THESIS

THE INFLUENCE OF PROPIONIBACTERIA ON *IN VIVO* RUMEN FERMENTATION CHARACTERISTICS AND *IN VITRO* LACTIC ACID CLEARANCE RATE IN FISTULATED STEERS FED MODERATELY HIGH CONCENTRATE DIETS

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ABSTRACT

THE INFLUENCE OF PROPIONIBACTERIA ON *IN VIVO* RUMEN FERMENTATION CHARACTERISTICS AND *IN VITRO* LACTIC ACID CLEARANCE RATE IN FISTULATED STEERS FED MODERATLY HIGH CONCENTRATE DIETS

The objective of this experiment was to determine the impact of a direct fed microbial (DFM) supplementation on rumen fermentation characteristics and *in vitro* lactic acid clearance. Fistulated steers (n = 6) were sorted into two groups of three steers, randomly assigned to one of two treatments, and fed a moderately high concentrate diet (14.9% CP, 1.17 Mcal/kg NEg, and 28.3% NDF) for 21 d prior to beginning the experiment. Treatments consisted of: 1) Control (No DFM; carrier only) or 2) DFM (0.225g·animal^{-1}·day^{-1} of 4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88). Treatments were administered daily, directly into the rumen via the cannula as a single bolus dose at the time of feeding. Immediately after treatment administration, the rumen contents were thoroughly mixed by hand. Two hours post feeding, rumen pH was determined, and rumen contents were sampled and analyzed for short chain fatty acids (SCFA), daily. On d 7 and 14, rumen fluid was collected from all steers and subjected to an *in vitro* lactic acid clearance challenge. Lactic acid and SCFA concentrations were determined at 0, 3, 6 and 9 h post-incubation. After d 14, all cattle received the basal diet for 21 d. On d 22, treatment crossover was implemented, and the experiment repeated. Data were analyzed by a mixed effects completely randomized block design (Proc Mixed, SAS Inst. Carey, NC). There were no treatment x block, treatment x time, or treatment x block x time interactions for any *in vivo* or *in vitro* rumen variables measured. Propionic acid concentrations were greater ($P < 0.05$) and total

SCFA tended ($P < 0.06$) to be greater in rumen fluid from steers receiving DFM compared to controls. Other *in vivo* rumen fermentation characteristics were similar. D- and total lactic acid concentrations but not L+ lactic acid concentrations were lesser ($P < 0.05$) at 3 h post incubation *in vitro*, for steers receiving DFM. D-, L+, and total lactic acids concentrations were similar between control and DFM treatments at 0, 6, and 9 h post incubation. *In vitro* molar proportions of propionic acid and total SCFA concentrations were greater ($P < 0.05$) and acetic acid molar proportions were lesser ($P < 0.05$) in steers receiving DFM. Collectively, under the conditions of this experiment, these data indicate that the DFM test article (*P. acidipropionici* - CP88) used in this experiment alters rumen fermentation characteristics *in vivo*, and *in vitro*, and lactic acid utilization *in vitro*.

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CHAPTER 1

INTRODUCTION

The United States beef cattle industry is heavily reliant upon efficiency and sustainability to deliver the safest and most nutritious product to consumers. In attempt to expand capabilities of the industry, cattle feeders have adopted technologies and nutritional supplements to aid in enhancing growth performance, feed efficiency, and end-product quality. Ruminants, such as beef cattle, rely on various bacteria, archaea, fungi, and protozoa in the rumen to convert plant material indigestible by humans into a nutrient-dense protein source (Li and Guan, 2017). The adoption of high-concentrate rations in feedlots has resulted in the availability of starches increasing fermentation in the rumen which can disrupt gas production and alter pH resulting in acidosis (Russell, 2001). Ruminal acidosis can threaten the integrity of the ruminal epithelium lining and allow bacteria to translocate into the blood stream which can be a direct cause of liver abscesses (Brent, 1976). Liver abscesses have had major economic and performance impacts for cattle producers.

Cattle feeders have implemented several different techniques to battle acidosis but among the most common is the use of antibiotics, such as tylosin phosphate. The use of antibiotics in food animal industry has caused a growing concern among consumers due to the possibilities of antibiotic resistance in both humans and livestock. The Food and Drug Administration (FDA) has since banned the use of any antibiotics that may be used as a growth promotant or to prevent disease outbreaks in groups of live animals. As of 2019, the only exception for using antibiotics in feedlot cattle is under the direction of a licensed veterinarian and producers must follow strict

protocols related to administering antibiotics as well as following the required withdrawal periods (VFD, 2015).

In a consumer-driven industry, cattle feeders have been able to adapt to new practices to maintain the health and efficiency of fed cattle. The use of natural supplements and direct-fed-microbials (DFM) have come to the forefront of recent studies as acceptable alternatives to antibiotics in cattle feed, however the impact of DFM on rumen fermentation characteristics are not well defined. Therefore, a topic of further understanding the rumen microbiome and fermentation characteristics through the inclusion of a direct fed microbial by *in vivo* and *in vitro* methods will serve as the focus of the current review and research.

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CHAPTER 2

LITERATURE REVIEW

The beef cattle industry in the United States has prided itself on the ability to efficiently and sustainably convert plant material, indigestible by humans, into beef, a nutrient dense protein source consumed globally. Efficiency in beef cattle production is largely measured by feed conversion rates. Compared to industry averages in 1977, U.S. beef cattle producers utilized 70 percent of the cattle, 81 percent of the feedstuffs, 88 percent of the water, and just 67 percent of the land to produce 1 billion kg of beef in 2007 (Capper, 2011). The majority of feedlot cattle in the United States are finished in the Great Plains region with over two-thirds of these cattle being fed in Nebraska, Kansas, and Texas (Drouillard, 2018). With so much of the country's beef being produced in such a concentrated area, cattle feeders have been forced to adapt their feeding techniques in order to produce the same amount of beef with fewer animals and less resources. This has led to a number of growth promoters being utilized, but these new technologies have not come without concern (NRC, 1980).

