THESIS

PROPIONIBACTERIAIUM ACIDIPROPIONICI CP 88 DOSE ALTERS IN VIVO AND IN VITRO RUMINAL FERMENTATION CHARACTERISTICS

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ABSTRACT

PROPIONIBACTERIAIUM ACIDIPROPIONICI CP 88 DOSE ALTERS IN VIVO AND IN VITRO RUMINAL FERMENTATION CHARACTERISTICS

Twelve steers, fitted with rumen canulae were used in a 4 x 4 Latin square design to examine the impact of the direct fed microbial Propionibacteria acidipropionici (PA) on rumen fermentation characteristics. All steers were housed together in one pen equipped with GrowSafe feed intake monitoring stations and one Greenfeed system used to estimate *in vivo* methane production. Steers were fed a corn silage-based diet throughout the experiment. Treatments consisted of PA administered at: 1) 0.0; 2) 1.0 x 10^8; 3)1.0 x 10^9; and 4) 1.0 x 10^{10} CFU·animal-1·day-1. Treatments were administered directly into the rumen as a single bolus dose daily. On day 7 and 14 of each period, rumen fluid was collected from each steer 2 h post treatment administration for VFA analysis and for determining *in vitro* fermentation characteristics. Following a 14-d washout period, animal treatments were switched, and the experiment repeated. Data were analyzed as a 4 x 4 Latin square design. *In vivo* propionic acid molar proportions (25.4 vs 23.6±0.24 mM) and total VFA concentrations (125.2 vs 121.3±1.87 mM) were greater ($P < 0.05$) in steers receiving PA when compared to controls. *In vitro* DM disappearance ($P < 0.05$; 63.3% vs 59.2%±1.12) and total VFA (P < 0.05; 147.9 vs 145.2±1.76 mM) were greater and methane (ml/g DMD) lesser ($P < 0.04$; vs 13.1 vs 15.6±0.11) in fermentation vessels incubated with rumen fluid from animals receiving PA when compared to controls. Dry matter disappearance ($P < 0.03$) and propionic acid ($P < 0.04$) increased linearly as dose of PA increased. *In vitro* total VFA tended ($P < 0.08$) to increase linearly and methane production tended ($P < 0.09$) to decrease quadratically in response PA dose. *In vivo* methane production was similar across

treatments. In conclusion, PA addition improved *in vivo* and *in vitro* rumen fermentation, gas production, and rumen microbial manipulation.

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CHAPTER 1: LITERATURE REVIEW

Introduction:

The United States has had a recent boom in human growth and development within the last couple decades. With powerplants being used to supply power by using fossil fuels, motorized vehicles are the main use of transportation, and agriculture being used as the main supply of food in the country. In turn to meeting the demand of a growing country more negative pressure has been put on global warming than years prior. Many researchers and scientists from across the globe recognize that this is a global environmental issue and have come together to combat the rising atmospheric temperature, release of reactive greenhouse gases into the environment, and the impact on the marine populations. Greenhouse gases are those that absorb infrared radiation in the atmosphere, trapping heat and warming the surface of the earth (Snyder et al.,2009). Greenhouse gas sources have been categorized based on the source they originate from. In 2007, Synder and others categorized the sources of greenhouse gases by sector stating that industry, transportation, commercial, residential, and agricultural sectors make up the majority of sources for greenhouse gases in the environment (Snyder et al.,2009). "Methane is described as a trace gas and is estimated to have a total global concentration of 1774 ±1.8 parts per billion (ppb), with a total increase of 11 ppb from 1998-2018" (Forster et al., 2007). An 11ppb increase might not seem like a significant increase over a 12-year period however, methane has a global warming potential of 25 times that of carbon dioxide. McMichael and others (2007) describe "that domesticated ruminants, such as cattle, sheep, and goats produce as much as 86 million metric tons (Tg) of methane per year." With an increasing human population, these numbers heavily influence the greenhouse gas effect. According to McMichael and others (2007) as much as 35% of all global greenhouse-gas emissions are derived from agriculture and land use.

Table 1: Greenhouse-gas emissions per year from livestock.

	Carbon dioxide (global, 2002)	Methane, enteric (global, 2004)	Methane, manure (global, 2004)
Cattle	1906	75[*†]	8[‡]
Small ruminants (sheep and goats)	514	9	0·3
Pigs	590	1	8
Camels	18	..	..
Horses	71	..	..
Poultry	61	..	1
Total	3161	86	18

Data are million tonnes of gas.*

[2]Dairy cattle account for a quarter of enteric methane emissions from cattle.†
Adapted from McMichael et al., 2007.

Table 1 represents livestock animals and their contribution to three different types of greenhouse gases. Cattle contribute 60.3% of total livestock carbon dioxide emissions, 87.21% of enteric methane admissions, and 44% of manure methane according to McMichael and others (2007).

Ruminant Methanogenesis:

A topic that has been at the forefront of feedlot cattle research has been mitigating enteric methane production to improve animal production efficiency and reduce greenhouse gas emissions. In order to create a reduction in the environmental impact of the beef cattle industry, it is important to understand how beef cattle convert feed into useful products for humans. In a study conducted by Murray and others in 2007, they report that 89% of methane emitted from ruminants is eructated through the nose. Balch and others (1979) indicated that “methane is

produced in the rumen by a group of *Archaea* known collectively as methanogens, which belong to the phylum *Euryarcheot.* Overall methane output from a ruminant animal can fluctuate based on the types of feedstuff consumed by the animal. A study done by Sharp and others in 1998 showcased that methane-creating groups of bacteria are in the rumen at different populations depending on their availability to grow and reproduce. Feed, pH, bioavailability, and rumen environment. In the liquid portion of the rumen, methanogens capture hydrogen that ultimately helping to reduce fluctuations in pH. In the same study, Sharp showed methane-producing microorganisms utilize hydrogen from enteric carbohydrate fermentation. This process is why researchers have labeled methanogenesis as a hydrogen sink.

