THESIS

INFLUENCE OF BOVAMINE DEFEND® PLUS ON GROWTH PERFORMANCE, CARCASS CHARACTERISTICS, ESTIMATED DRY MATTER DIGESTIBILITY, RUMEN FERMENTATION CHARACTERISTICS, AND IMMUNE FUNCTION IN FINISHING BEEF STEERS.

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ABSTRACT

INFLUENCE OF BOVAMINE DEFEND® PLUS ON GROWTH PERFORMANCE, CARCASS CHARACTERISTICS, ESTIMATED DRY MATTER DIGESTIBILITY, RUMEN FERMENTATION CHARACTERISTICS, AND IMMUNE FUNCTION IN FINISHING BEEF STEERS.

One hundred and eighty crossbred beef steers (406.0 ± 2.2 kg) were used to determine the impact of a novel direct-fed microbial (DFM) on growth performance, carcass characteristics, rumen fermentation characteristics, and immune response in finishing beef cattle. Steers were blocked by body weight and randomly assigned, within block, to 1 of 2 treatments (3 replicates/treatment: 30 steers/replicate). Treatments included: 1) no DFM (control) and 2) DFM supplementation at 50 mg·animal^{-1}·d^{-1} (BOVAMINE DEFEND® Plus). All steers were fed a high-concentrate finishing diet and individual feed intake was recorded daily via the GrowSafe® system. Body weights were collected every 28 d. On d 55, 10 steers per pen were injected with ovalbumin (OVA). Jugular blood samples were collected from each steer on d 0, 7, 14, and 21 post-injection. On d 112, the same steers were injected again with OVA and intramuscularly with a pig red blood cell solution. Jugular blood samples were collected from each steer on d 0, 7, 14, and 21 post-injection. On d 124 rumen fluid was collected from 3 steers per treatment and used to estimate in vitro rumen fermentation characteristics. Equal numbers of steers per treatment were transported to a commercial abattoir on d 145, 167, and 185 of the experiment, harvested, and carcass data collected. Initial body weight (BW) was similar across treatments. On d 28 and 55, steers receiving DFM had heavier BW ($P < 0.01$) compared to controls. Average daily gain was greater in DFM-supplemented steers from d 0 to 28 ($P < 0.01$) and d 0 to 55 ($P < 0.01$) of the

experiment compared to controls. Overall dry matter intake was greater ($P < 0.04$) and overall feed efficiency was similar in DFM-supplemented steers compared to controls. Dressing percentage ($P < 0.02$) was greater in steers receiving DFM compared to controls. Antibody titers to injected antigens were similar across treatments. However, red blood cell superoxide dismutase activity was greater ($P < 0.05$) in DFM-supplemented steers compared to controls. In vitro molar proportions of isobutyric and butyric acid were greater ($P < 0.01$) and dry matter (DM) digestibility tended ($P < 0.07$) to be greater in rumen fluid obtained from steers supplemented with DFM. These data suggest that BOVAMINE DEFEND® Plus supplementation improves growth performance during the initial period of the finishing phase, increases overall dry matter intake and dressing percentage, and may impact antioxidant status in beef cattle.

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CHAPTER 1

INTRODUCTION

The beef industry in the United States is a consumer driven industry that has focused on the efficiency and quality of the products being produced for many years. While the beginning systems of feeding cattle were simple, the current day operations are larger and more complex. The feeding of cattle developed into a productive industry when nutritional and managerial technologies became more available around the twentieth century (Mintert, 2003). As the cattle feeding industry grew, the demand for more efficient production practices grew. Producers knew ruminant animals had the ability to produce valuable products through the utilization of starch in their feeds (Huntington, 1997). But these animals, specifically cattle, ability to produce quality products is dependent upon the availability of the starch in grain products. Grain processing became a common practice for many cattle feeding operations. Grain processing helps to make starches in the feed, especially from corn, more accessible to the animal and their microbes.

With the constant demand for beef, the evolution of technologies aimed at improving animal growth and feedlot performance has become prominent. Products such as hormonal implants, beta agonists, ionophores, and in-feed antibiotics have been developed and extensively tested (Crawford et al., 2022). Many of these growth promoting technologies have been found to be successful in a variety of cattle feeding operations (Crawford et al., 2022). Increased concerns, from consumers, about the safety of some growth promoting products has lead the cattle feeding industry to find alternative products (McGuffey, 2017; Firkins and Mitchell, 2023). These alternative products can help promote health and efficient growth of cattle, while not causing potential risks for the animals or consumers. This demand has led to increased research into rumen modifiers, specifically direct-fed microbials (DFM). Direct-fed microbials are live

microorganisms that are fed to feedlot cattle with the aim of improving gut health and overall performance of the animals (Brashears et al., 2005).

Direct-fed microbials can be classified into three categories bacterial, fungal, or combination products (Elghandour et al., 2015). Bacterial DFM are the most common type; this review and project will mostly focus on bacterial DFM. The mode of action for bacterial DFM products are classified either in the rumen or post ruminal gastrointestinal tracts of cattle, but still is not fully understood. Bacterial DFM products have the potential to benefit the intestinal microorganism balance while modulating immune function and preventing pathogen adherence (Salimen et al., 1996; Holzapfel et al., 1998). The amount of variation in the bacterial strains used in bacterial DFM products is large, and results from studies have varied greatly. New products are still being developed and tested. The current review and research will focus on a new combination of four bacterial strains in a DFM product for feedlot cattle.

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CHAPTER 2

LITERATURE REVIEW

The current beef cattle industry in the United States is a multifaceted system which produces large amounts of high-quality products. This industry consistently faces feedback from consumers to be safer and more efficient in their production of beef products. While the industry has come a long way since its inception, there is always room for improvements. Beef production in the United States started around the beginning of the nineteenth century when crop production exceeded demand by consumers (Wagner et al., 2014). These beginning cattle feeding systems were very simple, and the growth of cattle systems was highly dependent on the growth of crop surpluses (Wagner et al., 2014). Cattle feeding became more productive around the twentieth century when technologies related to nutrition and feed management of cattle improved (Mintert, 2003). The improvement of beneficial technologies was not just limited to the cattle industry. The development of a steel plow and hybrid corn seeds resulted in large increases in the production of corn (Wagner et al., 2014).

As access to developing technologies grew, farmers/producers reported increased benefits of production when cattle were intensely managed (Mintert, 2003). This led to the cattle industry developing production sectors. As the industry began to specialize, regions where cattle were raised and fed changed, and regions of feeding operations became predominant (Mintert, 2003; Drouillard, 2018). The location of cattle feeding operations followed the productive crop lands. This resulted in the commercial cattle feeding industry being developed by the end of World War II (Mintert, 2003). In the 1960's the three largest cattle producing states were now located in the corn belt region of the United States (Featherstone et al., 1993). By the 1980's over 65% of cattle

feeding companies were operating in the plain's region, with this number increasing to 70% by the early 2000's (Featherstone et al., 1993). As the geographical location of cattle feeding operations began to concentrate in the plain's regions, the number of feedyards began to decrease, but the number of cattle being fed was increasing. In other words, the cattle industry was increasing the number of cattle per feedyard and improving their capacity to feed cattle. As the number of cattle being fed increased, demand for more efficient feedstuffs and feed processing technologies grew.

