Implications of the ability of *Enterococcus* spp. to survive the ensiling process and bovine gastrointestinal tract on the risk of bovine mastitis

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ABSTRACT

Three studies were conducted to assess if the ability of *Enterococcus* spp. surviving the ensiling process and bovine gastrointestinal tract could impact risk of bovine mastitis. The first study determined ability of enterococci to survive 3 wk ensiling. Grass and corn crops were divided into 3 treatments: 2 commercial silage inoculants, 1 negative control. After wk 1, 2, and 3 of ensiling, dry matter and bacterial enumeration were performed. Addition of silage inoculant led to greater levels of enterococci in grass silage compared with negative control levels, but showed less difference in inoculated corn silage. The second study quantified enterococci shedding rates in lactating dairy cows. Using a 4 x 4 Latin Square design, lactating, ruminally fistulated Holsteins were inoculated with enterococcal isolates from silage inoculants, ensiled forages, or clinical mastitis cases. Over the 4-period study, each period consisted of rumen and fecal sampling (2 wk) followed by a wash period (10 d). There were no significant differences in rumen or fecal enterococci levels between the 4 treatments. Both rumen and fecal enterococci levels showed significant differences between baseline and treatment periods (period 3, 4). The third study analyzed similarity in enterococcal isolates of silage and bovine origin using pulsed-field gel electrophoresis patterns from *Sma*I restrictions. Dendogram analysis showed none of the isolates met or were greater than a 75% genetic similarity and

produced a genetically diverse population. Results suggest *Enterococcus* spp. from silage inoculants are not likely to contribute to an increased risk of enterococcal bovine mastitis.

Keywords: *Enterococcus* spp., silage inoculant, shedding, bovine mastitis

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"Cows are my passion. What I have ever sighed for has been to retreat to a farm, and live entirely surrounded by cows" – Charles Dickens.

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INTRODUCTION

Silage, feed produced through lactic acid fermentation, is a major component in the diets of dairy cows. The National Research Council 2001 report of nutrient requirements for dairy cattle suggests that a lactating dairy cow's diet consists of 19-32% corn silage and 2-6% grass hay (NRC, 2001). To produce the high quality forage necessary for productive cows, dairy farmers often use silage inoculants to reduce the risk of protein degradation and loss of dry matter (**DM**). *Enterococcus* spp. are included in many commercially available silage inoculants, acting as lactic acid bacteria to protect feed nutrient value during fermentation by increasing lactic acid production and rapidly decreasing pH within a silo (Cai, 1999).

Helpful for silage preservation, enterococci are also known environmental mastitis-causing pathogens on dairy farms (Devriese et al., 1999, Green et al., 2002). Reports suggest *Enterococcus* spp. account for up to 11% of pathogens isolated from mastitis cases and possess a high affinity for sharing antimicrobial and virulence gene information (Gianneechini et al., 2002, Rossitto et al., 2002, Schaberg and Zervos, 1986). These bacteria are able to enter the mammary gland through teat end exposure to manure-soiled bedding and improperly managed pastures. Enterococcal mastitis results in physical damage to the mammary gland, impaired animal health, decreased milk production, and loss of product quality. Recent literature suggest that *Enterococcus* spp. isolates from silage inoculants have the ability to survive in bovine rumen fluid *in vivo* (Weinberg et al., 2003). If these mastitis-causing bacteria are able to survive in the rumen, there is a possibility of subsequent fecal shedding into a herd's environment.

Exploring the ability of enterococci originating from commercial silage inoculants to survive the ensiling process and bovine gastrointestinal tract will allow for the estimation of possible enterococcal mastitis risk to a dairy herd. Epidemiologic study of these bacteria from silo to milking barn is essential in gathering more information about the *Enterococcus* genus and assessing the possibility of using different bacteria as silage inoculants for continued animal feed preservation.

CHAPTER 1: Review of Literature

Description of mastitis and impact on the dairy industry

Mastitis is defined as an inflammation of the mammary gland (Brouillette et al., 2003, Kehrli and Shuster, 1994, Zhang et al., 2007). This inflammation can either be caused by physical injury to the teat or teat end and/or infection with a foreign organism, such as bacteria, yeasts, or fungi. Common to many mammals, including humans, mastitis has a significant impact on the dairy industry. The disease appears in either a clinical or subclinical state, with varying degrees of severity. Both states can affect the animal's health and decrease milk quality. Mastitis results in serious ramifications for both the animal and producer. Failure to efficiently identify and treat this disease can cause damage to the mammary gland, a prolonged immunocompromised state, and cow discomfort through pain and swelling (Harmon, 1994). The dairy producer faces the economic challenges of discarded milk due to poor quality, treatment cost for affected animals, loss of future milk production of the herd, culling, replacement costs, and in severe cases, death of the animal (Seegers et al., 2003).

Considering these challenges, mastitis is one of the most expensive problems that dairy farmers face (Harmon, 1994). Based on a 2008 study in New York State, a case of clinical mastitis costs on average $179 (Bar et al., 2008). Milk loss represents $115 of the total cost, $50 to treatment costs, and $14 because of increased herd mortality (Bar et al., 2008). The cost of mastitis to U.S. dairy farmers is approximately two billion dollars per year (Harmon, 1994). Areas like farm management and animal health should be considered when looking for factors that affect new intramammary infections (**IMI**). Some of these factors are teat and udder condition, milking protocols used, and stage of

lactation. One of the more important factors and a focus of this thesis is the effect of the animal's surrounding environment. As presented in a recent study from the University of Minnesota, more than 75% of clinical and subclinical mastitis cases identified by microbial testing are due to pathogens found in the cow's environment (Reneau and Bey, 2005).

Bacterial pathogens associated with mastitis (major and minor pathogens)

Bacterial pathogens able to cause bovine mastitis are usually isolated from milk in the mammary gland, streak canal of the teat, or both locations. These bacteria are classified as major or minor mastitis pathogens (Eberhart, 1986). Pathogens are categorized based on frequency of occurrence and pathogenicity of the bacteria.

Major pathogens

Major pathogens are defined as organisms that result in severe immune responses, cause the most compositional changes to milk, and lead to larger economic losses. In lactating dairy cows ranging from 0 to > 300 DIM, somatic cell counts (**SCC**) associated with infections caused by minor pathogens were 75% lower than the SCC associated with infections caused by major pathogen (Harmon, 1994). Within this category, the organisms are further divided into 'contagious' and 'environmental' groups. Classification of these groups is based on the means of spread to other lactating cows (Harmon, 1994).

Contagious pathogens are spread from cow to cow at milking time. Some commonly recognized contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*. The spread of contagious pathogens can be related to poor milker hygiene, poor vacuum and pulsation levels, poor sanitization of equipment, and

ineffective milking time protocols. The combination of these conditions can lead to infection in a dairy cow's mammary gland easily being spread to the udder of a herd-mate.

The source of environmental pathogens is, simply, the dairy cow's environment. This includes pasture, bedding, feed, water, manure, etc. These pathogens are able to gain access to the mammary gland at any point during the cow's daily life, but usually in-between milkings (Harmon, 1994). Some commonly recognized environmental pathogens are gram-negative coliforms, such as *Klebsiella* spp. and *Escherichia coli*, and gram-positive bacteria, such as *Streptococcus* spp. (other than *S. agalactiae*) and *Enterococcus* spp.

Minor pathogens

Minor mastitis pathogens are defined as organisms that are isolated from milk culture analysis but do not cause a large increase in SCC, cause lesser compositional changes within the milk, and have a very small effect on milk production (Harmon, 1994). Minor mastitis pathogens include 'opportunistic' bacteria, coagulase-negative *Staphylococcus* spp., and *Corynebacterium bovis*.

Opportunistic pathogens are bacteria normally present in a cow's environment but only infect the mammary gland at times of injury or immunosuppression. Primary sources of many of these pathogens are healthy teat skin and milker's hands. The majority of opportunistic pathogens are *Staph.* spp. other than *S. aureus*, such as *S. hyicus* and *S. epidermidis*. Many opportunistic pathogens can be eliminated with the use of effective postmilking hygiene and dry cow therapy.

Although all of these mastitis pathogens warrant attention, contemporary research

has shown that one genus is increasing in challenging milk quality. *Enterococcus* spp. were responsible for 19.0 % of clinical environmental streptococci IMI within a single dairy herd observed over 7 years at the William E. Kraus Dairy Research Center at The Ohio State University (Todhunter et al., 1995). With close to one fifth of all IMI's within a herd potentially associated with these bacteria, it is important to understand their role in mastitis infections.

Brief history of *Enterococcus spp.*

At first, the *Enterococcus* genus appeared as a categorical mystery to the scientific community. An unknown microorganism associated with both streptococci and fecal contamination, pinpointing a single identity for this bacteria proved challenging for microbiologists. Observed from human fecal samples, these bacteria appeared in short chains or pairs under the microscope and were titled "*Enterococcus*" by Thiercelin and colleagues in 1899 (Murray, 1990). "*Enterococcus*" comes from the Greek words "enteros" meaning "from within" and "kokkos" or "berry", thus describing the bacterial origin from within the body and its round or berry-like shape. In the same year, similar bacteria that caused severe endocarditis were termed "*Micrococcus zymogenes*" to describe their heartiness (MacCullum and Hastings, 1899). The term "*Micrococcus*" was adopted in the scientific world and thus began years of nomenclature confusion. It was not until 1906 when these "*Micrococcus*/*Enterococcus*" were associated with a well-established bacterial genus, streptococci. For the next 64 years, the microbiological world generally accepted these bacteria to be Lancefield Group D streptococci (Facklam, 1972).

This identification theory was eventually challenged and resulted in the suggestion of categorizing *S. faecalis* and *S. faecium* within the new genus

"*Enterococcus*". Surprisingly, the confusion continued until genetic technology had advanced enough to support the observed difference between *S. faecalis* and *S. faecium* compared to the rest of the *Streptococcus* genus. Thus, in 1984, nearly 100 years since the first observation, the mystery was solved and the microbial community accepted *Enterococcus* as a valid genus for this historically enigmatic bacteria (Schleifer and Kilpper-Bälz, 1984).

Microbial and biochemical characteristics of *Enterococcus* spp.

Bacteria classified in the *Enterococcus* genus are gram-positive cocci. They are facultative anaerobes, having the ability to survive in decreased oxygen environments as low as 5% O_2, switching from respiration to fermentation. Under the microscope, these bacteria are usually seen in pairs or short chains. Similar to streptococci, enterococci appear as gray, translucent colonies on esculin blood agar (**EBA**) plates and are catalase negative due to a lack of cytochrome enzymes. Unique to enterococci are the abilities to survive in high salt concentrations and within large temperature and pH ranges. In 1937, Sherman first noted these characteristics for survival in 6.5% NaCl, a pH of up to 9.6, and growth between 10-45°C (Sherman, 1937).

Enterococci are identified based on phenotypic reactions to medias chosen for these unique properties. In addition to a catalase test and gram stain, possible enterococci are identified by being streaked on a modified media with esculin to assess esculin hydrolysis, an EBA plate for the CAMP test to assess an increased presence of beta-hemolysis, and a high salt media to assess survival at high concentrations. It is widely accepted that bacteria displaying dark colonies on an esculin plate under short-wave UV light, negative for the CAMP test, and yellow colonies on a salt enriched plate are

phenotypically enterococci. It is important to note that the discussed identification methods are not entirely precise and require reevaluating due to enterococcal evolution. The Teixeira research group, out of the United States Centers for Disease Control and Prevention (**CDC**), tested for a better method of enterococcal identification when looking at phenotypic results between strains of *E. faecium*. Using 39 bacterial strains (35 *E. faecium* or *faecium*-like and 4 *E. faecalis*) from the American Type Culture Collection, researchers tested the pathogens for both phenotypic characteristics (ability to form acid in mannitol, glycerol, raffinose, and sorbitol, and susceptibility to vancomycin) and genotypic characteristics (SDS-PAGE genetic profiles). Results showed that an arabinose test could be included for more accurate identification due to the presence of sucrose negative strains (Teixeira et al., 1995). This study demonstrated that the genus is evolving and phenotypic tests should aim to be as inclusive of all probable outcomes as possible.

Diagnostic tests

Non-molecular identification methods

There are phenotypic methods for enterococci identification that are not agar plates and aid in taking the guesswork out of subjective media reactions. To reduce time and media usage, tests were developed with biochemical reagents en mass in one strip or card. After inoculation and incubation with bacterial isolates, any color change in wells on the strip can be read to relate to a specific genus and species.

One example is the API 20 STREP test (bioMerieux Industry, Hazelwood, MO). In 1982, before *Enterococcus* spp. received their own genus, British researchers examined 111 streptococci isolates from the National Collection of Type Cultures (**NCTC**), Colindale, London. The NCTC had previously identified five of these isolates

as *S. faecium*. After using the API 20 STREP test, researchers found a 100% correlation with the NCTC results and reported confidence in using the strip for identification of streptococci, including Lancefield Group D streptococci (Tillotson, 1982). Much of the work with the API 20 STREP test has been done with human and food isolates, however, this method can also analyze bovine isolates (Devriese et al., 1992, Petersson-Wolfe et al., 2008). Bacterial isolates from mammary secretions and the environment around an Ohio dairy farm were collected to identify mastitis-causing enterococci. Media-based and biochemical tests resulted in positive reactions with 81 of the original 111 isolates characterized as enterococci. Those strains that were initially identified as enterococci correlated with the positive *Enterococcus* spp. identification from the API 20 STREP test with greater than 95% confidence (Petersson-Wolfe et al., 2008).

Molecular methods

Although media colorimetric changes and biochemical reactions have proven effective for cost and methodology when identifying *Enterococcus* spp., technology has allowed for faster and more complete typing of strain profiles. There are many molecular methods that can be used, such as multilocus enzyme electrophoresis (**MLEE**), randomly amplified polymorphic DNA (**RAPD**), or restriction fragment length polymorphism (**RFLP**). However, two particular molecular methods are becoming the gold standards of distinguishing bacterial traits and strains within a specific species.

The first method is polymerase chain reaction (**PCR**). This molecular method has become a gold standard of bacteria classification within clinical laboratories. One study conducted with this method was performed to assess if there could be a rapid PCR assay to solely detect the *Enterococcus* genus. Primers were created from the highly conserved

region of the housekeeping *tuf* gene that would correspond with areas in *E. avium*, *E. faecalis*, *E. faecium*, and *E. gallinarum*. A total of 159 isolates from the Central Hospital in Quebec, Canada were used. Of these clinical isolates, the PCR assay with *tuf* gene primers was able to identify 100% of the enterococcal strains (Ke et al., 1999). PCR has also been proven effective for comparing isolates of the bovine origin. In 1995, researchers from the University of Tennessee were able to use PCR to fingerprint *E. faecium* isolates associated with IMI. A total of 42 *E. faecium* isolates were collected from 20 dairy cows (28% of the herd) that presented with mastitis during early lactation from November 1990 to June 1991. With the use of PCR-based DNA fingerprinting and plasmid analysis, researchers were able to trace infections specifically associated with subtypes of *E. faecium* over a period of time. *E. faecium* Subtype 2 caused the most clinical mastitis cases (5 out of 6) and Subtype 1 was most associated with calving season and mammary secretion (Keane et al., 1995). This PCR based method is a useful informational tool for future research in looking at the epidemiology of *E. faecium* mastitis.

The second popular molecular method, useful for typing and epidemiologic evaluation of a disease, is pulsed-field gel electrophoresis (**PFGE**). Unlike general gel electrophoresis, PFGE provides specific fingerprints by the large separation of DNA bands due to a pulsed electrical field that flows through the gel. Usually a lengthy process, scientists from Linkoping University in Sweden worked on a time-efficient pulsed-field protocol for typing enterococci (Saeedi et al., 2002). Concerned with enterococci as human pathogens, researchers wanted a faster way to run bacterial fingerprinting. Compared to the protocol suggested by the Bio-Rad Company (Bio-Rad

Laboratories, Inc., Hercules, CA), a modified PFGE protocol was created for clinical human isolates using less bacterial suspension, shorter washing time and restriction digestion time. No negative effects on the DNA plugs or the resulting gels were observed, leading researchers to conclude that this protocol could effectively be performed in 5 d rather than the 7 d Bio-Rad procedure (Saeedi et al., 2002).

This procedure was adopted and successfully performed with bovine isolates (Petersson-Wolfe et al., 2008). The objective of this research was to utilize PFGE to explore the epidemiology of enterococcal mastitis infections on dairy farms. The origins of the enterococcal isolates were mammary secretions from high SCC milk, postpartum milk, and clinical mastitis cases along with environmental bedding and feed sources. Following the PFGE methods of Saeedi et al. (2002) and calculating relatedness at 75% similarity with a dendogram, DNA fingerprints indicated that isolates from mammary secretions came from different sources, grouped with isolates from bedding sources, and were genetically diverse. In contrast, the fingerprints showed that the feed isolates clustered and were genetically non-diverse (Petersson-Wolfe et al., 2008). This research emphasized that PFGE can be extremely helpful in exploring the epidemiology of *Enterococcus* spp. Using this technique, comparisons could potentially be made between enterococcal isolates to identify a shared location of origin or genetic profile changes incurred through stress of the pathogen.

Pathogenesis

The pathogenesis of enterococcal mammary infections is not entirely known. Currently, there is a more complete understanding of coliform bacteria or *S. aureus* within the gland when compared to existing knowledge of enterococci. Like many

mastitis causing pathogens, *Enterococcus* spp. gain access to the mammary gland from the environment through the teat end. Hence, health of the teat end is extremely important as the first line of defense against bacterial infections. Once inside the mammary gland, enterococci rely upon virulence factors to proliferate, advantageously utilize the mammary environment, and establish an infection. Strains displaying both intrinsic and acquired antibiotic resistance allow *Enterococcus* spp. to better sustain an infection within a treated gland.

Virulence factors

Much of the current information related to enterococcal virulence factors has been gathered from studies comparing nosocomial and food isolates (Eaton and Gasson, 2001). However, the roles of these identified factors may translate to the environment of a mammary gland and will be portrayed as such in this paper. Known enterococcal virulence factors could affect adhesion to and change in condition of mammary epithelial cells, proliferation of the pathogen, and evasion of the bovine immune system within the mammary gland. Three factors which could help enterococci withstand flushing from the gland during milking are aggregation substance (*agg*), and two adhesion factors (*efaAfs* and *efaAfm*) for *E. faecalis* and *E. faecium*, respectively (Eaton and Gasson, 2001). Four virulence factors which may play a role in impairment of epithelial cells and proliferation of bacteria are the bacteriocin cytolysin (*cyl*), gelatinase (*gelE*) responsible for bioactive peptide degradation, acetoin, and siderophores. The latter two virulence factors aid in enterococcal proliferation. Acetoin allows the bacteria to degrade sugars and mucin as a food source (Gilmore, 2002). Siderophores may be used by the bacteria to bind iron within the mammary gland and use it as an energy source for growth (Gilmore, 2002).

Although more research is needed, the sex pheromone-responding plasmids of enterococci are thought to play a role in a quorum-sensing ability similar to that of *S. aureus* (Gilmore, 2002). These pheromones have been generally referred to as "sex pheromones" due to the ability of initiating "mating" (plasmid transfer from host to recipient). The sex pheromone determinants *cpd*, *cob*, *ccf*, and *cad* are believed to relate to plasmid acquisition that aid in the proliferation and conjugation of enterococci (Eaton and Gasson, 2001).

Antibiotic resistance

Enterococcus spp. are at the forefront of discussions on antibiotic resistance due to the well publicized and well researched discovery of vancomycin resistant strains and the knowledge of a unique affinity for conjugation. However, only a small portion of the public probably realizes that enterococci have intrinsic resistance to some antibiotics as well as the ability to acquire new resistances to medical drugs. These intrinsic resistances are mediated by genes that are located on the bacterial chromosome whereas acquired resistances are usually associated with plasmids or transposons (Murray, 1990).

Intrinsic resistance

Enterococcus spp. have intrinsic, or natural, resistance to beta-lactams, clindamycin, aminogylcosides, and possibly trimethoprim-sulfamethoxazole (Murray, 1990). The resistance to beta-lactams is a characteristic feature of *Enterococcus* spp. Enterococci found in a remote population on the Solomon Islands even showed basic levels of resistance to beta-lactams (Moellering and Krogstad, 1979). As with most of the inhibitory/resistance data, these values were obtained from a human population rather than an animal one. These penicillin resistance levels ranged from 25 μg/ml to < 100

μg/ml and gave bacteriostatic results instead of bactericidal ones. It is thought that this intrinsic resistance is due to a low affinity of penicillin-binding proteins (Williamson et al., 1985).

Acquired resistance

Enterococcus spp. possess the ability to acquire antibiotic resistance through conjugation. Enterococci, as previously mentioned, have an affinity for gene transfer and this is reflected in both nosocomial and on-farm antibiotic resistance. As Murray describes in a 1990 paper, there are three methods of conjugation utilized by the pathogen; broad-host range plasmids, narrow-host range plasmids, and conjugative transposons. Broad-host range plasmids depend on direct cell contact and an oligopeptide transport system. This is believed to be the mechanism by which genetic resistance information has been shared between *E. faecalis* and *S. aureus* (Schaberg and Zervos, 1986). Narrow-host range plasmids require the aid of an aggregation substance induced by pheromones. This aggregation substance clumps both the donating and receiving bacteria together and allows for genetic transfer within clusters. This transfer system has only been recognized in *E. faecalis* (Wirth, 1994). Conjugative transposons allow for free floating, extra-chromosomal DNA to attach to a membrane and induce uptake into the genome.

Using these methods of genetic transfer, *Enterococcus* spp. have acquired resistance to many common and last-resort antibiotic treatments. These treatments include non-beta-lactam penicillin, chloramphenicol, erythromycin, tetracycline, and vancomycin. Vancomycin, from the glycopepetide family, was commonly used in hospitals as a last-resort drug to treat severe bacterial infections. After noticing decreased

efficacy of treatment with this drug, researchers began looking closely at bacterial pathogens like *Enterococcus* spp. to find an explanation for failed patient recovery. A group of six genes was discovered to be associated with vancomycin resistance termed *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. Of these six genes, *vanA* and *vanB* appear to code for high and low levels of vancomycin resistance, respectively, due to conjugation by the Tn*1546* transposon (Kak and Chow, 2002). These genes are thought to have been acquired due to the lengthy and numerous uses of antibiotics. For example, avoparcin was added to animal feeds for years in Europe, which in turn acted as an instigator for these new vancomycin-resistant enterococci (**VRE**) and led to subsequent usage bans. Using the European history with VRE as a model, researchers from the CDC explored VRE in both agricultural and domestic settings. A review of VRE literature suggested farm animals, household communities, and home food preparation as possible non-medical reservoirs for VRE (McDonald et al., 1997).

Two additional research studies echo the need to concentrate on drug resistant enterococci in farm animals, not only as a possible transmission source but from an animal health perspective as well. The first study used 146 *E. faecium* and 166 *E. faecalis* strains from farm animals and pets in Belgium. Using agar plates with double dilutions of the antibiotics avoparcin, virginiamycin, acitracin, tylosin, avilamycin, and narasin, isolates were tested for minimum inhibitory concentrations (**MIC**). MIC levels for isolates of food-animal origin (from swine and ruminants combined) were higher overall than those for pets. Pet MIC levels were only high for beta-lactams (Butaye et al., 2001). Researchers concluded that farm animals are a large reservoir of resistant enterococci.

In the second study, researchers showed how resistant strains can compound mastitis infections. There were 362 environmental bacteria isolates collected from bovine mastitis cases from the San Joaquin Valley in California over a period of three years. Organisms were first indentified using the API 20 STREP test and then MIC susceptibility tests were performed using a micro-dilution plate according to methods described by the National Committee for Clinical Standards. Of the 40 *Enterococcus* spp. isolates, 97.5% were susceptible to 8 μg/ml of penicillin, 100% were susceptible to 8 μg/ml of ampicillin, 77.5% were susceptible to 4 μg/ml of tetracycline, 40% were susceptible to 8 μg/ml of ceftiofur, 22.5% were susceptible to 2 μg/ml of oxacillin, and only 2.5% were susceptible to 256 μg/ml of sulfadimethoxine (Rossitto et al., 2002). Due to the impressively large number of ineffective antibiotics, researchers concluded that *Enterococcus* spp. were resistant to the most antibiotics when compared to other pathogens that were tested. However, these researchers used MIC levels from human interpretive criteria to categorize pathogens as susceptible or not (Rossitto et al., 2002). More research needs to successfully be done in this area to develop standard susceptibility levels for dairy cows. In doing this, more efficacious treatments can be created in order to lessen the damage caused by harmful enterococcal mammary infections.

Epidemiology of intramammary infections caused by *Enterococcus* spp.

There is less knowledge regarding enterococcal mastitis when compared to contagious pathogens such as *S. aureus* or other environmentals, like coliforms. In one research study conducted with 29 dairy herds, close to 11% of all bovine mastitis cases (2.5% clinical and 8.2% sub-clinical) were caused by enterococci (Gianneechini et al.,

2002). Interestingly, the prevalence (the number of cases) of enterococcal mastitis is commonly known, but not the incidence (the rate of new cases). This is due to the fact that many veterinarians and laboratories do not differentiate between streptococcal and enterococcal species. As previously discussed, the shared history and similar phenotypic traits of these two genera make them difficult to distinguish and allow for similar management control practices (Smith and Hogan, 2003). While this lack of specificity is easier for general diagnoses or treatments, it does not allow for a clear epidemiologic representation of *Enterococcus* spp. on dairy farms. As technology advances and more molecular techniques are being used for rapid identification, more information may be gained about enterococcal infection incidences.

Recent studies have looked for temporal trends of mastitis pathogens and have taken research closer to defining the range of incidences of enterococcal mastitis. Green and colleagues assessed the risk of mastitis infection at dry off. Results from this research suggested that cows with enterococci in mammary secretions at dry off were at a significantly higher risk for mastitis in the next lactation (Green et al., 2002). Another study used surveys and milk samples to identify causative agents of mastitis infections. *Enterococcus* spp. and *Streptococcus* spp. were isolated from the 3,033 milk samples taken from Canadian farms in similar amounts, 2.2 % of all samples and 2.1% of all samples, respectively (Olde Riekerink et al., 2008). Calculations suggested that the incidence rate of streptococcal clinical mastitis (**IRCM**) was 1.21 per 100 cow-years for cows in a tie stall, 0.37 per 100 cow-years for cows in a free stall, and 0.56 per 100 cow-years for cows in other housing (Olde Riekerink et al., 2008). Although the incidence rate

for enterococci was not calculated, it could be suggested that the incidence rates would be similar based on the prevalence of the two genera.

There is still additional research needed to quantify the case fatality, duration of cases, and other epidemiological factors relating to enterococcal mastitis. However, the discussed studies assessing both prevalence and possible incidence only serve to reinforce the ability of *Enterococcus* spp. to cause reduction in milk quality and animal comfort.

Possible sources of enterococci on dairy farms

Similar to identifying the best management protocols for enterococci, possible farm sources of enterococci also need to be explored. Although a ubiquitous environmental pathogen, there may be specific areas within a dairy farm where enterococci are concentrated. Two recent studies have successfully gathered information about on-farm locations associated with enterococci.

The first study, from researchers in Ireland and Belgium, examined possible *Enterococcus* spp. contamination of raw milk from the dairy farm environment. Outside of the United States, raw milk is used for many different cheeses and dairy products. Researchers addressed issues of raw milk contaminated with enterococci and lactobacilli entering processing plants. Samples consisted of feces from 26 dairy cows, a fecal sample from the milker, teat rinse, plant rinse, bulk tank rinse, hand-washing rinse, water supply, water in the yard of the dairy, and silage. All samples were diluted and plated on Kanamycin Esculin Azide Agar (**KEA**). *Enterococcus* spp. were identified phenotypically and relation between strains and sources was evaluated using PFGE. Results showed that bovine feces had the most strains of enterococci (76 strains),

followed by the rinses, human feces, and milk samples from the bulk tank (11 strains) (Kagkli et al., 2007). Researchers were not able to detect any enterococci in the silage or yard water samples. Out of all 76 enterococcal strains identified, only one isolate from bovine feces shared similar PFGE patterns with the milk isolates. Isolates found in milk samples shared similar PFGE patterns with those from the bulk tank and rinses (Kagkli et al., 2007). Thus, researchers concluded enterococci from bovine feces were not a source of the previously observed milk contamination.

As previously mentioned, in 2008, Petersson-Wolfe et al. conducted a similar study but focused on assessing the sources of enterococci that could cause disease, rather than affect food production. A total of 102 isolates were identified from various bovine sources. There were 81 enterococcal isolates from mammary secretions and 21 isolates from environmental sources on the farm. Isolates obtained from mammary secretions were specified within three sources, high somatic cell count milk, postpartum milk samples, or clinical mastitis cases while environmental samples were divided into bedding and feed sources. Presumptive identification as *Enterococcus* spp. was based on results from the API 20 STREP test. After presumptive identification, PFGE using *Sma*I restriction patterns was tested on all selected isolates and a dendogram was created to assess relatedness of strains between sources. Results showed that the majority of isolates were *E. faecium*, followed by *E. faecalis*, and *E. casseliflavus* (Petersson-Wolfe et al., 2008). Analysis of the dendogram revealed that isolates from mammary sources were genetically diverse, grouping with each other as well as bedding isolates. Isolates from feed samples were genetically similar and grouped together (Petersson-Wolfe et al.,

2008). This study supports the presence of enterococci in feedstuffs and suggests that silage could be a viable reservoir of *Enterococcus* spp. in the cow's environment.

Silage inoculants and enterococci

Exploring forage as a source of enterococci leads to investigating two non-animal origins responsible for the presence of the bacteria on dairy farms. The first is the general environment. These are categorized as environmental pathogens and can originate from general organic matter and decay to more specific sources like use of bio-solids on crop fields. The second origin may be the use of silage inoculants.

Silage inoculants are used to preserve the nutrient value of forage crops necessary for animal feed. It is not standard for all farmers to use silage inoculants, but the additives are popular worldwide as an extra step towards silage preservation. Preservation is key once the forage is within a silo. During growth, native crop bacteria can cause harmful acetic acid production. Silage inoculants are added to create an acidic environment in an anaerobic setting. Homofermentive lactic acid bacteria (**LAB**) use fermentable carbohydrates available from the crop and convert them into organic acids, such as lactic acid (Weiss and Underwood, 2006). Additional acid is produced as fermentation occurs, which causes the pH to drop and thereby inhibits growth of any spoilage microorganisms.

There are different types of silage additives available to achieve nutrient preservation and minimize storage loss. Non-protein nitrogen made of urea and anhydrous ammonia can be added to increase the final crude protein of forages, especially corn silage, which can bypass the expense for future protein supplements. Two more additives that impact the nutritional value are adding other feedstuffs, like grain, to increase DM or supplementing with minerals, such as phosphorus, to benefit the animal.

Additives which lower the pH to preservation levels are acids, such as formic acid, along with the aforementioned microbial inoculants containing lactic acid bacteria (Weiss and Underwood, 2006).

In 1999, researchers from Japan investigated characteristics of microbial inoculants containing *Enterococcus* spp. Using selective media, alfalfa, rye-grass, sorghum, corn, and guinea grass were initially sampled for enterococci. From these forages, strains of enterococci were chosen and then applied to second-cut alfalfa and guinea grass. Additives were applied at 1.0×10^5 colony forming units (**cfu**)/g of fresh matter (Cai, 1999). After 60 d, there were 1.0×10^5 cfu/g DM of enterococci on both the alfalfa and grass (Cai, 1999). Researchers did not differentiate between the enterococci that were added and those that may have already been present on the forage. Therefore, the exact number of enterococci that survived from the original inoculant is unknown. Researchers also concluded that the bacteria did not improve forage quality and were not able to survive below a pH of 4.5 (Cai, 1999). Despite the lack of crop enhancement, this study is beneficial in showing enterococci are included in forage plant matter.