Cattle`s contribution to methane production:

As discussed previously, cattle digest through feedstuffs in the rumen through fermentation. Byproducts of the fermentation process are what the animals use as energy sources to maintain overall homeostasis. As a consequence of enteric fermentation, ruminants produce gases such as carbon dioxide, methane, and nitrogen. Johnson and others showcased a table (Table V below) that compared cattle methane emissions per hundred head of cattle (Johnson et al., 1996).

Table 2: Countrys Methane emissions per 1000 head of cattle

ESTIMATES OF ANIMAL METHANE EMISSIONS

Table V
Cattle methane emissions per hd for representative areas and the world total

Country	Cattle × 1000 hd	Methane emissions (kg/hd/yr)	Total methane (Tg/yr)
FSU	118	63	7.5
Brazil	140	50	7.0
Western Europe	100	64	6.4
Africa	188	33	6.1
India	197	28	5.6
U.S.	98	54	5.3
China	77	45	3.4
Australia	23	54	1.2
Other	338	46	15.6
World total	1279	45.4	58.1

Adapted from Johnson et al.1996.

This table shows the breakdown of how many large, domesticated ruminants each country has, methane emissions (kg/animal/yr), and total methane (Tg/yr). The beef cattle industry in the United States produces 9.1% of the entire worlds methane production (Johnson et al., 1996). This number fluctuates depending on the type of feed cattle are eating. On a higher forage diet, methane output will be higher than cattle on a finishing higher grain diet. On a forage diet, cattle will produce more hydrogens in the liquid portion of the rumen that are incorporated in methane by methanogens. Overall, each production stage of the livestock industry contributes methane admissions at different rates, depending on the stage of production of the animals.

Efforts towards methane reduction in livestock:

In the past century, there has been a growing effort to mitigate the emissions of greenhouse gases by the livestock industry. A true ruminant animal requires enteric fermentation

to sustain life. As a consequence of enteric fermentation, undesirable by-products are produced such as methane that contribute to greenhouse gases in the environment as well as a loss in energy retention in the animals. Since methane output from ruminant livestock are heavily influenced by nutritional factors, there has been a great effort to tackle this problem through nutrition. Nutritional methane mitigation techniques have proven to be useful in the cattle industry to help reduce the environmental impact of greenhouse gas release due to enteric fermentation. One effort that researchers have focused on to mitigate methane output is to provide concentrate (nonstructural carbohydrate) supplementation. By feeding less roughage and more concentrate, rumen fermentation is shifted from a fiber-based fermentation to a starch-based (non-structural carbohydrate) fermentation. This in turn, provides an alternative hydrogen sink inhibiting the growth of methanogens (Hoque et al.,2018). While this has proven to be effective it is only practical when cattle are intensively managed. According to Drouillard (2018), there are 94 million head of cattle across all 50 states. 55% of cattle are maintained in the central region of the United States, 20% are in the western region, 20% are in the southeastern region, and the remaining 5% are spread throughout Alaska and Hawaii (drouillard, 2018). 60% of cattle are on a grass-based system often called a cow-calf operation. The other 40% of cattle are in a feedlot system.

Another option that researchers have been exploring to reduce enteric methane production in beef cattle is through lipid supplementation. Lipid fermentation in the rumen, specifically fermentation of unsaturated fatty acids via biohydrogenation, has proven to be useful in decreasing methanogenesis. Biohydrogenation works by providing an alternative hydrogen sink so as to compete for hydrogen with methanogens. The process of biohydrogenation converts double bonds into single bonds, or unsaturated fats into saturated fats. However, this method of

enteric methane reduction through nutrition is limited in scope because increasing total dietary fat supplementation reduces fiber digestion. (Polan et al. 1964). According to Drackley in 2007, unsaturated fats can be toxic to many of the ruminal bacteria that help with fiber digestion. As explained by Drackley (2007): As you increase the amount of fat in the diet, many of the microbial populations become less effective at fermenting fiber. Therefore, limiting total fat inclusion in beef cattle diets to approximately 8% or less is important to maintain fiber digestion and animal productivity (NRC, 2016).

Ionophores are a topic of great interest to researchers due to their methane reduction potential. Ionophores, in general, help increase feed efficiency, weight gain and reduce methane production in the rumen. According to Marques and Cooke (2021), ionophores are an important dietary tool to enhance the efficiency and profitability of grazing feedlot cattle in a pasture/forage-based setting. Ionophores are carboxylic polyether antibiotics that work by targeting and interacting with gram-positive bacteria (Marques and Cooke, 2021). Ionophores have several different modes of action. They can work by interacting with metal ions, serving as a carrier through the lipid bilayer of bacteria, trade hydrogens for potassium or sodium to help maintain pH in the rumen and reduce formation of methane from hydrogens in the rumen, and can have selective ion-binding preferences. Ultimately ionophores shift microbial populations to bacterial that produces greater proportions of propionate (a hydrogen sink). Junior and others (2020) reported a shift from acetic acid and butyric acid to more propionic acid when fed ionophores for a short period of time. They utilized 72 beef cattle in this study separated into two groups. One group was fed an ionophore supplement dosage as fallows: doses were 25.0, 8.75 and 25.0 mg/kg dry matter, respectively and the other group served as the control with no ionophore supplementation. Blood D-lactate was collected from all animals at the day of

slaughter. This study provided results showcasing that ionophore supplementation can provide an increase in gene expression of lipoprotein lipase and CD36 over time and lower lactate production at time of slaughter and overall methane production was reduced due to the ionophore properties of the feed. Methane from the same study saw a decrease in enteric production. Overall enteric methane production was reduced by 5% compared to the control. This has proven to be effective in enteric methane reduction because methanogens utilize acetic and butyric acid instead of propionic acid as a hydrogen source.