2.10 Feed Processing Advancements

Producers have been searching for more efficient ways to feed cattle since the beginning of the cattle feeding industry. This started by feeding cattle on the corn stalks and byproducts that were left in the field after harvest. Then, when crop surpluses began to grow, they were fed to cattle. The original corn that was fed to cattle in the nineteenth century was a very dry and flinty variety. This resulted in many producers/farmers soaking their corn before feeding it to cattle (Matsushima, 2006). This was the beginning of feed processing in the cattle industry. While both forages and grains are commonly processed, grain processing has become more prominent. The production of animal products is dependent upon the amount of starch a ruminant animal is able to utilize from the grains being fed (Huntington, 1997). Grain processing, regardless of method, helps to make the starch of grains, specifically corn, more accessible for the animal to efficiently digest.

Grain processing has separated into two categories: dry processing or wet processing. Dry processing methods include, but are not limited to, grinding, crimping, pelleting and roasting. While wet processing can include soaking, boiling, or steam flaking/rolling. Zinn and Owens (2008) compared different dry methods of processing corn and found that dry rolled and

cracked corn resulted in similar digestion. These authors also reported grinding corn increased the initial rate of digestion but did not improve overall digestion when compared to dry rolled corn. Dry rolling corn has also been reported to increase total tract digestion when compared to whole corn in the diets of feedlot cattle (Murphy et al., 1994; Corona et al., 2005; Gorocica-Buenfil and Loerch, 2005).

The process of steam flaking corn was developed in the 1950's, to help feedlot operators find ways to increase feed efficiency (Matsushima, 2006). Steam flaking is a wet processing method that has been reported to increase the availability of energy within corn, when compared to feeding whole corn kernels (Zinn et al., 2002). In a review of starch utilization in ruminants by Huntington (1997), steam-flaking decreased the variation of digestibility when compared to dry rolled corn. This suggests that the alterations to the grain are more uniform in the steam flaking process. Steam flaking was found to increase average daily gain (ADG) and decrease dry matter intakes (DMI) of cattle when compared to dry rolled corn (Zinn, 1987; Zinn et al., 1998; Ward, 2000). When comparing steam-flaked corn (SFC) to whole kernel corn, SFC inclusion into feedlot cattle diets resulted in greater ADG, lower DMI, (Lee et al., 1982; Ramirez et al., 1985) and improved gain:feed and dietary net energy in cattle (González-Vizcarra et al., 2017). Steam-flaking corn and sorghum were also reported to increase digestibility when compared to dry rolling (Huntington, 1997). As much as feed processing advancements have improved the efficiency of the cattle feeding industry in the United States, the global demand for beef continues to grow. Leading producers to push for additional technologies which have the ability to improve the feed efficiency and growth of the animals in their operations.

2.11 Growth Promoting Technologies in the Feedlot Industry

As the global demand for beef grows, the management practices of producers have evolved to use technologies to improve animal growth performance, while still meeting the ever-growing demand of the consumer. Over the last 30 years the research focusing on the development of new growth promoting products and safe administration of existing products has become prominent (Crawford et al., 2022). Growth promoting technologies in the beef feedlot industry include, but are not limited to, hormonal implants, β-agonists, and ionophores. Growth promoting implants can contain a variety of natural or synthetic compounds that aim at increasing rate and efficiency of growth through producing physiological responses. The physiological responses produced are similar to those caused by the animals' natural hormones. Through the use of growth promoting implants cattle feeding operations have seen increased body weights (BW), ADG, feed efficiency, intakes, and hot carcass weights (Crawford et al., 2022). The growth implants most commonly used in modern day cattle feeding operations use a combination of trenbolone acetate and estrogen.

Ionophores are a commonly used feed additive in the feedlot cattle industry aiming to improve the feed efficiency of animals (Goodrich et al., 1984). The economic benefits from feeding ionophores have been found to include, but are not limited to, increased weight gains, improved feed efficiencies, and reduced gaseous emissions, morbidity, and mortality (McGuffey et al., 2001). Ionophores are able to create these economic benefits for operations through creating metabolic advantages within the animals. The biological benefits of ionophores, for cattle, can be classified into three main effect areas (Bergen and Bates, 1984). The first main effect being that ionophores increase the energy metabolism efficiency of the cattle through increasing the efficiency of the rumen bacteria. Through a similar process the ionophores can

also improve the nitrogen metabolism of cattle. The final main biological effect of ionophores are they have the ability to decrease the incidence of digestive disorders caused by abnormal environments within the rumen. Cattle are able to transform these metabolic advantages into an increase in efficiency and production. Ionophores are one of the most commonly studied feed additives in the cattle industry.

Throughout the research there has been consistent evidence which suggests the use of ionophores in cattle production systems can alter the rumen microbiome and reduce rates of digestive disorders, while optimizing ruminal fermentation (Nagaraja et al., 1997; McGuffey et al., 2001). Several ionophores are commercially available for use in cattle diets, including monensin, lasalocid, salinmycin, and laidlomiycin. Monensin is the most commonly used ionophore in the feedlot industry (Samuelson et al., 2016) and was originally approved for use in beef cattle in 1975 (Goodrich et al., 1984). Including ionophores into forage or grain-based diets has been shown to help optimize efficiency and performance of beef cattle (Marques and Cooke, 2021). Supplementation of laidlomiycin resulted in similar performance to cattle being fed monensin (Thompson et al., 2016). Feeding a combination of laidlomiycin and monensin resulted in an increased final BW, ADG, feed efficiency, and hot carcass weight in feedlot cattle when compared to a negative control (Thompson et al., 2016). Ionophores can be a good tool for producers to help improve the performance of cattle on feed.

Beta agonists are an approved feed additive for cattle that helps the animals to utilize more of the energy from feed. Most of the beta agonists fed to cattle are beta-adrenergic agonists (β-AA). Muscle mass in cattle fed β-AA is thought to increase because the synthesis of myofibrillar proteins is promoted while protein turnover is inhibited (Johnson et al., 2014). Beta-AA increase lean tissue growth by binding to β-AA receptors (β-AR) which are present in the

plasma membrane of both muscle and adipose tissue cells (Lynch and Ryall, 2008). This binding initiates a signaling cascade that results in lean tissue hypertrophy. The interaction of β-AA with the β-AR stimulates adenylate cyclase in the plasma membrane of the muscle cells (Mersmann, 1998). Adenylate cyclase stimulation increases the concentrations of cAMP in the muscle cells. Resulting in the binding of cAMP to regulatory subunits of protein kinase A, which has the ability to phosphorylate serine residues of several metabolic regulators (Mersmann, 1998). Activation of metabolic regulators can lead to the phosphorylation of hormone sensitive lipase, which activates multiple enzymes which have the ability to inhibit de novo biosynthesis of fatty acids. Ricks et al. (1984) proposed that beta agonists can reduce the accretion of adipose tissue because of the combined effects of increasing lipolysis and inhibiting de novo fatty acid biosynthesis. The inhibition of de novo biosynthesis of fatty acids is the overriding biological response of adipose tissue to *β*-AA (Ricks et al., 1984).