An earlier study from British researchers in 1990 also found enterococci on animal feed. Broiler mash, consisting of finely ground corn and soybeans, and wheat straw were used in the study. The original forage was sterilized at 121ºC in an autoclave for 15 min and then inoculated with two streptococci strains along with *E. faecalis* and *E. faecium* strains (Mackey and Hinton, 1990). Samples were taken from each inoculated forage once every week for four weeks. Results showed enterococcal strains had the second and third highest bacteria levels across the four weeks, only below *Streptococcus bovis*. Researchers reported *E. faecium* survived significantly better on straw at week four

(Mackey and Hinton, 1990). This study solidifies that enterococci bacteria do inhabit livestock environments on feed crops.

Enterococci as starter cultures and direct-fed microbials

For decades, *Enterococcus* spp. have been used as starter cultures and direct-fed microbials in food products and animal feed without explicit approval from the United States government. In 1995, the United States Food and Drug Administration (**FDA**) officially defined the term 'direct-fed microbial' for animal feed as products that are "purported to contain live (viable) microorganisms (bacteria and/or yeast). The labeling and promotional materials, including advertisements, for direct-fed microbial products state, suggest, or imply beneficial effects in animals associated with the products' content of viable microorganisms" (FDA, 1995). The FDA does not have a clear statement on the use of enterococci or list of approved feed ingredients. Currently, the FDA uses lists gathered by the Association of American Feed Control Officials (**AAFCO**). The AAFCO feed ingredient list defines enterococci as a direct-fed microbial that "has been reviewed by the FDA, Center for Veterinary Medicine and found to present no safety concerns when used in direct-fed microbial products" (AAFCO, 2003). However, as the FDA explicitly states, the AAFCO is not a government organization and does list feed ingredients that might be questionable (FDA, 1995). Although referring to enterococcal use as probiotics for humans, the World Health Organization (**WHO**) and the United Nations Food and Agriculture Organization (**FAO**) do plainly address the risk of including these bacteria in food. In 2002, a joint FAO/WHO guideline paper stated, "*Enterococcus* is emerging as an important cause of nosocomial infections and isolates are increasingly vancomycin resistant. The working group recognizes that some strains of

Enterococcus display probiotics properties, and may not at the point of inclusion in a product display vancomycin resistance. However, the onus is on the producer to prove that any given probiotics strain is not a significant risk with regard to transferable antibiotic resistance or other opportunistic virulence properties" (FAO/WHO, 2002). With differing safety opinions on the use of *Enterococcus* spp. by the FDA and FAO/WHO, one can understand the confusion and controversy that surrounds using this genus as additives at any point in the food chain.

Enterococcus spp. are commonly viewed internationally as beneficial for cheese making. The ability of these bacteria to degrade caseins is extremely useful in producing a variety of well-ripened cheeses. *E. faecium* is a frequently isolated species (20.5% of isolates) from cheese curds (Sarantinopoulos et al., 2002). One group of Greek researchers examined if the use of common enterococcal starter strains had any impact on the final physiochemical and sensory characteristics of Greek Feta cheese. Using two *E. faecium* strains acquired from Feta and Sardinian cheeses, researchers conducted Feta-making trials, pasteurized the cheese, and then evaluated the final products. Results showed that vats made with the enterococcal starters in combination with other lactic acid bacteria and even alone had between 4.0 – 6.6 log cfu/ml of enterococci during processing compared to the sizeable range of 0- 4.5 log cfu/ml seen in the control vat (Sarantinopoulos et al., 2002). As far as the physiochemical changes, the pH in all vats declined the first day post inoculation and remained near 4.5 for the rest of the trial (Sarantinopoulos et al., 2002). Sarantinopoulos and colleagues also noticed an increase in moisture and degraded caseins when both enterococcal strains were used together in a vat. There were no significant differences during the sensory evaluation of the cheeses

from different vats. Therefore, enterococci had the largest impact as starter cultures on the amount of degraded casein in the finished product. This exhibits the potential of *Enterococcus* spp. as starters in cheese production.

Enterococci are also used as feed additives in dairy calves and cows. In 2003, Nocek and researchers looked at the performance effect of feeding direct-fed microbials (**DFM**) to transition period dairy cows. They fed two diets consisting of no DFM and DFM containing 90 g of 5 x 10^9 cfu yeast and 5 x 10^9 cfu of two *E. faecium* strains in the total ration fed (Nocek et al., 2003). Holstein cows were fed from 3 wk prepartum to 10 wk postpartum, and blood samples were taken daily. Results conferred no significant difference in dry matter intake (**DMI**) between treatments during the prepartum period. However, the DFM diet did have significant impacts during the postpartum period with increased DMI, milk yield, and milk protein content as compared to the no DFM control diets (Nocek et al., 2003). Another research group also reported beneficial results when feeding *E. faecium* to dairy calves. This group examined the combination of lactulose, a disaccharide known to stimulate beneficial intestinal bacteria, with *E. faecium* and the impact on pre-ruminant calves. Working under the premise that separation stress from the mother effects milk consumption and animal health, 42 calves were divided into 3 groups. Two groups were fed milk replacer: one with a 1% concentration lactulose, the other with a 3% concentration of lactulose. The third group served as a negative control with no lactulose. All groups were fed milk replacers containing 10^9 cfu enterococci/kg (Fleige et al., 2009). After 19 wk of treatment, the calves were slaughtered and their intestinal morphology was examined. The group that received the disaccharide and bacteria combinations had an increased weight gain, decreased ileum villi height, and a

decrease of lymph follicle surface area (Fleige et al., 2009). Although this study was focused on lactulose, the research premise could be explored further to understand the effects of direct-fed enterococcal microbials and their interaction with other feed additives.

Reviewing both studies, it is suggested that *Enterococcus* spp. may assist in the health improvement of a dairy herd. The manner in which this occurs has been largely explored within the past 10 years (Wallace, 1994). The positive effects of improved feed intake are linked with the higher rate of fiber breakdown and duodenal flow associated with absorbable nitrogen (Martin and Nisbet, 1992, Williams et al., 1991). This is the cause of probiotic bacteria adhering and proliferating on the mucus layer of the gastrointestinal tract. The thought behind the proliferation is that the lactic acid bacteria from these probiotics become stimulated by dicarboxylic acids present throughout the gastrointestinal tract to result in increased fiber breakdown and better overall fermentation in the gut (Nisbet and Martin, 1991, Williams et al., 1991). However, the extent of these probiotic effects and the long-range implications of enterococcal usage on-farm remain unspecified.

Possible implications of enterococcal use on dairy farms

With the popularity of *Enterococcus* spp. use in on-farm products increasing, attention needs to be given to possible end results. Of the many potential outcomes, there are two of current human and animal importance; increasing antibiotic resistance and bacterial exposure from unintended mediums.

According to Kenner and researchers in 1960, the average daily enterococcal density of bovine feces would be 0.16 million enterococci/g moist feces, which is less

than the densities for humans, chickens, sheep, and pigs. With a potential for environmental contamination, it is a concern whether dairy cows are becoming a reservoir for antibiotic resistant strains. As aforementioned, Butaye and colleagues had identified farm animals as a greater source of multi-drug resistant enterococci than household pets (Butaye et al., 2001). Similarly in 2003, researchers from the United States Environmental Protection Agency (**EPA**) examined fecal samples from animals and humans to quantify enterococci levels and determine the level of vancomycin resistance in the isolates. Samples came from 14 animal species and human volunteers. Isolates were plated on selective media, confirmed with the API 20 STREP test and PCR, and were assessed for MIC using the E-test procedure with BHI agar (Rice et al., 2003). There were a total of 12 cows and each had a positive VRE isolated from their feces with mean *van C-2* MIC values of 8µg/ml (Rice et al., 2003). European studies also observed high levels of VRE from animals that were fed glycopeptides as growth promoters (Devriese et al., 1996). These studies support the fact that dairy cows are a possible and probable source of multi-drug, especially vancomycin, resistant enterococci.

Thus, there is a large likelihood of farm inhabitants coming into contact with enterococci, perhaps even from silage inoculants. Weinberg and colleagues have been exploring this possibility for the past six years looking at microbial inoculants and rumen fluid *in vitro*. In 2003, the researchers collected rumen fluid from two fistulated Holstein cows, strained it, inoculated it with different commercially available lactic acid bacteria silage inoculants, and sampled every 6-12 h until 72 h (Weinberg et al., 2003). After plating on selective media and taking pH readings, results showed that inoculants containing solely enterococci had both high and low ending pH readings but had one of

the highest levels of ending lactic acid bacteria (6.6-7.4 $\log_{10}$ cfu/ml) (Weinberg et al., 2003). Researchers felt comfortable concluding that these LAB silage inoculants survived and may have grown in strained rumen fluid. These researchers continued this work in 2004 when they took a closer look at inoculant effects on volatile fatty acids. Commercially available silage inoculants containing *E. faecium* were applied to wheat and corn forages that, post ensiling for 4-8 mo, were then placed into sterile and non-sterile strained bovine rumen fluid. After ensiling, *E. faecium* inoculants in the sterile wheat silage had a pH of 4.0 and a LAB level of 6.3 $\log_{10}$cfu/g DM while the nonsterile group had a pH of 4.5 and a LAB level of 7.0 $\log_{10}$ cfu/g DM (Weinberg et al., 2004). Inoculated corn had pH values of 4.4 in the sterile and 4.2 in the nonsterile as well as LAB levels of 7.4 $\log_{10}$cfu/gDM in the sterile and 7.6 $\log_{10}$ cfu/g DM in the nonsterile group (Weinberg et al., 2004). Researchers attributed these values to the varying ability of enterococcal inoculants to compete with native forage microflora. Once these silages were placed in the strained rumen fluid, both groups showed enterococcal levels between 7.7-9.0 $\log_{10}$ cfu/g DM post 48 h incubation for the wheat and 6.8-8.9 $\log_{10}$ cfu/g DM post 48 h incubation for the corn (Weinberg et al., 2004). This data supports the possible idea that bacteria from silage inoculants could survive from silo to cow and cause health concerns, like mastitis, when excreted into the environment. It is the aim of this study to see if this proposed pathway is feasible.

SUMMARY AND RESEARCH OBJECTIVES

The scientific world has yet to fully understand the pathology of *Enterococcus* spp. These bacteria have served as scientific conundrums and continue to confuse researchers when exploring current, possible uses or harms. This is especially true when addressing the problem of bovine mastitis. The impact of mastitis on animal health, milk quality, and farm economics needs to be addressed by researchers. As science has progressed and contagious pathogens are better controlled, a microbial void remains for environmental pathogens to fill and become larger on-farm problems. The current management protocols for enterococcal mastitis are not specifically tailored, and instead, lump it together with the streptococcal species it has gained independence from in the laboratory. Currently, hygienic milking protocols, proper bedding choices, and knowledge of susceptible times during lactation are the best ways to battle enterococcal mastitis. It is clear that if a point source of *Enterococcus* spp. could be indentified and controlled, it would help prevent resulting intramammary infections.

Consequently, the main objectives of this study were to determine the survivability and similarity of *Enterococcus* spp. from silage inoculants during the ensiling process and subsequently through the bovine gastrointestinal tract to indentify a possible on-farm source of enterococci. This was done by applying commercially available silage inoculants containing *E. faecium* to both corn and grass forages and ensiling each for three weeks. Isolates indentified and saved as *Enterococcus* spp. were then dosed to fistulated cows in a 4 x 4 Latin square design with fecal and rumen samples taken during four 2 wk periods. Finally, isolates saved from ensiling, fecal, and rumen samples were evaluated for genetic similarity using pulsed-field gel electrophoresis.

The first objective of this study was to determine the ability of *Enterococcus* spp. to survive the harsh environment of the ensiling process. Enterococci are naturally found in the environment, so an added objective was to observe any growth differences between enterococci on untreated forage and forage treated with commercially available silage inoculants.

The second objective of this research was to determine the fecal shedding rate of *Enterococcus* spp. from dairy cows. A pre-treatment period was included for all trial cows to serve as a baseline to which the inoculated fecal levels were compared. Additionally, rumen samples were taken to explore the translocation of these bacteria through the bovine gut.

The third and final objective was to assess strain diversity using pulsed-field gel electrophoresis. Moreover, this molecular technique could be used to look at any genetic differences in strains before and after ensiling and before and after passage through the bovine gastrointestinal tract to assess if *Enterococcus* spp. can viably come from silage inoculants and subsequently cause mastitis.

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CHAPTER 2: The ability of *Enterococcus* spp. to survive the ensiling process

ABSTRACT

A forage-based study was conducted to determine the ability of enterococci to survive a 3 wk ensiling process. Harvested grass and corn crops were respectively divided into 3 treatment groups consisting of 2 commercially available silage inoculants and 1 negative control group. Within 24 h of harvest, a uniform amount of forage was added to each of 18 vacuum sealable freezer bags. Inoculants 1 and 2 were applied (according to manufacturer directions) to each of 6 bags and the remaining 6 bags were not inoculated and served as negative controls. An industry grade vacuum sealer was used and nitrogen gas was pumped into the bag to create a pillow pack (1 atmosphere gases: 9 N_2). Dry matter and bacterial enumeration were performed on the forage prior to ensiling as well as after each week of ensiling. Bacterial enumeration was conducted according to standard bedding sampling procedures with the addition of the Kanamycin Esculin Azide Agar for the enumeration of enterococci. At wk 1, 2, and 3 of the ensiling process, a total of 6 bags were opened; 2 from each of the 3 treatment groups. Data suggest an increased number of enterococci on inoculated grass samples compared with the negative control at 3 wk post-ensiling. Inoculant 2 (7.3 ± 0.1 log cfu/g DM) displayed a greater count than Inoculant 1 (4.2 ± 1.7 log cfu/g DM) after a 3 wk process and both were greater than the negative control (1.2 ± 1.2 log cfu/g DM). Significant differences in enterococci levels were found between the negative control and Inoculant 2 at wk 3. Inoculation of corn silage did not appear to greatly change the enterococci count at 3 wk post-ensiling. Enterococci counts for Inoculant 1, Inoculant 2, and the negative control group were 4.3 ± 0.1 log cfu/g DM, 4.4 ± 0.1 log cfu/g DM, and 4.0 ± 0.1 log cfu/g DM,

respectively. Significant differences in enterococci levels were found between Inoculant 1 and 2 at wk 1 and between the negative control and Inoculant 2 at 1 wk and 3 wk post-ensiling. The addition of a silage inoculant led to greater levels of enterococci in grass silage compared with the negative control at the end of a 3 wk ensiling period. Enterococci levels did not show as large a difference in the inoculated corn silage samples at the end of the ensiling period. These data suggest that enterococci are able to survive the harsh conditions of an ensiling period.

INTRODUCTION

Found in human intestines and ubiquitous in the environment, enterococci possess the ability to cause a wide range of health conditions from urinary tract infections to meningitis. For the dairy industry, enterococci take on a more specialized role and are considered prevalent environmental mastitis pathogens (Sobiraj et al., 1997). Mastitis is an expensive disease, costing United States dairy farmers close to two billion dollars annually (Harmon, 1994). Finding sources of mastitis-causing pathogens could alleviate some of this financial burden by better identifying where to focus on-farm management and treatment. When looking for enterococcal sources on Ohio dairy farms, Petersson-Wolfe and colleagues found *Enterococcus faecium* strains in the cows' environment and also in silage feed samples (Petersson-Wolfe et al., 2008).

Enterococcus spp., categorized as environmental pathogens, have had a long-standing association with plant life and matter. However, pinpointing the mechanism by which these bacteria appear in outdoor reservoirs has proved challenging. One theory is that the presence is due to the weather. In 1961, Mundt looked at the buds and blossoms of both non-agricultural and agricultural plants for the presence of enterococci. While enterococci were recovered from a variety of both non-agricultural and agricultural plants, the bacteria were mostly temporary residents. Mundt concluded this was due to environmental factors such as wind, rain, and insects. Consequently, in 1972, Mundt collaborated with Martin to explore the possibility of insects as an enterococcal source. Although they found enterococci-positive insects, the numbers were not large enough to attribute insects being a major source of enterococci on forage crops and plants. A third theory is that enterococci are present on forage crops due to the use of biosolids on

agriculture fields. Cools and colleagues looked at microbial populations from soils treated with pig slurry. They found that *Enterococcus* spp. were able to survive in soil for at least eighty days (Cools et al., 2001).

It is clear that enterococci are expected pathogens on forage crops, even if the route of acquisition is still unknown. More attention is currently being given towards this bacterial genus by researchers and the United States Food and Drug Administration (**FDA**) with concern for animal feed being a vehicle for antimicrobial resistance. Da Costa and colleagues found that poultry feed consisting of soybean meal, cereal products, and additives had ubiquitous presence of enterococci over the course of six months (Da Costa et al., 2007). When testing for antimicrobial resistance, only 202 out of 723 enterococcal feed ingredient strains and 38 out of 414 enterococcal broiler strains were susceptible to all antimicrobials. In a similar vein, the FDA has been monitoring animal feed ingredients to assess the potential of selection for antimicrobial resistant strains. Researchers found that of the derived protein ingredients, *Enterococcus* spp. were isolated from 84% of the animal-derived samples and 91% of the plant-derived samples (Headrick et al., 2004). Resistances of these positive strains were to four or more current antimicrobials (Headrick et al., 2004).

Enterococci clearly pose a threat to animal health when found in feed crops and products. However, the magnitude of that threat still remains unknown. Another possible source for on-farm contamination is the use of silage inoculants. Some *Enterococcus* spp., such as *E. faecium*, are included in many of the commercially available silage inoculants used to preserve the nutrient value of silage during ensiling (Cai, 1999). It is currently unclear if use of silage inoculants can have any association with mastitis by

increasing the enterococcal load in the cows' environment. Studies have been done using forages and specific strains of *Enterococcus* spp., usually obtained from a centralized microbiological source such as the National Type Culture Collection (**NTCC**). One of these studies has resulted in determining that enterococci are present in inoculated forage crop for at least four weeks and only decreased by a 1-2 log10 cfu/ g forage change from beginning to end of the ensiling period (Mackey and Hinton, 1990). However, no studies have been done to assess the presence of enterococcal strains from commercially available silage inoculants after a period of ensiling. It would be extremely helpful to producers if a possible enterococcal source could be indentified and managed appropriately. Thus, it was the objective of this study to quantify the survivability of silage inoculant enterococcal strains on dairy animal forage crops over a three week ensiling period.

MATERIALS AND METHODS

Experimental Forages

Grass and corn crops were harvested at the Virginia Tech Dairy Center in August and November 2008, respectively. The grass crop was harvested with a farm mower, raked, and placed into 33-gallon black, draw-string garbage bags. The corn crop was harvested with a combine and placed into 55-gallon garbage cans, which were then covered with black garbage bags for transport. Forages were taken to the laboratory for ensiling immediately post-harvest.

Study Design

Eighteen ensiling bags were filled with each forage (2700g/bag grass and 4095g/bag corn) after harvest. The treatment groups consisted of 2 commercially available silage inoculants as well as 1 negative control group. Inoculant 1 was applied to 6 bags, Inoculant 2 was applied to 6 bags and the remaining 6 bags were not inoculated and served as negative controls. There was a total of 3 ensiling durations, consisting of post one, two, and three weeks ensiling. Within each treatment group and time period were duplicate samples of silage, with two bags for the negative control to be opened at wk 1, two treated with Inoculant 1 to be opened at wk 1, and two treated with Inoculant 2 to be opened after wk 1. This same design was continued for wk 2 and 3 (Appendix A).

Silage Inoculant Profiles and Dosing

The two commercially available silage inoculants contained dried *E. faecium*, and will be referred to as Inoculant 1 and Inoculant 2. According to manufacturer specifications, Inoculant 1 contained 2.1 billion cfu of *E. faecium* per gram within a 1.1lb bag. There was no specification for the amount of *E. faecium* within the 50 g jar of

Inoculant 2. Dosing was administered according to manufacturer's directions and adjusted for the volume of forage within each bag (Appendix A).

Ensiling Procedure

Ensiling procedure methods were tested using pasture grass from the Virginia Tech Dairy Center (Virginia Tech, Blacksburg, VA) prior to the beginning of the treatment periods. The harvested forages were immediately taken to the ensiling laboratory at the Food Science and Technology Building (Virginia Tech, Blacksburg, VA). A 3.5-gallon bucket holding a 16" x 26" plastic bag was placed on a scale and tared. The plastic bags were 3-mil high performance film vacuum pouches made of both nylon and polyethylene (Bunzl-Koch Supplies, Kansas City, MO). Silage was taken from the middle of the bag or can and placed into a labeled bag until the appropriate volume was reached. The largest volume the bags could hold and still be sealed was determined. For grass forage, bags were filled with 2700g of grass, and for corn silage, the bags were filled with 4095g of corn forage. Bags were labeled with treatment group information and week at which the bag should be opened. If a bag was in the negative control group, it received no inoculant treatment and was immediately sealed. If the bag was receiving one of the two inoculant treatments, the inoculant was applied into the bag with a 500mL polyethylene wash bottle, the forage was then mixed with a gloved hand for even coverage, and the bag was sealed.

All bags were sealed using a Koch UHravac Sealer (Bunzl-Koch Supplies, Kansas City, MO) and a nitrogen tank. The pillow-pack setting used to seal all treatments and forages were a vacuum of 96%, a vacuum plus for 10 s, a heat seal for 0.7 s, and a gas setting of 10% to result in a bag with a mix of 10% general atmosphere and 90% nitrogen

gas within each bag. Once sealed, the bags were lightly pressed to check for hissing air indicating a leak from puncturing or damage. If a leak was found within the first 24 h after the initial seal, the contents were placed into a new bag and resealed. After the forages had been sealed in appropriate bags with specified treatments, the bags were placed vertically upright into large crates holding 4 bags per crate. The crates were held in a dark, room temperature storage space in the Food Science and Technology building and were not disturbed until the designated week of opening. At 1 wk post-ensiling, a total of 6 bags were opened; 2 from each of the 3 treatment groups. Similarly, another 6 bags were opened at 2 wk post-ensiling and the final 6 bags opened at 3 wk post-ensiling.

Parameter Analyses

Gas and pH Analysis

On the first day of the designated week of opening, the bags were taken from the storage space and brought to the ensiling laboratory. Using a modified atmosphere packaging (**MAP**) gas analyzer, each bag was punctured with the small probe and levels of O_2 and CO_2 were recorded. The bags were then placed on a cart and transported to the Mastitis and Immunology Lab (Dairy Science Department, Virginia Tech). Within a fume hood, each bag was inverted, cut open, and had 5g of contents taken from the middle of the bag. The 5g were placed into a corresponding stomacher bag containing 45mL of sterile 1X Phosphate Buffered Saline (**PBS**) at 7.4 pH. Stomacher bags were then pressed flat to remove most of the air and placed into the Stomacher 400 Circulator (Seward, United Kingdom) at 230 rpm for 60 s. After the samples had been stomached, pH readings were taken using a pH electrode and values were recorded.

Serial Dilutions of Samples

Using labeled 96-well sterile plates, liquid from the supernatant of each post-stomached bag was serially diluted out to 10^6 dilutions (Appendix A). From each bag, 250µL of the samples were pipetted into 2 wells on column 1 on the plate. The other wells contained 225µL of sterile PBS. Using a multi-channel pipette, 25µL of the original silage sample from column 1 was transferred to column 2 and mixed 4 times. Then 25µL were transferred from column 2 to 3 and mixed, and subsequently repeated until column 5.

Microbial Analysis

The selective media used was tested with confirmed positive strains before use in the silage trials. Microbial analysis was conducted according to standard bedding sampling procedures with the addition of the Kanamycin Esculin Azide Agar (**KEA**) plates for the enumeration of enterococci (Hogan and Smith, 1997, Petersson-Wolfe et al., 2009). The standard bedding sample medias were Maconkeys agar (**MAC**) plates, Edwards modified media agar (**EMM**) plates, and Carbenicillin plates. From each well with samples on the 96-well plates, 40µL were removed and four 10µL drops were plated onto a half of an appropriately labeled plate for all four types of media. Once dry, the plates were inverted and placed in an incubator at 37°C for 24 h. After 24 h, colonies were enumerated visually per 10µL spot and recorded. The countable range used for the 4 drops was 2 – 25 cfu per drop. Microbial analysis was performed on samples from all bags at wk 0, 1, 2, and 3. All isolates indentified as enterococci were cryogenically saved for later use.

Freezing of Bacterial Cultures

Enterococcal isolates from the different weeks and treatments were identified from the KEA plates and streaked for isolation on Tryptic Soy Agar (**TSA**) plates. The plates were placed in an incubator overnight at 37°C. After 24 h, a single colony was selected using a sterile inoculating loop and placed into 5mL of sterile Tryptic Soy Broth (**TSB**). The inoculated tubes were placed in the incubator at 37°C for 24 h. The bacterial suspension was centrifuged in an Eppendorf 5810R Centrifuge for 10 min at 1620 *g* and 4°C to obtain a bacterial pellet. Within a sterile biosafety cabinet, the supernatant was decanted and replaced with 5mL of sterile skim milk (Becton, Dickinson, and Company, Sparks, MD). Tubes were vortexed to resuspend the pellet in the milk and 850µL of the culture solution was placed into two appropriately labeled 2mL cryovials, each containing 150µL of sterile 100% glycerol. Cryovials were vortexed and one vial was placed in a box for -20°C storage and the duplicate was placed in a box for -80°C storage.

Dry Matter Analyses

Dry matter analysis was performed on forage samples at wk 0, 1, 2, and 3. The analysis was conducted according to standard bedding samples procedures (Hogan and Smith, 1997). In brief, twelve-centimeter aluminum pans were labeled and weighed. Sufficient amounts of sample were placed into the pans to fill them to the brim and then the weights of the pans and samples were recorded as the 'wet weights'. Each pan was covered with aluminum foil lifted to one side to allow for airflow. All covered pans were put on a metal tray and placed in a drying oven at 110°C for 24 h. After 24 h, the pans were removed and uncovered. Pan and sample were weighed and the weight recorded as 'dry weight'. All samples were run in duplicate. The DM of the silage was then calculated, using the equation: ((dry silage weight – pan weight)/(wet silage weight – pan

weight)) x 100, and the average of the duplicates was determined. Silage samples from the 3 wk ensiling group were also sent to Cumberland Valley Analytical Services, Inc. (Hagerstown, MD) for additional analyses.

Data Management and Statistical Analyses

All values recorded from parameter analyses and calculations of DM were done within the Microsoft Excel program, version 2008 (Microsoft Corporation, WA). Graphs and charts were created from the stored data within Excel. The values of $\log_{10}$ colony forming units per gram of DM were calculated using formulas within Microsoft Access, version 2007 (Microsoft Corporation, WA). Microsoft Access also held records of isolates cryogenically frozen. Statistical analyses were performed using SAS Statistical Software, version 9.2 (SAS Institute Inc., NC). A MIXED procedure with repeated measures was run with bag within treatment as the repeated variable for each week with the following model:

$Y_{ijk} = \mu + T_i + B_{(i)j} + W_k + WT_{ik} + e_{ijk}$

where:

μ = mean of Y;

T_i = effect of treatment (i = 1 to 3);

$B_{(i)j}$ = effect of ensiling bag within treatment (j = 1 to 2);

W_k = effect of week of ensiling (k =1 to 3);

WT_{ik} = interaction of week of ensiling and treatment; and

e_{ijk} = error (bag within treatment and week interaction)

To account for multiple two-way comparisons, Tukey's adjustments were performed on the main effects of 'week' and 'treatment', and on the treatment-week

interactions, with week as the slice. Additionally, contrasts were done between treatments at each week. Significance was considered to be at a $P < 0.05$.

RESULTS

Parameter Results

At the end of the 3 wk ensiling period, all treatments associated with grass silage bags had O_2 levels ranging from 3.7% O_2 to 4.8% O_2. The amount of O_2 within the corn 3 wk post-ensiling had percentages between 6.6% O_2 and 6.9% O_2. The CO_2 level for the negative control grass bags were from 26.1% CO_2 to 39.9 % CO_2, while the levels for the Inoculant 1 bags were 27.6% CO_2 to 28.3% CO_2 and Inoculant 2 bags were from 27.2% CO_2 to 34.3 % CO_2. Within the corn silage treatments, the negative control bags and Inoculant 2 bags had levels of CO_2 from 25.6% CO2 to 35.1% CO_2 and 18.8% CO_2 to 31.8% CO_2, respectively. Levels in the Inoculant 1 corn bags over the 3 wk period ranged from 27.9% CO_2 to 37.8% CO_2. Three days post-ensiling, the corn bags became extremely full and were poked with a small gauge needle to release some pressure. The minuscule holes were then taped over and the bags remained on the normal ensiling schedule. Dry matter percent for all weeks and bags is listed in Table 2.1. Forage analysis from Cumberland Valley for the 3 wk corn silage is shown in Table 2.2. The pH levels of both silages over the course of the 3 wk ensiling period are shown in Figures 2.1-2.

Microbial Enumeration

Samples taken from original silage from the field were not used for statistical comparison due to fewer original samples than the number of trial bags tested. Average bacterial levels in each trial for each type of pathogen investigated at the end of each week are displayed in Appendix A.

At the end of ensiling, there were no detectable levels (< 10000 cfu/g DM) of gram negative bacteria on the grass (Appendix A). Statistical analyses revealed 'week' remained significant in the model. Significant changes in levels of gram negative bacteria were between the negative control and Inoculant 1 as well as Inoculant 1 and Inoculant 2 at wk 1, with both significances valued at $P < 0.01$. Similarly, there were no detectable levels of lactose positive bacteria on the grass at 3wk post-ensiling (Appendix A). 'Week' remained significant in the model. Significant changes in lactose positive bacteria levels were between the negative control and Inoculant 1 and Inoculant 1 and Inoculant 2 at wk 1, with both significances at $P < 0.01$. At the end of ensiling, there were no detectable levels of *Klebsiella* spp. on the grass (Appendix A). There were also no significant differences between any of the treatments at the different weeks of ensiling. For levels of *Streptococcus* spp., statistical analyses revealed a significant difference between the negative control and Inoculants 1 and 2 at wk 3 of ensiling, with $P < 0.01$ (Appendix A). However, in the corn silage, the only bacteria with any detectable levels were *Streptococcus* spp. (Appendix A). Statistical analyses revealed 'treatment' remained significant in the model. Additionally, there was a statistically significant difference between *Streptococcus* spp. levels associated with Inoculants 1 and 2 at wk 1, with $P < 0.05$ (Appendix A).

Enterococcus spp. Enumeration

Data suggest an increased number of enterococci on inoculated grass samples compared with the negative control (1.2 ± 1.21 $\log_{10}$cfu/g DM) after the 3 wk ensiling process (Figure 2.3). Additionally, Inoculant 2 (7.3 ± 0.11 $\log_{10}$cfu/g DM) displayed a higher count than Inoculant 1 (4.2 ± 1.75 $\log_{10}$fcu/g DM). Statistical results for the grass

silage indicated the main effects of 'treatment' and 'week' remained significant in the model. Also, there was a statistically significant difference in the amount of enterococci between the negative control and Inoculant 2 at 3 wk post-ensiling with $P = 0.0031$. Conversely, inoculation of corn silage did not appear to change the enterococci count following 3 wk of ensiling (Figure 2.4). Enterococci counts for Inoculant 1, Inoculant 2 and the negative control group were 4.3 ± 0.10 $\log_{10}$cfu/g DM, 4.4 ± 0.07 $\log_{10}$cfu/g DM and 4.0 ± 0.13 $\log_{10}$cfu/g DM, respectively. For the corn silage, the main effects of 'treatment' and 'week' remained significant in the model. Additionally, there were significant differences in the enterococcal levels between the negative control and Inoculant 2 ($P < 0.0001$) and between Inoculant 1 and Inoculant 2 ($P < 0.0001$) at one week post ensiling. At three weeks post-ensiling, there was a significant difference in enterococcal levels between the negative control and Inoculant 2, with $P = 0.0427$.

