DISSERTATION

AN *IN VITRO* SYSTEM EVALUATION OF THE RUMEN MICROBIOME AND RUMEN FERMENTATION CHARACTERISTICS AS A RESULT OF DIFFERING FEED ADDITIVES

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ABSTRACT

AN *IN VITRO* SYSTEM EVALUATION OF THE RUMEN MICROBIOME AND RUMEN FERMENTATION CHARACTERISTICS AS A RESULT OF DIFFERING FEED ADDITIVES

The objective of these studies was to screen for an alternative to antibiotics use to reduce liver abscess by evaluating the effects of probiotics on other feed additives on rumen fermentation characteristics and rumen microbiota composition in a closed *in vitro* system design to mimic fermentation of feedlot diets. The experiment in chapter 3 was conducted to affirm the understanding of differing microbiome compositions in three different matrices (fluid, lining, and digesta) of beef cattle consuming a high energy diet using 16S rRNA gene amplicon sequencing. Twelve crossbred feedlot steers (450 kg; ~ 3.0 years of age), fitted with ruminal fistulas, were utilized in this experiment. Across all sample regions, *Bacteroidetes* and *Firmicutes* were the predominant phyla present. The relative abundance of *Bacteroidetes* detected in rumen fluid was less ($P < 0.05$) when compared to bacteria from the rumen lining and digesta. In contrast, the relative abundance of *Firmicutes* was greater ($P < 0.05$) in rumen fluid and the rumen lining when compared to digesta samples. Additionally, the order *Actinomycetales* was found in the rumen fluid and the lining of the rumen, but not the digesta. Our results further affirm that the rumen ecosystem contains a core microbiome; however, within each region different bacteria are present. The presence of *Actinomycetales* have been associated with liver abscesses. As a result, it is important that the differences in rumen microbial communities are taken into consideration when evaluating the rumen.

The experiment in chapter 4 evaluated effects of encapsulated butyric acid and zinc (ButiPEARL™Z), a probiotic (CLOSTAT®500), and a novel feed additive (Compound K) and compare them to an antibiotic (Tylan®) on the alteration of rumen fermentation characteristics and the rumen microbial community, *in vitro*. Rumen fluid from three steers, fitted with a fistula that consumed a high energy diet, was collected and mixed in a 1:1 ratio with McDougall's buffer. Vaccine bottles were used as representations of rumen environments. Samples were collected at 6 h and 12 h post fermentation. Gas samples were measured for N_2, CO_2, and CH_4. The liquid portion was analyzed for volatile fatty acids (VFA) and the pellet fraction was analyzed for microbial protein. Propionic acid production ($P < 0.05$) increased with the inclusion of ButiPEARL and CLOSTAT (BPZ/CLO) and Compound K + ButiPEARL + CLOSTAT (K/BPZ/CLO) at 6 h of incubation. Furthermore, these treatments reduced ($P < 0.05$) butyric acid production. Methane was reduced ($P < 0.05$) at 6 and 12 h for the K/BPZ/CLO treatment compared to Control and Tylan. In addition, a homogenize samples of the rumen was used for microbiome analysis. No differences ($P > 0.05$) in alpha or beta diversity of the microbial community were observed between treatments. Similarly, the number of sequence variants was not affected by treatment inclusion ($P > 0.05$). Nonetheless the absence of microbial differences might be due to fermentation peaking at 6 hours as explain by the absence of pH change between 6 h and 12 h. Overall the inclusion of the tested compounds can improve fermentation factors. However, in order to fully understand their effects on the microbiota composition earlier time points should be tested.

The experiments of Chapter 5 were performed to investigate the *in vitro* system efficacy as a method of screening for feed additives, as well as to determine collection time points. In addition, the use of tannins, saponins, and direct-fed-microbials (DFM) as an alternative to

antibiotics to reduce liver abscess was evaluated. Rumen fluid was collected from steers fitted with ruminal fistulas and adjusted to a high energy finishing diet for 21 d. Gas samples were measured for N_2, CO_2, and CH_4. The liquid portion was analyzed for volatile fatty acids (VFA) and the pellet fraction was analyzed for microbial protein. Experiment A evaluated the effects of condensed and hydrolysable tannin, in which an increased ($P < 0.05$) propionic acid production was observed with the inclusion of tannins regardless of type at 18 h of fermentation. These same treatments reduced ($P < 0.05$) gas pressure at 18 h. In addition, microbial protein was not significant ($P = 0.226$), though a numerical increase was observed for all tannin types at 18 h of fermentation. While experiment B, evaluated a commercial tannin, DFM (*Lactobacillus animalis* + *Propionibacterium acidipropionici*), and saponin. Propionic acid production increased ($P < 0.05$) with all treatment inclusions at 6 h, 12 h and 18 h of fermentation. These same treatments reduced ($P < 0.05$) acetic acid production at 12 h, but no significant effects were seen at 18 h, though a numerical reduction was observed. Methane was reduced ($P < 0.05$) at 12 h and 18 h for Saponin, commercial tannin and Sap+DFM treatments as compared to Control. For both experiments a sterile swab was dip in a homogenize mixture of the rumen content of each treatment bottle for microbiome analysis. Alpha did not differ ($P > 0.05$) for both experiments at 18 h of fermentation. However, there was difference ($P > 0.05$) in beta diversity of the microbial community between treatments at 18 h for experiments A and B. The preliminary results of both experiments demonstrated that the close *in vitro* system is successful at screening for feed additives. Additionally, our results suggested that commercial blend of tannin, hydrolysable tannin, saponin and the combination of this treatments with the direct-fed-microbial offer potential benefits as an antibiotic alternative and that they need to be further tested at 12 h and 18 h.

Chapter 6 experiment evaluated a commercial tannin, hydrolysable tannin, saponins and direct-fed-microbials (DFM) as potential antibiotic alternatives to reduce liver abscess in feedlot cattle consuming a high energy diet in an *in vitro*. Three crossbred feedlot steers (450 kg; ~ 3.0 years of age), fitted with ruminal fistulas and adjusted to a high energy finishing diet for 21 d, were utilized in this experiment. Samples were collected at 12 h and 18 h post fermentation. Propionic acid production increased ($P < 0.05$) with the inclusion of commercial tannin + DFM, hydrolysable tannin + DFM and Saponin + DFM at 6 h of incubation. These same treatments reduced ($P < 0.05$) acetic acid production at 12 h, but no effects were seen at 18 h. Methane was reduced ($P < 0.05$) at 12 h for the hydrolysable tannin treatment compared to Control. Treatments of hydrolysable tannin + DFM and Saponin + DFM exhibited increased ($P < 0.05$) pH at 12 h of fermentation. In addition, DNA was extract for microbiome analysis. Alpha and beta diversity were different ($P > 0.05$) at 12 h. However, there was no differences ($P > 0.05$) in alpha or beta diversity of the microbial community between treatments at 18 h of fermentation. Across all samples at 18 h of fermentation the genus *Dialister* was present, however treatments Saponin+DFM and TCH+DFM had the greatest relative abundance. The presence of genus *Dialister* indicates association with energy harvesting bacteria indicating that the inclusion of these feed additives allowed shifts of certain bacteria and should be further tested in live cattle to investigate growth performance benefits.

Key words: Feed additives, rumen nutrition, probiotics, rumen microbiome

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CHAPTER I

INTRODUCTION

Today's consumers demand all beef products to be safe and wholesome. As a result, the beef industry in the United States has striven to meet the demand as efficiently and sustainably as possible. They have adopted technologies that have allowed for optimized growth performance and quality of the end product. Cattle are a unique aspect of the feedlot industry, as ruminant animals have a four chambered stomach that harbors a complex microbial ecology; making it ideally suited for fermentation (Russell, 2001). This complex gastrointestinal system utilizes bacteria, archaea, fungi, and protozoa to ferment and digest fibrous plant materials that have no nutritional value to humans into a product that rich in nutrients and vitamins – beef (Li and Guan, 2017). In addition to being able to digest fibrous materials, cattle can also break down starches from non-fibrous materials. Diets that contain high levels of available starches increase fermentation in the rumen and can disrupt gas production and pH regulations causing it to drop to an acidotic level (Russell, 2001). Rumen acidosis can damage/weaken the epithelium lining of the rumen allowing for bacteria to colonize the rumen wall, which later allows for entrance of bacteria into the portal blood and infecting the liver (Nagaraja and Chengappa, 1998).

Liver abscesses can have an unfavorable economic impact on cattle production. To reduce the incidence of liver abscesses the best tool the fed cattle industry has relied on is a macrolide antibiotic known as, tylosin phosphate. However, the use of antibiotics in the food industry is being scrutinized by the consumer, as the concerns for antibiotic resistance continue to grow. The Food and Drug Administration (FDA) has banned the use of antibiotics as growth

promoters and (as of 2019) the use of antibiotics to prevent disease outbreaks in live animals, other than approved use that falls under the prescription and supervision of a licensed veterinarian (Veterinary Feed Directive, 2015).

The increased restrictions of the use of antibiotics and the increased scrutiny by consumers has led to investigations of natural feed additives and direct-fed-microbials (DFM) as alternatives of antibiotics. Therefore, a central topic of advancing the understanding of the rumen microbiome and altering the microbial community by inclusion of natural feed additives as method of antibiotic alternatives will be the focus of the current review and research.

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CHAPTER II

LITERATURE REVIEW

The livestock industry in the United States strives for sustainability and ranchers and farmers take pride and care for their stock. Cattle possess a unique four chamber compartment that is able to digest fibrous plant material that has no nutrient value for humans into a nutrient and vitamin dense protein source that humans can digest – beef. The beef industry has been able to improve its production practices throughout the years. In fact, the industry has been able to produce the same amount of beef with fewer animals since 1977 (Capper, 2011). As the industry continues to compete for energy, land and water supply to produce a safe and affordable beef supply, new systems and practices need to be adapted.

Even though the beef industry strives for sustainability, the use of antibiotics is growing global concern (Zhang et al., 2014). In recent years, there has been a push to decrease the misuse and overuse of antibiotics by bringing them under the supervision of licensed veterinarians (Veterinary Feed Directive, 2015). The true path of how resistance can be passed from animals to humans is not fully understood. However, the current concerns for antibiotic resistance have led to opportunities to develop new technologies, such as non-culture-based methods, to screen for feed additives that can improve beef production and alter the microbial community in food production animals.

2.10 History of Antibiotic Use

Antibiotics have been utilized for over a century and are perhaps the most important discovery made by man. Documents show that in ancient Egypt, moldy bread was deemed as the

best treatment to treat a wound or diseases (Sipos et al., 2004). However, it wasn't until early 1900 that arduous research and an extensive screening process allowed for the synthesis of Salvarsan, a drug used to treat syphilis (Aminov, 2010). Salvarsan was the first widely used antibiotic. In 1928, Alexander Fleming unexpectedly discovered penicillin from plates that had been exposed to ambient air (Fleming, 1929). Following this discovery, it wasn't until the 1940's that Howard Florey collaborated with U.S researchers to mass produce penicillin to treat wounded soldiers (Zaffiri et al., 2012). The commercial production of penicillin spearheaded future research which came to be known as the golden age of antibiotic discovery, whereby the 1960s, over half of the viable antibiotics used today were discovered (Davies, 2006). The beginning of the golden age focus was primarily on isolating new antibiotics from naturally occurring microorganisms. By 1944, *Streptomycin* was unearthed from an organism found in soil that initiated a mass pursue for other potential organisms worldwide (Gould, 2016). This quest let researchers to discover antibiotics that previously were only found in certain soils around the world. Although world changing antibiotics were initially discovered from natural organisms found in soil, new antibiotic-resistant infections called for new methods of developing antibiotics. These new methods yielded antibiotics, such as methicillin and ampicillin (Gould, 2016).

Antibiotics discovered during the golden age up to 2015 were made by employing the original or slightly modified testing methods developed by Fleming in 1928. Even though these methods transformed medicine by providing effective treatments and cures for many diseases, they were limited to only culturable bacteria. Over 99% of microbial species in the world are unculturable. Therefore, new methods to discover antibiotics are being tested (Lewis, 2013). In 2015, the antimicrobial teixobactin was successfully isolated using a new culture-free method

(Ling et al., 2015). The viable utilization of new culture free techniques holds promise for new antibiotic discoveries to help combat diseases.

2.11 Concerns of Antibiotic Resistance

From the utilization of moldy bread to cure wounds to our most current discovery, teixobactin, antibiotics have transformed the medical industry. Their discovery changed the way diseases and injuries are treated, improved the wellbeing of humans and animals, and saved countless lives. Nevertheless, the overutilization of these novel antibiotics has led to a rise in antibiotic-resistant bacteria strains (Davies and Davies, 2010). The increasing population of drug resistant pathogens have closely followed the increased use of antibiotics by human and nontherapeutic animal use (Davies, 2006). Soon after the introduction of sulfonamides in 1937, specific mechanisms of resistance were discovered. Some cases, for example penicillin resistance, were observed before antibiotics were commercially used (Davies and Davies, 2010). This individual resistance has led to the more serious problem of resistance to multiple types of antibiotics. During the golden age in the late 1950s and 1960s, resistance to multiple drugs was first identified in gram-negative bacteria *Escherichia coli* and *Salmonella enterica* (Levy and Marshall, 2004). Even though drug resistant pathogens are considered a modern phenomenon, microbes that contain resistance have been collected that predate the antibiotic era, with some bacteria dating over 4 million years old (Wright, 2007; D'Costa et al., 2011; Bhullar et al., 2012). In the beginning, resistance was localized to hospitals were there was a greater amount of immunocompromised patients and most antibiotics were being used (Levy, 1998). Today, this is no longer the case. Resistant strains have expanded beyond the boundaries of the hospital and are no longer localized (Levy and Marshall, 2004).

Antibiotics are not only used in humans but are also heavily utilized in the agricultural industry, especially in the animal sector. This brings the concern of antibiotic-resistant strains being passed from the animal to the consumer. This creates a present need to discover new technology that can replace the use of therapeutic antibiotics in animal production settings.

2.12 Antibiotic Mechanisms of Resistance

Antibiotics have proven to be effective growth promoters in livestock operations. Even though they have been beneficial to produce a valuable food source, there are still concerns about antibiotic resistance. These concerns are the result of the method of application. Antibiotics are effective methods of treatment if they are able to induce cell death or inhibit cellular growth of a target pathogen; this occurs due to the inhibition of DNA, RNA, protein or cell wall synthesis (Kohanski et al., 2010). Antibiotics utilized as growth promoters are given for long periods of time at very small doses. This method increases the chance of selecting for resistant strains (Levy, 1998). While antimicrobials that are efficient should kill bacteria, there are some microbes that adapt and create resistance. The surviving bacteria are less likely to be affected by any of the antibiotics, hence become resistant. Many mechanisms of resistance exist. One mechanism of acquiring resistance is through horizontal gene transfer (HGT; Davies, 2006), a process by which bacteria that counteract the effects of an antimicrobial agent will transfer their resistance to other bacteria. Kapoor et al. (2017) stated that the biochemical resistance mechanisms that bacteria use to gain resistance include: antibiotic inactivation, target modification, altered permeability, and protection or avoidance of the metabolic pathway. Each of these biochemical resistance mechanisms affects different antibiotics. Beta-lactams, aminoglycosides, and chloramphenicol are affected by the enzymes beta-lactamases, aminoglycoside-modifying and chloramphenicol acetyltransferases respectably (Kapoor et al.,

2017). In the case of beta-lactamase, a majority of the beta-lactams that contain ester and amide bonds are hydrolyzed, which includes affected antibiotics of penicillin, cephalosporins, monobactams, and carbapenems (Kapoor et al., 2017).

Another mechanism of antibiotic resistance includes alterations in ribosomal subunits. The bacterial 70S ribosomes consist of two ribonucleoproteins subunits (30S and 50S), which are the targets of the inhibitors of protein biosynthesis (Vannuffel and Cocito, 1996). Aminoglycoside-modifying enzymes are known inhibitors of subunit 30S, while chloramphenicol acetyltransferases are inhibitors of subunit 50S (Kapoor et al., 2017). Modifications of the ribosomal subunit that prevent binding to the subunits can result in antibiotic resistance. It has been stated that tetracyclines and aminoglycosides act upon the conserved sequences of the 16S rRNA of the 30S ribosomal subunit preventing binding, whereas macrolides, chloramphenicol, and lincosamides affect the early stages of protein synthesis, by targeting the 23S rRNA of the 50S ribosomal subunit (Vannuffel and Cocito, 1996; Yoneyama and Katsumata, 2006).

Understanding the mechanism of resistance is an important factor as the cattle industry uses a variety of antibiotics. The cattle industry relies on a tylosin, a macrolide antibiotic, to prevent or reduce liver abscesses. The concerns of resistance are endless; therefore, the discovery of alternatives for critically important antibiotics, such as tylosin, that can be used to treat the medical concerns of the animals without the threat of increasing antibiotic resistance are of the utmost importance.

2.13 Antibiotic Use in Animal Agriculture

Animals get sick and depending on severity may require medical treatment. Intensive feeding operations run the risk of having all animals in their facilities become ill with an

infection due to the close proximity in which these animals are housed. To reduce mortality and morbidity due to subclinical and clinical infections, intensive feeding operations use antibiotics (Gersema and Helling, 1986). However, antibiotic use in animal agriculture has not been limited to the treatment or prevention of infections only. Antibiotics are also used to improve growth and improve feed efficiency (Gersema and Helling, 1986). Administration of antibiotics varies depending on the species and on the desired outcome. Use may also vary by treatment size; antibiotics targeting a specific animal may be different than if targeting the entire herd. This use in food animals raises concerns about antimicrobial resistance due to their method of application. Antibiotics utilized as growth promoters are given for months at a time in small doses to combat infection which increases the chance of selecting for resistant strains, which can affect people that consume undercooked meat (Levy, 1998). Aminov (2010) states that the distribution of antibiotic use has been 50% for humans and 50% for animal production, agriculture/horticulture, and aquaculture. The number of antibiotics approved for agriculture production increased the concern of antibiotic resistance. Some antibiotics used in human medicine are also utilized in animal production (Levy, 1998). Around the time of world war II, farmers observed improved weight gain on animals that had been treated with antibiotics. This observation led to the approval of the use of antibiotics in food animals for growth promotion by the Food and Drug Administration (FDA) in 1951 (Hao et al., 2014). Following this approval, research was initiated, including Hewes (1955) that performed a trial in guinea pigs and reported significant weight gains were found in animals treated with chlortetracycline.

As population continues to increase and land that was once used for animals or agriculture use becomes urbanized, the need to produce more protein with less land and less water become eminent. Antibiotics and new growth technologies have become a common

practice in the industry. These methods are allowing producers to increase their production. Each time a food animal gets sick the animal's performance is affected and can have unfavorable future production yields; therefore, antibiotics are used in an attempt to combat the occurrence of illness and disease. The mode of action of antibiotics in growth promotion is not well established. However, there are some theories that have been mentioned in previous research. These theories range from 1) nutrients may be protected from being destroyed by bacteria, by reducing growth-depressing metabolites produced by bacteria such as (ammonia and bile degradation products); 2) the absorption of nutrients may be enhanced as result of a thinning of the small intestinal wall; 3) a reduction of toxins via the reduction of certain bacteria; and 4) the reduction on incidences of subclinical intestinal infections, which in turn reduce the metabolic cost of fighting an infection (Butaye et al., 2003; Niewold, 2007).

Even though the use of subtherapeutic antibiotics have allowed us to improve animal production, concerns of increasing resistance in bacteria and the threat of cross-contamination from consuming animal products are still critically relevant. Gram-negative bacteria such as *Salmonella spp*. and *Escherichia coli* are the primary concern for resistance as livestock can harbor these pathogens (Elder et al., 2000). Nonetheless, if antibiotics are supplied to animals, the presence of certain pathogens are reduced, lowering the likelihood of transmission to humans. For example, application of virginiamycin decreases contamination of *Clostridium perfringens* and other food-borne pathogens in animal carcasses (Hao et al., 2014).

As food production continues to expand and livestock operations become increasingly complex, establishing an understanding of the negative effects of antibiotic use in animals and the potential pathway to antibiotic resistance is critical. Regulatory agencies across the world have started to push back on antibiotic use in animals deemed for food production. Banning the

use of antibiotics in animals started as early as the 1960's in some European countries. However, the early antibiotic bans only targeted specific antibiotics used as growth promoters that were also important in human medicine (Wielinga et al., 2014). In 1986, Sweden banned all growth-promoting antibiotics to combat antimicrobial resistance (Casewell, 2003). The trend on banning antibiotics continued until 1999 when the European Union (EU) banned the last remaining antibiotics that belonged in the classes used for humans (bacitracin, spiramycin, virginiamycin and tylosin [macrolite]) for use as a growth promoter in food animal production (Casewell, 2003). In addition, the EU phased out any other antibiotics (not in the same classes used for humans) used for growth promotion in 2006 (Cogliani et al., 2011).

The United States decided to address concerns of antibiotic use in food animal production by implementing the Veterinary Feed Directive (VFD) Final Rule, which includes measures to prohibit the feed and water use of medically important antibiotics in animals in the food production chain and bringing the remaining therapeutic antibiotics under the supervision of a licensed veterinarian (Veterinary Feed Directive, 2015). The VFD is intended to reduce the rates of misuse and overuse of drugs in livestock production settings in the United States. However, even with the VFD implementation, growing concerns of antibiotic resistance still remain. These concerns can only be addressed with extensive research on antibiotic replacements or alternatives that can help the industry to improve the animal production and simultaneously reduce health issues, specifically pertaining to important microbials.

2.14 Critically Important Antibiotics

Critically important antibiotics are defined as 1) antibiotics that are sole, or one of the limited available therapies, to treat serious bacterial infections in people; 2) antibiotics that are used in the human medical field to treat infection caused by either bacteria that was transmitted

from nonhuman sources or that has acquired resistance genes from nonhuman sources (WHO, 2017). The World Health Organization (WHO) has categorized Cephalosporins (3rd, 4th, and 5th generations), glycopeptides, macrolides, ketolides, polymyxins and quinolones as highest priority classes of antibiotics for human medicine.

Of the antibiotics classified as highest priority, cephalosporin is used to treat serious infections from food-borne enteric pathogens. Therefore, if resistance to this drug is developed, human health will encounter a substantial threat. To combat this threat, the FDA has limited the use of cephalosporins by prohibiting certain extra-label7/10/19 1:43:00 PM uses of the drug in food animal production (FDA, 2018). On the contrary, macrolides are still heavily utilized in the beef industry as a prevention method for liver abscesses, as well as, for treatment of enteric pathogens in humans (Nagaraja and Chengappa, 1998; Schwarz et al., 2001).

Although macrolides were discovered in the early 1950s, the first macrolides intended for animal use was spiramycin, developed in the early 1960s, followed by erythromycin and tylosin (Prescott, 2017). Tylosin has shown success in reducing liver abscesses in feedlot cattle (Nagaraja and Chengappa, 1998). The use has been scrutinized as the threat of resistance continues to exist. This concern is derived from *Campylobacter spp.* infections and the potential for resistance to be transmitted to humans that consume animal products (Lin et al., 2007). To prevent contamination of antibiotic-resistant strains from beef products, it is important to stay diligent and explore other methods and alternatives to antibiotics in food animal production.

2.20 Etiological Agents

Liver abscess infections in the liver cause a pus-filled blister that can occur anywhere in the liver, which eventually becomes sterile and later enclosed by scar tissue and reabsorbed (Nagaraja and Chengappa, 1998). Liver abscesses are a consequence induced by acidosis and

ruminitis in cattle that are consuming diets high in fermentable carbohydrates (Amachawadi and Nagaraja, 2016). The infection of liver abscesses is known to be primarily caused by Gram-negative anaerobes such as *Fusobacterium necrophorum* (previously named *Sphaerophorus necrophorus*); however, studies have shown that the abscesses are polymicrobial (Amachawadi and Nagaraja, 2016; Weinroth et al., 2017). Because liver abscesses are polymicrobial, the bacteria flora in the purulent material are both aerobic and anaerobic, as well as, culturable and non-culturable (Nagaraja et al., 1996a; Nagaraja et al., 1996b; Weinroth et al., 2017). In some studies, they have been able to isolate *Fusobacterium necrophorum* from the purulent material of liver abscess from 71% to 100% (Amachawadi and Nagaraja, 2016) and have concluded that *F. necrophorum* is the primary etiologic agent. In most studies, *Trueperella pyogenes* (previously known as *Actinomyces pyogenes* or *Corynebacterium pyogenes*) is the second most frequent bacteria isolated from liver abscesses (Nagaraja and Lechtenberg, 2007; Amachawadi and Nagaraja, 2015). Additionally, there are other bacteria that have been associated with liver abscesses such as *Bacteroides* spp. (Weinroth et al., 2017), *Clostridium* spp., *Staphylococcus*, *Pasteurella* spp. (Simon and Stovell, 1971), *Prevotella* spp., *Streptococcus* spp., and other unidentified gram-negative and gram-positive bacteria (Nagaraja and Lechtenberg, 2007). Even though many bacteria reside in liver abscesses, *F. necrophorum* and *T. Pyogenes* have been found in a higher frequency suggesting that they work synergistically to cause liver abscesses (Takeuchi et al., 1983; Nagaraja et al., 1996a; Doré et al., 2007).

2.20.1 *Fusobacterium necrophorum*

The true etiology of liver abscesses is not yet clear. However, it has been stated that *Fusobacterium necrophorum* is one of the causative organisms of liver abscesses (Nagaraja et al., 1996a). *Fusobacterium necrophorum* is a nonmotile, non-spore forming, rod-shaped, Gram-

negative bacteria, anaerobic organism (Langworth, 1977). This organism is anaerobic, as well as, somewhat aerotolerant and is a normal inhabitant of the gastrointestinal tract of animals and humans (Nagaraja and Lechtenberg, 2007). Additionally, *F. necrophorum* is found in foot rot of domesticated animals, calf diphtheria, necrotic lesion in the oral cavity and the soil of pastures (Langworth, 1977; Nagaraja et al., 2005; Bennett et al., 2009). Besides being deemed the main cause of liver abscesses in cattle, *Fusobacterium necrophorum* is the predominant organism that causes gingivitis and periodontal diseases in humans (Nagaraja et al., 2005). Being a primary causative organism for a human disease adds to the layer of concerns regarding possible antibiotic resistance.

Historically, *Fusobacterium necrophorum* has been categorized into four biotypes: A, B, AB, and C (Langworth, 1977). Biotype A is known as *F. necrophorum* subspecies *necrophorum*, while biotype B is subspecies f*unduliforme*. Biotype A is known to be more pathogenic than B (Amoako et al., 1994; Nagaraja et al., 2005). Additionally, biotype AB has been isolated from foot lesions of cattle and ovines and possesses similar characteristics of A and B (Emery et al., 1985). Biotype C strains are classified as *Fusobacterium pseudonecrophorum* and are nonpathogenic (Bailey and Love, 1993; Nicholson et al., 1994). Biotypes A and B are often isolated from liver abscesses due to their toxin and other virulence factors that facilitate the survival to form the abscesses. Tan et al. (1992) described the virulence factors that are comprised of endotoxic lipopolysaccharides, leukotoxin, and hemolysin, with leukotoxins being highlighted as important in the establishment of liver abscesses.

Even though *F. necrophorum* is found in the rumen, the bacteria does not ferment carbohydrates, instead it utilizes lactic acid as an energy source, which later ferments to produce acetate, propionate, and butyrate (Lechtenberg et al., 1988). This organism has been associated

with diets high in starches (Smith, 1940; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007). *Fusobacterium necrophorum* has been isolated from ruminal contents of cattle, as well as, from the ruminal wall (Narayanan et al., 1997), suggesting that this organism is part of the flora that adheres to the rumen wall to create rumen lesions. In the rumen, the concentration of *F. necrophorum* is low and ranges from (7×10^5/g to 7×10^6/g) depending on the diet's proportion of roughage or grain (Tan et al., 1994; Nagaraja and Chengappa, 1998). The higher abundance of *F. necrophorum* in high grain diets might be due to the increased lactate available (Nagaraja and Lechtenberg, 2007). An increased quantity of available lactate can result in a lower ruminal pH, increasing the concentration of *F. necrophorum*, while at the same time lowering the integrity of the rumen wall.

2.20.2 *Trueperella pyogenes*

Trueperella pyogenes (formerly known as *Arcanobacterium pyogenes*) is a Gram-positive, rod-shaped, facultative-anaerobic organism (Amachawadi and Nagaraja, 2016). This organism can grow under aerobic and strictly anaerobic conditions; however, its optimum growth is under a carbon dioxide enriched environment (Jost and Billington, 2005). *Trueperella pyogenes* is the second bacteria that has been found to be a causative agent of liver abscesses (Nagaraja and Chengappa, 1998), due the presence being ranked as the second most common bacterium isolated from liver abscesses of cattle. This organism has been found to be a normal inhabitant of the mucous membranes of the respiratory and digestive tracts of animals (Jost et al., 2002). *T. pyogenes* has also been isolated from the ruminal wall of cattle (Narayanan et al., 1998), indicating that it inhabits the ruminal lining and can further explain its pathway to the liver. A previous liver abscess review cited Lechtenberg (1993) stating that *T. pyogenes* is able to induce abscesses in the liver when injected intraportally with *F. necrophorum* or its leukotoxin;

however, *F. necrophorum* was able to induce abscesses by itself (Nagaraja and Lechtenberg, 2007). This synergistic behavior further emphasizes that *T. pyogenes* is probably not the primary inducer of liver abscess, but rather an organism that assists with the development. It is believed that *T. pyogenes* utilizes the oxygen, creating an anaerobic environment allowing *F. necrophorum* to thrive, while the leukotoxins from *F. necrophorum* provide protection to prevent phagocytosis of *T. pyogenes* (Tadepalli et al., 2009). Additionally, the lactic acid produced by *T. pyogenes* serves as an energy source for *F. necrophorum*. Even though *T. pyogenes* is able to act as a primary pathogen of infection, the colonization of this bacteria usually follows a previous physical or microbial trauma, which further emphasizes the classification of *T. pyogenes* as one of the most common opportunistic pathogens (Jost et al., 1999; Jost and Billington, 2005)

Trueperella pyogenes has been found to express several putative virulent factors that aid in adherence and colonization of infected tissue (Jost and Billington, 2005). Some of the virulence factors consist of cholesterol-dependent cytoloysin, pyolysin (PLO), extracellular matrix-binding proteins collagen-binding proteins (CbpA), fibrinogen-binding protein, fibronectin-binding protein, exoenzymes, DNase, proteases and others (Jost and Billington, 2005). Pyolysin is thought to be the primary virulence factor of *T. pyogenes*, as it forms pores that result in cytolis, and promotes haemolysis of immune cells (Jost and Billington, 2005). Nonetheless, the precise role of *T. pyogenes* in liver abscesses is not fully understood.

2.20.3 *Salmonella enterica*

Salmonella enterica was previously only isolated from liver abscesses in humans (Qu et al., 2013). However, Amachawadi and Nagaraja (2015) were the first to isolate anaerobic *Salmonella enterica* was from liver abscesses in Holstein steers. From these isolations, 100% of them were a match for the serotype Lubbock. *Salmonella* has been previously isolated from the

lymph nodes of healthy cattle (Arthur et al., 2008), which indicates that the presence in the immune system is systemic to beef cattle. A more recent study was able to isolate different *Salmonella* serotypes, including Agona, Cerro, Give, Lubbock and Muenster, from liver abscesses of cattle from 22 feedlots in three different regions of the United States (Central Plains, Southwest, High Plains) that were slaughtered in 6 different commercial abattoirs (Arizona, California, Colorado and Kansas; Amachawadi et al., 2017). It is widely understood that diets and husbandry methods vary between different regions of the United States due to individually, unique climates. Amachawadi et al. (2017) performed two isolation during the experimental sampling. The first isolation of *Salmonella* was from beef liver abscesses of cattle from the same source, whereas the later experiment that reported a greater diversity had investigated multiple regions, which shows the impact of climate and management based upon region. Weinroth et al. (2017) utilized nonculturable methods to investigate the diversity of the microbial community in the purulent material of liver abscesses from dairy type cattle and beef type cattle. They concluded that feedlot location and supplementation (antibiotic; tylosin) both had an effect on the diversity of the liver abscess communities.

Even though *S. enterica* have been isolated from liver abscesses of bovines, at this time it is not well understood if it is one of the etiologic agents of liver abscesses or how the bacteria travels to the liver. Previous studies state that bacteria may enter the portal blood through rumen lesions (Nagaraja and Chengappa, 1998). *Salmonella* is present in the gut as are other bacteria to be known to be a primary and secondary causative organism, which presents the possibility that the presence of *Salmonella* in the gut could bridge the epithelial layer, enter the portal blood and travel to the liver to initiate infection. *Salmonella enterica* are facultative intracellular pathogens that are capable of inhabiting and colonizing a variety of hosts, as well as, surviving different

environments with fluctuations of oxygen (Amachawadi and Nagaraja, 2015; Bugarel et al., 2015). Therefore, there is a possibility that *Salmonella* plays a large role in the formation of liver abscesses in feedlot cattle. Nevertheless, further research is needed to understand the role of *Salmonella* in liver abscesses.

