[4]Calculated as NFC = 100 – [CP+ ash+ fat (NRC, 2001 values) +NDF]

Table 4-2. Effect of inoculant treatment[1] on the quantity and chemical composition of 'spoiled'[2] silage removed daily from silos and the associated nutrient and energy losses

	Control	Inoculant	SEM[3]	P value treatment	P value time	P value treatment x time
Quantity of spoiled silage, kg /d	12.88	5.69	1.53	0.002	0.40	0.49
Percentage of spoiled silage	7.83	3.39	1.08	0.004	0.76	0.71
Chemical composition of spoiled silage						
DM, %	31.6	29.8	1.36	0.36	0.12	0.07
CP, %	10.4	10.1	0.22	0.35	0.02	0.32
ADF, %	28.7	27.9	0.67	0.41	0.22	0.67
NDF, %	47.1	47.5	0.71	0.63	0.05	0.90
Ash, %	5.05	5.01	0.41	0.94	0.03	0.63
GE, KJ/g	20.5	20.5	0.08	0.99	0.02	0.60
Energy and nutrients lost in spoiled silage						
CP kg/d	0.92	0.23	0.20	0.03	0.02	0.39
ADF, kg/d	2.53	0.80	0.54	0.04	0.01	0.51
NDF, kg/d	4.12	1.34	0.88	0.04	0.01	0.51
Ash, kg/d	0.13	0.03	0.03	0.03	0.02	0.30
GE, KJ/d	7699	1809	168	0.02	0.02	0.29

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri.*
[2]Silage that was visibly moldy, dark, heating or slimy.
[3]SEM = standard error of mean.

Table 4-3. Effect of inoculant treatment[1] on fermentation indices and temperature during ensiling in 'good'[2] corn silages

	Control	Inoculant	SEM[3]	P value treatment	P value time	P value treatment x time
pH	3.99	3.91	0.20	0.01	0.04	0.25
Ammonia nitrogen, % of DM	1.25	1.58	0.2	0.34	0.01	0.13
Lactic acid, % of DM	7.86	7.63	0.32	0.63	0.60	0.09
Acetic acid, % of DM	3.56	5.11	0.50	0.07	0.05	0.10
Lactate: acetate	2.53	1.58	0.32	0.08	0.19	0.20
Propionic acid, % of DM	0.94	1.10	0.07	0.17	0.15	0.86
Butyrate, % of DM	0.19	0.25	0.06	0.52	0.70	0.54
Mean temperature, ^{o}C	22.70	22.13	0.22	0.08	NA[4]	NA
Maximum temperature, ^{o}C	30.16	28.89	1.41	0.51	NA	NA
Minimum temperature, ^{o}C	20.05	20.62	0.67	0.57	NA	NA

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri.*

[2]Silage that was not visibly moldy, dark, heating or slimy.

[3]SEM = standard error of mean.

[4]NA = not applicable.

Table 4-4. Effect of inoculant treatment[1] on fungal counts, aerobic stability and temperature of 'good'[2] corn silage

	Control	Inoculant	SEM[3]	P value treatment	P value time	P value treatment x time
Yeasts and molds, log cfu/g	4.62	2.59	0.62	0.01	0.08	0.29
Aerobic stability (h)	9.5	14.7	10.6	0.71	0.63	0.35
Area under the temperature curve	2212	1916	81	0.02	0.01	NA[4]

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri.*
[2]Silage that was not visibly moldy, dark, heating or slimy.
[3]SEM = standard error of mean.
[4]NA = not applicable.

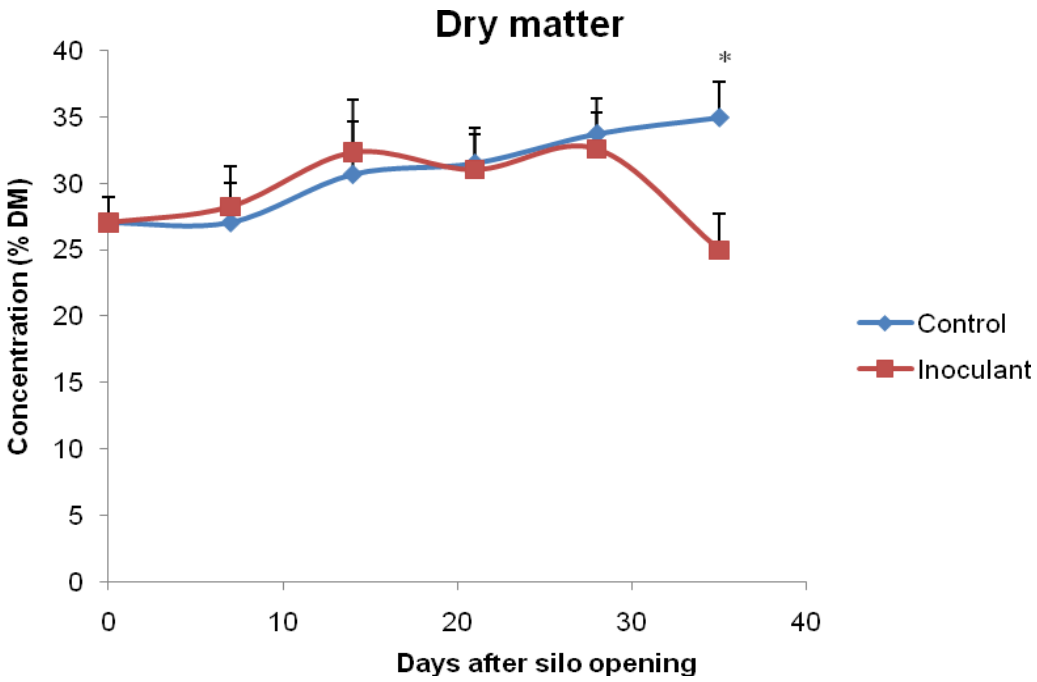

Figure 4-1. Changes in dry matter concentration of spoiled silage with time. * = P<0.05.

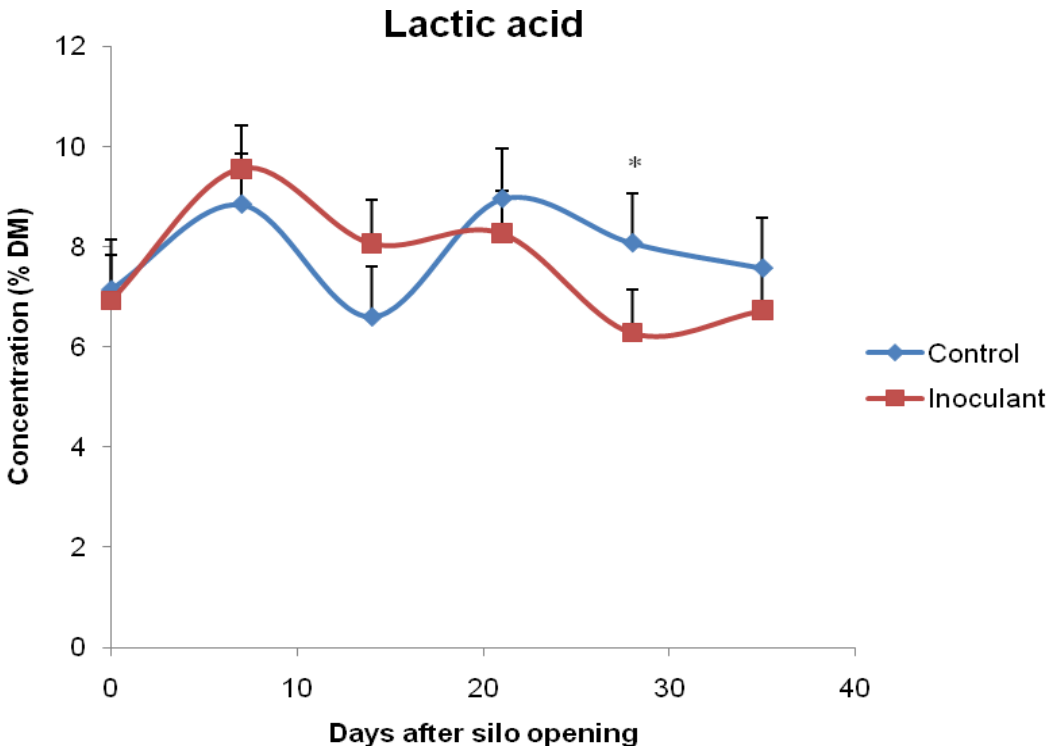

Figure 4-2. Changes in lactic acid concentration of corn silage with time. * = P<0.05.

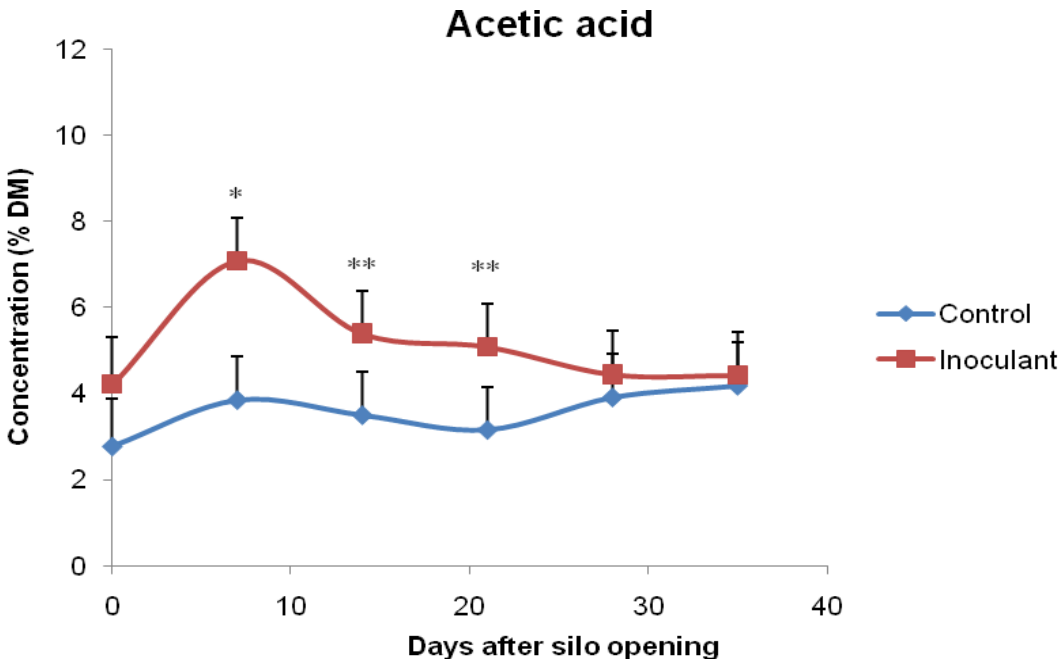

Figure 4-3. Changes in acetic acid concentration of corn silage with time. * = P<0.05, ** = 0.05<P<0.10.