DISCUSSION

The final enterococcal counts from grass at 3 wk post ensiling was 1.21 $\log_{10}$cfu/g DM for the negative control and 5.76 $\log_{10}$cfu/g DM for the treated groups. The final enterococcal counts from corn at 3 wk post ensiling were 4.02 $\log_{10}$cfu/g DM for the negative control and 4.37 $\log_{10}$cfu/g DM for the treated groups. In both types of silage, the treated groups had higher enterococci counts at the end of the ensiling period than the negative controls. These values are slightly higher than those previously reported in a study looking at the efficacy of applying silage inoculants containing *Enterococcus* spp. and ensiled forage quality (Cai, 1999).

At the end of a 60 d ensiling period with alfalfa and guinea grass, Cai reported levels of enterococci between 10^3- 10^5 cfu/g fresh matter. The microbial enumeration for Cai's 1999 study was done with fresh matter, which represents the amount of bacteria native to that plant at the moment of testing. The methods for enumeration used in the present ensiling trials were based on DM values for two reasons. The first was that DM is the accepted unit of feed for forages used in dairy research and the dairy industry. The second was that the DM method is a more conservative assessment for bacterial levels (Blok et al., 2000).

The final $\log_{10}$cfu/g DM for both grass and corn silages never reached a non-detectable level. Although non-detectable levels would not have been expected, it could be postulated that the results observed are due to a short ensiling period. However, previous research investigating bacterial inoculant effects on silage factors have used ensiling periods ranging from 3 wk – 6 mo, which have resulted in viable lactic acid bacteria counts at the end of each experiment (Ashbell et al., 2001, Kent et al., 1989,

Mackey and Hinton, 1990). Since a goal of this research was to ultimately be useful to producers, the ensiling timeline used had to reflect likely on-farm conditions. Literature and farm practices have suggested that silage can be taken from a silo and fed to animals as early as 3 wks post ensiling. Thus, the timeline was chosen to mimic an early end of the storage spectrum. This timeline was also chosen so that the bacterial populations would have enough time to make an impact on their surroundings. Enterococci, like any other bacteria, have a four stage growth process consisting of the lag, log, stationary, and death phase. The 3 wk period was thought to ensure that the bacterial populations would be examined at the end of their log phase, but more likely during their stationary phase on the forage crops.

Survival of the non-enterococcal populations on both forage crops was not surprising. *Streptococcus* spp. followed a similar survival pattern as the *Enterococcus* spp. for both corn and grass. This result is to be expected due to the well-documented similarity between the two bacterial genera. Mackey and Hinton observed similar results in 1990 when looking at enterococcal and streptococcal levels on inoculated animal feed and straw. In both the animal feed and straw, researchers observed that enterococcal and streptococcal bacteria counts were usually within 1-2 log differences of each other over 4 wk (Mackey and Hinton, 1990). The decline, and even absence, of gram negative bacteria throughout the ensiling trial was also expected. As Gibson et al. saw in 1958, gram negative populations were the first to lose viability over 1-8 d of ensiling. As the bacteria are placed within a silo, the aerobic bacteria, such as many gram negative pathogens, grow until the successive community of anaerobic and facultative anaerobic bacteria shift the environment to one deprived of oxygen. With the addition of silage inoculants that

contain more anaerobic fermentative bacteria, it is no surprise that gram negative and lactose positive bacteria frequently decreased within the first week of ensiling for both grass and corn crops.

As seen from the forage analysis, the silage that resulted after three weeks was a viable option for animal feed. Levels recommended for corn silage for dairy cows by the National Research Council (**NRC**) are 8.3% CP, less than 26% ADF, and around 44% NDF (NRC, 2001). Levels from the trial corn silage samples were 7.8-8.3% DM CP, 25.5-26.0% DM ADF, and 47.4-47.9% DM NDF. These results are matching or close to the recommended NRC values and allow for the conclusion that the method of ensiling in plastic bags yielded quality silage. Ashbell et al. also found this to be true in their 2001 paper looking at the technology for silage making in plastic bags. The ending pH values for the current research study were fairly similar to Ashbell's findings. At the end of 4 wk of ensiling, Ashbell's bags were ranging between 3.7-4.0 pH and the bags from both the corn and grass trails ranged from 4.0-5.0 pH. Thus, the bacterial counts and pH trends seen from these bags were an adequate representation of activity within a silo.

Looking over the course of this study, there were four areas that could have improved the quality of results. The first is the amount of inoculant put onto each forage type. It is possible that due to human error, not every bag was mixed appropriately or was dosed with the correct amount of silage inoculant. Also, perhaps if one of the other many commercially available silage inoculants containing *Enterococcus* spp. was used, the results may have varied. The second area for improvement would have been during ensiling of the corn. The corn fermented so quickly during the first 3 d, which required the bags to be punctured to release pressure. This may have impacted the results seen for

the next 3 wk. The third area is that more bags could have been used for each week and treatment to increase the number of replicates. The addition of more bags might have allowed for a more robust statistical analysis. One may suggest that the ensiling period could have been longer than 3 wks. However, ensiling the bags for over a month might have resulted in enterococci levels representative of a death phase versus a log or stationary bacterial growth phase. Finally, the last area of improvement could have been using a more precise method of microbial identification. All bacteria identifications were done visually based on previous identification practice and experience. If biochemical testing or even PCR could have been run on the isolates before they were saved, the reported number of bacteria may have changed. However, the feasibility of these methods was low due to the large time commitments and cost.

CONCLUSIONS

These data show the addition of a silage inoculant led to higher levels of enterococci in grass silage compared with the negative control at the end of a 3 wk ensiling period. Enterococci levels did not show a large, marked difference in the inoculated corn silage samples. Therefore, the silage inoculants had a larger impact on the bacterial load of the grass as compared with corn. Other parameter analyses resulted in a desirable decreased pH and increased DM percentage over 3 wks. There were significant effects of treatment and week in both forage types. This is especially true for Inoculant 2, where the longer the silage was in the bags, the more enterococci were found over the ensiling period. These data support that enterococci are able to survive the harsh conditions of a 3 wk ensiling period in both grass and corn forages. Further studies

should focus on a larger scale trial and potentially feed the resulting silage to dairy cows to assess digestibility and milk production effects.

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Table 2.1. Average dry matter percentages for trial grass and corn silages after each week of a 3 wk ensiling period.

Week	Grass DM (%)	Corn DM (%)
0	30.4	35.0
1	27.3	30.0
2	24.7	27.7
3	54.3	28.0
Total	**32.2**	**29.2**

Table 2.2 Cumberland Valley Forage Analysis averages for trial corn silage post-3 wk ensiling.

Corn Silage	As Fed	Dry Matter	Unit
Moisture	67.1		%
Dry Matter	33.0		%
Crude Protein	2.7	8.05	% DM
Adjusted Protein	2.7	8.05	% DM
Soluble Protein		41.65	% CP
Degradable Protein (calc.)		70.8	% CP
TDN	23.0	69.9	% DM
Net Energy Lactation	0.24	0.73	Mcal/lb
Net Energy Maintenance	0.25	0.74	Mcal/lb
Net Energy Gain	0.15	0.47	Mcal/lb
Acid Detergent Fiber	8.5	25.8	% DM
Neutral Detergent Fiber	15.7	47.7	% DM
Ash	1.0	2.9	% DM
NFC	12.9	39.2	% DM
Calcium	0.08	0.25	% DM
Phosphorus	0.06	0.19	% DM
Magnesium	0.07	0.20	% DM
Potassium	0.37	1.13	% DM
Sodium	0.005	0.013	% DM
Iron	20	61	PPM
Manganese	11	31.5	PPM
Zinc	9	25.5	PPM
Copper	2	6	PPM

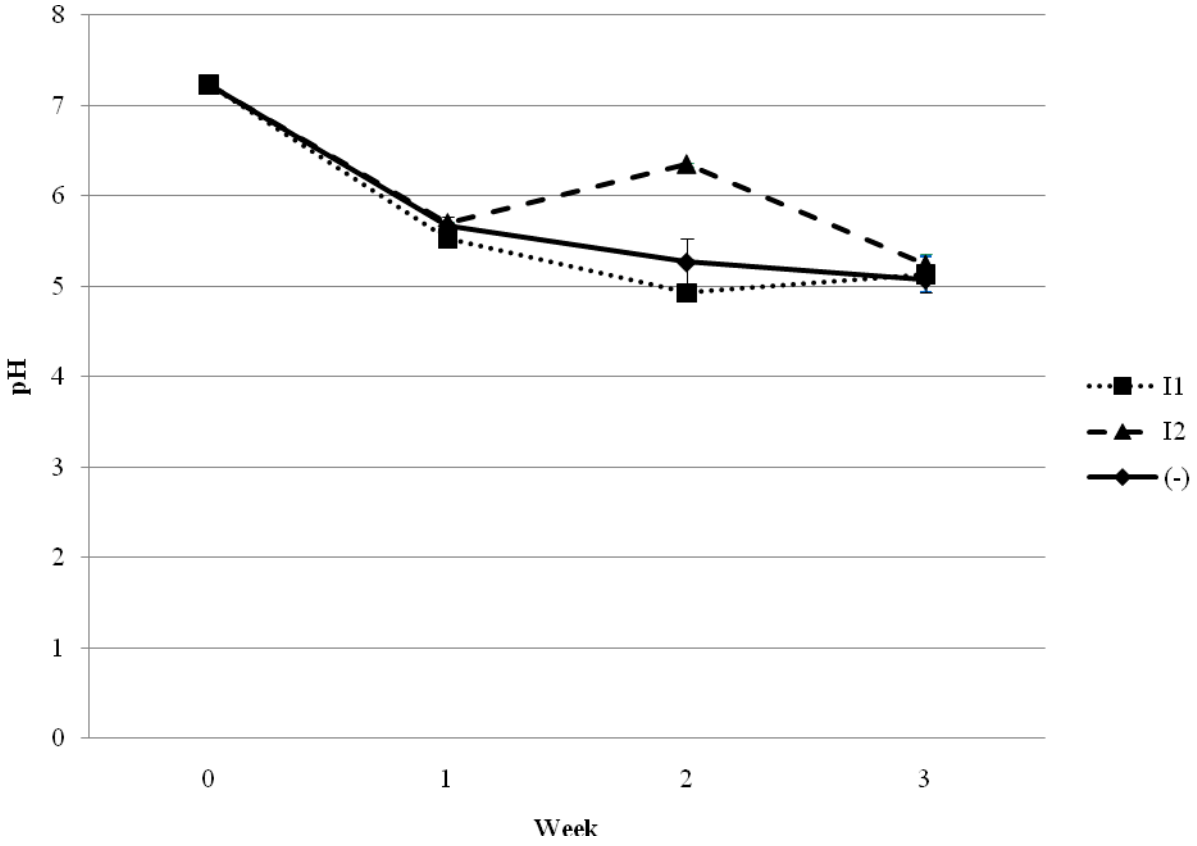

Figure 2.1 pH of trial grass per week over 3 wk ensiling period with treatments (shown in legend). Values shown are mean ± SE.

(-) (Negative control of non-inoculated grass)
I1 (Commercially available silage inoculant 1)
I2 (Commercially available silage inoculant 2)

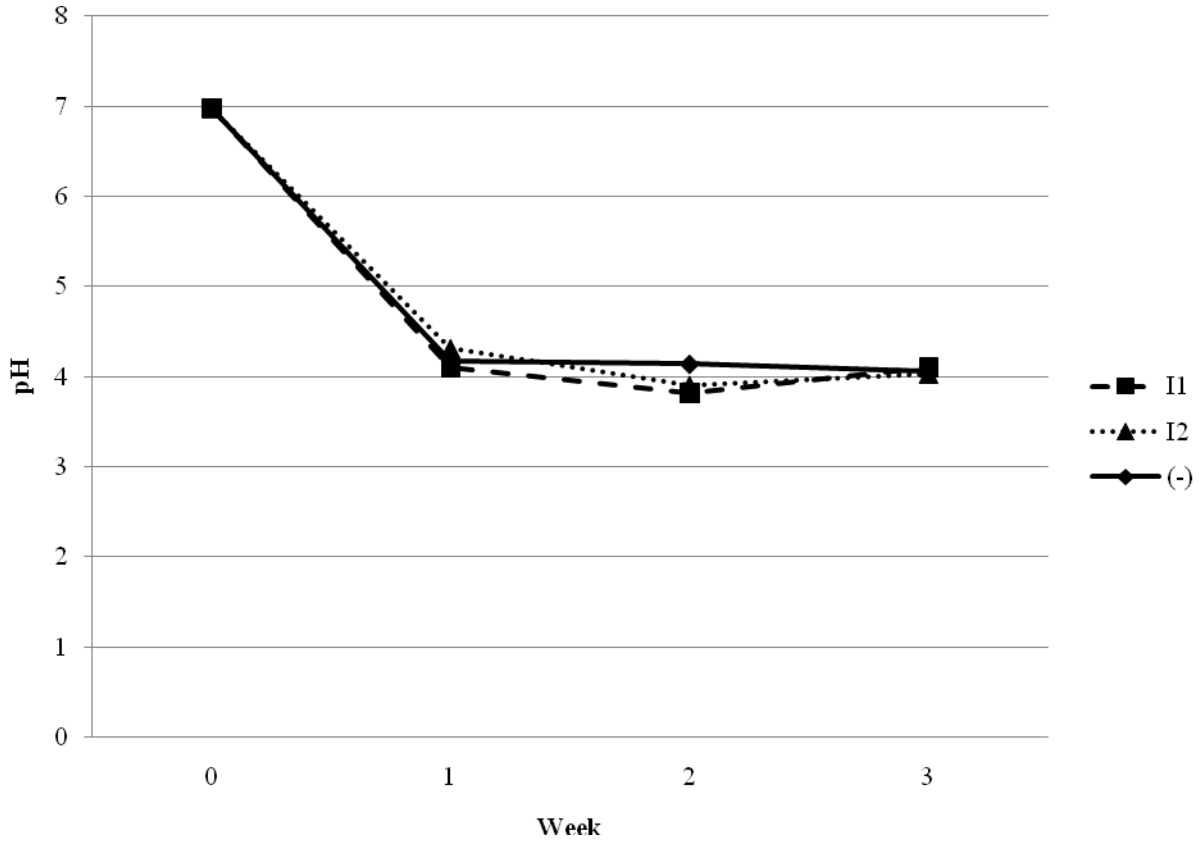

Figure 2.2 pH of trial corn per week over 3 wk ensiling period with treatments (shown in legend). Values shown are mean ± SE.

(-) (Negative control of non-inoculated corn)
I1 (Commercially available silage inoculant 1)
I2 (Commercially available silage inoculant 2)

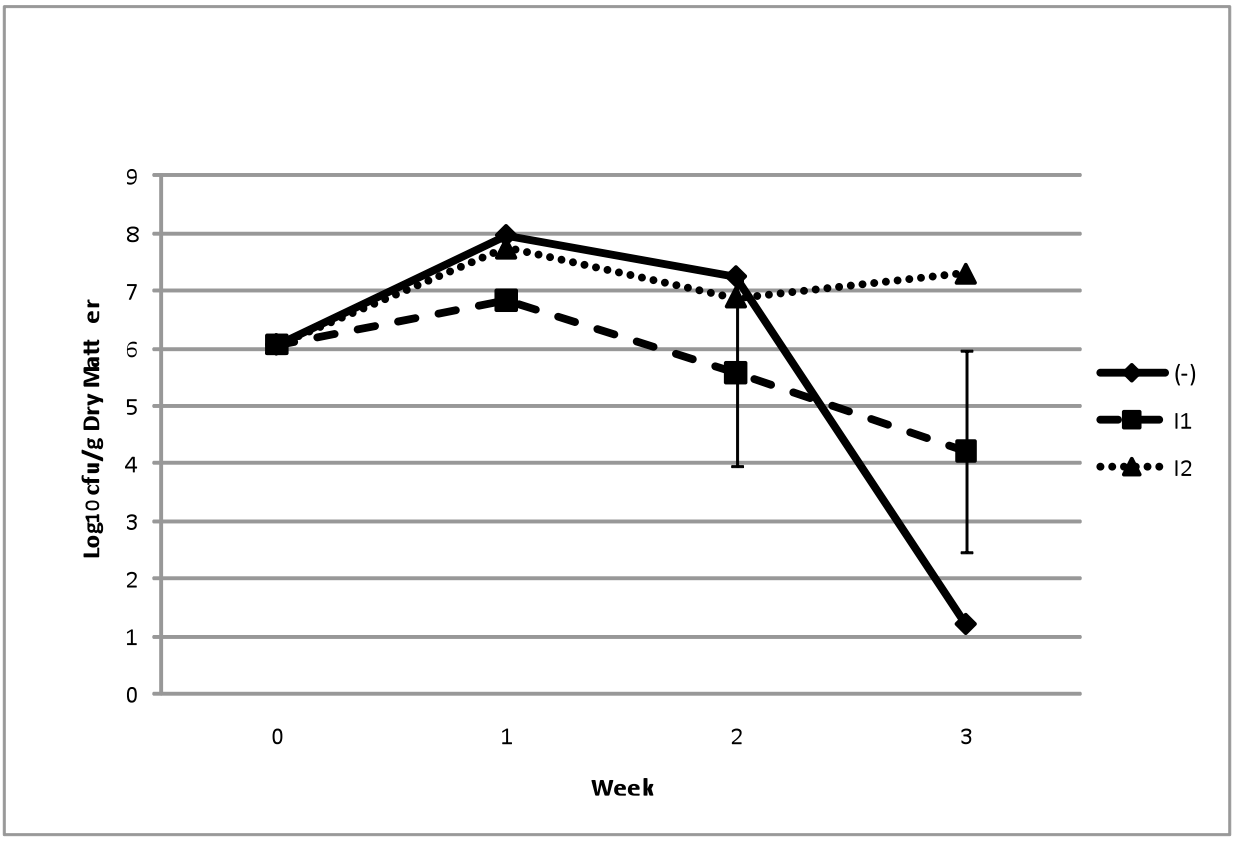

Figure 2.3 *Enterococcus* spp. levels in trial grass per week over 3 wk ensiling period with treatments (shown in legend). Values shown are mean ± SE. Treatment I2 > (-) at wk 3 ($P < 0.05$) using the MIXED model procedure in SAS with repeated measures.

(-) (Negative control of non-inoculated grass)
I1 (Commercially available silage inoculant 1)
I2 (Commercially available silage inoculant 2)

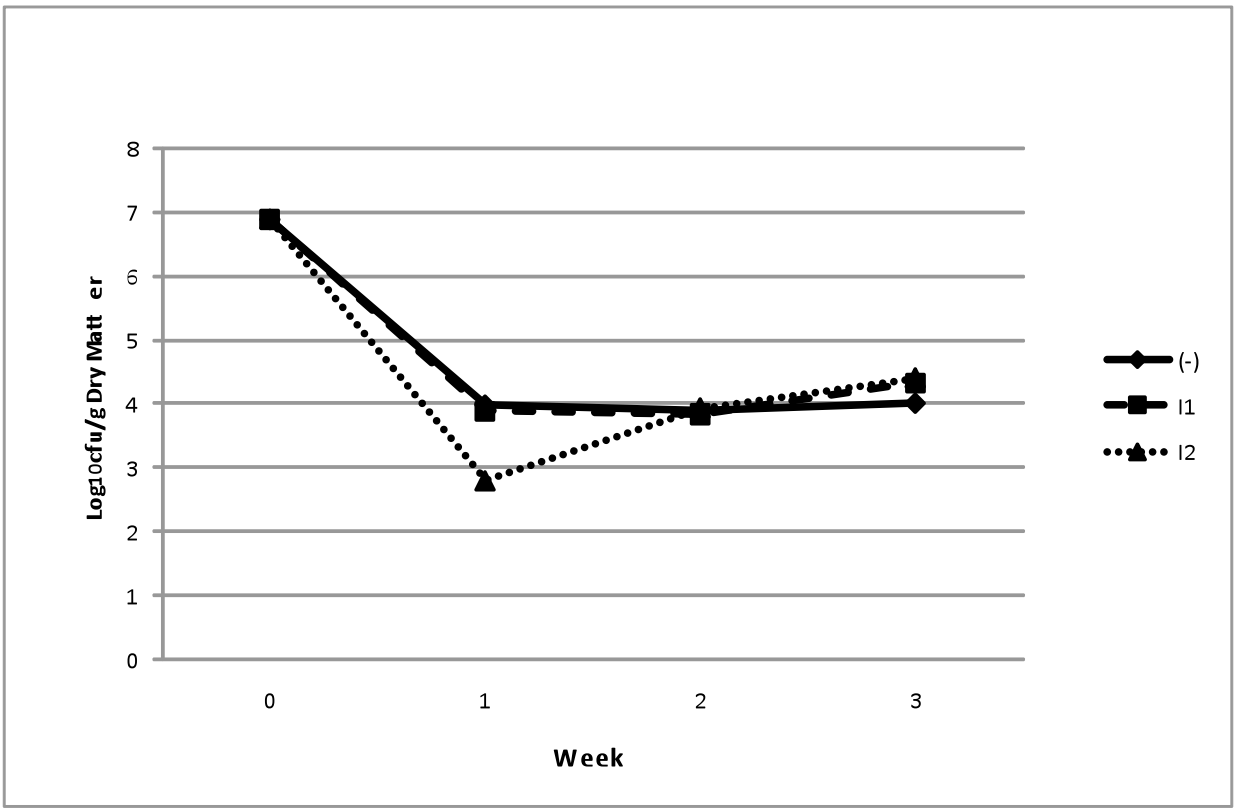

Figure 2.4 *Enterococcus* spp. levels in trial corn per week over 3 wk ensiling period with treatments (shown in legend). Values shown are mean ± SE. Treatment (-) > I2 at wk 1 ($P < 0.05$) and Treatment I1 > I2 at wk 1 ($P < 0.05$) using the MIXED model procedure in SAS with repeated measures.

(-) (Negative control of non-inoculated corn)
I1 (Commercially available silage inoculant 1)
I2 (Commercially available silage inoculant 2)

CHAPTER 3: Fecal shedding of *Enterococcus* spp. in lactating dairy cows

ABSTRACT

An *in vivo* trial was conducted to enumerate enterococci shedding rates in lactating dairy cows. Using a 4 x 4 Latin Square design, 4 lactating, ruminally fistulated cows were inoculated with 4 enterococcal isolates over 4 mo. Isolates came from a commercially available silage inoculant (n=1), ensiled forages (n=2), and a clinical mastitis case (n=1). All cows were housed in the same pen and received the same diet for the duration of the study. Each period consisted of 2 wks followed by a wash period of 10 d before starting the next period. Samples for baseline enterococci levels were obtained over 2 d prior to inoculation during seven samples times: -48 h, -24 h, -6 h, -4 h, -2 h, -1 h, and time 0 pre-inoculation. Inoculation occurred at time 0 with 1 x 10^8 cfu/mL of inoculant dosed into the rumen within 10 mL of phosphate buffered saline. Following treatment administration rumen and fecal samples were collected during 8 sampling times for each period: 1 h, 2 h, 4 h, 6 h, 24 h, 48 h, 72 h, and 168 h post-inoculation. Samples were serially diluted and bacterial enumeration was conducted according to standard bedding sampling procedures with the addition of Kanamycin Esculin Azide Agar plates for the enumeration of enterococci. *Enterococcus* spp. enumerated from rumen samples ranged from 2.0 – 5.3 $\log_{10}$cfu from -48 h to 168 h post inoculation over all periods. Statistical analyses revealed that the main effects of 'period' and 'time', the interaction of period and time, and the interaction of treatment and time remained significant in the model. There was also a significant increase in enterococcal levels averaged for all treatments between the baseline period and periods 3 and 4. *Enterococcus* spp. enumerated from fecal samples ranged from 3.0 – 6.7 $\log_{10}$cfu from -48 h to 168 h post

inoculation over all periods. Statistical analyses revealed that the main effects of ‘period’ and ‘time’, as well as the interaction of period and time remained significant in the model. There was also a significant increase in the levels of fecal enterococci averaged for all treatments between the baseline period and periods 3 and 4. These data suggest that the amount of enterococci shed in bovine feces differed from baseline levels over time, but cannot conclude that it was due to ruminal inoculation with enterococcal isolates.

INTRODUCTION

As the control methods for contagious mastitis pathogens are improved and implemented on dairy farms, a remaining void is being filled by environmental pathogens and their resulting IMI (Oliver and Sordillo, 1988). The large cost associated with mastitis drives the need for indentifying environmental pathogen sources and implementing reasonable controls. When looking to create a model to estimate cost of clinical mastitis cases in high yielding (11,000kg/cow/yr) dairy cows, Bar and colleagues found that there were substantial monetary losses for all parities within a lactating herd. It is estimated that the first event of clinical mastitis in primiparous animals results in a 116 kg loss in milk. For multiparous cows, that milk loss has been estimated to be 186 kg of milk. The average cost of a clinical mastitis case was $179, which mostly comprised costs of treatment and milk losses (Bar et al., 2008).

Within a Tennessee dairy herd, 20/71 dairy cows that calved within a 2 mo period had clinical or subclinical *Enterococcus faecium* mastitis during early lactation (Keane et al., 1995). Thus, researchers found that enterococcal IMI play a significant role post calving and into early lactation. Petersson-Wolfe and colleagues found a higher number of IMI in early lactation cows than late lactation cows when challenged with *E. faecium* mammary infusions. After challenging 19 cows with 1.24×10^4 cfu *E. faecium*, 80% (16/20 quarters) of early lactation cows were infected, 50% (9/18 quarters) of late lactation cows were infected (Petersson-Wolfe et al., 2009). Understanding the possible transfer mechanisms of this bacterial genus from original source to mammary gland could help producers better manage the incidence of these mastitis infections and alleviate some of the associated financial burden.

As explored in the preceding chapter, animal feed treated with silage inoculants containing *Enterococcus* spp. is a viable source of bacterial presence on dairy farms. In 2004, Weinberg and colleagues discovered that enterococci, along with other LAB from silage inoculants, were able to survive *in vitro* in strained bovine rumen fluid for at least 48 h. After inoculating and ensiling a variety of forage products, such as wheat, early dent corn, and half-milk line corn, a 2.5g sample of each silage was placed within 25mL of either autoclaved, strained rumen fluid or non-autoclaved, strained rumen fluid. Enterococci levels in both rumen fluids reached as high as 9.0 $\log_{10}$cfu/mL with wheat silage, 8.5 $\log_{10}$cfu/mL with early dent corn, and 6.7 $\log_{10}$cfu/mL with half-milk line corn after 48 h incubation at 39ºC (Weinberg et al., 2004). These results lead to a logical thought process; enterococci in the feed could result in the presence of enterococci in the gastrointestinal tract and feces of dairy cows.

There have been very few studies to enumerate the amount of enterococci shed by dairy cows. In 1960, the average daily enterococcal density of bovine feces was calculated at 0.16 million enterococci/g moist feces from 21 bovine fecal samples. This number was the lowest, with enterococcal densities higher for humans, chickens, sheep, and pigs (Kenner et al., 1960). In 2007, researchers found 1.75 $\log_{10}$ cfu/g feces of enterococci isolated from all 26 bovine fecal samples but concluded that bovine feces were not a significant enterococcal contamination factor of raw milk (Kagkli et al., 2007). While looking at the impact of sprinkler systems on shedding rates of various environmental pathogens in dairy cattle, researchers observed a decrease in enterococci shedding associated with the heat stress relief. Researchers found 20 enterococcal isolates obtained from the feces of cows with the sprinkler system and 46 enterococcal isolates

obtained from the feces of cows without a sprinkler system (Edrington et al., 2009). These diverse studies provide the little knowledge currently available about enterococcal shedding in dairy cows.

There have been no studies to date that directly relate the ingestion of *Enterococcus* spp. to fecal shedding in dairy cattle. Many of these studies have used gram negative bacteria as causative agents, such as the pathogenic *Escherichia coli* O157:H7. Grauke and colleagues used four ruminally fistulated steers to look at translocation and shedding of a single dose of 2×10^{11} cfu *E. coli* O157:H7. Researchers found that the bacteria rapidly moved through the rumen, duodenum, and feces, resulting in no bacteria detected in sample sources after 14 d post inoculation without enrichment (Grauke et al., 2002). If similar studies were done with enterococci, it would allow a better estimate of the risk for dairy cows of acquiring enterococcal mastitis. Therefore, it was the objective of this study to model an experiment after Grauke's work to explore the translocation and shedding rate of enterococcal isolates obtained from silage inoculants.

MATERIALS AND METHODS

Experimental Animals

A total of 4 ruminally fistulated Holstein cows (2nd lactation or greater) housed at the Virginia Tech Dairy Center (Blacksburg, Virginia) were used for the current study. The cows chosen for this study were 4016, 4093, 4150, and 4205. Cows were allowed 1 wk to transition to the new pen social dynamics. All animals were fed the high group TMR once daily between 9-10am. The farm had ensiled the TMR with a different enterococci-containing silage inoculant than ones used in the previous ensiling trial. All cows remained in the same pen for the duration of the study, except for one cow (4150) that suffered a foot injury during the third period. She was moved to the sick pen and taken care of following IACUC protocol. All other cows on study remained healthy and received no other treatment other than the designated inoculant dose. Cows were milked twice daily in a double-eight, herringbone parlor with automatic takeoffs, flush system, and AfiMilk monitoring system. All cows on study, bar any illness, were subjected to regular herd management practices.

Study Design

Following a 4 x 4 Latin Square design, 4 cows were assigned to 4 treatments (A-D) in 4 time periods (Figure 3.1). Prior to inoculation, cows were sampled seven times for baseline measurements at -48 h, -24 h, -6 h, -4 h, -2 h, -1 h, and time 0 pre-inoculation. Treatment periods consisted of 2 wk, with 10 sampling times during that period at 1 h, 2 h, 4 h, 6 h, 24 h, 48 h, 72 h, 168 h, 240 h, and 336 h post-inoculation (Figure 3.2). The timeline was shortened after period 2 to 168 h since it was observed that enterococci were returning near baseline levels around 48 h. Only values to 168 h are

discussed from periods 1 and 2. Between each period was a 10 d wash period to decrease any possible crossover between treatments. The 4 mo study began on July 18th, 2009 and finished on October 18th, 2009.

Inoculant Profiles and Dosing

Four *Enterococcus faecium* isolates were chosen for use in this study. Two were from a previous ensiling trial, one from a silage inoculant, and one from previous mastitis research. The first enterococcal isolate from the ensiling trial was #35 (Treatment A), which was isolated from grass crop that had been ensiled with a commercially available silage inoculant for 3 wks. The second enterococcal isolate from the ensiling trial was #60 (Treatment D), which was isolated from corn crop that had been ensiled with a second commercially available silage inoculant for 3 wks. An enterococcal isolate, #6 (Treatment C), was used from a commercially available inoculant itself after it had been streaked on enterococci-selective media. Finally, isolate AZ 67 (Treatment B) was from previous mastitis research done by Petersson-Wolfe and colleagues in 2008 and was isolated from a clinical case of bovine mastitis in Ohio. The concentration of inoculum for each treatment was 1 x 10^8 cfu/mL for the four main periods. During the last 7 d of the period 4, a higher concentration of 1 x 10^{11} cfu/mL was administered as a repeat treatment from that same period due to a lack of significance on fecal shedding observed with the lower dose. This concentration was calculated based on levels of enterococci resulting after 3 wks on corn silage, as seen in the ensiling trial, and a cow's average daily intake of 45 lb DM/d. Sampling was treated as if it were the first 7 days of a new period (Higher Dose, HD period). All doses were administered within 10 mL of sterile

phosphate buffered saline (**PBS**), for total concentrations of 1 x 10^9 cfu and 1 x 10^{12} cfu, respectively.