In summary, the feeding of *β*-AA can result in varying physiological effects including, increased energy usage and reduced amounts of adipose tissue (Yang and McElligott, 1989). The feeding of a β-AA for the last 28 to 42 d of the finishing period has become a common practice in the feedlot industry (Samuelson et al., 2016). Ractopamine hydrochloride is the most common βAA fed to cattle, this product was first approved for use in cattle diets in 2003 (Crawford et al., 2022). The feeding of β-AA to feedlot finishing cattle has consistently shown increased muscle growth and improved cattle performance. Cattle supplemented with β-AA exhibited increased ADG, feed efficiency, hot carcass weight, dressing percentage, longissimus muscle cross section area and mass (Avendaño-Reyes et al., 2006; Vasconcelos et al., 2008b; Montgomery et al., 2009). In a study comparing β-AA to steroidal implants, it was proposed that β-AA products have the ability to increase the ratio of protein to DNA, therefore increasing muscle mass

(Johnson et al., 2014). Whereas steroidal implants have been shown to increase the accumulation of DNA in cells. While many new technologies have been developed to help improve the growth and efficiency of feedlot cattle, not all the outcomes are positive.

As cattle continue to consume more high energy feedstuffs and are kept in feedyards longer, the incidence of liver abscesses is increasing. Liver abscesses in feedlot cattle are a source of major economic loss because of reduced body weights, feed intake, feed efficiency, and carcass yield (Nagaraja and Lechtenberg, 2007). To help control the prevalence of liver abscess in feedlot cattle the use of feed grade antibiotics, specifically tylosin, is common. The inclusion of feed grade antibiotics into the receiving diets of feedlot cattle have been attributed to a tendency to improve average daily gain and feed efficiency when compared to negative control cattle (Perry et al., 1986; Birkelo, 2003; Duffield et al., 2012; de Souza et al., 2018). While tylosin is an in-feed antimicrobial which is approved for usage to reduce the incidence of liver abscesses in feedlot cattle, there are concerns about the formation of antibiotic resistance in these animals. When tyolisn is fed at the labeled dosage and duration the proportions of macrolide resistant enterococci increased in cattle gastrointestinal tracts (Müller et al., 2018; Cazer et al., 2020). Additional studies have reported that as the duration of tyolsin feeding increases the proportion of resistant enterococci also increases (Molitoris et al., 1986; Alexander et al., 2008; Jacob et al., 2008; Beukers et al., 2015; Müller et al., 2018). Because of increased concern from consumers and producers, the feedlot industry has been searching to find alternatives to help promote health and growth of beef animals without causing potential problems for the animals and consumers. This demand has led to increased research involving rumen modifiers.

2.12 Rumen Modifiers

Rumen modifiers can be loosely defined as feed additives that potentially have ruminal mechanisms of action when fed to cattle (McGuffey, 2017; Firkins and Mitchell, 2023). Rumen modifiers are metabolic modifiers which act within the rumen environment of cattle's digestive tracts. Rumen modifiers have been added to the diets of cattle to improve the efficiency of microbial protein synthesis while optimizing fiber degradation and improving the efficiency of nutrient usage (Firkins and Mitchell, 2023). All of these improvements within the rumen environment have the potential to lessen the environmental impact of ruminants. Potential sources of metabolic modifiers include, but are not limited to, feed additives, hormones, nutrients in feed, or microorganisms and the products of microorganisms (McGuffey, 2017). More specifically, rumen modifiers are often supplemented as feed additives that contain organic acids, enzymes, dietary lipids, plant secondary compounds (i.e., Tannins) or direct-fed microbials (Cobellis et al., 2015).

Certain modifiers require exhaustive studies to demonstrate the safety and efficacy of the modifier within the environment and target animal (McGuffey, 2017). These studies are generally completed by regulatory agencies. There are also feed additives used as modifiers that are classified as "generally regarded as safe" (GRAS; McGuffey, 2017), meaning that these GRAS substances have little to no oversight by regulatory agencies. There are also products that are referred to as complete rumen modifiers (CRM). A CRM is a multifunctional feed additive with properties such as bacterial growth factors, hydrogen sinks, defaunation, or inhibition of methanogenesis (Thalib et al., 2011). The majority of the rumen modifiers discussed in the literature have the mechanistic possibility to change fermentation pathways. Multiple research studies have focused on high fiber diets and the relationships between rumen modifiers and fiber

digestion (Firkins and Mitchell, 2023). Specifically, the research is focused on finding rumen modifiers which can be effective within the rumen while not limiting the amount of fiber degradation occurring. Little information is available about the use of rumen modifiers and their influence on rumen fermentation in cattle diets containing moderately high levels of starch (Firkins and Mitchell, 2023).

Joffe (1918) described salt as the first metabolic modifier. This demonstrates substances which have the ability to affect fermentation within the rumen have been used in the cattle industry for many decades. But the research into specific rumen modifiers and their full relationships within the rumen environment is a newer area of study. Improved feed efficiency has been reported while dosing cattle with a propionate-producing probiotic rumen modifier (Weiss et al., 2008). Additionally, the use of an additive lactate source resulted in decreased DMI while maintaining milk production levels, leading to improved milk efficiency in dairy cattle (Caputo Oliveira et al., 2020). Direct-fed microbial (DFM) products are one of the most common rumen modifiers being mentioned in the literature currently. Direct-fed microbial products, yeast or bacterial based, are GRAS substances which have been shown to improve digestive health while increasing DMI, feed efficiency, and milk production in cattle (McGuffey, 2017).

2.20 Direct-Fed Microbials

The first recorded theory about probiotics was proposed by Metchnikoff in 1908. Metchnikoff was a scientist who published writings about health and life extension. He published a theory that the properties of fermented milk products could lead to longevity in life. This theory was later termed probiotic diets. In 1989 Fuller defined probiotic as a live microbial supplement, with beneficial affects to the host animal by improving intestinal balance of microbes. Later, Kmet et al. (1993) redefined probiotics as cultures of live microorganisms that are introduced

into the rumen with the intention of improving nutrition and animal health (FAO/WHO, 2001). Over the years, the terminology has transitioned to direct-fed microbials in the livestock industry (Elghandour et al., 2015). The office of Regulatory Affairs of the Food and Drug Administration (FDA) define DFM as products fed to livestock that contain live microorganisms from naturally occurring sources (Brashears et al., 2005). Throughout time in the literature, the terms DFM and probiotic have been used interchangeably, but these two words no longer mean the same thing in terms of cattle feeding. Probiotics can contain enzymes or crude extracts in combination with live microorganisms (McAllister et al., 2011) whereas DFM only contains live microorganisms from naturally occurring sources. Direct-fed microbials have been used to aid in cattle production systems for over two decades (LeJeune and Wetzel, 2007). DFM have been reported to improve feed conversion efficiency and average daily gain (Swinney-Floyd et al., 1999; Elam et al., 2003; Cull et al., 2015).

There are three types of direct-fed microbials which are used in cattle supplements: bacterial, fungal, and a combination of both (Elghandour et al., 2015). This review will focus predominantly on bacterial DFM, which are the most common type of DFM used in cattle production. Within the bacterial DFM category there are three types: lactic acid producing bacteria (LAB), lactic acid utilizing bacteria (LUB), and other microorganisms (Elghandour et al., 2015). Common bacteria that are used in cattle DFM products are, but not limited to, *Lactobacillus*, *Propionibacterium*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Bacillus*. Based on years of similar research in humans, bacterial DFM products have the potential to positively benefit the balance of intestinal microorganisms while preventing pathogen adherence, modulating immune function, and influencing permeability of gut tissues (Salimen et al., 1996; Holzapfel et al., 1998).