CHAPTER 5
RELATIONSHIP BETWEEN RUST INFESTATION AND THE QUALITY AND SAFETY OF CORN SILAGE TREATED WITH OR WITHOUT A BACTERIAL INOCULANT

Introduction

Southern corn rust is an aggressive disease of corn caused by *Puccinia polysora* Underw. Pustules produced during the growth of the fungus cause severe damage to the leaf cuticle causing increased transpiration, premature leaf desiccation, and reduced photosynthesis and nutrient translocation (Lucas, 1998). The fungus can kill the hosts and yield losses of up to 45% have been reported in cases with severe epiphytotic contamination in the US (Rodriguez-Ardon et al., 1980) and other areas of the world (Rhind et al., 1952; Liu and Wang, 1999). The disease has several similar symptoms and effects to common rust, which is caused by *Puccinia sorghi;* however, cool wet conditions favor Common rust whereas hot humid conditions favor Southern rust. Despite concerted research efforts that have produced resistant commercial hybrids, the unpredictable occurrence of Southern rust and its fast and aggressive development make it a real threat to dairy operations in the Southeast that rely on corn silage for dietary energy and fiber.

Infestation of corn plants by *Puccinia polysora* can affect negatively the chemical composition of corn forage. Johnson et al. (1997) reported increases of DM, NDF, and ADF and a decrease in DM digestibility of corn forage infested by Southern rust. Yet, mycotoxins are important food safety hazards that are commonly produced after the growth of opportunistic fungi on previously stressed or challenged plants (Whitlow and Haggler, 2005).

Undesirable microorganisms can survive during ensiling in inadequately packed silage particularly in the top layer or around the sides of bunkers or in air pockets in the

silage mass. Such areas can contain sufficient oxygen to favor the survival or growth of mycotoxin-producing fungi. High concentrations of aflatoxins, which are carcinogenic secondary metabolites of *A. parasiticus* and *A. flavus*, were detected in aerobically exposed corn silages (Gonzales Pereyra, 2008).

Dual-purpose inoculants containing homofermentative and heterofermentative bacteria are used to improve silage fermentation and aerobic stability, respectively (Driehuis et al., 2001; Filya, 2003; Adesogan et al., 2004). To our knowledge, no study has evaluated if such inoculants can improve the quality and safety of disease-infested silage. Yet the safety of feeding silage to animals depends on the bacterial and fungal population particularly because fungi may introduce significant amounts of harmful mycotoxins to animals (Mallman et al., 2009).

This study aimed to examine the relationship between increasing severity of rust infestation on corn plants and the fermentation, nutritive value, aerobic stability, and safety of the resulting silage. A second objective was to determine if bacterial inoculation could mitigate adverse effects of rust infestation on the quality, aerobic stability, and safety of corn silage. We hypothesized that rust infestation would decrease the quality of corn silage and inoculation would mitigate adverse effects of the disease on measures of silage quality and safety.

Materials and Methods

Silage, Treatments and Design

A corn hybrid was grown on a 65-ha field in August 2007 as a summer or second planting silage crop. The corn was infested with Southern rust at tasselling and treated with Abound fungicide (Syngenta Crop Protection, LLC, Greensboro, NC) by aerial application using a crop duster airplane. However, uneven coverage of the fungicide

allowed rust to persist in some parts of the field. Consequently, in some areas, plants were completely disease free (no rust, NR) whereas in other areas, leaves in the lower half (medium rust, MR) or all leaves (high rust, HR) were infested as shown by dense occurrence of small orange pustules on the leaves as well as yellowish brown discoloration (Figure 5-1). Corn forage from representative parts of the field with each rust classification were harvested when the DM of NR plants was 40% DM, and chopped using a Jaguar 900 forage harvester (Claas of America Inc., Omaha, NE). The forages were separated into piles and either treated with 100 mL of deionized water (Control) or 100 mL of a solution of Buchneri 500 inoculant (LB500 - Lallemand Animal Nutrition, Milwaukee, WI) that delivered 1×10^5 cfu/g of *Pediococcus pentosaceus* 12455 and 4×10^5 cfu/g of *Lactobacillus buchneri* 40788 . The inoculant also contained enzymes resulting from *Trichoderma reesei* fermentation and their minimum activities were 400 mg of glucose/min/g (β-glucanase), 1.140 mg of glucose/min/g (xylanase), and 18 mg of glucose/min/g (galactomannanase). Four replicates of each treatment were ensiled in 20 L laboratory silos at a density of 250 kg of DM/m^3 for 97 days at ambient temperature (25^{o}C) in an enclosed barn. Each of the 24 silos was sealed with airtight plastic lids and lids were secured with heavy-duty duct tape. Samples of freshly-treated unensiled forage were stored at -20ºC for chemical analyses. Weights of empty and full silos at ensiling were recorded for estimation of DM loss. At silo opening, silo weights were recorded, and the top slimy or moldy layer of silage was disposed of where present, as is the norm in practice. The remaining forage was transferred to a 20 L plastic bag for proper mixing, after which subsamples were collected for analysis of nutritive value, microbial counts, aerobic stability, and mycotoxins.

Laboratory Analysis

Corn forage and silage samples were dried at 60ºC for 48 h in a forced-air oven. Dried samples were ground to pass the 1-mm screen of a Wiley mill (A. H. Thomas, Philadelphia, PA) and analyzed for DM (105^{o}C for 16 h) and ash (512^{o}C for 8 h). Concentrations of NDF and ADF were measured in an ANKOM 200 Fiber Analyzer (Ankom Technologies, Macedon, NY) using the method of Van Soest et al. (1991). Heat-stable α-amylase and sulfite were used in the NDF assay. Nitrogen (N) was determined by rapid combustion using a Macro elemental N analyzer (Vario MAX CN, model ID 25.00-5003; Elementar, Hanau, Germany) and CP was calculated by multiplying N concentration by a factor of 6.25. In vitro DM digestibility (IVDMD) was measured with the method of Van Soest et al. (1966) and NDFD was estimated after analysis of dried samples and residues from the IVDMD assay for NDF. Silage samples were also analyzed for alflatoxin, zearalenone, deoxynivalenol, and fumonisin by thin-layer chromatography (Scott et al., 1970) at Dairyland Laboratories, Arcadia, WI).

Silage extract was prepared by mixing 25 g of corn silage with 225 mL of 0.1% peptone water in a stomacher for 3 minutes. The solution was filtered through 2 layers of cheesecloth and an aliquot was immediately used for fungal counts as described by Schmidt and Kung Jr. (2010). Yeasts and molds were enumerated after serial 10-fold dilutions by spread-plating on malt extract agar (Oxoid CM59, Oxoid Inc.). Silage pH was measured using a pH meter (Corning Model 12, Corning Scientific Instruments, Medfield, MA). An aliquot of 100 mL of silage extract was stored at -20ºC, after its pH was reduced to 2 with 50% (wt./vol.) sulfuric acid. Subsequently, such samples were used to quantify lactate and VFA with a high performance liquid chromatograph (Hitachi®, FL 7485, Tokyo, Japan) coupled to a UV Detector (Spectroflow 757, ABI

Analytical Kratos Division, Ramsey, NJ) set at 210 nm. The column was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) with a 0.015M sulfuric acid mobile phase and a flow rate of 0.7 mL/min at 45°C (Arriola et al., 2011).

Aerobic stability was measured by placing 2 kg of silage in an open-top polystyrene box. Temperature sensors (HOBO temperature data logger 64k, Onset computer corporation, Cape Cod, MA) were placed in the geometric center of each silage sample and temperatures were recorded every 30 min for 14 d. Four additional sensors were placed in the room to record ambient temperature. Silages were covered with 2 layers of cheesecloth to avoid drying. Aerobic stability was denoted by the time that elapsed before the difference between silage and ambient temperature was above 2ºC (Huisden et al., 2009). Samples of unensiled corn forage also were analyzed for DM, CP, NDF, ADF, IVDMD, NDFD, alflatoxin, zearalenone, deoxynivalenol, and fumonisin as described above.

Statistical Analysis

This experiment was analyzed as a completely randomized design with 3 (severity of rust: NR, MR, and HR) × 2 (inoculation treatments: inoculated vs. Control) factorial arrangement of treatments. The REG procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) was used to evaluate relationships between rust levels and the measures of the fermentation, nutritive, shelf life, and safety of corn silage. The GLM procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC) and a model containing inoculant, rust and interactions of these terms, was used to analyze the data. Mean separation was performed using the PDIFF procedure of SAS, which employs the Fisher's F-protected least significant difference test. Polynomial contrasts were used to examine effects of

increasing severity of rust infestation on measures of silage quality and safety. Significance was declared at $P \leq 0.05$ and tendencies at $0.1 \geq P > 0.05$.

Results and Discussion

The chemical composition of the freshly treated corn forage is shown in Table 5-1. The chemical composition of the forage was similar to that of corn forage in other studies in Florida (Kim and Adesogan, 2006; Huisden et al., 2009) except that concentrations of NDF and ADF of MR and HR silages were greater.