2.21 Pathogenesis

The manner of the development of liver abscesses has been studied for centuries. In fact, research was performed as early as the 1940's when liver abscesses started to economically impact the meat industry. Smith (1944) was one of the first to observe relationships between lesions in the rumen wall and liver abscesses in cattle. This research was also the first to associate source (geographical region) with liver abscesses of cattle that were fed diets with excess of starch. Later, Jensen (1954a, b) confirmed the correlation between liver abscesses and ruminal pathology which resulted in the term “ruminitis”. Nonetheless, Weiser et al. (1966) found no correlation between the lesions in the rumen and liver abscesses. From these historical correlations, researchers continue to investigate the true pathogenesis of liver abscess (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007). Even though research has not led to a precise answer, the idea that acidotic conditions predispose and leads to liver abscesses is a well-accepted concept (Jensen 1954b, (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007).

Sudden changes of diets from a low energy to a high energy diet, changes in feeding patterns and feeding very little roughage have been associated with ruminitis and the damage to the proactive layer of the rumen (Rowland et al., 1969; Nagaraja and Chengappa, 1998; Elam, 2007). The initial damage of the rumen lining may become drastically aggravated by sharp feed particles, foreign objects in the feed and even hair from licking or grooming incidents (Nagaraja

and Lechtenberg, 2007; Amachawadi and Nagaraja, 2016). Nagaraja and Chengappa (1998) stated that the further damage created by the penetration of foreign objects becomes vulnerable to invasion and colonization by bacteria such as *F. necrophorum.* Since *T. pyogenes* is found to reside on the ruminal wall of ruminants, this may be a portal of entry for *T. pyogenes* and *F. necrophorum* to gain access to the portal blood and eventual infect the liver and cause liver abscesses.

Undoubtedly, the virulence factors of both *F. necrophorum* and *T. pyogenes* play a vital role in infecting the rumen epithelium, eventually allowing the bacteria to enter into the portal blood. Given the interconnectivity of the circulatory system and the blood filtration responsibility of the liver, there is the possibility that these bacteria will eventually end up infecting the liver and forming liver abscesses. The liver is one of the most resilient organs, due to the richly oxygenated environment that contains numerous phagocytic cells (leukocytes and Kupffer cells) as defense mechanisms (Nagaraja and Chengappa, 1998). Thus, an anaerobic organism, such as *F. necrophorum,* is forced to survive the high levels of oxygen and phagocytic mechanism of the liver, in order to colonize and form abscesses. In order to survive *F. necrophorum* relies on virulence factors, such as leukotoxin, which may protect *F. necrophorum* from phagocytosis by leukocytes (Tan et al., 1996). Additionally, *T. pyogenes* assists in the survival of *F. necrophorum,* as it utilizes oxygen creating more favorable anaerobic conditions while also producing lactic acid, which is a major energy substrate of *F. necrophorum* (Tadepalli et al., 2009*)*. Even though the pathogenesis of liver abscess is not well understood, all of these factors may contribute to the development of an environment favorable to bacteria growth and formation of liver abscesses.

2.22 Diagnosis

Being able to diagnose cattle with liver abscesses will benefit the feedlot industry significantly. If producers and veterinarians are able to diagnose cattle at early stages of liver abscess development, interventions can be applied to reduce abscess severity. However, the downfall lies in that cattle with liver abscess do not express any clinical or physiological signs, leading to abscesses only being detected after evisceration of carcass during the slaughtering process (Nagaraja and Lechtenberg, 2007). Not being able to detect liver abscesses presents multiple challenges to producers. To begin with, liver abscesses negatively impact the live animal and carcass performance. In addition, as a method of prevention producers apply antibiotics in the cattle diets which is negatively impacting the consumer's perception. Since liver abscesses cannot be diagnosed until the animal has been eviscerated post-slaughter, multiple techniques to diagnose liver abscesses have been investigated.

One technology that has been investigated is the use of ultrasonography (ultrasound). Ultrasonography works by transferring electrical pulses to high-frequency sound waves. These waves are reflected back and the reflectance of the waves is projected onto a screen, dependent upon tissue density (Houghton and Turlington, 1992). Ultrasound allows us to create non-invasive views of the interior of an animal to gauge the presence of a liver abscess. Nagaraja and Chengappa (1998) stated that the liver is an ideal organ for ultrasonography due to its location and consistency of the tissue. In the past, several researchers have used ultrasonography to visualize soft tissues, including the liver (Lechtenberg and Nagaraja, 1991; Braun, 2009). Saginala et al. (1997) utilized ultrasound to determine that all steers were abscess free prior to being induced with *F. necrophorum* to test the efficacy of a potential vaccine. Although the liver is an ideal organ because of its location and tissue consistency, ultrasonography cannot visualize

the whole liver, as part of the liver is facing the internal cavity and cover by other organs such as the lungs and kidneys (Nagaraja and Lechtenberg, 2007; Braun, 2009). Nevertheless, ultrasound has been able to provide information such as the onset and progression of experimentally induced and naturally occurring abscesses (Lechtenberg and Nagaraja, 1991).

Ultrasound; however, is an expensive technology to use in feedlot cattle. This technology is often deemed an impractical tool due to the speed and amount of cattle processed at a feedlot. Due to its limitation, ultrasound its rarely utilized. Other parameters such as blood metrics have been studied to attempt to early diagnose liver abscesses. When cattle were induced with *F. necrophorum* to prompt liver abscess formation, a decrease in serum albumin concentration was evidence of hepatic dysfunction (Lechtenberg and Nagaraja, 1991). In addition, Doré et al. (2007) noted that complete blood cell counts and serum biochemistry of cattle that have liver abscess were consistent with chronic inflammation. A recent study performed by Macdonald et al. (2017) examined the blood parameters of 29 bulls throughout the finishing phase (only 9 out of 29 had liver abscesses). Animals with liver abscesses had elevated plasma cortisol and aspartate aminotransferase, along with decreased levels of albumin cholesterol and testosterone (Macdonald et al., 2017).

Although methods have been studied to try to predict early onset of liver abscesses, these methods are not widely used, as these methods have their limitations in a feedlot setting, as well as, economic disadvantages. More research is needed to understand possible methods to prevent or diagnose abscesses in cattle

2.23 Prevalence

The prevalence of bovine liver abscesses can have a wide range of variability and can range from 0% to an excess of 95%; however, in recent years rates have been reported from 10%

to 20% (Nagaraja and Lechtenberg, 2007; Brown and Lawrence, 2010; Amachawadi and Nagaraja, 2016). In 2012, the National Beef Quality Audit (NBQA) reported that 20.9% of livers were condemned, specifically with 5.4% of livers containing severe abscesses and 8.3% exhibiting minor abscesses (McKeith et al., 2012). The most recent NBQA also reported similar condemnation values (20.7%) that were associated with liver abscesses; both of which are higher than the 1994 and 1999 NBQA's (Harris et al., 2018). The increase in liver condemnations may be due to an increase in Holsteins audited. Although liver abscess can occur in all types of cattle, they are influenced by a number of factors such as diet (forage type, grain type and amount fed), the number of days on feed, and cattle type and breed (dairy type or beef type), as well as, geographical locations and seasons (Nagaraja et al., 1996b; Reinhardt and Hubbert, 2015). Amachawadi and Nagaraja, (2016) reported that the incidence of liver abscesses is greater in Holsteins fed for beef than beef breeds and greater in steers than heifers among beef breeds. In the same publication, the incidence of liver abscesses based on the Elanco Liver Check is 13.9 % in beef heifers, 16.0 % in beef steers and 28.3% on Holsteins.

Previously, liver abscesses had been associated with only feedlot cattle (beef breeds). However, the feeding industry has started to raise and feed more Holstein steers that are entering the beef production system (Reinhardt and Hubbert, 2015). The most recent NBQA reported that fed Holsteins have become a considerable proportion of the total cattle slaughtered (Eastwood et al., 2017). In addition, cull dairy cows and Holsteins steers fed for beef have a greater proportion of severe liver abscesses (A+). The increase in severity observed in Holstein steers might be related to the increased days on feed, while the increase of incidence in dairy cows might be the result of tylosin free diets being that this antimicrobial is not approved for use in dairy cows (Amachawadi and Nagaraja, 2016). The increased number of days on feed can result in a greater

incidence of acidosis that can damage the integrity of the ruminal wall, resulting in subsequent increases in the probability that liver abscesses develops. The lack of intervention, such as tylosin, in dairy cows might be one of the reasons for the higher incidence of liver abscesses; however, dairy cows experience multiple ration changes annually as they proceed throughout lactation and dry cycles. These factors can also contribute to digestive upsets that lead to the formation of liver abscesses.

Abscesses in the liver can also develop in range cattle that are not fed a concentrated diet prior to slaughter. As reported in The National Market Cow and Bull Beef Quality Audit, liver abscesses accounted for 27.3% of liver condemnations of range cattle (Roeber et al., 2001). The incidence of liver abscesses in non-fed cattle might be due to other factors that lead to damage of the rumen epithelial. Due to the fact that *F. necrophorum* is ubiquitous to the gastrointestinal tract of animals, the bacteria may gain access to the portal blood. Rezac et al., (2014a) cited data from (Nagaraja and Smith, 2000), stating that liver abscesses can form due to traumatic reticuloperotinitis ("hardware disease"). Range cattle are not supplemented with tylosin and graze throughout vast pastures. These pastures may contain debris and other feeds that can create punctures in the rumen epithelial further leading to liver abscesses. Regardless of cattle type or feeding strategies, liver abscesses can still cause financial losses to producers. Hence, there is great critical need to develop alternative feed additives that can help improve efficiency while reducing the incidence of liver abscesses.

2.24 Economic Impact

Liver abscesses are considered abnormalities in the liver that result in livers being condemned under federal inspection. Rezac et al. (2014b) stated that even healed scars from previous liver abscesses may result in condemnation. In the early days of slaughter, monetary

losses were not as serious as they are today. However, Smith (1940) stated that condemnation of livers were a direct loss to the packing industry, due to the resulting lower general prices on live cattle. In addition, the losses due to condemnation are small in comparison to the losses from low performance in feedlot cattle that have liver abscesses. This statement is true today: liver abscesses incur a cost for the packers of over $20 dollars per head, while the producers continue to see a decline in animal performance from cattle that have liver abscesses (Nagaraja et al., 1996b; Brown and Lawrence, 2010).

Liver abscesses can range from 0, A and A+, based on severity, with 0 = No abscesses – a normal, healthy liver, A = one or two small abscesses, (in some cases up to four well-organized abscesses under one inch in diameter and finally A+ = one or more large abscesses are present, with inflammation of liver tissue (Elanco Liver-Check Service, 2019). In the past, Brown et al. (1975) documented liver scores into four categories 0, A-, A, A+. A score of A- represented one or two small abscesses or abscess scars present and a score of A involved two or four well-organized abscesses less than 1 inch in diameter. The latter definition combines A- and A into one category (A).

The impact in performance and gain of the animal depends on the severity of the liver abscesses. The impact can be related to the impact on liver abscess activity as the number of abscesses increase the liver incurs additional tax. In addition, adhesions and the likelihood of an abscess bursting and contaminating other parts of the carcass that will need to be trimmed can also translate to further losses. Nagaraja and Chengappa (1998) theorize that cattle with severe liver abscesses and scored as A+ may require extra carcass trimming as a result of the adhesion of abscesses to the diaphragm and other organs, which may result in the complete condemnation of the entire viscera. Recent work by Rezac et al. (2014b) stated that cattle that had severe liver

abscesses (A+), 43% of them also displayed pulmonary lesions. In addition to packing plants incurring economic losses due to the physical condemnation of the liver, producers also incur losses due to decreased feed efficiency, reduction in feed intake, lower weight gain and ultimately decreased carcass dressing percentage (Nagaraja and Chengappa, 1998). In a review of literature by Nagaraja and Chengappa (1998) multiple studies (Smith, 1944; Wieser et al., 1966) were mentioned that reported no negative performance effects from animals with abscesses. However, Brink et al. (1990) reported a depression of daily gain (11%) and reduction in feed efficiency (9%). The severity of the abscesses can influence the correlation of financial losses. Smaller abscesses (score of A) have minimal effect on cattle performance (rate of gain, feed efficiency) and carcass yield, whereas severe abscesses (score of A+) can significantly reduce cattle performance by reducing feed intake up to 13%, weight gain up to 11.4 %, feed efficiency about 29.5% and carcass weight up to 4.6% (Nagaraja et al., 1996b). Reinhardt and Hubbert, (2015) stated that severe liver abscesses that are associated with adhesion to the internal body-cavity wall of carcass, result in a greater reduction of hot carcass weight (HCW), due to extensive trimming. These adhesions and contaminations cost extra money in labor and product loss due to condemnation. In addition, carcasses that continue through production have lower weights due to the intense trimming resulting in less saleable yield. To further explain the economic impact, Brown and Lawrence (2010) reported decreased carcass weights, 12^{th} rib subcutaneous fat and reduced longissimus muscles of cattle with severe liver abscesses as compared carcasses with normal livers.

Liver abscesses are a concern of the packers and their value depends on the market demand. The meat export federation reports a peak in global beef liver exports in 2014 of 215,000 mt (USMEF, 2014). If liver abscesses continue to be a major reason for

condemnation, packers would continue to experience a significant monetary loss due to fewer livers being exported.

Liver abscesses occur in all types of cattle; however, the incidence and severity are greater in fed Holstein cattle than they are fed beef cattle (Amachawadi and Nagajara, 2016). In recent years fed Holsteins contribute to a higher proportion of cattle being slaughter, which can cause even higher losses to packers as more livers will be condemned (Reinhardt and Hubbert, 2015). This can even result in some packers not slaughtering Holstein type cattle as their losses due to liver abscess and adhesions can cause a significant financial loss. Even though Holsteins play a big role in the food industry, there is not much data quantifying economical losses as a result of liver abscesses.

2.25 Prevention and Control

Liver abscesses can develop in all types of cattle. Unfortunately, the current diagnoses for liver abscesses are at slaughter. As a result, the industry has largely relied on antimicrobial compounds to prevent and control liver abscesses.

2.25.1 Antimicrobials

The rumen is a complex compartment of the digestive tract of ruminant animals and it functionally depends on a variety of microbes. The bacteria that live in the rumen and help break down feed are also found to be causative of liver abscesses. Therefore, the industry has relied on antimicrobials to minimize the incidence of liver abscess in feedlot cattle.

The primary causative organism, *F. necrophorum*, has been reported to be susceptible to tetracyclines, penicillins, and macrolides; however, is resistant to aminoglycosides and ionophores (Baba et al., 1989; Lechtenberg and Nagaraja, 1989). Lundeen (2013) stated in the Feed Additive Compendium, that six antibiotics (chlortetracycline, neomycin sulfate in

combination with oxytetracycline, oxytetracycline, bacitracin methylene disalicylate, viginiamycin, and tylosin) are approved for use in the feed as methods of reducing the incidence of liver abscesses. These antibiotics differ in the inhibition methods of *F. necrophorum* and T. *pyogenes* (Nagaraja and Chengappa, 1998). Of the antibiotics mentioned above, tylosin has been found to be the most successful at combating liver abscesses, thus is the most commonly used in feedlots (Amachawadi and Nagaraja, 2016).

Tylosin phosphate is a macrolide and is believed to have an inhibitory effect on *F. necrophorum* (Nagaraja and Lechtenberg, 2007). Nagaraja and Lechtenberg (2007) cited literature that explained that tylosin is primarily effective against gram-positive bacteria and even though *F. necrophorum* is a gram-negative organism, the macrolide seems to have an effect. Tylosin inhibitory effects on *F. necrophorum* are believed to be in the rumen, liver or both (Nagaraja and Chengappa, 1998). Literature reviews have demonstrated that feeding tylosin reduces liver abscess incidence by 40% to 70% (Brink et al., 1990; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007). The reduction of liver abscesses might be a result of the reduction of *F. necrophorum* in the rumen. However, tylosin does not reduce liver abscesses completely as cattle that are fed tylosin still exhibit 12% to 18% liver abscesses (Reinhardt and Hubbert, 2015). Furthermore, since tylosin is a macrolide and consumers have raised concerns of antibiotic resistance, new alternative methods are being investigated to understand possible efficacy of reducing liver abscesses.

2.25.2 Dietary Management and Acidosis Reduction

Other areas that the beef industry have explored to reduce the incidence of liver abscesses are proper dietary management practices, including proper nutrition and bunk management, as these help to minimize rumen imbalance. Reinhardt and Hubbert, (2015), stated that increasing

course roughage in the total mix ration is an effective method of reducing liver abscesses. Liver abscesses have been alleged to be second to acidosis in the rumen. Acidosis is a result of over-consumption of readily fermented carbohydrates and is typically classified in several form: acute or sub-acute types, often called clinical and sub-clinical, respectively (Owens et al., 1998; González et al., 2012). The severity of acidosis is dependent on the amount, frequency, and duration of grain feeding with varying classifications ranging from acute acidosis (as a result of lactic acid accumulation) to subacute acidosis (due to an accumulation of volatile fatty acids (VFA's) in the rumen) (Nagaraja and Titgemeyer, 2007). Acute and subacute acidosis are measured by the pH of the rumen fluid. Measured pH levels between 5.0 and 5.5 are categorized as subacute acidosis, while a pH value below 5.0 is considered to be acute acidosis (Nagaraja and Titgemeyer, 2007). Since acidosis can compromise the integrity of the rumen wall, feedlots have relied on dietary management practices that can decrease the incidence of acidosis or duration of acidosis. Rumen acidosis has been well known and extensively investigated since grain feeding has become a common practice in feedlot cattle (Owens et al., 1998; Nagaraja and Titgemeyer, 2007; González et al., 2012). Due to the highly energy intensive nature and composition of cereal grains with high readily fermentable carbohydrate of feedlot diets (González et al., 2012), higher amounts of dietary roughage and less processed grains need to be fed to reduce the incidence of acidosis (Owens et al., 1998). Roughages are less fermentable than grains. Inclusion of higher proportions of roughage at an increased particle size increases eating time and increases rumination, leading to more saliva production (González et al., 2012). Saliva contains bicarbonates and phosphates which help to buffer ruminal pH (Bailey and Balch, 1961).

Although the inclusion of roughage can reduce the incidence of acidosis, the inclusion of this feedstuff in feedlot diets still has tradeoffs in beef production. The increased amount of

roughage in the diet results in a less energy dense diet, which will lead to diminished animal efficiency (Owens et al, 1998). Nutritionists in feedlots work to formulate balanced rations that maintain animal efficiency and reduce the incidence of rumen acidosis. The feedlot industry depends on these well-balanced diets and other feed additives to reduce the incidence of liver abscesses. Moreover, the feedlot industry practices strict management protocols, such as gradual adjustment to concentrate diets and feeding/bunk management, which assist in reducing the incidence of acidosis and ultimately liver abscesses. (Schwarz et al., 2001) stated that smaller more frequent feeding and a uniform intake of fermentable carbohydrates resulted in less digestive upsets. Nevertheless, feeding programs and inclusion of roughage are other avenues of approach that might reduce the incidence of rumen acidosis and liver abscess.

2.30 Feed Ingredient Alternatives

Since the approval of antibiotics for growth promotion in the early 1950s, they have been heavily used in intensive feeding operations. Antibiotics have proven to be effective at improving growth, feed efficiency and overall animal performance (Niewold, 2007). However, the emergence and spread of antibiotic resistance have created the need for new options to replace antibiotics. It is important to minimize the use of antibiotics as regulations are becoming stricter. The concern for resistance does not stop at the worry of consuming animal products. Hao et al. (2014) commented that a large portion of the antibiotics consumed by animals is excreted and can create resistance in soil and waterways. A variety of alternatives have been tested including prebiotics, probiotics, vaccines, immune modulators, organic acids, herbal extracts, and phytochemicals as potential alternatives of antibiotics. Prebiotics consists of non-digestible ingredients that, if consumed in sufficient amounts, simulate growth and activity of a number of host microbes in the gut (Uyeno et al., 2015). Probiotics are live microorganisms, that

in order to be considered efficient, must survive, colonize and persist (transiently) in the gastrointestinal tract (Mingmongkolchai and Panbangred, 2018). Some of these alternatives have been found to have other benefits. Uyeno et al. (2015) stated that direct-fed-microbials (DFM) can be fed to animals that are expose to stressful environments to reduce the risk or severity of scours caused by a disturbance of the microflora in the gut.

One of the alternatives is the use of essential oils. Essential oils, such as canola oil and fumaric acid, have variable results in performance in cattle (Beauchemin and McGinn, 2006). The variability may be due to the diverse ecology which resides in the gastrointestinal tract of ruminant animals. Essential oils have been found to have some antibiotic properties that can inhibit the growth of certain microorganisms (Nazzaro et al., 2013). Trombetta et al. (2005) stated that that essential oils are more effective against Gram-positive than Gram-negative bacteria. The effectiveness against Gram-positive might be due to the outer membrane of Gram-positive bacteria being more conducive to allow for hydrophobic compounds to permeate the membrane (Nazzaro et al., 2013). Some essential oils, such as limonene (20 or 100 μg/mL) and thymol (100 μg/mL), are effective as inhibiting *F. necrophorum* growth in an *in vitro* setting (Elwakeel et al., 2013). Even though, some alternatives have shown to be potential alternatives to antibiotics, currently vaccines have the most potential and are the most widely used alternative (Johnston, 2001).

Overall, a variety of products have been tested to determine their efficacy as alternatives for antibiotics in animal agriculture. The results of these tests have been highly variable. In certain studies, specific products have been shown to be beneficial while others have not. Further studies are required to determine the correct doses, administration methods and timing of administration (calf, weaning, stokers, or feedlot). Each of the alternatives that have been tested

focus on altering different aspects of the animal, specifically related to health or growth performance.

2.31 Tannins

Tannins are phenolic compounds and are widely distributed through the plant kingdom (Min et al., 2003). Tannins are classified into two major categories: condensed and hydrolysable (Krueger et al., 2010). They are classified as plants secondary compounds, as well as, saponins. A literature review written by Krueger et al. (2010) stated that term "tannin" was originally given to substances that were able to tan leather but is now used to denote naturally occurring substances of high molecular weight that contain phenolic hydroxylic groups to enable it to form cross-links with proteins.

Since tannins are ubiquitous to plants (Barbehenn and Peter Constabel, 2011) and the diets of ruminants consist of plant based materials, it is important to understand their functionality in the rumen. Tannins in high concentration can be toxic to ruminants and can reduce feed intake, growth, and gastrointestinal tract damage (Hervás et al., 2003). However, at low to moderate concentration, tannins can shift the site of protein degradation resulting in increased absorption of essential amino acids from the small intestine and reduced incidences of frothy bloat in cattle (Barry and McNabb, 1999). Rivera-Méndez et al., (2017) studied the effects of tannin supplementation and found that condensed tannins led to increased weight gain, gain efficiency and dietary net energy. Another benefit of tannin supplementation is the reduction of methane production (Patra and Saxena, 2011; Goel and Makkar, 2012). Reductions in methane can be a result of increased propionic acid, which has been found to favor rumen papillae growth. A marked reduction in methane is beneficial for the beef industry, specifically related to the scrutiny from consumers and others about the industry’s contribution to greenhouse gases.

2.32 Saponins

Saponins are naturally derived detergents found in plants such as *Yucca schidigera* that grows in the arid Mexican desert and *Quillaja saponaria* from a tree that grows in arid areas of Chile (Cheeke, 2000). Saponins contain antifungal and antibacterial properties and are currently used as dietary additives for livestock for ammonia and odor control (Cheeke, 2000). Miyakoshi et al. (2000) reported that saponins from *Yucca schidigera* exhibited anti-yeast activity against brewer's yeast (*Saccharomyces cerevisiae*). *Saccharomyces cerevisiae* is a live yeast that improves milk yield and composition, as well as, rumen digestibility by increasing ruminal cellulolytic bacteria numbers in cattle (Anjum et al., 2018). Additionally, the inclusion of saponin from *Y. shidigera in vitro* decreased ammonia concentration and extended the lag phase of *Streptococcus bovis* (Wallace et al., 1994). Extending the lag phase of S. bovis can reduce the likelihood of pH dropped during peak fermentation. Readily available starch in the diet of cattle allows for *S. bovis* to grow at a faster rate, which increases the amount of lactate production (Russell and Hino, 1985). The increase in lactate production results in a decreased in rumen pH. The reduction of rumen pH can increase the likelihood of rumen acidosis and the integrity of the rumen wall might be compromised. Therefore, the inclusion of saponins can slow down the rate of growth of *S. bovis* reducing the likelihood of rumen acidosis.

2.40 Volatile Fatty Acids in the Rumen

The volatile fatty acids (VFA) utilized for energy in the rumen are short-chain fatty acids. These VFAs are absorbed through the rumen wall and the molecular proportion of each VFA is related to the energy utilization (Cottyn and Boucque, 1968). Volatile fatty acids originate from the bacterial fermentation in the rumen with acetic, propionic, and butyric acids making up roughly 95% of the total VFAs present in the rumen (Peters et al., 1990). Volatile fatty acids

absorbed in rumen make their way to the liver were they undergo gluconeogenesis providing energy to the animal (Wang et al., 2012). Wang et al, (2012) stated that propionate is the most important VFA for gluconeogenesis and the concentration is dependent on propionate-generating bacteria such as *M. elsdenii* and *S. ruminantium*. In lambs that were grazing high-quality pastures, a higher concentration of VFAs was found in the rumen, specifically of the proportion of VFA's propionate and butyrate made up the highest portion (Levy and Marshall, 2004). Propionate production is increased with the inclusion of high grain, low fiber diets (Bauman et al., 1971); however, the increase in propionate can result in lower in milk fat (50% reduction). A decrease in propionic acid to acidic and butyric acid can decrease liver glucose (Miettinen and Huhtanen, 1996). The reduction in liver glucose can result in less energy for the animals to utilize. As the amount of grain increases in the diet, the fermentation capacity of the microbes increases. The increase in fermentation results in a higher proportion of VFAs as microbes convert starches into VFAs. The increase in VFAs can lead to a decrease in rumen pH. The drop in ruminal pH can damage the rumen epithelial allowing access of *F. necrophorum* into the portal vein. Propionate and butyrate can also support ruminal papillae growth (Sander et al., 1959; Shen et al., 2005).

Butyric acid is usually synthesized in the rumen and is an important VFA that helps maintain intestinal integrity (Sander et al., 1959). Sander et al. (1959) found that the inclusion of sodium propionate and sodium butyrate stimulated papillae growth. In a study by Mentschel et al. (2001), the supplementation of propionate and butyric acid increased papillae length 2.2 mm and 4 mm respectively when compared to control length of 1.0 mm. Furthermore, a study by Shen et al. (2005) reported that the addition of n-butyric acid resulted in an increase of the papillae surface of 20% to 40% for steers feed low and high nutritional levels. The increase in

length and total surface area of the rumen papillae by the presence of propionate and butyrate can be of importance to reduce the incidence of liver abscesses. As the production of these VFAs increases, the amount of energy intake by the animal will increase. Additionally, stimulated papillae growth can result in a healthier animal.

2.50 Summary

Liver abscesses have been studied since the 1940s and until this day there is not a definite answer regarding the specific pathogenesis and causative factors of this metabolic disease. With the increased concern of antibiotic resistance, there is a heightened necessity for alternative feed additives that may reduce liver abscesses while also upholding animal efficiency and production. Measuring rumen characteristics, such as VFAs, is a common method of assessing the efficiency and efficacy of different feed additives in a ruminant's diet. Hence, the reason for these experiments. The goal of these experiments are to characterize the rumen microbiota utilizing 16S DNA sequencing and utilize natural feed additives to alter the rumen ecology in a way that can reduce the activity of the bacteria believed to be causative organism without the utilization of antibiotics.

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CHAPTER III

CHARACTERIZATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE RUMEN LINING, DIGESTA, AND RUMEN FLUID FROM BEEF CATTLE CONSUMING A HIGH ENERGY DIET USING 16S rRNA GENE AMPLICON SEQUENCING

SUMMARY

The objective of this study was to affirm the understanding of differing microbiome compositions in three different matrices (fluid, lining, and digesta) of beef cattle consuming a high energy diet using 16S rRNA gene amplicon sequencing. Twelve crossbred feedlot steers (450 kg; ~ 3.0 years of age), fitted with ruminal fistulas, after consuming high energy finishing diets (1.43 Neg, Mcal/kg DM) for 21 d, were utilized in this experiment. Microbial DNA from samples collected from three regions within the rumen (lining [dorsal sac], digesta, and fluid [geometric center of rumen]) was extracted and the V4 region of the 16S rRNA gene was amplified and sequenced. Across all sample regions, bacterial sequences were classified into 34 phyla, 76 classes, 143 orders, and 254 families. Bacteroidetes and Firmicutes were the predominant phyla present across all samples. The relative abundance of Bacteroidetes detected in rumen fluid was lesser ($P < 0.05$) when compared to bacteria from the rumen lining and digesta. In contrast, the relative abundance of Firmicutes were greater ($P < 0.05$) in rumen fluid and the rumen lining when compared to digesta samples. Additionally, the order *Actinomycetales* was found in the rumen fluid and the lining of the rumen, but not the digesta. Our results further affirm that the rumen ecosystem contains a core microbiome; however, within each region different bacteria are present. The presence of *Actinomycetales* have been associated with liver

abscesses. As a result, it is important that the differences in rumen microbial communities are taken into consideration when evaluating the rumen.

Key words: 16S rRNA, rumen, feedlot cattle, microbiome

INTRODUCTION

The bovine rumen microbiome plays an important role in the development of calves as well as wellbeing in adulthood. The rumen's unique and complex microbiome is vital for the animals nutritional development as it allows for conversion of human-indigestible plant biomass into energy for growth and other metabolic processes (Shabat et al., 2016). The high population density, diversity, and complexity all play a role in this digestion and conversion of feedstuffs to energy (Brulc et al., 2009). While the rumen is important for animal wellbeing and growth, the microorganism populations that reside in this organ that can fluctuate due to a variety of factors including dietary changes (Tajima et al., 2000).

Bacteria found in the rumen of cattle consuming high concentrate diets have been associated to liver abscess (Nagaraja and Chengappa, 1998). Although the true etiology of liver abscess is not yet clear, previous researchers have linked certain organisms as causative of liver abscesses; namely *Fusobacterium necrophorum* and *Trueperella pyogenes.* These bacteria are thought to inhabit lesions in the rumen wall and gain access to the liver, ultimately causing an infection (Nagaraja and Chengappa, 1998). However, Weinroth et al. (2017) reported the presence of five phyla of bacteria (*Baceroidetes, Proteobacteria, Fusobacteria, Firmicutes,* and *Actinobacteria*) in active liver abscesses at the time of slaughter—indicating that multiple bacteria serve an important role in liver abscess formation. The beef cattle industry has implemented various approaches to reduce the incidence of liver abscess. However, the most effective tool to reduce incidence of liver abscess is the inclusion of antibiotics in finishing diets.

Antibiotic inclusion in food animals has come under increased concern in regards to the transmission of antibiotic resistant bacteria (Center for Disease Control and Prevention, 2017). Since rumen microbial ecology is diverse and liver abscesses are polymicrobial, understanding microbial ecology in location within the rumen (e.g. rumen lining, digesta, and rumen fluid) is needed. Therefore, this study attempted to characterize the microbial communities in the rumen lining, digesta, and fluid using 16S rRNA gene amplicon sequencing to further understand the complex interplay of the microbiome. Allowing for deeper insight into how the rumen microbiome works synergistically and how it can affect or benefit the host.

MATERIALS AND METHODS

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 16-6550A). Steers were housed at CSU's Agricultural Research, Development and Education Center.

Cattle Population

Twelve crossbred feedlot steers (450 kg; ~ 3.0 years of age) fitted with ruminal fistulas, were utilized in this study. Prior to the sample collection, cattle were adjusted to a high energy finishing diet consisting of 90 % concentrate and 10 % roughage (1.43 NEg, Mcal/kg DM) for 3 weeks (21 d).

Rumen Content Collection

At the completion of the 21d diet adjustment period, three samples were taken via the ruminal fistulas from each steer approximately 1 h after morning feeding: a sample of the aqueous portion of the rumen "fluid", a sample of the bolus "digesta", and a sample of the lining of the wall of the rumen "lining". Rumen fluid was collected by hand-compressing a small amount of rumen digesta collected from the geometric center of the rumen into a 50 ml conical

tube. Rumen lining samples were collected by gently running a 50 ml conical tube across the rumen wall membrane of the dorsal sac. The digesta sample was collected by obtaining approximately 85 grams of digesta from the solid portion of the rumen contents (fiber mat). Once samples were collected, they were immediately placed on ice and transported to the Microbial Ecology Laboratory at Colorado State University for DNA isolation.

DNA Isolation and Sequencing

DNA isolation of fluid samples (0.16 to 0.18 g) was performed with the QIAamp PowerFecal DNA Kit (QIAGEN; Venlo, Netherlands) while lining (2 to 5g) and digesta (5 g) DNA was isolated via DNeasy PowerMax Soil Kit (QIAGEN), both following the manufacture's protocols. DNA concentration was evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) with an average concentration of 235 ng/µl per sample. Each sample was diluted so that 600ng of DNA was present in 30µl of sterile water. Aliquots of DNA from all samples were shipped to Novogene Bioinformatics Technology Company (Chula Vista, CA) for library preparation and sequencing. The V4 region of the 16S rRNA subunit was amplified using the 515F-806R primers pair (Caporaso et al., 2011). Paired-end sequencing (2 x 250 base pairs) was completed on the Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA).