PCR Confirmation of Inoculant Strains

Each selected isolate was confirmed as an *Enterococcus* spp. using PCR. Bacteria were plated for isolation on Tryptic Soy Agar (**TSA**) and placed in a 37ºC incubator overnight. A single colony was transferred from the plate to 5mL of sterile Tryptic Soy Broth (**TSB**) with a sterile loop and incubated overnight at 37ºC. After incubation, 1mL of each bacterial solution was transferred to a sterile microcentrifuge tube and centrifuged at 14000 *g* and 4ºC for 5 min. Pellets were washed with sterile PBS and centrifuged again. Supernatants were decanted, replaced with 500µL sterile ddH_2O, and tubes were vortexed. The tubes were placed on a heat block set at 105ºC for 20 min. After boiling, the tubes were centrifuged once more at 14000 *g* and 4ºC for 5 min and stored at 4ºC until further use. Before isolates could be analyzed with PCR, the bacterial concentration was analyzed using the Nano Drop 1000 (Bio-Rad, Hercules, CA). Any isolate with a DNA concentration higher than 90.0 ng/µL was considered sufficient to be analyzed by PCR.

The genes selected for identification were the housekeeping genes *tuf* from streptococci (*tuf*-strep) and *tuf* from enterococci (*tuf*-entero). The following primers (Integrated DNA Technologies, Inc., Coralville, IA) were used for both genes: *tuf*-entero forward (5'-TAC TGA CAA ACC ATT CAT GAT G-3'), *tuf*-entero reverse (5'-AAC TTC GTC ACC AAC GCG AAC-3'), *tuf*-strep forward (5'-GTA CAG TTG CTT CAG GAC GTA TC-3'), and *tuf*-strep reverse (5'-ACG TTC GAT TTC ATC ACG TTG-3').

The 'master mix' used consisted of 10μL of 5x Buffer (Promega, Madison, WI), 4μL 2.5 mM dNTP mix (Fermentas, Glen Burnie, MD), 2μL 5 μM forward primer (Integrated DNA Technologies, Inc., San Diego, CA), 2μL 5 μM reverse primer (Integrated DNA Technologies, Inc., San Diego, CA), 2μL 25 mM $MgCl_2$ (Promega, Madison, WI), 24μL nuclease-free dH_2O, 1μL Go TAQ Hot Start (Promega, Madison, WI), and 5μL of template DNA. The amounts of master mix were multiplied by the number of isolates plus one to ensure enough reagents for analysis. For this investigation, all master mix amounts were multiplied by 8 since there were 7 isolates tested. PCR was done on a BioRad Thermocylcer (Bio-Rad Laboratories, Inc., Hercules, CA) with a standardized run protocol for the *tuf* primers. This protocol consisted of an initial hot start at 95°C for 3 min, denaturation at 95°C for 30 min, annealing temperature of 55ºC for 1 min, extension at 72°C for 1 min, denaturation to extension step for 30 cycles, final extension at 72°C for 7 min, and then tubes remained at 4°C until retrieved from the machine.

Prior to running the agarose gel, 5μL of dye was added to each product tube for visualization. Each gel was run with a ladder for about 4 h and was subsequently exposed to UV light. Pictures were taken of the gel to quantify fluorescent bands. The presence of a band at 112 base pairs was considered positive for the *Enterococcus* genus.

Standard Curves for Dose Inoculants

After PCR confirmation of bacterial isolates, standard growth curves were analyzed for the isolates to be used as inoculums. The selected dose isolates were streaked out for isolation onto TSA plates from their -80ºC stock vials and incubated overnight at 37ºC. One colony from each isolate was inoculated into labeled 25mL tubes

filled with sterile TSB. Post overnight incubation at 37ºC, serial dilutions were made. After blanking a spectrophotometer with 1mL of sterile PBS, 1mL samples for each dilution and isolate were read in sterile cuvettes at 600nm. A sterile 96-well plate was prepared with varying amounts of sterile PBS. From each OD dilution tube, 100µL of the bacterial culture was added to the first row (Row A) on the 96-well plate. Then, 50µL from Row A were transferred to Row B for a 1:10 dilution. Next, 25µL were transferred to Row C for a 1 x 10^{-2} dilution. Another 25µL was transferred from Row C to D, and similarly continued down the plate in 10-fold dilutions until Row H. Each dilution was plated onto EBA plates with 3 consecutive 25µL drops of suspension and incubated overnight at 37ºC. After incubation, the number of colonies in the 3 drops was counted and the final concentration was calculated with the equation: (colony count/3) x 40 x (1/ final dilution) = cfu/mL.

Sampling Procedure

Both protocols for rumen and fecal sampling can be seen in Appendix B. In brief, cows were sampled in freestalls to ensure minimal movement and stress of the animal. For rumen samples, thermoses were filled with warm water and brought to the barn. Immediately prior to sampling, the warm water was dumped out and the cap of the thermos was replaced to keep the warm atmosphere. Wearing gloves, the cannula was pushed open, and 500g of rumen content were collected. Rumen contents were directly placed into the thermos and returned to the laboratory (Grauke et al., 2002). For fecal samples, lubricant was applied to an artificial insemination glove and fecal grab samples were obtained. 500g of feces were placed into a sterile specimen cup and immediately returned to the laboratory (Grauke et al., 2002).

Laboratory Analysis

Isolation of Bacteria from Samples

Within a fume hood, thermoses and cups were opened and 10g of contents taken from the middle of each container. The 10g were weighed in a sterile, tared weigh boat and placed into a corresponding stomacher bag containing 90mL of sterile 1X PBS at 7.4 pH. Stomacher bags were then pressed flat to remove most of the air and placed into the Stomacher 400 Circulator (Seward, United Kingdom) at 230 rpm for 60 s.

Serial Dilutions

Using labeled 96-well sterile plates, each bag was serial diluted out to 10^6 dilutions. From each bag, 250µL of the samples were pipetted into 2 wells on column 1 on the plate. The other wells contained 225µL of sterile PBS. Using a multi-channel pipette, 25µL of the original silage sample from column 1 was transferred to column 2 and mixed 4 times. Then 25µL were transferred from column 2 to 3 and mixed, and subsequently repeated until column 5 (Appendix B).

Microbial Analysis

The selective media used was tested with confirmed positive strains before use in the enumeration trial. Microbial analysis was conducted according to standard bedding sampling procedures (Hogan and Smith, 1997)with the addition of the Kanamycin Esculin Azide Agar (**KEA**) plates for the enumeration of enterococci. The standard bedding sample medias were Maconkeys agar (**MAC**) plates, Edwards modified media agar (**EMM**) plates, and Carbenicillin plates. From each well with samples on the 96-well plates, 40µL were removed and four 10µL drops were plated onto a sixth of an appropriately labeled plate for all four types of media. Once dry, the plates were inverted and placed in an incubator at 37°C for 24 h. After 24 h, colonies were enumerated

visually per 10µL spot and recorded. The countable range used was 2 – 25 cfu per drop. Microbial analysis was performed on samples from all cows and all treatment weeks, as well as on the covariate samples prior to inoculation. All isolates indentified as enterococci were cryogenically saved.

Freezing of Bacterial Cultures

Enterococcal isolates from the various weeks and treatments were identified from KEA plates and streaked for isolation on TSA plates. The plates were placed in an incubator overnight at 37°C. After 24 h, a single colony was selected using a sterile inoculating loop and placed into 5mL of sterile TSB. The inoculated tubes were placed back in the incubator at 37°C for 24 h. The tubes were centrifuged in an Eppendorf 5810R Centrifuge for 10 min at 1620 *g* and 4°C to obtain a bacterial pellet. In a sterile biosafety cabinet, the supernatant of the TSB tubes was poured out and replaced with 5mL of sterile skim milk (Becton, Dickinson, and Company, Sparks, MD). Tubes were vortexed to resuspend the pellet in the milk and 850µL of the culture solution was placed into two appropriately labeled 2mL cryovials, each containing 150µL of sterile 100% glycerol. Cryovials were vortexed and one vial was placed in a box for -20°C storage and the duplicate was placed in a box for -80°C storage.

Data Management and Statistical Analysis

All values recorded from drop plate counts were managed within the Microsoft Excel program, version 2008 (Microsoft Corporation, WA). Graphs and charts were created from the stored data within Excel. The values of $\log_{10}$ colony forming units were calculated using formulas within Excel. Microsoft Access, version 2007 (Microsoft Corporation, WA) held records of cryogenically frozen isolates. Statistical analysis was

performed using SAS Statistical Software, version 9.2 (SAS Institute Inc., NC). For statistical analyses, a MIXED procedure with repeated measures was run with 'cow' as the random variable and 'cow*treatment*period' as the repeated variable for each time using the following model:

$Y_{ijkl} = \mu + T_i + C_j + Tm_k + TTm_{ik} + P_l + PTm_{kl} + e_{ijkl}$

where:

μ = mean of Y;

T_i = effect of treatment (i = 1 to 4);

C_j = effect of cow (j = 1 to 4);

Tm_k = effect of sampling time (k = 1 to 8);

TTm_k = interaction of treatment and sampling time;

P_l = effect of period (l = 1 to 4);

PTm_{kl} = interaction of period and sampling time; and

e_{ijkl} = error (cow, sampling time, and period interaction)

Least squares means of the main effects of 'period', 'treatment', 'time', as well as the interactions between 'period and time' and 'time and treatment' were analyzed. Significance was considered to be at a $P < 0.05$. For period analyses, a MIXED procedure was run with 'cow' and 'cow*period' as random variables using the following model:

$Y_{ij} = \mu + C_i + P_j + e_{ij}$

where:

μ = mean of Y;

C_i = effect of cow (i = 1 to 4);

P_j = effect of period (j = 1 to 4); and

e_{ij} = error (cow and period interaction)

Dunnett adjusted comparisons were performed on the least squares means of 'period' for comparison of individual treatment periods (1, 2, 3, 4, and HD) to the baseline period. Significance was considered to be at $P < 0.05$.

RESULTS

Microbial Enumeration and Analysis

Samples compared in results were from 48 h prior to treatment during the baseline period and 168 h into each treatment period. Average bacterial levels in each period for each treatment investigated were calculated. Only statistical analyses of period effect included the baseline period and higher dose (**HD**) period. Bacteria levels across sampling times as well as period differences for gram negative bacteria, *Klebsiella* spp., and *Streptococcus* spp. are displayed in Appendix B.

Gram negative bacteria enumerated from MAC plates for rumen samples ranged from below detectable levels (< 1000 cfu) to 5.0 $\log_{10}$cfu over periods 1-4. Statistical analyses of treatments showed effects of 'period', 'time', and 'period*time' with a significance of $P > 0.0001$ for each. Period statistical analyses revealed rumen gram negative bacteria levels in period 3 significantly increased (+1.2 $\log_{10}$cfu, $P > 0.0219$) from the baseline period and levels in period 2 significantly decreased (-1.5 $\log_{10}$cfu, $P > 0.0036$) from the baseline period. Gram negative bacteria enumerated from fecal samples ranged from below detectable levels to 6.5 $\log_{10}$cfu over periods 1-4. Statistical analyses of treatments revealed an effect of 'period' with significance at $P < 0.0160$. Period statistical analyses showed significant increase (+1.5 $\log_{10}$cfu, $P < 0.0376$) in fecal gram

negative bacteria levels between the baseline period and period 4. Levels of *Klebsiella* spp. enumerated from rumen samples on Carbenicillin plates ranged from below detectable levels to 4.4 $\log_{10}$cfu over periods 1-4. Treatment statistical analyses revealed effects of 'period', 'time', and 'period*time' with significance at $P < 0.0001$ for each. Statistical analyses of periods showed significant increase in rumen *Klebsiella* spp. between the baseline period and periods 3 (+1.0 $\log_{10}$cfu, $P < 0.0216$) and 4 (+1.0 $\log_{10}$cfu, $P < 0.0248$). There was significant decrease in levels between the baseline period and period 2 (-1.4 $\log_{10}$cfu, $P < 0.0019$). *Klebsiella* spp. enumerated from fecal samples ranged from below detectable levels to 5.2 $\log_{10}$cfu over periods 1-4. Statistical analyses of treatments revealed effects of 'period' and 'period*time' with significances of $P < 0.0010$ and $P < 0.0009$, respectively. Period statistical analyses showed significant increase in fecal *Klebsiella* spp. between the baseline period and period 2 (+2.3 $\log_{10}$cfu, $P < 0.0003$). Finally, for *Streptococcus* spp. enumerated from EMM plates, rumen sample levels ranged 1.5 – 5.2 $\log_{10}$cfu over periods 1-4. Treatment statistical analyses revealed effects of 'period', 'time', and 'period*time' with significances of $P < 0.0103$, $P < 0.0001$, and $P < 0.0169$, respectively. Statistical analyses of periods showed significant increase in rumen *Streptococcus* spp. between the baseline period and periods 2 (+0.5 $\log_{10}$cfu, $P < 0.0262$), 3 (+0.9 $\log_{10}$cfu, $P < 0.0006$), 4 (+0.7 $\log_{10}$cfu, $P < 0.0036$), and HD (+1.0 $\log_{10}$cfu, $P < 0.0003$). *Streptococcus* spp. enumerated from fecal samples ranged 2.2 – 7.0 $\log_{10}$cfu from periods 1-4. Statistical analyses of treatments revealed effects of 'period' and 'period*time' with significances at $P < 0.0019$ and $P < 0.0308$, respectively. Period statistical analyses showed significant increase in fecal *Streptococcus*

spp. between the baseline period and periods 2 (+1.1 $\log_{10}$cfu, $P < 0.0235$), 4 (+1.4 $\log_{10}$cfu, $P < 0.0122$), and HD (+1.2 $\log_{10}$cfu, $P < 0.0314$).

Enterococci Enumeration

Enterococcus spp. enumerated from rumen samples on KEA plates ranged 2.0 – 5.3 $\log_{10}$cfu from periods 1-4 (Figure 3.3). The highest enterococcal level, 5.3 $\log_{10}$cfu, resulted from a sample taken during the HD period at 1 h from cow 4205 who had received treatment C. The lowest enterococcal level, 2.0 $\log_{10}$cfu, was seen twice and resulted from a sample taken during period 4 at 1 h from cow 4016 who had received treatment B and a sample taken during period 2 at 6 h from cow 4150 who had received treatment A. Statistical analyses of treatments revealed effects of 'period', 'time', and 'period*time' with significance at $P < 0.0001$ for each. Period statistical analyses showed significant increase in ruminal enterococci between the baseline period and periods 3 (+0.8 $\log_{10}$cfu, $P < 0.0009$), 4 (+0.9 $\log_{10}$cfu, $P < 0.0013$), and HD (+1.0 $\log_{10}$cfu, $P < 0.0003$) (Figure 3.5). *Enterococcus* spp. enumerated from KEA plates for fecal samples ranged 3.2 – 6.7 $\log_{10}$cfu from periods 1-4 (Figure 3.4). The highest level of enterococci, 6.7 $\log_{10}$cfu, was obtained from a sample taken during the HD period at 48 h post inoculation from cow 4016 who had received treatment B. The lowest enterococcal level, 3.2 $\log_{10}$cfu, was obtained from a sample taken during the period 1 at 24 h post inoculation from cow 4205 who had received treatment D. Treatment statistical analyses showed effects of 'period', 'time', and 'period*time' with significances at $P < 0.0045$, $P < 0.0006$, and $P < 0.0125$, respectively. Statistical analysis of periods revealed significance increase in fecal enterococci between the baseline period and periods 3 (+1.1

$\log_{10}$cfu, $P < 0.0016$), 4 (+0.7 $\log_{10}$cfu, $P < 0.0474$), and HD (+1.6 $\log_{10}$cfu, $P < 0.0001$) (Figure 3.6).

DISCUSSION

The average log counts for enterococci from rumen samples over the course of the current 4 mo study were 2.0 – 5.3 $\log_{10}$cfu. The average log counts for enterococci from fecal samples over the course of the study were 3.2 – 6.7 $\log_{10}$cfu. There was a significantly higher level of enterococci isolated from both rumen and fecal samples during treatment periods 3, 4, and HD when compared to the baseline period. However, there was no significant treatment difference in enterococci levels from either rumen or fecal samples.

Since there has been no previous research conducted directly looking at shedding rates of enterococci from dairy cows, it was hard to specifically assess if the results seen were within a normal bacterial range. However, looking at two previous studies conducted on natural enterococcal prevalence in bovine feces, the resulting amounts of enterococci from the current study appear to agree with preceding data.

In a 2008 USDA study, the natural fecal shedding of enteric pathogens, including *Enterococcus* spp., were enumerated in beef heifers every month for 12 mo. The samples resulted in an 88.5% prevalence of enterococci in the cattle (Riley et al., 2008). This was the highest prevalence, exceeding those of *Campylobacter* spp., *E. coli*, and *Salmonella* spp. (Riley et al., 2008). This study supports the current findings that enterococci are shed by bovines, as seen by the bacterial presence during the baseline period. While examining survival of enteric bacteria in bovine feces on pasture during winter, spring, summer, and fall months, New Zealand researchers found that the enterococci collected ranged from 1-

3.5 x 10^6 cfu/g feces throughout all four seasons (Sinton et al., 2007). The highest level of fecal enterococci observed during this study nearly matched the highest level of fecal enterococci observed in the current study, with 3.5 x 10^6 cfu equated to 6.5 $\log_{10}$ cfu.

Comparing the data to the Grauke et al. study with *E. coli* translocation, the results of the current study were not as conclusive do to lack of specific isolate identification. Using 4 ruminally fistulated steers and inoculating a single dose of 2 x 10^{11} cfu *E. coli* O157:H7, researchers found that the bacteria rapidly moved through the rumen and feces (Grauke et al., 2002). There was a 1 x 10^4 cfu *E. coli* increase from the baseline levels to 7 d post inoculation. There were no detectable levels of *E. coli* O157:H7 by 14 d post inoculation without enrichment (Grauke et al., 2002). In the current study, the amount of enterococci isolated from fecal samples during treatment periods was significantly different than the amount isolated during the baseline period. However, it is unclear as to whether this was because of the ruminal inoculants, the cows already eating inoculated TMR, a washout period not being long enough, or enterococci coming from a different source. Thus, it cannot be concluded that the fecal results of the current study fully support the original hypothesis.

Levels of *Enterococcus* spp. isolated from rumen contents during periods 3, 4, and HD were significantly higher than those isolated during the baseline period. In 2003, Weinberg and colleagues saw similar results using a study model with strained bovine rumen fluid inoculated with lactic acid bacteria (**LAB**), including *Enterococcus* spp., from silage inoculants. They reported a 10-fold increase of LAB from the non-inoculated rumen fluid to the inoculated fluid at the end of the 72 h trial using an inoculation dose of 1 x 10^7 cfu LAB (Weinberg et al., 2003). The researchers felt confident that their model

mimicked a natural setting, since the LAB would have come into contact and competed with the natural microflora within the rumen fluid.

Enterococci levels shed in the feces only changed a little over 1 log from the baseline period to the HD period, values at 4.3 $\log_{10}$cfu to 5.9 $\log_{10}$cfu respectively. Larger shedding amounts were hypothesized, believing a large increase could have been logically attributed to the ruminal inoculants. Based on Weinberg's conclusions and results that the bacteria would have most likely been able to compete with the native rumen microflora, a non-bacterial explanation might account for the small increase observed in fecal shedding. In 2007, Gutierrez and Davis assessed the ability of ruminal protozoa to ingest ruminal bacteria. Stains of diluted rumen contents showed that the protozoans *Entodinium caudatum, E. minimum, E. dubardi*, *E. longinucleatum, E. bursa, E. nanellum, E. exiguum*, and *E. vorax* all contained gram-positive cocci. The bacterial strains were isolated by washing of the protozoa and culture on a starch feed-extract agar. These cultures were then provided to *Entodinium* spp., which had been starved for 24 h. Results showed that the protozoans did ingest the provided bacteria, which were soon after identified as *Streptococcus bovis* (Gutierrez and Davis, 2007). Although not conducted with *Enterococcus* spp., we propose a similar outcome would occur based on the microbial and physiological similarities between streptococci and enterococci that have been well documented. Perhaps this is why the enterococcal inoculums used in the current study, even at a high dose, were not able to drastically increase the amount of enterococci shed into the environment.

For the current study, there were 4 areas that could have been improved to increase the quality of results. The first is the amount of inoculant in each cow. It is

possible that due to human error, not every cow had their rumen contents mixed appropriately. Also, perhaps if enterococcal isolates other than the ones chosen were used, the shedding results might have varied. The second area for improvement would have been the concentration of the enterococcal dose. Since there had been no literature support for enterococcal dosing into a rumen, the doses were based on previous literature for different bacterial genera. The volume of the dose could have been lost in the cavernous expanse of the rumen or the concentration, though seemingly high, could have been too low to create a large microbial impact within the cow. The third area is that more cows could have been used for each period and treatment to increase the number of replicates. The addition of more cows might have allowed for a more robust statistical analysis. Finally, the last area of improvement could have been using a more precise method of microbial identification. Creating mutants of the isolates that were susceptible to a certain antimicrobial or fluorescently tagged would have made enumeration easier, may have resulted in a more accurate number of enterococci reported, and would serve as a better indicator of study design quality. However, the feasibility of these methods was low due to the expensive nature of the techniques and animal care and use concerns.

CONCLUSIONS

These data suggest that the bovine fecal shedding rate of enterococci may not be directly increased by ruminal inoculation with enterococcal isolates. Enterococci levels in rumen and fecal samples from treatment periods did show marked increase when compared to the levels isolated during the baseline period. However, due to lack of specific bacterial identification, it is unclear if the observed increases were due to ruminal inoculants or due to inadequate washout period lengths, cows eating already inoculated

TMR, or enterococci from the greater environment. These data support that enterococcal isolates are able to survive in the ruminal environment post-inoculations, but do not appear to be shed in drastically greater amounts into the cow's environment than what is naturally observed. Further studies should focus on a larger scale trial with increased dose concentration and potentially tag the microbial dose strains for better isolation and enumeration.

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Cow	Period 1	Period 2	Period 3	Period 4
4016	A	D	C	B
4093	B	C	D	A
4150	C	A	B	D
4205	D	B	A	C

Figure 3.1 Latin Square design for *in vivo* shedding trial with 4 ruminally fistulated cows, 4 sampling periods (2 wk) with wash periods (10 d), and 4 treatments (*Enterococcus* spp. isolates A-D).

*A = isolate from ensiled grass
B = isolate from clinical mastitis case
C = isolate from silage inoculant
D = isolate from ensiled corn

Figure 3.2 Shedding *in vivo* trial sampling timeline (times presented in h pre and post rumen inoculation with enterococcal isolate). Inoculation occurred at time 0 h. Diamond at 168 h indicates the end of shortened timeline used for sampling after period 2.

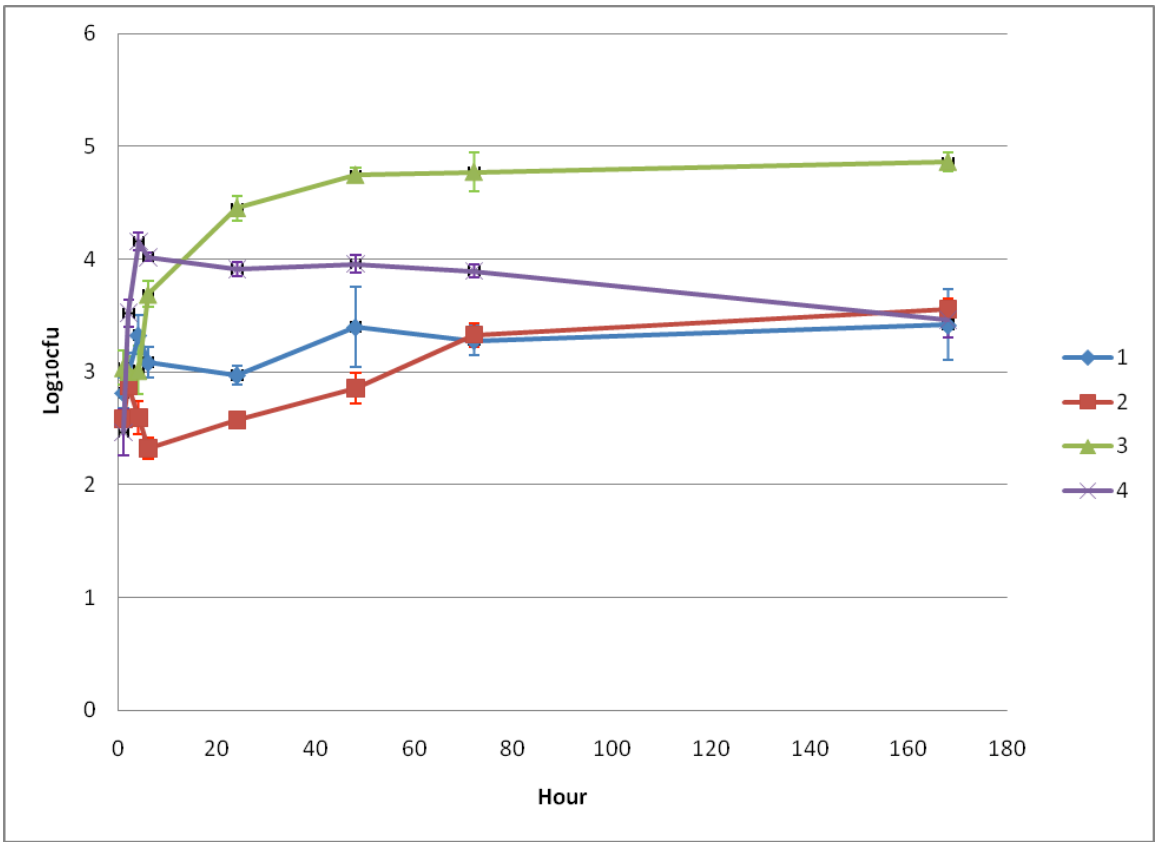

Figure 3.3. Average rumen *Enterococcus* spp. levels for all 4 trial periods over 0 to 168 h from ruminal inoculation with 1 x 10^8 cfu/mL enterococci. Treatments are collapsed within each period due to no significant differences within periods. Period, hour, and period*hour are all significant at $P < 0.0001$. Values shown are mean ± SE.

1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)

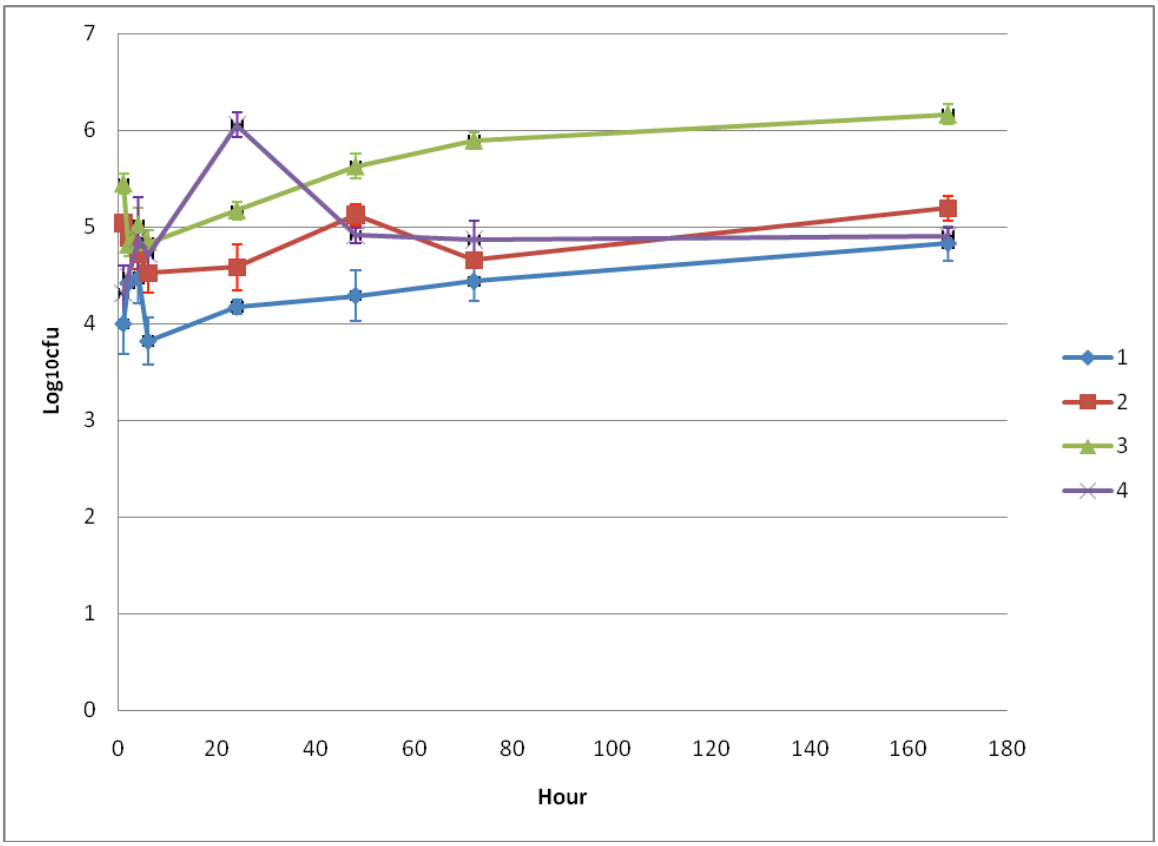

Figure 3.4. Average fecal *Enterococcus* spp. levels for all 4 trial periods over 0 to 168 h from ruminal inoculation with 1 x 10^8 cfu/mL enterococci. Treatments are collapsed within each period due to no significant differences within periods. Period, hour, and period*hour are significant at $P < 0.0045$, $P < 0.0006$, and $P < 0.0125$, respectively. Values shown are mean ± SE.

1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)

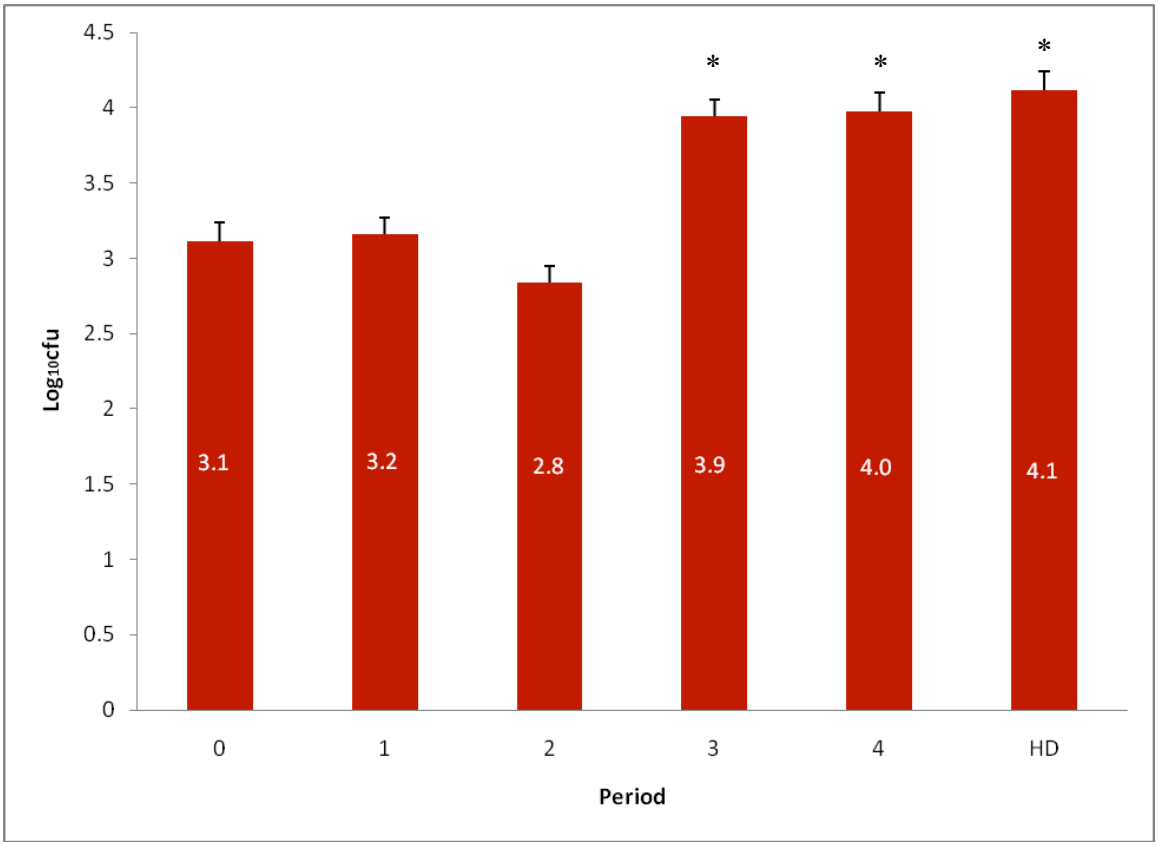

Figure 3.5 Average rumen *Enterococcus* spp. levels over 0 to 168 h during the baseline period, 4 treatment periods (ruminal inoculation with 1 x 10^8 cfu/mL enterococci), and the higher dose period (ruminal inoculation with 1 x 10^{11} cfu/mL enterococci). Treatments are collapsed within each period due to no significant differences within periods. Sampling times are collapsed within periods to display period effect. Values shown are mean ± SE.