As indicated by the adjusted r^2 value, rust infestation accounted for ($P < 0.05$) most of the variability in DM concentration (82%), DM loss (76%), pH (76%), and DMD (65%), almost half of that in NDF concentration (41%) and between 20 and 24% of that in ADF, butyrate, and NDFD (Table 5-2). Weak relationships (adjusted $r^2 < 20\%$) also existed ($P < 0.05$) between the level of rust infestation and mold counts (15%) and concentrations of acetate (14%) and ammonia-N (15%). The strong relationship between DM concentration and the level of rust infestation was visually evident as increasing rust infestation made the crops drier. It is noteworthy that rust infestation was also highly correlated with pH, DM loss, and DMD, which are some of the most important measures of silage quality. The respective slopes and adjusted r^2 of these relationships indicate that as rust infestation increased, pH and DM concentration increased, and IVDMD decreased. Consequently, increasing rust infestation worsened the fermentation of corn silage, increased the associated losses, and reduced the nutritive value of the silage. Although no relationship was found between rust infestation and aerobic stability, it is pertinent to note that mold counts ($P = 0.03$, adjusted $r^2 = 0.15$) and aflatoxin concentration ($P = 0.07$, adjusted $r^2 = 0.09$) had weak but significant relationships with rust infestation. This is likely because rust infestation by *Puccinia*

polysora allowed opportunistic *Aspergillus* molds to infest corn plants and produce the mycotoxin. Like lodging, and insect injury, disease infestation often allows opportunistic fungi to invade plant tissues and produce mycotoxins (Whitlow and Haggler, 2005; Adesogan and Quieroz, 2009).

The chemical composition, IVDMD and NDFD of silages are shown in Table 5-3. Increasing rust infestation increased ($P < 0.001$, quadratic) the DM concentration of the silages and resulted in a 20-percentage unit difference between NR and HR silages. Crude protein and ash concentrations decreased and then increased ($P < 0.001$, quadratic) with increasing rust infestation, with the nadir occurring in MR silage. The NDF and ADF concentrations increased linearly ($P <0.01$), whereas IVDMD decreased linearly ($P < 0.001$) as rust infestation increased. The decreases in the nutritive value of corn silage with increasing rust infestation reflect the effects of the disease on corn forage and indicate that ensiling did not mitigate adverse effects of rust infestation on silage nutritive value. *Puccinia polysora* induces a premature desiccation of the plant (Rodriguez- Ardon et al., 1980), which could explain the adverse effects of increasing rust infestation on forage and silage DM concentration and nutritive value in this study. Johnson et al. (1997) reported that the DM, NDF and ADF concentrations of 5 corn hybrids were increased by Southern rust infestation leading to decreased IVDMD. Potter (1987) and Wilson et al. (1991) reported that rust infestation decreased the digestibility of two cultivars of ryegrass and pearl millet, respectively. In agreement, this study shows that rust infestation decreased the digestibility of corn forage and silage, largely by increasing the relative proportion of fiber in the silage. Johnson et al. (1997) reported that rust infestation had no effect on the CP concentration of corn plants.

Although a quadratic CP response to increasing rust infestation was detected in this study, the differences in CP concentration were very small. Based upon a literature review Dimmock and Gooding (2002) stated that the effect of rust on wheat protein is variable even though rust may have a deleterious impact on N accumulation and partitioning within the plant.

The progressive increase in fiber concentration as rust infestation increased is similar to findings of Johnson et al. (1997) and is attributable to premature desiccation of the plant or increased lignin deposition (Hammerschmidt, 1984), which is a defense mechanism against the invading fungus and the associated disease (Ride, 1978). Increases in fiber contamination with rust infestation may have also been caused by utilization of digestible nutrients in the plant by the fungus.

Inoculation slightly reduced ($P < 0.001$) the DM concentration and increased ($P = 0.02$) the ADF concentration of silages, perhaps reflecting the use of water-soluble carbohydrates as substrates by the bacteria in the inoculant. Inoculation also increased the NDFD of silages ($P = 0.023$), reflecting the action of the fibrolytic enzymes in the inoculant as well as those released by *L*. *buchneri* (Kang et al., 2009**).**

All silages had pH equal to or less than 4, indicating that the fermentations were satisfactory (Table 5-4). However, increasing rust infestation increased the pH values (quadratic, $P < 0.001$). The latter occurred largely because increasing rust infestation progressively decreased concentrations of lactate, acetate, propionate, and total VFA in Control forages but these trends were mostly reversed by inoculation (rust × inoculant interaction, $P < 0.01$). These results demonstrate perhaps for the first time that rust infestation worsens the fermentation of corn silage but inoculant application can mitigate

such effects. It is also noteworthy that inoculation was most effective at improving the fermentation of HR silages, probably because uninoculated HR silages had had fewer epiphytic bacteria or less fermentable substrate.

Ammonia-N concentrations decreased to a nadir in MR silages as rust infestation increased in Control silages but increased progressively in inoculated silages (rust × inoculant interaction, $P < 0.001$). Butyrate was only detected in HR silages and Control HR silages had slightly less ($P = 0.023$) butyrate than inoculated HR silages. Butyrate is primarily produced by Clostridia in silages with relatively high moisture concentrations, slow acidification rates or high final pH values (>4.8). Clostridia typically increases proteolysis and deamination in silages resulting in greater ammonia-N concentrations (McDonald, 1991). In this study, inoculation decreased the DM concentration, increased the pH, and tended to increase ammonia-N concentration. Therefore, inoculation may have increased Clostridial growth. Nevertheless, the difference in butyrate and ammonia-N concentrations between inoculated and Control silages were minimal and these disadvantages were outweighed by the benefits of inoculation.

Yeasts were not detected in the silages and inoculation did not affect DM losses. Mold counts decreased (quadratic, $P < 0.05$) as the level of rust infestation increased likely because of the concomitant increases in DM concentration reduced the water activity of the silage and thereby curtailed growth of molds. Aerobic stability increased (quadratic, $P < 0.01$) as the level of rust infestation increased reflecting the corresponding decrease in mold counts. Inoculation had no effect ($P = 0.13$) on mean mold counts though counts were 3.5 times greater in Control HR silages versus inoculated HR silages (3.40 vs. 0.95 cfu/g). The latter explains the 75% improvement in

the aerobic stability of Control versus inoculated HR silages (44 vs. 77 h). The increase in aerobic stability with increasing rust infestation was more pronounced in inoculated than Control silages (rust × inoculant interaction, $P = 0.01$) further justifying inoculation of the silages.

Aflatoxin was only detected in the Control HR silage (rust x inoculant interaction, $P = 0.10$) and the level (5.2 mg/kg) was 260 times greater than the Action Level of 20 µg/ kg stipulated by the US Food and Drug Administration for dietary ingredients for dairy cattle. Consequently, the Control HR silage would be unsafe to feed. High levels of aflatoxin in corn silage are notable but not unique. Gonzales Pereyra et al. (2008) reported an aflatoxin concentration of 156 µg/ kg in corn silage. Yet silage is expected to have low concentrations of aflatoxin because the toxin is primarily produced by *Aspergillus flavus* and *A. parasiticus*, which have low tolerance for the anaerobic conditions and low pH in silage (Holmquist et al., 1983). However, the latter factors do not prevent the survival of these organisms in silage. Garon et al. (2006) successfully isolated *A. parasiticus* from corn silage samples with a high aflatoxin concentration (34 µg/kg).

That no toxin was found in the inoculated HR silage suggests that inoculation prevented accumulation of the toxin in the rust infested silage. Future research should validate this finding and determine effects of rust infestation on populations of *A. parasiticus and A. flavus* on corn forages before and after ensiling.

No deoxynivalenol or fumonisin was detected in the silages. However, unlike rust-infested silages, Control and inoculated NR silages had high concentrations of zearalenone (0.65 vs. 0.47 mg/kg). The FDA does not have action, advisory or

guidance levels established for zearalenone; however, the concentrations of zearalenone in this study were below the guidance level (2000 µg/ kg) stipulated by the European Commission legislation (European Commission, 2006). Nevertheless, the presence of zearalenone in the uninfested corn silages indicates that summer or second planting corn silage produced in Florida could be infested with this mycotoxin, perhaps because the hot, humid conditions allow the fungi that produce this mycotoxin to thrive.

No data was found in the literature on how inoculation affects the quality of disease-infested silage. However, effects of bacterial inoculation on the quality of ensiled healthy corn plants are well documented (Pedroso et al., 2010; Schmidt and Kung 2010; Arriola et al., 2011). Huisden et al. (2009) reported that the aerobic stability of corn silage was increased by the same inoculant used in this study (LB500; 143 vs. 389 h). Schmidt and Kung (2010) demonstrated that *L. buchneri* alone or in combination with *Pediococcus pentosaceus* increased the concentration of acetate, which decreased the population of spoilage fungi and increased aerobic stability of corn silage. In this study, mean concentrations of antifungal acids (acetate, and butyrate) were increased by inoculation and hence mean aerobic stability was increased. The inoculated HR silage had the greatest acetate concentration, the lowest mold counts, and the best aerobic stability. These results reflect the ability of the *L. buchneri* in the inoculant to synthesize the antifungal organic acid, acetate, by using lactate as a substrate (Oude Elferink et al., 2001).

Conclusions

Rust infestation was positively correlated with pH, DM concentration, and DM recovery and negatively correlated with IVDMD and it explained most of the variability in these measures. Therefore, increasing rust infestation reduced the nutritive value,

worsened the fermentation, and increased losses from the silage. Silages with the highest level of rust infestation had aflatoxin concentrations that exceeded the FDA Action Level for livestock feeds by 260%, implying that such silage is unsafe to feed. Inoculation with the dual-purpose inoculant decreased most negative effects of rust infestation on the nutritive value and fermentation of the silage, prevented accumulation of aflatoxin in the silage, and increased aerobic stability.

Table 5-1. Chemical composition of untreated and inoculated corn forage with different levels of rust infestation (% of DM)[1]

	Control			Inoculant		
	NR	MR	HR	NR	MR	HR
DM	39.3	40.7	58.2	39.0	42.1	58.6
CP	7.40	7.41	8.07	7.96	6.82	8.32
NDF	44.7	49.1	55.1	45.3	51.1	54.6
ADF	25.5	29.3	32.9	26.4	28.4	31.2
ASH	4.43	3.76	4.79	4.66	3.86	4.40

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri*, NR = no-rust level; MR = medium rust level; HR = high rust level.