Bioinformatics and Statistical Analysis

Analyses were conducted in QIIME2 v. 2018.11 (Bolyen et al., 2018). Raw reads were imported in to QIIME2 via the 'tools import' command and exact sequence variants (ESV) were classified using the DADA2 plugin (Callahan et al., 2016) with the first 20 base pairs from the 5' end trimmed off and the 3' end and truncated to 228 and 235 for the forward and reverse reads respectively. Phylogenetic assignment was performed using 'feature-classifier classify-sklearn' with a pretrained pre-trained Naïve Bayes classifier trained with the 515-806R primers on the

Greengenes database (DeSantis et al., 2006). Reads that were assigned to mitochondria and chloroplasts were removed from the analysis. For alpha and beta diversity measurements, the data was rarified to 132,775 reads. A rooted phylogenetic tree was constructed using 'align-to-tree-mafft-fasttree' (Price et al., 2010; Katoh and Standley, 2013) for further analysis. The command 'diversity core-metrics-phylogenetic' was used to calculated Faith's diversity for assessment of alpha diversity and weighted and unweighted UniFrac Distances for beta diversity.

Differences in alpha diversity were assessed with a Kruskal-Wallis test while differences in beta diversity were evaluated with a PERMANOVA test. Phylum level differences were investigated with a feature tables collapsed to phyla level with an added Pseudo count using ANCOM.

RESULTS AND DISCUSSION

Across all samples, 5.8 M reads were used in the analysis (average 160.4 K, range 132.8 to 179.0 K). Read number was influenced ($P = 0.03$) by sample location. On average, rumen fluid samples had a greater number ($P < 0.05$) of reads than lining samples; though the number of reads associated with digesta samples were similar ($P > 0.05$) to both fluid and lining samples. The bacterial communities were sequenced to an appropriate depth as indicated by the leveling off of a rarefaction curve (Figure 3.1). Across the entire dataset, 4,447 features (Exact Sequence Variants) belonging to 34 phyla, 76 classes, 143 orders, 254 families were identified. Regardless of sample location or animal, Bacteroidetes and Firmicutes were the predominant phyla present (Table 3.1).

Differences in sampling locations

The microbiome differed in both alpha ($P = 0.002$) and beta ($P = 0.001$) diversity between sampling locations within the rumen, as assessed by Faith's phylogenetic diversity (Fig

3.2A) and weighted (Fig 3.2B) and unweighted UniFrac (not pictured). The rumen fluid had the highest alpha diversity, followed by the digesta and finally the lining. These results are reasonable, as the rumen fluid functions to transport metabolites and growth medium for microorganisms and will therefore contain a combination of all bacteria present in the rumen (Caldwell and Bryant, 1966). When accounting for sampling location, individual animal was a significant ($P < 0.05$) source of variation for microbiome measurements as measured by both weighted and unweighted UniFrac distances. However, there appeared to be a core microbiome that was found in all animals and locations representing genera. This agrees with previous literature, including Jami and Mizrahi, (2012) who reported that 50% of OTUs were common across all tested Israeli Holstein Friesian lactating cows and 30% were in less than 10% of animals.

Variation in rumen composition between individual cattle is multifaceted and could be influenced by a variety of external forces (such as diet, location of feeding, stage of life, source of cattle, seasonality, etc.) as well as the animals own genetics. The variation in rumen microbial ecology might explain the results of Li and Guan, (2017) which explains that certain bacteria in the rumen result in a more or less efficient animal. As we further understand these differences between individual cattle, correlation of commensal bacteria to a disease state (such as liver abscesses) could be determined and allow for more comprehensive treatment options.

Common versus specific microbiome

Of the 493 genera identified, 302 (61%) were identified in all three sample locations collected (Fig 3.3B). Bacteria belonging to the class Alphaproteobacteria and the order Actinomycetales and Clostridiales, as well as *Clostridium perfringens* were identified in both the fluid and lining but not the digesta. McGuirk, (2015) reported that Clostridial organism are

common flora in cattle, and *Clostridium perfringens* occurs widely in the environment and in the gastrointestinal tract of most mammals. Furthermore, the presences of Actinomycetales in the rumen lining is notable as it has been identified in the purulent material of liver abscesses (Weinroth et al., 2017). All sampling locations share the phyla Bacteroidetes and Firmicutes, which suggests that these phyla play an important role in the digestive track of ruminant animals. A highly abundant member in the phylum Bacteroidetes was the family *Prevotellaceae* which has been found to utilize various substrates, such as starch, protein, hemicellulose, pectides and pectin (Carberry et al., 2012). The presence of this bacteria in the rumen may aid in digestion of the high concentrate diets.

IMPLICATIONS

Understanding the rumen microbiome in feedlot cattle continues to be of importance for the cattle industry. The ruminal microbiome is diet and animal dependent. As such, a more thorough understanding of the digestive microbiota and the relative importance of each class of microorganisms may lead to more efficient and sustainable management strategies to improve efficiency and our understanding of the etiology of liver abscess formation. The detected differences in the microbiome present in different locations further demonstrate that even though there is a rumen core microbiome, the different matrices of the rumen contain specific bacteria that have been associated with liver abscesses. These results provide opportunities for future research in the reduction of antibiotic use and prevention of liver abscesses.

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Table 3.1. Phyla that were log fold different across sample locations as determined by an analysis of composition test (ANCOM)

Phyla	**Fluid**	**Lining**	**Digesta**	**SEM[1]**
Bacteroidetes	48.31[b]	61.83[a]	57.92[a]	1.20
Firmicutes	37.88[a]	29.34[b]	22.88[c]	1.14
Archaea, Euryarchaeota	2.37[a]	0.51[b]	0.41[b]	0.26
Spirochaetes	1.54[b]	1.86[b]	2.83[a]	0.20
Planctomycetes	0.46[a]	0.18[b]	0.13[b]	0.05
Lentisphaerae	0.08[b]	0.05[b]	0.21[a]	0.01
Synergistetes	0.04[b]	0.05[b]	0.09[a]	0.00
Cyanobacteria	0.04[b]	0.03[b]	0.16[a]	0.02

[1]Standard Error of the Mean

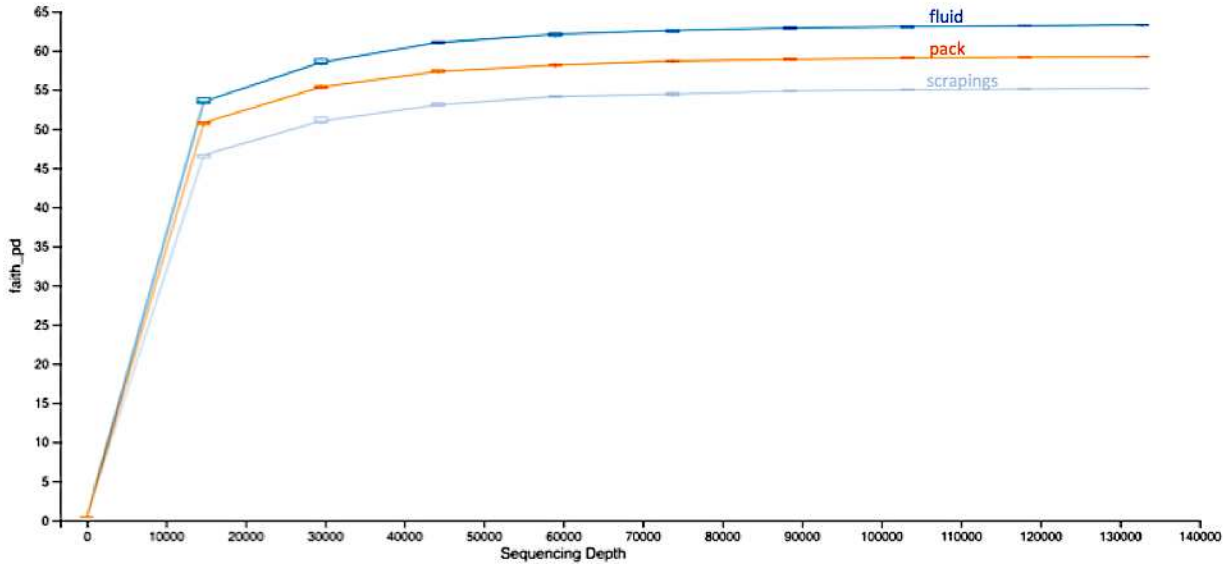

Figure 3.1: Rarefaction curve of all samples grouped by location of collection within the rumen – the leveling off of the curve suggests sampling was conducted at an appropriate depth and additional sampling would not have increased understanding of rumen bacterial biodiversity.

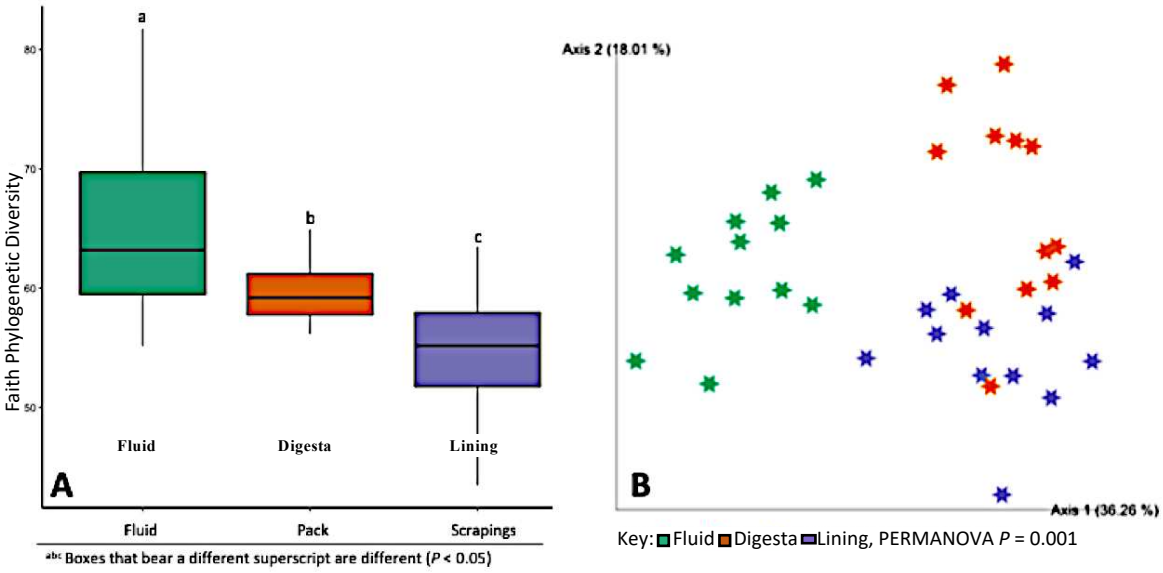

Figure 3.2: (A) Alpha diversity (as measured by Faith's phylogenetic diversity) and (B) beta diversity (as measured by weighted UniFrac distances) colored by location of sample collection across animals. Alpha and beta diversity was different ($P < 0.05$) between all sample types.

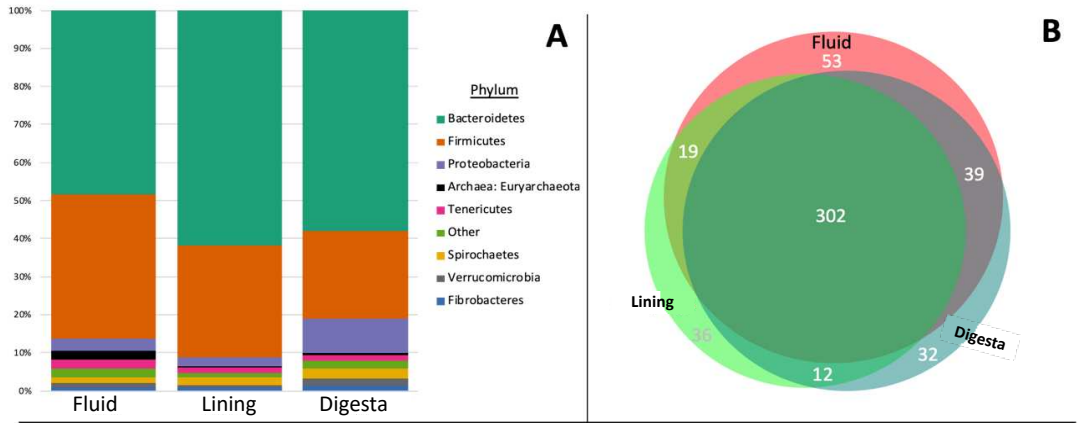

Figure 3.3: (A) Relative abundance of phyla present (note phyla present in less than 1% of the entire microbiome were grouped into "other") in each area of the rumen sampled. (B) Number of genera present in all three, two of the three, or unique to individual location in the rumen.

CHAPTER IV

IN VITRO ASSESSMENT OF NOVEL FEED ADDITIVES ON RUMEN FERMENTATION CHARACTERISTICS AND MICROBIOME

SUMMARY

The objective of this experiment was to evaluate the effects of encapsulated butyric acid and zinc (ButiPEARL™Z), a probiotic (CLOSTAT®500), a novel feed additive (Compound K), and compare them to an antibiotic (Tylan®) on the alteration of rumen fermentation characteristics and the rumen microbial community, *in vitro*. Three crossbred feedlot steers (450 kg; ~ 3.0 years of age), fitted with ruminal fistulas and adjusted to a high energy finishing diet for 21 d, were utilized in this experiment. Rumen fluid was collected and mixed in a 1:1 ratio with McDougall's buffer. A set of vaccine bottles (N = 120; n = 10/treatment) containing 1 g of feed plus dietary treatments were filled with 50 ml of rumen fluid McDougall's buffer solution, sealed and placed in a warm water bath (39˚C). Treatments included: 1) Control (feed + rumen fluid/McDougall's buffer mixture); 2) Tylosin phosphate (Tylan; 90 mg/hd/d); 3) Compound K (K; 30 ppm/hd/d); 4) ButiPEARL™ Z+ CLOSTAT® 500 (BPZ/CLO; BPZ 2lb/T + CLO 0.5g/hd/d; and 5) Combination (K/BPZ/CLO). Samples were collected at 6 h and 12 h post fermentation. Gas samples were measured for N_2, CO_2, and CH_4. The liquid portion was analyzed for volatile fatty acids (VFA) and the pellet fraction was analyzed for microbial protein. Propionic acid production ($P < 0.05$) increased with the inclusion of BPZ/CLO and K/BPZ/CLO at 6 h of incubation. Furthermore, these treatments reduced ($P < 0.05$) butyric acid production. Methane was reduced ($P < 0.05$) at 6 and 12 h for the K/BPZ/CLO treatment compared to

Control and Tylan. In addition, microbial DNA was extracted from the liquid portion and the V4 region of the 16S rRNA gene was amplified and sequenced using a lllumina MiSeq. Similarly, the number of sequence variants was not affected by treatment inclusion ($P > 0.05$). Nonetheless the absence of microbial differences might be due to fermentation peaking at 6 hours as explain by the absence of pH change between 6 h and 12 h. Overall the inclusion of the tested compounds can improve fermentation factors. However, in order to fully understand their effects on the microbiota composition earlier time points should be tested.

Key words: 16S rRNA, rumen, feed additives, feedlot cattle, microbiome

INTRODUCTION

The feedlot industry in the United States utilizes antibiotics, such as tylosin phosphate, to reduce the incidences of liver abscesses. However, the use of antibiotics in feedlot diets have become unwanted because of the concern of antibiotic resistance. Therefore, the Food and Drug Administration (FDA) banned the use of antibiotics as growth promoters in cattle feed and drinking water, so that these products can now only be used for therapeutic purposes under veterinary oversight (Veterinary Feed Directive, 2015). As a result of antibiotics being banned and constant pressure from consumers, the benefits of other feed additives as alternative compounds have been a topic of critical research (Rode et al., 2002; Beauchemin and McGinn, 2006; Dehghani et al., 2019).

For decades, researchers have tried to manipulate the microbial ecology of the rumen to improve production and efficiency, as well as, replace antibiotic use (Burroughs et al., 1960; Linehan et al., 1978; Meyer et al., 2009; Ponce et al., 2011). However, results of these types of experiments have been highly variable. The introduction of culture free approaches has revolutionized research techniques and crafted new ways to investigate the composition,

diversity and structure of bacteria in relation to their host (Gilbert et al., 2014). Due to the complexity of the rumen ecology, culture free approaches can help further our understanding of the interaction of the rumen microbiome on growth performance and health benefits. The microbial ecology is a community of host-associated symbiotic microbes that are suited to help with metabolism and immune function by converting human-indigestible plant biomass into fuel for growth and development (Shabat et al., 2016). Both beneficial and pathogenic bacteria thrive in the gut of healthy animals in a subclinical manner without displaying symptoms. The supplementation of direct-fed-microbials (DFM) and other feed additives in livestock have been studied (Meyer et al., 2009; Ponce et al., 2011; Ferraretto and Shaver, 2015; Mingmongkolchai and Panbangred, 2018) but have provided little information on resulting microbial shifts.

CLOSTAT™ (Kemin Industries, Des Moines, IA) is an anticlostridial probiotic, derived from a strain of *Bacillus subtilis* (PB6), and has been found to inhibit *Clostridium perfringens*. *Clostridium perfringens* is a commensal inhabitant of the rumen (McGuirk, 2015) and is known to cause problems under digestive upsets, favoring the growth of toxins. Hemorrhagic bowel syndrome (HBS) or jejunal hemorrhage syndrome (JHS) is one of the diseases of adult cattle that can be associated with *C. perfringens* (Ceci et al., 2006). CLOSTAT™ has been found to be effective in replacing antibiotics like colistin as shown by an improved growth rates in swine (Nguyen and Carter, 2018). In feedlot cattle, PB6 tended to impact feed efficiency (Kemin, 2019). Other methods used to impact the rumen microbiota involve the inclusion of short chain fatty acids and mineral supplementation. ButiPearl™Z (Kemin Industries, Des Moines, IA) is an encapsulated feed additive containing calcium butyrate and zinc. Supplementation of butyric acid and zinc stimulates intestinal health, rumen papillae growth and cattle performance (Spears and Kegley, 2002; Shen et al., 2005; Ma et al., 2012). Even though the complexes have been shown

to improve performance and alleviate potential illnesses, further research is necessary to understand their mechanisms within a rumen environment. Therefore, the objective study was to utilize a closed *in vitro* system to screen the influence of different feed additives on rumen fermentation characteristics and microbial communities.

MATERIALS AND METHODS

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 16-6550A). Steers were housed at CSU's Agricultural Research, Development and Education Center.

Rumen Content Collection

Three crossbred feedlot steers (450 kg; ~ 3.0 years of age) fitted with ruminal fistulas, were utilized in this study. Prior to sample collection, cattle were adjusted to a high energy finishing diet consisting of 71% concentrate and 20% roughage (1.43 NEg, Mcal/kg DM) for 3 weeks (21 d; Table 4.1). At the completion of the 21-d diet adjustment period, rumen fluid was collected at a single time point, approximately 2 h post feeding, as described by Ward and Spears (1993). Briefly, rumen fluid (~ 4 L) from all three steers was filtered twice through four layers of cheesecloth and combined into one pre-warmed (39°C) thermos. A modified McDougall's (McDougall, 1948) buffer solution (19.60 g $NaHCO_3$, 7.40 g Na_2HPO_4, 1.14 g KCl, 0.94 g NaCl, 0.24 $MgSO_4*7H_2O$ per 2 L H_2O) was mixed with rumen fluid at a 1:1 ratio, simulating saliva production during rumination (Tilley and Terry, 1963). Rumen fluid pH was recorded before and after being mixed with McDougall's buffer solution.

In vitro Chambers

Approximately 2 kg (wet weight) of the high concentrate diet fed to the steers was collected upon discharge from the feed truck and dried in a forced air-drying oven at 60°C for 72

h and ground through a 2.0 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). The ground ration was weighed and dispensed (1.000 ± 0.005 g) into pre-labeled 100 ml vaccine bottles containing the appropriate dose of each treatment. Treatments consisted of: 1) Control (Feed + rumen fluid/McDougall's buffer mixture); 2) Tylosin phosphate (Tylan; 90 mg/hd/d); 3) Compound K (K; 30 ppm/hd/d); 4) ButiPEARL™ Z + CLOSTAT® 500 (BPZ; 2lb/T + CLO 500; 5g/hd/d); 5) Compound K/ ButiPEARL™ Z/CLOSTAT® 500 (K/BPZ/CLO 500). Two sets of 10 vaccine bottles were prepared for each treatment per time point. One set of vaccine bottles was used to evaluate rumen fermentation characteristics at two time points (6 h and 12 h), while the second set was used for microbiome analysis at the same time points (Figure 4.1). Sampling times for rumen fermentation characteristics were set every six hours to determine where the major shifts occur. This process was repeated twice on two different days with different temperatures to account for environmental effect on the rumen fermentation.

The McDougall's buffer/rumen fluid mixture was dispensed into the vaccine bottles (50 ml) containing the pre-weighed feed and treatment. The bottles were capped and sealed immediately after the McDougall's buffer/rumen mixture was dispensed. The vaccine bottles remained sealed to maintain anaerobic conditions and were incubated at 39°C in a circulating water bath for the remainder of the experiment. Vaccine bottles were removed for sampling purposes as required by the experimental design.

Sample Collection

To simulate rumen motility, vaccine bottles were gently swirled every 4 h. Samples were removed at each predetermined time point, gas pressure was recorded, and a 10 ml gas sample was collected and immediately analyzed for nitrogen (N), methane (CH_4) and carbon dioxide (CO_2) concentrations (ml) via gas chromatography. At each collection time, the pH of the

contents of each vaccine bottle destined for analysis of rumen fermentation characteristics was measured. Then, contents of each vaccine bottle were transferred to individual pre-weighed 50 ml conical tubes and centrifuged at 1,000 x *g* at 5°C for 30 min (Beckman Model TJ-6; Beckman Coulter, Indianapolis, IN). A 2 ml aliquot of the supernatant was extracted from the conical tube post centrifugation, acidified with meta-phosphoric acid, and frozen at -80°C until analyzed for volatile fatty acid (VFA) concentrations via gas chromatography. The remaining supernatant was aspirated, and the indigestible residue was dried in a forced air-drying oven at 60°C for 120 h to determine *in vitro* dry matter disappearance (DMD). The second set of paired vaccine bottles were immediately removed at each collection time period and dispensed into 50 ml conical tubes for 16S rRNA gene amplicon sequencing.

Volatile Fatty Acid Analysis

After thawing at room temperature, samples designated for VFA analysis were centrifuged at 28,000 x *g* at 5°C for 15 min and the supernatant was removed and placed into a 1.5 ml gas chromatography vial and analyzed for VFA's. The VFA concentrations were determined via gas chromatography (Agilent 6890N, Santa Clara, CA) fitted with a fused silica capillary column (30 m x 0.25 µm x 0.25 µm) and a flame ionization detector. The following instrument parameters were used: injection mode = split less; injection volume = 1.0 µl; carrier gas = helium; carrier gas flow = 1.0 ml/min; injector temperature = 250°C; oven ramping program = 100°C for 3 min, 185°C for 11 min; detector temperature 250°C.

In vitro Gas Production Analysis

Gas pressure of each vaccine bottle was determined using a digital pressure gauge fitted with a 20-gauge needle (Dwyer Instruments Inc., Michigan City, IN). Gas composition (N, CH_4, and CO_2) was determined by withdrawing 10 ml of gas from the vaccine bottle headspace using a

10 ml syringe. The gas samples were immediately injected into the injection port of a gas chromatograph (Shimadzu GC – 14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector set at 100°C.

Dry Matter Disappearance

The dry matter disappearance (DMD) was determined at 6 h and 12 h by weighing the 50 ml conical tubes prior to dispensing the vaccine bottle rumen contents into the tube. An aliquot of the supernatant was used for VFA analysis, as described above, while the solid undigested material was allowed to dry in a forced air-drying oven at 60°C for 120 h. Once the sample was dry, the conical tube was weighed again to determine the remaining feed that was not digested. The DMD was calculated as follows:

$$DMD, \% = \left(\frac{\text{initial substrate DM mass } - (\text{undigested DM mass} - \text{microbial DM residue mass})}{\text{initial substrate DM mass}}\right) * 100$$

After the DMD% was calculated the microbial growth was calculated to account for true digestibility, by subtracting the amount of microbial growth from each treatment.

Protein Analysis

Protein analysis of the remaining residue post *in vitro* incubation was performed by utilizing a Quick Start™ Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). The dry residue contents from the DMD tubes were ground into a powder with a mortar and pestle. Then 0.1 g was dispensed into a 2 ml microcentrifuge tube and mixed with 1 ml of a 25% sodium chloride (NaCl) solution. The tubes were then placed on a plate shaker for 30 min at room temperature. After 30 min, samples were vortexed for 30 s. The samples were then centrifuged at 1,200 x *g* at 5°C for 15 min. Once centrifuged, 25 µl of supernatant was removed and placed into one well of a 96-well plate that contained 125 µl of a 25% NaCl solution. Each sample assayed

was run in duplicate. A 150 µl portion of the Quick Start™ Bradford assay was added to each of the 96 wells containing samples and a standard ranging from 0 to 90 µg of protein/µl was used to determine protein concentration.

Rumen Fermentation Characteristics Statistical Analysis

This experiment was designed as a randomized complete block design replicated over two days. Data were blocked by hour of sample collection. Least squares means (LSmeans) and pooled standard error of the mean (SEM) were reported for all response variables. Data were evaluated using the Mixed Procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Differences were reported at a significance level of $\alpha = 0.05$.

16S rRNA Amplicon Sequencing

At each time point, artificial rumen samples were removed from the water bath and the content of the bottles was transferred to 50 ml conical tubes and frozen (-20˚C). Once samples were frozen they were transported to the Metcalf lab at Colorado State University and stored at -80°C until DNA extraction. Prior to DNA extraction, samples were thawed at 4°C. A sterile cotton swab (BD SWUBE™ Becton, Dickinson and Company, Franklin Lakes, NJ) was used to obtain a swab of a homogeneous mixture (after inverting of the conical tubes) of the rumen fluid. DNA was extracted from the swab sample using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). DNA concentration was evaluated using Qubit fluorometric quantification (Thermo Fisher Scientific, Inc., Waltham, MA) with an average concentration of 235 ± 5 ng/µl per sample. Library preparation (16S rRNA) was performed and transported to the Colorado State University Microbiology Immunology & Pathology Next Generation Sequencing Illumina Core (Fort Collins, CO) for sequencing. The V4 region of the 16S ribosomal subunit was amplified with the 515f-806r primer set per the Earth Microbiome Project standard protocol

(http://www.earthmicrobiome.org/protocols-and-standards/16s/). The 16S amplicons were sequenced on an Illumina Miseq (Illumina Inc., San Diego, CA) at the Colorado State University Microbiology Immunology & Pathology Next Generation Sequencing Illumina Core (250 x2). Along with biological samples, 7 extraction blanks were included as negative controls and 1 Zymo Mock Community (Zymo Research, Irvine, CA) was included as a positive control per plate.

Bioinformatics and Statistical Analysis

Analyses were conducted in QIIME2 v. 2019.1 (Bolyen et al., 2018). Raw reads were imported into QIIME2 via the 'tools import' command and exact sequence variants (ESV) were assigned using the DADA2 plugin (Callahan et al., 2016) with the first 20 base pairs from the 5' end and the 3' end trimmed. Phylogenetic assignment was performed using 'feature-classifier classify-sklearn' with a pretrained pre-trained Naïve Bayes classifier trained with the 515-806R primers on the Greengenes database (DeSantis et al., 2006). Reads that were assigned to mitochondria and chloroplasts were removed from the analysis. Six of the negative control samples all contained fewer than 114 sequences; a single negative control contained 31037 sequences, but all reads were assigned to a two phylum (Proteobacteria; 94.89 % and Firmicutes; 5.1% which indicated that it was not likely contaminated by adjacent wells and so it was filtered out of the data set. The positive control was used to asses accuracy and reliability of the run. For alpha and beta diversity measurements, the data was rarified to 14,909 reads. A SEPP phylogenetic tree was constructed for further analysis (Janssen et al., 2018). The command 'diversity core-metrics-phylogenetic' was used to calculated Faith's diversity for assessment of alpha diversity (Faith, 1992), and weighted and unweighted UniFrac Distances (Lozupone and Knight, 2005) for beta diversity.

Differences in alpha diversity were assessed with a Kruskal-Wallis test while differences in beta diversity were evaluated with a PERMANOVA test. Phylum level differences were investigated with a feature tables collapsed to phyla level with an added Pseudo count using an analysis of composition of microbiomes (ANCOM; Mandal et al., 2015). Data was block by day to account for variability.

RESULTS

Fermentation Characteristics of 6 h of fermentation

The results of analyses of rumen fermentation characteristics for 6 h of fermentation are presented in Table 4.2. Acetic acid production was similar across treatments. Propionic acid production was greater ($P < 0.05$) for the combination of BPZ/CLO and K/BPZ/CLO when compared to Tylan and Control. Treatment was a significant ($P = 0.0058$) source of variation for butyric acid. Butyric acid concentrates were lower ($P < 0.05$) for BPZ/CLO compared to Control and TYL treatments. However, K/BPZ/Col had similar butyric acid concentration compared to Tylan. Meanwhile, valeric acid concentrations were lesser ($P < 0.05$) in K, BPZ/CLO, and K/BPZ/CLO when compared to Control and Tylan treatments. Dry matter disappearance (DMD) was greater ($P < 0.05$) in Control and K treatments when compared to all other treatments. There were no differences in microbial protein between samples; however, numerical increases were observed in all treatments when compared to Control. The treatments K, BPZ/CLO and K/BPZ/CLO exhibited a lower ($P = 0.0240$) production of methane when compared to Control. Additionally, these treatments also demonstrated a significant ($P < 0.0001$) reduction in total gas production (Table 4.2).

Fermentation Characteristics of 12 h of fermentation

Table 4.3 shows the fermentation characteristics at 12 hours of fermentation. There were no differences ($P > 0.05$) in acetic acid production across treatments. Propionic acid concentrations were greater for K/BPZ/CLO when compared to all other treatments. Moreover, the increase in propionic acid explains the significant reduction in methane by the same treatment. Butyric acid production for Tylan and K/BPZ/CLO was lower ($P < 0.05$) compared to all other treatments. In addition, the effects on DMD were similar to 6 hours of fermentation being that Control and K had a significantly higher DMD than other treatments.

16S amplicon sequence results for 6 h and 12 h of fermentation

Although differences were present in the rumen fermentation characteristics (e.g. propionic acid production increased and there was a reduction in methane production), the microbiome between treatments at both 6 and 12 h did not differ in both alpha ($P > 0.05$) and beta ($P > 0.05$) diversity between treatments, as assessed by Faith's phylogenetic diversity (Figure 2) and unweighted and weighted unifrac distances (Figures 4.3). Moreover, the predominant orders represented (more than 1% of entire microbiome) across treatments were Bacteroidales, Clostridiales, Aeromonadales, Erysipelotrichales, and Bifidobacteriales (Figure 4.4).

DISCUSSION

Rumen Fermentation Characteristics

This study investigated the effects of *in vitro* supplementation of direct-fed-microbials and other feed additives as alternatives to antibiotics, such as tylosin phosphate in beef cattle diets. Many studies have reported increased performance effects with the supplementation of different strains of *Bacillus subtilis* in dairy cattle and ewes (Kritas et al., 2006; Sun et al., 2013). Kritas et al.

(2006) reported that the supplementation of *B. subtilis* increased milk yield, fat, and protein content in ewes. Additionally, Sun et al. (2013) reported increased milk yield and milk components in dairy cattle. However, the strains of *B. subtilis* in the previous studies are not the same strain (PB6) that was used in this study. *Bacillus subtilis* PB6 has demonstrated performance attributes in chickens and pigs, but has not yet been studied in feedlot diets (Kritas et al., 2006;Nguyen and Carter, 2018). Rumen volatile fatty acids (VFAs), DMD, and pH concentrations are the indicators that reflect rumen performance in the present study. Our results showed an increased production of propionic acid for treatments supplemented with PB6 when compared to Control and Tylan, with no effect on pH concentration. Propionic acid is considered as a major glucogenic precursor that increases blood glucose levels for gluconeogenesis (Sauer et al., 1989). Perry et al. (1976) observed an increase in the production of propionate and the requirement of 10% less dry matter per kilogram gain. An interesting find in our experiment was that the *in vitro* supplementation of butyric acid and zinc with the addition of BP6 (BPZ/CLO) decreased the production of butyric acid and DMD, especially at 6 h of fermentation when compared to Control and Tylan. The reduction in butyric acid is not well understood; however, butyric acid fermentation is characteristic of anaerobic bacteria, such a *Clostridium*, which by means of glycolysis produces pyruvate and subsequent ATP via butyric acid fermentation (Ciani et al., 2013). It is possible that the variable results in microbial protein production, VFA production, and the decrease in DMD at 12 h was due to fermentation peaking at 6 h. This theory is supported by pH staying stable at around 5.1 at 6 h and 12 h as well as the lack of nitrogen gas production between 6 h and 12 h of fermentation.

16S amplicon sequences

Regardless of the time or day of collection, *Bacteroidetes* and *Firmicutes* were the predominant phyla present. These phyla have been found to be part of the rumen core microbiome (Petri et al., 2013). The order *Bacteroidales* was majorly composed by the genus *Prevotella*. *Prevotella* has been found to utilize starches, protein, hemicellulose, pectides and pectin (Al-Fataftah and Abdelqader, 2014). These ingredients were readily available for fermentation in the *in vitro* system. The family *Ruminococcaceae* of the order *Clostridiales* was also present in all treatments. *Ruminococcaceae* has been stated to be a probiotic-type bacteria and a butyrate producer (Byerley et al., 2017).