There are several nutritional supplements that are currently used in feedlot cattle production to aid in improving feed conversion ratios. Beta agonists, hormonal implants, ionophores, and antibiotics are among the most popular options utilized in feedlot production (BCRC, 2013). Several antibiotics, such as tylosin phosphate, are being incorporated into rations to help prevent cases of liver abscesses, which ultimately improves overall feed efficiency. Consumers have since expressed a number of concerns about the persistent use of antibiotics in cattle feeding, especially as it relates to developing antibiotic resistance in both livestock and

humans. This has brought forth the creation of the Veterinary Feed Directive that is designed to prevent the abuse and overuse of antibiotics in feedlots (VFD, 2015). While feedlots have had to adapt to new legislation, it has opened the door for new opportunities such as direct-fed-microbials (DFM) to be substituted into ration formulation and help maintain the current efficiency of cattle feeding (Seo et al., 2010).

2.10 History of Antibiotic Use

Antibiotics have changed the course of modern medicine, not only for humans, but for animals as well. Throughout human history, humans have used different techniques to treat diseases and infections. However, it was not until the late 19th and early 20th Century that research into producing a widely available antibiotic was initiated. There are many references from the ancient societies of Egypt, China, Serbia, Greece, and Rome indicating the benefits of applying moldy bread to affected areas (Gould, 2016). Several hundred years later, the mold in bread was determined to contain *penicillium fungi* which was crucial in fighting various bacterial infections (Keyes et. al., 2003). By the turn the turn of the 20th Century, Salvarson, a drug used to treat syphilis, was the first broadly used antibiotic (Aminov, 2010). The availability of antibiotics changed drastically in 1928 when Alexander Fleming discovered penicillin through a contaminated petri dish containing staphylococci (Lobanovska and Pilla, 2017). While Fleming was unable to purify penicillin at the time, his discovery started a major revolution in antibiotic production known as the golden age of antibiotic discovery (Gould, 2016). Through further research and development, penicillin was made widely available by 1945 and opened the door for further antibiotic discoveries in the 1960's.

Many of the antibiotics discovered during the golden age are derived from microorganisms. Of the many theories as to why soil microorganisms play such a crucial role in

antibiotic production, the most likely reason is that antibiotics produced by certain microorganisms are acting as "biological weapons" to inhibit the growth of competing microorganisms (Hutchings et al., 2019). *Streptomycin*, an aminoglycoside antibiotic derived from *Streptomyces* genus, discovered in soil microorganisms in 1944 allowed for scientists to isolate other naturally occurring antibiotic producing microorganisms (National Library of Medicine, 2021). Of the numerous antibiotics discovered between 1945 and 1978, 55% of those antibiotics originated from the genus *Streptomyces* (Hutchings et al., 2019). As new strains of antibiotics were being discovered and tested, there was a growing concern over the increase of antibiotic-resistance in microorganisms. This ultimately led to the need of developing new methods and technologies to search for additional antibiotic types.

There have been over 140 antibiotics developed in the past 80 years (Spellberg, 2014). Many of the antibiotics that were discovered during the golden age of modern medicine in the 1950's and 1960's were identified using very similar techniques used by Alexander Fleming in 1928 (Gould, 2016). While his methods undoubtedly have had a profound effect on medicine, the introduction of new technologies in the 21st Century has expanded research capabilities. The use of culture free techniques and the recent emergence of synthetic antibiotics have been significant in continuing the advancement of widely available antibiotics (Ling et al., 2015).

2.11 Antibiotic Use in Feedlot Cattle

As much of an impact as antibiotics have had on human medicine, they have had an equally important impact in animal agriculture. Specifically in feedlot cattle production, antibiotics have played a critical role in not only treating and preventing illness in feedlot cattle, but also by improving feed efficiency and average daily gain as well. In the last several decades, there has been an exponential increase in antibiotic usage in feedlot diets. In 2017, a USDA

stewardship report on US feedlots determined that over 85% of cattle feedlots administered antibiotics by feed, water, or injection at some point during an animal's life, of which only 50% of feedlots used antibiotics for medically important reasons, such as preventing bovine respiratory disease (USDA, 2019; Dall, 2019). Nearly one third (29%) of the feedlots included in the studies used antibiotics simply as a growth promotant to improve feed efficiency, weight gain, or carcass yield (USDA, 2019).

Antibiotics have a tremendously broad range of applications aside from treating illness. Preventing the incidence of liver abscesses has become extremely important to cattle feeders feeding high concentrate diets. *Fusobacterium necrophorum*, a gram-negative bacterium that is a major proteolytic species of bacteria in the rumen, has been long associated as the primary etiological agent of liver abscesses (Veloso and Drouillard, 2020; Amachawadi and Nagaraja, 2016). According to the 2012 National Beef Quality Audit Report, approximately 21% of livers were condemned at slaughter and nearly 65% of these were due to liver abscesses (McKeith et al., 2012). The majority of feedlots in the United States report liver abscess rates between 12% and 32% (Nagaraja and Lechtenberg, 2007). While the liver is not a significant financial loss at slaughter depending on the current markets, the decrease in feed efficiency and consequent carcass yield reduction is far more concerning to both cattle feeders and packers (Nagaraja and Lechtenberg, 2007). Antibiotics, such as tylosin, have been proven to help reduce the incidence of liver abscesses in feedlot cattle finishing on high concentrate diets (Amachawadi and Nagaraja, 2016). There are currently five available antibiotics that are approved for the use in feedlot cattle to reduce the incidence of liver abscesses. These include bacitracin methylene disalicylate, chlortetracycline, oxytetracycline, tylosin, and virginiamycin (Nagaraja and Lechtenberg, 2007). Tylosin is the most widely used in the United States' cattle feeding industry

and has also proved to be among the most effective feedgrade antibiotics in reducing the incidence of liver abscesses by 40% to 70% in feedlot cattle consuming high concentrate finishing diets (Nagaraja and Lechtenberg, 2007).