The early research to test efficacy and effectiveness of probiotics was done in humans before direct-fed microbial product research was completed within livestock production systems. Immune function in humans was improved by increasing the circulation of Immunoglobulin (Ig) A and IgM producing cells and the production of proinflammatory cytokines when supplemented with DFM (Dicks and Botes, 2010). These increases have been credited to colonization of beneficial microbes, sourced from the probiotic supplement, within the gastrointestinal tract of humans. Colonization within the intestines allows for the beneficial bacteria to competitively attach to intestinal mucosa. This binding inhibits harmful pathogens from binding to the intestinal surface when they are introduced to the environment (Dicks and Botes, 2010). It has also been proposed that adherence of microorganism components from probiotics is believed to increase the proliferation of beneficial bacteria (Salminen et al., 1996). The amount of research on probiotics in humans is expansive (Salimen et al., 1996; Holzapfel et al., 1998). The general consensus is that the most reliable research results came from clinical trials when compared to in vitro models (Elghandour et al., 2015).

The differences between current day probiotics and DFM were discussed previously. The early research using probiotics can still be related to DFM products because of the type of probiotic products being used. This early research tested the efficacy and effectiveness of probiotics composed of microorganism-based products, which are more similar to current day DFM than current day probiotics. Meaning that conclusions drawn from these studies can potentially be related to DFM products. From the extensive research conducted with probiotics, three key criteria for an effective probiotic product were presented by Havenaar and Huis In't Veld, (1992). First, the components of an effective probiotic product need to meet the following general microbiological criteria (Havenaar and Huis In't Veld, 1992). They need to be safe

(nonpathogenicity), survive the defenses within the gastrointestinal tract (i.e., saliva, pH, digestive enzymes, competitive pathogens), have host specificity, and genetic stability (Holzapfel et al., 1998). In vitro models are commonly used to test the safety of products within animal systems. These models are also used to test the survival of different bacteria in the varying environments of an animal's GI tract (Holzapfel et al., 1998). The second criteria for effective probiotics are the technological aspects (Havenaar and Huis In't Veld, 1992). This means the ability to grow the bacterial components on a commercial scale. This criterion also helps to determine which strain of the bacteria will be used in the probiotic product. This decision is often made based off which strain has the best viability and survivability within the shelf stable product (Havenaar and Huis In't Veld, 1992). The final criterion is the functional effects and the underlying mechanisms. In humans the functional effects of probiotics have been concluded as, but not limited to, immune modulation and strengthening of the intestinal mucosa. These effects are attributed to the adherence of beneficial bacteria to prevent pathogen adherence, influence on mucosal permeability, gut microflora modification, and changes in the bacterial enzyme's capacity of the host (Havenaar and Huis In't Veld, 1992).

2.21 Mode of Action of DFM in Ruminants

The effects of DFM can differ within a ruminant animal, when compared to humans. These differences are mainly attributed to the differences in the anatomy of the digestive tract. Some bacteria from a DFM product can act within the rumen environment while others will bypass the rumen and become active in the post ruminal gastrointestinal (GI) tract. Direct-fed microbials can grow in the rumen, therefore benefiting the fermentation and microbial ecosystem (Seo et al., 2010). Lactic acid production and utilization by DFM bacteria has been related to feed efficiency and health of animals (Seo et al., 2010). The mode of action for direct-fed

microbials in the ruminant animal is not well described within the digestive tract as a whole unit. When mode of action has been studied and reported, it is split up by mode of action in the rumen and in the post ruminal digestive tract of the ruminant animal (Seo et al., 2010; Elghandour et al., 2015).

2.22 Ruminal Mode of Action

Mode of action for bacterial DFM in the rumen is largely contingent on the type of bacteria present in the product. Many lactic acid producing bacteria (LAB) are native in the rumen, but they are also common additives to bacterial DFM products. The LAB mechanism produces a constant supply of lactic acid, leading to an adaptation of the microbiome of the rumen, which then adapts to the accumulation of lactic acid (Yoon and Stern, 1995). This accumulation of lactic acid stimulates the lactate utilizing bacteria (LUB) which are present in the rumen, therefore stabilizing the pH of the rumen (Seo et al., 2010). This mechanism has led to the belief that LAB is pivotal in the health and composition of the gut flora within animals (Brashears et al., 2005). Lactic acid producing bacteria, *Lactobacilli* and *Enterococci*, have been reported to potentially prevent ruminal acidosis in dairy cattle (Nocek et al., 2002). Acidosis can be prevented by LAB facilitating the growth of native ruminal microbes that have been adapted to the presence of lactic acid in the rumen (Yoon and Stern, 1995).

Lactate utilizing bacteria are often used in direct-fed microbials for cattle because of their ability to decrease lactate concentration in the rumen while maintaining ruminal pH (Kung and Hession, 1995; Seo et al., 2010). The mechanisms of LUB in the rumen have been proposed to convert lactate into volatile fatty acids (VFA; Seo et al., 2010). This favors the production of propionic acid rather than lactic acid in the rumen. Increased propionate production in the rumen will lead to an increase in hepatic glucose levels (Stein et al., 2006), resulting in increased feed

efficiency of the animal. Increased propionate production leads to decreased methane production and an increase of ruminal pH away from acidosis (Stein et al., 2006). A common LUB used in DFM products is *Propionibacteria*. *Propionibacteria* is naturally found in rumens, in high concentrations, of cattle on high forage and mixed forage/concentrate rations, but not in the rumens of cattle on high concentrate diets (Stein et al., 2006). Certain species of *Propionibacteria* were reported to increase cow milk production (Stein et al., 2006) and calf weight gain (Adams et al. 2008) while increasing propionate production in the rumen (Lehloenya et al., 2008; Luo et al., 2017; Stein et al., 2006). Propionate is the most important precursor of glucose synthesis in the ruminant animal (Nagaraja et al., 1997).

Another common LUB in DFM products marketed to cattle is *Megasphaera elsdenii*. When cattle are fed a highly fermentable diet *Megasphaera elsdenii* helps to prevent drastic changes in pH that can be caused by accumulation of lactate in the rumen (Yang et al., 2009). Studies showed that inoculation of *Megasphaera elsdenii* into the rumen resulted in modified fermentation and prevented accumulation of lactate during the transition phase of feedlot cattle, in both in vitro and in vivo studies (Greening et al., 1991; Kung and Hession, 1995). The modulation of the rumen environment can help to reduce the occurrences of lactic acidosis in feedlot steers (Robinson et al., 1992). This bacterium has the ability to use lactate, glucose, and maltose simultaneously while competing with lactate producing organisms (Russell and Baldwin, 1978). Robinson et al. (1992) derived that inoculation of *Megasphaera elsdenii* had effects on ruminal pH, feed intake, osmolarity, and VFA concentrations in steers induced with acute acidosis on a 90% concentrate ration. In this same study the steers inoculated with *Megasphaera elsdenii* ate 24% more dry matter (DM). While some DFM products contain

bacteria that are able to positively influence the environment in the rumen, the majority of benefits from DFM are in the post ruminal gastrointestinal tract.