Bromochloromethane is a type of anti-methanogenic compound that has been a heavy topic of interest in reducing enteric methane production from livestock. Due to the ozone depletion properties of bromochloromethane chemicals and its ability to react in the atmosphere to create forever chemicals, scientists have come to a consensus to ban this product for use in livestock feeds. Scientists have been working with red seaweed due to its reduction ability in enteric methane production. The issue is that feeding these products are not a permanent fix. We see a reduction in methane but for only the first month or two of feeding, then the enteric methane production spikes backup. Bromochloromethane works by "hindering the combamide-dependent methyltransferase step" in methanogenesis (Matsui, 2020). As described in this study and a study conducted by Matthews and others in 2008, bromochloromethane inhibits methane production by methanogens by inhibiting methyl transfer.

Essential oils have also been shown to reduce enteric methane production by inhibiting the growth of certain bacteria, protozoa, and fungi that produce methane (Benchaar, 2007) Organic Acid supplementation has been proven to provide assistance in mitigating enteric methane production. From a methane production standpoint, this is a popular feed additive due to the low toxicity levels and natural origin. Newbold and others (2006) examined the impacts of

organic acid supplements that provide an alternative hydrogen sink. These experiments focused on three different approaches to decrease ruminal methanogenesis in ruminants. Yeast additives, organic acid supplementation, and plant extracts were studied as alternatives to antibiotics. In these studies they reported that all three treatments reduced methane production with no impact on growth performance.

The last topic of interest to decrease enteric methane production from livestock is to feed a direct fed microbial. All probiotics that we feed to livestock are considered to be direct fed microbials. Known positive properties of feeding probiotics include an increased immune response, steady development of the microbial populations, prevention of feed-related allergies, improved fiber digestibility, overall increase in feed intake, and overall enteric methane reduction (Bibarkar et al, 2014). Probiotics, when compared to antibiotics, shift the microbial population through substrate competition with other microbial populations in the gastrointestinal tract of ruminants, whereas antibiotics work by inhibiting the growth of specific microbial populations in the gastrointestinal tract of ruminants.

Propionibacteria:

Proprionibacteria has been a heavily researched area in animal science. Researchers have been interested in exploring the impact of Proprionibacteria on rumen fermentation characteristics. Probiotics and especially Proprionibacteria have been used in the cattle industry for over 20 years, helping producers increase feed efficiency, growth efficiency, total VFA production and milk production in dairy cattle (McAllister et al., 2011). All probiotics work In one of six different ways, they can modify the balance of intestinal cells, adhere to intestinal mucosa, prevent pathogenic adherence or activation, influence gut permeability, enhance immune response, or convert lactic acid to propionic acid in the rumen. Simply, there are two

common practices when feeding cattle a probiotic: options include creating and supplementing an encapsulated bolus to cattle or feeding it in a total mixed ration. Propionibacteria are typically gram-positive bacteria that do not produce spores. This is important because when Propionibacteria produces spores that means that the environment is not suitable for growth. While having a lot of added benefits in the cattle production system, propionibacteria have been shown to reduce methane production by indirectly serving as an alternative hydrogen sink (McAllister et al., 2011). In general, propionibacteria convert feedstuff into propionic acid. As the propionic acid concentration increases, butyric and acetic acid production decreases. As a result of this process, propionic (a H^+ sink) helps to reduce methane production. There are many different strains of propionibacteria, all having different impacts on ruminal fermentation. Examples include *Propionibacteria freudenreichii, Lactobacillus acidophilus, Propionibacterium enterococcus faecium, and Propionibacteria acidipropionici* to name a few. *Propionibacteria acidipropionici* (C88) has been a topic of interest due to its ability to increase the concentration of propionic acid production while still maintaining feed efficiency and overall rumen health. Figure 1 showcases the pathway propionibacteria use to convert feedstuff into propionic acid. Some key steps in this reaction are the creation of pyruvate from glucose, the creation of oxaloacetate that enters the tricarboxylic acid cycle. Then the product get converted into succinate and then through other reaction is turned into propionate to be used by propionic bacteria.