The basis of most feedlot operations is to maximize efficiency and profitability, which is a large reason why antibiotic growth promoters (AGP) have played such a crucial role in the livestock feeding industry. While antibiotics such as tylosin are primarily used to reduce instances of liver abscesses, ionophores are antimicrobials that focus on improving weight gain and feed conversion by altering the rumen microflora to help prevent ruminal acidosis and improve fermentation efficiency (Russell and Strobel, 1989).

The Food and Drug Administration (FDA) has approved the usage of ionophore and non-ionophore antibiotics in beef cattle diets (Bretschneider et al., 2008). There are currently 3 different ionophores that are commercially available for use in feedlot diets, including monensin, lasalocid, and laidlomycin propionate (Hersom and Thrift). Ionophores are very versatile compounds utilized in cattle feeding in that they can be fed at nearly every stage of production. However, their primary role is still to alter rumen fermentation to improve feed efficiency by reducing dry matter intake while maintaining average daily gain (DiCostanzo et al., 1996). Ionophores function by negatively affecting the metabolism of gram-positive bacteria in the rumen, which allows for the growth of bacteria to maximize digestive efficiency by increasing propionic acid production and decreasing the amount of acetic and lactic acid being produced (Hersom and Thrift). Not only do ionophores generally depress dry matter intake while maintaining or improving average daily gain, but by increasing the proportions of propionic acid and decreasing the amount of acetate and butyrate in the rumen, beef cattle have shown to be more energetically efficient. Because of the reduction in acetic acid, methane production is also

reduced resulting in the expectation that animal performance will be improved due to carbon and energy being retained during rumen fermentation (Bergen and Bates, 1984).

2.12 Economics of Antibiotic Use

One purpose for administering antibiotics to beef cattle has always been to maximize animal health in the feedlot, however the incentive of added growth performance provided by antimicrobials has recently become a significant reason for their usage. According to the USDA (2015), 49 percent of cattle at large feedlots in the United States have been given antibiotics for production purposes, and about 75 percent of feedlots carrying 1,000 head or more have provided antibiotics in either feed or water (USDA, 2015). The restrictions placed on production-purpose antibiotics by the VFD and the FDA has since impacted cost of production for both beef producers and consumers. The USDA (2015) was able to develop estimates for the effects that antibiotic restrictions had on production and beef prices. When production costs are increased by 1-3 percent due to limiting growth-promoting antibiotics, consumers can expect wholesale prices to increase by 1-3 percent as well.

In addition to using antimicrobials to improve feed efficiency and animal performance, there are also several preventative purposes for including antibiotics, such as tylosin, in rations. Liver abscesses are one of the major disorders in beef feedlot cattle that have caused economic losses to both producers and packers. Abscesses are currently the number one reason that livers are condemned in U.S. packing plants (Nagaraja and Lechtenberg, 2007). While the liver itself is not a significantly valuable commodity, the decrease in animal performance causes much more economic concern. Cattle with liver abscesses typically experience decreased feed intake and decreased weight gain, which results in lighter weight carcasses and lower carcass yield (Nagaraja and Lechtenberg, 2007). Brown and Lawrence (2010) stated that liver abscesses can

decrease carcass value anywhere from $20 to $80 USD per animal. In 2020, there were 32.8 million head of cattle slaughtered for meat (USDA, 2021). With the average liver abscess prevalence around 12 percent, this equates to between $78 million and $314 million USD in losses annually for beef cattle feeders and packers (Nagaraja and Lechtenberg, 2007).

2.13 Concerns of Antibiotic Use

While the use of antibiotics in cattle feeding programs may seem like a viable option to ensure maximum efficiency, the consumer is who ultimately drives production of a product. Consumers today are more focused about what goes into producing their food and far more interested in where their food comes from. There has been growing concern about the ability of antibiotics in cattle feed to transmit antibiotic resistant genes to pathogens that infect humans, therefore, making antibiotics used in human medicine less effective in treating disease. This has led to a number of changes in cattle feeding strategies including the development of the Veterinary Feed Directive and a surge in all-natural beef production (described previously and below).

Although antibiotic therapies have changed the efficiency of beef cattle production across the globe, there has been increasing worry about the overuse of certain antimicrobials leading to the development of antibiotic resistant pathogens. *Salmonella spp.* and *Escherichia coli* O157H7 are typically the most relevant food-borne pathogens and raise the threat of not being able to treat food born illnesses caused from cross-contamination with *Salmonella spp., Escherichia coli* O157H7, and in appropriate food handling and preparation (Elder et al., 2000). Humans are capable of experiencing antibiotic-resistant intestinal infections through consuming contaminated food products or coming in contact with animal waste that harbors a resistant pathogen (CDC, 2020). The number of cases of antibiotic-resistant bacteria being identified as the etiology of

infection in humans has been increasing rapidly across the world for the past several decades (Sobur et al., 2019). This has forced global regulatory agencies to impose restrictions on the use of antibiotics in livestock production. The majority of restrictions placed on antibiotics, beginning as early as the 1960's, was on therapies used as growth promoters (Wielinga et al., 2014). Sweden was one of the first countries to ban growth-promoting antibiotics in 1986 (Casewell, 2003). The European Union followed suit quickly thereafter with the ban of antibiotics used in livestock production that belonged in the same class as antibiotics used in human medicine (bacitracin, spiramycin, virginiamycin, and tylosin) in 1999 and eventually phased out other growth-promoting antimicrobials by 2006 (Casewell, 2003; Cogliani et al., 2011)