Table 5-2. The relationship between the severity of rust infestation and measures of the quality of the fermentation, nutritive value, safety, and shelf life of corn silage

Parameter	Intercept	Slope	R^2	Adjusted[1] R^2	RMSE	P value
pH	3.46	0.16	0.77	0.76	0.07	<.0001
NH_3-N,	0.16	0.05	0.19	0.15	0.08	0.03
Lactate	3.86	-0.24	0.04	0.001	0.97	0.32
Total VFA	4.75	0.07	0.003	-0.04	1.16	0.80
Acetate	1.06	0.31	0.17	0.14	0.56	0.04
Propionate	1.15	-0.11	0.12	0.08	0.26	0.08
Butyrate	2.94	-0.42	0.24	0.20	0.64	0.01
DM loss	3.46	0.16	0.77	0.76	0.07	<0.001
Molds, log cfu/g	0.16	0.50	0.19	0.15	0.08	0.03
Aerobic stability, h	<0.001	0.32	0.04	0.002	0.97	0.32
DM	25.3	9.88	0.83	0.82	3.74	<0.001
CP	9.03	0.11	0.06	0.02	0.38	0.25
NDF	41.2	3.06	0.44	0.41	2.95	0.001
ADF	22.0	1.88	0.24	0.20	2.84	0.02
Ash	4.89	-0.20	0.11	0.07	0.48	0.11
IVTDMD[2]	72.6	-4.55	0.66	0.65	2.76	<0.001
NDFD[3]	45.0	-3.00	0.28	0.24	4.17	0.02
Aflatoxin	-1.74	1.30	0.13	0.09	2.79	0.07

[1]Adjustment of the r^2 value for degrees of freedom following analysis of variance
[2]IVTDMD = 48-h in vitro true dry matter digestibility, %.
[3]NDFD = NDF obtained after 48-h of in vitro true dry matter digestibility, %.

Table 5-3. Effect of rust infestation and inoculant application on chemical composition of corn silage (% of DM or as stated)[1]

	Control			Inoculant			SEM[2]	Inoculant	Rust	Inoculant x Rust	Rust level	
	NR	MR	HR	NR	MR	HR					Linear	Quadratic
DM	38.0	40.6	58.3	37.2	39.6	56.5	0.29	<0.001	<0.001	0.24	<0.001	<0.001
CP	9.4	8.8	9.6	9.3	8.8	9.6	0.11	0.82	0.01	0.96	0.05	<0.001
NDF	44.1	47.7	48.5	43.4	48.8	51.3	1.50	0.38	0.002	0.51	<0.001	0.30
ADF	23.1	25.1	25.3	25.3	25.4	30.7	1.21	0.02	0.02	0.13	0.006	0.42
Ash	4.7	4.1	4.5	5.1	4.0	4.8	0.14	0.44	0.001	0.35	0.06	0.01
IVTDMD[3]	66.9	63.2	60.1	68.9	64.2	57.5	1.41	0.93	<0.001	0.25	<0.001	0.80
NDFD[4]	38.1	39.8	36.2	43.4	45.7	33.0	1.3	0.02	<0.001	0.004	0.001	0.001

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri*, NR = no-rust level; MR = medium rust level; HR = high rust level.
[2]SEM = standard error of mean.
[3]IVTDMD = 48-h in vitro true dry matter digestibility, %.
[4]NDFD = NDF obtained after 48-h of in vitro true dry matter digestibility, %.

Table 5-4. Effect of rust infestation and inoculant application on fermentative parameters, microbial counts, aerobic stability and mycotoxins in corn silage (% of DM or as stated)[1]

	Control			Inoculant			SEM[2]	Inoculant	Rust	Inoculant x Rust	Rust level	
	NR	MR	HR	NR	MR	HR					Linear	Quadratic
pH	3.65	3.71	3.97	3.68	3.69	4.00	0.02	0.314	<0.001	0.340	<0.001	<0.001
NH_3-N	0.29	0.19	0.25	0.17	0.27	0.42	0.03	0.074	0.001	<0.001	0.002	0.034
NH3, % total N	3.05	2.18	2.55	1.87	2.98	4.24	0.28	0.07	0.007	<0.001	0.004	0.175
Lactate	4.99	4.02	2.28	2.27	3.17	3.50	0.21	<0.001	0.006	<0.001	0.004	0.077
Total VFA	6.37	4.59	4.02	4.04	5.31	6.31	0.36	0.441	0.740	<0.001	0.916	0.449
Acetate	1.53	1.35	1.38	1.27	1.89	2.67	0.20	0.006	0.022	0.005	0.007	0.615
Propionate	1.16	0.90	0.58	0.94	0.91	0.74	0.05	0.694	<0.001	0.004	<0.001	0.253
Butyrate	0.00	0.00	0.51	0.00	0.00	0.71	0.03	0.023	<0.001	0.009	<0.001	<0.001
DM loss, %	1.13	1.26	0.93	1.07	0.97	0.78	0.24	0.415	0.514	0.904	0.334	0.536
Molds, log cfu/g	5.24	4.96	3.40	4.93	5.20	0.95	0.64	0.127	<0.001	0.117	<0.001	0.018
Aerobic stability, h	26.0	27.5	44.0	27.5	23.8	77.3	3.77	0.05	<0.001	0.012	<0.001	0.005

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri*, NR = no-rust level; MR = medium rust level; HR = high rust level. [2]SEM = standard error of mean.

Table 5-5. Effect of rust severity and inoculant application on mycotoxin concentration of corn silage (mg/kg of DM)[1]

Items	Control			Inoculant			SEM[2]	Inoculant	Rust	Inoculant x Rust
	NR	MR	HR	NR	MR	HR				
Aflatoxin	0.00	0.0	5.20	0.00	0.0	0.00	1.50	0.109	0.109	0.109
Zearaleneone	0.646	0.0	0.0	0.471	0.0	0.0	0.080	0.303	<0.001	0.303
Deoxynivalenol	0.00	0.0	0.00	0.00	0.0	0.00	0.00	NA[3]	NA	NA
Fumonisin	0.00	0.0	0.00	0.00	0.0	0.00	0.00	NA	NA	NA

[1]Control = no inoculant added, Inoculant = 1 x 10^5 cfu of *P. pentosaceus* and 4 x 10^5 of *L. buchneri*, NR = no-rust level; MR = medium rust level; HR = high rust level.
[2]SEM = standard error of mean.
[3]NA = Not applicable.

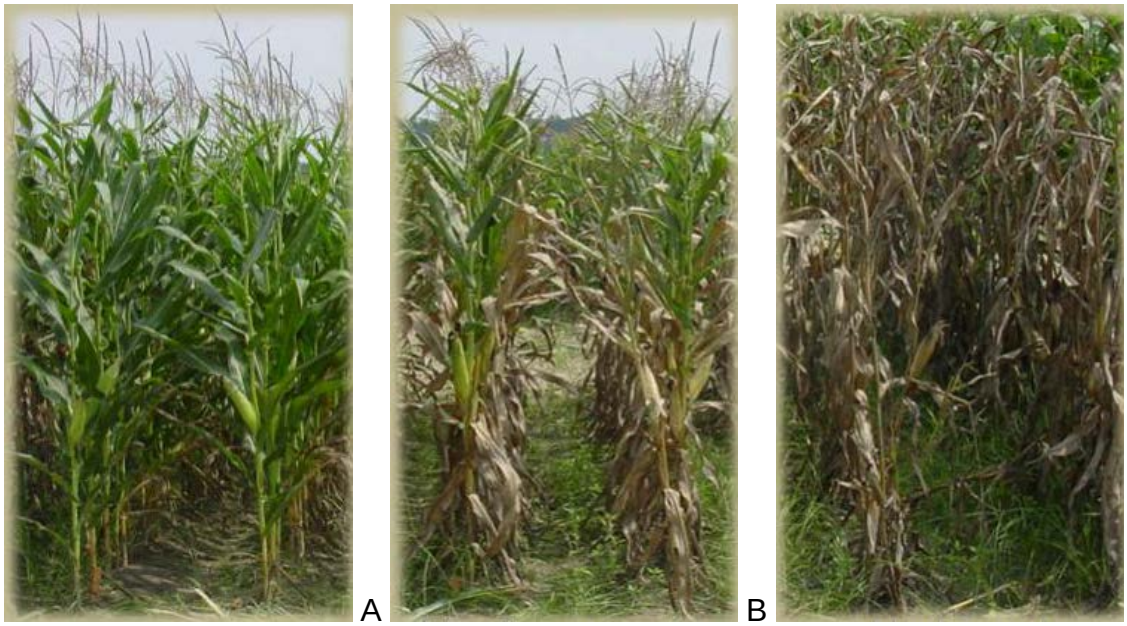

Figure 5-1. Corn plants affected by different severities of Southern Rust. A) no rust, B) medium rust and C) high rust.

CHAPTER 6
EFFECT OF ADDING A MYCOTOXIN-SEQUESTERING AGENT ON MILK AFLATOXIN M1 CONCENTRATION AND THE PERFORMANCE AND IMMUNE RESPONSE OF DAIRY CATTLE FED AN AFLATOXIN B1 - CONTAMINATED DIET

Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus parasiticus*, *A. flavus* and the rare *A. nomius* (Creppy, 2002). They occur in nature as the B_1 (AFB_1), B_2, G_1, G_2, and M_1 forms (Masoero et al., 2007). Aflatoxins can negatively affect animal health, performance and reproduction if consumed in sufficient quantities (Whitlow and Haggler, 2005). The toxic and carcinogenic M_1 form, which results from conversion of AFB_1 by hepatic metabolism, can be secreted into milk (Masoero et al., 2007). Due to the high amount of milk and milk products consumed by humans, keeping the concentration of aflatoxin M_1 (AFM_1) in milk within safe levels is critical. To avoid the risk of aflatoxin ingestion and intoxication, agencies around the world have established acceptable limits for aflatoxin concentration in milk and feeds. In the US, the Food and Drug Administration (FDA) stipulated Action Levels for aflatoxin in raw milk and lactating cow feeds are 0.5 and 20 µg/kg, respectively. The maximum allowable concentration set by the European Commission is 0.05 µg/kg of milk (Creppy, 2002). The goal of keeping feed and milk aflatoxin concentration below these limits can be difficult to achieve because mycotoxin-producing molds infect crops and grains before and after harvesting (Garcia- Lopez and Phillips., 1999; Gonzales-Pereyra et al., 2008; Richard et al., 2009). Furthermore, damage from insects, hail, lodging, and diseases (Queiroz et al., 2009) can predispose plants to mycotoxin contamination.