0 (Baseline Period, no inoculation)
1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)
HD (Higher Dose Period)

* = Statistical significance ($P \leq 0.05$) between period indicated and Period 0 (baseline period).

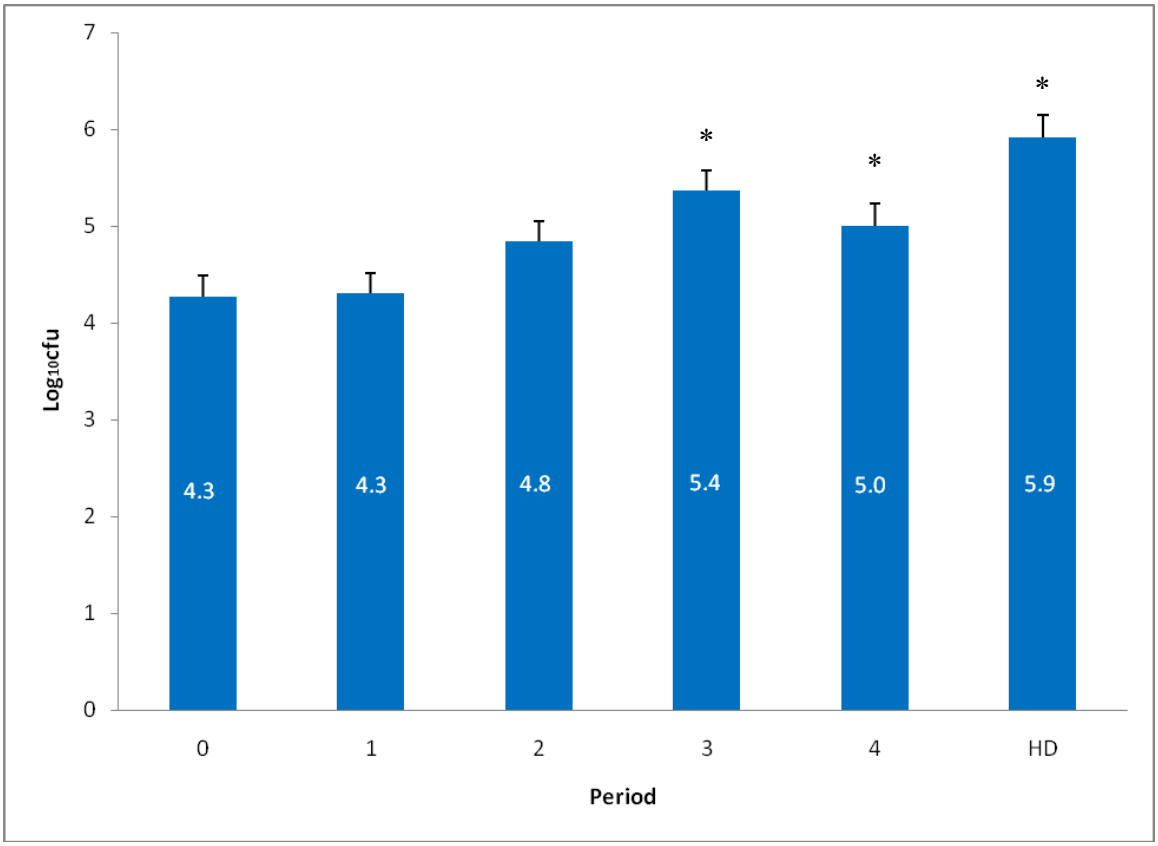

Figure 3.6 Average fecal *Enterococcus* spp. levels over 0 to 168 h during the baseline period, 4 treatment periods (ruminal inoculation with 1 x 10^8 cfu/mL enterococci), and the higher dose period (ruminal inoculation with 1 x 10^{11} cfu/mL enterococci). Treatments are collapsed within each period due to no significant differences within periods. Sampling times are collapsed within periods to display period effect. Values shown are mean ± SE.

0 (Baseline Period, no inoculation)
1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)
HD (Higher Dose Period)

* = Statistical significance ($P \leq 0.05$) between period indicated and Period 0 (baseline period).

CHAPTER 4: Pulsed-field gel electrophoresis examination of enterococcal strains isolated from silage inoculants, bovine rumen contents, and bovine feces

ABSTRACT

Pulsed-field gel electrophoresis patterns from *Sma*I restrictions were used to analyze genetic similarity of enterococcal isolates (n = 80) of environmental and bovine origin. Isolates originated from enterococci-inoculated silage (n = 2), enterococci isolated from silage inoculants (n = 1), clinical enterococcal mastitis cases (n = 3), bovine rumen samples (n = 42), and bovine fecal samples (n = 32). Isolates obtained from bovine rumen and fecal samples were collected prior to inoculation with an enterococcal isolate and post inoculation during a 4 mo shedding trial. All isolates were identified to the genus level with Kanamycin Esculin Azide agar for preliminary identification and standard milk culturing biochemical testing for further identification. A dendogram was used for pictorial analysis of genomic relatedness between the enterococcal isolates with a 75% cutoff value for similarity. With this cutoff value, there was no obvious clustering by trial period, cow, or time sampled that occurred within the isolates. None of the isolates met or were greater than the 75% cutoff value set for analysis. Similarity ranged from 0 to 70.6%. The two most similar isolates (70.6%) were a rumen sample at 9 h and a fecal sample at 10 h that were obtained from the same cow during the same trial period. These results suggest the enterococci from environmental and bovine origin used in this study are genetically diverse with no valued similarity or obvious relational clustering.

INTRODUCTION

Enterococci have been well-researched and documented to cause human disease (McDonald et al., 1997, Murray et al., 1990). They are of great concern to the health community for their ability to share and obtain genetic material with surrounding organisms (Kak and Chow, 2002, Schaberg and Zervos, 1986, Wirth, 1994). Over a period of seven years, *Enterococcus* spp. were responsible for 19.0 % of clinical environmental streptococci intramammary infections (**IMI**) within a single dairy herd (Todhunter et al., 1995). Also, after a 60 d ensiling trial using enterococcal microbial silage inoculants, researchers found 1.0×10^5 cfu/g DM of enterococci remaining on both the alfalfa and grass crops tested (Cai, 1999). Since an IMI could be associated with these pathogens and presence in silage inoculants is common, it is important to gather epidemiologic knowledge of this genus from silage to cow to farm environment.

A gold standard for genetic comparison in microbial epidemiology is pulsed-field gel electrophoresis (**PFGE**). PFGE is a molecular typing method which uses a pulsed, multi-directional electrophoresis field to separate large DNA fragments (30-10,000 kb). Since its first conception in the early 1980s, PFGE has been a DNA fingerprinting technique widely used and is the basis of PulseNet surveillance at the CDC (Schwartz and Cantor, 1984). There are four commonly used PFGE instruments: field inversion gel electrophoresis (**FIGE**), transverse alternating field electrophoresis (**TAFE**), rotating gel electrophoresis (**RGE**), and contour-clamped homogeneous electric field (**CHEF**). FIGE separates the selected DNA by inverting the polarity between two electrodes, thus moving the fragments forwards and backwards (Carle et al., 1986). TAFE uses a vertically placed gel and angle geometry between two electrodes to ‘zig-zag’ the DNA

fragments down the gel (Gardiner et al., 1986). RGE uses two electrodes, whose polarities remained fixed while the gel is placed on a circular running plate that is rotated by magnetic force (Gemmill, 1991). The final and most popular PFGE method, CHEF, involves two electrode pairs that switch polarity with a homogeneous electric field to push the DNA down the agarose gel (Chu, 1990).

Within the past 20 years, CHEF PFGE has helped researchers investigate epidemiologic relationships for restriction endonucleases, antimicrobial resistance, and even mastitis pathogen sources. In 1990, Murray and colleagues began to delve into this work when they evaluated which PFGE protocol would give the best representation of enterococcal fingerprints. Using 27 enterococcal isolates from human sources in the United States, Thailand, and Chile, researchers tested a PFGE protocol originally used for *Escherichia coli* analysis and the restriction enzyme *Sma*I to gain DNA fingerprints. The protocol and the G + C rich restriction enzyme were used to separate and depict fragments ranging from 14 to 1,000 kb (Murray et al., 1990). Later in 2000, researchers adopted Murray's method to design a rapid protocol for subtyping vancomycin-resistant enterococcal isolates. Using 80 enterococcal isolates from human sources, investigators found that a reduced lysis duration, restriction digestion, and staining time still resulted in valid identification of vancomycin-resistant strains of enterococci (Turabelidze et al., 2000).

Recognizing that CHEF PFGE could be used for valid enterococcal identification and antimicrobial resistance recognition, Petersson-Wolfe and colleagues used this instrumentation to look at the profile of enterococcal isolates from mammary secretions (n = 81) and animal bedding and feed on dairy farms (n = 21) (Petersson-Wolfe et al.,

2008). Presumptive identification as *Enterococcus* spp. was based on results from the API 20 STREP test. CHEF PFGE using *Sma*I restriction was run on all selected isolates and a dendogram was created to assess relatedness of strains between sources. API 20 STREP test results showed that the majority of isolates were *Enterococcus faecium*, followed by *E. faecalis*, and *E. casseliflavus* (Petersson-Wolfe et al., 2008). Dendogram analysis revealed that isolates from mammary sources and bedding were genetically diverse, while isolates from feed samples were genetically similar (Petersson-Wolfe et al., 2008). This research suggests that different enterococcal isolates found in the same animal feed environment would be similar to one another when genetically profiled.

A previously described *in vivo* shedding trial was conducted to assess if enterococcal isolates which survived the ensiling process on forages ensiled with enterococci-containing microbial inoculants would survive the bovine gastrointestinal tract. It was the goal of the current study to use CHEF PFGE to evaluate the genomic relatedness between silage inoculants and the subsequent rumen and fecal isolates. The results of this study would help to explore the validity of an epidemiologic path of *Enterococcus* spp. from silo to lactating dairy cow environment.

MATERIALS & METHODS

Bacterial Isolates

Isolates were collected between October 2008 and September 2009. The isolate from a silage inoculant was collected from a commercially available product and ensiled forage isolates were collected post 3 wk ensiling. Rumen and fecal isolates were collected at the Virginia Tech Dairy Center from 4, ruminally-fistulated lactating dairy cows. Mastitis case isolates and PCR-confirmed *E. faecium* isolates were obtained from previous work by Petersson-Wolfe, Stewart, and colleagues (2008). All isolates were grown in pure culture, cryogenically frozen, and saved at -80ºC.

Isolate Identification

Bacterial isolates were preliminarily identified at the time of original sampling using Kanamycin Esculin Azide agar (**KEA**). KEA is selective for enterococci, with a positive culture surrounded by a distinctive black halo on the agar after a 24 h incubation at 37ºC. Isolates saved as enterococci were streaked for isolation and tested using standard milk culturing biochemical medias. An isolate was considered to be an *Enterococcus* spp. if the selected colonies were esculin positive, CAMP test negative, salt positive, catalase negative, and identified as gram positive cocci under 100X magnification.

Pulsed-field Gel Electrophoresis

Bacterial DNA was prepared using a modified protocol based on previous work in 2002 by Saeedi and colleagues. In brief, bacterial isolates were streaked for isolation on Esculin Blood Agar (**EBA**) plates and incubated overnight at 37ºC. After 18 h, a single colony was inoculated into appropriately labeled, sterile Eppendorf tubes containing 2mL

sterile Tryptic Soy Broth (**TSB**). The inoculated tubes were incubated overnight at 37ºC. After incubation and gentle mixing, 500µL of each bacterial suspension was transferred to a new 2mL Eppendorf tube and placed in a microcentrifuge for 5 min at 7200 *g*. The supernatant was decanted and the pellet was resuspended in 150µL of TE buffer (10mM Tris, pH 7.5, 1mM EDTA, pH 7.5). Each tube received 6µL of a lysozyme/lysostaphin (25mg/ml, 2mg/ml) mixture and was incubated at 37ºC for 30 min. The lysozyme solution was prepared fresh on the day of its intended use and the lysostaphin solution was previously aliquotted and stored at -20ºC until use. Following incubation, 150µL of cooled 1.6% InCert agarose/1.0% sodium dodecyl sulfate was mixed into each bacterial suspension and pipette into appropriately labeled disposable plug molds (2 molds/isolate). The two plugs from each isolate were then transferred into one sterile 2mL Eppendorf tube containing 1mL ES lysis buffer (0.5M EDTA, pH. 9.0, 1.0% sodium-lauroyl-sarcosine). Once the plugs had been transferred, 40µL lysozyme/lysostaphin (25mg/mL, 2mg/mL) mixture was added to each tube and incubated for 1h at 37ºC. Post-incubation, all liquid within each tube was removed and 1mL fresh ES lysis buffer followed by 100µL Proteinase K solution (20mg/mL) was added to each tube. Tubes were incubated for 4 h in a 50ºC water bath. Proteinase K solution had been previously prepared, aliquotted, and stored at -20ºC until use. Following the 4 h incubation, each set of plugs was transferred to individual strainers and washed 4 times with TE buffer for 10 min in a 50ºC water bath. After the washes, each set of plugs was transferred to a new, sterile 2mL Eppendorf tube filled with 1mL fresh TE buffer and stored at 4ºC until further use.

On the day of electrophoresis, restriction digestion was done with fresh enzymatic solution using 40 units of *Sma*I (New England Biolabs, Ipswich, MA), sterile ddH_20, and enzyme buffer. A 1mm slice of the desired isolate plug was transferred into a sterile 1.5mL Eppendorf tube containing 100µL of the enzymatic solution which incubated on the bench top at RT for 4h. After incubation, the plugs were affixed to the gel comb with a drop of 1.0% Seakem Gold agarose (Cambrex, East Rutherford, NJ). A lambda ladder (Promega, Madison, WI) was used as a size marker in the first lane on each gel. The comb was placed in the gel apparatus while more agarose was poured and allowed to harden for 15 min at RT. Once the comb was removed, each well was filled with the remaining agarose. The gel was then covered and transported to the CHEF DR II PFGE (Bio-Rad, Hercules, CA) apparatus in the Virginia Tech Life Sciences Building. PFGE was performed at 4°C with a 0.5X TBE (0.9M Tris base, 0.9M boric acid, 0.02M EDTA, pH 8.0) buffer recirculation. The PFGE run settings were as follows: initial switch time at 5s, final switch time at 35s, run time for 14h, start ratio at 1, and voltage at 180V. Gels were stained with ethidium bromide for 30 min, UV illuminated, and photographed. Each photograph was saved as a JPEG file.

Dendogram Creation and Analysis

The JPEG files of gel pictures were converted into TIFF files using the Microsoft Paint program, version 2008. The files were then uploaded to FPQuest Software (Bio-Rad, Hercules, CA). All photos were analyzed using the Dice coefficient-UPGMA (unweighted pair-group method with arithmetic averages) with 1.0% band tolerance. A dendogram with a 75% cutoff value was created to compare genetic relatedness between all isolates across all gels (Petersson-Wolfe et al., 2008).

RESULTS

Seventy-four of the 80 examined enterococcal isolates produced PFGE fingerprint patterns (Appendix C). There were no clusters that appeared at, or above the similarity cutoff value of 75% (Figure 4.1). Additionally, there was no obvious clustering by the factors of trial period, cow, or time sampled that occurred within the isolates. Similarity ranged from 0 to 70.6%. The two most similar isolates (70.6%) were a rumen sample at 1 h post ruminal inoculation with enterococci and a fecal sample at 2 h post inoculation that were obtained from the same cow (4016), on the same day, during the same trial period (Higher Dose). The least similar isolates (0%) were between a single fecal isolate from cow 4505 sampled at 14 h during Period 1 and a cluster of isolates that was diverse in cow, trial period, and time sampled. Please note that dendogram results are presented with the time of sampling instead of the hours pre/post inoculation and ‘cov’ representing isolates from baseline sampling prior to ruminal inoculation with enterococci.

DISCUSSION

Enterococcal isolates of inoculant, silage, and bovine gastrointestinal tract sources were genetically diverse when analyzed by the widely accepted interpretive method of PFGE. The isolates (n = 74) did not show any obvious clustering within the generated dendogram and there was no single common clone identified. Similarly, there was no significant genetic relatedness between isolates when using a 75% genetic similarity cut-off. The 6 isolates that did not display PFGE patterns may have not had a high enough concentration of DNA within the agarose plugs or may have required a longer incubation with the restriction enzyme. The similarity of the ladder with enterococcal isolates was most likely due to the presence of same-sized bands in enterococcal profiles. PCR-confirmed enterococcal strains from clinical mastitis cases were analyzed by PFGE multiple times, resulting in similar patterns and confirming the current PFGE technique. These results were not expected but also not surprising, based on 2 previous epidemiologic studies analyzing bacterial isolates from cattle environments.

The first study compared enterococcal isolates from a dairy farm environment and numerous mastitis cases (Petersson-Wolfe et al., 2008). These researchers assessed a total of 102 isolates from high somatic cell count cows, postpartum cows, clinical mastitis cases, bedding samples, and feed samples. Dice-UPGMA analysis of the dendogram revealed that isolates from mammary and bedding sources were genetically diverse while isolates from feed sources were similar at a genetic similarity cut-off at 75% (Petersson-Wolfe et al., 2008). These results showed that if enterococcal isolates came from the same dairy farm, there was still much heterogeneity between the samples. However, this was not the case when it came to analyzing the feed samples, which all clustered

together. Thus, the implication from this study is that *Enterococcus* spp. isolated from a single herd and similar environment can produce a heterogeneous population. This observation held true for the current study, where an extremely heterogeneous population was seen after PFGE analysis.

The second study, by Yoshii and colleagues in 2009, evaluated PFGE profile changes in four young Holstein steers that were inoculated by stomach tube with *Escherichia coli* O157:H7. After collecting fecal samples for 40 d, *E. coli* isolates shed from the steers were compared to the original inoculation strain and each other using CHEF PFGE. Researchers found that of the 905 recovered isolates analyzed by PFGE, 289 isolates had a PFGE profile different than that of the original strain (Yoshii et al., 2009). They were also able to visually indentify 12 distinct PFGE profiles within these isolates. The differences between these 12 profiles were only as few as one to three band variations. Researchers then took the investigation a step further, and performed whole genome PCR to confirm that the variation seen in PFGE profiles was due to deletion of 5 chromosomal regions within the bacterial genome (Yoshii et al., 2009). Thus, researchers concluded that various PFGE profiles could be seen in isolates from a single animal due to strain mutation while passing through the gastrointestinal tract.

In the current study, the data agreed with Petersson-Wolfe et al.'s work, resulting in a heterogeneous population of isolates. However, this population differed from Yoshii et al.'s work in that there were no obvious repeated PFGE profiles. There are two proposed reasons why the current data presented itself in such a fashion. The first is the lack of an identification step for the isolates that were to be analyzed by PFGE. Petersson-Wolfe and colleagues used the API 20 STREP test to confirm and identify

enterococcal species. Yoshii and researchers used a known *E. coli* O157:H7 strain with nalidixic acid resistance to be able to properly isolate and identify the desired resulting strains. Biochemical testing paired with the selective media of KEA are not a perfect system, and another technique like the API 20 STREP test or PCR could have led to better genus and species confirmation.

The second reason for such differing PFGE profiles is that the enterococcal population within the cow's environment could be that diverse. As seen in Petersson-Wolfe's work and that of Garcia-Migura and colleagues on poultry and swine farms, enterococcal genetic homogeneity in food animals can be a common result (Garcia-Migura et al., 2005, Petersson-Wolfe et al., 2008). The highest degree of similarly (70.6%) was observed from a set of isolates which came from the same cow on the same day during the same period. Although close to the genetic similarity cut-off (75.0%), this set of isolates would not allow for the conclusion that a single enterococcal strain could pass through the bovine gastrointestinal tract. Also, the fact that isolates had a 0% similarity might have meant that they were not enterococci. Since genomic analysis comparing purposely-inoculated strains of *Enterococcus* spp. to shed strains had never been done before this study, improvements to future methods would be needed. Thus, as Yoshii and colleagues also concluded, how these environmental bacteria change and what causes these genomic changes requires much more investigation.

CONCLUSIONS

This data shows the genetic heterogeneity of enterococcal samples isolated from silage inoculants, silage, and the bovine gastrointestinal tract when analyzed by PFGE. There was no obvious clustering within the generated dendogram and there was no single

genetic clone identified within the patterns. Similarly, there was no significant genetic relatedness seen between any isolates when using a standard 75% similarity cut-off value. Therefore, the inoculants produced a heterogenic population between the factors of dose strain, cow, period, or time sampled. This data did not support the original hypothesis that enterococcal stains from silage inoculated with enterococci-containing microbial inoculants would be able to pass through the gastrointestinal tract of lactating dairy cows. Further studies should focus on more specific microbial and molecular identification techniques and a larger enterococcal isolate pool to analyze.

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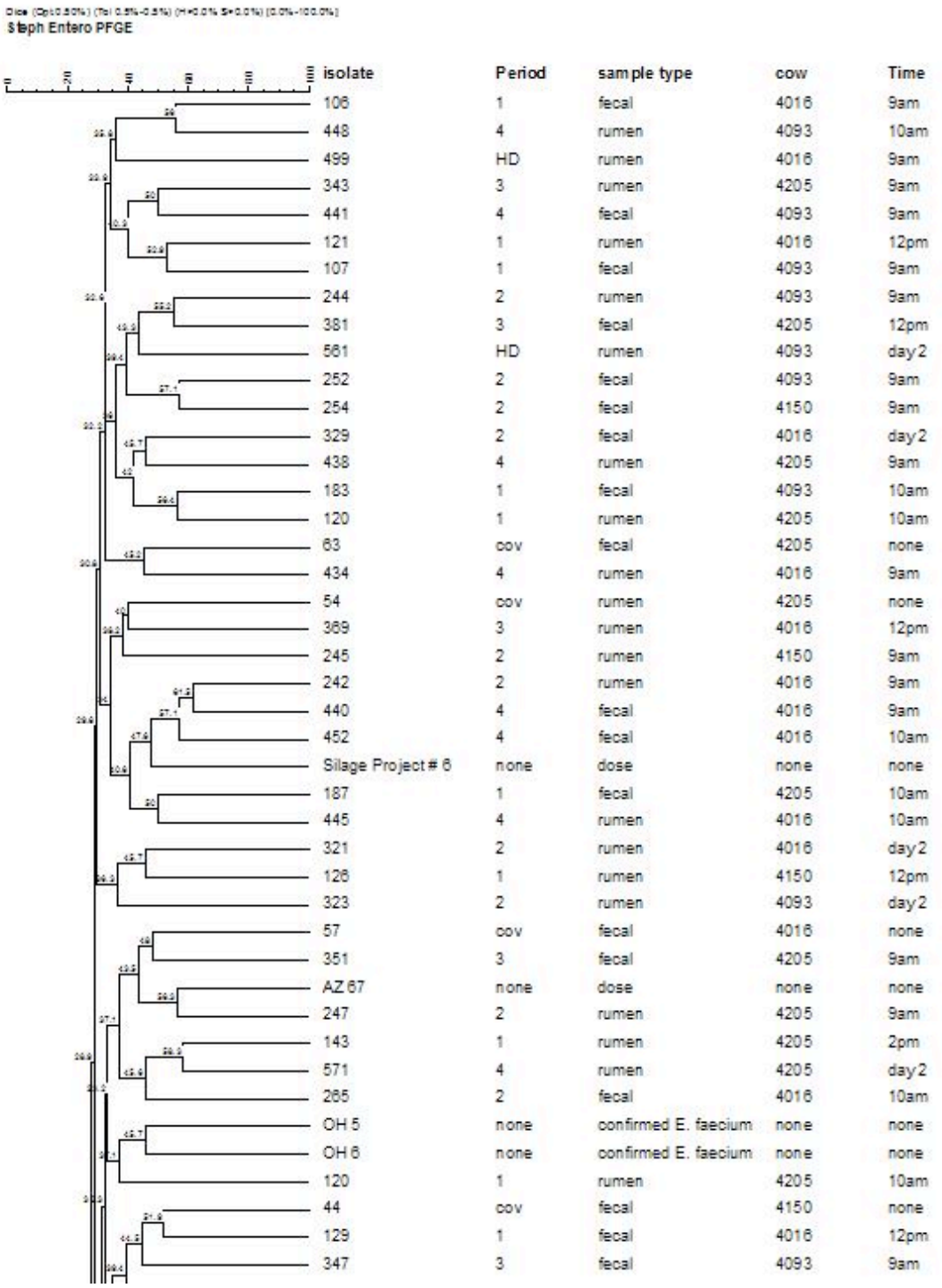
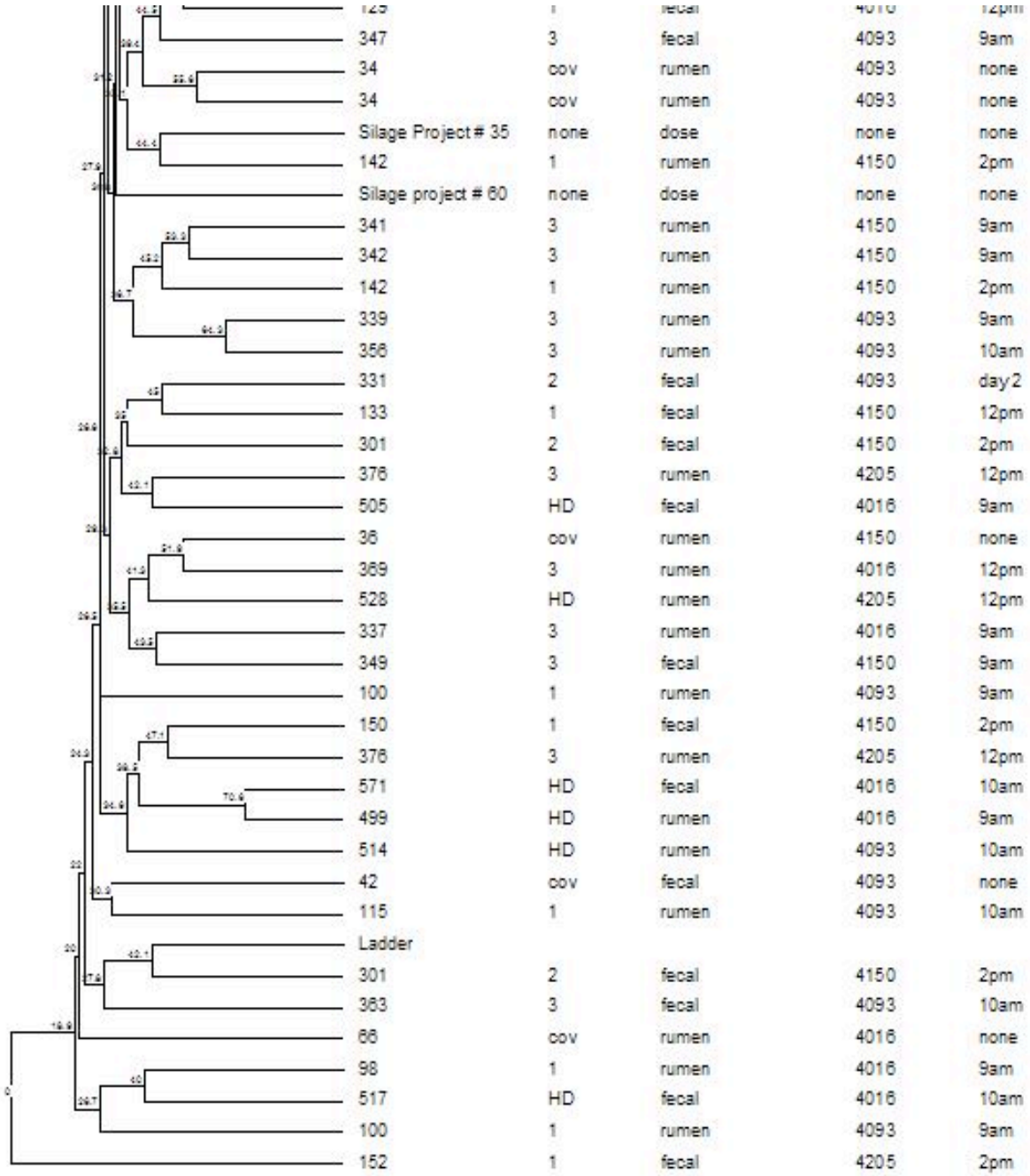


isolate	Period	sample type	cow	Time
106	1	fecal	4016	9am
448	4	rumen	4093	10am
499	HD	rumen	4016	9am
343	3	rumen	4205	9am
441	4	fecal	4093	9am
121	1	rumen	4016	12pm
107	1	fecal	4093	9am
244	2	rumen	4093	9am
381	3	fecal	4205	12pm
561	HD	rumen	4093	day2
252	2	fecal	4093	9am
254	2	fecal	4150	9am
329	2	fecal	4016	day2
438	4	rumen	4205	9am
183	1	fecal	4093	10am
120	1	rumen	4205	10am
63	cov	fecal	4205	none
434	4	rumen	4016	9am
54	cov	rumen	4205	none
369	3	rumen	4016	12pm
245	2	rumen	4150	9am
242	2	rumen	4016	9am
440	4	fecal	4016	9am
452	4	fecal	4016	10am
Silage Project # 6	none	dose	none	none
187	1	fecal	4205	10am
445	4	rumen	4016	10am
321	2	rumen	4016	day2
126	1	rumen	4150	12pm
323	2	rumen	4093	day2
57	cov	fecal	4016	none
351	3	fecal	4205	9am
AZ 67	none	dose	none	none
247	2	rumen	4205	9am
143	1	rumen	4205	2pm
571	4	rumen	4205	day2
265	2	fecal	4016	10am
OH 5	none	confirmed E. faecium	none	none
OH 6	none	confirmed E. faecium	none	none
120	1	rumen	4205	10am
44	cov	fecal	4150	none
129	1	fecal	4016	12pm
347	3	fecal	4093	9am

Continued

Figure 4.1 PFGE dendogram obtained by using pulsed-field gel electrophoresis profiles for 74 strains of *Enterococcus* spp. isolates of silage inoculant, ensiled forage, and bovine origin by the unweighted pair group algorithm with arithmetic averages. Isolate clusters were assessed with a 75% similarity cutoff value.