The United States Food and Drug Administration first introduced legislature to combat antibiotic resistance in the early 1990's when the administration required that medically important antimicrobials be prescribed by a licensed veterinarian (FDA, 2021). The United States furthered their regulation on antibiotic use in livestock production when the Veterinary Feed Directive (VFD) Final Rule was put into effect in October of 2015. In 1996, the United States Congress passed the Animal Drug Availability Act that allowed for new marketing and production possibilities for animal pharmaceuticals and medicated feeds (VFD, 2015). Since then, there has been a surge in antibiotic usage in animal agriculture, more specifically the beef and pork sectors. This has forced the Food and Drug Administration (FDA) to tighten regulations in order to avoid the overuse of specific antibiotic treatments. Antibiotics are still widely available for feedlots to utilize; however, they require a more in-depth application process and require a prescription from a certified veterinarian. The primary goals of the final VFD ruling were to promote the judicious use of antibiotics, protect public health, and to help limit the

development of antimicrobial resistance (Pyatt et al., 2016). The VFD ruling requires feedlot operators to work closely with their veterinarian to gain approval to use a number of antibiotic therapies (FDA, 2021). The VFD focuses primarily on shared-class antibiotics that are approved for use in both humans and animals, such as penicillin, tetracyclines, and macrolides. Requiring VFD approval for tetracyclines and macrolides have had the biggest impact on the cattle industry, however animal-grade antibiotics such as ionophores are still available and do not require a prescription from a certified veterinarian (Pyatt et al., 2016).

The demand for beef has been steadily decreasing since the 1970's, largely due to the lack of information available to customers about the product they are consuming (Boland and Schroeder, 2002). Consumers are determined to put the healthiest ingredients into their body. This has created a rise in the amount of natural or organic beef that is being produced annually. While natural and organic beef production is very similar, there are subtle differences that differentiate the two. If beef products from cattle are to be labeled as "natural", the cattle are not permitted to have any antibiotics, ionophores, or implants administered during any stage of their life. However, natural cattle producers are allowed to use feedstuffs that are conventionally produced in ration formulation. Typically, certified natural cattle can bring premiums as much as $0.35 per kg on a live-weight basis (Troxel, 2012). Certified organic cattle production also prohibits the use of antibiotics, ionophores, and growth implants, and also requires that the cattle are fed organically raised feedstuffs throughout their lifetime. The process to certify and market organic beef products is much more rigorous as it is overseen by the USDA. Organic beef cattle operations are required to go through an intensive USDA inspection in order to qualify as a certified organic animal production facility (Troxel, 2012). The increase in availability of both natural and organic beef products in stores has initiated questions as to whether consumers would

be willing to pay premiums for these products. According to a survey conducted by Jennifer Grannis (2000), 51 percent of consumers indicated they would pay at least 10 percent price premiums for natural steak, while 94 percent would pay at least 12 percent price premiums for natural ground beef.

2.14 Direct Fed Microbials

While the current legislation on limiting feed-grade antibiotics in feedlot rations has certainly reduced the concern over antibiotic resistance in beef products, the industry is still searching for alternatives to maximize beef cattle production efficiency. Direct fed microbials (DFM) have been examined as a possible option to be incorporated in feedlot ration formulation to promote rumen health and improve feed efficiency.

Direct fed microbials are a nutritional supplement that limit gastrointestinal infection and promotes healthy microbial populations in the digestive tract (Seo et al., 2010) Moore et. al. (1946) recorded the first documented research on DFM for use in animals with the study of sulfasuxidine, streptothricin, and streptomycin in chicks, and by 1999, the American Association of Feed Control Officials had listed 42 different microbials as acceptable "food" products opening the door for their use in food animal rations (Moore et. al., 1946; Buntyn et. al., 2016) Currently, there are over 3,000 scientific journal articles regarding the use of DFM to improve livestock production (Buntyn et. al., 2016) The response of direct fed microbials has largely varied in studies due to the differences in production scenario, diet concentration, dosage amount, and strain of DFM (Elghandour et. al., 2015). There have been several studies concerning the use of DFM in lactating dairy cows (Krehbiel et. al., 2003). Jaquette et. al. (1988) and Ware et. al. (1988a) reported findings that dairy cows produced an additional 1.8 kg/d in total milk yield when fed a diet containing *L. acidophilus*. While the original purpose of DFM in

beef cattle was to enhance rumen health and to establish gastrointestinal tract microflora in young calves, their use in finishing cattle has been steadily increasing (McAllister et al., 2011). Ware et. al. (1988b) was among the first to report that *L. acidophilus* improved weight gain and feed conversion in feedlot cattle, and a report from Swinney-Floyd et. al. (1999) further confirmed that including a combination of *L. acidophilus* and *P. freudenreichii* in the ration led to improvement in total feed efficiency. With recent bans on antibiotic usage in feedlots across Europe, cattle feeders have begun to adopt the use of DFM. There are several different forms of DFMs, including lactic acid producing bacteria (LAB), lactic acid utilizing bacteria (LUB), yeast products, etc. (Seo et al., 2010). Many DFM act to maximize the potential healthy bacteria in the gut. Through the promotion of a healthy and stable rumen, feedlots have seen trends of increased feed efficiency and reduced incidents of ruminal acidosis (Seo et al., 2010).

Among the most popular species of DFM thus far in cattle feeding, *Propionibacterium* has provided some of the most promising results. The first studies on *Propionibacterium* were conducted by Albert Fritz (1879) and it has since been developed into a viable feed additive to feed-grade antibiotics (Zarate, 2012). *Propionibacterium* is a lactic acid utilizing DFM that works by fermenting lactate to propionate. Propionate is the major precursor for gluconeogenesis in beef cattle (Reynolds et al., 2003). By increasing the production of propionate in the rumen, hepatic glucose production is increased, which in turn provides more substrates for animal growth (Stein et al., 2006; Weiss et al., 2008). The mode of action for *Propionibacterium* is to: 1) convert lactate to volatile fatty acids, 2) produce propionic acid rather than lactic acid, 3) increase feed efficiency, 4) decrease methane production, and 5) increase ruminal pH (Seo et al., 2010). Propionibacterium has been given Generally Regarded as Safe (GRAS) status by the FDA, which further opens to door for usage in cattle diets (Vyas et al., 2014).