Nevertheless, no significant differences were observed in both alpha and beta diversity between treatments. It was expected that tylosin (a macrolide antibiotic) will alter the microbial diversity. However, no studies have evaluated microbial diversity within 24 h of a subtherapeutic dose of an antibiotic application in animals. In a study by Zaheer et al., (2013) the inclusion of subtherapeutic tylosin for 28 d in the diet had very little effect on *Mannheimia haemolytica* when compared to single injections of therapeutic antibiotics (tilmicosin and tulathromycin). This suggests no differences were observed in alpha diversity for the Tylan may have been due to the subtherapeutic amount and the short amount of time the antibiotic had to adjust the rumen microbial community. In a study by Horton and Nicholson (1980), they reported no differences in feedlot performance were found in cattle supplemented with tylosin, although a reduction in liver abscess was observed.

Feed additives (probiotics/prebiotics) have the ability to modulate the balance of the microbiota of animals. Nonetheless, the functionality of each DFM is dependent on its specific mode of action, application method, and environmental parameters. The lack of differences

found between the feed additives and direct-fed-microbials (DFM) could also be due to the short time duration from application to collection. Antibiotic alternatives, such as DFM, can promote health and optimize productivity in cattle; however, such results are dependent on the product administration, as well as, must exhibit predictability and consistency (McAllister et al., 2011).

CONCLUSION

The use of feed additives, such as Compound K, ButiPEARL™ Z and CLOSTAT® 500, resulted in a significant ($P < 0.05$) increase in the production of propionic acid, reduction ($P < 0.05$) in methane and increase in microbial protein production. However, there were no differences in the microbiome within and between samples. Therefore, further research is needed to determine the appropriate feeding regimen of feed additives that would allow for bacterial shifts. Future metabolism studies with the use of live fistulated cattle is also warranted to determine the extent of the impact of these feed additives on the rumen bacteria and the overall animal performance.

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Table 4.1**.** Ingredients and chemical composition of the basal diet (DM basis).

Item	Percentage
Ingredient Composition (% DM)	
Steam Flaked Corn	61.00
Corn Silage	10.00
Alfalfa Hay	10.00
Dry Distillers Grain (DDG)	10.00
Fat (Tallow)	5.00
Chemical Composition	
Dry Matter, %	69.86
Crude Protein, %	12.90
ADF, %[1]	9.45
NDF, %[2]	16.27
NEg, Mcal/kg[3]	1.43

Macro- and microminerals included in diet: calcium = 0.71%, phosphorus = 0.33%, salt 0.51%, potassium 0.62%, sulfur 0.16%, magnesium 0.18%, zinc 19.50 ppm, iron 169.42 ppm, copper 6.04 ppm, manganese 11.80 ppm, cobalt 0.12 ppm, iodine 0.50 ppm, selenium 0.13 ppm, sodium 0.24%, and chlorine 0.44%

[1] ADF = Acid detergent fiber

[2] NDF = Neutral detergent fiber

[3] NEg = Net energy for growth

Table 4.2. Effects of feed additives on rumen fermentation characteristics at 6 h of *in vitro* fermentation

	Treatment						
	Control	**Tylan**	**Compound K[1]**	**BPZ/CLO[2]**	**K/BPZ/CLO[3]**	**SEM**	***p*-value**
				VFA* Mol/100 mol			
Acetic	31.34^{ab}	30.99^{b}	31.11^{b}	31.84^{a}	31.57^{ab}	0.229	0.084
Propionic	42.87^{c}	43.64^{bc}	45.12^{ab}	45.91^{a}	46.12^{a}	0.555	<0.001
Butyric	17.65^{a}	16.79^{ab}	16.20^{abc}	15.05^{c}	15.37^{bc}	0.516	0.006
Valeric	8.14^{a}	8.58^{a}	7.57^{b}	7.20^{bc}	6.94^{c}	0.159	<0.001
				Proportion of total gas pressure			
% Methane	7.78^{a}	7.49^{ab}	7.04^{b}	6.92^{b}	6.97^{b}	0.213	0.024
% Nitrogen	28.73^{ab}	28.28^{b}	28.70^{ab}	28.83^{ba}	29.59^{a}	0.390	0.223
% CO_2	63.50^{bc}	64.23^{ab}	64.26^{a}	64.25^{a}	63.45^{c}	0.215	0.008
Gas pressure (pa)	163013.00^{a}	157635.00^{b}	155504.00^{b}	151671.00^{c}	146134.00^{d}	1255.87	<0.001
				Fermentation Characteristics			
pH	5.20^{a}	5.16^{a}	5.17^{a}	5.16^{a}	5.23^{a}	0.038	0.627
% DMD**	71.18^{a}	66.73^{b}	70.33^{a}	62.45^{c}	62.33^{c}	0.651	<0.001
MB protein µg/ml***	258.20^{a}	264.55^{a}	263.39^{a}	264.10^{a}	272.28^{a}	7.877	0.801

[1]Compound K (Kemin Inc. proprietary DFM)

[2]ButiPEARL™Z + CLOSTAT®500

[3]Compound K + ButiPEARL™Z + CLOSTAT®500

* Dry Matter digestibility (Total DMD of each treatment was adjusted by subtracting the total microbial growth)

** Volatile fatty acid

***Microbial Protein (determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium chloride; NaCl). Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit.

Significance was recorded at $P < 0.05$

Table 4.3. Effects of feed additives on rumen fermentation characteristics at 12 h of *in vitro* fermentation.

	Treatment						
	Control	**Tylan**	**Compound K**	**BPZ/CLO**	**K/BPZ/CLO**	**SEM**	***p*-value**
				VFA* Mol/100 mol			
Acetic	29.93^{ab}	29.68^{b}	30.18^{ab}	30.28^{ab}	30.35^{a}	0.226	0.217
Propionic	43.28^{c}	44.83^{b}	43.08^{c}	43.71^{bc}	46.81^{a}	0.522	<0.001
Butyric	18.20^{a}	16.63^{b}	18.33^{a}	17.88^{a}	15.56^{b}	0.430	<0.001
Valeric	8.59^{ab}	8.86^{a}	8.41^{ab}	8.13^{b}	7.26^{c}	0.168	<0.001
				Proportion of total gas pressure			
% Methane	8.45^{a}	7.96^{ab}	8.43^{a}	8.34^{a}	7.50^{b}	0.173	<0.001
% Nitrogen	23.67^{b}	23.76^{b}	23.72^{b}	24.54^{a}	25.15^{a}	0.242	<0.001
% CO_2	67.88^{a}	68.28^{a}	67.85^{a}	67.12^{b}	67.35^{b}	0.166	<0.001
Gas pressure (psi)	201679.00^{a}	197176.00^{a}	197969.00^{ab}	193426.00^{b}	182670.00^{c}	1627.33	<0.001
				Fermentation Characteristics			
pH	5.22^{a}	5.10^{a}	5.12^{a}	5.20^{a}	5.13^{a}	0.070	0.719
% DMD**	60.82^{a}	57.07^{b}	60.49^{a}	50.78^{c}	50.73^{c}	0.604	<0.001
MB protein μg/ml***	276.71^{a}	303.73^{b}	313.87^{b}	315.17^{b}	313.51^{b}	5.983	<0.001

[1]Compound K (Kemin Inc. proprietary DFM)

[2]ButiPEARL™Z + CLOSTAT®500

[3]Compound K + ButiPEARL™Z + CLOSTAT®500

* Dry Matter digestibility (Total DMD of each treatment was adjusted by subtracting the total microbial growth)

** Volatile fatty acid

***Microbial Protein (determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium chloride; NaCl). Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit.

Significance was recorded at $P < 0.05$

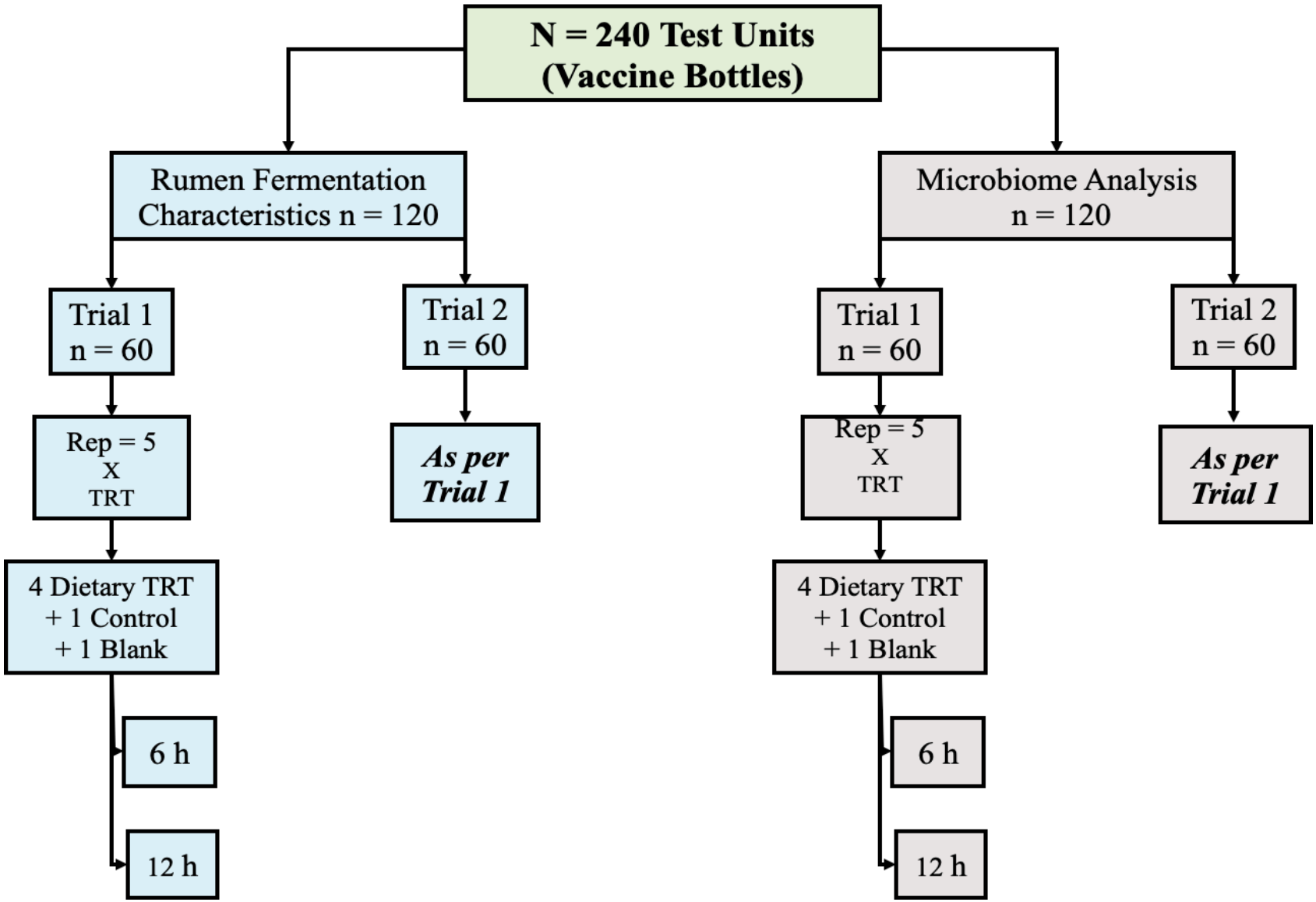

Figure 4.1. Study experimental design.

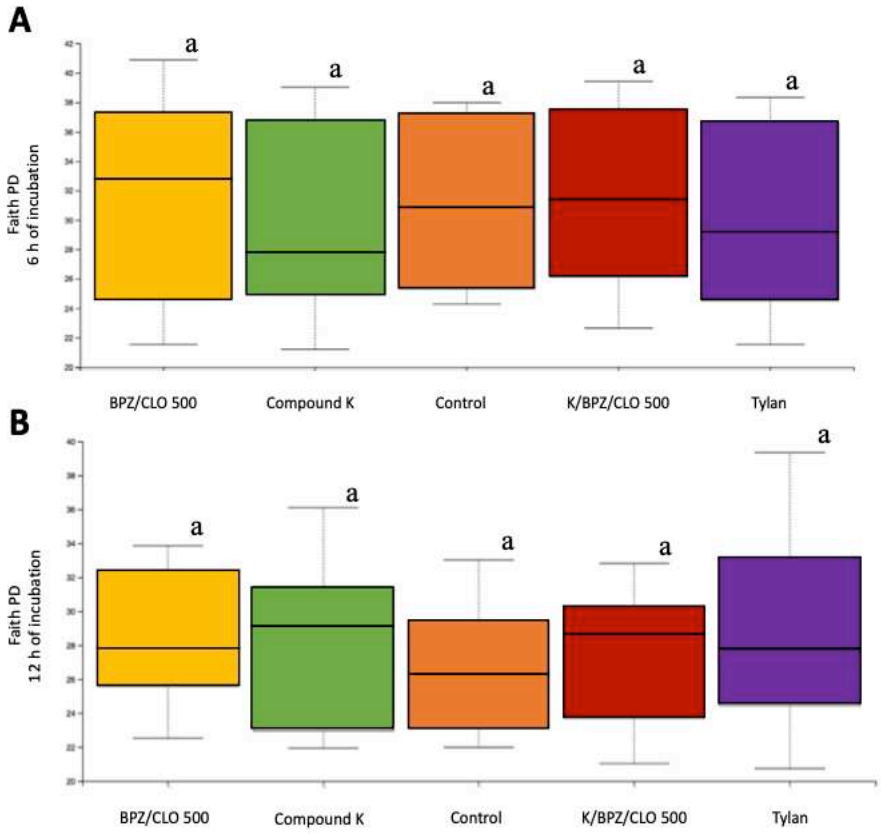

Figure 4.2. Alpha diversity as measure by Faith's phylogenetic diversity Index. Alpha diversity was not significant ($P = 0.945$) at 6 h of incubation **(A)** and not significant ($P = 0.872$) at 12 h of incubation **(B)** between all sample types. Statistical differences for alpha diversity were determine using non-parametric Kruskal-Wallis test.

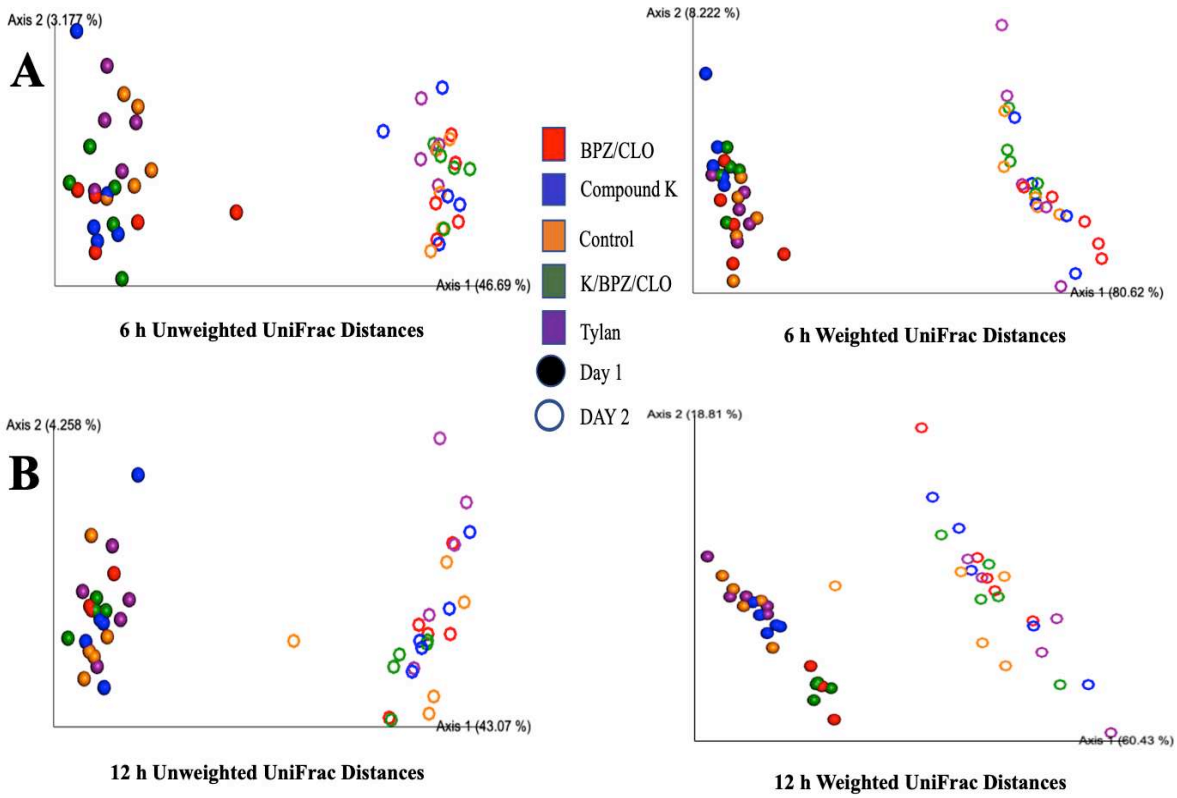

Figure 4.3. Beta diversity or microbial community composition, visualized as principal coordinates analysis ordination bases on 16S rRNA gene sequence by Unweighted and Weighted UniFrac distances color by treatment and shape by day, differed by day (**A** & **B**) but did not differ by treatment group at 6 h of incubation (**A**) and at 12h of incubation (**B**).

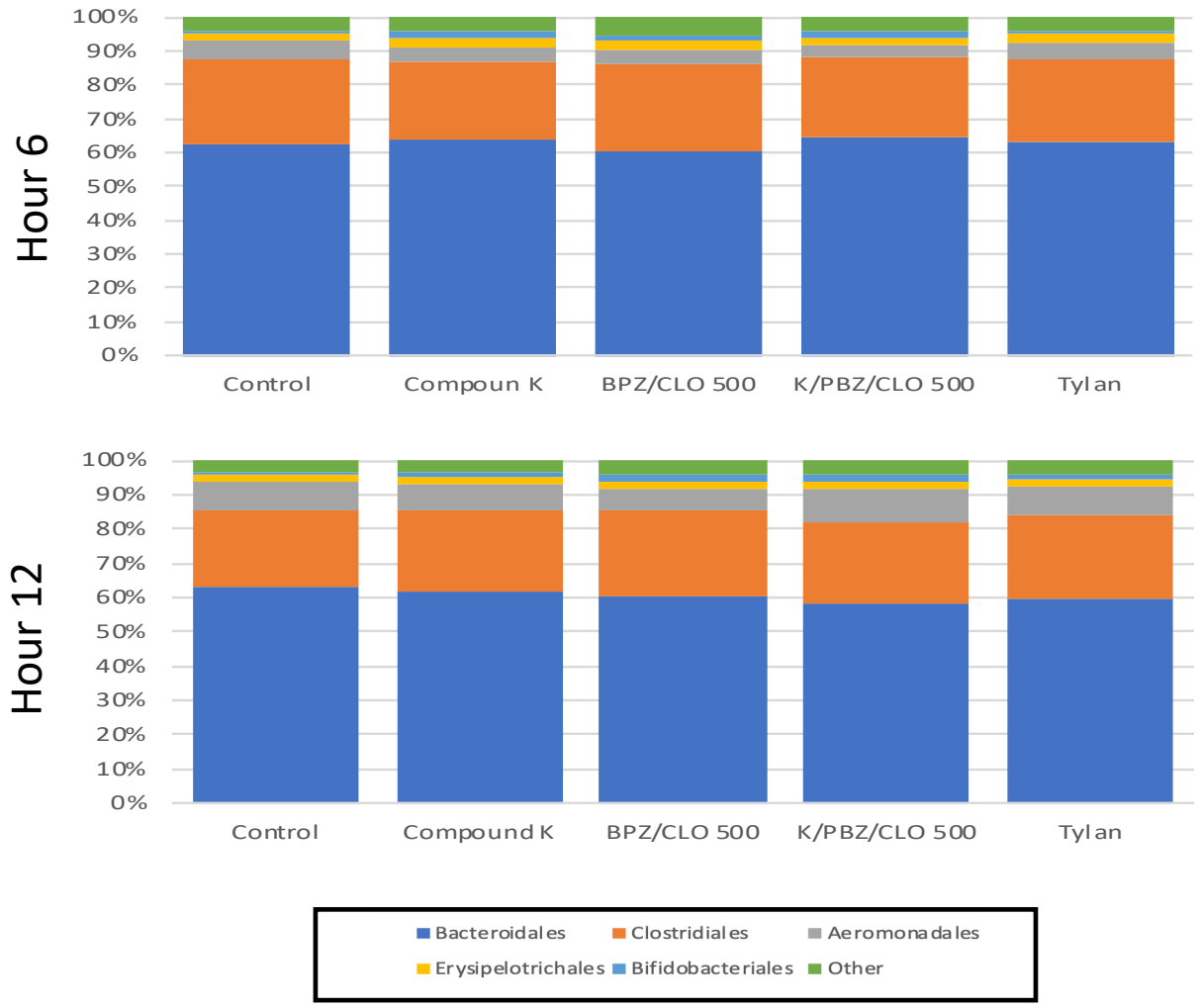

Figure 4.4. Normalize relative abundance of orders of bacteria (combine across treatment day and hour; order present in less than 1% of the entire microbiome were grouped into "other").

CHAPTER V

INFLUENCE OF SAPONINS DOSE, DIRECT-FED-MICROBIALS AND TANNIN DOSE AND SOURCE ON RUMEN *IN VITRO* FERMENTATION CHARACTERISTICS AND MICROBIOTA COMPOSITION

SUMMARY

Two studies were performed to investigate the *in vitro* system efficacy as a method of screening for feed additives, as well as to determine collection time points. In addition, the use of tannins, saponins, and direct-fed-microbials (DFM) as an alternative to antibiotics to reduce liver abscess was evaluated. Rumen fluid was collected from steers fitted with ruminal fistulas and adjusted to a high energy finishing diet for 21 d. Vaccine bottles (experiment A: N = 280, n = 5/treatment, containing 0.500 g of feed plus dietary treatment; experiment B: N = 128, n = 2/treatment, containing 1 g of feed plus dietary treatments) were filled with 50 mL of rumen fluid McDougall's buffer solution, sealed and placed in a warm water bath (39˚C). Experiment A treatments consisted of: 1) Control (Feed + rumen fluid/McDougall's buffer mixture); 2) Condensed tannin (CDl/h; low and high dose; l = 15g/hd/d, h = 30 g/h/d); 3) Hydrolysable tannin (HDl/h; low and high dose; l = 15g/hd/d, h = 30 g/h/d;); 4) Commercial tannin (condensed + hydrolysable; CHl/h; low and high dose; l = 15g/hd/d, h = 30 g/h/d;), while experiment B treatments consisted of: 1) Control (Feed + rumen fluid/McDougall's buffer mixture); 2) Commercial tannin (TCH; condensed + hydrolysable, 15g/hd/d); 3) Saponin (Sap; 2 g/h/d); 4) direct-fed-microbial (DFM; $2x10^{05}$ + $2.50x10^{04}$ colony-forming units (CFU)/g/hd/d of *Propionibacterium acidipropionici* and *Lactobacillus animalis*); 5) Commercial tannin + direct-fed-microbial (TCH+DFM; fed as described above); 6) Saponin + DFM (Sap+DFM; fed as

described above); 7) Commercial tannin + saponin + DFM (TCH+Sap+DFM; fed as described above). Samples were collected at 0 h, 6 h, 12 h, and 18 h post fermentation for both experiments. Gas samples were measured for N_2, CO_2, and CH_4. The liquid portion was analyzed for volatile fatty acids (VFA) and the pellet fraction was analyzed for microbial protein. In experiment A, increased ($P < 0.05$) propionic acid production was observed with the inclusion of tannins regardless of type at 18 h of fermentation. These same treatments reduced ($P < 0.05$) gas pressure at 18 h. In addition, microbial protein was not significant, though a numerical increase was observed for all tannin types at 18 h of fermentation. In experiment B, propionic acid production increased ($P < 0.05$) with all treatment inclusions at 6 h, 12 h and 18 h of fermentation. These same treatments reduced ($P < 0.05$) acetic acid production at 12 h, but no significant effects were seen at 18 h, though a numerical reduction was observed. Methane was reduced ($P < 0.05$) at 12 h and 18 h for Sap, TCH and Sap+DFM treatments compared to Control. In addition, microbial DNA was extracted from the liquid portion and the V4 region of the 16S rRNA gene was amplified and sequenced using a lllumina MiSeq (experiment A) and a lllumina HiSeq (experiment B). Alpha diversity did not differ ($P > 0.05$) for both experiments at 18 h of fermentation. However, there was difference ($P > 0.05$) in beta diversity of the microbial community between treatments at 18 h for experiments A and B. The preliminary results of both experiments demonstrated that the close *in vitro* system is successful at screening for feed additives. Additionally, our results suggested that commercial blend of tannin, hydrolysable tannin, saponin and the combination of this treatments with the direct-fed-microbial offer potential benefits as an antibiotic alternative and that they need to be further tested at 12 h and 18 h.

Key words: feed additives, feedlot cattle, rumen microbiome, saponins, tannins, 16S rRNA,

INTRODUCTION

There has been an interest in decreasing the use of feed grade antibiotics in the cattle feeding industry to prevent production related diseases. The Food and Drug Administration (FDA) has been able to reduce the use of antibiotics by bringing them under the supervision of licensed veterinarians (Veterinary Feed Directive, 2015). The increased regulations and concerns about antibiotic resistance from consumers has led to an increase in the investigation of natural feed additives as alternatives to antibiotics.

Feed additives vary in their chemical compound and in their mode of action. Due to the fact that plants utilize tannins as a method of defense against herbivores via deterrence and/or toxicity (Barbehenn and Peter Constabel, 2011), they can cause a shift in the ruminal microbiome. Tannins are phenolic compounds that are the most abundant secondary metabolites created by plants (Min et al., 2003; Barbehenn and Peter Constabel, 2011). Tannins are derived from multiple plants (i.e. chestnut tree and Quebracho tree), and are classified into two major groups: condensed and hydrolysable (Krueger et al., 2010). Tannins are naturally occurring substances of high molecular weight that contain phenolic hydroxylic groups, which enable the compound to form cross-links with proteins (Krueger et al., 2010). These characteristics also explains tannins high affinity to bind to protein. Due to the ability of tannins binding to protein, their supplementation should improve feed efficiency as protein would not be degraded in the rumen. Low to moderate concentration of tannins can shift the site of protein degradation, resulting in increased absorption of essential amino acids from the small intestine and reduced incidences of frothy bloat in cattle (Barry and McNabb, 1999). In addition, Rivera-Méndez et al. (2017) found that the supplementation of condensed tannins led to increased weight gain, gain efficiency and dietary net energy. Another benefit of tannin supplementation is the reduction of

methane production (Patra and Saxena, 2011; Goel and Makkar, 2012). Improvement in weight gain and reduction of methane can result in beneficial outcomes to the cattle feeding industry as the scrutiny of greenhouse gasses continue, while the demand for protein is growing.

Saponins are naturally derived detergents found in plants, such as *Yucca schidigera*, that grows in the arid Mexican desert and, *Quillajas saponaria*, from a tree that grows in arid areas of Chile (Cheeke, 2000). Saponins provide antifungal, antibacterial, and other beneficial properties that extend the bacterial lag phase, which complement the current use as dietary additives for livestock associated with ammonia and odor control (Wallace et al., 1994; Cheeke, 2000). The antibacterial benefits of saponins can impact the rumen microbial community by acting as a bacterial inhibitor to allow for a reduction of ammonia that further reduces the work load of the liver to serve as a detoxifier via the urea cycle. Furthermore, saponins from *Yucca schidigera* exhibited anti-yeast activity against brewer's yeast (*Saccharomyces cerevisiae*; Miyakoshi et al., 2000). *Saccharomyces cerevisiae* is a live yeast that improves milk yield and composition, as well as, rumen digestibility by increasing ruminal cellulolytic bacteria numbers in cattle (Anjum et al., 2018). Additionally, (Wallace et al., 1994) stated that the inclusion of saponin from *Y. shidigera in vitro* extended the lag phase of *Streptococcus bovis*, a bacteria that is readily available in diets containing ample amounts of fermentable starch. The growth of *S. bovis* can increase the amount of lactate produced (Russell and Hino, 1985). The increase in lactate production results in decreased rumen pH, which can increase the likelihood of rumen acidosis and the likelihood of compromise rumen wall integrity. Therefore, the inclusion of saponins can slow down the rate of growth of *S. bovis,* reducing the likelihood of rumen acidosis.

Direct-fed microbials (DFM) have been reported to enhance animal efficiency by altering ruminal bacterial communities (Krehbiel et al., 2003). (Stein et al., 2006) supplemented a DFM

containing *Propionibacteria* to dairy cows and reported an increase in propionic production. Propionic production has been stated to be the most important substrate of gluconeogenesis (Wang et al., 2012). Even though DFM and plant compounds have been shown to positively benefit the animal, their impacts on the whole rumen microbial ecology are challenging to interpret. As antibiotic resistance continues to be a factor by which consumers make food choices due to perceived human health concerns, new technologies that allow us to explore unculturable bacteria can allowed for new discoveries of alternatives that can prevent liver abscesses and antibiotic use. Therefore, the objective of this study was to evaluate the effects of saponins, tannins and DFM on ruminant volatile fatty acid production and ruminal microbiota composition *in vitro* utilizing 16S rRNA gene amplicon sequencing.

MATERIAL AND METHODS

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 16-6550A). Steers were housed at CSU's Agricultural Research, Development and Education Center (ARDEC).

Treatments for Experiment A (all tannin types and doses):

1. Control = Feed + rumen fluid/McDougall's buffer mixture (25% rumen fluid:75% McDougall's buffer)
2. CHl = Condensed + Hydrolysable Tannin: Normal Dose (15 g/hd/d)
3. CHh = Condensed + Hydrolysable Tannin: High Dose (30 g/hd/d)
4. CDl = Condensed Tannin: Normal Dose (15 g/hd/d)
5. CDh = Condensed Tannin: High Dose (30 g/hd/d)
6. HDl = Hydrolysable Tannin: Normal Dose (15 g/hd/d)
7. HDh = Hydrolysable Tannin: High Dose (30 g/hd/d)

Treatments for Experiment B (all treatment combinations):

1. Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)
2. DFM = Direct-fed-microbial; *Lactobacillus animalis* ($2x10^{05}$ colony forming units (CFU)/g/hd/d) + *Propionibacterium acidipropionici* ($2.50x10^{04}$ colony forming units (CFU)/g/hd/d)
3. Saponin = Yucca *shidigera* extract (2 g/hd/d)
4. TCH = Commercial tannin (Condensed + Hydrolysable) (15 g/hd/d)
5. Sap+DFM = Saponin + DFM (dose as above)

6. TCH+DFM = Commercial tannin (Condensed + Hydrolysable Tannin) + DFM (dose as above)
7. TCH+Sap+DFM = Commercial tannin + Saponin + DFM (dose as above)

Rumen Fluid Collection

Three crossbred feedlot steers (680 ± 10 kg; approximately 7 years of age) fitted with ruminal fistulas, were adjusted to a high concentrate finishing diet for three weeks prior to sample collection (Table 5.1). After this adjustment period, rumen fluid was collected at a single time point, approximately 2 h post feeding, as described by (Ward and Spears, 1993). Briefly, rumen fluid (~ 4 L) from all three steers was filtered twice through four layers of cheesecloth and combined into one pre-warmed (39°C) thermos. A modified McDougall's (McDougall, 1948) buffer solution (19.60 g $NaHCO_3$, 7.40 g Na_2HPO_4, 1.14 g KCl, 0.94 g NaCl, 0.24 $MgSO_4*7H_2O$ per 2 L H_2O) was mixed with rumen fluid at a 1:3 and 1:1 ratio for experiment A and B respectively, simulating saliva production during rumination (Tilley and Terry, 1963). Rumen fluid pH was recorded before and after being mixed with McDougall's buffer solution.

In vitro Chambers

Approximately 2 kg (wet weight) of the high concentrate diet fed to the steers was collected upon discharge from the feed truck and dried in a forced air-drying oven at 60°C for 72 h and ground through a 2.0 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). The ground ration was weighed and dispensed (0.500 ± 0.005 g for experiment A, and 1.000 ± 0.005 g for experiment B) into pre-labeled 100 ml vaccine bottles containing 1.0 ml of each of the appropriate dietary treatments. For experiment A, two sets of 5 vaccine bottles were prepared for each treatment per time point; one set of 5 vaccine bottles was used to evaluate rumen fermentation characteristics at four time points (0, 6, 12, 18 h; N = 20), while the second set of 5 was used for microbiome analysis at two time points (0 and 18 h; N = 10) (Figure 5.1).

Moreover, for experiment B, two sets of 2 vaccine bottles were prepared for each treatment per time point; one set of 2 vaccine bottles was used to evaluate rumen fermentation characteristics at four time points (0, 6, 12, 18 h; N = 8), while the second set of 2 vaccine bottles was used for microbiome analysis at three time points (0, 12, 18 h; N = 8) (Figure 5.2). The number of vaccine bottles was different per experiment due to the number of treatments and times points which increase the number of total bottles.

The McDougall's buffer/rumen fluid mixture was dispensed into the vaccine bottles (50 mL) containing the pre-weighed feed and treatment. The bottles were capped and sealed immediately after the McDougall's buffer/rumen mixture was dispensed. The vaccine bottles remained sealed to maintain anaerobic conditions and were incubated at 39°C in a circulating water bath for the remainder of the experiment. Vaccine bottles were removed for sampling purposes as required by the experimental design.