Figure 4.1 Continued



CHAPTER 5: General Conclusions

Physical consequences of bovine mastitis for dairy cows and associated economic stresses for farmers are ever-present. Not only has research publicized how costly a problem mastitis can be, it has also shown that as management of contagious mastitis pathogens improves, environmental pathogens are becoming more prevalent. One such group of environmental pathogens is the *Enterococcus* genus. Still relatively new in their classification independence from the *Streptococcus* genus, enterococci await to be fully defined in the scientific community. One of the areas in most need of definition is how these bacteria relate to bovine mastitis. Current management protocols for enterococcal mastitis are good milking hygiene, sanitary housing and bedding conditions, and knowledge of susceptible times during lactation periods. If specific on-farm sources of *Enterococcus* spp. could be identified, it would help design more specific control methods and combat resulting intramammary infections.

The main objectives of this study were to determine the survivability and similarity of *Enterococcus* spp. from silage inoculants during the ensiling and through the bovine gastrointestinal tract to indentify a possible on-farm source of enterococci. The production and preservation of quality animal feed is paramount to farm management. Producers use silage inoculants to reduce the risk of protein degradation and loss of dry matter. *Enterococcus* spp. are included in many commercially available silage inoculants due to the ability to produce lactic acid. Enterococci protect feed nutrient value during fermentation by increasing lactic acid production and rapidly decreasing silo pH. Exploring the ability of these enterococci originating from commercial silage inoculants to survive the ensiling process and bovine gastrointestinal tract would allow for

assessment of the risk of enterococcal mastitis. An epidemiologic study of these bacteria is essential in gathering more information about the bacterial genus. Despite current ensiling strains having general approval from the FDA, different bacteria could be proposed if enterococcal inoculants pose a significant risk to animal health.

The first aim of this study was to determine the ability of *Enterococcus* spp. to survive a 3 wk ensiling process. This research was conducted by applying commercially available silage inoculants containing *E. faecium* to both corn and grass forages and ensiling each for three weeks. The second objective of this research was to determine the fecal shedding rate of *Enterococcus* spp. from dairy cows. Isolates indentified and saved as *Enterococcus* spp. were then dosed to fistulated cows in a 4 x 4 Latin square design with fecal and rumen samples taken over the course of 4 mo. A pre-treatment period was done for all trial cows to serve as a baseline to which the inoculated fecal levels were compared. Rumen samples were also examined to observe the translocation of these bacteria through the bovine gut. The third and final objective was to assess strain diversity using pulse field gel electrophoresis. Isolates saved from ensiling, fecal, and rumen samples were evaluated for diversity using pulsed-field gel electrophoresis. This molecular technique was used to look at any genetic differences in strains before and after ensiling and before and after passage through the bovine gastrointestinal tract to achieve an assessment if *Enterococcus* spp. can viably come from silage inoculants and subsequently cause mastitis.

The ensiling study data showed that the addition of a silage inoculant led to higher levels of enterococci in grass silage compared with the negative control at the end of the 3 wk ensiling period. Enterococci levels did not show a marked difference in the inoculated corn silage samples by the end of the ensiling period. Thus, it was concluded that the silage inoculants had a larger impact on the bacterial load of the grass than the corn. Parameter analyses resulted in a desirable drop in pH and increased DM percentage over 3 wk. There were significant effects of treatment and week in both forage types. These data support that enterococci are able to survive the harsh conditions of a 3 wk ensiling period in both grass and corn forages. Future ensiling studies should focus on a larger scale trial and feed the resulting silage to dairy cows to assess digestibility and milk production effects.

The shedding trial data suggested that the bovine fecal shedding rate of enterococci is not increased by ruminal inoculation of enterococcal isolates. Enterococci levels in both rumen and fecal samples from treatment periods did show a marked increase when compared to the levels isolated during the baseline period. However, these results cannot be directly correlated to ruminal inoculation with enterococci. Enterococci observed could have also been due to the herd already being fed TMR ensiled with a silage inoculant, not having a long enough washout period between treatments, or enterococci coming from a different environmental source. The amount of enterococci observed from fecal shedding was not as large as hypothesized. This might have been due to inability of the enterococci to compete with native rumen microflora for resources, being killed by acid produced in the lower gastrointestinal tract, or being ingested by ruminal protozoa. Thus, enterococci were not shed in strikingly larger amounts into the

cow's environment than the observed natural amount. Further studies should consider an increased dose concentration and the use of microbial tags of the dosed bacteria for better translocation records. The PFGE analysis showed the genetic heterogeneity of enterococcal samples isolated from silage inoculants, silage, and the bovine gastrointestinal tract. A generated dendogram depicted no obvious clustering or identified a single clone within investigated patterns. There was no significant genetic relatedness seen between any isolates when using a standard 75% similarity cut-off value. This similarity value was not increased due to lack of similar clusters. The value was not decreased so results from the current study would be comparable with the extensive dendogram literature which uses a 75% similarity cut-off value. It was concluded that the inoculants produced a heterogenic population between the factors of dose strain, cow, period, or time sampled. Future research should focus on more specific microbial and molecular identification techniques, such as fluorescently tagged enterococci, and analyze a larger isolate population.

Based on the results of these three studies, it was realized that the inclusion of *Enterococcus* spp. in silage inoculants and their use on dairy farms do not appear to significantly contribute to an increased risk of enterococcal mastitis. Although there was not a large increase from natural enterococci shedding levels, the fact that these bacteria are present in the farm environment should be of concern to animal, food, and immunology researchers. It has been well documented that *Enterococcus* spp. have a high affinity for sharing virulence traits. Much of the genetic information researched relates to antimicrobial resistance. Future epidemiologic research should aim to gain better antibiotic and antimicrobial resistance profiles for enterococci isolated from dairy farm

environments. This information would allow the scientific community to gain an improved understanding of the conjugative sharing of genetic materials and prepare both the dairy industry and human medical profession with enhanced treatment methods for these rapidly adapting precarious pathogens.

APPENDIX A

Protocol for enumeration of bacteria.

Procedure: Bacterial enumeration.

Use: General __X__ Specific _____, Project Name _____

Personel: Laboratory Technician

Description:

I. Microbiology

A. 5 g of silage is measured in a disposable plastic weigh boat and transferred to a stomacher bag.

B. 45 ml sterile PBS is added to the silage in the stomacher bag.

C. Remove air from bag, put in stomacher. Turn on stomacher for 60 seconds. Be sure to pick out any sharp pieces of contents that could puncture the bag.

D. Silage mixture is diluted in PBS and plated onto the surface of selective media.

E. Dilution of silage mixture.
   a. Dilutions are done in sterile 96-well plate and are serial 10 fold dilutions up to a -5 or 100,000 dilution.
   b. Samples are run in duplicate.
   c. 225 µl of sterile PBS is added to columns 2 through 5 on 96-well plate.
   d. 250 µl of solution from stomacher bag is added to column 1 on 96-well plate.
   e. 25 µl is transferred from wells in column 1 to wells in column 2. Mix well. 25 µl from column 2 are then transferred to column 3 and mixed. Continue on through column 5.
   f. Remove 40 µl from each well and deliver four 10 µl spots onto one-half of an appropriately labeled agar plate.
   g. Agar plates should be labeled with date, cow, sampling period, and dilution. Dilutions are indicated by -1,-2,-3,-4,-5. Petri dishes are labeled on the bottom plate.

F. Incubate petri dishes overnight at 37°C.

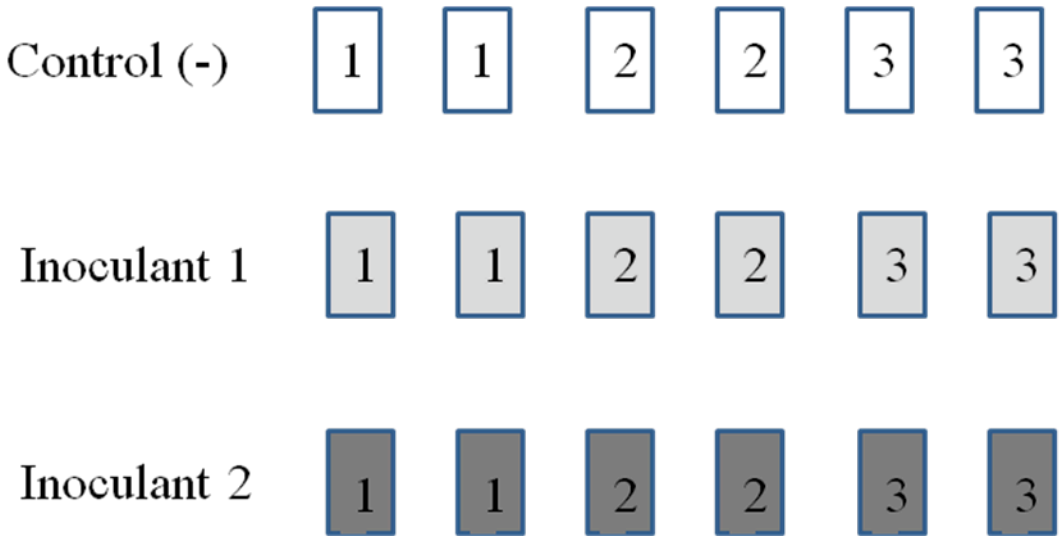
G. Count number of bacterial colonies per 10 µl spot and record number for each spot in notebook. Also record the dilution that was counted.

Table A.1 Bacterial enumeration of gram negative bacteria, lactose positive bacteria, *Klebsiella* spp., *Streptococcus* spp., and *Enterococcus* spp. from trial grass and corn over a 3 wk ensiling period.

Grass						
Week	**Treatment**	**Gram Negative** (log10cfu/g DM)	**Lactose Positive** (log10cfu/g DM)	**Klebsiella spp.** (logc10fu/g DM)	**Streptococcus spp.** (log10cfu/g DM)	**Enterococcus spp.** (log10cfu/g DM)
0	**none**	7.3	ND	7.3	5.1	6.1
1	**Neg C**	5.4	5.2	3.7	8.1	8.0
1	**I 1**	ND	ND	ND	6.7	6.8
1	**I 2**	5.1	5.0	3.0	7.9	7.7
2	**Neg C**	ND	ND	ND	6.6	7.2
2	**I 1**	ND	ND	ND	6.3	5.6
2	**I 2**	4.9	4.9	3.2	6.4	6.9
3	**Neg C**	ND	ND	ND	4.3	1.2
3	**I 1**	ND	ND	ND	7.6	4.2
3	**I 2**	ND	ND	ND	7.0	7.3
Corn						
Week	**Treatment**	**Gram Negative** (log10cfu/g DM)	**Lactose Positive** (log10cfu/g DM)	**Klebsiella spp.** (log10cfu/g DM)	**Streptococcus spp.** (log10cfu/g DM)	**Enterococcus spp.** (logc10fu/g DM)
0	**none**	8.3	7.9	8.2	5.5	6.9
1	**Neg C**	ND	ND	ND	4.7	4.0
1	**I 1**	ND	ND	ND	5.2	3.9
1	**I 2**	ND	ND	ND	4.0	2.8
2	**Neg C**	ND	ND	ND	4.2	3.9
2	**I 1**	ND	ND	ND	4.7	3.8
2	**I 2**	ND	ND	ND	4.1	3.9
3	**Neg C**	ND	ND	ND	4.1	4.0
3	**I 1**	ND	ND	ND	4.2	4.3
3	**I 2**	ND	ND	ND	4.1	4.4

Neg C (Negative control of non-inoculated forage)
I1 (Commercially available silage inoculant 1)
I2 (Commercially available silage inoculant 2)
* ND (level below detection limit (< 10000 cfu/g DM))

Figure A.1 Study design for grass and corn silage within three treatment groups over a 3 wk ensiling period.



* 1 = Bag opened after one week of ensiling
  2 = Bag opened after two weeks of ensiling
  3 = Bag opened after three weeks of ensiling

Figure A.2 Calculations of inoculant amounts based on volume of grass and corn forages within ensiling bags.

Grass

Inoculant 2: (1 g / 0.0022046 lbs) x (2 lbs / 1 ton) = (907194.05 g / 1 ton)
(0.5 g / 907194.05 g) x 2700 g = 0.0014881 g inoculant

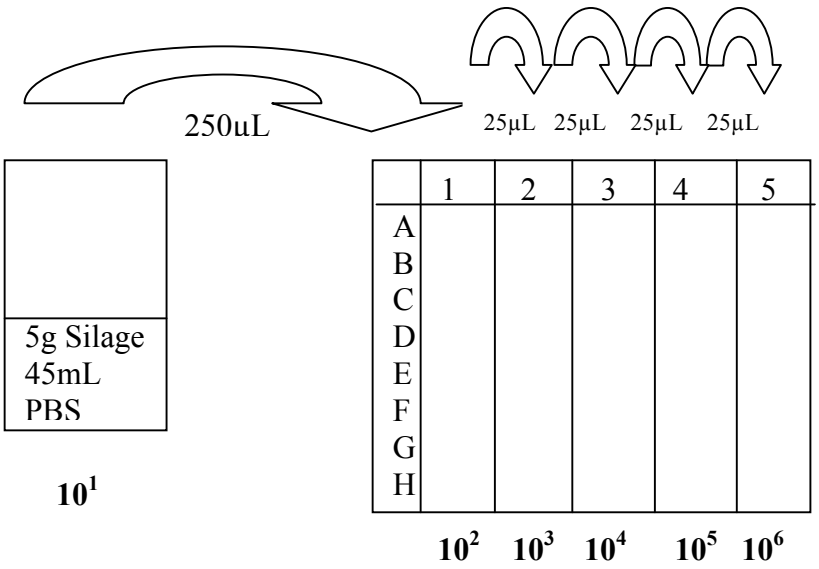
Inoculant 1: (0.005 ton / 1 lb) x (1 gallon / 1 ton) x 5.947 lb = 0.0029735 gallons
0.0029735 gallons x (9.09 g / 1 gallon) = 0.0270291 g inoculant

Corn

Inoculant 2: (0.5 g / 907194.05 g) x 4095 g = 0.002257 g inoculant

Inoculant 1: (907194.05 g / 1 ton) x (0.0005 ton / 1 lb) = 453.59703 g / 1 lb
(453.59703 g / 1 lb) x 4095 g = 9.028 lbs
0.004514 gallons x (9.09 g / 1 gallon) = 0.0410323 g inoculant

Figure A.3 Dilution scheme of silage samples to 10^6 dilutions in phosphate buffered saline (PBS) within a 96-well plate.



APPENDIX B

Rumen sampling protocol.

Procedure: Rumen contents sampling and bacterial enumeration.

Use: General __X__ Specific _____, Project Name _____

Personel: Laboratory Technician

Description:

I. Rumen Sampling

A. Cows will be caught in headlocks.
B. Take designated thermos and fill with warm water before taking out to barn. Dump out warm water out when ready to sample.
C. Wear artificial insemination (AI) gloves while collecting rumen samples.
D. Calmly approach the cow and push in the cannula plug, remove it, and place in a bucket of warm water out of the way. Be sure to remember which cannula goes to which cow.
E. Gently enter through the cannula and grab a handful of rumen contents.
F. Place contents into designated thermos.
G. Repeat until the 1 liter thermos is full.
H. Once done with sampling, gently place cannula plug back into place and dispose of trash in appropriate bins.
I. Change gloves and designated thermoses between cows.
J. Mark down on sampling sheet the time the sample was taken and any observations or remarks about the cows.

II. Microbiology

A. 10 ml of rumen contents (RC) is measured in a disposable plastic weigh boat and transferred to a stomacher bag.
B. 90 ml sterile PBS is added to the RC in the stomacher bag.
C. Remove air from bag, put in stomacher. Turn on stomacher for 60 seconds. Be sure to pick out any sharp pieces of contents that could puncture the bag.
D. RC is diluted in PBS and plated onto the surface of selective media.
E. Dilution of RC.
    a. Dilutions are done in sterile 96-well plate and are serial 10 fold dilutions up to a -5 or 100,000 dilution.
    b. RC samples are run in duplicate.
    c. 225 µl of sterile PBS is added to columns 2 through 5 on 96-well plate.
    d. 250 µl of RC solution from stomacher bag is added to column 1 on 96-well plate.

e. 25 µl is transferred from wells in column 1 to wells in column 2. Mix well. 25 µl from column 2 are then transferred to column 3 and mixed. Continue on through column 5.
f. Remove 40 µl from each well and deliver four 10 µl spots onto one-half of an appropriately labeled agar plate.
g. Agar plates should be labeled with date, cow, sampling period, and dilution. Dilutions are indicated by -1,-2,-3,-4,-5. Petri dishes are labeled on the bottom plate.

F. Incubate petri dishes overnight at 37°C.
G. Count number of bacterial colonies per 10 µl spot and record number for each spot in notebook. Also record the dilution that was counted.

Fecal sampling protocol.

Procedure: Fecal grab sampling and enumeration of bacteria.

Use: General __X___ Specific ____, Project Name ___

Personel: Laboratory Technician

Description:

I. Fecal Grab Sampling

A. Cows will be caught in headlocks to ensure minimal movement of the animal.
B. Remember to release any cows in headlocks that are not in the study.
C. Wear artificial insemination (AI) gloves while collecting fecal samples.
D. Squeeze a generous amount of lubricant on AI glove.
E. Rub a small amount of the lubricant around the hand of the glove and begin to gently insert the glove into the rectum of the cow while holding the tail up and to the side.
F. While gently inserting the hand into the rectum, scoop feces into the hand and draw feces out of the rectum gently. Scoop enough to fill 2 containers per cow.
G. Place fecal sample in the properly labeled container and place in holding bin.
H. Ensure that gloves are changed between cows.
I. Once the sample has been taken, please dispose of any trash (i.e. paper towels, gloves) in the appropriate bins.
J. Mark down on sampling sheet the time the sample was taken and any observations or remarks about the cows.

II. Microbiology

A. 10 g of feces is weighed in disposable plastic weigh boat and transferred to stomacher bag.
B. 90 ml sterile PBS is added to feces in stomacher bag.
C. Remove air from bag, put in stomacher. Turn on stomacher for 60 seconds.
D. Feces is diluted in PBS and plated onto the surface of selective media.
E. Dilution of feces.
    a. Dilutions are done in sterile 96-well plate and are serial 10 fold dilutions up to a -5 or 100,000 dilution.
    b. Fecal samples are run in duplicate.
    c. 225 µl of sterile PBS is added to columns 2 through 5 on 96-well plate.
    d. 250 µl of feces solution from stomacher bag is added to column 1 on 96-well plate.
    e. 25 µl is transferred from wells in column 1 to wells in column 2. Mix well. 25 µl from column 2 are then transferred to column 3 and mixed. Continue on through column 5.
    f. Remove 40 µl from each well and deliver four 10 µl spots onto one-half of an appropriately labeled agar plate.

g. Agar plates should be labeled with date, cow, sampling period, and dilution. Dilutions are indicated by -1,-2,-3,-4,-5. Petri dishes are labeled on the bottom plate.

F. Incubate petri dishes overnight at 37°C.

G. Count number of bacterial colonies per 10 µl spot and record number for each spot in notebook. Also record the dilution that was counted.

Table B.1 Average rumen and fecal gram negative bacteria, *Klebsiella* spp., and *Streptococcus* spp. levels for all 4 trial periods over 1 to 168 h from ruminal inoculation with 1 x 10^8 cfu enterococci/mL PBS. Treatments are collapsed within each period due to no significant differences within periods. Values shown are $\log_{10}$cfu. (ND, not detectable = cfu < 1000 cfu)

Rumen									
Period	**Bacteria**	**1**	**2**	**4**	**6**	**24**	**48**	**72**	**168**
1	Gram negative bacteria	2.7	4.0	3.1	2.4	0.6	2.5	1.0	0.7
	Klebsiella spp.	0.4	1.9	2.1	ND	ND	1.9	0.3	0.3
	Streptococcus spp.	3.3	3.3	3.3	3.4	3.9	3.7	4.0	4.1
2	Gram negative bacteria	2.0	2.4	1.6	0.4	0.4	0.4	ND	0.3
	Klebsiella spp.	0.3	1.5	ND	ND	ND	ND	ND	ND
	Streptococcus spp.	3.5	3.4	3.6	3.5	4.1	3.9	4.0	4.1
3	Gram negative bacteria	3.7	4.2	3.0	4.3	3.5	3.4	3.3	3.2
	Klebsiella spp.	3.1	2.2	3.7	3.3	3.3	2.6	2.2	0.3
	Streptococcus spp.	3.7	3.2	3.4	3.7	4.5	4.2	4.8	4.7
4	Gram negative bacteria	2.7	4.3	4.6	4.3	3.7	1.5	3.3	ND
	Klebsiella spp.	1.7	3.6	3.9	4.2	3.1	1.3	2.5	0.7
	Streptococcus spp.	3.7	3.7	4.2	4.0	4.5	3.2	4.3	3.5
Fecal									
Period	**Bacteria**	**1**	**2**	**4**	**6**	**24**	**48**	**72**	**168**
1	Gram negative bacteria	4.5	3.9	4.4	3.7	4.0	4.7	4.9	2.7
	Klebsiella spp.	2.4	0.5	1.4	1.8	0.9	1.1	4.4	2.8
	Streptococcus spp.	4.5	5.0	5.0	3.7	4.0	4.7	5.1	5.3
2	Gram negative bacteria	4.6	3.5	3.1	1.8	2.6	5.4	3.8	4.5
	Klebsiella spp.	3.7	4.1	3.7	3.7	3.4	2.6	2.4	3.2
	Streptococcus spp.	5.6	5.3	5.4	5.8	5.5	5.7	5.4	5.8
3	Gram negative bacteria	5.4	4.3	4.1	4.5	4.9	5.1	4.6	4.8
	Klebsiella spp.	2.6	2.2	1.9	1.7	1.8	2.7	1.7	0.5
	Streptococcus spp.	5.9	5.4	4.8	4.6	4.7	5.5	5.8	6.2
4	Gram negative bacteria	5.0	5.2	5.2	5.2	5.8	4.5	4.0	5.0
	Klebsiella spp.	2.5	2.6	1.0	1.6	2.7	0.7	2.5	0.3
	Streptococcus spp.	5.3	6.4	5.6	5.5	6.4	5.0	5.6	5.1

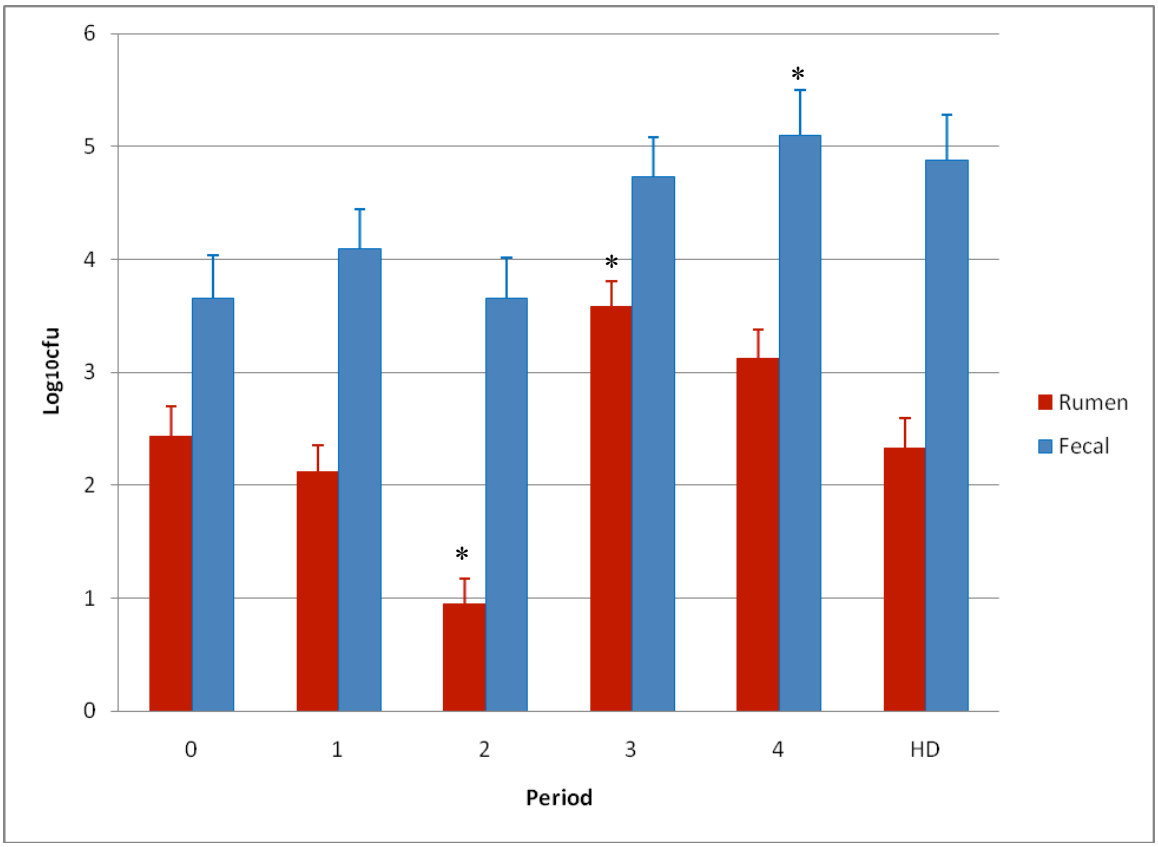


Figure B.1 Average rumen and fecal gram negative bacteria levels over 0 to 168 h during the baseline period, 4 treatment periods (ruminal inoculation with 1 x 10^8 cfu/mL enterococci), and the higher dose period (ruminal inoculation with 1 x 10^{11} cfu/mL enterococci). Treatments are collapsed within each period due to no significant differences within periods. Sampling times are collapsed within periods to display period effect. Values shown are mean ± SE.

0 (Baseline Period, no inoculation)
1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)
HD (Higher Dose Period)

* = Statistical significance ($P \leq 0.05$) between period indicated and Period 0 (baseline period).

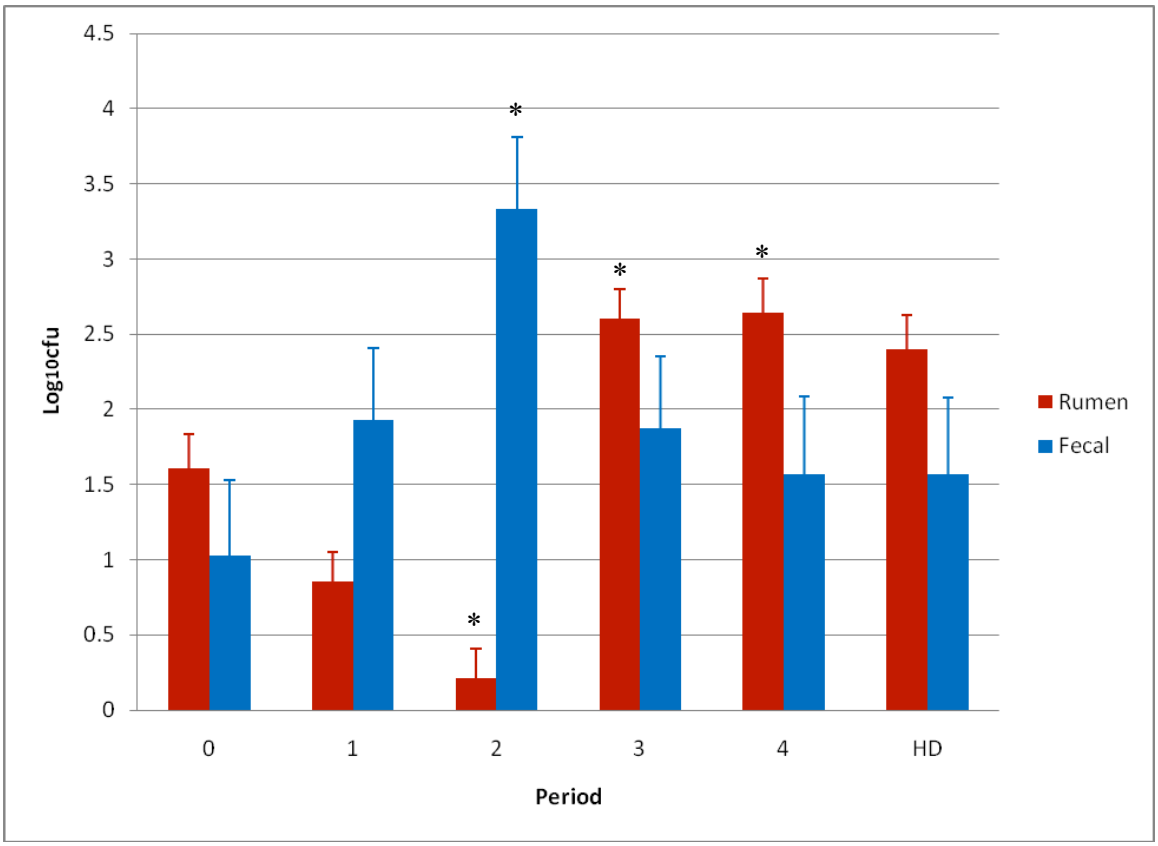


Figure B.2 Average rumen and fecal *Klebsiella* spp. levels over 0 to 168 h during the baseline period, 4 treatment periods (ruminal inoculation with 1 x 10^8 cfu/mL enterococci), and the higher dose period (ruminal inoculation with 1 x 10^{11} cfu/mL enterococci). Treatments are collapsed within each period due to no significant differences within periods. Sampling times are collapsed within periods to display period effect. Values shown are mean ± SE.

0 (Baseline Period, no inoculation)
1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)
HD (Higher Dose Period)

* = Statistical significance ($P \leq 0.05$) between period indicated and Period 0 (baseline period).