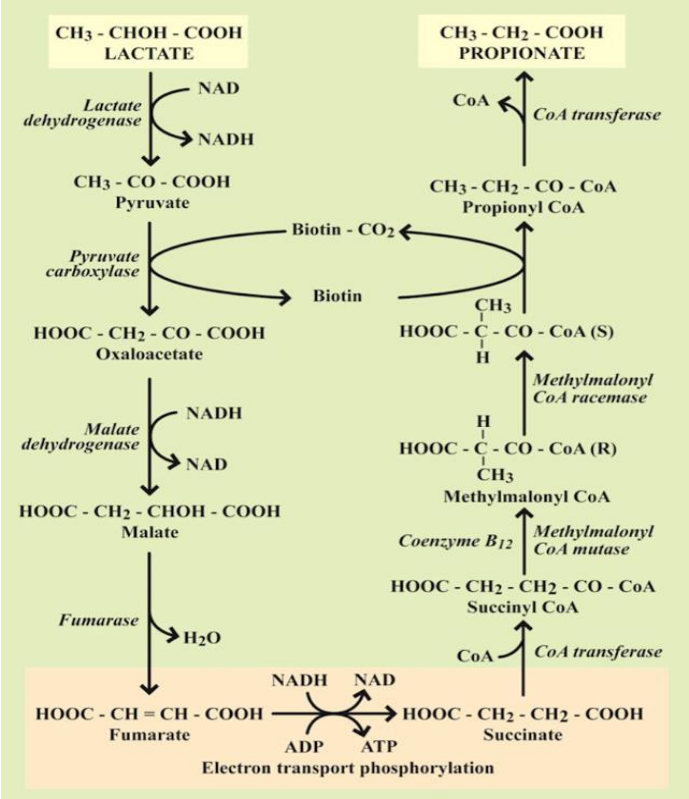

Figure 1: Propionate Production

Adapted from Dr. Nagaraja (DMP 925: CHAPTER IX-19)

Propionibacteria acidipropionici (C88):

Propionibacteria acidipropionici (C88) is a fairly new propionibacteria that has been shown to have minimal impact on ruminal pH while simultaneously increasing propionic acid production in the rumen when cattle are fed a high concentrate diet along with *Propionibacteria acidipropionici* (C88) supplementation (Vyas et al., 2014). The trade name for this product is called Propionibacterium. Feed efficiency in a production system is vital for the success of the operation. The known strains of *Propionibacteria acidipropionici* studied are P169, P5, P54. Vyas and others explored the methane inhibiting properties of *Propionibacteria acidipropionici* (P169, P5, and P54). The numbers of the probiotic indicate the subspecies of *Propionibacteria*

acidipropionici. In this experiment the researchers fed 20 fistulated cows. The authors reported that direct fed microbial supplementation decreased DMI and therefore, reduced enteric methane production. They suggested that the results occurred due to a decrease in fiber digestion [1]and not due to the modes of action of the *Propionibacteria acidipropionici* strain.

Conclusion:

Since the effect of greenhouse gas emissions is a global environmental issue, there has been strides to reduce methane production in ruminant production system. As stated previously, since methane is 25 times more reactive in Earth's atmosphere compared to carbon dioxide. The half-life of methane is about 9 years. Then it converts to CO_2 and reverts back into the soil over the next 100 years. Great efforts from the livestock industry to mitigate methane emissions and overall global greenhouse gas emissions has been the driver for the United States cattle industry to look at unique alternatives to reduce methanogenesis in the rumen. There are many unknown properties of feed additives yet to be researched and how they affect the rumen microbial population, ruminal health, bovine physiological properties, and methane reduction properties. In the following chapter, properties of *Propionibacteria acidipropionici* (C88) were tested to identify its role in the reduction of enteric rumen methane production.

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CHAPTER 2: PROPIONIBACTERIAIUM ACIDIPROPIONICI CP 88 DOSE ALTERS IN VIVO AND IN VITRO 2

Overview: Twelve beef steers, fitted with rumen canulae were used in a 4 x 4 Latin square design to examine the impact of the direct fed microbial Propionibacteria acidipropionici CP 88 (PA) on rumen fermentation characteristics, *in vitro* CH_4, CO_2, and N_2 production, and *in vivo* CH_4 and CO_2 production. All steers were housed in the same pen equipped with 8 GrowSafe feeding stations to monitor individual animal feed intake and one GreenFeed System to estimate individual animal CH4 and CO2 production. Steers were fed a corn silage based diet throughout the experiment. Treatments consisted of PA administered at: 1) control (0.0); 2) 1.0 x 10^8; 3)1.0 x 10^9 ; and 4) 1.0 x 10^{10} cfu·animal-^{1}·d-^{1}. Treatments were administered directly into the rumen as a single bolus dose daily. On d 7 and 14 of each period, rumen fluid was collected from each steer 2 h post treatment administration for VFA analysis and *in vitro* DM digestibility determination. Following a 14-d washout period, animal treatments were switched and the experiment repeated until the 4 x 4 Latin square was complete. *In vivo* propionic acid molar proportions and total VFA concentrations were greater ($P < 0.05$) in steers receiving PA when compared to controls. All other *in vivo* rumen fermentation characteristics were similar across treatments. *In vitro* DM disappearance ($P < 0.05$) and total VFA ($P < 0.05$) were greater and CH4 lesser ($P < 0.04$) in fermentation vessels incubated with rumen fluid from animals receiving PA when compared to controls. Dry matter disappearance ($P < 0.03$) and propionic acid molar proportions ($P < 0.04$) increased linearly as dose of PA increased. *In vitro* total VFA tended ($P < 0.08$) to increase linearly and CH4 production per unit of DM digested tended ($P < 0.09$) to

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decrease quadratically in response PA dose. All other *in vitro* rumen fermentation characteristics were similar across treatments. These data indicate that PA impacts *in vivo* and *in vitro* rumen fermentation production, and rumen microbial manipulation.

Introduction:

Direct fed microbial (DFM) supplementation to ruminants has been reported to alter the ruminal bacterial populations and improve animal production efficiency (Krehbiel et al., 2003). However, the overall impacts of DFM supplementation on ruminal fermentation characteristics are not well defined. Nagaraja et al. (1997) described how Propionibacterium are a lactate utilizing bacteria, but their use as a DFM has been focused on the production of propionate, the primary glucose precursor in ruminants. Fistulated steers fed a high concentrate diet supplemented with a range of *P*. acidilactici-DH42 doses (10^7 to 10^{10} cfuf·animal^{-1}·d^{-1}) for 7 d had increases in rumen propionic acid at the expense of acetic acid across the entire range of *Propionibacteriaium acidipropionici* dosages (Kim et al., 2000).