Sample Collection

To simulate rumen motility, vaccine bottles were gently swirled every 4 h. Samples were removed at each predetermined time point per the experiment being conducted (i.e., experiment A and B) and the gas pressure was recorded, and a 10 ml gas sample was collected and immediately analyzed for nitrogen (N), methane (CH_4) and carbon dioxide (CO_2) concentrations (ml) via gas chromatography. At each collection time, the pH of the contents of each vaccine bottle destined for analysis of rumen fermentation characteristics was measured. Then, contents of each vaccine bottle were transferred to individual pre-weighed 50 ml conical tubes and centrifuged at 1,000 x *g* at 5°C for 30 min (Beckman Model TJ-6; Beckman Coulter, Indianapolis, IN). A 2 ml aliquot of the supernatant was extracted from the conical tube post centrifugation, acidified with meta-phosphoric acid, and frozen at -80°C until analyzed for

volatile fatty acid (VFA) concentrations via gas chromatography. The remaining supernatant was aspirated, and the indigestible residue was dried in a forced air-drying oven at 60°C for 120 h to determine *in vitro* dry matter disappearance (DMD). The second set of paired vaccine bottles were immediately removed at each collection time period and dispensed into 50 ml conical tubes for 16S rRNA gene amplicon sequencing.

Volatile Fatty Acid Analysis

After thawing at room temperature, samples designated for VFA analysis were centrifuged at 28,000 x *g* at 5°C for 15 min and the supernatant was removed and placed into a 1.5 ml gas chromatography vial and analyzed for VFA's. The VFA concentrations were determined via gas chromatography (Agilent 6890N, Santa Clara, CA) fitted with a fused silica capillary column (30 m x 0.25 µm x 0.25 µm) and a flame ionization detector. The following instrument parameters were used: injection mode = spitless; injection volume = 1.0 µl; carrier gas = helium; carrier gas flow = 1.0 ml/min; injector temperature = 250°C; oven ramping program = 100°C for 3 min, 185°C for 11 min; detector temperature 250°C.

In vitro Gas Production Analysis

Gas pressure of each vaccine bottle was determined using a digital pressure gauge fitted with a 20-gauge needle (Dwyer Instruments Inc., Michigan City, IN). Gas composition (N, CH_4, and CO_2) was determined by withdrawing 10 ml of gas from the vaccine bottle headspace using a 10 ml syringe. The gas samples were immediately injected into the injection port of a gas chromatograph (Shimadzu GC – 14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector set at 100°C.

Dry Matter Disappearance

The dry matter disappearance (DMD) was determined (18 h for experiment A and B) by weighing the 50 ml conical tubes prior to dispensing the vaccine bottle rumen contents into the tube. An aliquot of the supernatant was used for VFA analysis, as described above, while the solid undigested material was allowed to dry in a forced air-drying oven at 60°C for 120 h. Once the sample was dry, the conical tube was weighed again to determine the remaining feed that was not digested. The DMD was calculated as follows:

$$DMD, \% = \left(\frac{\text{initial substrate DM mass } - (\text{undigested DM mass} - \text{microbial DM residue mass})}{\text{initial substrate DM mass}}\right) * 100$$

Protein Analysis

Protein analysis of the remaining residue post *in vitro* incubation was performed by utilizing a Quick Start™ Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). The dry residue contents from the DMD tubes were ground into a powder with a mortar and pestle. Then 0.1 g was dispensed into a 2 ml microcentrifuge tube and mixed with 1 ml of a 25% sodium chloride (NaCl) solution. The tubes were then placed on a plate shaker for 30 min. After 30 min, samples were vortexed for 30 s. The samples were then centrifuged at 1,200 x *g* at 5°C for 15 min. Once centrifuged, 25 µl of supernatant was removed and placed into one well of a 96-well plate that contained 125 µl of a 25% NaCl solution. Each sample assayed was run in duplicate. A 150 µl portion of the Quick Start™ Bradford assay was added to each of the 96 wells containing samples and a standard ranging from 0 to 90 µg of protein/µl was used to determine protein concentration.

Rumen Fermentation Characteristics Statistical Analysis

Experiment A and B were designed as a randomized complete block design replicated over two days. Data were blocked by day of the experiment. Least squares means (LSmeans) were reported for all response variables of both experiment A and B with treatment contrasts

reported in experiment A. Least square means (LSmeans) and pooled standard error of the mean (SEM) were reported for all response variables in experiment B. Data were evaluated using the Mixed Procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Differences were reported at a significance level of $\alpha = 0.05$.

16S rRNA Amplicon Sequencing

At each time point, artificial rumen samples were removed from the water bath and the content of the bottles was transferred to 50 ml conical tubes. The samples were placed on ice and transported to a -80°C freezer until DNA extraction. Prior to DNA extraction, samples were thawed at 4°C. A sterile cotton swab was used to obtain a swab of a homogeneous mixture (after inverting of the conical tubes) of the rumen fluid, and DNA was extracted from the swab sample using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). After DNA extraction, qualified DNA libraries from each sample were either transported to the Metcalf lab and Colorado State University Microbiology Immunology & Pathology Next Generation Sequencing Illumina Core (Fort Collins, CO; for samples collected from experiment A) or Novogene Corporation (Beijing, China; for samples collected from experiment B) to perform 16S rRNA library preparation and sequencing. The V4 region of the 16S ribosomal subunit was amplified with the 515f-806r primer set per the Earth Microbiome Project standard protocol (http://www.earthmicrobiome.org/protocols-and-standards/16s/). Paired-end sequencing (2 x 250) was either conducted on an Illumina MiSeq (Illumina Inc., San Diego, CA; for experiment A) or on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA; for experiment B).

Bioinformatics and Statistical Analysis

Amplicon samples were processed through the QIIME2 (2018.8) pipeline (Caporaso et al., 2010). Samples were demultiplexed and assigned exact sequence variance (ESV) using the

DADA2 plugin with primers and lower quality ends trimmed off (Callahan et al., 2016). Multiple sequence alignment of the sequences were completed with MAFFT and filtered to remove highly variable positions (Katoh and Standley, 2013). FastTree 2 was used to construct and root a phylogenetic tree (Price et al., 2010). Taxonomic classification was conducted using a pretrained Naive Bayes classifier trained on the Greengenes database trained with a dataset trimmed using the 515f/806r primers (DeSantis et al., 2006). Reads assigned to mitochondria and chloroplasts were removed from downstream analysis, as well as those reads that did not have an assignment to phylum.

Alpha diversity was assessed via Faith's Phylogenetic Diversity and beta diversity was measured using weighted UniFrac distances. Alpha diversity was compared using the lm and ANOVA functions in R and compared using the CLD function from the emmeans package. Beta diversity was evaluated using the PERMANOVA puggin in QIIME2. Differential abundance was conducted at the aggregated phylum level via ANCOM (Mandal et al., 2015). In all comparisons, $\alpha = 0.05$ and an FDR adjustment was used when appropriate.

RESULTS

Experiment A

Fermentation characteristics

Results describing the influence of tannin type and dose are shown in Table 5.2 and 5.3. No differences were observed for propionic acid production at 2 h and 12 h of fermentation (Table 5.2). A significant ($P = 0.003$) difference in propionic acid production was observed by treatment at 18 h. There was a significant reduction ($P < 0.05$) in methane production for CDh with 10.11 mL and HDl with 10.14 mL when compared to the Control with 12.77 mL (Table 5.3). HDl also exhibited the highest pH at 18 h. In addition, HDl had a lower gas pressure and

higher ($P < 0.05$) microbial protein production at 18 h of fermentation when compared to the Control (Table 5.3). Furthermore, dry matter disappearance (DMD) was significantly higher ($P < 0.05$) for CDh over Control and other tannin treatments.

Microbiome analysis at 18 h of fermentation

Microbiome data for rumen samples at 18 h, including Tannin combinations (CH1/h, CDl/h, HDl/h) and the Control, which included only rumen fluid and feed, were compared. Beta-diversity (comparison of microbial community composition) between the different treatment groups revealed clustering of samples by treatment ($P = 0.001$; Figure 5.3). These treatments, particularly HDl, resulted in rumen microbial communities that differed in proportion or presence/absence of microbes. Alpha diversity was not statistically ($P = 0.19$: Figure 5.4) different between treatments, meaning that although the composition of the microbial communities differed (beta diversity) between treatments, there was not one treatment that had measurably more or less numbers of types of microbes (alpha diversity). Across samples *Bacteroides*, *Firmicutes*, and *Proteobacteria* dominated the microbial community (Figure 5.5). Taxonomy plots allowed for the visualization of microbial composition between treatments that was captured by beta diversity statistical approaches. For example, at 18 h, CHl had a numerically lower amount of *Proteobacteria* and HDl had the numerically highest amount when compared to other treatments, 11.99% and 28.68% respectively (Table 5.4). The treatment of HDl appears to cluster away from the other treatments as seen by the beta diversity pattern (Figure 5.3). Based on these results, it was decided to further test HDl (hydrolysable tannin at a normal dose), along with CHl (commercial blend between hydrolysable and condensed tannin at a normal dose).

Experiment B

Fermentation characteristics

Propionic acid and butyric acid production increased ($P < 0.05$) at 12 h of fermentation for all treatments when compared to Control (Table 5.5). Acetic acid production decreased ($P < 0.05$) for all treatments at 12 h of fermentation when compared to Control. Moreover, at 12 h of fermentation, the total VFA production increased ($P < 0.05$) by all treatments when compared to Control. Additionally, no changes in pH ($P > 0.05$) were observed for any of the treatments (Table 5.6). All treatments had a lower ($P < 0.05$) nitrogen production at 18 h, with the exception of Sap+DFM that did not have any readable values and TCH+DFM that exhibited higher nitrogen production ($P < 0.0001$; Table 5.6). In addition, methane production was reduced ($P < 0.05$) for all treatments at 12 h in relation to Control. The same reduction ($P < 0.05$) in methane for all treatments was observed at 18 h of fermentations, with the exceptions of DFM that was numerically higher at 14.98 mL when compared to Control at 14.79 mL. Moreover, DMD was increased ($P < 0.05$) for all treatments, especially Sap when compared to the Control (Table 5.6). An increased ($P < 0.05$) in CO_2 production was observed for all treatments, except TCH when compared to Control.

Microbiome analysis

The microbiome was analyzed at 0, 12, and 18 h of fermentation; however, only the 18 h fermentation results were analyzed in depth. These data are shown in Figures 5.6, 5.7, 5.8, 5.9, 5.10 and Table 5.7. The treatments were compared back to the Control, which consisted of just rumen fluid and feed to determine shifts in the microbiome (Figure 5.6). Beta diversity (comparison of microbial community composition) revealed differences between the treatment groups ($P = 0.021$; Figure 5.7) with TCH+DFM samples visibly separating from other samples

in the experiment. When looking at alpha diversity, while there was no statistical difference (P = 0.11), TCH+DFM showed visibly lower alpha diversity (fewer types of microbes) which likely contributed to its distinctive beta diversity pattern (Figure 5.8). The taxonomy composition for experiment B varied in abundance and can be seen in Figure 5.9 and Table 5.7. TCH+DFM and Sap were observed to have a higher percentage of the phyla *Firmicutes* and *Actinobacteria* present when compared to the rest of the treatments. The treatment of TCH had the lowest percentage of the phylum *Cyanobacteria*. In addition, with the acknowledgement of a small sample size and lack of formal testing, a subjective microbiome shift was observed between 0 h, 12 h, and 18 h of fermentation within treatments (Figure 5.10).

DISCUSSION

The objective of both experiments was to evaluate the effects of alternative feed additives on rumen fermentation parameters and microbiota *in vitro* as possible substitutes for antimicrobials. Experiment A was conducted as a pilot study to determine the type of tannins to test in further experiments, while experiment B was conducted to determine ideal time points for collections. Past studies have shown some promise in terms of positively altering the rumen as a result of alternative feed additives. This study also demonstrated changes in the rumen through several different metrics as a result of feed additives.

In experiment A, CDh and HDl resulted in the reduction of methane, as well as, an increase in propionic acid production while maintaining a steady acetic acid production as compared to the Control at 18 h of fermentation. This shows that CDh or HDl may be beneficial in reducing greenhouse gases and improving fermentation efficiency. The reduction of methane is also consistent with the reduction found by Pellikaan et al. (2011) in an *in vitro* study where condensed and hydrolysable tannins were used. Furthermore, CDh had a greater DMD as

compared to the other treatments, which may indicate improved feed conversion potential. Barry and McNabb (1999) concluded that forages that contain condensed tannins can increase efficiency in protein digestion and increase animal productivity. CDh also had the least nitrogen production of all the treatments. This reduction is in agreement with McSweeney et al. (2001) that describes that the use of a condensed tannins complex with protein resulted in a reduction of available nitrogen for the rumen microorganisms. The relationship between dosage and tannin types observed in this experiment suggest that both condensed and hydrolysable tannins play a role in altering rumen fermentation characteristics.

Treatment differences of rumen microbiota were observed. At 18 h, CHl had a lower relative abundance of taxa in the *Proteobacteria* phylum and HDl had the higher relative abundance of *Proteobacteria* when compared to other treatments. These differences were driven mainly by the order *Aeromonadales* and *Desulfovibrionales*, which accounted for 50% and 14% of the *Proteobacteria* phyla, respectively. Taxa in the diverse *Proteobacteria* phylum dominate many free-living (non-host associated) environments, such as soils and freshwater, as well as, associated with plants and in the oral cavity of mammals (Costello et al., 2009; Lauber et al., 2009; Redford and Fierer, 2009; Pascault et al., 2014). *Proteobacteria* is also a common phyla that contributes to the rumen core microbiome (Petri et al., 2013). It is important to note that *Proteobacteria* in the gastrointestinal tract are facultative anaerobes (Shin et al., 2015). This is imperative as the etiology of liver abscesses involves facultative anaerobes to decrease the reductive oxidation reaction to allow *Fusobacterium necrophorum* (obligate anaerobe) to colonize the liver (Nagaraja and Chengappa, 1998).

Sequences matching the *Actinobacteria* phylum, which includes the family *Actinomycetaceae* and species, such as *Trueperella pyogenes*, were detected. *Trueperella*

pyogenes have been previously associated as bacteria that are thought to cause liver abscesses (Nagaraja and Chengappa, 1998). However, no genus level *Trueperella* was identified. Other microorganism previously linked to liver abscesses, such as the *Fusobacterium* genus, were not identified for any of the treatments, including the Control. The lack of presence of the *Fusobacterium* and *Trueperella* genera may be due to the small sample size.

For experiment B, the production of propionic acid was significantly higher for 2 h, 6 h, and 12 h of fermentation and numerically higher for 18 h of fermentation for all treatments when compared to the Control. This fluctuation in propionic acid demonstrates more favorable rumen digestion. The increased production of propionic acid is beneficial for the animal energy metabolism. Propionic acid has been defined as the most important VFA in the rumen, due to its critical role as a gluconeogenesis substrate (Wang et al., 2012). The DFM treatment had a numerically greater difference in propionic acid as compared to the Control at 18 h of fermentation, which is expected as DFM improves microbial efficiency. The increase in propionate can also be due to the DFM being a mixture of *Lactobacillus animalis* and *Propionibacterium acidipropionici.* Supplementing *Propionibacterium* to cattle has demonstrated an increase in propionic acid production and a decrease in the acetate: propionate ratio (Stein et al., 2006). Additionally, treatments that included saponin resulted in a statistical increase in gas pressure at 12 h, proceeded by a gradual increase until 18 h of fermentation. These results are consistent with Wang et al. (2000), who reported that saponin extracted from yucca increased gas production from a highly fermentable diet during the first 10 h of *in vitro* incubation. Dry matter disappearance was greater for all treatments, especially saponin when compared to the Control. The improvement in DMD is critical to digestion, as it shows that the

inclusion of these dietary treatments could improve feed conversion. It has been reported that *in vitro* DMD is increased by the inclusion of saponin (Wang et al., 2000).

For experiment B, TCH+DFM was strikingly different than the other treatments with a lower alpha diversity and distinct beta diversity. This is of particular interest as antibiotic treatments are also known to decrease alpha diversity of a microbial community while altering beta diversity (Looft et al., 2012). Furthermore, all treatments also included sequences matching the *Actinobacteria* phylum. The treatments TCH and Saponin had the highest hits for *Actinobacteria*. This is critical as *Trueperella pyogenes* have been previously associated as causative bacteria in liver abscesses due to being a facultative anaerobe (Nagaraja and Chengappa, 1998). While the *Trueperella* genus was not identified in these samples, the family *Actinomycetaceae* was, which *Trueperella* belongs to; however, due to sequence homology *Actinomycetaceae* could not be further classified (meaning their genus remains unknown). The lower number of samples did not allow us to make significant conclusions and further testing is required.

CONCLUSION

The changes observed in rumen characteristics and microbiota shifts over time for both experiments indicate that the close in vitro system was successful at screening for feed additives. Saponins and different types of tannins had diverse effects on the magnitude and rate of total gas and methane production, especially during the final stages of fermentation. Moreover, these results corroborate that hydrolysable tannins and saponins when mixed with DFM improved propionic acid production and increased pH up to 12 h of i*n vitro* fermentation. The screening of bacteria via 16S rRNA sequencing was diverse for all treatments. These data demonstrate a proof of concept for manipulating the rumen microbial community and the digestive activity for cattle

consuming a high concentrated diet and the benefits of these antibiotic alternative need to be further tested at 12 h and 18 h of fermentation. It is possible that these changes in ruminal microbiota could reduce liver abscess rates and improve rumen dry matter digestibility. Further research should be conducted to evaluate these compounds *in vivo* to determine how cattle digestive behavior, general health and eating habits affect live animal performance and liver abscess formation.

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Table 5.1**.** Ingredients and chemical composition of the basal diet in dry matter (DM) basis.

Item	Percentage
Ingredient Composition (% DM)	
Steam Flaked Corn	61.00
Corn Silage	10.00
Alfalfa Hay	10.00
Dry Distillers Grain (DDG)	10.00
Fat (Tallow)	5.00
Chemical Composition	
Dry Matter, %	69.86
Crude Protein, %	12.90
ADF, %[1]	9.45
NDF, %[2]	16.27
NEg, Mcal/kg[3]	1.43

Macro- and microminerals included in diet: calcium = 0.71%, phosphorus = 0.33%, salt 0.51%, potassium 0.62%, sulfur 0.16%, magnesium 0.18%, zinc 19.50 ppm, iron 169.42 ppm, copper 6.04 ppm, manganese 11.80 ppm, cobalt 0.12 ppm, iodine 0.50 ppm, selenium 0.13 ppm, sodium 0.24%, and chlorine 0.44%

[1] ADF = Acid detergent fiber

[2] NDF = Neutral detergent fiber

[3] NEg = Net energy for growth

Table 5.2. The influence of tannin type and dose on *in vitro* volatile fatty acids (VFA) production in experiment A.

	Treatment							Contrast[1]						
	Con[a]	CHl[b]	CHh[c]	CDl[d]	CDh[e]	HDl[f]	HDh[g]	Trt	Dose[h]	Type	Con* Tan[e]	T1* T2[j]	T1* T3[k]	T2* T3[l]
	VFA[2] Mol/100mol							*P*-value						
Acetic														
2 h	59.00	59.45	59.53	63.57	58.84	57.64	56.74	0.001	0.025	0.643	0.807	0.071	0.023	0.001
12 h	54.32	53.92	54.46	53.79	52.56	52.99	53.78	0.043	0.925	0.017	0.154	0.023	0.066	0.624
18 h	53.38	54.82	54.38	54.03	54.20	53.13	48.98	0.003	0.032	0.037	0.890	0.522	0.002	0.001
Propionic														
2 h	24.99	24.68	24.39	22.95	23.75	24.20	24.39	0.114	0.559	0.044	0.144	0.017	0.623	0.071
12 h	25.84	25.74	25.49	25.33	26.68	25.85	26.47	0.131	0.064	0.181	0.840	0.293	0.148	0.680
18 h	24.93	25.40	24.36	25.36	27.35	26.85	22.68	0.003	0.162	0.163	0.737	0.021	0.351	0.002
Isobutyric														
2 h	1.48	1.68	1.60	1.75	1.68	1.70	1.70	0.679	0.545	0.384	0.127	0.408	0.533	0.869
12 h	3.67	4.39	4.43	4.32	3.70	3.84	3.89	0.452	0.516	0.133	0.286	0.231	0.111	0.676
18 h	5.02	4.88	5.14	4.76	4.17	4.40	5.13	0.004	0.374	0.009	0.207	0.005	0.207	0.118
Butyric														
2 h	8.32	8.17	8.35	9.08	9.11	9.74	10.19	0.194	0.663	0.042	0.328	0.173	0.011	0.182
12 h	9.74	9.60	9.35	9.91	10.20	10.35	9.46	0.489	0.381	0.132	0.871	0.146	0.275	0.703
18 h	9.92	8.74	9.52	9.46	8.53	10.03	13.99	0.005	0.059	0.214	0.884	0.855	0.001	0.001
Isovaleric														
2 h	2.12	2.15	2.23	2.43	2.49	3.02	3.23	0.001	0.374	0.001	0.028	0.086	0.001	0.001
12 h	3.31	3.25	3.09	3.25	3.35	3.39	3.07	0.472	0.217	0.355	0.608	0.322	0.637	0.599
18 h	3.15	2.59	2.93	3.06	2.78	3.43	4.77	0.006	0.086	0.055	0.767	0.599	0.000	0.001
Valeric														
2 h	4.08	3.86	3.90	4.22	4.12	3.70	3.74	0.877	0.975	0.591	0.706	0.337	0.625	0.177
12 h	3.12	3.09	3.18	3.41	3.51	3.57	3.33	0.188	0.886	0.021	0.209	0.038	0.045	0.935
18 h	3.61	3.56	3.67	3.33	2.97	3.18	4.47	0.000	0.024	0.117	0.660	0.009	0.270	0.001
T-VFA														
2 h	68.92	68.80	68.84	71.51	69.19	69.48	69.74	0.813	0.574	0.306	0.716	0.283	0.592	0.626
12 h	79.34	79.66	79.41	80.06	80.20	80.40	79.35	0.568	0.335	0.239	0.384	0.224	0.488	0.593
18 h	90.65	88.31	90.01	89.34	93.75	89.96	98.76	0.008	0.001	0.028	0.575	0.143	0.005	0.101

[1] Contrast: explains treatment significance
[2] VFA = Volatile fatty acids
[a] Con = control; Feed + rumen fluid/McDougall's buffer mixture (25% rumen fluid:75% McDougall's buffer)
[b] CHl = commercial tannin normal dose
[c] CHh = commercial tannin high dose
[d] CDl = condense tannin normal dose
[e] CDh = condensed tannin high dose
[f] HDl = hydrolysable tannin normal dose
[g] HDh = hydrolysable tannin high dose
[h] Dose = Normal; (15 g/hd/d) or high; (30 g/hd/d)
[e] Contrast of control vs. tannins
[j] Contrast of commercial tannin vs. condensed tannin
[k] Contrast of commercial tannin vs. hydrolysable tannin
[l] Contrast of condensed vs. hydrolysable
Significance was recorded at $P < 0.05$

Table 5.3. The influence of tannin type and dose on *in vitro* fermentation characteristics in experiment A.

	Treatment							Contrast[1]						
	Con[a]	CHl[b]	CHh[c]	CDl[d]	CDh[e]	HDl[f]	HDh[g]	Trt	Dose[h]	Type	Con* Tan[e]	T1* T2[j]	T1* T3[k]	T2* T3[l]
	Accumulated volume (ml) of nitrogen, methane, and CO_2							*P*-values						
Nitrogen														
2 h	2.93	2.22	3.21	2.32	3.30	4.65	1.39	<0.001	0.046	0.453	0.796	0.703	0.247	0.431
12 h	5.72	5.63	5.53	5.67	5.45	5.64	5.47	0.054	0.004	0.708	0.063	0.764	0.696	0.928
18 h	5.86	5.60	7.15	5.63	4.93	5.07	5.21	0.245	0.546	0.057	0.726	0.099	0.083	0.832
Methane														
2 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12 h	8.16	10.33	10.23	10.58	10.27	9.97	10.01	0.420	0.831	0.997	0.023	0.845	0.690	0.553
18 h	12.77	13.09	10.37	10.72	10.11	10.14	13.11	0.034	0.863	0.220	0.122	0.116	0.902	0.166
CO_2														
2 h	0.23	0.19	0.27	0.19	0.27	0.37	0.11	<0.001	0.066	0.965	0.823	0.885	0.702	0.613
12 h	55.60	70.23	70.46	72.12	71.82	67.87	67.96	0.394	0.998	0.951	0.022	0.748	0.632	0.427
18 h	92.80	94.20	77.86	94.04	82.26	81.54	92.52	0.302	0.284	0.753	0.429	0.732	0.880	0.863
pH														
2 h	7.28	7.33	7.32	7.23	7.28	7.28	7.20	0.309	0.748	0.051	0.866	0.102	0.064	0.763
12 h	5.89	5.94	5.86	5.98	5.97	6.07	6.00	0.023	0.117	0.012	0.108	0.077	0.002	0.129
18 h	5.76	5.69	5.74	5.66	5.79	5.92	5.71	0.001	0.761	0.194	0.914	0.747	0.011	0.019
Gas Pressure (pa)														
2 h	2232.53	1668.53	2413.17	1737.48	2454.53	3392.22	1034.21	<0.001	0.171	0.684	0.683	0.834	0.513	0.656
12 h	95754.00	94403.00	93755.00	96154.00	94155.00	92555.00	91480.00	0.028	0.134	0.973	0.070	0.284	0.046	0.004
18 h	113846.00	111516.00	110095.00	111888.00	269695.00	98126.00	106648.00	0.517	0.308	0.384	0.768	0.228	0.898	0.184
Microbial Protein (µg/ml)[*]														
2 h	32.85	28.82	33.06	30.78	34.35	31.03	49.74	0.019	0.014	0.251	0.682	0.701	0.024	0.073
12 h	38.24	36.39	34.18	35.16	31.96	40.95	53.19	0.004	0.405	0.339	0.908	0.602	0.001	0.001
18 h	43.19	53.52	55.77	52.92	73.00	74.96	69.60	0.029	0.337	0.060	0.007	0.221	0.020	0.226
% DMD														
18 h	40.75	41.33	42.92	44.08	62.50	44.06	40.43	0.001	0.001	0.001	0.006	0.001	0.867	0.001

[1] Contrast: explains treatment significance
[a] Con = control; Feed + rumen fluid/McDougall's buffer mixture (25% rumen fluid:75% McDougall's buffer)
[b] CHl = commercial tannin normal dose
[c] CHh = commercial tannin high dose
[d] CDl = condense tannin normal dose
[e] CDh = condensed tannin high dose
[f] HDl = hydrolysable tannin normal dose
[g] HDh = hydrolysable tannin high dose
[h] Dose = Normal; (15 g/hd/d) or high; (30 g/hd/d)
[e] Contrast of control vs. tannins
[j] Contrast of commercial tannin vs. condensed tannin
[k] Contrast of commercial tannin vs. hydrolysable tannin
[l] Contrast of condensed vs. hydrolysable
[*] Microbial protein was determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium bicarbonate). Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit.
Significance was recorded at $P < 0.05$

Table 5.4. Simple means of phyla relative abundance by treatment in experiment A.

Phyla	Con[1]	CHl[2]	CHh[3]	CDl[4]	CDh[5]	HDl[6]	HDh[7]
Proteobacteria	12.35%	11.99%	13.72%	8.51%	18.22%	28.68%	14.39%
SR1[a]	0.09%	0.10%	0.08%	0.07%	0.05%	0.04%	0.11%
Spirochaetes	6.04%	6.68%	5.39%	4.31%	4.92%	4.84%	3.85%
Bacteroidetes	58.29%	56.13%	58.41%	59.77%	53.21%	44.51%	54.37%
Lentisphaerae	0.24%	0.26%	0.24%	0.30%	0.22%	0.11%	0.21%
LD1[a]	0.02%	0.05%	0.03%	0.04%	0.03%	0.01%	0.04%
Fibrobacteres	0.05%	0.09%	0.05%	0.11%	0.12%	0.17%	0.59%
Chloroflexi	0.15%	0.17%	0.09%	0.11%	0.15%	0.15%	0.22%
Synergistetes	0.08%	0.07%	0.06%	0.10%	0.08%	0.12%	0.16%
TM7[a]	0.01%	0.01%	0.01%	0.02%	0.02%	0.00%	0.03%
Planctomycetes	0.05%	0.06%	0.06%	0.05%	0.04%	0.02%	0.07%
Verrucomicrobia	0.85%	0.98%	0.85%	0.74%	0.82%	0.73%	1.16%
Euryarchaeota, Archaea	0.26%	0.35%	0.31%	0.36%	0.33%	0.26%	0.48%
Cyanobacteria	0.83%	0.80%	0.73%	0.73%	0.71%	0.53%	0.72%
Tenericutes	1.93%	2.19%	1.80%	2.11%	1.83%	1.64%	2.67%
Acidobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
WPS-2[a]	0.01%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%
Actinobacteria	0.12%	0.16%	0.13%	0.10%	0.15%	0.11%	0.13%
Armatimonadetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Elusimicrobia	0.06%	0.07%	0.07%	0.07%	0.07%	0.05%	0.14%
Fusobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Firmicutes	18.49%	19.76%	17.93%	22.45%	18.96%	17.96%	20.51%

[a] Candidate phyla
[1] Con = control; Feed + rumen fluid/McDougall's buffer mixture (25% rumen fluid:75% McDougall's buffer)
[2] CHl = commercial tannin normal dose
[3] CHh = commercial tannin high dose
[4] CDl = condense tannin normal dose
[5] CDh = condensed tannin high dose
[6] HDl = hydrolysable tannin normal dose
[7] HDh = hydrolysable tannin high dose
Dose = Normal; (15 g/hd/d) or high; (30 g/hd/d)

Table 5.5. Effect of direct-fed microbials, saponin and tannin on *in vitro* volatile fatty acids (VFA's) production in experiment B.

	Treatment							
	Control[1]	**DFM[2]**	**Saponin[3]**	**TCH[4]**	**Sap+ DFM[5]**	**TCH+ DFM[6]**	**TCH+Sap + DFM[7]**	**Trt**
				VFA Mol/100 mol				
Acetic								
2 h	54.93^{a}	53.90^{b}	54.68^{ab}	54.48^{ab}	39.17^{c}	54.28^{ab}	54.38^{ab}	<0.001
6 h	54.32^{a}	41.46^{b}	46.12^{c}	45.53^{c}	47.32^{c}	45.10^{c}	46.59^{c}	<0.001
12 h	51.40^{a}	35.40^{b}	40.31^{c}	38.97^{bc}	43.49^{ce}	39.47^{cf}	39.18^{bcf}	0.005
18 h	52.96^{ac}	31.20^{bd}	37.69^{acd}	36.35^{acd}	44.29^{acd}	27.90^{bd}	37.88^{acd}	0.244
Propionic								
2 h	24.50^{a}	29.64^{b}	29.09^{b}	29.28^{b}	41.77^{c}	29.48^{b}	29.70^{b}	<0.001
6 h	27.14^{a}	34.54^{b}	32.88^{b}	33.72^{b}	29.78^{a}	33.88^{b}	31.50^{a}	0.011
12 h	28.09^{a}	35.57^{b}	33.17^{c}	37.73^{bf}	30.98^{c}	33.43^{be}	33.30^{cg}	<0.001
18 h	25.99^{a}	49.60^{a}	33.42^{a}	34.80^{a}	29.73^{a}	37.90^{a}	32.57^{a}	0.509
Isobutyric								
2 h	5.50^{a}	5.39^{b}	5.47^{ab}	5.45^{ab}	3.92^{c}	5.43^{ab}	5.44^{ab}	<0.001
6 h	5.44^{a}	4.67^{ab}	4.61^{b}	4.56^{b}	4.76^{ab}	4.51^{b}	4.54^{b}	0.205
12 h	5.14^{a}	5.35^{ae}	5.05^{a}	3.90^{b}	4.66^{cf}	4.97^{af}	4.97^{abf}	<0.001
18 h	3.73^{a}	3.49^{a}	5.29^{a}	5.04^{a}	4.86^{a}	3.84^{a}	5.35^{a}	0.461
Butyric								
2 h	6.67^{a}	5.15^{b}	5.01^{b}	5.06^{b}	7.19^{c}	5.07^{b}	5.19^{b}	<0.001
6 h	6.19^{a}	8.37^{b}	7.40^{c}	7.79^{d}	7.92^{d}	7.48^{c}	7.79^{d}	<0.001
12 h	6.88^{a}	10.32^{b}	9.48^{c}	9.05^{c}	9.12^{c}	9.75^{bc}	9.81^{bc}	<0.001
18 h	7.35^{a}	6.53^{a}	9.79^{a}	10.14^{a}	8.84^{a}	7.01^{a}	10.12^{a}	0.358
Isovaleric								
2 h	4.57^{a}	3.74^{b}	3.61^{b}	3.60^{b}	5.07^{c}	3.71^{b}	3.63^{b}	0.002
6 h	3.77^{a}	6.39^{b}	5.16^{c}	4.70^{c}	6.22^{b}	5.30^{c}	5.52^{c}	0.002
12 h	4.08^{a}	6.78^{b}	6.22^{ce}	5.26^{d}	6.35^{be}	6.33^{be}	6.78^{b}	<0.001
18 h	4.76^{a}	4.72^{a}	7.11^{a}	6.77^{a}	6.34^{a}	5.19^{a}	7.00^{a}	0.376
Valeric								
2 h	2.84^{a}	2.20^{b}	2.15^{b}	2.14^{b}	2.90^{a}	2.05^{b}	1.66^{c}	<0.001
6 h	3.16^{a}	4.57^{b}	3.85^{c}	3.71^{c}	4.02^{cd}	3.73^{ce}	4.07^{cd}	<0.001
12 h	4.51^{a}	6.61^{b}	5.78^{cd}	5.11^{ade}	5.40^{def}	6.06^{bdf}	5.97^{bdf}	0.003
18 h	5.23^{a}	4.49^{a}	6.72^{a}	6.91^{a}	5.94^{a}	4.67^{a}	7.10^{a}	0.327
T-VFA mM)								
2h	70.16^{a}	69.66^{b}	69.50^{b}	69.52^{b}	72.38^{c}	69.55^{b}	69.43^{b}	<0.001
6h	84.04^{a}	87.80^{b}	86.19^{c}	86.14^{c}	86.51^{c}	86.36^{c}	86.36^{c}	<0.001
12h	92.27^{a}	97.58^{b}	95.98^{c}	95.34^{c}	95.17^{c}	96.31^{c}	96.51^{bc}	<0.001
18h	99.41^{a}	103.17^{ab}	104.70^{a}	104.93^{ac}	102.53^{a}	109.54^{bcd}	104.85^{ad}	0.139

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

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] DFM = Direct-fed-microbial; *Lactobacillus animalis* ($2x10^{05}$ colony forming units (CFU)/g/hd/d) + *Propionibacterium acidipropionici* ($2.50x10^{04}$ colony forming units (CFU)/g/hd/d)

[3] Saponin = Yucca *shidigera* extract (2 g/hd/d)

[4] TCH = Commercial tannin (condensed + hydrolysable) (15 g/hd/d)

[5] Sap+DFM = Saponin + DFM

[6] TCH+DFM = Commercial tannin (condensed + hydrolysable) + DFM

[7] T/S/DFM = Commercial tannin + Saponin + DFM

Table 5.6. Effect of direct-fed microbials, saponin and tannin on i*n vitro* rumen fermentation characteristics in experiment B.