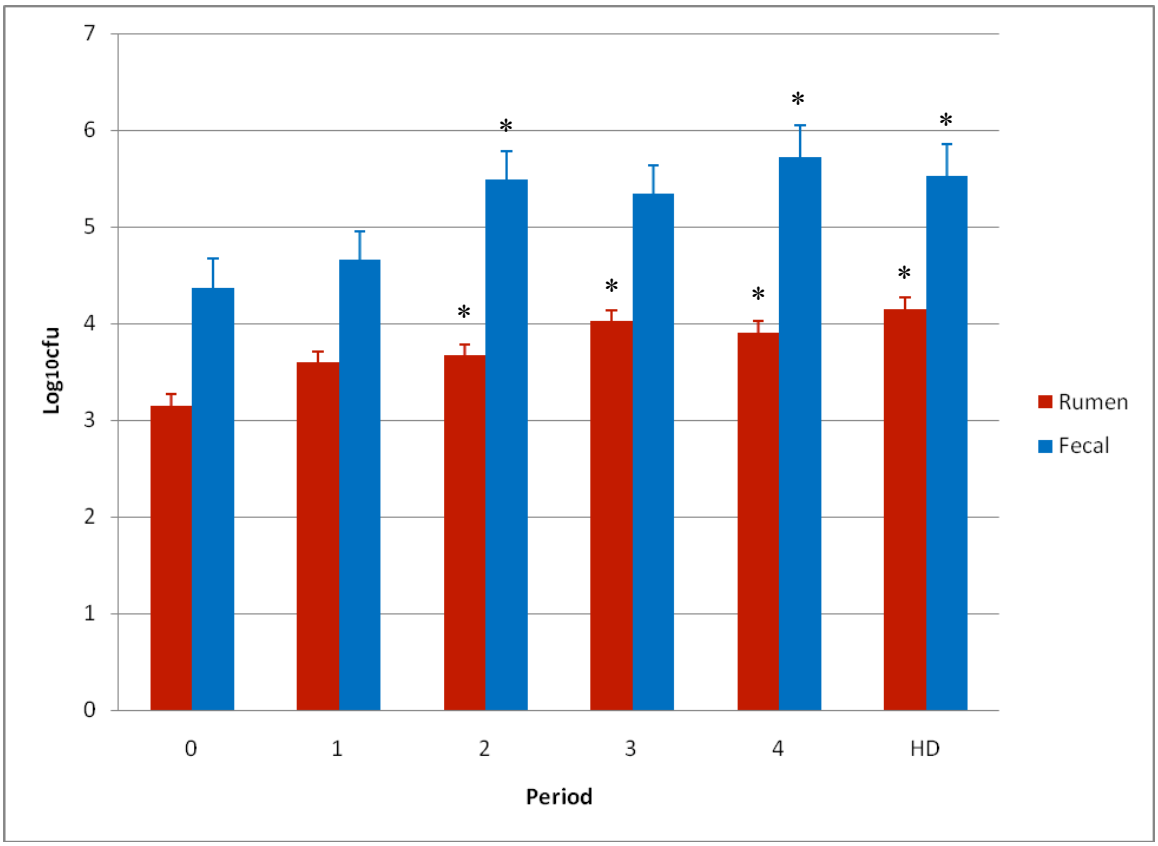
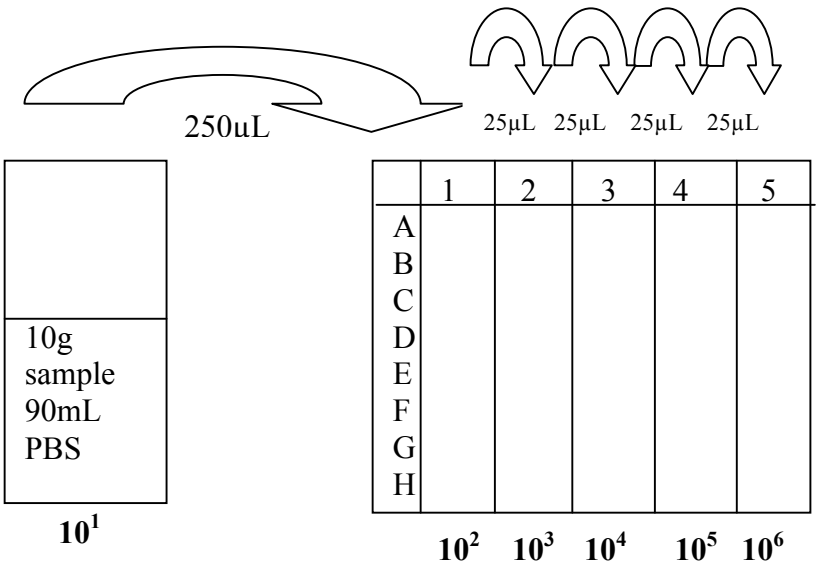


Figure B.3 Average rumen and fecal *Streptococcus* spp. levels over 0 to 168 h during the baseline period, 4 treatment periods (ruminal inoculation with 1 x 10^8 cfu/mL enterococci), and the higher dose period (ruminal inoculation with 1 x 10^{11} cfu/mL enterococci). Treatments are collapsed within each period due to no significant differences within periods. Sampling times are collapsed within periods to display period effect. Values shown are mean ± SE.

0 (Baseline Period, no inoculation)
1 (Period 1)
2 (Period 2)
3 (Period 3)
4 (Period 4)
HD (Higher Dose Period)

* = Statistical significance ($P \leq 0.05$) between period indicated and Period 0 (baseline period).

Figure B.4 Dilution scheme of rumen and fecal samples to 10^6 dilutions in phosphate buffered saline (PBS) within a 96-well plate.



APPENDIX C

Pulsed-Field Gel Electrophoresis protocol.

Procedure: Pulsed-field gel electrophoresis for *Enterococcus* spp.

Use: General __X__ Specific _____, Project Name _____

Personel: Laboratory Technician

Description:

Day 1:

1. Streak bacterial samples for isolation on blood plates and incubate (37° C, 18 h)

Day 2:

1. Fill 2 mL Eppendorf tubes with sterile tryptic soy broth (TSB)
2. Using sterile loop transfer one bacterial colony to labeled tube containing TSB and incubate (37° C, 18 h)

Day 3:

1. Remove tubes from incubator
2. Mix contents of tube using 1 mL pipette
3. Transfer 500 µL of bacterial suspension to a new, sterile, labeled 2 mL Eppendorf tube
4. Spin 500 µL of bacterial suspension in microfuge (7142 g, 5 m)
    a. 7142 g corresponds to setting of '5' on Marathon Micro A microcentrifuge
5. Pour off supernate
6. Resuspend bacterial pellet in 150 µL TE buffer and mix well with pipette
7. Add 6 µL of lysozyme/lysostaphin (25 mg/mL, 2 mg/mL) mixture to each tube, gently tap to mix and incubate (37° C, 30 m)
    a. Make lysozyme solution fresh daily and combine with lysostaphin solution (in 1:1 ratio prior to use)
    b. Lysostaphin can be thawed from storage in -20° C freezer
    c. Store on ice until use
8. Melt InCert agarose for plug making in microwave and cool in 50° C water bath
    a. See 'Stock Solutions' for directions regarding making of InCert agarose
9. Label plug molds (2 per isolate)
    a. Separation of plug molds can be done by placing gap between molds on edge of counter and using heel of hand to apply pressure to molds
10. Label sterile 2 mL tubes (1 per isolate) and fill with 1 mL lysis (ES) buffer
11. Following incubation with lysozyme/lysostaphin, add 150 µL cooled InCert agarose to bacterial suspension, mix well with pipette and fill each of two plug molds with molten suspension
12. Allow plugs to harden on ice (5 m)

13. Transfer 2 plugs per isolate into labled tube containing lysis buffer
14. Add 40 µL lysozyme/lysostaphin (25 mg/mL, 2 mg/mL) mixture to each tube, gently tap to mix and incubate (37° C, 1 h)
15. Following incubation, pipette off liquid from each tube into beaker containing supernatant from step 5
    a. Autoclave and discard liquid
16. Add 1 mL fresh lysis buffer to each tube
17. Add 100 µL Proteinase K solution (20 mg/mL), gently tap to mix and incubate (50° C water bath, 4 h)
    a. Proteinase K can be thawed from storage in -20° C freezer
    b. Store on ice until use
18. Transfer each set of plugs to 1 BioRad strainer and cap with 50 mL conical tube filled with TE buffer
19. Wash plugs gently by inverting stack of strainers several times and incubate with TE buffer (50° C water bath, 10 m).
20. Pour out TE buffer and fill with fresh TE buffer
21. Repeat steps 19 and 20 for a total of 4 times
22. Label and fill new, sterile, Eppendorf tubes with 1 mL of fresh TE buffer
23. Transfer each set of plugs to labeled tubes and store (4° C) until digestion
24. To conduct digestion on same day, allow plugs to harden on ice (10 m) and continue with step 1 on Day 4.

Day 4

1. Make enzymatic solution following guidelines provided in Table 1
    a. Make enough solution for total number of plugs plus 1 (i.e. if running 14 isolates, make enough solution for 15)
2. Label sterile 1.5 mL Eppendorf tubes and fill with 100 µL of enzymatic solution
3. Transfer 1 plug from tube of TE buffer onto clean surface of ruler and cut 1 mm section of plug using clean, sharp razor blade
    a. Use alcohol pads to clean ruler, blade and transfer tools
4. Transfer plug slice to labeled digestion tube and return remaining portion of plug to TE buffer
5. Store remainder of plugs (4° C) until further use
6. Allow digestion to occur (RT, 4 h)
7. Mix 0.5X TBE buffer and save 100 mL for agarose
    a. See ‘Stock Solutions’ for directions on making 0.5X TBE buffer
8. Melt SeaKem gold agarose (1 g) in 100 mL 0.5X TBE buffer in microwave and allow to cool in 50° C water bath
9. Transfer each plug slice to gel comb along with a 1 mm slice of Lambda ladder
10. Seal each plug to comb using 1 drop of cooled, molten agarose
11. Place comb in gel apparatus and pour agarose leaving 2 mL to seal wells
12. Return remaining agarose to water bath and allow gel to harden (RT, 15 m)
13. Once hardened, remove comb slowly and fill each well with remaining agarose
14. Remove gel from apparatus and carefully place in electrophoresis machine
15. Pour remaining 0.5X TBE buffer in electrophoresis machine
16. Turn on machine and pump

17. Start run
18. Allow run to go overnight
    a. Parameters:
        i. Initial switch time 5.00 s
        ii. Final switch time 35.00 s
        iii. Run time 14 h
        iv. Start ratio 1
        v. Voltage 180 V

Day 5

1. Fill 9x9" glass dish with 200 mL sterile water
2. Remove gel from electrophoresis machine and place gel in sterile water
3. Add 20 µL of 10 mg/mL ethidium bromide to water and allow to rock (30 m) in dark
4. Dry pump lines and drain electrophoresis machine using drain tube port
5. Following incubation, rinse gel using dH_2O and place in Gel Doc. Press 'Select scanner' then 'Gel Doc XR' in the next window
6. Using 'Live Focus' in 'Quanity One' software center gel on screen and then close door to Gel Doc
7. Press 'UV Trans' on Gel Doc and click 'Manual Acquire' on computer screen to obtain image. Increase/decrease exposure time to optimize the picture (approx 0.3-0.4 s). Ideally the background is as dark as possible and the bands are as light as possible.
8. Following image acquisition click 'Annotate' to label lanes by clicking text box button ('ABC') and put cursor where text box is desired and enter information (isolates in each lane, date) and save image ('File' – 'Save as').
9. Image will be saved in Quanity One format, to save as a .jpg file click 'file' then 'Export to JPEG'. Then click 'export' and finally save the image.

Stock solutions for PFGE.

0.5M EDTA pH 7.5, and pH 8.0
Preparation:
186.1 g $Na_2EDTA\text{-}2\ H_2O$
Dissolve in 800 mL dH_2O
Adjust pH to 7.5 or 8.0 with 10 N NaOH
Dilute to 1000 mL with dH_2O
Autoclave (121° C, 15 m), store at RT

1M Tris HCl, pH 7.5
Preparation:
121.1 g Trizma base
Dissolve in 650 mL dH_2O
Adjust pH to 7.5 with HCl (uses a lot of HCl)
Dilute to 1000 mL with dH_2O
Autoclave (121° C, 15 m), store at RT

20% SDS
Preparation:
20 g SDS
Dilute in 100 mL dH_2O
Store at RT

10X TBE (Tris-Borate EDTA)
(0.9M Tris base, 0.9M boric acid, 0.02M EDTA, pH 8.0)
Preparation:
108 g Trizma base
55 g boric acid
40 mL 0.5M EDTA, pH 8.0 (from above)
Dilute to 1000 mL with dH_2O
Re-check pH
Autoclave (121° C, 15 m), store at RT
Discard if precipitate forms

10N NaOH
Preparation:
200 g NaOH
Dissolve in 400 mL dH_2O and warm
Cool to RT
Dilute to 500 mL with dH_2O
Store at RT

Plug Wash TE (Tris-EDTA) buffer
(10mM Tris pH 7.5 and 1mM EDTA, pH 7.5)
Preparation:

10 mL 1M Tris, pH 7.5
2 mL 0.5M EDTA, pH 7.5
Dilute to 1000 mL with dH_2O
Autoclave (121° C, 15 m), store at RT

1.6% InCert/SDS agarose mix
(1.6% InCert, 1.0% SDS)
Preparation:

0.16 g InCert agarose
Add to 10 mL sterile dH_2O
Melt agarose in microwave (avoid boil over)
Add 50 μL 20% SDS and mix well by inverting by hand
Cool to 50° C in water bath
For future use melt in microwave and cool in water bath, store at RT

Lysis ES buffer
(0.5M EDTA, pH 9.0; 1% sodium-lauroyl-sarcosine)
Preparation:

93.5 g EDTA
Add to 350 mL warmed dH_2O
While stirring, add NaOH pellets until pH is 8.5, adjust to 9.0 with 10N NaOH
Cool to RT and re-check and adjust pH if necessary
Add 5 g sodium-lauroyl-sarcosine
Adjust volume to 500 mL
Autoclave (121° C, 15 m), store at RT

0.5X TBE (Tris borate EDTA)
Preparation:

100 mL 10X TBE
1900 mL sterile dH_2O

1% SeaKem Gold Agarose
Preparation:

1 g SeaKem Gold Agarose
Dissolve in 100 mL 0.5X TBE
Heat in microwave until fully dissolved, avoid boil over
Cool in 50° C water bath prior to use

Lysozyme (25 mg/mL)
Preparation (immediately prior to use):
0.0125 g lysozyme
Add to 0.5 mL sterile dH_2O
Gently tap to mix
Store on ice until use

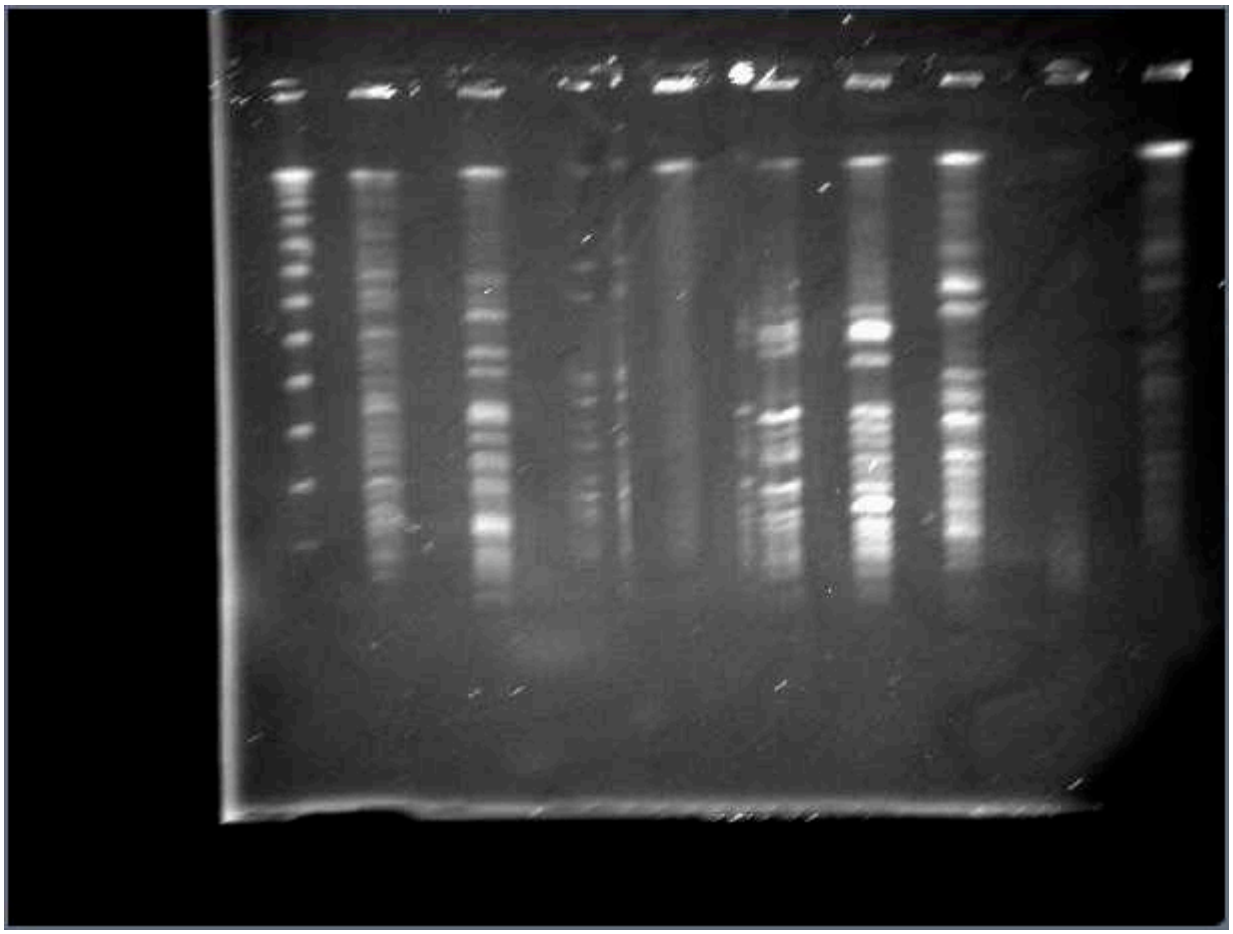
Lysostaphin (2 mg/mL)
Preparation:
14 mg lysostaphin
Add to 7 mL sterile dH_2O
Gently tap to mix
Aliquot 0.5 mL into 2 mL Nalgene tubes
Store at -20° C for up to 3 months until use

Proteinase K (20 mg/mL)
Preparation:
500 mg Proteinase K
Dilute to 25 mL with sterile dH_2O
Aliquot 1.5 mL per 2 mL microcentrifuge tube and store at -20° C

Table C.1 Quantities for enzymatic digestion using *Sma*I (20,000U/mL) for PFGE.

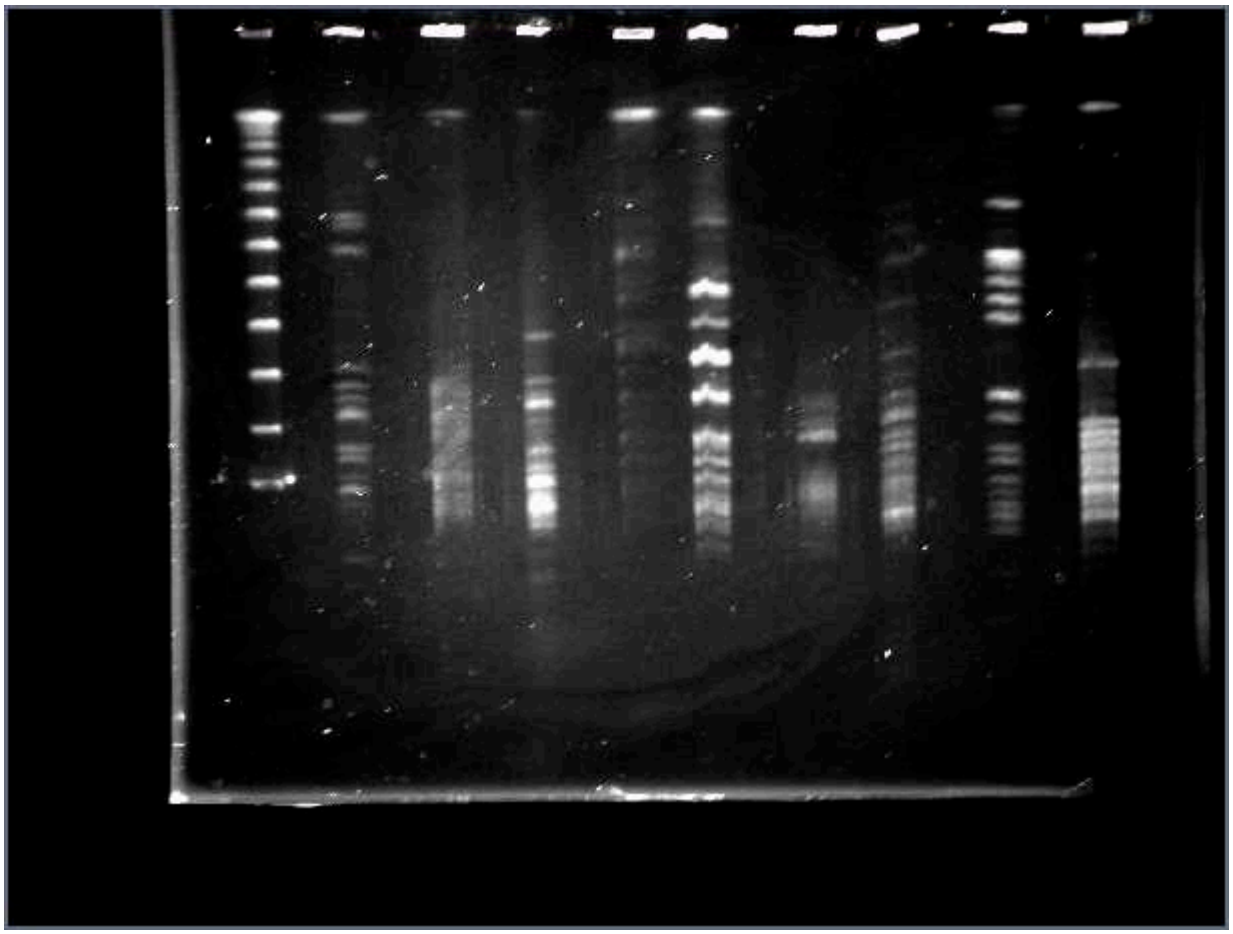
# of plug slices	dH_2O (µL)	10X buffer (µL)	Enzyme (µL)	Total volume (µL)
1	88	10	2	100
2	176	20	4	200
3	264	30	6	300
4	352	40	8	400
5	440	50	10	500
6	528	60	12	600
7	616	70	14	700
8	704	80	16	800
9	792	90	18	900
10	880	100	20	1000
11	968	110	22	1100
12	1056	120	24	1200
13	1144	130	26	1300
14	1232	140	28	1400
15	1320	150	30	1500
16	1408	160	32	1600

Figure C.1 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



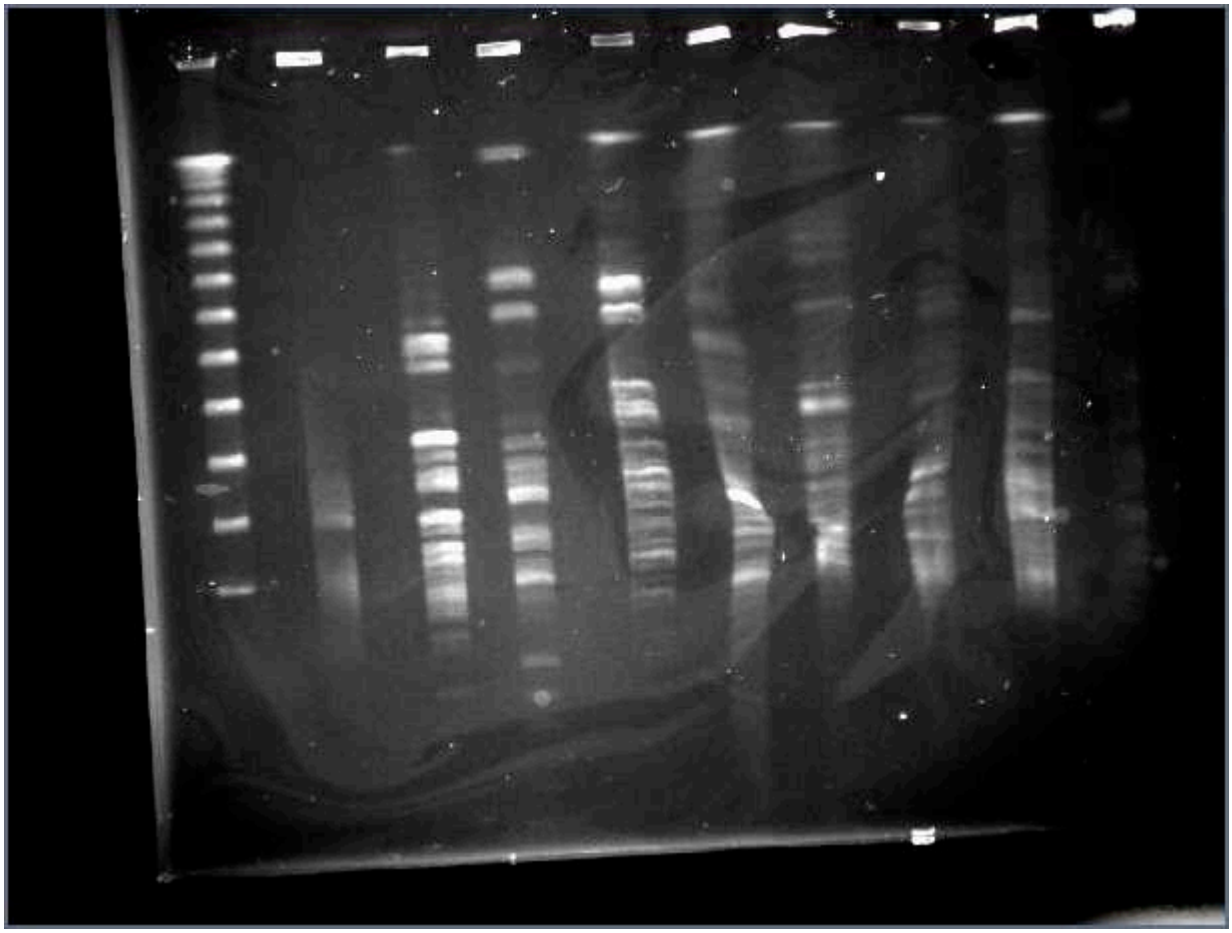
Lane	Isolate #	Origin
1	Ladder	
2	OH 5	Confirmed *E. faecium*
3	OH 6	Confirmed *E. faecium*
4	Silage Project # 35	Used for dose
5	Silage Project 3 6	Used for dose
6	AZ 67	Used for dose
7	57	Covariate 4016 fecal
8	42	Covariate 4093 fecal
9	Silage Project # 60	Used for dose
10	66	Covariate 4016 rumen

Figure C.2 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



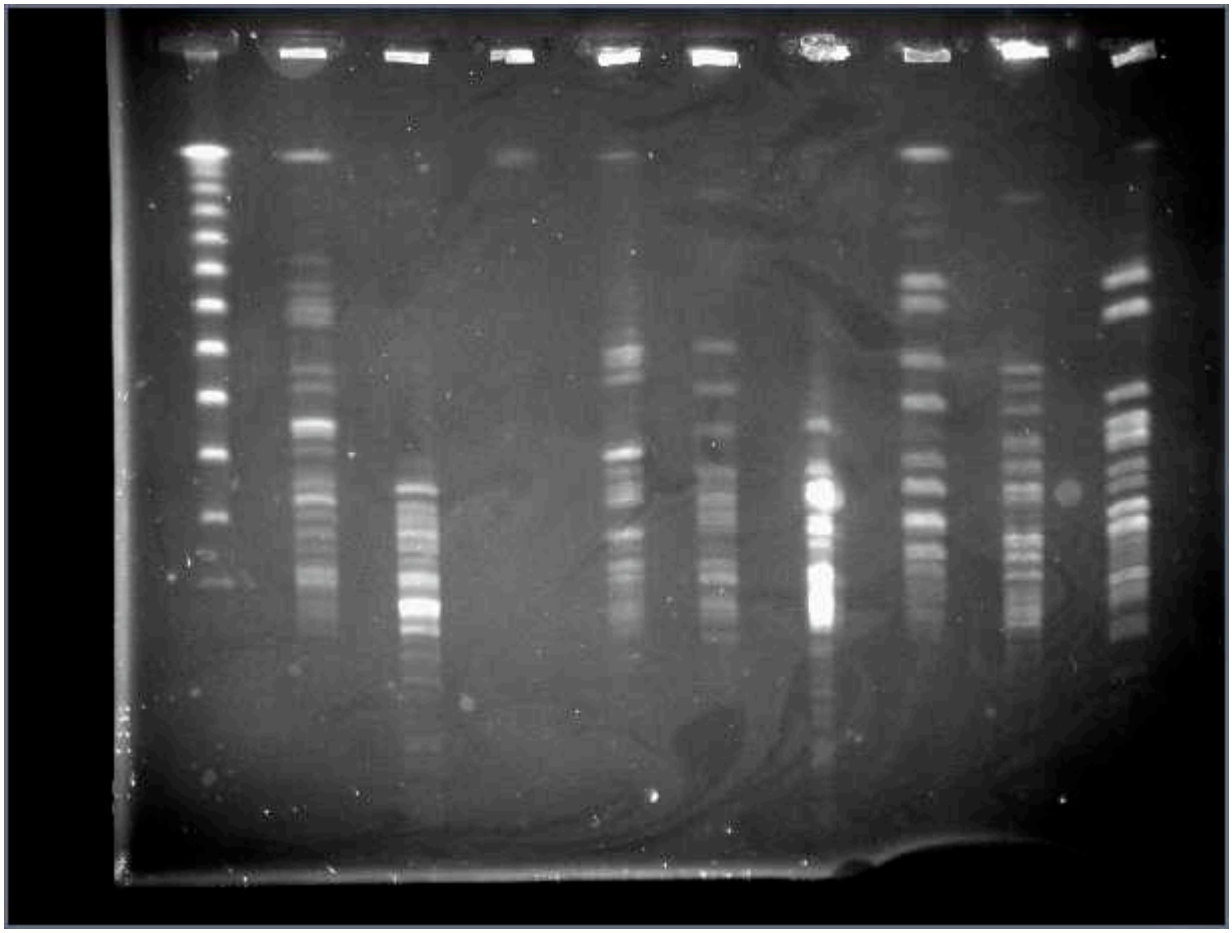
Lane	Isolate #	Origin
1	Ladder	
2	34	Covariate 4093 rumen
3	36	Covariate 4150 rumen
4	44	Covariate 4150 fecal
5	54	Covariate 4205 rumen
6	63	Covariate 4205 fecal
7	98	P1 4016 rumen 9am
8	106	P1 4016 fecal 9am
9	121	P1 4016 rumen 12pm
10	129	P1 4016 fecal 12pm

Figure C.3 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



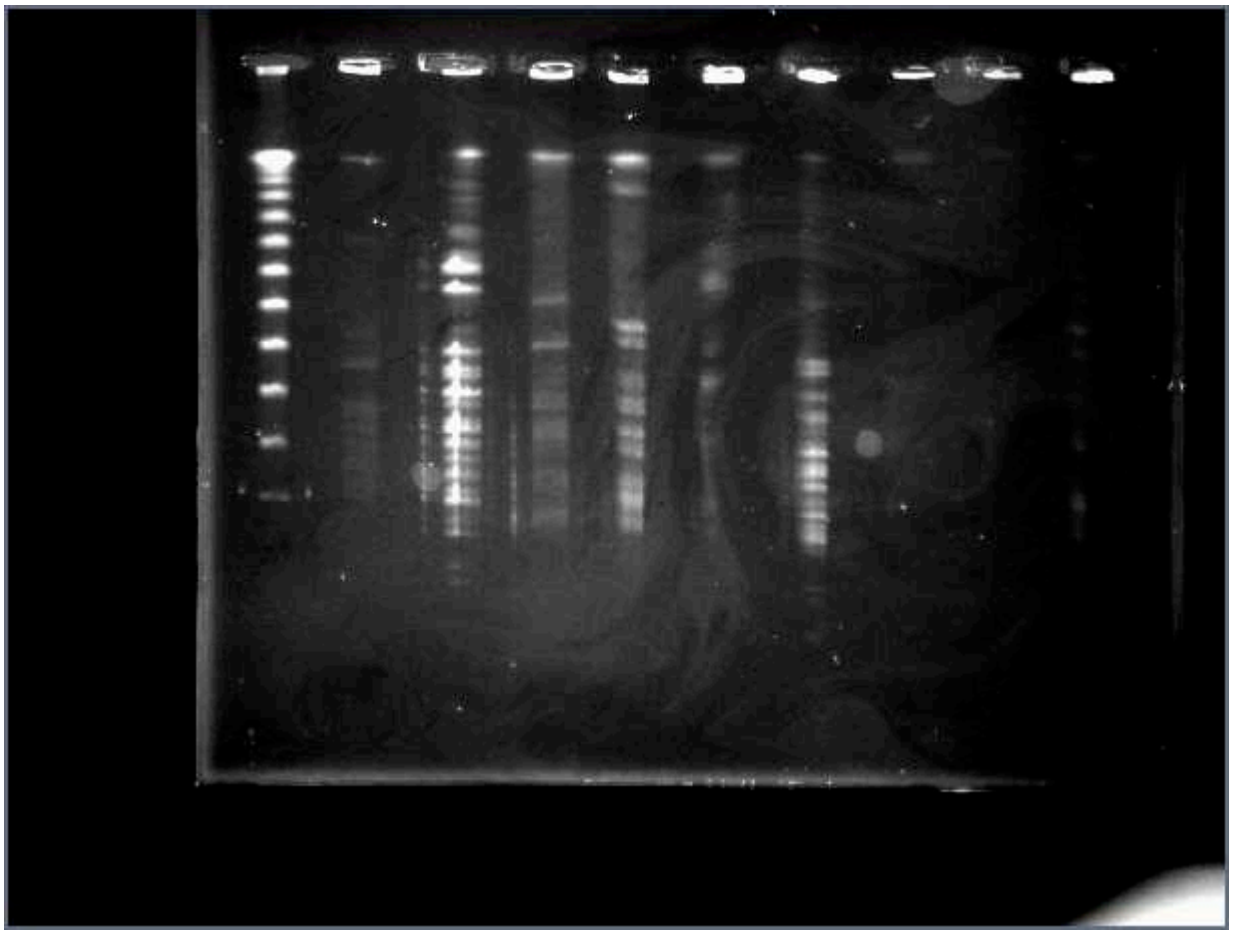
Lane	Isolate #	Origin
1	Ladder	
2	100	P1 4093 rumen 9am
3	107	P1 4093 fecal 9am
4	115	P1 4093 rumen 10am
5	183	P1 4093 fecal 10am
6	126	P1 4150 rumen 12pm
7	133	P1 4150 fecal 12pm
8	142	P1 4150 rumen 2pm
9	150	P1 4150 fecal 2pm
10	120	P1 4205 rumen 10am