2.20 Summary

The occurrence of liver abscesses in beef cattle and the implication of antibiotics to reduce financial and performance losses have been studied for decades. The pathogenesis and direct cause of liver abscesses is still not clearly defined, but the application of antibiotics, such as tylosin phosphate, has greatly reduced the incidence of abscesses in recent years. Currently there is a growing concern of antibiotic resistance in both beef cattle and humans, leading to a call for alternative feed additives. Direct fed microbials have been studied as possible solutions to reduce the probability of liver abscesses while also maintaining rumen characteristics important to feed efficiency and production. Therefore, the objectives of the subsequent experiment were to study the impact of a direct fed microbial (DFM; Summitt 10X: 4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88) on *in vivo* and *in vitro* rumen propionic acid production in rumen fistulated steers and *in vitro* lactic acid clearance from rumen fluid following exogenous lactic acid addition.

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CHAPTER 3

THE INFLUENCE OF PROPIONIBACTERIA ON *IN VIVO* RUMEN FERMENTATION CHARACTERISTICS AND *IN VITRO* LACTIC ACID CLEARANCE RATE IN FISTULATED STEERS FED MODERATLY HIGH CONCENTRATE DIETS

SUMMARY

The objective of this experiment was to determine the impact of a direct fed microbial (DFM) supplementation on rumen fermentation characteristics and *in vitro* lactic acid clearance. Fistulated steers (n = 6) were sorted into two groups of three steers, randomly assigned to one of two treatments, and fed a moderately high concentrate diet (14.9% CP, 1.17 Mcal/kg NEg, and 28.3% NDF on a dry matter basis) for 21 d prior to beginning the experiment. Treatments consisted of: 1) Control (No DFM; carrier only) or 2) DFM (0.225g·animal^{-1}·day^{-1} of 4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88). Treatments were administered daily, directly into the rumen via the cannula as a single bolus dose at the time of feeding. Immediately after treatment administration, the rumen contents were thoroughly mixed by hand. Two hours post feeding, rumen pH was determined, and rumen contents were sampled and analyzed for short chain fatty acids (SCFA), daily. On d 7 and 14, rumen fluid was collected from all steers and subjected to an *in vitro* lactic acid clearance challenge. Lactic acid and SCFA concentrations

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This chapter is a published paper completed by R. J. Gifford[*] as the primary experimenter and with the assistance of M. P. Thorndyke[*], O. Guimaraes[*], H. Hallmark[*], S. Crane[*], T. A. Thomas[*], S. R. Goodall[†], J. J. Wagner[*], and T. E. Engle[*]. Experiments were conducted at [*]Colorado State University, Department of Animal Sciences, Fort Collins, CO, 80523, USA through funding provided by [†]MicroBios, Houston, TX, 77002, USA.

were determined at 0, 3, 6 and 9 h post-incubation. After d 14, all cattle received the basal diet for 21 d. On d 22, treatment crossover was implemented, and the experiment repeated. Data were analyzed by a mixed effects completely randomized block design (Proc Mixed, SAS Inst. Carey, NC). There were no treatment x block, treatment x time, or treatment x block x time interactions for any *in vivo* or *in vitro* rumen variables measured. Propionic acid concentrations were greater ($P < 0.05$) and total SCFA tended ($P < 0.06$) to be greater in rumen fluid from steers receiving DFM compared to controls. Other *in vivo* rumen fermentation characteristics were similar. D- and total lactic acid concentrations but not L+ lactic acid concentrations were lesser ($P < 0.05$) at 3 h post incubation *in vitro*, for steers receiving DFM. D-, L+, and total lactic acids concentrations were similar between control and DFM treatments at 0, 6, and 9 h post incubation. *In vitro* molar proportions of propionic acid and total SCFA concentrations were greater ($P < 0.05$) and acetic acid molar proportions were lesser ($P < 0.05$) in steers receiving DFM. Collectively, under the conditions of this experiment, these data indicate that the DFM test article (*P. acidipropionici* - CP88) used in this experiment alters rumen fermentation characteristics *in vivo*, and *in vitro*, and lactic acid utilization *in vitro*.

Key words: fermentation, short chain fatty acids, lactic acid, direct fed microbial

INTRODUCTION

Ruminal acidosis can threaten the integrity of the ruminal epithelium and permit bacteria translocation to the blood stream which may ultimately lead to liver abscess formation (Brent, 1976). Liver abscesses can have a major economic impact on beef cattle production efficiency (Amachawadi and Nagaraja, 2016). Hicks (2011) reported that liver abscesses contribute

approximately $15.8 million in losses to the beef cattle industry, annually. To help prevent liver abscess formation, feed-grade antibiotics can be added to the diets fed to beef cattle (Lundeen, 2013).

The increasing public concerns surrounding antibiotic resistance has led to investigation of alternative technologies for decreasing the incidence of liver abscesses without the use of antibiotics. The use of direct fed microbials (DFM) has been reported to enhance animal efficiency by altering ruminal bacterial communities (Krehbiel et al., 2003). Even though DFM have been shown to positively benefit the animal, the impacts of DFM on ruminal fermentation characteristics are not well defined. Therefore, the objectives of this experiment were to study the impact of a direct fed microbial (DFM; Summitt 10X: 4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88) on *in vivo* and *in vitro* rumen propionic acid production in rumen fistulated steers and *in vitro* lactic acid clearance from rumen fluid following exogenous lactic acid addition.

MATERIALS AND METHODS

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 20-9823A). Steers were housed at the CSU Agricultural Research, Development and Education Center.