	Treatment							
	Control[1]	**DFM[2]**	**Saponin[3]**	**TCH[4]**	**Sap+ DFM[5]**	**TCH+ DFM[6]**	**TCH+Sap +DFM[7]**	**Trt**
				Accumulate Volume (ml)				
Nitrogen								
2 h	5.72^{a}	2.78^{ac}	2.28^{ac}	3.14^{ac}	2.04^{bc}	2.04^{bc}	6.99^{a}	0.075
6 h	1.84^{a}	6.82^{bdf}	2.96^{ce}	7.17^{bd}	2.77^{ce}	2.10^{f}	1.87^{f}	<0.001
12 h	2.09^{ae}	1.93^{ae}	6.72^{b}	1.88^{ae}	7.86^{c}	2.97^{d}	2.16^{e}	<0.001
18 h	3.34^{a}	2.10^{b}	2.14^{b}	2.34^{c}	N/A	6.87^{d}	2.89^{e}	<0.001
Methane								
2 h	0^{a}	0.01^{a}	0.01^{a}	0.01^{a}	0.74^{a}	0.02^{a}	0.02^{a}	0.479
6 h	11.93^{a}	12.38^{b}	10.60^{c}	10.85^{d}	11.25^{e}	10.77^{d}	11.60^{f}	<0.001
12 h	13.89^{a}	13.62^{b}	11.76^{b}	12.32^{c}	12.68^{c}	12.21^{cde}	11.90^{be}	<0.001
18 h	14.79^{a}	14.98^{a}	12.20^{b}	12.83^{b}	12.90^{b}	N/A	12.51^{b}	0.031
CO_2								
2 h	0.45^{a}	0.77^{a}	0.43^{ab}	0.33^{ab}	0.95^{bc}	0.91^{bc}	0.88^{bc}	0.011
6 h	65.86^{a}	75.86^{b}	72.99^{be}	57.61^{c}	80.08^{d}	75.14^{b}	71.71^{e}	<0.001
12 h	66.18^{a}	71.49^{b}	71.98^{b}	62.69^{c}	72.97^{bd}	70.62^{bd}	69.73^{b}	0.001
18 h	75.87^{a}	80.97^{ab}	83.98^{b}	71.03^{a}	85.11^{bc}	N/A	80.53^{abc}	0.009
pH								
2 h	6.96^{a}	6.99^{a}	7.06^{a}	7.02^{a}	7.15^{ab}	7.10^{a}	7.10^{a}	0.219
6 h	6.54^{a}	6.35^{b}	6.31^{b}	6.47^{d}	6.34^{b}	6.38^{c}	6.36^{b}	0.001
12 h	5.98^{a}	5.87^{a}	5.91^{a}	5.91^{a}	5.94^{a}	5.96^{a}	5.97^{a}	0.861
18 h	5.80^{a}	5.82^{a}	5.83^{a}	5.82^{a}	5.79^{a}	5.82^{a}	5.71^{a}	0.886
Microbial Protein (µg/ml)*								
2 h	12.99^{a}	40.02^{a}	12.21^{a}	55.63^{a}	34.90^{a}	30.92^{a}	30.87^{a}	0.719
6 h	19.37^{a}	19.34^{a}	28.98^{a}	20.87^{a}	33.24^{a}	28.75^{a}	23.67^{a}	0.237
12 h	18.97^{a}	28.51^{a}	22.94^{a}	22.84^{a}	26.78^{a}	29.37^{a}	32.56^{a}	0.964
18 h	15.00^{a}	12.32^{a}	17.99^{a}	52.79^{a}	24.13^{a}	31.52^{a}	48.82^{a}	0.459
Gas Pressure (psi)								
2 h	11238.00^{bcd}	15996.00^{abc}	9687.13^{dc}	8618.45^{d}	19236.00^{a}	18478.00^{a}	17271.00^{ab}	0.036
6 h	153615.00^{c}	168267.00^{b}	166853.00^{b}	140343.00^{d}	179850.00^{a}	170507.00^{b}	167267.00^{b}	<0.001
12 h	232664.00^{dc}	241799.00^{bc}	246177.00^{ab}	223425.00^{d}	253348.00^{a}	243350.00^{abc}	248004.00^{ab}	0.004
18 h	261036.00^{bc}	271516.00^{ab}	282478.00^{ab}	248315.00^{c}	289890.00^{a}	N/A	280168.00^{ab}	0.021
% DMD								
18 h	54.54^{a}	59.70^{b}	61.73^{c}	59.31^{b}	58.87^{b}	56.91^{d}	60.10^{b}	0.001

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

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] DFM = Direct-fed-microbial; *Lactobacillus animalis* ($2x10^{05}$ colony forming units (CFU)/g/hd/d) + *Propionibacterium acidipropionici* ($2.50x10^{04}$ colony forming units (CFU)/g/hd/d)

[3] Saponin = Yucca *shidigera* extract (2 g/hd/d)

[4] TCH = Commercial tannin (condensed + hydrolysable) (15 g/hd/d)

[5] Sap+DFM = Saponin + DFM

[6] TCH+DFM = Commercial tannin (condensed + hydrolysable) + DFM

[7] T/S/DFM = Commercial tannin + Saponin + DFM

* Microbial protein was determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium bicarbonate). Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit

Table 5.7. Simple means of phyla relative abundance by treatment at 18 h of fermentation in experiment B.

Phyla	Control[1]	TCH+DFM[2]	Sap+DFM[3]	DFM[4]	TCH[5]	Saponin[6]	TCH+Sap +DFM[7]
Actinobacteria	0.45%	2.28%	0.49%	0.53%	0.45%	0.93%	0.30%
WPS-2[a]	0.07%	0.00%	0.06%	0.11%	0.05%	0.06%	0.03%
SR1[a]	0.00%	0.04%	0.05%	0.05%	0.00%	0.05%	0.01%
Firmicutes	20.45%	54.95%	28.40%	30.26%	18.33%	37.56%	20.95%
Elusimicrobia	0.14%	0.00%	0.13%	0.11%	0.12%	0.08%	0.12%
Bacteroidetes	60.49%	27.92%	53.82%	47.53%	65.79%	37.38%	55.74%
Euryarchaeota, Archaea	1.41%	5.42%	1.88%	2.20%	1.00%	3.01%	1.79%
Verrucomicrobia	4.96%	0.33%	2.80%	4.90%	3.21%	5.50%	3.21%
LD1[a]	0.04%	0.03%	0.08%	0.11%	0.07%	0.12%	0.10%
Cyanobacteria	4.73%	0.41%	4.43%	3.98%	4.74%	4.91%	4.53%
Lentisphaerae	1.20%	0.00%	1.22%	1.28%	1.07%	1.58%	1.20%
Chloroflexi	0.32%	0.41%	0.71%	0.68%	0.27%	1.00%	0.50%
Planctomycetes	0.11%	0.20%	0.24%	0.19%	0.14%	0.31%	0.16%
TM7[a]	0.04%	0.09%	0.04%	0.13%	0.02%	0.15%	0.08%
Fusobacteria	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.01%
Tenericutes	2.51%	2.76%	3.46%	4.08%	1.76%	4.18%	2.79%
Armatimonadetes	0.04%	0.01%	0.04%	0.04%	0.03%	0.02%	0.03%
Synergistetes	0.00%	0.22%	0.05%	0.04%	0.02%	0.03%	0.04%
Proteobacteria	1.80%	2.14%	1.10%	1.97%	1.62%	2.22%	1.31%
Spirochaetes	1.17%	1.18%	0.84%	1.66%	1.16%	0.82%	1.01%
Fibrobacteres	0.00%	0.00%	0.02%	0.03%	0.02%	0.02%	0.02%

[a] Candidate phyla
[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)
[2] TCH+DFM = Commercial tannin (condensed + hydrolysable) + DFM
[3] Sap+DFM = Saponin + DFM
[4] DFM = Direct-fed-microbial; *Lactobacillus animalis* ($2x10^{05}$ colony forming units (CFU)/g/hd/d) + *Propionibacterium acidipropionici* ($2.50x10^{04}$ colony forming units (CFU)/g/hd/d)
[5] TCH = Commercial tannin (condensed + hydrolysable) (15 g/hd/d)
[6] Saponin = Yucca *shidigera* extract (2 g/hd/d)
[7] T/S/DFM = Commercial tannin + Saponin + DFM

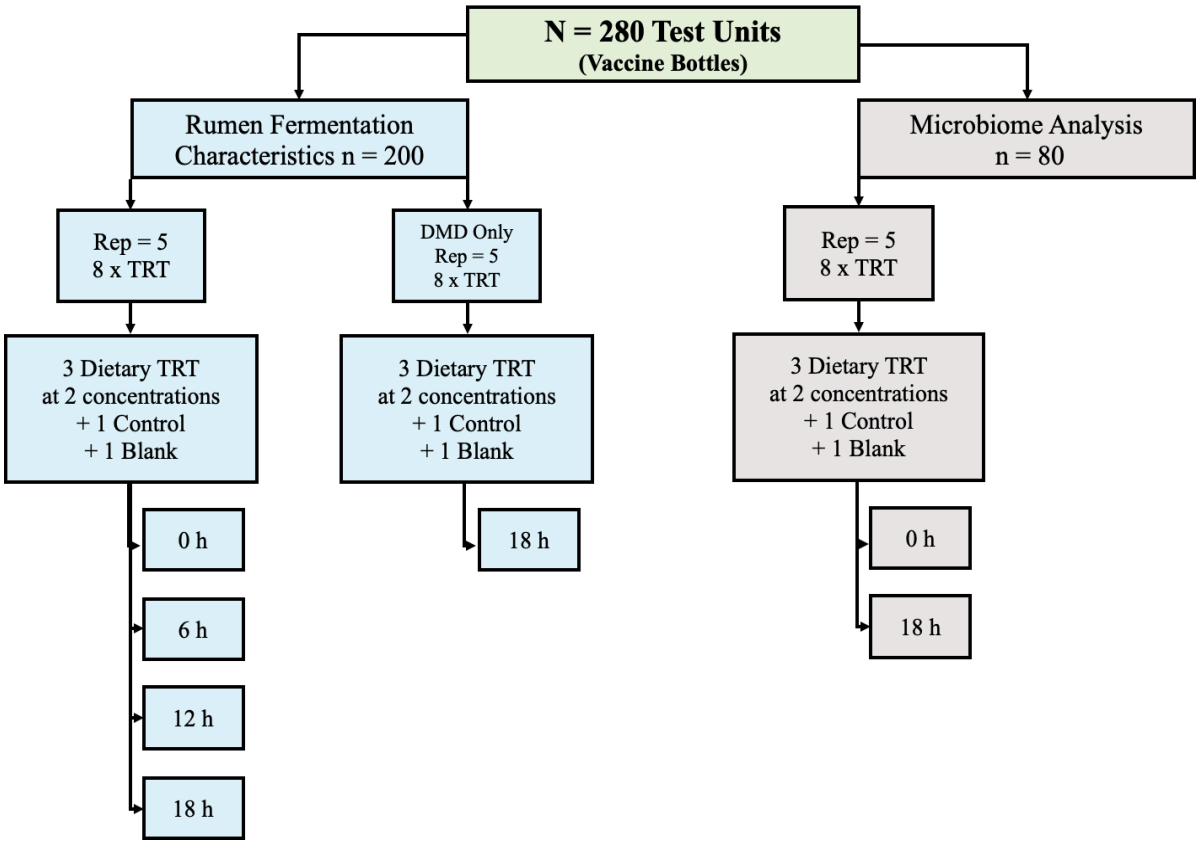

Figure 5.1. Experimental design for experiment A.

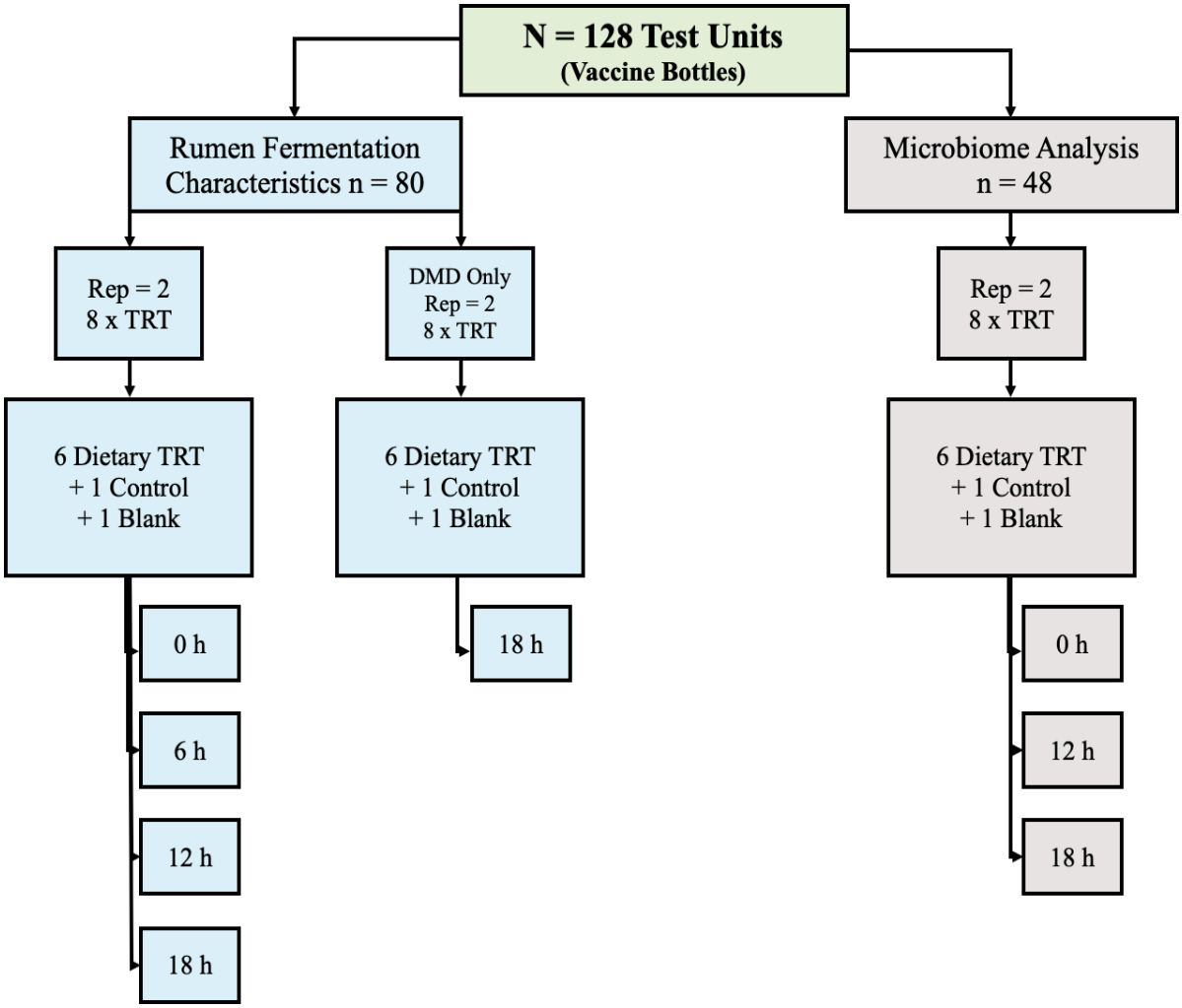

Figure 5.2. Experimental design for experiment B.

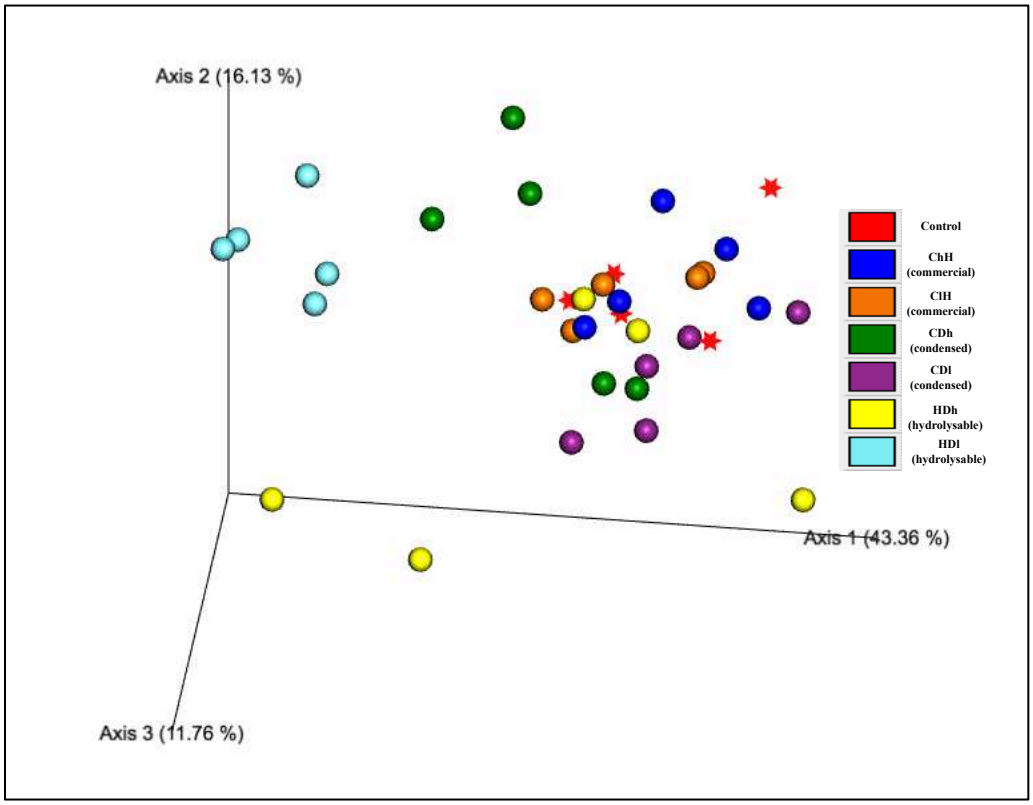

Figure 5.3. Weighted Unifrac distances at 18 h by treatment based on 16S rRNA gene amplicon data in experiment A. There was a significant difference between the treatment groups (P = 0.001). Each sphere represents the diversity of the microbial community of a single sample. The closer together two spheres, the more similar the microbial communities. Samples are colored by treatment. HDL (hydrolysable at 15 g/hd/d) samples appear distinct from other samples meaning that they have different relative abundances of microbes than samples in other treatments.

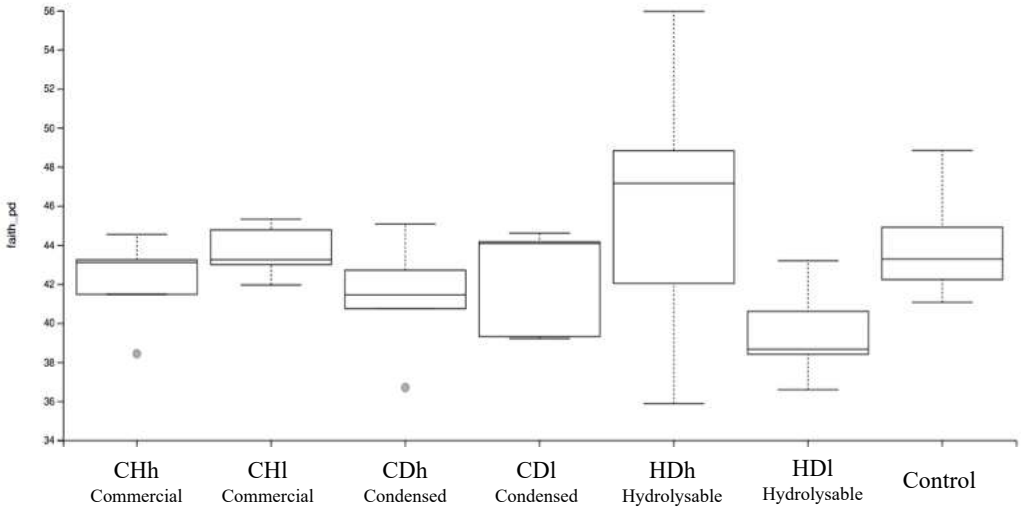

Figure 5.4. Faith's Phylogenetic Diversity (alpha diversity) at 18 h in experiment A by treatment of 16S rRNA gene amplicon data. There was not a difference between the treatment groups (P = 0.19).

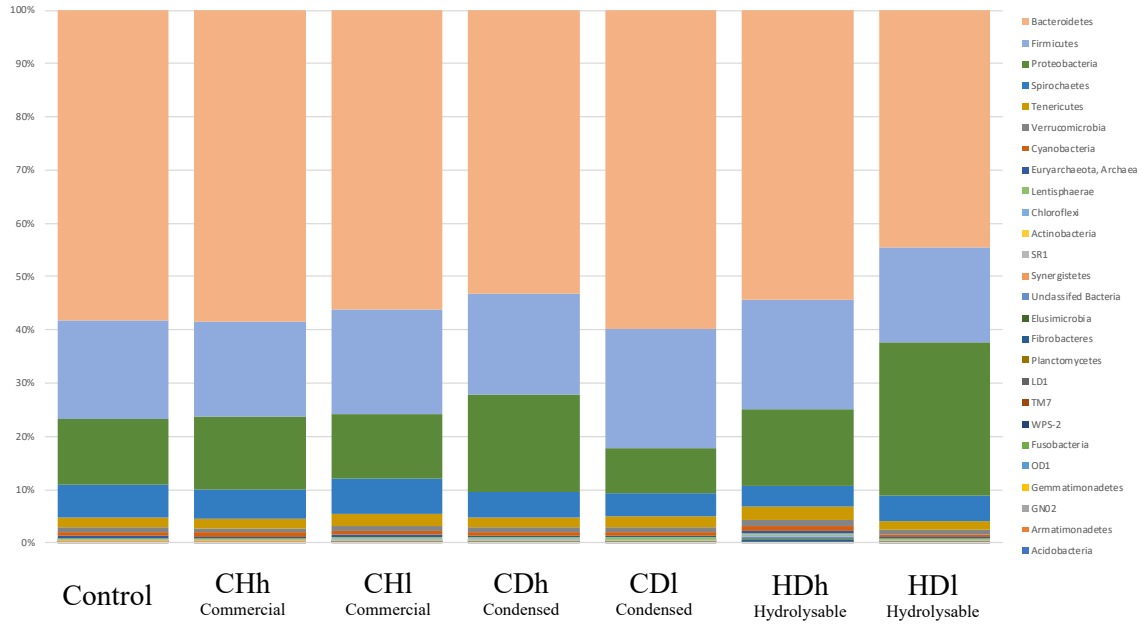

Figure 5.5. Microbial taxonomic composition of samples at 18 h by phyla present in experiment A.

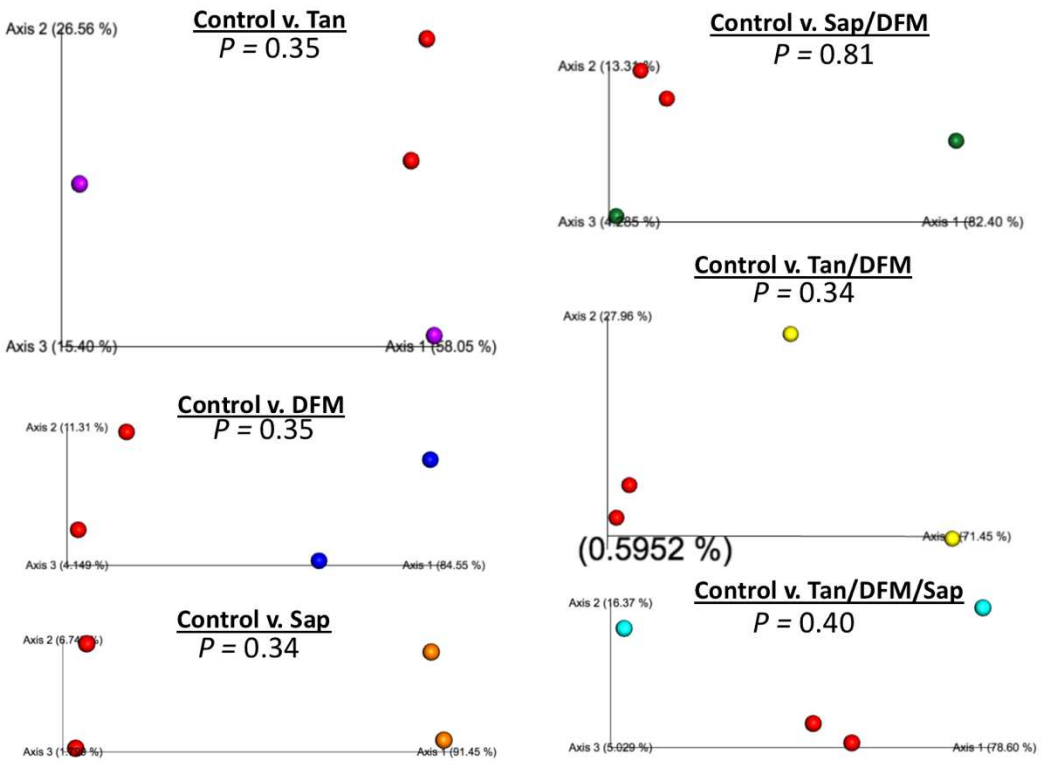

Figure 5.6. Comparison of treatments to controls individually in experiment B.

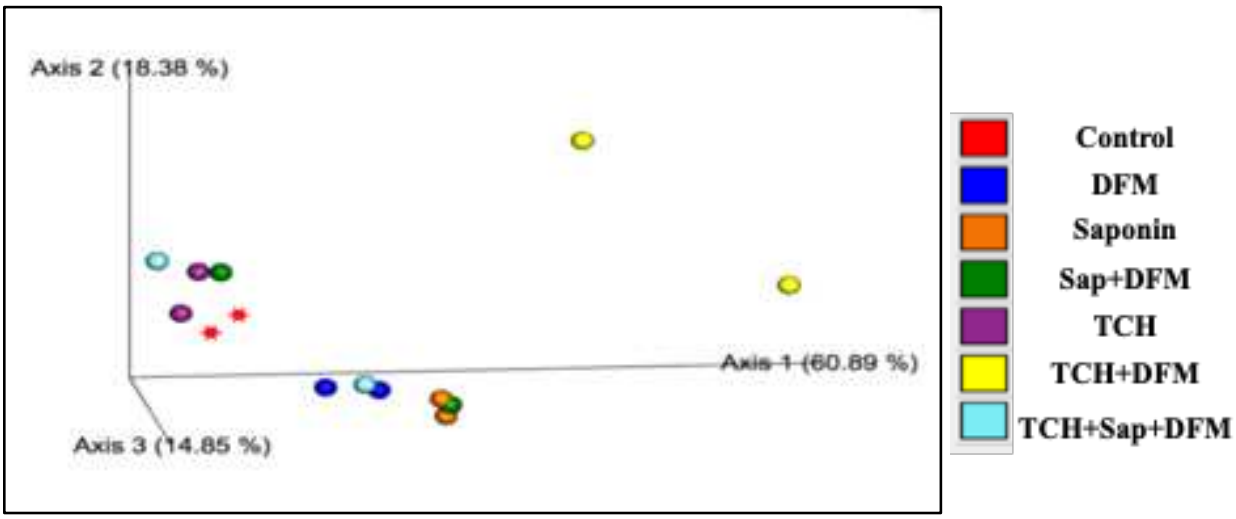

Figure 5.7. Weighted Unifrac distances at 18 h by treatment based on 16S rRNA gene amplicon data in experiment B. Each sphere represents the diversity of the microbial community of a single sample. The closer together two spheres, the more similar the microbial communities. Samples are colored by treatment. There was a significant difference between the treatment groups (P = 0.021).

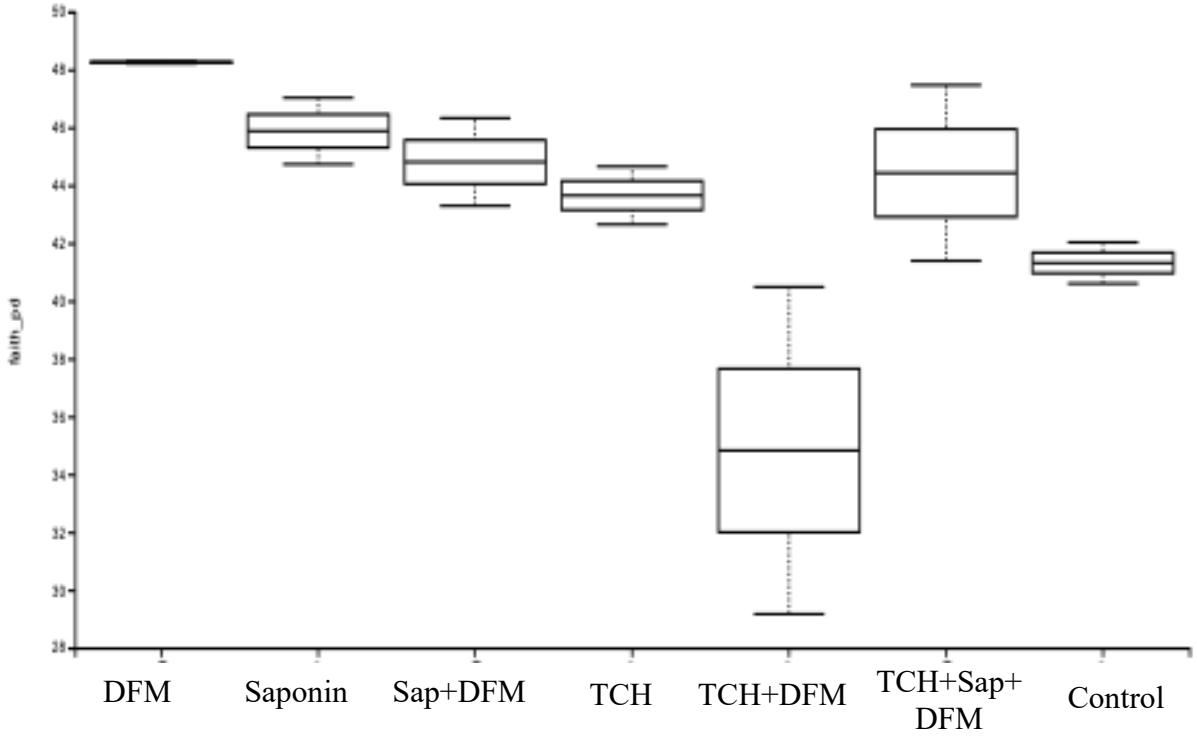

Figure 5.8. Faith's Phylogenetic Diversity at 18 h by treatment of 16S rRNA gene amplicon data in experiment B. There was not a difference between the treatment groups ($P = 0.11$).