Many post-harvest treatments are used for detoxifying feeds contaminated with mycotoxins including thermal inactivation, irradiation, fermentation, ammoniation, and

nixtamalization (Lopez-Garcia and Phillips., 1999). Most of these methods are costly, time consuming or partially effective (Kurtz et al., 2009), and most are impractical for detoxification of the large quantities of feed used by many US dairies. Studies have demonstrated that dietary addition of adsorbent clays is a promising and effective way to prevent aflatoxin intoxication by livestock on farms (Stroud, 2006). However, few ofsuch studies have examined the effect of the clay dose on efficacy in dairy cows and fewer still have examined dose effects on markers of the immune response in dairy cows.

The objectives of this study were to determine the effect of adding two doses of a montmorillonite hydrated sodium-calcium-aluminosilicate-clay- based mycotoxin adsorbent on milk aflatoxin M_1 (AFM_1) concentrations and the performance and immune response of dairy cows fed a diet contaminated with aflatoxin B_1 (AFB_1).

Materials and Methods

Cows, Treatments and Design

Animals used in this study were cared for according to protocols approved by the University of Florida Institutional Animal Care and Use Committee. Eight lactating Holstein cows in late lactation (295 ± 45 DIM) were stratified by milk production and randomly assigned to one of four treatments arranged in a balanced, replicated 4 x 4 Latin square design. Cows were housed in an open-sided, free-stall barn bedded with sand and equipped with Calan gates (American Calan Inc.) for individual feeding and misters and fans to minimize heat stress. The following treatments were investigated: 1) Control diet (C), 2) Toxin diet (T) containing C and 75 µg/kg of AFB_1 in the TMR, 3) Low-clay (LC) diet containing T and the adsorbent added at 0.2% of the TMR DM, and 4) High-clay diet (HC) containing T and the adsorbent added at 1% of the TMR DM. The

adsorbent was Calibrin A from Amlan International, Chicago, IL. Diets were formulated to meet or exceed the nutrient requirements of Holstein cows in late lactation producing 20 kg/d of milk (NRC, 2001). The ingredient and chemical compositions of the basal TMR fed to all cows are shown in Table 6-1. Cows were fed the toxin and adsorbent based on an estimated average DMI of 23 kg/d, resulting in a daily intake of 1725 µg of AFB_1 for T, LC, and HC treatments, and 46 and 230 g of adsorbent in the LC and HC diets, respectively. Dietary AFB_1 was obtained from an *Aspergillus parasiticus* (NRRL-2999) culture at the University of Missouri Diagnostic Laboratory and it contained 640 mg/kg of AFB_1, 22 mg/kg of aflatoxin B_2, 333 mg/kg of aflatoxin G_1, and 3 mg/kg of aflatoxin G_2. Appropriate doses of the adsorbent were mixed into the TMR and fed daily to cows fed LC and HC diets. The AFB_1 was added only to diets on d 6 to 9 of each period and d 10 to 12 and 1 to 5 of the current and subsequent periods were for clearance of the toxin from the cow's milk. Therefore, the toxin was dosed for 4 d and it cleared from the system over 8 d. The daily dose of AFB_1 was divided into 2 portions and each was mixed with 20 mL of molasses and 400 g of corn silage to facilitate consumption. The mixture was fed to cows in a plastic container before the rest of the TMR was fed at the a.m. and p.m. feeding times (0700 and 1700 h). Intake of the TMR was restricted to 95% of that in a two-week pretrial period when a common diet was fed to all cows. This feeding strategy ensured complete consumption of the clay adsorbent and AFB_1 in the relevant diets and prevented contamination of equipment and the feed bunk with the toxin.

Analytical Procedures

Cows were milked twice a day at 0100 and 1300 h and milk weights were recorded. Two milk samples were collected from a.m. and p.m. milkings on d 5, 9, 10,

11, and 12 in each period. Samples were analyzed by Southeast Dairy labs (Belleview, FL) for fat, protein and somatic cell counts (SCC) using a Bentley 2000 Near Infrared Reflectance Spectrophotometer (Bentley Instruments Inc., Chaska, MN). Somatic cell scores were generated as described by Norman et al. (2000) for statistical analysis of SCC. Values for 3.5% fat-correct milk yield were calculated according to the equation: [(0.4324 × milk yield) + (16.218 × milk fat yield)] (NRC, 2001). Milk AFM_1 concentration was quantified using the radioimmunoassay test (CHARM II test, Charm sciences Inc., Maiden, MA) described by Diaz et al. (2004), which had been validated against High Performance Liquid Chromatography measurements in a ring test (Salter et al., 2006).

Weights of feed offered and refused by each cow were recorded daily. Dietary ingredients (corn silage, alfalfa hay, and concentrate mix) were sampled representatively daily. Four composites of the daily samples in each period were subsampled, dried at 60ºC 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 analyzed for DM (105^{o}C for 16 h) and ash (512^{o}C for 8 h). Concentrations of NDF and ADF were measured using the method of Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Nitrogen was determined by rapid combustion using a Macro elemental N analyzer (Vario MAX CN, model ID 25.00-5003; Elementar, Hanau, Germany) and CP was calculated as N x 6.25. Blood samples (10 mL) were collected at 5 and 9 d from the coccygeal vein into vacutainer tubes containing sodium heparin anticoagulant (Becton, Dickinson and Co., Franklin Lakes, NJ), stored on ice during transport, centrifuged at 2,500 × *g* for 20 min at 4°C to separate plasma, and stored at –20°C until analyzed. 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 in duplicate samples using the colorimetric procedure described by Demetriou et al. (1974). Plasma fibrinogen concentrations were determined using a kit (Sigma procedure No. 880; Sigma Diagnostics, St. Louis, MO) as described by Arthington et al. (2003). Neutrophil phagocytic activity was measured by monitoring the uptake of *E.coli* particles labeled with a pH-sensitive dye from pHrodo™ *E. coli* BioParticles® Conjugate (Invitrogen Life Sciences, Carlsbad, CA). Briefly, 40 µL of pHrodo ™ E.coli were added to a 100 µL aliquot of blood containing no more than 5×10^3 cell/µL. The solution was incubated for 2 h at 37^oC and cell membranes were disrupted by incubation in 2.5 mL of lysis buffer for 15 min at room temperature. The suspension was centrifuged at 2000 × g for 5 min, and washed with fluorescence-activated cell sorting (FACS) buffer before a final centrifugation at 2000 × g for 5 min. The pellet was kept on ice and phagocytotic activity was measured using a flow cytometer with a 488 nm excitation wavelength (FACSort; Becton Dickinson, San Jose, CA). Neutrophil adhesion molecules, CD62 (L-selectin) and CD18 (β-integrin) were quantified by flow cytometry as described by Silvestre et al. (2011). Complete blood counts (CBC) were conducted at the University of Florida Clinical Pathology Laboratory using a Bayer Advia 120 hematology analyzer (Bayer Avia 120 hematology analyzer; Bayer Diagnostics (Siemens), Deerfield, IL).

Statistical Analysis

The experiment consisted of a balanced, replicated Latin square design with 4 treatments and 4 periods. The MIXED procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) was used to analyze the data. The model for analyzing the data included treatment, period, square, treatment x period, and treatment x square interactions, and

cow within square as the random effect. Data from blood and milk samples collected on d 5 were used as covariates for analyzing data obtained during the toxin-dosing period. Blood data were not normally distributed, therefore they were log transformed and analyzed with the GLIMMIX procedure of SAS. The PDIFF statement of SAS was used to compare Least Squares means. Contrast statements were used to compare the Control treatment to each of the other treatments. Significance was declared at $P \leq 0.05$ and tendencies were declared if $P > 0.05 < 0.10$.

Results and Discussion

Dry matter intake and milk yield were not affected by dietary treatments ($P > 0.05$) and averaged 19.2 kg/d (Table 6-2). Feeding diet T tended to reduce FCM yield (19.0 vs. 20.8 kg/d, $P = 0.08$). Kutz et al. (2009) detected no changes in DMI or milk yield when 112 µg of AFB_1/kg of TMR DM was fed to dairy cows in early to midlactation for 7 d. Stroud (2006) reported no changes in milk yield due to feeding 170 µg of AFB_1 /kg of TMR DM for 11 days, but the toxin decreased DMI by 1.5 kg of DM/d compared to cows no supplemented with AFB_1. Whitlow and Hagler (2005) suggested that diets contaminated with aflatoxin levels above 100 µg/kg may adversely affect animal performance and health, therefore it is noteworthy that feeding 75 µg/kg of the toxin in this study also reduced 3.5% FCM yield. Stroud (2006) noted that dosing cows with AFB_1 concentrations less than 150 µg/kg is more common than using higher rates because natural contamination rates are much lower (28 ±19 µg/kg; Whitlow, 2005).

Milk composition was reduced by feeding the toxin. Compared to diet C, diet T reduced milk protein concentration (3.28 vs. 3.36%, $P = 0.01$) and milk fat yield (0.67 vs. 0.74 kg/d, $P = 0.04$). A previous study demonstrated binding of aflatoxin to some fractions of milk protein but no changes in milk composition were reported (Barbiroli et

al., 2007). Smith et al. (1994) did not detect changes in milk fat and protein percentages due to feeding 200 μg of AFB_1/kg of diet DM to goats. Kutz et al. (2009) reported that milk protein and fat percentage were unaffected when dairy cows were fed an aflatoxin-contaminated diet. The reason why the toxin adversely affected milk quality in this study but not others may be partly due to the combination of different aflatoxins fed in the current study. Natural dietary mycotoxin contamination of diets typically results in more adverse effects on cows than purified mycotoxins because of synergistic effects of different toxins (Applebaum et al., 1982). The similarity in milk quality and FCM yield measures among diets C, LC, and HC indicate that both doses of the adsorbent prevented adverse effects of the toxin on milk production and composition.