Animals and Treatment

Six steers, fitted with ruminal cannula, were used in this experiment. Steers were weighed and sorted into two groups of three steers per group. Groups were then randomly assigned to one of two treatments. Treatments consisted of: 1) Control (0.0 cfu·hd^{-1}·day^{-1}; carrier only) or 2) DFM (0.225g·head^{-1}·day^{-1} of 4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* yielding a daily dose of 1.0 x 10^{10} CFU·hd^{-1}·day^{-1}). Steers were housed in feedlot pens (7 m x 40 m)

equipped with a concrete feed bunk, a 3 m x 7 m concrete bunk pad, and an automatic waterer. Prior to beginning the experiment, a basal feedlot transition diet (Table 1) was fed to all steers for 21 d. After the 21-day adjustment period, treatments were initiated.

All steers were fed the basal ration, once daily, at approximately 0800h. Rations were delivered to supply 12 kg DM to each steer. The basal diet (Table 1) was formulated to meet or exceed the National Academy of Science, Engineering, and Medicine (NASEM, 2016) requirements for moderate growth cattle. At the time of feed delivery, steers received either the control or DFM treatments directly through the rumen fistula via a single bolus dose. For treatment delivery, 901 mL of water containing either the Control (water plus DFM carrier) or 901 mL of the DFM added to the water (dose = 1.00×10^{10} CFU·animal^{-1}·d^{-1} in 901 mL of water).

Pens were checked daily to ensure that cattle were in the appropriate pens and that all gates were secure. Furthermore, all cattle were monitored for health and locomotion problems daily. Steers exhibiting significant symptoms of respiratory disease were removed from the pen and rectal body temperatures were recorded. Steers exhibiting body temperatures greater than 39.4°C were considered moribund. All moribund steers were treated according to the appropriate treatment schedule and immediately returned to their original pen and allowed a chance to recover. If problems persisted concerning the health status of a specific steer, the steer was removed from the experiment. If a steer was removed from the experiment, the steer was weighed, the feed in the feed bunk was weighed and a feed sample was obtained for DM determination.

Rumen Sampling

Immediately after treatment addition to the rumen, rumen contents were thoroughly mixed by hand. Daily rumen pH was determined by inserting a portable pH meter (EcoTestr pH 2+; Oaktron 153 Instruments, Vernon Hills, IL) into the geometric center of the rumen at 2 h post feeding. Following rumen pH determination rumen contents were thoroughly mixed by hand and a sample was obtained from the geometric center of the rumen (approximately 250 g). After each collection, ruminal samples were centrifuged at 28,000 x *g* at 5°C for 30 min. A 2.0 ml aliquot of the supernatant was acidified with 25% (vol/vol) meta-phosphoric acid and frozen at -80°C until analyzed for SCFA concentrations via gas chromatography.

On day 7 and 14 of the experiment, rumen fluid was collected from all steers 2 hours post-feeding as described by Ward and Spears, (1993). Briefly, rumen fluid (~ 4 L) from each steer was filtered once through four layers of cheesecloth and combined into individual pre-warmed (39°C) thermoses. A modified McDougall's buffer solution (39.20 g NaHCO3, 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, as a means of 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 basal 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 (0.50 ± 0.005 g) into pre-labeled 50 mL conical tubes (12 tubes per animal). Then 1 mL of an 85% racemic lactic acid solution was added to each conical tube to provide an equivalent of 5 mg of lactic acid/mL of rumen fluid. Immediately after lactic

acid addition, 30 mL of McDougall's buffer/rumen fluid mixture was dispensed into the appropriate conical tubes. The tubes were capped with one-way pressure release valves, to maintain anaerobic conditions, and were incubated at 39°C in a circulating water bath. To simulate rumen motility, all tubes were gently swirled every 3 h. At 0, 3, 6, and 9 hours three tubes per animal were removed from the water bath and centrifuged at 28,000 x *g* at 5°C for 30 min. A 2.0 ml aliquot of the supernatant was acidified with 25% (vol/vol) meta-phosphoric acid, and frozen at -80°C until analyzed for lactic acid and chain fatty acid (SCFA) concentrations.

Volatile Fatty Acid and Lactic Acid Analysis

After thawing at room temperature, samples designated for SCFA 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 SCFA. The SCFA 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. L+ lactate and D-lactate were analyzed by via enzyme linked immunosorbent assay (Sigma Chemical, St. Louis, MO).

After day 14 of the experiment, control and DFM treatments were stopped and all cattle received the basal diet only, for 21 d. This period served as the washout period of the experiment. After the 21 d washout period, cattle previously receiving the control treatment were switched to the DFM treatment and cattle previously receiving the DFM treatment were switched to the control treatment and the above described experiment was repeated.

Statistical Analysis

A mixed effects model repeated measures analysis for a completely randomized block design was used to analyze daily measurements of rumen pH and SCFA concentrations. The fixed effects were treatment, time, and the treatment x time interaction. For all response variables measured, individual animal was considered the experimental unit. Several covariance structures were compared to determine the most appropriate covariance structure for data analysis. In vitro lactic acid concentrations were analyzed separately by time using a mixed effects model (PROC MIXED, SAS Inst. Inc., Cary, NC) for a completely randomized block design. If a treatment x block, treatment x time, or a treatment x block x time were not significant, data were pooled across block, time or block and time where appropriate. For all response variables, significance was determined at $P \leq 0.05$ and tendencies were determined at $P > 0.05$ and ≤ 0.10. When a significant treatment × time interaction was detected, treatment means were separated using the PDIFF option of the LSMEANS statement of SAS.

RESULTS

***In Vivo* Fermentation Characteristics**

The influence of *in vivo* daily dosing of a DFM on rumen pH and SCFA composition in fistulated steers two hours post feeding over a 14-day period is described in Table 2. There were no treatment x block, treatment x time, or treatment x block x time interactions for any response variables measured. Therefore, overall main effects are presented. Propionic acid concentrations were greater ($P < 0.05$) and total SCFA tended ($P < 0.06$) to be greater in steers receiving DFM when compared to controls. All other rumen fermentation characteristics were similar across treatments.