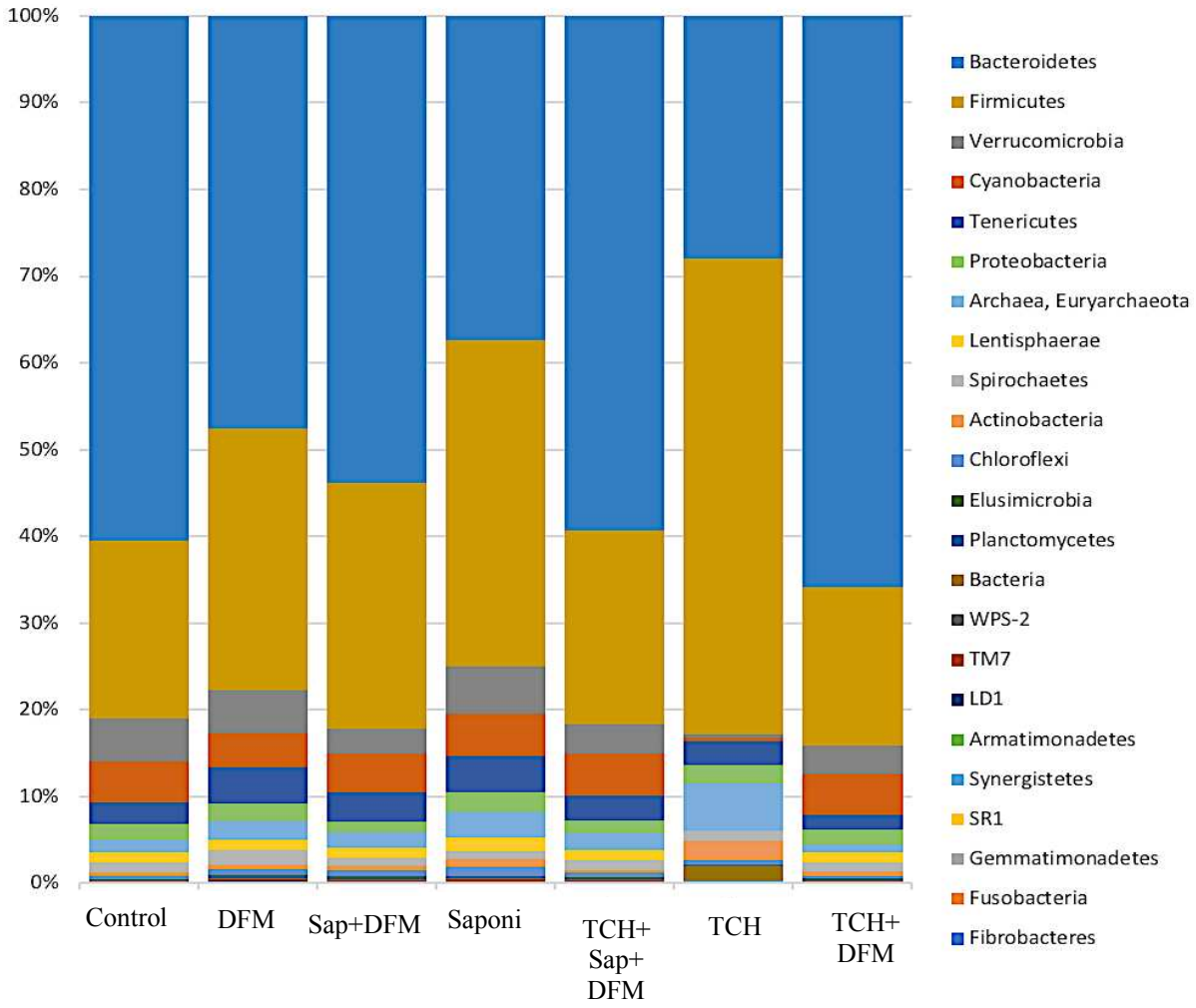

Figure 5.9. Taxonomic composition of samples at 18 h by phyla present in experiment B.

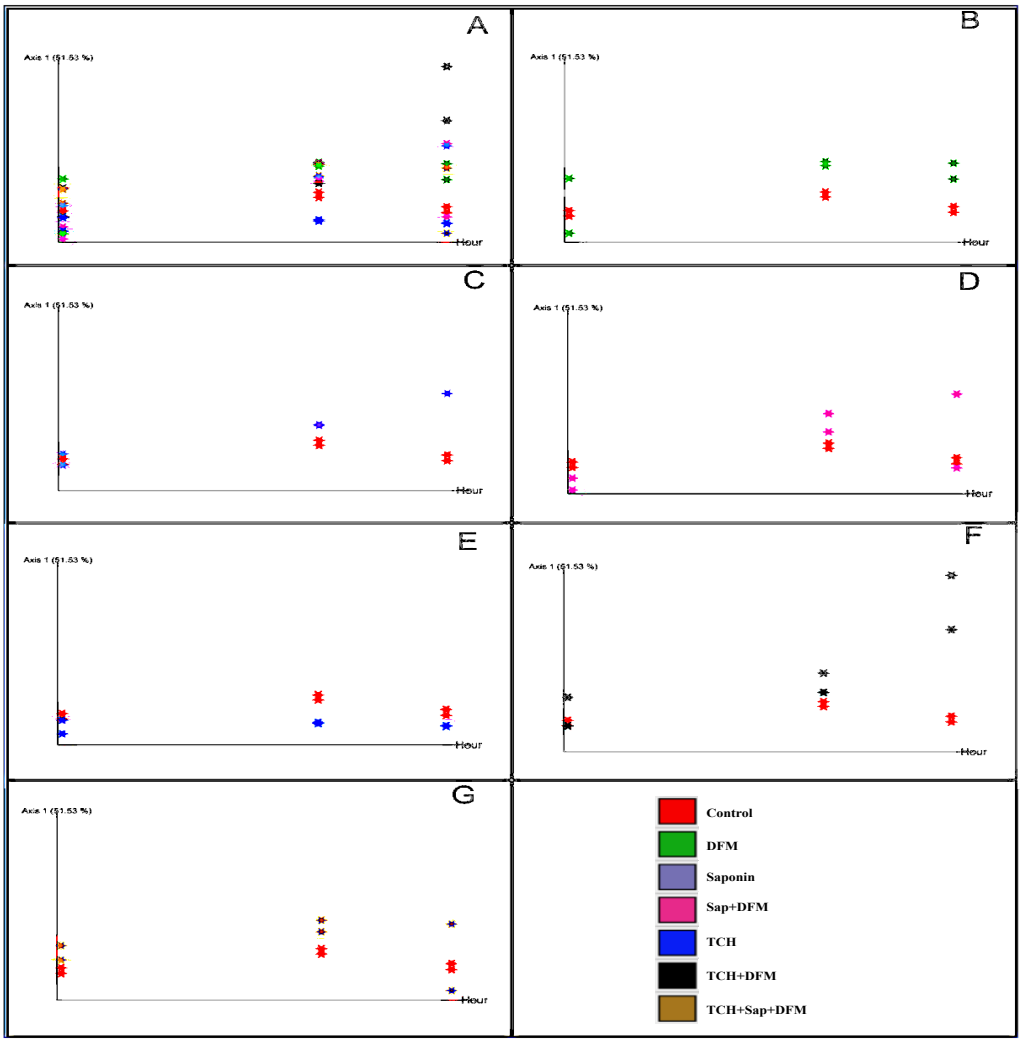

Figure 5.10. Experiment B beta diversity of samples forced to an Axis of time. (A) All treatments compared to the control, (B) DFM compared to the control, (C) Saponin compared to the control, (D) Sap/DFM combination compared to the control, (E) Tannins compared to the control, (F) Tan/DFM combination compared to the control, and (G) Tan/Sap/DFM compared to the control. The time points represented are 0 h, 12 h, and 18 h.

CHAPTER VI

CHANGES OF *IN VITRO* BOVINE RUMEN MICROBIAL DIVERSITY DUE TO INCLUSION OF NOVEL, NATURAL FEED ADDITIVES AND DIRECT-FED-MICROBIALS

SUMMARY

Tannins, saponins, and direct-fed-microbials (DFM) were assessed as potential replacers for antibiotics to reduce liver abscesses in feedlot diets in an *in vitro* tests. Three crossbred feedlot steers (450 kg; ~ 3.0 years of age), fitted with ruminal fistulas and adjusted to a high energy finishing diet for 21 d, were utilized in this experiment. A set of vaccine bottles (N = 120; n = 10/treatment) containing 1 g of feed plus dietary treatments were filled with 50 mL of rumen fluid McDougall's buffer solution, sealed and placed in a warm water bath (39˚C). Treatments consisted of: 1) Control (Feed + rumen fluid/McDougall's buffer mixture); 2) Commercial Tannin (TCH; Condensed + Hydrolysable; 15 g/hd/d); 3) Hydrolysable Tannin (HT; 15 g/hd/d); 4) Saponin (Saponin; 2g/hd/d); 5) Commercial tannin + direct-fed-microbial (TCH+DFM; DFM was fed at $2x10^{05} + 2.50x10^{04}$ colony-forming units (CFU)/g/hd/d of *Propionibacterium acidipropionici* and *Lactobacillus animalis* respectably for all treatments); 6) Hydrolysable Tannin + DFM (THD+DFM; same dose as described above); 7) Saponin + DFM (Saponin+DFM; same dose as described above). Samples were collected at 12 h and 18 h post fermentation. Gas samples were measured for N_2, CO_2, and CH_4 while the liquid portion was analyzed for volatile fatty acids (VFA) and the pellet fraction was analyzed for microbial protein. Propionic acid production increased ($P < 0.05$) with the inclusion of TCH+DFM, THD+DFM and Saponin+DFM at 6 h of incubation. These same treatments reduced ($P < 0.05$) acetic acid

production at 12 h, but no effects were seen at 18 h. Methane was reduced ($P < 0.05$) at 12 h for THD treatment compared to Control. Treatments of THD+DFM and Saponin+DFM exhibited increased ($P < 0.05$) pH at 12 h. In addition, microbial DNA was extracted from the liquid portion and the V4 region of the 16S rRNA gene was amplified and sequenced using a lllumina HiSeq. At 12 h, alpha and beta diversity were different ($P > 0.05$). However, there was no difference ($P > 0.05$) in alpha or beta diversity of the microbial community between treatments at 18 h. Across all samples at 18 h of fermentation the genus *Dialister* was present, however treatments Saponin+DFM and TCH+DFM had the greatest relative abundance. The presence of genus *Dialister* indicates association with energy harvesting bacteria indicating that the inclusion of these feed additives allowed shifts of certain bacteria.

Key words: feed additives, feedlot cattle, rumen microbiome, saponins, tannins, 16S rRNA,

INTRODUCTION

A ruminant animal's stomach is comprised of a complex four-chambered stomach allowing for digestion of a variety of feed sources, in part, via microbial fermentation generating volatile fatty acids (VFA; Cunha et al., 2011). Certain by-products of fermentation are absorbed through the rumen epithelium of the host animal and provide the animal with substrates for energy metabolism. As dietary fermentable starch increases, there is a shift in the rumen microbial community from cellulolytic digesting bacteria to more amylolytic digesting bacteria (Wang et al., 2012). In this ruminal environment, propionic acid production is increased, ultimately reducing rumen pH. This shift decreases methane production, as well, which ultimately increases animal production efficiency. Many other factors can contribute to rumen pH changes, such as changes in barometric pressure or ambient temperature, and animal stress

(Yadav et al., 2013). These changes in pH can cause alterations in digestion leading to acute reductions in rumen pH below 5.1— resulting in acidosis.

Ruminal acidosis compromises the integrity of the ruminal epithelium and permits bacterial translocation to the blood stream, which ultimately may cause liver abscesses (Brent, 1976). Liver abscesses result in substantial economic loss to the industry (Amachawadi and Nagaraja, 2016). Producers incur losses due to decrease feed efficiency, reduction of feed intake, lower weight gain and ultimately decreased carcass dressing percentage while the packing plants incurring economic losses due to the physical condemnation of the liver (Nagaraja and Chengappa, 1998). Brown and Lawrence (2010) stated that a 3.5 % incidence rate of liver abscesses would cost the industry over $7 million annually. To prevent liver abscess formation, the beef cattle industry depends on the utilization of tylosin phosphate, an antibiotic. Tylosin phosphate falls into the macrolide class of antibiotics, which are important for clinical treatment of human infections (WHO, 2017). Therefore, there is a concern that feeding tylosin phosphate to beef cattle could lead to increased levels of macrolide antibiotic resistance in humans, ultimately leading to treatment failures (Wegener, 2003).

The public concern surrounding antibiotic resistance has led to an on-going investigation of alternative technologies for decreasing the incidence of liver abscesses without the use of antibiotics. Plant compounds, such as saponins and tannins, are naturally used by plants as self-defense mechanisms to prevent predation and disease (Śliwiński et al., 2002) and may be efficacious in preventing liver abscesses in beef cattle. These compounds have been reported to improve rumen health (Wallace et al., 1994; Makkar et al., 1995; Wang et al., 2000; Anjum et al., 2018) by optimizing starch digestibility and maintaining rumen pH, ultimately improving ruminal wall integrity and reducing formation of liver abscesses.

Tannins, are compounds found in plants that are classified into two categories: 1) hydrolysable and 2) condensed tannins (Min et al., 2003; Min et al., 2006; Krueger et al., 2010). When fed at low concentrations, tannins can increase ruminal protein degradation, resulting in increased metabolizable amino acid flow to the small intestine (Barry and McNabb, 1999; Min et al., 2003); although, high amounts of tannin supplementation can be toxic and reduce efficiency. Saponins and tannins have also been reported to reduce methane production in ruminants, thus improving energy retention and overall animal production efficiency (Patra and Saxena, 2011; Goel and Makkar, 2012; Sun et al., 2017). Śliwiński et al. (2002) reported that beef cattle diets supplemented with saponins and tannins reduced ammonia production in the rumen without altering protein digestion, which may improve nitrogen retention and growth efficiency by the animal.

The use of direct-fed microbials (DFM) has been reported to enhance animal efficiency by altering ruminal bacterial communities (Krehbiel et al., 2003). Furthermore, research has demonstrated that the inclusion of DFM in feedlot diets can reduce shedding of enteric bacteria by feedlot cattle (Younts-Dahl et al., 2005). Even though DFM and plant compounds have been shown to positively benefit the animal, the impacts of DFM and plant compounds on ruminal fermentation characteristics are difficult to elucidate. Nonetheless, as antibiotic resistance continues to be a factor by which consumers make food choices due to perceived human health concerns, it is critical to explore new alternatives in preventing liver abscesses. Therefore, the objective of this study was to evaluate the effects of saponins, tannins and DFM on ruminant volatile fatty acid production and ruminal microbiota composition *in vitro* as possible substitutes for dietary antimicrobials.

MATERIAL AND METHODS

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 16-6550A). Steers were housed at CSU's Agricultural Research, Development and Education Center (ARDEC).

Rumen Content Collection

Three crossbred feedlot steers (450 kg; ~ 3.0 years of age) fitted with ruminal fistulas were utilized in this study. Prior to sample collection, cattle were adjusted to a high energy finishing diet consisting of 71% concentrate and 20% roughage (1.43 NEg, Mcal/kg DM) for 3 weeks (21 d; Table 6.1). At the completion of the 21-d diet adjustment period, rumen fluid was collected at a single time point, approximately 2 h post feeding, as described by Ward and Spears, (1993). Briefly, rumen fluid (~ 4 L) from all three steers was filtered twice through four layers of cheesecloth and combined into one pre-warmed (39°C) thermos. A modified McDougall's buffer solution (39.20 g $NaHCO_3$, 14.80 g Na_2HPO_4, 2.28 g KCl, 1.88 g NaCl, 0.48 $MgSO_4*7H_2O$ per 2 L H_2O) was mixed with rumen fluid at a 1:1 ratio, simulating saliva production during rumination (Tilley and Terry, 1963). Rumen fluid pH was recorded before and after being mixed with McDougall's buffer solution.

In vitro Chambers

Approximately 2 kg (wet weight) of the high concentrate diet fed to the steers was collected upon discharge from the feed truck and dried in a forced air-drying oven at 60°C for 72 h and ground through a 2.0 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). The ground ration was weighed and dispensed (1.000 ± 0.005 g) into pre-labeled 100 mL vaccine bottles containing the appropriate dose of each treatment. Treatments consisted of: 1) Control (Feed + rumen fluid/McDougall's buffer mixture); 2) Commercial Tannin (TCH; Condensed + Hydrolysable; 15 g/hd/d); 3) Hydrolysable Tannin (HT; 15 g/hd/d); 4) Saponin

(saponin; 2g/hd/d); 5) Commercial tannin + direct-fed-microbial (TCH+DFM; DFM was fed at $2x10^{05} + 2.50x10^{04}$ colony-forming units (CFU)/g/hd/d of *Propionibacterium acidipropionici* and *Lactobacillus animalis* respectably for all treatments); 6) Hydrolysable tannin + DFM (THD+DFM; same dose as described above); 7) Saponin + DFM (Saponin+DFM; same dose as described above). Two sets of 10 vaccine bottles were prepared for each treatment per time point. One set of vaccine bottles was used to evaluate rumen fermentation characteristics at two time points (12 h and 18 h), while the second set was used for microbiome analysis at the same time points (Figure 6.1). Sampling times for rumen fermentation characteristics were set every six hours to determine where the major shifts occur. This process was repeated twice on two different days.

The McDougall's buffer/rumen fluid mixture was dispensed into the vaccine bottles (50 mL) containing the pre-weighed feed and treatment. The bottles were capped and sealed immediately after the McDougall's buffer/rumen mixture was dispensed. The vaccine bottles remained sealed to maintain anaerobic conditions and were incubated at 39°C in a circulating water bath for the remainder of the experiment. Vaccine bottles were removed for sampling purposes as required by the experimental design.

Sample Collection

To simulate rumen motility, vaccine bottles were gently swirled every 4 h. Samples were removed at each predetermined time point, gas pressure was recorded, and a 10 mL gas sample was collected and immediately analyzed for nitrogen (N), methane (CH_4) and carbon dioxide (CO_2) concentrations (mL) via gas chromatography. At each collection time, the pH of the contents of each vaccine bottle destined for analysis of rumen fermentation characteristics was measured. Then, contents of each vaccine bottle were transferred to individual pre-weighed 50 mL conical

tubes and centrifuged at 1,000 x *g* at 5°C for 30 min (Beckman Model TJ-6; Beckman Coulter, Indianapolis, IN). A 2 mL aliquot of the supernatant was extracted from the conical tube post centrifugation, acidified with meta-phosphoric acid, and frozen at -80°C until analyzed for volatile fatty acid (VFA) concentrations via gas chromatography. The remaining supernatant was aspirated, and the indigestible residue was dried in a forced air-drying oven at 60°C for 120 h to determine *in vitro* dry matter disappearance (DMD). The second set of paired vaccine bottles were immediately removed at each collection time period and dispensed into 50 mL conical tubes for 16S rRNA gene amplicon sequencing.

Volatile Fatty Acid Analysis

After thawing at room temperature, samples designated for VFA analysis were centrifuged at 28,000 x *g* at 5°C for 15 min and the supernatant was removed and placed into a 1.5 mL gas chromatography vial and analyzed for VFA's. The VFA concentrations were determined via gas chromatography (Agilent 6890N, Santa Clara, CA) fitted with a fused silica capillary column (30 m x 0.25 μm x 0.25 μm) and a flame ionization detector. The following instrument parameters were used: injection mode = spitless; injection volume = 1.0 μL; carrier gas = helium; carrier gas flow = 1.0 mL/min; injector temperature = 250°C; oven ramping program = 100°C for 3 min, 185°C for 11 min; detector temperature 250°C.

In vitro Gas Production Analysis

Gas pressure of each vaccine bottle was determined using a digital pressure gauge fitted with a 20-gauge needle (Dwyer Instruments Inc., Michigan City, IN). Gas composition (N, CH_4, and CO_2) was determined by withdrawing 10 mL of gas from the vaccine bottle headspace using a 10 mL syringe. The gas samples were immediately injected into the injection port of a gas

chromatograph (Shimadzu GC – 14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector set at 100°C.

Dry Matter Disappearance

The dry matter disappearance (DMD) was determined at 6 h and 12 h by weighing the 50 mL conical tubes prior to dispensing the vaccine bottle rumen contents into the tube. An aliquot of the supernatant was used for VFA analysis, as described above, while the solid undigested material was allowed to dry in a forced air-drying oven at 60°C for 120 h. Once the sample was dry, the conical tube was weighed again to determine the remaining feed that was not digested. The DMD was calculated as follows:

$$DMD, \% = \left(\frac{\text{initial substrate DM mass } - (\text{undigested DM mass} - \text{microbial DM residue mass})}{\text{initial substrate DM mass}}\right) * 100$$

Protein Analysis

Protein analysis of the remaining residue post *in vitro* incubation was performed by utilizing a Quick Start™ Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). The dry residue contents from the DMD tubes were ground into a powder with a mortar and pestle. Then 0.1 g was dispensed into a 2 mL microcentrifuge tube and mixed with 1 mL of a 25% sodium chloride (NaCl) solution. The tubes were then placed on a plate shaker for 30 min at room temperature. After 30 min, samples were vortexed for 30 s. The samples were then centrifuged at 1,200 x *g* at 5°C for 15 min. Once centrifuged, 25 µL of supernatant was removed and placed into one well of a 96-well plate that contained 125 µL of a 25% NaCl solution. Each sample assayed was run in duplicate. A 150 µL portion of the Quick Start™ Bradford assay was added to each of the 96 wells containing samples and a standard ranging from 0 to 90 µg of protein/µL was used to determine protein concentration.

Rumen Fermentation Characteristics Statistical Analysis

This experiment was designed as a randomized complete block design replicated over two days. Data were blocked by hour of sample collection. Least squares means (LSmeans) and pooled standard error of the mean (SEM) were reported for all response variables. Hour after feeding prior to rumen fluid collection was used as a covariate, due to the event of the steers being fed three hours earlier on the second day of rumen collection as compared to the first rumen fluid collection. Data were analyzed using the Mixed Procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Differences were reported at a significance level of $\alpha = 0.05$.

16S Amplicon Sequencing

At 12 and 18 hours, 10 artificial rumen samples per treatment were removed from the hot water bath and transferred into a 50 mL conical tube. The samples were placed on ice and transported to a -80 ˚C freezer until DNA extraction. Prior to DNA extraction, samples were thawed. A sterile cotton swab was used to swab a homogeneous mixture of rumen fluid and the swab was used in the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). After DNA extraction, qualified DNA libraries from each sample were shipped to Novogene Corporation (Beijing, China) for 16S rRNA library preparation and sequencing. The V4 region of the 16S subunit was amplified with the 515/806R primer set. Paired-end sequencing (2 x 250) was conducted on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA).

Bioinformatics and Statistics

Amplicon samples were processed through the QIIME2 (2018.8) pipeline (Caporaso et al., 2010). Samples were demultiplexed and assigned exact sequence variance (ESV) using the DADA2 plugin with primers and lower quality ends trimmed off (Callahan et al., 2016). Multiple sequence alignment of the sequences were completed with MAFFT and filtered to remove highly variable positions (Katoh and Standley, 2013). FastTree 2 was used to construct

and root a phylogenetic tree(Price et al., 2010). Taxonomic classification was conducted using a pretrained Naive Bayes classifier trained on the Greengenes database trained with the 515/806 primers (DeSantis et al., 2006). Reads assigned to mitochondria and chloroplast were removed from downstream analysis as well as those reads that did not have an assignment to phylum.

Alpha diversity was assessed via Faith's Phylogenetic Diversity and beta diversity was measured using weighted UniFrac distances. Alpha diversity was compared using the lm and anova functions in R and compared using the CLD function from the emmeans package. Beta diversity was evaluated using the PERMANOVA adonis puggin in QIIME2. Differential abundance was conducted at the aggregated phylum level via ANCOM (Mandal et al., 2015). In all comparisons, $\alpha = 0.05$ and an FDR adjustment was used when appropriate.xs

RESULTS

Rumen Fermentation Characteristics at 12 h of Fermentation

Treatment effects on VFA concentrations for 12 h of fermentation are presented in Table 6.2. Total VFA (TVFA) concentrations were not different ($P = 0.917$) across treatments. However, there was a difference ($P < 0.05$) between treatment acetic acid and propionic acid (A:P) ratios with the Saponin+DFM having the lowest A:P concentration. Moreover, an increase ($P = 0.016$) in propionic acid was observed for both THD+DFM and Saponins+DFM when compared to Control. Treatment had no effect ($P > 0.05$) on butyric and valeric acid. Both TDH+DFM and Saponins+DFM had a higher ($P < 0.05$) pH, while TCH had the lowest ($P < 0.05$) pH. In addition, dry matter digestibility (DMD) differed ($P < 0.05$) between treatments (Table 6.2). The treatments with direct-fed-microbial (DFM) had a higher percentage of DMD, even though the pH and A:P ratio were more basic and lower respectively. Saponin and treatments with DFM produced less ($P < 0.05$) gas when compared to Control (Table 6.3). Even

though, there was no statistical effect on methane production, THD had the lowest numerical production of methane. Furthermore, microbial protein was higher ($P < 0.05$) for Saponins+DFM when compared to other treatments (Table 6.3).

Rumen Fermentation Characteristics at 18 h of Fermentation

Results for rumen characteristics at 18 h of fermentation are shown in table 6.4 and 6.5. There was a significant effect of treatment inclusion on concentration TVFA and pH (Table 6.4). The pH was higher ($P < 0.05$) in THD+DFM and lower in Saponin+DFM. However, acetic acid, propionic acid, and DMD were not different ($P > 0.05$) between treatments. Nevertheless, THD and Saponin+DFM had a higher numerical DMD when compared to Control. Gas production across all treatments was different ($P < 0.05$; Table 6.5). THD+DFM and Saponin+DFM exhibited higher gas production when compared to Control. On the other hand, methane production was not ($P > 0.05$) affected by treatment. However, THD+DFM and Saponin+DFM had lower ($P < 0.05$) CO_2 concentrations when compared to Control.

16s Sequencing Between Times Points

The microbiomes of artificial rumens between time points was found to differ at the community level ($P = 0.003$; Fig. 6.2A) and in individual sample diversity ($P < 0.001$; Fig. 6.2B). As expected, this result indicated that microbial fermentation of the rumen significantly changes the composition of the bacterial communities present over the course of rumination.

Treatments at hour 12 altered alpha diversity ($P = 0.003$; Fig 6.3A), and beta diversity ($P = 0.037$; Fig 6.4A) in the microbiome samples. When phyla were compared, *Fusobacteria* was different ($P > 0.05$; Table 6.6) across treatments with the Control containing the highest levels of *Fusobacteria*. While these numbers were significant, the biological ramifications are less clear as the highest amount was still present in less than 1%. Moreover, the predominant orders

represented (more than 1% of entire microbiome) across treatments were *Lactobacillales*, *Clostridiales*, *Bacteroidales*, *Enterobacteriales* and *Erysipelotrichales* (Figure 6.5A). At 12 h of fermentation, Saponin+DFM and THD+DFM had higher amount of the order *Bacillales*. The genus *Clostridium* was present in a higher abundance in all treatments, except TCH and TCH+DFM (Figure 6.6). In addition, the genus *Proteus* and *Oscillospira* were present in all samples however all treatments had a lower abundance when compared to Control.

At 18 h of fermentation, the microbiome between treatments did not differ in both alpha ($P = 0.554$) and beta ($P > 0.05$) diversity as assessed by Faith's phylogenetic diversity (Figure 6.3B) and weighted unifract distances (Figure 6.4B). Moreover, the predominant orders represented (more than 1% of entire microbiome) across treatments were *Lactobacillales*, *Clostridiales*, *Bacteroidales*, *Enterobacteriales*, *Erysipelotrichales*, and *Bacillales* (Figure 6.5B). At 18 h of fermentation, the genus *Sharpea* was present in higher abundance for TCH+DFM followed by Saponin+DFM (Figure 6.7). In addition, the genus *Dialister* was present in Control, Saponin+DFM and TCH+DFM. Of these treatments, Saponin+DFM had a higher presence of *Dialister* when compared to all the treatments.

DISCUSSION

Rumen *In Vitro* Fermentation Characteristics

In this study the effects of tannins, saponins and DFM were evaluated as possible substitutes for antibiotic alternatives to reduce liver abscesses. The inclusion of DFM, in conjunction with the treatments, resulted in greater proportions of propionic acid, pH and higher DMD. The DFM use in this study consisted of a combination of *Lactobacilus animalis* and *Propionibacterium acidipropionici*. Our results agree with the theory and results of (Mandal et al., 2015). They reported that lactic acid producers can provide a tonic level of lactic acid to

stimulate the lactic acid utilizer that further results in an increase in ruminal pH. In addition, *Propionibacterium* explains the high levels of propionic acid. However, contradicting results were found by Yang et al. (2004) where *Propionibacterium* resulted in less propionate production. In our study, at 12 h of fermentation, the inclusion of saponin showed a slight increase in propionic acid production. In previous research, saponin (*Yucca schidigera* extract) has been found to increase propionate (Pen et al., 2007).

The *in vitro* supplementation of both commercial tannin and hydrolysable tannin resulted in less methane produced at 12 hours. However, no reduction of methane was reported with the feeding of Chestnut tannins (Śliwiński et al., 2002) and Quebracho tannins (Beauchemin et al., 2007). Although, some tannin extracts (Acacia cyanophylla; vetch-oat hay) have been found to reduce methane (Rira et al., 2015). At 18 h of fermentation in our study, saponin resulted in less methane production. The reduction of methane has been observed with supplementation of saponins (Rira et al., 2015). However, other research contradicts our findings, as methane production did not differ when a ryegrass hay and nonconcentrated (3:2, w/w DM basis) was fed (Pen et al., 2007). Saponin decreased gas production but also exhibited less efficient microbial protein production. This contrast published work (Blümmel et al., 1997) that suggested that a decrease in gas production should lead to higher microbial protein production. This inverse relationship was observed in tannin S + DFM and saponin + DFM, suggesting that the DFM addition plays a role in this relationship. Nonetheless, the inclusion of tannins, saponins and DFM show promising traits in an *in vitro* setting.

Effects of Supplementing Tannins, Saponin, and DFM Rumen Microbial Community

At both collection time points for all treatments, the majority phyla present were *Bacteroides*, *Firmicutes* and *Proteobacteria*. The presence of these phyla are important as they

have been determined to consistently be present in the rumen core microbiome (Petri et al., 2013). Even though comparisons between 12 h and 18 h were not conducted, due to the experimental design not being considered a repeated measures design, the order *Enterobacteriales* increased as fermentation increased. The presence of the genus *Proteus* is ubiquitous to the mammalian digestive tract (Drzewiecka, 2016).The reduction of this genus by the test treatments is important, as this bacteria has been found to be transmitted from the fecal matter of birds to cattle feed ,which may pose as a autoinfection and cross-infection threat (Rogers, 2006; Drzewiecka, 2016). *Oscillospira* is a common inhabitant of the rumen environment (Moir and Masson, 1952). Mackie et al. (2003) found that switching diets from Lucerne pellet to a 70% grain drastically reduced the presence of *Oscillospira* from $1.3x10^5$ to 50 per g of ingesta. Allison et al. (1976) stated that the toxins produced by *Clostridium* are proteins that result in a rapid degradation in the rumen. The presence of *Clostridium* in the hydrolysable tannin treatment is most likely due to the high affinity of tannins to bind to proteins (Asquith and Butler, 1986; Huang et al., 2018). Petri et al. (2013) found that *Sharpea* increased during acidotic challenges with a grain-based diet versus forage-based diet followed by sharp decline during the recovery period. Similarly, our study exhibited higher abundance of *Sharpea* for Saponin+DFM and TCH+DFM treatments in an *in vitro* grain based simulated diet. In addition, the increase of the genus *Dialister* has been associated with energy harvesting and increased average daily gain (Myer et al., 2015). The higher abundance in the Saponin+DFM treatment can be associated with higher energy available for growth.

CONCLUSION

The changes observed over time in both 12 and 18 hours indicate that the addition of feed additives in the form of DFM, tannins and saponin can alter the rumen composition and function.

These data demonstrate a proof of concept for ruminal manipulation resulting in ruminal homeostasis for rumen environments that are subjected to high concentration diets. As a result, there is a possibility that these changes could reduce bacterial translocation out of the rumen and decrease liver abscess rates.

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Table 6.1. Ingredients and chemical composition of the basal diet in dry matter (DM) basis.

Item	Percentage
Ingredient Composition (% DM)	
Steam Flaked Corn	61.00
Corn Silage	10.00
Alfalfa Hay	10.00
Dry Distillers Grain (DDG)	10.00
Fat (Tallow)	5.00
Chemical Composition	
Dry Matter, %	69.86
Crude Protein, %	12.90
ADF, %[1]	9.45
NDF, %[2]	16.27
NEg, Mcal/kg[3]	1.43

Macro- and microminerals included in diet: calcium = 0.71%, phosphorus = 0.33%, salt 0.51%, potassium 0.62%, sulfur 0.16%, magnesium 0.18%, zinc 19.50 ppm, iron 169.42 ppm, copper 6.04 ppm, manganese 11.80 ppm, cobalt 0.12 ppm, iodine 0.50 ppm, selenium 0.13 ppm, sodium 0.24%, and chlorine 0.44%

[1] ADF = Acid detergent fiber

[2] NDF = Neutral detergent fiber

[3] NEg = Net energy for growth

Table 6.2. Effect of direct-fed microbials (DFM), saponins and tannins on 12 h *in vitro* rumen fermentation characteristics.