Concentrations of AFM_1 in milk from cows fed diets T, LC, and HC (0.57, 0.64 and 0.46 μg/kg, respectively) were greater ($P < 0.05$) than those of cows fed diet C (0 μg/kg) and concentrations were lower ($P < 0.05$) in cows fed HC versus those receiving diet T or LC (Table 6-3). Relative to diet T, diet HC reduced the milk AFM_1 concentration by 20% but diet LC did not reduce the value. Milk AFM_1 concentrations were greater than the FDA Action Level (0.5 μg/kg) in cows fed T or LC, whereas those of cows fed HC were lower. Therefore, dietary addition of HC kept the milk safe and legal but addition of LC did not, indicating the ineffectiveness of the low dose of the adsorbent. Other studies have also reported that mycotoxin binders reduced the AFM_1 concentration of milk. Diaz et al. (2004) compared effects of adding activated carbon, esterified glucomannan, calcium bentonite and 3 sodium bentonite products at 1.2% of diet DM on concentrations of AFM_1 in milk of cows fed diets contaminated with 100 μg/kg of AFB_1. Respective reductions in milk AFM_1 concentrations were 59, 31, 65, 50 and 61%. Kutz

et al. (2009) evaluated the effect of adding two hydrated-sodium- calcium aluminosilicates (Novasil plus, Engelhard Corp, New Jersey, USA and Solis, Novus International Inc., St. Charles, MO) or an esterified glucomannan (MTB-100, Alltech, Nicholasville, KY) product at 0.5% of diet DM on concentrations of AFM_1 in milk of cows fed 100 µg of AFB1/kg of diet. The aluminosilicate products reduced milk AFM_1 concentration by 45 and 48%; however, the esterified glucomannan caused a reduction of only 4%.

The effects of the strategy used to administer the toxin and adsorbent on performance of cows and milk AFM_1 concentration are unknown. Adding the toxin to ingredients fed before the rest of the TMR was offered achieved the objective of minimizing contamination of equipment and ensuring complete consumption of toxin, but may have limited binding of the toxin by the adsorbent if the outflow rate of the toxin from the rumen was rapid. A similar approach was used by Kutz et al. (2009) to ensure complete ingestion of the toxin and minimize equipment contamination. However, Diaz et al. (2004) and Stroud (2006) mixed the toxin and adsorbent with the rest of the ingredients in the TMR, which is a more practically relevant strategy. That these authors changed their dosing strategy reflects the importance of minimizing equipment contamination with the toxin and the associated health and safety risks.

Excretion and transfer of aflatoxin into the milk were lower in cows fed HC than those fed LC but cows fed T had similar values to those fed LC or HC. Therefore, addition of either dose of the adsorbent did not reduce the transfer and excretion of the toxin in milk. This may have been because the dosing method did not maximize binding of the toxin by the adsorbent. Nevertheless, the numerical trend for lower transfer and

excretion of the toxin for diet HC versus T or LC is consistent with the lower AFM_1 concentration in milk of cows fed HC versus T or LC.

The LC adsorbent was less effective than HC at preventing transfer and excretion of AFB_1 to milk because adsorption of aflatoxin by aluminosilicate binders happens in a dose-dependent manner (Sarr, 1992). Even though application rates of mycotoxin adsorbents to ruminant diets range from 0.5 to 1.2% of dietary DM (Harvey et al., 1991; Stroud, 2006; Kutz et al., 2009), low rates have not been effective consistently at mitigating effects of aflatoxin on the performance, health and milk AFM_1 concentrations of dairy cows.

The rate of transfer of dietary aflatoxin into milk was reported to be between 0 and 4% by Sieber and Blanc (1978, as described by Van Egmond, 1989) and between 1 and 6% by the European Food Safety Authority (EFSA, 2004). Lafont et al. (1980) fed daily doses of 0.09, 0.18, 0.86, or 2.58 mg to cows in early and late lactation producing 20 L/d of milk on average and the respective transfer rates were 0.78 and 0.22%. The transfer rates in late lactation and the milk production level are similar to those in this study, which also involved cows in late lactation. Frobish et al. (1986) reported that high-producing cows have greater transfer rates than cows with moderate to low milk production. Cows in this study and that of Lafont et al. (1980) also had lower transfer rates than the mean value of 2% reported for cows producing 33.8 kg/d in a similar study (Kutz et al., 2009). In fact, after reviewing 14 studies on effects of adsorbents on aflatoxin transfer, Stroud (2006) reported that the mean transfer rate was about 1% as in this study, when diets were dosed with up to 150 μg/kg of aflatoxin.

Treatment effects on immune responses are summarized in Table 6-4. Haptoglobin concentrations in plasma were greater ($P < 0.01$) in cows fed diet T than other diets, whereas values for cows fed diets C, LC and HC did not differ. Stimulation of the acute-phase response due to inflammatory stress is characterized by increased secretion of acute-phase proteins such as ceruloplasmin and haptoglobin (Bertoni et al., 2008). Haptoglobin is often used in ruminants as a biomarker to identify immune-challenged animals (Heegaard et al., 2000; Arthington et al., 2003). Hiss et al. (2004) demonstrated the sensitivity of haptoglobin to immune stressors by showing that blood haptoglobin concentration was increased 11.3 folds after 12 h of intramammary administration of lipopolysaccharides. That blood haptoglobin concentrations were elevated when diet T was fed but not when diets C, LC, or HC were fed indicates that both doses of the adsorbent prevented the increased innate immune inflammatory stress response caused by the toxin. Fibrinogen and ceruloplasmin are also acute phase protein markers of the innate immune response, yet their concentrations did not differ among treatments. This is partly because these markers are often less sensitive and respond less consistently than haptoglobin to inflammatory stressors (Arthington et al., 2003). Furthermore, because concentrations of these proteins were only measured at a single time point, 96 h after toxin dosing was initiated, important trends in the early response of ceruloplasmin and fibrinogen to the toxin-induced stress may have been undetected.

The fluorescence intensity observed when evaluating the activity of adhesion molecules indicates the presence of receptors for β-integrin or L-selectin on the leukocyte membrane (Murphy et al., 2008). Both are adhesion molecules that facilitate

the interaction between leukocytes and the endothelium, which is needed to allow diapedesis, which is further cellular extravasation of leukocytes to the site of infection (Kenneth et al., 2008). In this study, feeding diet T instead of C tended to increase the expression of β-integrin (131 versus 220, *P* = 0.1), which agrees with the haptoglobin response. Collectively, the response to haptoglobin and β-integrin indicate that the toxin increased inflammatory stress, but feeding the adsorbent prevented this problem.

Dietary treatments did not affect the percentage of phagocytotic neutrophils or neutrophil phagocytosis (Table 6-4). The technique used to evaluate neutrophil phagocytosis is based on using *E.coli* cells stained with a pH-sensitive die, which becomes fluorescent when bacterial cells are exposed to the acidic environment within the leukocyte phagosome. This technique is more convenient, rapid, and less laborious than more traditional techniques (Silvestre et al., 2011). However, it does not involve using dihydrorhodamine 123 or other oxidase-peroxidase markers, therefore it cannot account for oxidative burst, which is the capacity of neutrophils to destroy the phagocytized bacteria.

The pattern of clearance of AFM_1 in the milk after toxin dosing was terminated on d 9 is shown in Figure 6-1. No AFM_1 was detected in the milk of any cow by d 12. Clearance of the toxin within 3 d agrees with published data. Frobish et al. (1986) reported that after 3 to 4 d of AFB_1 removal from diets, the AFM_1 concentration in milk should be reduced to normal levels. The studies of Applebaum et al. (1982) and Diaz et al. (2004) also confirmed that AFM_1 cleared from the milk of animals receiving an AFB_1-contaminated diet within 4 d. Masoero et al. (2007) detected that within 24 h of toxin withdrawal from the diet, milk AFM_1 levels had dropped below the FDA legislative limit

(0.5 µg/kg) and this decrease was significant ($P < 0.05$) within 48 h of withdrawing the toxin from the diet. In this study, no AFM_1 was detected in the milk of cows fed diet HC 24 h after the toxin was withdrawn from the diet. Therefore, the high dose of the adsorbent kept the milk legal and safe when the toxin was included in the diet and cleared toxin residues from the milk within a shorter period than the low dose.

Conclusions

Feeding AFB_1 tended to reduce FCM yield, reduced milk fat yield and milk protein concentration, increased the innate immune response, and increased milk AFM_1 concentration to levels that exceeded the FDA legislative limit. The low and high doses of the mycotoxin adsorbent prevented the adverse effects of the toxin on the immune response, milk quality, and FCM yield but only the high dose kept the AFM_1 concentration of the milk below the FDA Action Level.

Table 6-1. Ingredient and chemical composition of the experimental diet

Ingredient composition	% of DM
Corn silage	40.9
Alfalfa hay	8.05
Wet brewers grains	5.56
Distillers grains	6.97
Dried citrus pulp	3.34
Ground corn	18.2
SoyPlus[1]	4.03
Soybean meal	5.26
Sugarcane molasses	3.95
Mineral and vitamin mix	3.74
Chemical composition	
DM, %	46.7
Ash, % of DM	5.8
Crude protein, % of DM	15.5
Neutral detergent fiber, % of DM	39.1
Acid detergent fiber, % of DM	20.4
Aflatoxin B1, µg/kg	ND[2]
Afltoxin B2, µg/kg	ND
Aflatoxin G1, µg/kg	ND
Aflatoxin G2, µg/kg	ND
Deoxynivalenol, mg/kg	ND
T-2, mg/kg	ND
Zearalenone, mg/kg	ND

[1]Mineral mix contained 23.7% CP, 9.7% Ca, 8.0% Na, 6.7% K, 2.4% Mg, 0.4% S, 0.9% P, 2886 mg/kg of Mn, 3092 mg/kg of Zn, 886 mg/kg of Cu, 339 mg/kg of Fe, 31 mg/kg of Co, 30 mg/kg of I, 17.0 mg/kg of Se. 147,756 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis).
[2]ND concentrations of respective toxin were below lower detection limits (5 µg/kg for aflatoxins, and 0.5 mg/kg for deoxynivalenol, T-2, and zearalenone.