Huck et. al. (2000) reported the feeding Lactobacillus acidophilus for 28 d and *Propionibacteria freudenreichii* for the remainder of the finishing period resulted in improved performance compared to controls or Propionibacteria freudenreichii supplementation alone. In contrast, Krehbiel et. al. (2004) reported that feeding Lactobacillus acidophilus during the entire finishing period tended to increase feedlot performance and carcass merit when compared to feeding Lactobacilus acidophilus for the first 27 d of the feeding period followed by Propionibacteria freudenreichii for the remainder of the 140-d finishing period. Regardless of these effects on performance, limited published data exists examining the impacts of Propionibacteria species on rumen fermentation characteristics in feedlot cattle. Therefore, the objective of this experiment was to evaluate the effects of Propionibacteria acidipropionici (PA)

on rumen fermentation characteristics and *in vitro* methane (CH_4) , carbon dioxide (CO_2), and dinitrogen (N_2) and *in vivo* CH_4 and CO_2 production.

Materials and Methods

Prior to the initiation of this experiment, all animal use, handling, and sampling techniques described herein were approved by the Colorado State University Animal Care and Use Committee (Protocol # 1875).

Twelve beef steers, fitted with rumen canulae, were used in a 4 x 4 Latin square design to examine the impact of a direct fed microbial (DFM), *Propionibacteriaium acidipropionici* CP88 (PA), on rumen fermentation characteristics. All steers were housed together in a dirt-surfaced pen equipped with eight GrowSafe feeding stations (GrowSafe Systems, Ltd. Calgary, AB, Canada) to monitor individual animal feed intake and one GreenFeed System (C-Lock Inc. Rapid City, SD, USA.) to estimate individual animal CH_4 and CO_2 production over the course of the experiment. All steers were fed a corn silage based diet (Table 1) throughout the duration of the experiment. The basal diet was formulated to meet or exceed the nutrient requirements for growing feedlot cattle (NASEM, 2016).

Following a 2 week adaptation period to the basal diet, all steers were weighed and blocked by body weight (4 steers per weight block with 3 weight blocks). Steers within a weight block were randomly assigned to one of 4 treatments. Treatments consisted of 1) control (0.0); 2) 1.0×10^8; 3)1.0×10^9 ; and 4) 1.0×10^{10} cfu·animal^{-1}·d^{-1} of *Propionibacteriaium acidipropionici* CP88. Immediately prior to treatment administration, rumen pH was determined inserting a portable pH meter (EcoTestr pH 2+; Oaktron 153 Instruments, Vernon Hills, IL, USA) into the geometric center of the rumen as described by Gifford et al. (2021). Following pH determination,

treatments were administered into the rumen through the canulae and the rumen contents were thoroughly mixed by hand. Treatment delivery was performed as described by Gifford et al. (2021). Briefly, appropriate dilutions of the DFM treatments were made in deionized water and administered directly into the rumen via the cannula as a single bolus dose at 0700 h daily. For the control treatment, water and the DFM carrier were administered. The same volume of water (901 mL per animal) was used to deliver all daily treatment doses.

On d 7 and 14, rumen fluid was collected from each steer 2 h post treatment administration. Approximately 250 g of rumen contents were collected from each animal and centrifuged at 28,000 x g at 5°C for 30 min. Supernatant was acidified with 25% meta-phosphoric acid and frozen at -20°C until volatile fatty acids (VFA) analysis could be performed as described by Gifford et al. (2021). After the 14 d treatment administration, all treatments were discontinued for 14 d (washout period). Following the 14 d washout period, animal treatments were switched and the experiment repeated until the 4 x 4 Latin square was complete.

During each treatment period, enteric CH_4 and CO_2 production were estimated using a GreenFeed emission measurement system (C-Lock Inc., Rapid City, SD). Weekly calibrations were completed and CO_2 recoveries were completed monthly (Hristov et al., 2015). Beauchemin et al.(2012) examined the use of cannulas for estimating emissions using the sulfur hexafluoride technique and determined that gas leaking could be minimized if tight-fitting cannulas, such as the ones utilized in this experiment, were used. Prior to starting the experiment, the animals were acclimated to the GreenFeed System. The GreenFeed System was then left in the pen for the entire duration of the experiment. The bait feed used in this experiment was an alfalfa pellet feed (approximately 35 g per discharge). Animals were allowed a maximum of 6 discharges per visit to the GreenFeed System with 30 second intervals between each discharge. The GreenFeed

System was programed to require 4 hours between each visit and allowed each animal to visit a maximum of 6 times per day. Methane and CO_2 production (g/kg DMI) per animal per period, were calculated by dividing total CH_4 and CO_2 production estimated by the GreenFeed System for the last 7 days in each period by the total kg of DMI consumed by each animal as determined by the GrowSafe feeding system for the last 7 days in each period.

Animal husbandry:

The feeding location was checked daily to ensure that all gates were secure and that all equipment was functioning properly. Health status was monitored daily as described by Gifford et al. (2021). Briefly, all animals were monitored for health and locomotion problems daily. Any animal exhibiting symptoms of respiratory disease or locomotion problems was removed from the pen for more a more thorough assessment by trained personnel. If an animal was determined to be moribund, the animal was treated according to the facility treatment protocol and immediately returned to their original pen. If problems persisted concerning the health status of a specific animal, the animal was removed from the experiment. If an animal was removed from the experiment, the animal was weighed at the time of removal.