2.23 Post Ruminal Mode of Action

The proposed mode of action for bacterial DFM in the post ruminal environment can vary, but there are four different modes of action that bacteria can have within the gastrointestinal tract of cattle. The first being, competitive exclusion or attachment of pathogens (Reuben et al., 2022). Beneficial bacteria from the DFM will enter the intestine and bind to the mucosal linings of the gut. This binding does not allow harmful pathogens such as *Salmonella* and *E. Coli* to bind (Reuben et al., 2022). Meaning that bacterial DFM could compete with pathogens at binding sites on the intestinal surface (Krehbiel et al., 2003). Studies have shown that the inclusion of DFM in the diets of feedlot cattle can decrease the concentrations of *E. coli* in the carcasses of these cattle at harvest (LeJeune and Wetzel, 2007). Ohya et al. (2000), tested *Streptococcus bovis* LCB6 and *Lactobacillus gallinarum* LCB 12 ability to eliminate *E. Coli* O157:H7 from inoculated Holstein calves. These authors suggested that bacterial DFM products could reduce fecal shedding of *E. Coli* O157:H7, because of diminished counts found in study animals. Another study found that LAB adhered to the intestinal tracts in mice, to protect the animals against a *Salmonella* infection (Frizzo et al., 2010).

Another mode of action proposed is that the DFM could have the ability to secrete antimicrobial substances (Reuben et al., 2022). Some bacteria have the capability to secrete bacteriostatic or bactericidal substances that are harmful to intestinal pathogens (Reuben et al., 2022). Bacteriostatic substances will prevent the growth of pathogens (Pankey and Sabath, 2004) by interacting with the DNA replication pathways, limiting their abilities to reproduce (Dicks and Botes, 2010). Bactericidal substances will kill the pathogens it interacts with (Pankey and

Sabath, 2004). Cotter et al. (2005) documented in detail the LAB bacteriocins. One of which is reuterin, a bacteriocin that inhibits the binding of substrates to ribonucleotide reductase to interfere with the DNA synthesis of pathogenic microorganisms (Dobrogosz et al., 1989).

Lactic acid producing bacteria have also been reported to produce hydrogen peroxide, which is important in adding to the bacterial DFM's competitive exclusion properties (Carlsson et al., 1983). Hydrogen peroxide has the ability to oxidize the sulfhydryl groups on bacteria cell proteins (Dicks and Botes, 2010). This results in glycolysis being blocked because of oxidation affecting the enzymes included in the glycolysis process, such as glucose transport enzymes, glycerol aldehyde-3-phosphate dehydrogenase, and hexokinase (Carlsson et al., 1983). Lactic acid from LAB cells have been reported to decrease the counts of coliforms in the GI tracts of pigs (Ratcliffe et al., 1986). Another study showed a decrease in pH, which could reduce the growth of pathogens (Fuller, 1977).

The next major mode of action in the post ruminal GI tract is immune stimulation. Beneficial bacteria adhere to the lumen of the intestinal tract which helps to stimulate the immune system of the host animal (Reuben et al., 2022). This adherence will increase the secretion of lymphocytes and macrophages into the gastrointestinal tract of the animal (Yang et al., 2009). The secretion of these immune cells assists in stimulating the innate immune system within the gastrointestinal tract of the animal.

The final proposed mode of action of bacterial DFM in the post ruminal intestinal tract is colonization resistance (Reuben et al., 2022). The feeding of direct-fed microbials to neonatal ruminants has been shown to increase the natural populations of bacteria in their GI tract (Reuben et al., 2022). Lactic acid producing bacteria that travel past the rumen are able to produce bacteriocin compounds to fight pathogens in the intestinal tract (Cotter et al., 2005).

These LAB bacteria can also play critical roles in penetrating the pathogen cells to interfere with their normal functions (Holzapfel et al. 1995).

The results of the bacterial DFM actions in the lower GI tract are proposed to attach to the intestinal mucosal boarder and prevent the potential for pathogen adherence (Seo et al., 2010). Also, the DFM helps maintain a lower pH in the post ruminal gastrointestinal tract to inhibit the growth of pathogens, while producing antibacterial compounds, such as hydrogen peroxide and bacteriocins (Seo et al., 2010). Bacterial DFM can modulate immune cells and stimulate immune function while adapting microbial balance in the GI tract. This was demonstrated in an experiment by Frizzo et al. (2010), which inoculated LAB to young calves in aims to improve growth. These calves were fed milk replacer that contained a high quantity of spray-dried whey powder to cause an intestinal imbalance. Calves receiving the LAB had increased ADG, feed intake, and decreased fecal consistency index (Frizzo et al., 2010). Meaning that calves which were inoculated with LAB had stabilized microbial populations and reduced incidence of diarrhea.

2.24 DFM Mode of Action in the Immune System

The mode of action of bacterial DFM in the intestinal tract of ruminants is correlated to the immune response of the host animal. The gastrointestinal tract contains various immune cells, including but not limited to; macrophages, neutrophils, T and B lymphocytes, and dendric cells that are stimulated by intraepithelial regions, Peyer's patches, and lamina propria (Krehbiel et al., 2003). Antibiotics have the ability to be bacteriostatic or bactericidal and, as mentioned previously, some bacteria can secrete substances that can act similarly to antibiotics, but most DFM products have to function through indirect mechanisms. These include altering the intestinal microbiome of the host, modulating the host innate immune system, or enhancing

intestinal efficiency (Buntyn et al., 2016). Most DFM products that have the potential to influence the immune system of the host animal can do so through interacting with the innate immune system of the host. The innate immune system consists of cellular defenses, such as neutrophils, cytokines, macrophages, natural killer cells and dendritic cells, and chemical defenses that required prior exposure to be effective (Tizard, 1977). When wanting to evaluate immune response in animals there are three main models used: 1) a direct immune challenge with a substance which can cause an immune response; 2) a pathogen challenge using live pathogens; or 3) natural exposure to pathogens which are similar to those occurring in natural scenarios or introduction to an environment known to contain pathogens (Buntyn et al., 2016). While these studies can differ in execution, they all provide information which can help in understanding how cattle's immune systems respond to various challenges.

One of the most stressful times for beef cattle is during the receiving phase of the feedlot cycle. The incidences of sickness and infection, specifically bovine respiratory disease (BRD), is greatly increased for cattle during this transition time (Buntyn et al., 2016). The main type of pathogen challenge study using a DFM product which is referenced within the literature uses a yeast based DFM. Feedlot steers were supplemented with *Saccharomyces cerevisiae* during an infection of BRD (Cole et al., 1992). DFM supplemented steers had increased DMI for the first 13 days post infection and tended to have less BW loss when compared to non-supplemented animals (Cole et al., 1992). The amount of cattle studies using a direct immune challenge that report changes in animals' immune response are limited. In a study using heifers, animals were challenged with a lipopolysaccharide (LPS; Sanchez et al., 2014). Heifers who received a *S. cerevisiae* supplementation for 36 days prior to the LPS challenge demonstrated increased insulin concentrations and blood urea nitrogen (BUN) when compared to control heifers. After

the LPS challenge supplemented heifers had modified metabolic responses, shown by increased BUN, and decreased non-esterified fatty acids (NEFA) concentrations compared to the control animals (Sanchez et al., 2014). An example of a natural exposure study evaluated newly received steers during the initial receiving period and recorded the percentage of animals treated in a control group and a group supplemented with a multistrand DFM product (Kenney et al., 2015). These researchers reported the effect of DFM supplementation on the percentage of animals being treated for sickness. The DFM supplemented group did not show improved growth performance when compared to the control animals. However, there was a reported relationship between supplementation of DFM and concentrations of degradable intake protein. This relationship was explained as an increase in animals ADG as degradable intake protein increased, for treatment animals when compared to controls (Kenney et al., 2015).