Table 6-2. Effect of dietary addition of aflatoxin B_1 (AFB_1) with or without low (LC) or high (HC) doses of a mycotoxin binder[1] on the performance of dairy cows

Item	Control (C)	AFB_1 toxin (T)	LC+T (LC)	HC+T (HC)	SEM	Contrast *P values*		
						C vs. T	C vs. LC	C vs. HC
DMI, kg/d	20.3	18.0	18.1	20.4	1.24	0.12	0.14	0.96
Milk yield, kg/d	19.5	18.9	19.9	19.1	1.11	0.44	0.60	0.60
3.5% FCM, kg/d	20.8	19.0	20.5	19.4	0.79	0.08	0.80	0.12
Milk protein, %	3.36[ab]	3.28[c]	3.35 [b]	3.41[a]	0.095	0.01	0.72	0.08
Milk fat, %	3.75	3.78	3.68	3.69	0.180	0.77	0.55	0.61
Milk protein, kg/d	0.63	0.60	0.67	0.66	0.05	0.62	0.59	0.65
Milk fat, kg/d	0.74[a]	0.67[b]	0.73[ab]	0.69[ab]	0.03	0.04	0.73	0.09
SCC x 1000/mL	272	147	194	260	112	0.25	0.35	0.73

[a, b,c] Means within rows with no common superscript differed significantly ($P < 0.05$).
[1]Produced by Amlan International, Chicago, IL.

Table 6-3. Effect of dietary addition of aflatoxin B_1 (AFB_1) with or without low (LC) or high (HC) doses of a mycotoxin binder[1] on the aflatoxin M_1 (AFM_1) concentration in the milk

Item	Control (C)	AFB_1 toxin (T)	LC+T (LC)	HC+T (HC)	SEM	Contrast *P values*		
						C vs. T	C vs. LC	C vs. HC
Concentration, µg/kg	0^{c}	0.57^{a}	0.64^{a}	0.46^{b}	0.04	<0.001	<0.001	<0.001
Excretion, µg/d	0^{c}	10.1^{ab}	12.9^{a}	8.7^{b}	1.55	<0.001	<0.001	<0.001
Transfer, %	0^{c}	0.56^{ab}	0.75^{a}	0.50^{b}	0.05	<0.001	<0.001	<0.001
Reduction, %	100^{a}	0^{c}	-0.5^{c}	20^{b}	7.05	<0.001	<0.001	<0.001
Clearance rate, µg//h	0^{c}	0.011^{ab}	0.013^{a}	0.009^{b}	0.0008	<0.001	<0.001	<0.001

[a, b, c] Means within rows with no common superscript differed significantly (P<0.05).
[1]Produced by Amlan International, Chicago, IL.

Table 6-4. Effect of dietary addition of aflatoxin B_1 (AFB_1) with or without low (LC) or high (HC) doses of a mycotoxin binder[1] on markers of the innate immune response

Item	Control (C)	AFB_1 toxin (T)	LC+T (LC)	HC+T (HC)	SEM	Contrast *P values*		
						C vs T	C vs LC	C vs. HC
Acute phase proteins								
Ceruloplasmin, mg/100mL	21.0	21.5	20.2	22.2	1.18	0.74	0.64	0.48
Haptoglobin, arbitrary unit[1]	14.4[b]	22.0[a]	14.8[b]	16.0[b]	1.98	0.01	0.87	0.56
Fibrinogen, mg/100mL	270	301	278	275	25	0.38	0.81	0.89
Median fluorescence intensity of neutrophils and neutrophil-adhesion molecules								
β2-integrin (CD18)	131.1	219.7	154.7	138.2	32	0.10	0.55	0.85
L-selectin (CD62)	822.4	951.6	977.8	912.5	129	0.42	0.29	0.52
Neutrophils	100	113	110	102	14	0.29	0.42	0.87
Neutrophil phagocytosis, %	82.6	79.6	82.2	83.0	3.0	0.45	0.91	0.92

[a, b,c] Means within rows with no common superscript differed significantly ($P<0.05$).
[1]Produced by Amlan International, Chicago, IL.

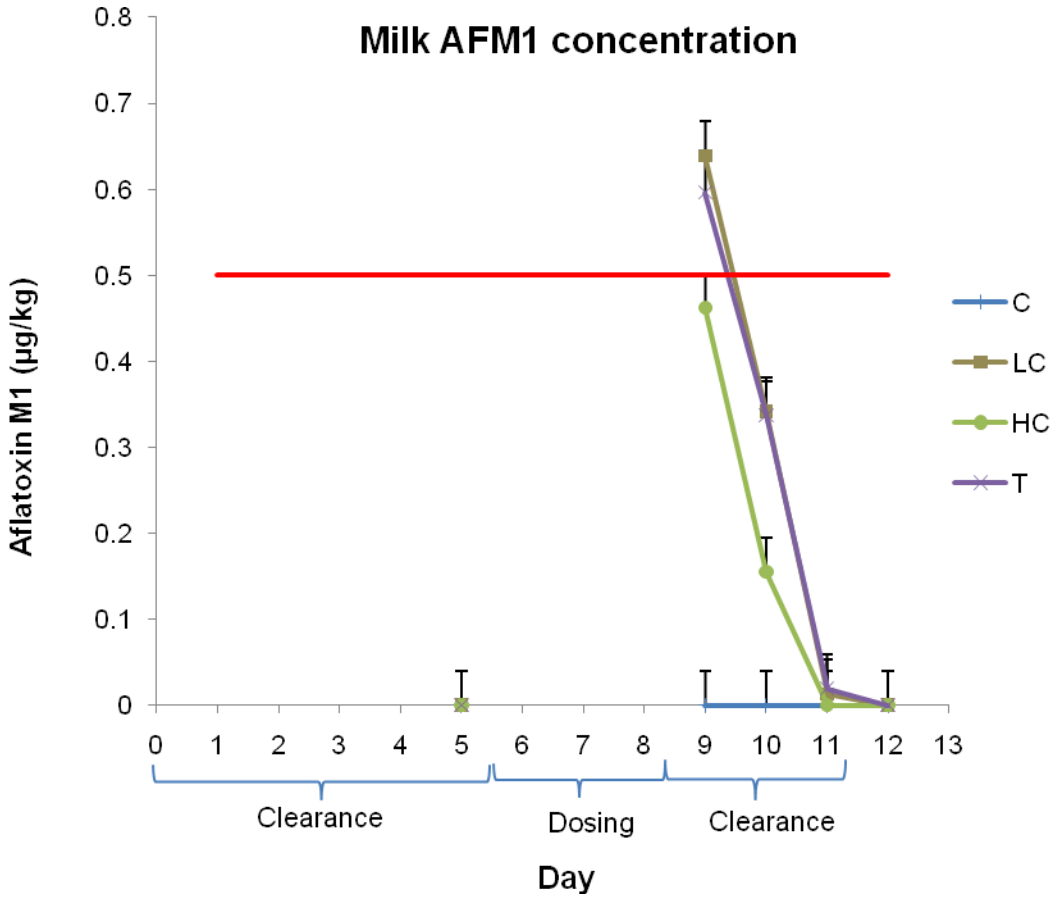

Figure 6-1. Effect of dietary addition of aflatoxin B_1 with or without low (LC) or high (HC) doses of a mycotoxin binder on clearance of aflatoxin M_1 (AFM_1) from the milk of dairy cows.

CHAPTER 7
SUMMARY, GENERAL CONCLUSIONS AND RECOMENDATIONS

The main objective of this series of studies was to evaluate the effect of different inoculants on the fermentation, aerobic deterioration, DM and nutrient losses, pathogenicity, and toxicity of corn silages. An additional objective was to evaluate the efficacy of using a montmorillonite-based adsorbent to prevent adverse effects of dietary AFB_1 on the performance and innate immune response of dairy cows and the transfer of dietary mycotoxins into milk. In order to achieve these objectives four studies were performed.

The first study aimed to evaluate the effectiveness of three commercial bacterial inoculants at controlling *E. coli* O157:H7 in corn silages during ensiling and feedout phases of silage production. A second objective was to determine if the inoculants exhibited and transferred antibacterial activity against *E. coli* O157:H7 to the silages. Chopped corn forage was ensiled after treatment with:1) distilled water (negative Control); 2) 5×10^5 cfu/g of *E. coli* O157:H7 (EC, positive control); 3) EC and 1×10^6 cfu/g of *Pediococcus pentosaceus* and *Propionibacterium freudenreichii* (EC+BII); 4) EC and 1×10^6 cfu/g of *Lactobacillus buchneri* (EC+LB); 5) EC and 1×10^6 cfu/g of *L. buchneri* and *P. pentosaceus* (EC+B500). *Escherichia. coli* O157:H7 *w*as not detected in silages after their pH dropped below 4. Applying inoculants containing *L. buchneri* resulted in a more heterolactic fementation, which increased acetic acid concentrations, reduced counts of spoilage fungi and thereby increased aerobic stability compared with control treatment. After 144 h of aerobic exposure only inoculants containing *L. buchneri* kept silage pH below 4 and thus prevented reestablishment of the pathogen. Applying EC+BII containing *P. freudenreichii* did not improve aerobic stability or inhibit the growth

of *E.coli* O157:H7. All pure cultures of bacterial inoculants exhibited antibacterial activity, independent of pH against *E. coli* O157:H7, but this trend did not persist in inoculated silages, suggesting that *E. coli* elimination from silages was mediated by pH reduction.