***In Vitro* Lactic Acid Clearance/Utilization Rates**

Table 3 describes the influence of *in vivo* daily dosing of a DFM on changes in vitro lactic acid concentrations (mM) at 0, 3, 6, and 9 hours post fermentation. There were no day (7 and 14 day) x treatment, block x treatment, or day x block x treatment interactions for any response variables measured. Therefore, overall means are presented in Table 3. D (-) and total lactic acid concentrations but not L (+) lactic acid concentrations were lesser ($P < 0.05$) at 3 h post incubation when compared to controls, suggesting a more rapid rumen clearance due to the DFM. D (-), L (+), and total lactic acids concentrations were similar between control and DFM treatments at 0, 6, and 9 h post incubation.

***In Vitro* Fermentation Characteristics**

The influence of *in vivo* daily dosing of a DFM on *in vitro* SCFA composition at 0, 3, 6, and 9 hours post *in vitro* incubation with lactic acid in rumen fluid collected from fistulated steers receiving either control or DFM treatments is shown in Table 4. There were no treatment x block, treatment x time, or treatment x block x time interactions for any response variables measured. Therefore, overall treatment means are shown in Table 4. Molar proportions of propionic acid and total SCFA concentrations were greater ($P < 0.05$) and acetic acid molar proportions were lesser ($P < 0.05$) in steers receiving DFM when compared to control steers. Isobutyric and butyric acid concentrations were similar across treatments.

DISCUSSION

This study investigated the effects of *in vivo* and *in vitro* supplementation of a DFM (*Propionibacteria acidipropionici* - CP88) on rumen fermentation characteristic and lactic acid disappearance rates. Narvaez et al. (2014) conducted a study focusing on the supplementation of *Propionibacteria acidipropionici* to beef cattle fed a 15.5 percent CP, 8.9 percent ADF, and 25.7 percent NDF dry-rolled corn and corn dried distiller's grain finishing diet. This study reported

that supplementing *Propionibacteria acidipropionici* in the finishing diet had no effect on the feed intake (10.55 kg vs. 10.53 kg), growth rate (1.81 kg vs. 1.77 kg), and feed conversion (0.168 kg/d vs. 0.169 kg/d) of steers receiving the DFM when compared to the control steers. Furthermore, steers supplemented with *Propionibacteria acidipropionici* had greater incidents of subacute ruminal acidosis compared to control steers (4.23 bouts/d vs. 2.22 bouts/d). Sanchez et al. (2014) reported that *Propionibacteria acidipropionici* increased the total volatile fatty acids (119.1 m*M* vs. 92.8 m*M*) while decreasing the ratio of acetate to propionate (3.1 vs. 3.6) in the rumen of cattle fed dormant, low-quality forages when compared to non-supplemented control cattle. The P169 strain of *Propionibacteria acidipropionici* used in the previous studies is different than the CP88 strain used in the current study. There are currently no published studies concerning the performance effect of *Propionibacteria acidipropionici* – CP88 in any species of livestock. However, Propionibacteria acidipropionici - strain DH42 administered to fistulated steers fed a high concentrate diet demonstrated similar SCFA effects to this study (Kim et. al., 2000). Propionate was increased at the expense of acetate across a range of *P. acidipropionici* doses (10^7 to 10^{10} CFU·animal^{-1}·d^{-1}).

Measurements of SCFA and pH were the parameters used in the present study to represent rumen fermentation characteristics. Steers receiving the DFM had increased concentrations of propionic acid both *in vivo* and *in vitro,* increased total SCFA *in vitro* and decreased concentrations of acetic acid *in vitro* compared to control steers, while pH was similar across treatments. Lewis and Yang (1992) reported that *Propionibacteria acidipropionici* can convert lactate and glucose to propionic and acetic acid, respectively, resulting in the increase of propionic acid and/or acetic acid with a concurrent decrease of lactic acid and/or glucose in the rumen (Lewis and Yang, 1992; Wilson and Krehbiel, 2011). L (+) and D (-) lactate are the two

isoforms of lactate that exist in the rumen of beef cattle (Hernández, 2014). L (+) lactate is produced from pyruvate during anaerobic glycolysis by bacterial L-lactate dehydrogenase when pyruvate is not being efficiently metabolized in the TCA cycle (Kovacic, 2009). Because most mammals lack D-lactate dehydrogenase, D (-) lactate is typically formed through the consumption of feedstuffs or by intestinal bacteria production (Ewaschuk et. al., 2005; Monroe et. al., 2019). Lactate can be converted to volatile fatty acids through two primary metabolic pathways (Vidra and Németh, 2017). The succinate pathway follows glycolysis where glucose is converted to pyruvate. Pyruvate is converted into oxaloacetate in the first steps of the TCA cycle where propionate is formed through the conversion of malate, succinyl-CoA, and propionyl-CoA (Liu et. al., 2016). Rather than producing propionate from glucose, the secondary acrylate pathway utilizes lactate to generate propionate, primarily through the consumption of the reducing equivalent, NADH (Gonzalez-Garcia et. al., 2017). The primary cause for subacute ruminal acidosis has largely been defined by low rumen pH and bicarbonate when the rumen is over-producing D (-) lactate (Hernández, 2014). Because of the larger proportions of L-lactate dehydrogenase in beef cattle and the greater binding affinity for L (+) lactate, D (-) lactate is typically metabolized at a slower rate when it is present in the gastrointestinal tract (Kovacic, 2009). Lactate and lactic acid are typically used interchangeably, however lactate must bind to a hydrogen ion to become lactic acid (Andersen et. al., 2013). The reduction of lactic acid in the rumen has shown positive results in reducing instances of acidosis (Calsamiglia et al., 2012) The lactic acid concentrations reported in the *in vitro* portion of this study, rapidly decrease from hour 0 to hour 3 (between 70 and 90% of the total lactic acid), where they then remained similar across both treatments through hour 9. In future studies regarding lactic acid disappearance,

more frequent measurements between hour 0 and hour 3 should be obtained to depict lactic acid disappearance rates more accurately between treatments.