In vitro rumen fluid collection:

As previously described, on d 7 and 14 of each period of the experiment, approximately 1 L of rumen fluid was collected from all steers 2 h post-feeding as described by Ward and Spears, (1993) and processed as described by Gifford et al. (2021). Rumen fluid from each steer was filtered once through four layers of cheesecloth into individual pre-warmed (39°C) thermoses. A modified McDougall's buffer solution was mixed with rumen fluid at a 1:1 ratio (Tilley and Terry, 1963) and pH was recorded.

In vitro Chambers: Approximately 4 kg (wet weight) of the basal diet was collected from the feed truck at the time of feed mixing. The basal diet ration sample was then dried at 60°C for 72 h in a forced air drying oven and ground to fit through a 2.0 mm screen (Thomas Scientific, Swedesboro, NJ; Gifford et al., 2021). The ground ration was added to pre-weighed 50 mL conical tubes (0.5 ±.001g; 3 conical tubes per animal per incubation time point) and 100 mL glass bottles (1.0±.0001 g; 3 glass bottles per animal per incubation time point). A 1:1 McDougall's buffer: rumen fluid mixture was then dispensed into all *in vitro* vessels (30 ml into the conical tubes and 60 mL into the glass bottles). The conical tubes were capped with one-way valves to maintain anaerobic conditions and were used to determine dry matter digestibility.

Glass bottles were capped and sealed with an airtight rubber stopper immediately after the buffer:rumen fluid mixture was dispensed into the glass bottle. The glass bottles remained sealed to maintain anaerobic conditions. Gas pressure of each glass bottle was determined, at the end of each incubation timepoint, using a digital pressure gauge (Dwyer Instruments Inc, Michigan City, IN, USA) fitted with a 20-gauge needle inserted through the rubber stopper. Gas composition (CH_4, CO_2, and N_2,) was determined by aspirating 10.0 ml of headspace gas from the glass bottle and immediately injecting the gas sample into the injection port of a gas chromatograph (Shimadzu GC – 14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector set at 100.0˚C.

All conical tubes and glass bottles were incubated in a circulating water bath at 39 °C for the appropriate lengths of time (0, 6 or 12 h) and swirled by hand every 3 h. At 0, 6, and 12 hours, three tubes per animal (3 conical tubes and 3 glass bottles) were removed from the water bath. Conical tubes were centrifuged at 28,000 x g at 5°C for 30 min and glass bottles were sampled (as described above) and then uncapped and discarded. Supernatant was removed from

all conical tubes and combined with 25% meta-phosphoric acid and frozen at -20°C until analyzed for VFA concentrations. The remaining pellet was dried in a forced air drying oven at 60°C for 72. Following drying, the pellet dry weight was used to determine dry matter disappearance at each time point. Blank tubes containing only the McDougall's rumen fluid mixture were used to adjust the dry matter disappearance calculation for initial microbial and digesta weight contributed from the McDougall's rumen fluid mixture.

Volatile Fatty Acid Analysis: Post thawing, rumen fluid samples were centrifuged at 28,000 x g at 5°C for 15 min. Supernatant was analyzed for VFA composition via gas chromatography as described by Gifford et al. (2021).

Statistical Analysis

A mixed effects model repeated measures analysis for a completely randomized 4 x 4 Latin square design was used to analyze *in vivo* and *in vitro* repeated measurements. The fixed effects were treatment, time, period, and all interactions. For all response variables measured, individual animal or *in vitro* vessel was considered the experimental unit. Several covariance structures were compared to determine the most appropriate covariance structure for data analysis. If interactions were not significant, data were pooled and main effects were reported. 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 or treatment × time interaction were detected, treatment means were separated using the PDIFF option of the LSMEANS statement of SAS. (SAS Inst. Inc., Cary, NC). Linear, quadratic, and cubic effects were determined to examine the impact of DFM dose on rumen fermentation characteristics.

Results

One animal was removed from the experiment due to a foot injury after the second period of the experiment. All data for this animal was removed from analysis. All other animals remained healthy throughout the experiment.

The influence of *in vivo* daily dosing of DFM on *in vivo* rumen fermentation characteristics in fistulated steers is described in Table 2. There were no treatment x time interactions for any response variables measured. Therefore, overall main effects are presented. Propionic acid concentrations and total VFA concentrations were greater ($P < 0.05$) in steers receiving DFM when compared to controls. All other rumen fermentation characteristics were similar across treatments.

The influence of *in vivo* daily dosing of DFM on *in vitro* fermentation parameters is also shown in Table 2. There were no treatment x time interactions for any response variables measured. Therefore, overall means are presented in Table 2. Dry matter digestibility ($P < 0.05$) and total VFA ($P < 0.05$) were greater, CH_4 production per unit of DM digested was lesser ($P < 0.04$), and molar proportions of propionic acid tended ($P < 0.06$) to be greater in fermentation vessels incubated with rumen fluid collected from animals receiving DFM when compared to controls. All other rumen fermentation characteristics were similar across treatments.

Linear, quadratic, and cubic effects were determined to compare the influence of DFM dose on rumen fermentation characteristics (Table 3). *In vitro* DM disappearance increased ($P < 0.03$) linearly as dose of DFM increased. *In vitro* production of propionic acid increased ($P < 0.04$) and total VFA tended ($P < 0.08$) to increase linearly in response to increasing DFM dose. Furthermore, CH_4 production per unit of DM digested tended ($P < 0.09$) to decrease quadratically in response to increasing doses of DFM.