Bacterial DFM products have been demonstrated to impact the humoral, cellular, and innate parts of the immune system in livestock (Lee et al., 2010; Roos et al., 2010; Seo et al., 2010). When the bacterial DFM makes it to the post ruminal GI tract, they have the ability to be absorbed by the intestinal cells via transcytosis (Seo et al., 2010). They are then engulfed by either macrophages, presenting cells, and/or dendritic cells, which stimulates an immune response (Dicks and Botes, 2010). A study feeding a combination DFM product to broiler chickens resulted in energy repartitioning, via changing concentrations of ATP, to the immune system (Qiu et al., 2012). Chickens being fed the DFM in this study also had faster rates of antibody production without observed changes in growth performance or whole-body metabolism. There have been reports of some strains of LAB that will activate the macrophages to release cytokines and begin to stimulate an immune response (Miettinen et al., 1996). *Lactobacilli* entering the GI tract via oral administration has shown an enhancement of the innate

immune system, specifically increased phagocytosis and natural killer cells (Erickson and Hubbard, 2000; Isolauri et al., 2001). These studies also reported increased production of IgA and decreased IgE in humans and animals (Erickson and Hubbard, 2000; Isolauri et al., 2001; Dicks and Botes, 2010). The influence of bacterial DFM products on T and B cells is reported with mixed results. It appears that the effects of DFM supplementation on immune response in cattle vary depending on dose, strain, and duration of intake of the DFM product (Krehbiel et al., 2003). The reported effects and modes of action of the products have been variable. This variation is most likely due to differences in bacteria and strains of bacteria being used. The following studies outline some of the performance results which have been reported while feeding bacterial DFM products.

2.30 Bacterial Direct-Fed Microbial Studies

Numerous studies have been conducted to evaluate the effectiveness of a large variety of bacterial direct-fed microbial products. While the amount of variation in bacterial DFM products used is large, many studies have reported positive results from the utilization of these products. Gill et all., (1987) reported that feeding a bacterial DFM in the beginning 28-d receiving period of feedlot cattle resulted in a 9.5% improved feed efficiency, a 10.9% reduced morbidity, and a 9.3% improved average daily gain. Overall, the results demonstrate improved animal performance during one of the most stressful times for feedlot cattle. Other researchers used a DFM containing *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* to evaluate morphology of the digestive tract of neonatal Holstein calves (Dick et al., 2013). They found greater average ileal villus height, crypt depth, and total (villus + crypt) height before weaning and greater average ruminal papillae width in DFM treated animals. No differences in final ADG or BW were reported when comparing DFM treatment and control animals. For this study, while

normal performance measures of the calves were not improved, the development of the digestive tract of DFM calves was better than non-supplemented calves.

A DFM product using a combination of *Enterococcus faecium* (6×10^9 cfu/d) and yeast resulted in increased propionate and decreased butyrate concentrations when fed to feedlot cattle receiving a high concentrate diet (Beauchemin et al., 2003). This same study also reported a tendency for increased flow of nitrogen from feed into the duodenum, while microbial nitrogen tended to decrease. These researchers stated that besides the propionate increase, these other metabolic changes could be seen as undesirable (Beauchemin et al., 2003). Additional researchers tested different bacterial strains on 30 rumen fistulated steers from the Nellore breed being fed a high concentrate diet, using the combination of *Enterococcus faecium* and *Saccharomyces cerevisiae* at 3.5×10^9 CFU/g or a combination of *Bacillus licheniformis* and *Bacillus subtilis* at a concentration of 3.2×10^9 CFU/g (Dias et al., 2022). These researchers reported the supplementation of a DFM, regardless of combination, reduced rumen ammonia levels and acetate:propionate ratio and tended to improve performance of steers when compared to control cattle. To contrast these studies, other researchers have reported no performance responses to DFM supplementation to receiving feedlot cattle (Kiesling and Lofgreen, 1981; Krehbiel et al., 2001) or newly weaned calves (Drew and Thomas, 1981; Kercher et al., 1985; 1986). Additionally, no treatment effects on performance parameters were reported when *Megasphaera elsdenii* was fed at the beginning of the feedlot period to *Bos indicus* bulls (Lopes et al., 2021).

Many other experiments have reported improved milk and meat production and increased growth in both beef and dairy cattle (Ghorbani et al., 2002; Nocek et al., 2002; Stein et al., 2006). Nocek et al., (2003) reported that DFM supplementation increased the milk yield, milk

protein and dry matter intake during the postpartum period of dairy cattle. Insulin and blood glucose concentrations were increased while non-esterified fatty acids (NEFA) levels decreased in these same cattle (Nocek et al., 2003). Supplementing a combined bacterial DFM product to lactating dairy cattle (120 d in milk) improved milk protein by 6.9%, milk yield by 7.6% and energy correlated milk by 6.0% when compared to non-supplemented dairy cattle (Boyd et al., 2011). The supplementation of a DFM containing *Enterococcus faecium* and *Saccharomyces cerevisiae* resulted in improved milk fat yield and total tract starch digestibility when compared to non-supplemented cattle (AlZahal et al., 2014). Dairy cows supplemented with a DFM (yeast and *Enterococcus faceium*) pre- and postpartum had increased DMI and milk production when compared to negative control animals (Nocek and Kautz, 2006). Early lactation dairy cattle supplemented with yeast culture (YC) and/or an enzymatically hydrolyzed yeast (EHY) produced more milk, fat corrected milk, energy corrected milk, milk protein percentage, and milk fat when compared to non-supplemented control cows (Nocek et al., 2011).

2.31 Bacterial Strains in BOVAMINE DEFEND® Plus Trials

A newer DFM product on the market within the cattle industry is BOVAMINE DEFEND® Plus (Chr. Hansen A/S, Hørsholm, Denmark). This product contains a combination of four different bacteria, *Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus licheniformis*, and *Bacillus subtilis*. The initial published research using this product reported improved health of newborn beef calves after inoculation with *Clostridium perfringens* type A (Guimaraes et al., 2023). Numerous studies have been completed investigating individual or combinations of the bacterial strains in BOVAMINE DEFEND® Plus. Animal health, cattle performance, and pathogenic growth results have been varied from these experiments. *Lactobacillus animalis* has been reported to positively affect the barrier function of the rumen

(Brashears et al., 2005; McAllister et al., 2011). While *Propionibacterium freudenreichii* has been proven to increase the proportions of propionate produced in a rumen environment (Brashears et al., 2005; McAllister et al., 2011). *Bacillus licheniformis* and *Bacillus subtilis* are both stable in the digestive tracts of animals and have been shown to act as pathogen competitors (Su et al., 2020; Mingmongkolchai and Panbangred, 2018).