These results suggest that the DFM (*Propionibacteria acidipropionici* - CP88) is fermenting lactic acid to propionic acid, most likely through either the acrylate or succinate pathways (Baldwin et. al., 1962; Schulmand and Valentino, 1976). While it is assumed that the DFM is converting lactic acid to propionate through either of the two pathways, it is unclear as to why D (-) lactate clearance was significantly different while L (+) lactate clearance was not.

CONLCUSION

Collectively, under the conditions of this experiment, our results indicate that the DFM test article used in this experiment (*Propionibacteria acidipropionici* - CP88) increases rumen fermentation efficiency through greater propionic acid acid production *in vivo* and *in vitro* while reducing acetic acid *in vitro*. Increases D (-) and total lactic acid clearance/utilization rates (reduced potential for lactic acidosis) were also demonstrated *in vitro*. Future research concerning the ability to incorporate the DFM efficiently and effectively into feedlot rations is also warranted.

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Table 1. Dry matter ingredient composition of basal cross.

Ingredient	%
Corn Silage	50.0
Cracked corn	23.6
Distiller's grains	8.5
Alfalfa hay	7.5
Wheat straw	5.5
Liquid Supplement.[1]	4.4
Limestone	0.40
Salt	0.10
Analyzed nutrient composition	
DM, % as fed	62.6
CP, %	14.9
ADF, %	18.3
NDF, %	28.3
Ether extract, %	6.02
NEg, Mcal/kg	1.17
NEm, Mcal/kg	1.86
Calcium, %	0.62
Magnesium, %	0.20
Phosphorus, %	0.35
Potassium, %	1.30
Sulfur, %	0.21
Cobalt, mg/kg	0.20
Copper, mg/kg	18.0
Manganese, mg/kg	71.9
Selenium, mg/kg	0.21
Zinc, mg/kg	60.8

[1]Liquid supplement provided in a molasses - fat suspension: 3.72% NPN (Urea), 0.61% Ca ($CaCO_3$), 0.56% Salt (NaCl), 2.75% K (KCl), 110,000 IU/kg Vitamin A, 9.4 IU/kg Vitamin E, and 440 g/metric ton of monensin (Rumensin 90, Elanco Animal Health, Greenfield, IN).

Table 2. Influence of *in vivo* daily dosing of a direct fed microbial (DFM) on rumen pH and short chain fatty acid (SCFA) composition in fistulated steers two hours post feeding over a 14 day period.

	Treatment			$P <$ [a]		
Item	Control	DFM[b]	SEM	Trt	Time	Trt x time
pH	6.66	6.69	0.04	0.63	0.0003	0.25
Acetic acid, mM/100mM	56.78	55.89	1.02	0.94	0.02	0.61
Propionic acid, mM/100mM	23.79	26.35	1.15	0.04	0.001	0.63
Isobutyric acid, mM/100mM	0.64	0.62	0.12	0.61	0.01	0.49
Butyric acid, mM/100mM	18.79	17.14	0.94	0.47	0.02	0.31
Total SCFA, mM	126.7	135.2	4.14	0.06	0.002	0.24

[a]Initial (time 0 hour) measurements for each response variable were used as covariates for statistical analysis. There were no treatment x block, treatment x time, or treatment x block x time interactions for any response variables measured. Therefore, overall treatment LS means are presented.

[b]4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88.

Table 3. Influence of *in vivo* daily dosing of a direct fed microbial (DFM) on *in vitro* lactic acid concentrations (mM) at 0, 3, 6, and 9 hours post fermentation.

	Treatment			$P <$ [a]
Item	Control	DFM[b]	SEM	Trt
0 hour				
L (+)	17.32	15.67	3.08	0.81
D (-)	18.77	19.95	3.70	0.85
Total	36.09	35.62	6.70	0.94
3 hours				
L (+)	4.89	1.74	1.41	0.12
D (-)	5.98	1.89	1.44	0.05
Total	10.87	3.62	3.01	0.04
6 hours				
L (+)	2.21	2.26	0.65	0.84
D (-)	2.82	2.85	0.77	0.98
Total	5.03	5.48	1.42	0.78
9 hours				
L (+)	3.43	2.68	0.96	0.65
D (-)	3.72	2.90	0.93	0.71
Total	7.14	5.58	1.99	0.84

[a]Initial (time 0 hour) measurements prior to lactic acid addition were used as covariates for statistical analysis. There were no day (7 and 14 day) x treatment, block x treatment, or day x block x treatment interactions for any response variables measured. Therefore, overall treatment LS means are presented.

[b]4.45x 10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88.

Table 4. Influence of *in vivo* daily dosing of a direct fed microbial (DFM) on *in vitro* short chain fatty acid (SCFA) composition at 0, 3, 6, and 9 hours post *in vitro* incubation with lactic acid in rumen fluid collected from fistulated steers two hours post feeding.

	Treatment			$P <$ [a]		
Item	Control	DFM[b]	SEM	Trt	Time	Trt x time
Acetic acid, mM/100mM	48.10	43.24	1.92	0.05	0.001	0.91
Propionic acid, mM/100mM	32.38	37.20	1.29	0.03	0.002	0.82
Isobutyric acid, mM/100mM	1.67	1.62	0.08	0.61	0.001	0.73
Butyric acid, mM/100mM	17.94	17.94	1.24	0.76	0.0003	0.86
Total SCFA, mM	134.33	146.31	5.37	0.04	0.002	0.74

[a]Initial (time 0 hour) measurements for each response variable were use as covariates for statistical analysis. There were no treatment x block, treatment x time, or treatment x block x time interactions for any response variables measured. Therefore, overall treatment LS means are presented.

[b]4.45×10^{10} CFU/g of *Propionibacteria acidipropionici* - CP88.