This experiment demonstrated that application of inoculants containing *L. buchneri* prevented establishment of *E.coli* O157:H7 on silage by keeping the pH low during the aerobic exposure phase. This effect was attributed to the antimycotic effect of the acetic acid produced by *L. buchneri,* which prevented the growth of yeasts that initiate spoilage by increasing the pH during aerobic exposure by metabolizing lactic acid. This suggests that application of *L. buchneri* inoculants at ensiling may prevent establishment of *E. coli* O157:H7 on silages during the feedout stage. Therefore, such inoculants may help to limit or inhibit the growth of *E. coli* O157:H7 in a TMR containing a mixture of ingredients contaminated with the pathogen and *L. buchneri*-treated silage. This may help to prevent the spread of the pathogen among dairy cows, which are known as the primary reservoir. Future research should validate these suggestions and examine if *L. buchneri* inoculants can increase *E. coli* shedding in cows and inhibit the growth of other pathogens that require neutral pH to thrive such as *Listeria monocytogenes, Bacillus aureus, Clostridia spp., Klebsiella* spp., and other *Enterobacteria*. Future work should also determine if inoculants can inhibit the growth of *E. coli* O157:H7 on silages that do not ferment as readily as corn silage because of high buffering capacities or low sugar concentrations such as legume silages and tropical grasses, respectively. Due to the discovery of antibacterial activity against the pathogen

by pure cultures of all inoculants, future work should also identify the source of such activity and develop methods of ensuring that it persists during ensiling.

The objective of the second study was to evaluate the effects of a dual-purpose inoculant, which was the most promising treatment in the previous study, on the fermentation, quality and aerobic stability of corn silages made in farm-scale silos. This trial was conducted because little information exists on effects of using dual-purpose inoculants to preserve silages in farm-scale silos. Corn forage was harvested at 34% DM and treated without (Control) or with 1 X 10^6 cfu/g of *Lactobacillus buchneri* and *Pediococcus pentosaceus*. Forty-five metric tons of corn forage were packed alternately into each of four replicate silo bags per treatment and ensiled for 166 days. During the feed-out, silage was removed from the bags, separated into good and spoiled (darkened, moldy, slimy, or heating) silage, and weighed daily for 35 d. Weekly samples were analyzed for chemical composition, aerobic stability and fungal counts. Inoculation did not affect the chemical composition of the spoiled or good silage but decreased the quantity and percentage of spoiled silage in the bags by over 50%, which resulted in a decrease of associated nutrient and energy losses. Inoculated silages tended to have a more heterolactic fermentation and therefore had lower yeast counts. However, aerobic stability was not different across treatments.

The main deduction from this experiment was that treatment with the dual-purpose inoculant markedly reduced nutrient and energy losses in the silages by inhibiting the growth of spoilage fungi and thereby curtailing the amount of spoiled silage. To our knowledge this is the first time the latter has been shown in farm-scale silos. These reductions in losses would directly improve profitability on dairies and increase

environmental stewardship and food safety by reducing the amount of discarded and toxigenic or pathogenic silage, respectively. That most measures of temperature were numerically but not statistically reduced by inoculation suggests that there may have been insufficient power to detect differences between treatments in aerobic stability or that the inoculant could not increase this measure under the relatively low packing density used and the prevailing hot, humid conditions, which predispose to the growth of spoilage organisms. Therefore, this study should be repeated with more replicates to conclusively indicate if the aerobic stability of silages made in farm scale silos can be improved by inoculation.

Plants grown under environmental stress are more susceptible to the growth of opportunistic epiphytic fungi that produce mycotoxins and cause undesirable fermentations. The third experiment aimed to evaluate 1) if increasing levels of southern rust infestation on corn plants reduces the nutritive value and safety of corn silage and 2) if application of the dual-purpose bacterial inoculant used in previous experiments could mitigate adverse effects of rust infestation on silage quality. Corn plants with three levels of rust infestation, none (NR), medium (half of the plant was infested, MR) and high rust (whole plant was infested, HR) were harvested at 40% DM. The forages were chopped and ensiled without (Control) or with application of a bacterial inoculant (INO) at a rate that delivered 1×10^5 cfu/g of *Pediococcus pentosaceus* 12455 and 4×10^5 cfu/g of *Lactobacillus buchneri* 40788. Each treatment was ensiled in quadruplicate 20 L laboratory silos for 97 days. As the level of rust infestation increased, concentrations of silage DM and NDF increased, whereas *in vitro* DM digestibility decreased by 9%. The NDF digestibility of NR and MR silages were similar among untreated silages or

inoculated silages but corresponding values for HR silages were lower particularly in inoculated silages. Concentrations of lactate decreased with increasing rust infestation in Control silages, but this trend was reversed in inoculated silages. Mold counts of NR and MR silages were similar among Control or inoculated silages; whereas corresponding values in HR silages were lower particularly in inoculated silages. Consequently, aerobic stability was greater in HR silages than NR or MR silages among Control or inoculated silages. Aflatoxin was only detected in Control HR silages and the level (5.2 mg/kg) exceeded the Food and Drug Administration (FDA) Action Level for feeds. Absence of the toxin in inoculated HR silages suggested that inoculation prevented accumulation of the toxin.

This study demonstrated for the first time that rust infestation severely reduced the nutritive value and fermentation of corn silage but dual-purpose inoculants could mitigate or reduce such adverse effects. Further, high levels of rust-infestation resulted in higher levels of aflatoxin in uninoculated corn silages than the FDA Action level, but inoculated silages were free of the toxin. Therefore, such inoculants can be used to improve the fermentation, nutritive value, and safety of rust-infested silages. These benefits are probably attributable to the antimycotic effects of the acetic acid produced by *L. buchneri*, which prevented the growth of aflatoxin-producing *Aspergillus* molds. Future studies are needed to confirm the latter, and to determine if the mode of action is acetate-induced inhibition or binding of aflatoxins to cell walls of bacteria in the inoculant. Additional studies should examine if such inoculants can be used to prevent adverse effects of other diseases and plant stressors such as insect damage and lodging on the quality and safety of corn silage.

The occurrence of mycotoxins in the silage in the previous study and the high corn silage intake by dairy cows in the USA highlighted the need to examine the efficacy of using adsorbents to minimize adverse effects of mycotoxins on dairy cows. The fourth trial aimed to examine the effects of adding two doses of a mycotoxin adsorbent on milk aflatoxin M_1 (AFM_1) concentration and the performance and immune response of dairy cows fed a diet contaminated with aflatoxin B_1 (AFB_1). Eight multiparous lactating cows were used in an experiment with a duplicated 4 x 4 Latin square design with 12-d periods. Treatments included the following: 1) Control diet (C); 2) Toxin diet (T) containing C and 75 µg/kg of AFB_1; 3) Low-clay (LC) diet containing T and 0.2% of a clay-based aflatoxin binder (Calibrin A- Amlan International, Chicago, IL); and 4) High-clay diet (HC) containing T and 1% of the binder. In each period, the toxin was dosed on d 6 to 9, whereas the clay was fed every day. Milk production and DMI were recorded daily and milk was sampled twice daily on d 5, 9, 10, 11, and 12 in each period. Blood samples were collected on days 5 and 9 of each period. Dietary treatments did not affect DMI, milk yield, or feed efficiency (kg FCM /kg DMI). Feeding T instead of C tended to reduce 3.5% FCM yield, reduced milk fat yield and milk protein concentration. Concentrations of AFM_1 in milk of cows fed the T and LC diets were similar and greater than those of cows fed the HC or C diets. Haptoglobin concentration in plasma was greater and β2-integrin expression tended to be greater in cows fed diet T instead of C, but values for cows fed LC, HC and C did not differ. Feeding diets T and LC resulted in greater milk AFM_1 concentrations than the FDA Action Level but feeding diets HC and C did not. Therefore, diet T made the milk unsafe, increased the innate immune response, tended to reduce FCM yield, and reduced milk quality. Feeding the HC or LC diets

prevented adverse effects of the toxin on the innate immune response and FCM yield and milk quality, but only the HC diet kept milk AFM_1 concentrations below the unsafe threshold.

This trial demonstrated that dietary addition of enterosorbents at an appropriate dose is an effective strategy to detoxify diets or silages contaminated with aflatoxin and thereby ensure the safety of milk from cows fed such diets or silages. The study also showed that enterosorbents can also reduce adverse effects of aflatoxin ingestion on milk yield and quality and they can prevent the energetically expensive immune response caused by the toxin. Future studies should compare different types of enterosorbents, determine optimum dose rates for each one, and examine long-term effects of their inclusion in diets on the performance, health, and reproduction of dairy cows.

The main conclusions from this series of studies are that in addition to traditionally known effects such as improving the fermentation, DM and nutrient recovery, and aerobic stability of silages, inoculants can be used strategically to mitigate the pathogenicity and toxicity of silages, and thereby prevent the spread of pathogens. These benefits provide additional justification for inoculant use for forage conservation that will improve profitability and environmental stewardship on dairies, enhance the safety of dairy products, and improve herd and human health.

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BIOGRAPHICAL SKETCH

Oscar Queiroz was born in Sao Paulo, Brazil. He enrolled at the University of Sao Paulo, Escola Superior de Agricultura “Luiz de Queiroz” in Brazil in 1999 and earned his Bachelor of Sciences in agricultural engineering 5 years later. This university is the oldest and perhaps the best institution for studying Agricultural Sciences in Brazil. Oscar obtained his Master of Sciences degree at the same university in 2007. His thesis focused on developing new methods to improve the quality and aerobic stability of sugarcane silage to facilitate its use as a livestock feed in Brazil. In August 2007, Oscar became a Doctor of Philosophy candidate in the Department of Animal Sciences at the University of Florida under Dr. Adegbola Adesogan’s supervision. While pursuing his doctoral research, Oscar also participated in or coordinated several additional research projects on various areas such as using fibrolytic enzymes to improve the digestion of forages and feeds and the performance of dairy cattle, evaluating effects of different films for covering bunker silos on the quality and shelf life of silage, determination of the influence of the culture medium, temperature, and duration on the growth of yeasts and molds on silage, examination of effects of the amount of silage and container type on the aerobic stability and shelf life of silage, examination of effects of species and bacterial inoculation on the fermentation and nutritive value of tropical grasses, etc. Oscar’s long-term plan is to become a faculty member at a university. His immediate plan is to hone and improve the skills and knowledge he acquired during his Ph.D. program in a postdoctoral research position where he can fulfill his passion to improve technologies involved in making silage.