2.32 *Lactobacillus animalis*

Lactobacillus animalis is a lactic acid-producing bacteria, which has been naturally found in cattle rumen samples, and has been reported to have gut barrier function capabilities within the rumen environment (Brashears et al., 2005; McAllister et al., 2011). In a study evaluating the functional activity in vitro of bacteria strains isolated from calves, *Lactobacillus animalis* demonstrated a large resistance to the effects of digestion, both gastric and intestinal (Ripamonti et al., 2011). This bacterium was able to survive in gastric fluid with a pH as low as 3 and intestinal fluid at a pH of 8.0. Meaning that *Lactobacillus animalis* showed properties that are essential for a DFM product (Ripamonti et al., 2011). Ware et al. (1988) completed a pooled summary of eight feedlot experiments, they reported improved ADG and feed efficiency in steers receiving *Lactobacillus acidophilus* (1×10^8 cfu·steer^{-1}·d^{-1}) when compared to non-supplemented controls. Improved weight gains, gain to feed ration, and hot carcass weights have been reported when *Lactobacillus animalis* was included as a DFM in the diet of finishing feedlot steers (Hanford et al., 2011; Cull et al., 2012 and 2015).

Brashears et al. (2003) observed that *Lactobacillus acidophilus* decreased the amount of *Escherichia coli* O157:H7 that was shed in the individually collected feces of feedlot cattle during the feeding period. Cattle used in this study were Angus cross beef steers being fed a high concentrate diet. Shedding of *E. coli* O157:H7 was detected twice as much in control animal

feces when compared to DFM supplemented animals' feces. Levels of *E. coli* O157:H7 on hide samples at time of slaughter were decreased for animals receiving *Lactobacillus acidophilus* supplementation, when compared to control animals. These authors reported no difference in final BW, ADG, DMI, or carcass traits for DFM supplemented animals when compared to controls. Peterson et al. (2007) conducted a study which fed *Lactobacillus acidophilus* to cattle known to be shedding *E. coli* O157:H7. The effect of DFM supplementation of *E. coli* O157:H7 shedding, and animal performance were reported. Steers receiving DFM treatment shed less *E. coli* O157:H7 than control steers. No differences in final BW, ADG, DMI, hot carcass weight or fat thickness were found when comparing to control animals. The animals receiving *Lactobacillus acidophilus* tended to be more efficient and have lower USDA yield grades (Peterson et al., 2007).

2.33 *Propionibacterium freudenreichii*

Propionibacterium freudenreichii, a gram-positive bacterium that has been reported to increase propionate production in the rumen (Brashears et al., 2005; McAllister et al., 2011). *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). Elam et al. (2003) reported that supplementing *Propionibacterium freudenreichii* at 1×10^9 cfu·animal^{-1}·day^{-1} to mixed breed beef steers fed a high concentrate finishing diet resulted in decreased levels of *E. coli* in feces of supplemented animals. The feeding of a DFM containing *Propionibacterium freudenreichii* has been reported to inhibit the penetration of *E. coli O157* into the intestinal mucosal layer, therefore decreasing instance of *E. coli O157* infections and shedding (Reid and Burton, 2002). Reduced total CH_4 production was explained as a result of increasing propionate production to finishing beef steers being supplemented with

Propionibacterium freudenreichii (Meale et al., 2014). The supplementation of *Propionibacterium* strains to forage fed cattle has been reported to reduce enteric methane emissions through the increased production of ruminal propionate (Jeyanathan et al., 2014).

2.34 *Lactobacillus animalis* and *Propionibacterium freudenreichii*

Many studies have been published using a combination of *Lactobacillus animalis* and *Propionibacterium freudenreichii* which report performance results in beef and dairy cattle. In feedlot cattle consuming a high concentrate diet, Galyean et al. (2000) investigated the inclusion of 1×10^6 cfu·animal^{-1}·day^{-1} or 1×10^9 cfu·animal^{-1}·day^{-1} *Lactobacillus animalis* with 1×10^9 cfu·animal^{-1}·day^{-1} *Propionibacterium freudenreichii*. The researchers reported heavier final body weights and greater average daily gains when cattle were supplemented with *Lactobacillus animalis* and *Propionibacterium freudenreichii*, irrespective of dose of *Lactobacillus animalis*. The daily supplementation of LAB and LUB bacterial DFM products demonstrated an improvement to average daily gain and feed to gain ratio of feedlot cattle (Swinney-Floyd et al., 1999; Elam et al., 2003; Rust et al., 2000a,b). Specifically, feeding a combination of *L. acidophilus* 53545 and *P. freudenreichii* P-63 was reported to increase the feed efficiency of feedlot steers (Swinney-Floyd et al., 1999).

Supplementing *Lactobacillus animalis* and *Propionibacterium freudenreichii,* at a combined dose of 4×10^9 cfu·animal^{-1}·day^{-1}, to lactating (120 d in milk) dairy cows, improved milk yield, protein yield and energy-corrected milk by 7.6, 6.9, and 6.0 percent, respectively, compared to non-supplemented cows (Boyd et al., 2011). This study also reported dry matter (DM) digestibility was increased by three percent in DFM supplemented dairy cows compared to controls. Treatment of *Lactobacillus animalis* (1 × 109 cfu/d) and *Propionibacterium*

freudenreichii (2 × 109 cfu/d; LAPF) to 30 multiparous cows, who were 75 ± 32 d in milk resulted in no difference in intake, fecal score, milk yield or composition when compared to control animals (Lawrence et al., 2021). The percentage of fecal starch decreased, and starch digestion tended to increase in treatment animals in the previous study. Raeth-Knight et al. (2007) fed *Lactobacillus acidophilus* and *Propionibacterium freudenreichi*i to mid-lactation Holstein cows to evaluate the effect of DFM supplementation on rumen fermentation, performance, and nutrient digestibility. Fecal grab samples were collected for the digestibility measurements and rumen fistulated multiparous cows were used to evaluate rumen fermentation characteristics. No differences were observed for cow performance, rumen fermentation, or diet digestibility when comparing DFM cattle to control cows.

To evaluate changes in ruminal digestion when feeding a *Lactobacillus animalis* and *Propionibacterium freudenreichii* supplement, 6 ruminal cannulated cows at approximately 123 d in milk were used (Lawrence et al., 2021). Treatment resulted in increased rumen pH and a decreased milk yield for treatment cattle when compared to controls after switching to a high-starch diet. These researchers also noted a few modest changes in the bacterial populations of the rumen and fecal samples of treatment animals. Conversely, additional researchers have reported no impacts on feedlot cattle growth performance and carcass characteristics for animals being fed combinations of *Lactobacillus animalis* and *Propionibacterium freudenreichii* or single bacterial strains of *Lactobacillus animalis* or *Propionibacterium freudenreichii* (Vasconcelos et al., 2008a; Luebbe et al., 2013; Thomspon et al., 2020; Cull et al., 2022). Specifically, ADG, DMI and carcass characteristics were not affected by DFM supplementation or dose (Vasconcelos et al., 2008a) and *Lactobacillus animalis* and *Propionibacterium freudenreichii* were not found to affect average daily gain or final body weights (Cull et al., 2022).