Figure C.4 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



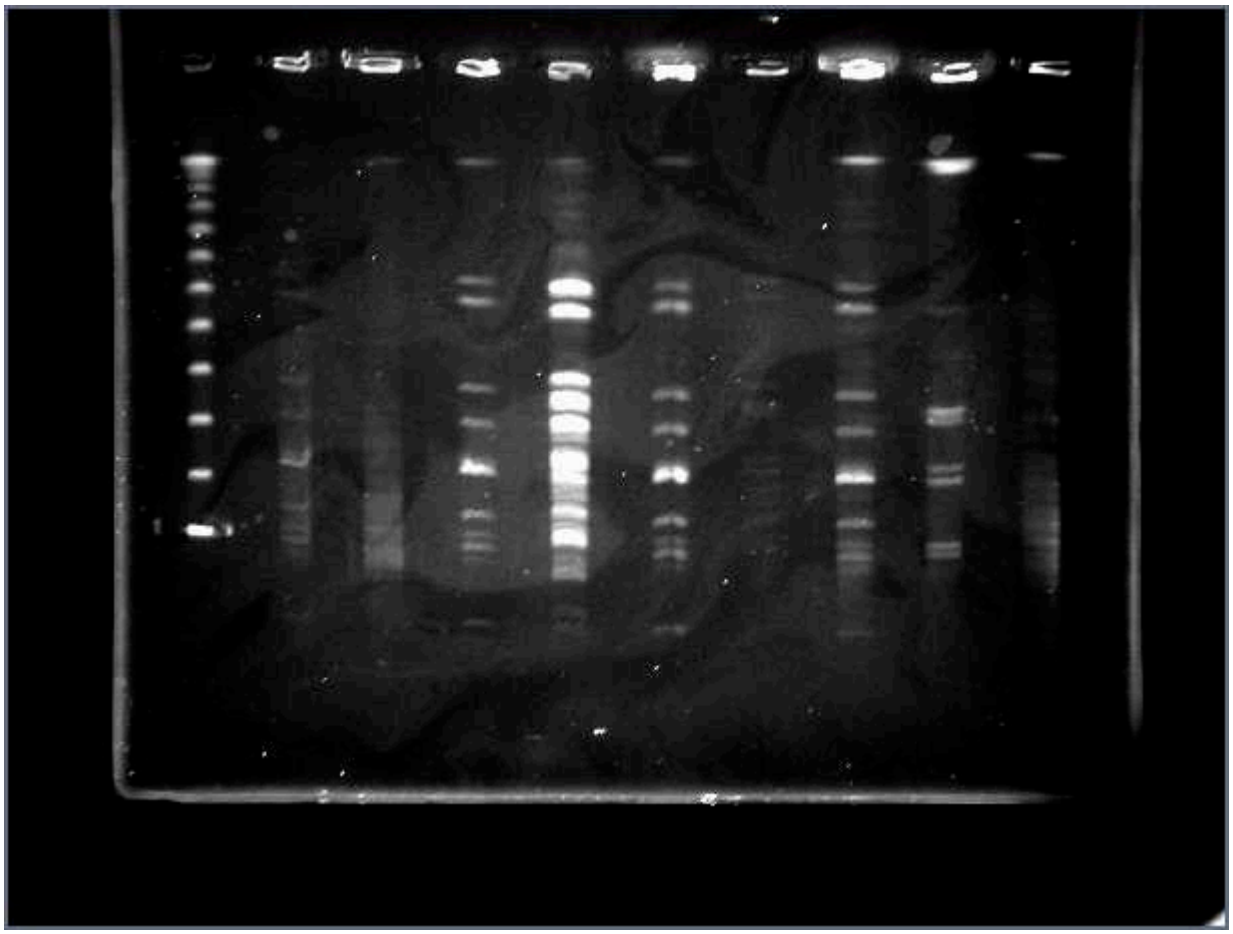
Lane	Isolate #	Origin
1	Ladder	
2	187	P1 4205 fecal 10am
3	143	P1 4205 rumen 2pm
4	152	P1 4205 fecal 2pm
5	242	P2 4016 rumen 9am
6	265	P2 4016 fecal 10am
7	321	P2 4016 rumen day 2
8	329	P2 4016 fecal day 2
9	244	P2 4093 rumen 9am
10	252	P2 4093 fecal 9am

Figure C.5 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



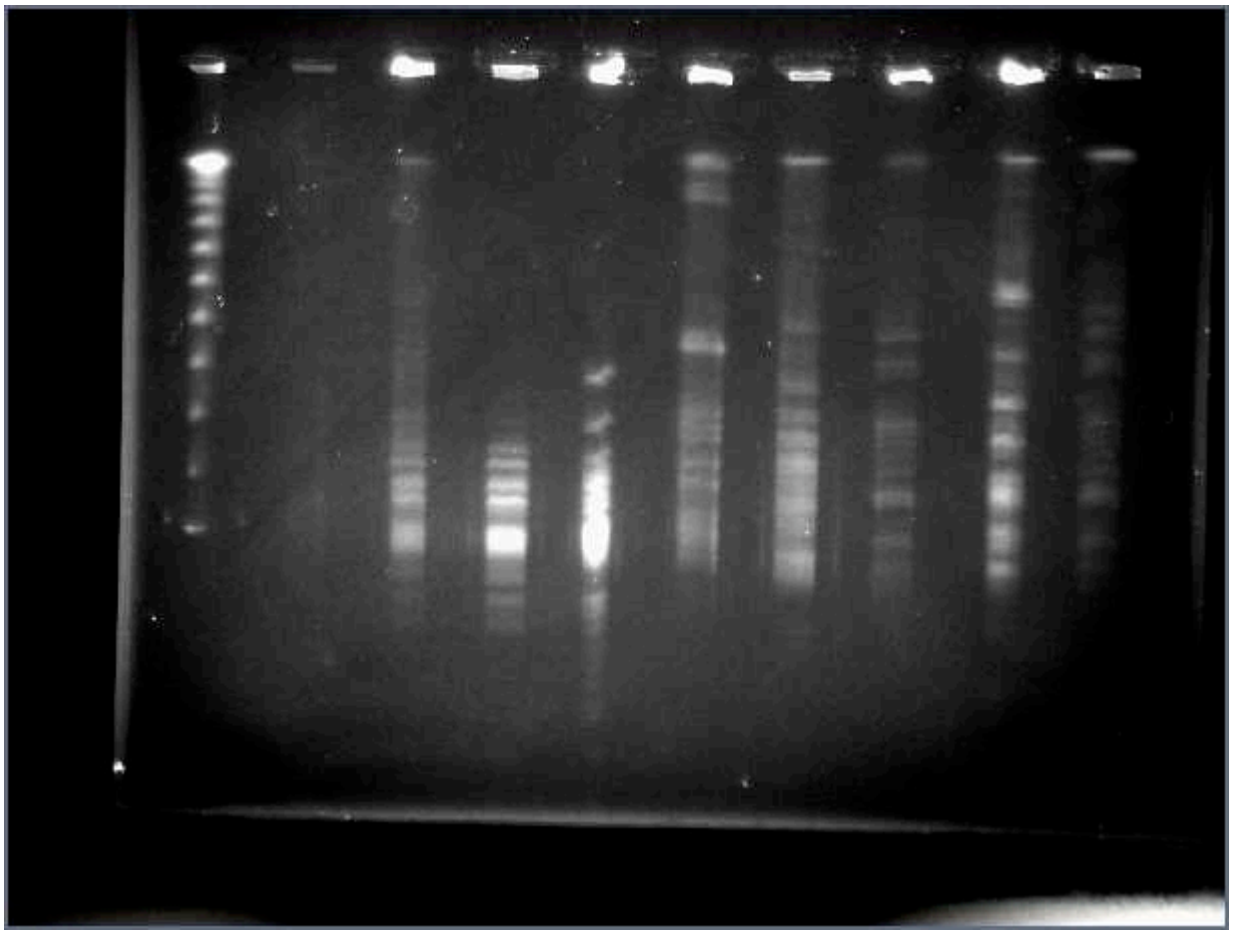
Lane	Isolate #	Origin
1	Ladder	
2	323	P2 4093 rumen day 2
3	331	P2 4093 fecal day 2
4	245	P2 4150 rumen 9am
5	254	P2 4150 fecal 9am
6	301	P2 4150 fecal 2pm
7	247	P2 4205 rumen 9am
8	256	P2 4205 fecal 9am
9	327	P2 4205 rumen day 2
10	336	P2 4025 fecal day 2

Figure C.6 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



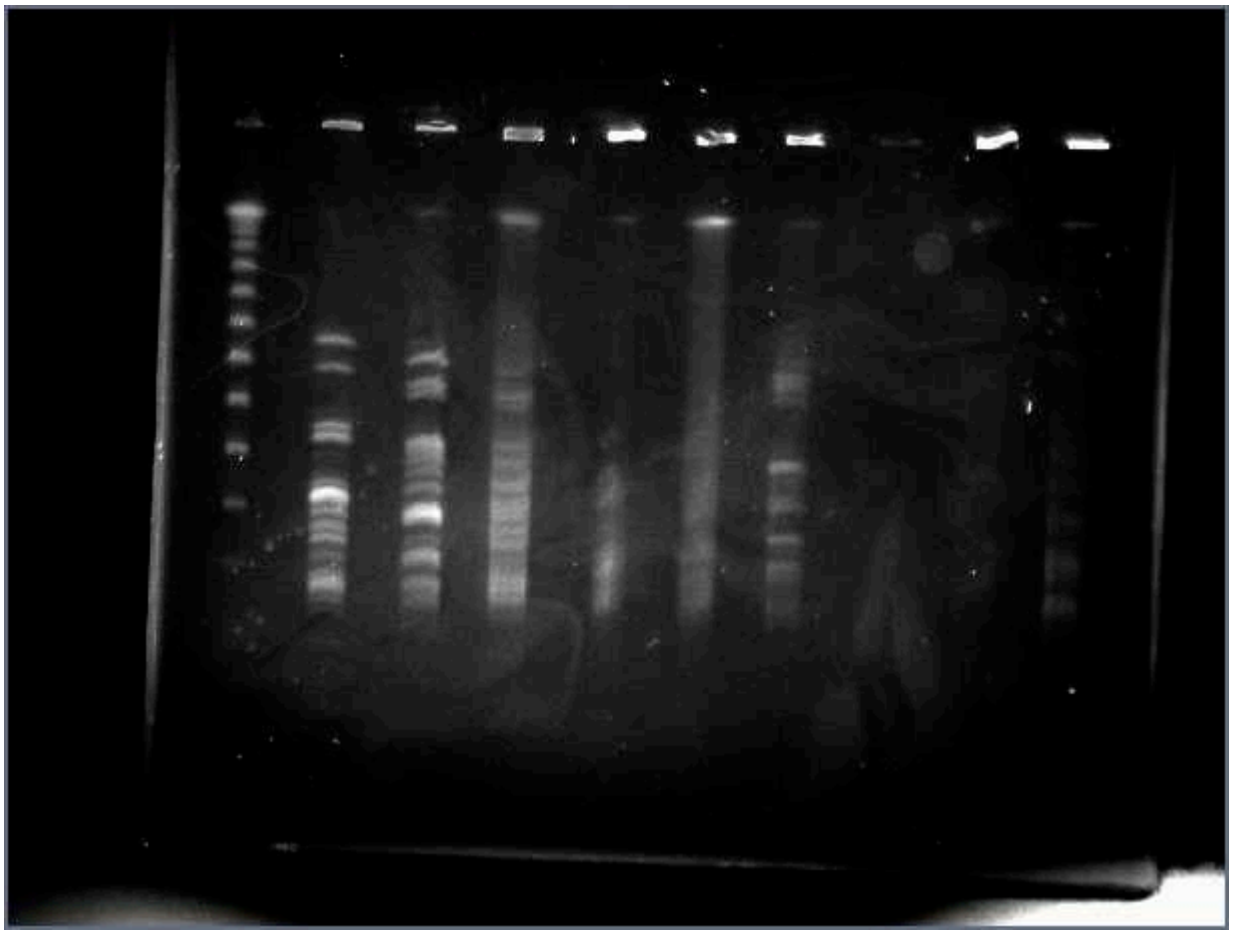
Lane	Isolate #	Origin
1	Ladder	
2	337	P3 4016 rumen 9am
3	369	P3 4016 rumen 12pm
4	339	P3 4093 rumen 9am
5	347	P3 4093 fecal 9am
6	356	P3 4093 rumen 10am
7	363	P3 4093 fecal 10am
8	341	P3 4150 rumen 9am
9	349	P3 4150 fecal 9am
10	342	P3 4150 rumen 9am

Figure C.7 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



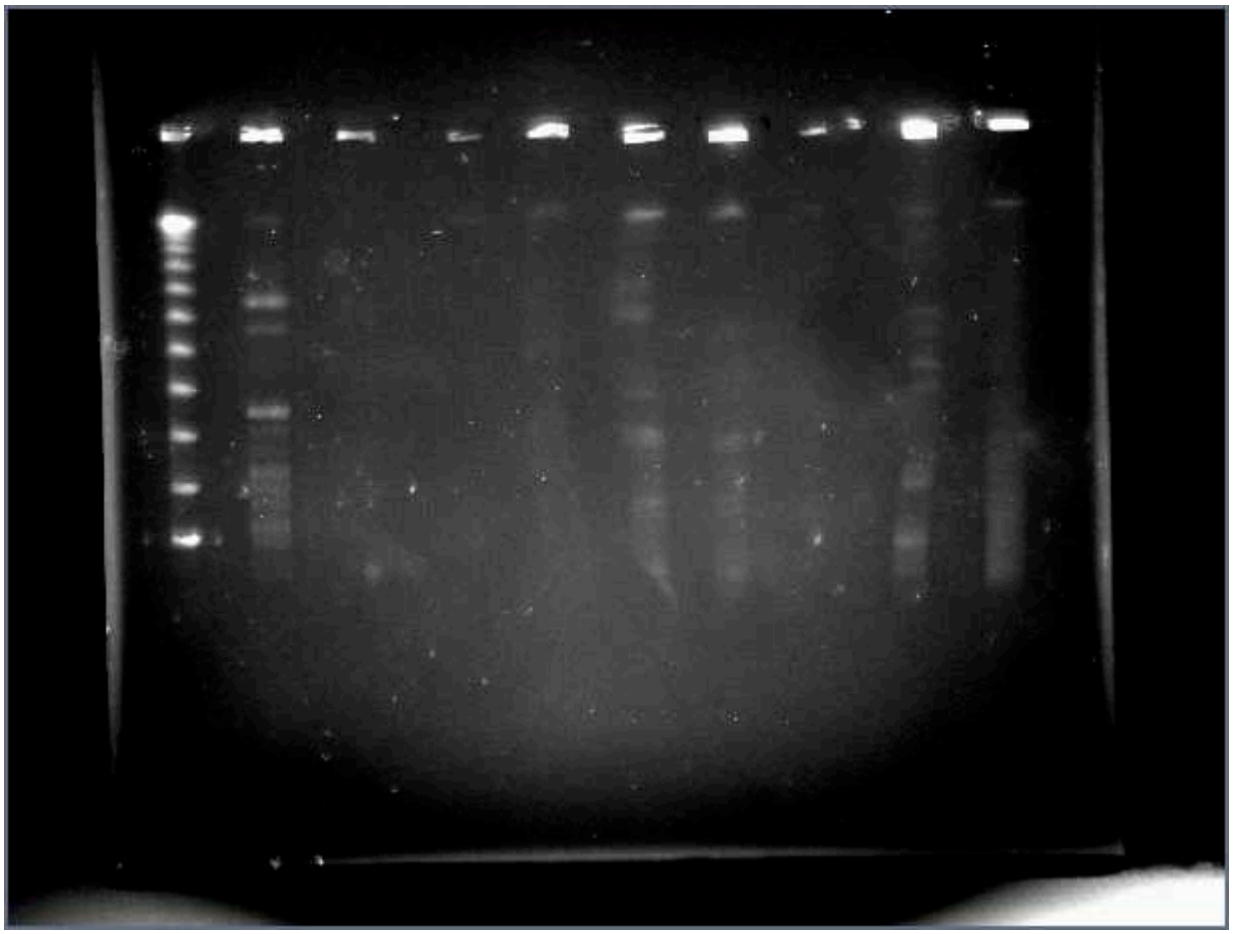
Lane	Isolate #	Origin
1	Ladder	
2	350	P3 4150 fecal 9am
3	343	P3 4205 rumen 9am
4	351	P3 4205 fecal 9am
5	376	P3 4205 rumen 12pm
6	381	P3 4205 fecal 12pm
7	434	P4 4016 rumen 9am
8	440	P4 4016 fecal 9am
9	445	P4 4016 rumen 10am
10	452	P4 4016 fecal 10am

Figure C.8 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



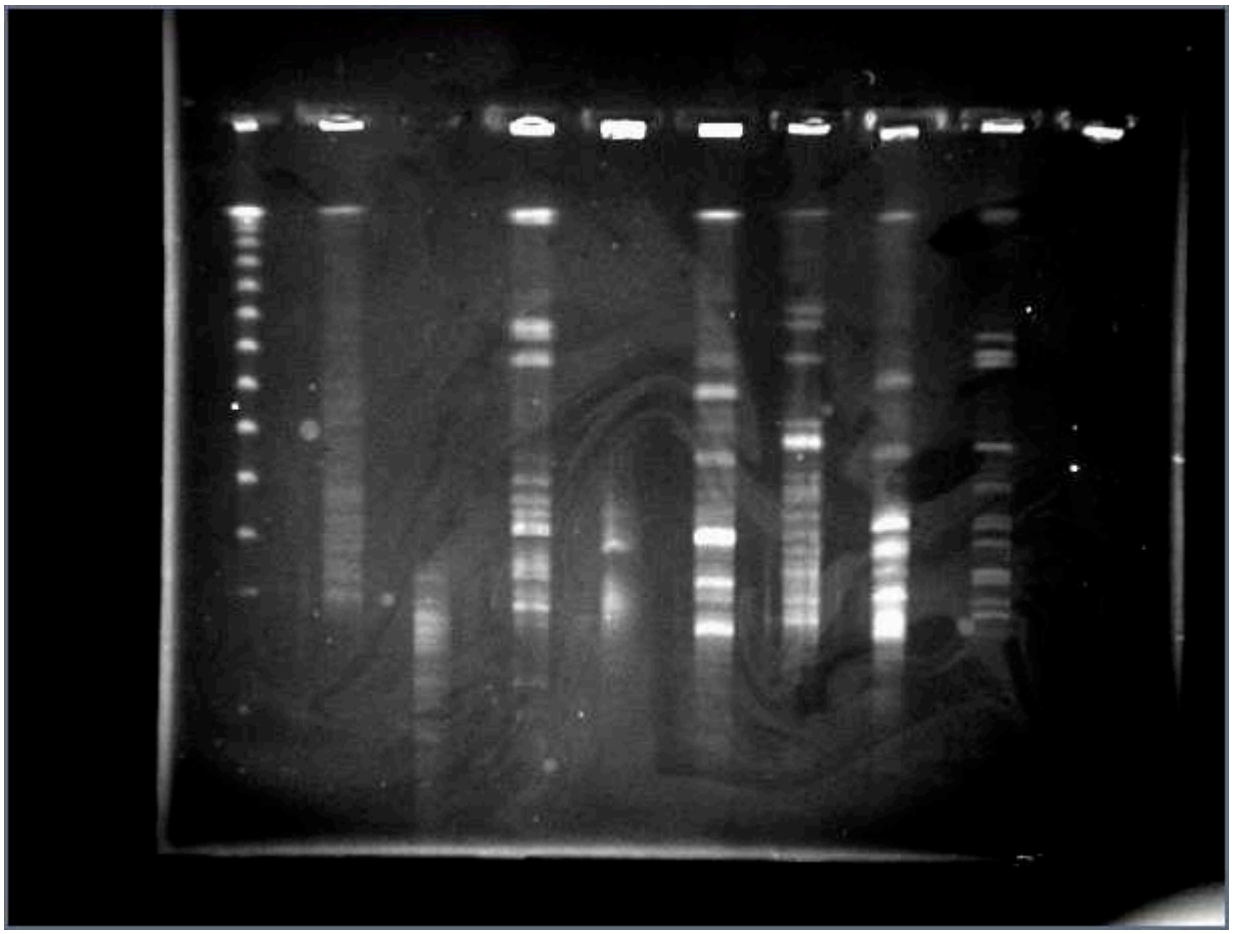
Lane	Isolate #	Origin
1	Ladder	
2	448	P4 4093 rumen 10am
3	441	P4 4093 fecal 9am
4	438	P4 4205 rumen 9am
5	571	P4 4205 rumen day 2
6	499	HD 4016 rumen 9am
7	505	HD 4016 fecal 9am
8	524	HD 4016 rumen 12pm
9	517	HD 4016 fecal 10am
10	514	HD 4093 rumen 10am

Figure C.9 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



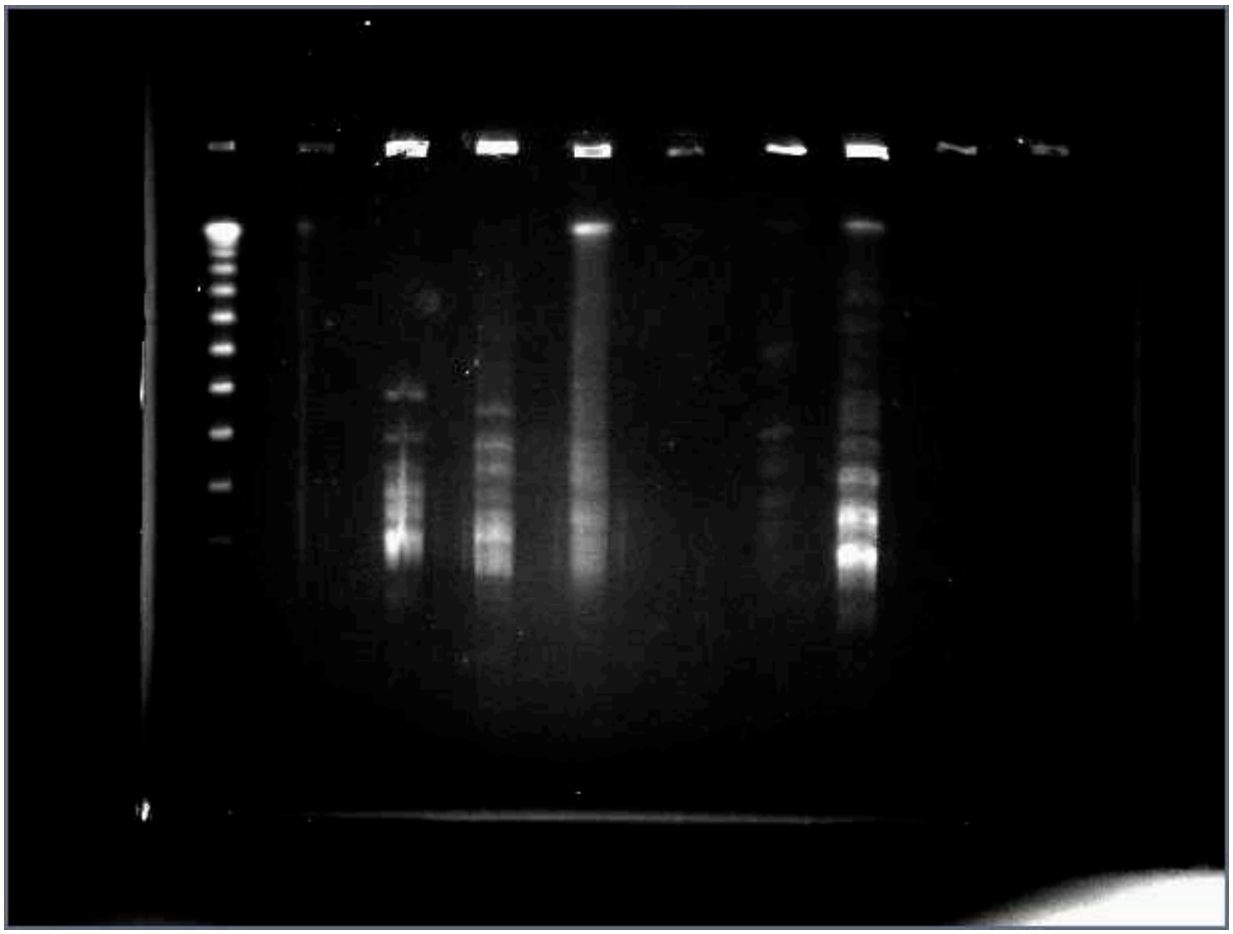
Lane	Isolate #	Origin
1	Ladder	
2	561	HD 4093 rumen day 2
3	503	HD 4205 rumen 9am
4	558	HD 4205 fecal day 1
5	528	HD 4205 rumen 12pm
6	301	P2 4150 fecal 2pm
7	256	P2 4205 fecal 9am
8	327	P2 4205 rumen day 2
9	336	P2 4205 fecal day 2
10	369	P3 4016 rumen 12pm

Figure C.10 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



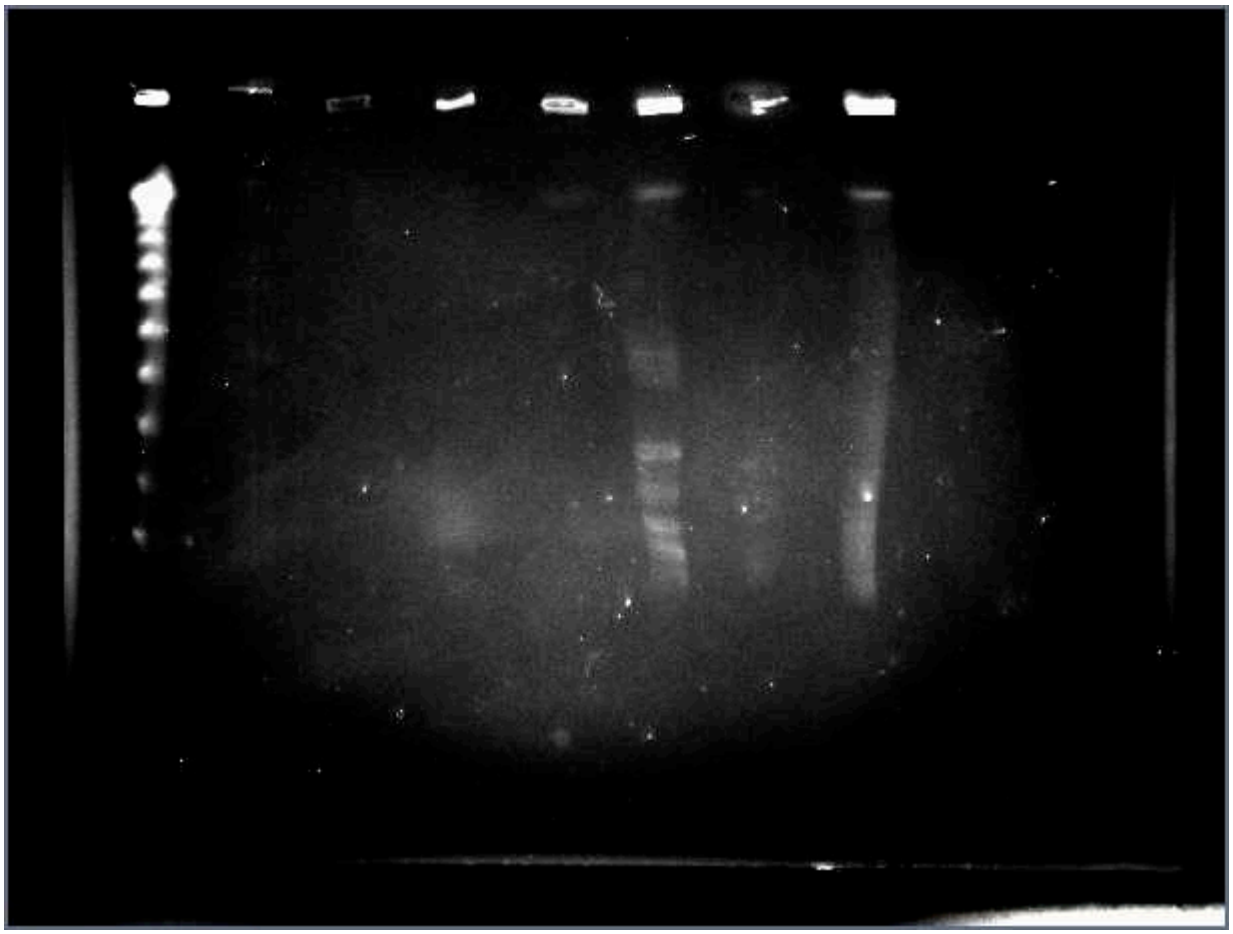
Lane	Isolate #	Origin
1	Ladder	
2	Silage Project # 6	Used as dose
3	Silage Project # 60	Used as dose
4	34	Covariate 4093 rumen
5	100	P1 4093 rumen 9am
6	126	P1 4150 rumen 12pm
7	133	P1 4150 fecal 12pm
8	142	P1 4150 rumen 2pm
9	120	P1 4205 rumen 10am
10	152	P1 4205 fecal 2pm

Figure C.11 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



Lane	Isolate #	Origin
1	Ladder	
2	350	P3 4150 fecal 9am
3	376	P3 4205 rumen 12pm
4	571	HD 4016 fecal 10am
5	499	HD 4016 rumen 9am
6	524	HD 4016 rumen 12pm
7	517	HD 4016 fecal 10am
8	514	HD 4093 rumen 10am
9	503	HD 4205 rumen 9am
10	558	HD 4205 fecal day 1

Figure C.12 Pulsed –field gel electrophoresis patterns of enterococcoal isolates of silage inoculant, ensiled silage, and bovine origin following *Sma*I digestions. (Isolates shown below).



Lane	Isolate #	Origin
1	Ladder	
2	350	P3 4150 fecal 9am
3	524	HD 4016 rumen 12pm
4	503	HD 4205 rumen 9am
5	558	HD 4205 fecal day 1
6	528	HD 4205 rumen 12pm
7	327	P2 4205 rumen day 2
8	369	P3 4016 rumen 12pm
9	Empty	
10	Empty	