Discussion

Results for rumen VFA obtained in the current experiment are similar to those reported previously by Gifford et. al., 2021. Briefly, Gifford et al. (2021) utilized 6 fistulated steers in a crossover design to investigate the impact of PA (1.0x 1010 cfu·animal-^{1}·day-^{1}) on *in vivo* and *in vitro* rumen propionic acid production and *in vitro* lactic acid clearance from rumen fluid following exogenous lactic acid addition. The authors reported that *in vivo* propionic acid concentrations were greater and total VFA tended to be greater in rumen fluid from steers receiving DFM. *In vitro* total lactic acid disappearance was greater at 3 h post incubation when rumen fluid collected from animals supplemented with DFM was incubated with lactic acid compared to incubation with rumen fluid collected form control. These data indicate that PA alters rumen fermentation characteristics. Fistulated steers supplemented with P. acidilactici-DH42 (10^{7} to 10^{10} cfu·animal-^{1}·d-^{1}; similar dose range to the current experiment) for 7 d/dose had increases in rumen molar proportions of propionic acid and decreases in molar proportions of acetic acid across the entire range of P. acidilactici-DH42 dosages. When the DFM was removed from the diet, butyrate production increased which suggests that DFM impacted butyrate production as well (Kim et al., 2000). However, the authors also reported that lactic acid and rumen pH were not influenced by DFM supplementation.

In an experiment by Vyas et. al. (2014), there were no differences in rumen pH, molar proportions of individual VFA, or total enteric CH_4 (g/d) production in heifers fed a 70% roughage:30% concentrate diet when supplemented with three different Propionibacterium strains (P169, P5, P54) compared to controls. Methane emission intensity expressed as g of CH_4 produced per kg DMI was reduced in animals receiving all 3 PA strains when compared to controls. This response was attributed to PA animals having a numerically greater DMI than

controls. However, the authors indicated that the lack of a reduction in CH_4 production in PA supplemented animals is most liked due to the inability of all three PA strains to integrate into the rumen microbiome. In the current experiment, there was no difference in DMI of rumen cannulated steers fed a 40% concentrate: 60% roughage-based diet due to *Propionibacteriaium acidipropionici* (CP88) supplementation and no reduction in CH_4 emission adjusted for DMI. However, *in vitro* CH_4 production, adjusted for DMD, was decreased due to *Propionibacteriaium acidipropionici* (CP88) supplementation, which improved *in vitro* DMD. There appears to be some level of disagreement between *in vivo* and *in vitro* CH_4 results in the current experiment, although *in vivo* DMD was not determined.

Narvaez et el. (2014) fed a corn and corn dried distillers grain diet to yearling steers during the finishing period with 1.0 x 10^{11} cfu·animal-^{1}·d-^{1} of *Propionibacteriaium acidipropionici* P169. There was no observed effect on feed intake, growth rate, feed conversion, rumen pH, total VFA production, propionate or the acetic:propionic ratio due to *Propionibacteriaium acidipropionici* P169 supplementation when compared to the controls. In contrast, Sanchez et al. (2014) supplemented low quality forages with a 36% CP supplement (454g·animal-^{1}·d-^{1}) containing 6 x 10^{10} 10^{11} cfu·anima-^{1}·d-^{1} of P*ropionibacteriaium acidipropionici* P169 to Brangus heifers and reported that molar proportions of propionic acid were increased and the acetic:propionic ratio was reduced in animals receiving PA. Lehloenya et.al., 2008 also supplemented *Propionibacteriaium acidipropionici* -P169 to rumen and duodenal cannulated steers fed a silage based diet for 21 d. At a rate of 1 x 10^{11} cfu·animal-^{1}·d-^{1} of P169, a trend toward increased rumen propionic acid molar proportions and a decrease in acetic acid molar proportions was observed. Collectively these data suggest that the response to PA supplementation may be diet dependent and/or strain dependent. In the current experiment, VFA

and CH_4 responses to increasing doses of *Propionibacteriaium acidipropionici* (CP88) were obtained in experimental diets containing approximately 40% concentrate:60% roughage with added monensin. Additional research evaluating feedlot cattle performance with P. acidipropionici- CP88 is warranted.

Table 3. Dry matter ingredient composition of basal diet.

Ingredient	%
Corn Silage	50.0
Cracked corn	23.9
Distiller's grains	8.7
Alfalfa hay	7.2
Wheat straw	5.0
Liquid Supplement1	4.4
Limestone	0.40
Salt	0.10
Analyzed nutrient composition	
DM, % as fed	62.4
CP, %	15.1
ADF, %	17.9
NDF, %	28.0
Ether extract, %	6.4
NEg, Mcal/kg	1.21
NEm, Mcal/kg	1.91
Calcium, %	0.63
Magnesium, %	0.22
Phosphorus, %	0.36
Potassium, %	1.41
Sulfur, %	0.24
Cobalt, mg/kg	21.8
Copper, mg/kg	18.0
Manganese, mg/kg	81.3
Selenium, mg/kg	2.1
Zinc, mg/kg	64.9

1Liquid supplement provided in a molasses 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 4. Influence of direct fed microbial dose on in vivo and in vitro fermentation characteristics.