	Treatment								
	Control[1]	**TCH[2]**	**THD[3]**	**Saponin**	**TCH+DFM[4]**	**THD+ DFM[5]**	**Saponin+ DFM**	**SEM[6]**	**Trt[7]**
	VFA[8] Mol/100 mol								
Total VFA (mM)	87.62^{abc}	88.23^{abc}	87.01^{bc}	86.37^{c}	87.99^{abc}	89.96^{a}	89.36^{ab}	0.9171	0.101
Acetic Acid	48.03^{a}	47.91^{a}	49.85^{a}	47.60^{ab}	45.77^{abc}	38.22^{c}	38.87^{bc}	3.0794	0.044
Propionic Acid	28.82^{bc}	26.59^{c}	28.46^{c}	29.28^{bc}	31.03^{abc}	35.62^{ab}	37.74^{a}	2.4245	0.016
Butyric Acid	21.10^{ab}	22.64^{ab}	21.66^{ab}	20.71^{b}	21.37^{ab}	23.83^{a}	20.88^{b}	1.0311	0.319
Valeric Acid	2.05^{a}	2.86^{a}	1.94^{a}	2.12^{a}	1.83^{a}	2.33^{a}	2.51^{a}	0.4750	0.749
Acetic: Propionic	1.71^{ab}	1.85^{a}	1.79^{a}	1.72^{ab}	1.74^{a}	1.33^{bc}	1.13^{c}	0.1372	0.003
% DMD[9]	55.91^{bc}	55.32^{bc}	49.48^{c}	62.65^{ab}	69.47^{a}	66.70^{a}	66.98^{a}	2.6209	<0.001
GP/DMD[10]	28.17^{a}	28.05^{ab}	27.52^{abc}	24.84^{e}	25.88^{de}	26.88^{bcd}	26.74^{cd}	0.4342	<0.001
pH	5.24^{b}	5.11^{c}	5.34^{ab}	5.31^{ab}	5.35^{ab}	5.40^{a}	5.40^{a}	0.0364	<0.001

[a,b,c,d,e] Means with different superscripts within a row differ ($P < 0.05$)

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] TCH = Condensed and Hydrolysable Tannin

[3] THD = Hydrolysable Tannin

[4] TCH+DFM = TCH+DFM (DFM = *Lactobacillus animalis* + *Propionibacterium acidipropionici*)

[5] THD+DFM = THD+DFM

[6] SEM = Pooled standard error of the mean

[7] Trt = Treatment

[8] VFA = Volatile fatty acids

[9] DMD = Dry Matter Disappearance

[10] GP/DMD = Gas pressure per gram of dry matter disappearance

Table 6.3. Effect of direct-fed microbials (DFM), saponins and tannins on 12 h *in vitro* rumen fermentation gas production and microbial protein production

	Treatment								
	Control[1]	**TCH[2]**	**THD[3]**	**Saponin**	**TCH+ DFM[4]**	**THD+ DFM[5]**	**Saponin+ DFM**	**SEM[6]**	**Trt[7]**
	Accumulated Volume (ml)								
Nitrogen	4.62[a]	4.67[a]	4.63[a]	4.30[b]	4.52[ab]	4.69[a]	4.63[a]	0.0979	0.069
Methane	16.29[ab]	15.24[bc]	14.67[c]	16.71[a]	1.16[ab]	15.93[abc]	15.91[abc]	0.4671	0.059
CO_2	79.10[b]	80.08[ab]	80.70[a]	78.98[b]	79.32[b]	79.38[b]	79.31[b]	0.4045	0.044
Gas Pressure (pa)	100953.00[a]	100519.00[ab]	98588.00[abc]	89011.00[e]	92707.00[de]	96299.00[bcd]	95803.00[cd]	1585.11	< 0.001
Microbial Protein (µg/ml)[8]	48.51[a]	41.98[a]	42.03[a]	48.29[a]	44.48[a]	54.64[a]	57.49[a]	5.5795	0.334

[a,b,c,d,e] Means with different superscripts within a row differ ($P < 0.05$)

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] TCH = Condensed and Hydrolysable Tannin

[3] THD = Hydrolysable Tannin

[4] TCH+DFM = TCH+DFM (DFM = *Lactobacillus animalis* + *Propionibacterium acidipropionici*)

[5] THD+DFM = THD+DFM

[6] SEM = Pooled standard error of the mean

[7] Trt = Treatment

[8] Microbial protein was determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium bicarbonate) Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit.

Table 6.4. Effect of direct-fed microbials (DFM), saponins and tannins on 18 h *in vitro* rumen fermentation characteristics.

	Treatment								
	Control[1]	**TCH[2]**	**THD[3]**	**Saponin**	**TCH+ DFM[4]**	**THD+ DFM[5]**	**Saponin+ DFM**	**SEM[6]**	**Trt[7]**
VFA[8] Mol/100 mol									
Total VFA (mM)	86.46^{b}	87.76^{b}	86.58^{b}	87.57^{b}	87.52^{b}	91.83^{a}	88.92^{ab}	1.1044	0.019
Acetic Acid	52.36^{a}	52.54^{a}	52.23^{a}	47.71^{ab}	49.64^{ab}	42.60^{b}	46.02^{ab}	2.5584	0.056
Propionic Acid	26.73^{ab}	26.87^{ab}	26.04^{ab}	30.56^{a}	27.25^{ab}	23.52^{b}	25.86^{ab}	1.9160	0.301
Butyric Acid	18.78^{c}	19.41^{bc}	19.92^{bc}	18.82^{c}	20.02^{bc}	24.79^{a}	21.66^{b}	0.8535	< 0.001
Valeric Acid	2.22^{b}	1.59^{b}	1.89^{b}	3.01^{b}	3.18^{b}	9.18^{a}	4.44^{b}	1.2538	0.001
Acetic: Propionic	2.34^{a}	2.39^{a}	2.33^{a}	2.01^{a}	2.22^{a}	2.09^{a}	2.15^{a}	0.1585	0.564
% DMD[9]	52.98^{ab}	51.79^{b}	56.78^{a}	52.45^{ab}	52.23^{ab}	54.89^{ab}	56.45^{ab}	1.6764	0.176
GP/DMD[10]	27.22^{abc}	26.09^{bc}	23.48^{c}	30.76^{ab}	29.51^{abc}	33.12^{a}	32.54^{ab}	2.4338	0.063
pH	5.56^{c}	5.71^{b}	5.62^{bc}	5.62^{bc}	5.70^{b}	5.90^{a}	5.51^{c}	0.0481	< 0.001

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

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] TCH = Condensed and Hydrolysable Tannin

[3] THD = Hydrolysable Tannin

[4] TCH+DFM = TCH+DFM (DFM = *Lactobacillus animalis* + *Propionibacterium acidipropionici*)

[5] THD+DFM = THD+DFM

[6] SEM = Pooled standard error of the mean

[7] Trt = Treatment

[8] VFA = Volatile fatty acids

[9] DMD = Dry Matter Disappearance

[10] GP/DMD = Gas pressure per gram of dry matter disappearance

Table 6.5. Effect of direct-fed microbials (DFM), saponins and tannins on 18 h *in vitro* rumen fermentation gas production and microbial protein production.

	Treatment								
	Control[1]	**TCH[2]**	**THD[3]**	**Saponin**	**TCH+DFM[4]**	**THD+ DFM[5]**	**Saponin+ DFM**	**SEM[6]**	**Trt[7]**
	Accumulated Volume (ml)								
Nitrogen	4.54^{bc}	4.37^{c}	4.18^{c}	5.30^{ab}	4.84^{bc}	5.77^{a}	4.83^{bc}	0.3027	0.006
Methane	20.84^{ab}	21.88^{ab}	24.32^{a}	19.79^{b}	20.04^{b}	22.09^{ab}	18.65^{b}	1.2633	0.064
CO_2	74.62^{ab}	73.75^{abc}	71.49^{c}	74.90^{ab}	75.11^{ab}	72.14^{bc}	76.52^{a}	1.0816	0.029
Gas Pressure (pa)	97326.00^{e}	93894.00^{f}	92617.00^{g}	113867.00^{c}	103959.00^{d}	130276.00^{a}	124016.00^{b}	11435.00	0.129
Microbial Protein (µg/ml)[8]	46.79^{a}	45.17^{a}	44.39^{a}	52.40^{a}	51.37^{a}	51.94^{a}	56.16^{a}	6.0267	0.334

[a,b,c,d,e,f,g] Means with different superscripts within a row differ ($P < 0.05$)

[1] Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer)

[2] TCH = Condensed and Hydrolysable Tannin

[3] THD = Hydrolysable Tannin

[4] TCH+DFM = TCH+DFM (DFM = *Lactobacillus animalis* + *Propionibacterium acidipropionici*)

[5] THD+DFM = THD+DFM

[6] SEM = Pooled standard error of the mean

[7] Trt = Treatment

[8] Microbial protein was determined by mixing (0.1 g of the dry pellet from the *in vitro* fermentation with 1 ml of sodium bicarbonate) Then samples were assayed for protein using a Quick Start Bradford Protein Assay Kit.

Table 6.6. Simple means of microbial phyla percentages relative abundance by treatment at 12 h of fermentation.

Phyla[1]	Control	TCH[2]	THD[3]	Saponin	TCH+DFM[4]	THD+DFM[5]	Saponin+DFM
Archaea, Euryarchaeota	0.78%	0.55%	0.65%	0.38%	0.36%	0.47%	0.36%
Actinobacteria	0.37%	0.58%	0.61%	0.50%	0.40%	0.54%	0.48%
Armatimonadetes	0.03%	0.02%	0.03%	0.02%	0.03%	0.03%	0.03%
Bacteroidetes	11.81%	9.93%	11.52%	8.96%	8.64%	9.15%	8.67%
Chloroflexi	0.06%	0.07%	0.09%	0.05%	0.06%	0.06%	0.06%
Cyanobacteria	0.02%	0.01%	0.02%	0.01%	0.01%	0.02%	0.01%
Firmicutes	83.18%	85.94%	83.19%	86.95%	87.23%	86.44%	86.83%
Fusobacteria	0.06%	0.02%	0.00%	0.10%	0.01%	0.00%	0.03%
LD1[a]	0.06%	0.04%	0.05%	0.04%	0.04%	0.04%	0.04%
Lentisphaerae	0.30%	0.17%	0.22%	0.16%	0.15%	0.18%	0.17%
Planctomycetes	0.04%	0.03%	0.06%	0.04%	0.04%	0.05%	0.05%
Proteobacteria	2.21%	1.92%	2.62%	2.06%	2.40%	2.22%	2.39%
Spirochaetes	0.05%	0.02%	0.02%	0.05%	0.02%	0.03%	0.02%
Synergistetes	0.05%	0.06%	0.07%	0.06%	0.06%	0.06%	0.06%
Tenericutes	0.43%	0.28%	0.40%	0.28%	0.21%	0.33%	0.36%
TM7[a]	0.12%	0.12%	0.14%	0.11%	0.12%	0.13%	0.14%
Unidentified Bacteria	0.02%	0.01%	0.02%	0.01%	0.02%	0.02%	0.02%
Verrucomicrobia	0.28%	0.14%	0.17%	0.14%	0.14%	0.14%	0.15%
WPS-2[a]	0.11%	0.06%	0.09%	0.07%	0.07%	0.09%	0.11%

[a] Candidate phyla

[1] Phyla that were present in fewer than two groups were excluded

[2] TCH = Condensed and Hydrolysable Tannin

[3] THD = Hydrolysable Tannin

[4] TCH+DFM = Condensed and Hydrolysable +DFM (DFM = *Lactobacillus animalis* + *Propionibacterium acidipropionici*)

[5] THD+DFM = Hydrolysable +DFM

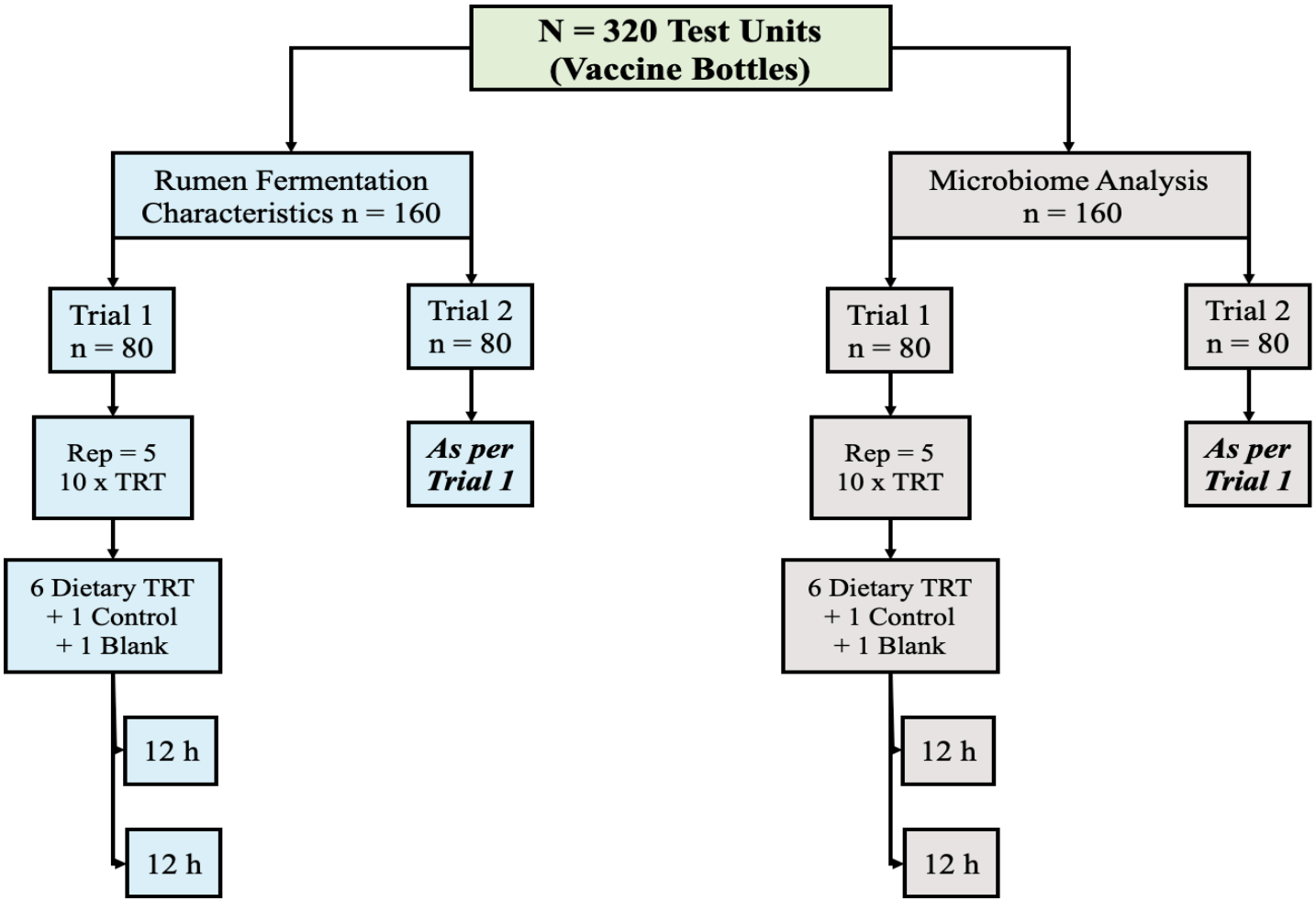

Figure 6.1. Experimental designed.

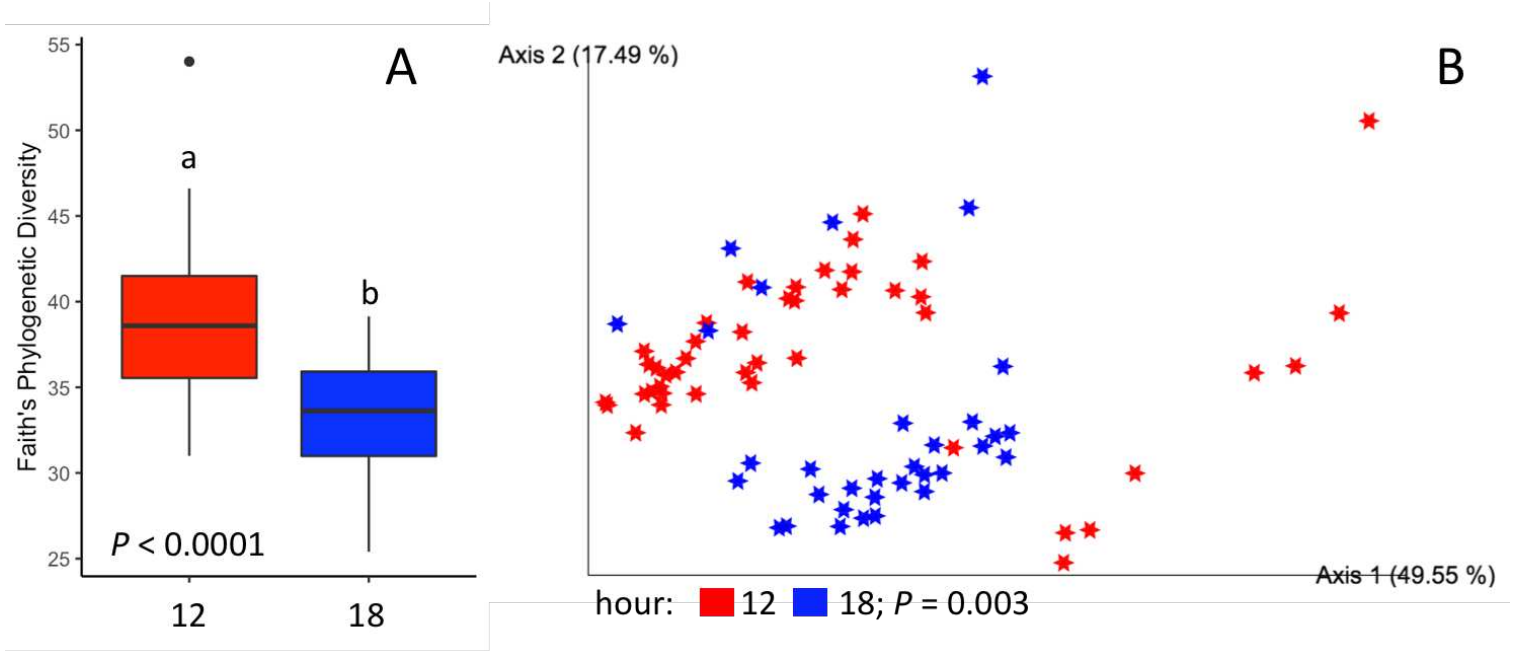

Figure 6.2. (A) alpha and (B) beta diversity of rumen samples colors by time of collection. Both were found to differ ($P < 0.0001$ and $P = 0.003$; respectively).

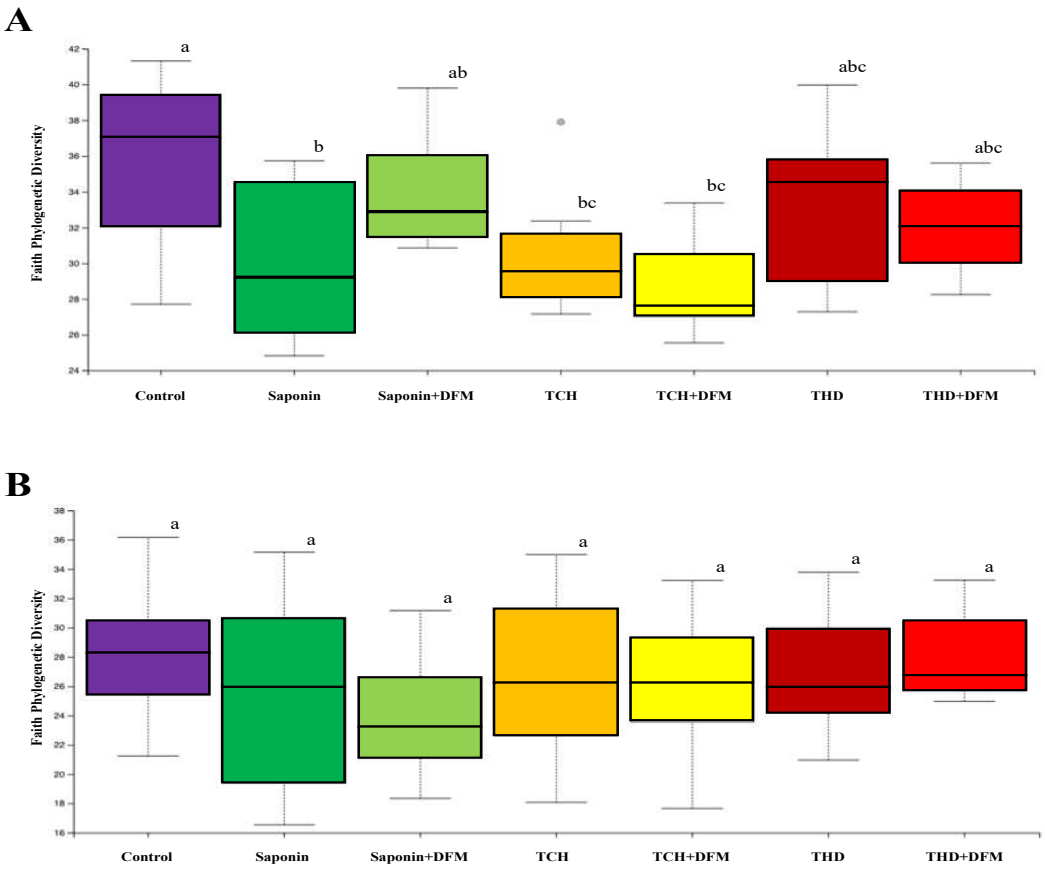

Figure 6.3. Alpha diversity as measure by Faith's phylogenetic diversity Index. Alpha diversity was significant ($P = 0.004$) different at 12 h of fermentation **(A)** and not significant ($P = 0.554$) at 18 h of fermentation **(B)** between all sample types. Statistical differences for alpha diversity were determine using non-parametric Kruskal-Wallis test.

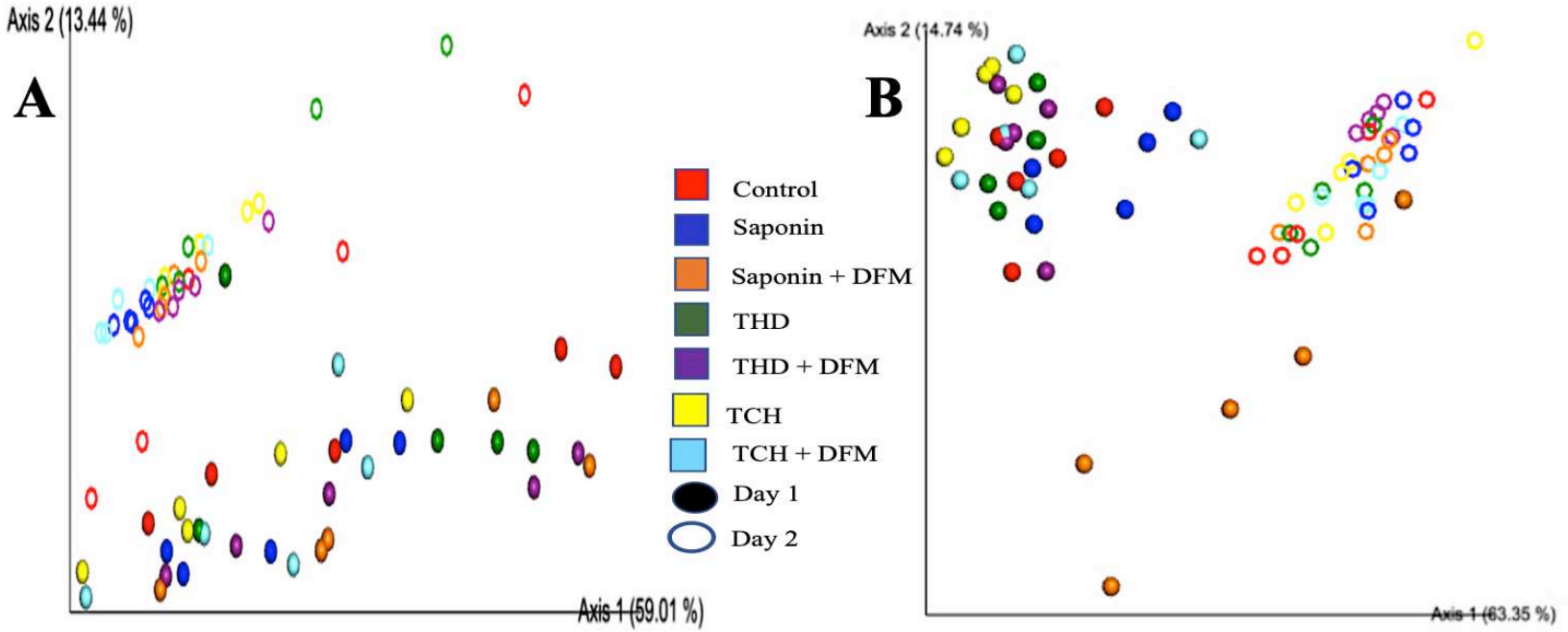

Figure 6.4. Beta diversity or microbial community composition visualized as principal coordinates analysis ordination bases on 16S rRNA gene sequence by Weighted UniFrac distances color by treatment and shape by day. Treatments at 12 h differ by treatment (P = 0.037; **A**) but did not differ by treatment group at 18 h of fermentation (P = 0.130; **B**).

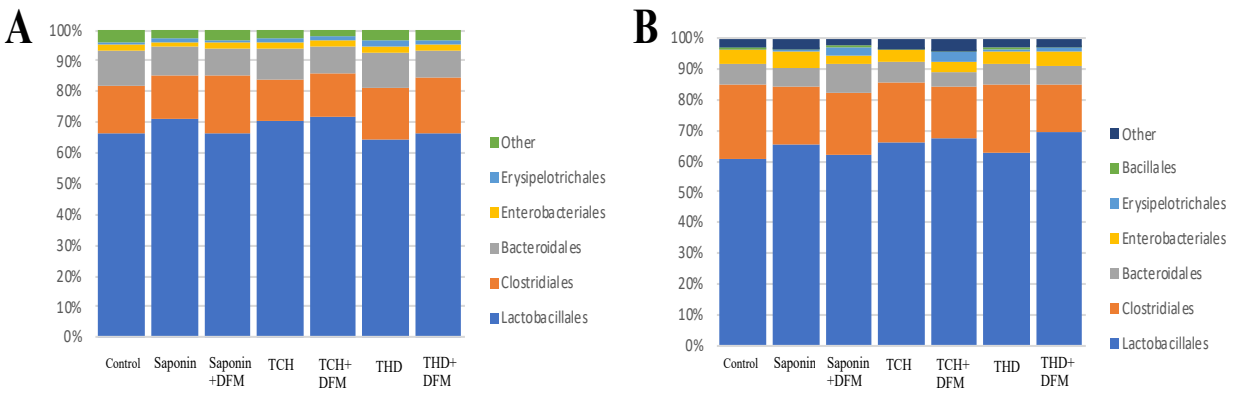

Figure 6.5. Normalize relative abundance of orders of bacteria (combine across treatment day and hour; order present in less than 1% of the entire microbiome were grouped into "other") 12 h are represented in (**A**) and 18 h are represented in (**B**)

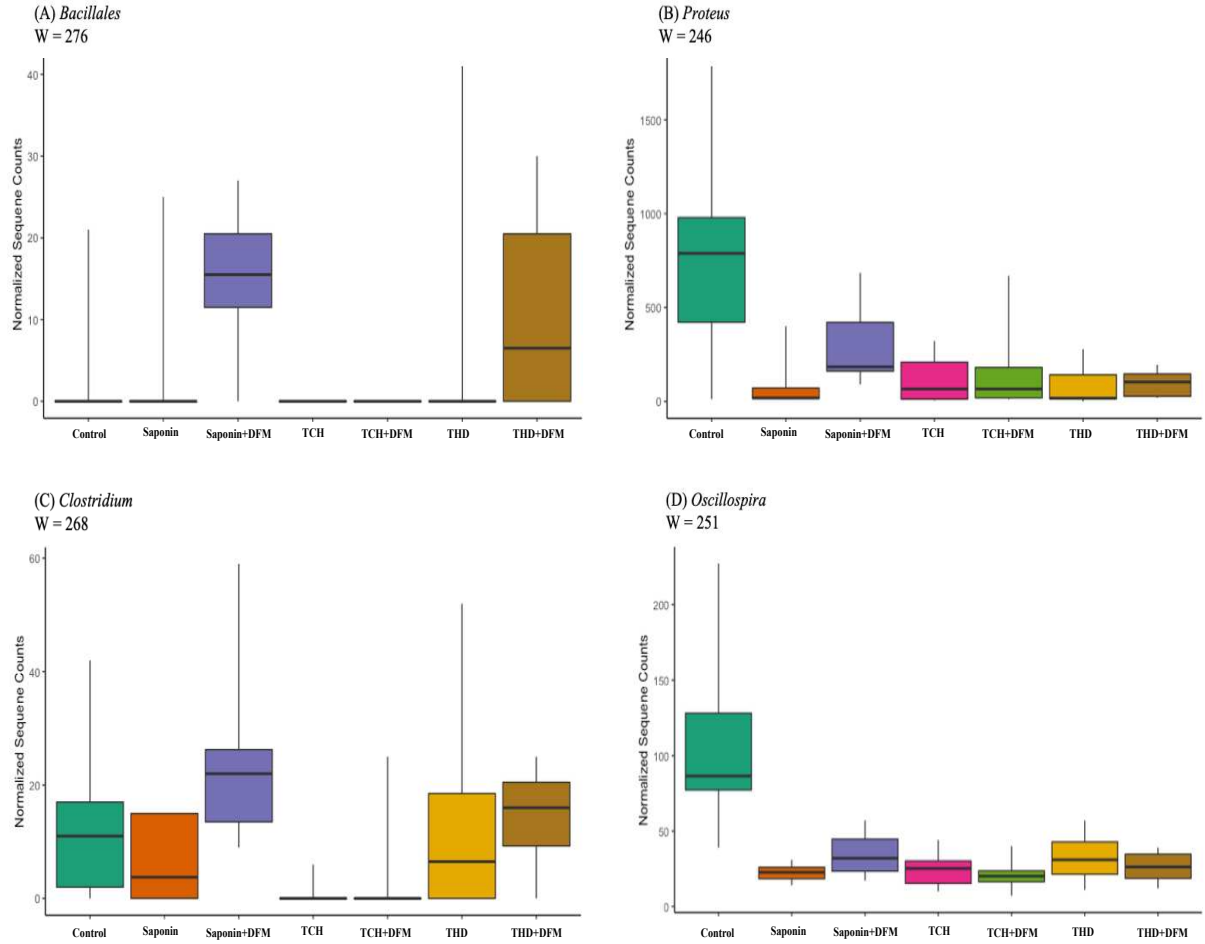

Figure 6.6. Relative abundance of the differentially abundant microbiota at 12 h of fermentation, (A) *Bacillales*, (B) *Proteus*, (C) *Clostridium*, and (D) *Oscillospira*, detected by ANCOM between treatments. Results are displayed as mean relative abundance for each treatment, with horizontal black lines delineating the abundance of unique SVs assigned within a given genus.

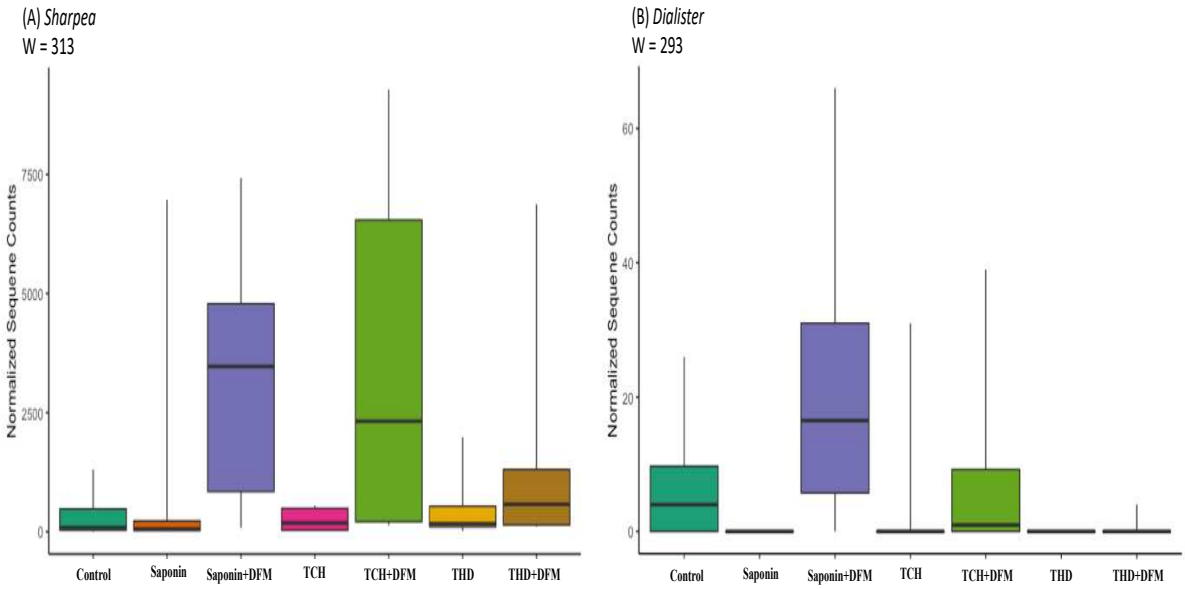

Figure 6.7. Relative abundance of the differentially abundant microbiota at 18 h of fermentation, (A) *Sharpea*, and (B) *Dialister*, detected by ANCOM between treatments. Results are displayed as mean relative abundance for each treatment, with horizontal black lines delineating the abundance of unique SVs assigned within a given genus.