Item	Treatment[a]				SEM	*P*<		
	0.0[b]	0.1[c]	1.0[d]	10.0[e]		Trt	Time	Trt x Time
In vivo								
n=	11	11	11	11	---	---	---	---
DMI[f], kg/d	12.5	12.8	12.3	12.8	0.34	0.93	0.02	0.73
Rumen pH, s.u.	6.55	6.63	6.54	6.61	0.05	0.72	0.01	0.84
Acetic acid, mM/100mM	57.1	57.6	56.2	56.1	0.97	0.78	0.08	0.78
Propionic acid, mM/100mM	23.6	24.8	25.3	26.3	0.24	0.05	0.02	0.61
Isobutyric acid, mM/100mM	0.51	0.49	0.47	0.42	0.09	0.87	0.01	0.84
Butyric acid, mM/100mM	18.8	17.1	18.0	17.2	0.84	0.54	0.03	0.81
Acetic acid/Propionic acid	2.4	2.3	2.1	2.2	0.21	0.34	0.06	0.78
Total VFA, mM	121.2	125.4	123.2	127.1	1.87	0.05	0.002	0.54
CO_2, g/kg DMI	587	591	601	589	12.3	0.84	0.01	0.71
CH_4, g/kg DMI	20.3	20.1	18.3	18.9	1.11	0.19	0.01	0.62
In vitro								
DMD[g], %	59.2	61.7	63.2	65.2	1.12	0.05	0.002	0.63
Acetic acid, mM/100mM	50.1	48.2	49.0	46.7	1.72	0.88	0.001	0.91
Propionic acid, mM/100mM	30.5	33.0	33.0	34.7	1.05	0.06	0.001	0.82
Isobutyric acid, mM/100mM	1.61	1.65	1.57	1.67	0.21	0.87	0.001	0.78
Butyric acid, mM/100mM	17.81	17.2	16.4	16.9	0.98	0.65	0.002	0.90
Acetic acid/Propionic acid	1.6	1.5	1.5	1.3	0.15	0.23	0.07	0.58
Total VFA[f], mM	145.2	147.3	147.9	148.7	1.76	0.05	0.001	0.76
CO_2, ml/g DMD	80.2	81.7	83.1	81.3	0.41	0.12	0.0001	0.67
CH_4, ml/g DMD	15.56	13.73	12.69	12.91	0.11	0.04	0.0001	0.67
N_2, ml/g DMD	4.24	4.57	4.21	4.22	0.07	0.62	0.001	0.82

[a]*Propionibacteria acidipropionici* dose.

[b]0.0 cfu·animal^{-1}·d^{-1}.

[c]1.0 x 10^8 cfu·animal^{-1}·d^{-1}.

[d]1.0 x 10^9 cfu·animal^{-1}·d^{-1}.

[e]1.0 x 10^{10} cfu·animal^{-1}·d^{-1}.

[f]Dry matter intake.

Table 5. Influence of direct fed microbial dose on in vivo and in vitro fermentation characteristics.

Item	Treatment[a]				SEM	*P*<		
	0.0[b]	0.1[c]	1.0[d]	10.0[e]		Linear	Quadratic	Cubic
In vivo								
n=	11	11	11	11	---	---	---	---
DMI[f], kg/d	12.5	12.8	12.3	12.8	0.34	0.89	0.81	0.91
Rumen pH, s.u.	6.55	6.63	6.54	6.61	0.05	0.92	0.87	0.95
Acetic acid, mM/100mM	57.1	57.6	56.2	56.1	0.97	0.46	0.71	0.92
Propionic acid, mM/100mM	23.6	24.8	25.3	26.3	0.24	0.39	0.34	0.62
Isobutyric acid, mM/100mM	0.51	0.49	0.47	0.42	0.09	0.18	0.84	0.71
Butyric acid, mM/100mM	18.8	17.1	18.0	17.2	0.84	0.84	0.76	0.84
Acetic acid/Propionic acid	2.4	2.3	2.1	2.2	0.21	0.19	0.21	0.81
Total VFA, mM	121.2	125.4	123.2	127.1	1.87	0.78	0.81	0.41
CO_2, g/kg DMI	587	591	601	589	12.3	0.21	0.38	0.74
CH_4, g/kg DMI	20.3	20.1	18.3	18.9	1.11	0.28	0.91	0.84
In vitro								
DMD[g], %	59.2	61.7	63.2	65.2	1.12	0.03	0.14	0.81
Acetic acid, mM/100mM	50.1	48.2	49.0	46.7	1.72	0.12	0.54	0.68
Propionic acid, mM/100mM	30.5	33.0	33.0	34.7	1.05	0.04	0.48	0.76
Isobutyric acid, mM/100mM	1.61	1.65	1.57	1.67	0.21	0.33	0.42	0.58
Butyric acid, mM/100mM	17.81	17.2	16.4	16.9	0.98	0.11	0.67	0.79
Acetic acid/Propionic acid	1.6	1.5	1.5	1.3	0.15	0.21	0.29	0.67
Total VFA[h], mM	145.2	147.3	147.9	148.7	1.76	0.08	0.73	0.97
CO_2, ml/g DMD	80.2	81.7	83.1	81.3	0.41	0.21	0.45	0.64
CH_4, ml/g DMD	15.56	13.73	12.69	12.91	0.11	0.37	0.09	0.37
N_2, ml/g DMD	4.24	4.57	4.21	4.22	0.07	0.78	0.67	0.86

[a]*Propionibacteria acidipropionici* dose.
[b]0.0 cfu·animal^{-1}·d^{-1}.
[c]1.0 x 10^8 cfu·animal^{-1}·d^{-1}.
[d]1.0 x 10^9 cfu·animal^{-1}·d^{-1}.
[e]1.0 x 10^{10} cfu·animal^{-1}·d^{-1}.

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