2.35 *Bacillus licheniformis*

Bacillus licheniformis is a potent competitor of potentially harmful bacteria within the rumen (Mingmongkolchai and Panbangred, 2018). *Bacillus licheniformis* naturally occurs and is normally found within soil. This strain of *Bacillus* is generally used in combination with other bacteria in probiotic products. Most research on *B. licheniformis* has been conducted in aquaculture environments (Swapna et al., 2015). *Bacillus licheniformis* is a commonly used microorganism in DFM products because of its extracellular enzymes and their properties which make it highly functional in industrial environments (Ferrari et al., 1993). One of the extracellular enzymes that *B. licheniformis* has been found to produce is alpha amylase. Alpha-amylase is one of the most commonly isolated commercial products from bacterial strains. While *B. licheniformis* is commonly used, it can be difficult to manipulate genetically, and mutants of this strain can be deficient in producing the important extracellular enzymes (Ferrari et al., 1993).

A study by Qaio et al. (2010) reported that *B. licheniformis* increased ruminal fiber digestion via the increase of cellulolytic bacteria in supplemented dairy cows compared to controls. The supplementation of *B. licheniformis* also increased total VFA concentration and decreased ammonia nitrogen concentration in rumen fluid samples. This study also reported increased microbial crude protein flow into the duodenum when comparing to control animals. Supplementation of *B. licheniformis* to dairy cattle increased milk protein and yield when compared to non-supplemented cattle (Qiao et al., 2010). In a study using lambs fed a high grain diet; starch, dry matter, and organic matter intake decreased, while dietary alpha-amylase increased in lambs supplemented with *B. licheniformis* (Rojo et al., 2005). This resulted in a quadratic increase of total tract dry matter, organic matter and starch digestibility and ruminal

starch digestion. Meaning that the inclusion of *B. licheniformis* into high concentrate diets of lambs increased the extent of ruminal starch digestion (Rojo et al., 2005). The authors concluded that the extra cellular enzyme of alpha-amylase might be an alternative to other practices to increase ruminal starch digestion. In crossbred dorper wethers the dietary supplementation of *B. licheniformis* resulted in improved apparent digestibility of neutral detergent fiber, organic matter nitrogen (N) and dry matter (Deng et al., 2018). This study also reported improvements in energy metabolism and N utilization and retention.

2.36 *Bacillus subtilis*

Bacillus subtilis is a growth inhibitor of potentially harmful bacteria within the GI tract of cattle (Mingmongkolchai and Panbangred, 2018). *Bacillus subtilis* has been reported to be a strong bacterial strain with steady genetics, which could be a leading candidate for DFM and probiotic products (Ferrari et al., 1993). *Bacillus subtilis* is a GRAS microorganism that can be found widespread in nature (Lee et al., 2019). *B. subtilis* has been found to grow efficiently in low-cost nitrogen and carbon sources (Olmos-Soto, 2014) and had been found to exhibit antiviral activity while also producing antibiotics (Lee et al., 2019). Certain strains of *B. subtilis* have been reported to have antiviral, antimicrobial, and anticancer effects.

Song et al. (2014) evaluated the effects of two different concentrations of *B. subtilis* (0.5×1011 cfu or 1.0×1011 cfu) on hind gut fermentation and microbiota concentrations in lactating dairy cattle. These authors reported a decrease in fecal ammonia concentrations without a change in fecal pH or VFA concentration when compared to control cattle. Changes in the microbial populations and diversity of cattle receiving the DFM supplement were observed, leading to the conclusion that the inclusion of DFM products led to positive changes in the animal. *Bacillus subtilis* supplementation increased ruminal nitrogen levels after feeding in dairy

cows but was found to have no effect on rumen fermentation characteristics, ruminal apparent nutrient digestibility, or non-ammonia nitrogen and microbial CP duodenal flow when compared to control cattle (Qiao et al., 2010). Based off the above findings, these authors proposed that *B. subtilis* could promote the growth of protein degrading microbes in the rumen. In a study feeding a *Bacillus subtilis* based DFM product to pigs the inclusion of the DFM product resulted in improved villi length of the duodenum and jejunum in young piglets (nursery age) (Cai et al., 2015). Pigs receiving the DFM had greater ADG and gain:feed, resulting in improved growth performance when compared to control animals. These authors also reported the supplementation of DFM increased nitrogen digestibility, lowered fecal ammonia release, and lowered BUN, meaning these animals had enhanced protein utilization compared to controls. This data helps to explain the greater apparent total tract digestibility of nitrogen that has been reported with the feeding of *Bacillus subtilis* to growing pigs (Cai et al., 2015).

In a study using 36 early lactation Holstein cow's supplementation of *Bacillus subtilis* resulted in a linear increase of energy correlated milk, milk production, milk fat, milk protein, and lactose yield when compared to negative control cows (Sun et al., 2013). The somatic cell counts of milk from supplemented cattle decreased, irrespective of dose of *B. subtilis*. Four rumen cannulated dairy cows were used to compare pre- and post- periods of *Bacillus subtilis* DFM supplementation (Sun et al., 2013). These researchers reported decreased ruminal pH and increased ammonia nitrogen, proportions of valerate and propionate, and total volatile fatty acids during the period of feeding *B. subtilis* (Sun et al., 2013). Acetate proportion and acetate to propionate ratio were lower in treatment animals during the trial period. When compared to the pre-trial period, proteolytic and amylolytic bacteria and total ruminal bacteria increased but protozoa decreased during the trial period. NDF digestibility was also reduced during the trial

and post-trial phases. Overall, these researchers concluded that the inclusion of a *Bacillus subtilis* DFM product has the ability to improve milk components and production, while promoting total ruminal bacteria, proteolytic, amylolytic bacteria growth, and decreasing somatic cell counts (Sun et al., 2013). Leading the researchers to state the *B. subtilis* bacterial strain has the potential to be an effective DFM supplement for cattle.

Colombo et al. (2021) evaluated the health and performance responses of newly received feedlot beef cattle who received a symbiotic *Bacillus subtilis* supplement. Feed intake of animals receiving the DFM supplement was greater during the first 3 weeks of the receiving period when compared to control cattle. No differences were reported for ADG, BW, and feed efficiency. The incidence of bovine respiratory disease also did not differ among treatments but steers that received the DFM supplement did have improved responses to BRD treatment when compared to control animals. The researchers stated that this suggested a heightened immunocompetence due to enhanced metabolism that resulted from the supplementation of the *Bacillus subtilis* based DFM product (Colombo et al., 2021).

2.37 *Bacillus licheniformis* and *Bacillus subtilis*

The number of studies using a combination of *Bacillus licheniformis* and *Bacillus subtilis* are limited. *Bacillus licheniformis* and *Bacillus subtilis* are both spore forming bacteria that are stable in the digestive tract of mammals. They can function as competitors of pathogens and improve nutrient digestion (Mingmongkolchai and Panbangred, 2018; Su et al., 2020). Cappellozza et al. (2023) reported improved dry matter and neutral detergent fiber digestion of dairy TMR and single feedstuffs in an in vitro setting using a *Bacillus licheniformis* and *Bacillus subtilis* DFM with in incubation vessels when compared to control vessels. Incubation with DFM also increased the production of gas in vitro. The authors concluded that the DFM containing a

combination of *Bacillus licheniformis* and *Bacillus subtilis* has the potential to improve nutrient utilization in a rumen environment. An additional study evaluated the effects of a DFM supplement on in vitro dry matter, neutral detergent fiber, and starch digestibility on a variety of common ruminant feedstuffs (Pan et al., 2022). The DFM used in this study contained *Bacillus licheniformis* and *Bacillus subtilis*, at a total dose of 3.2×10^9 cfu·g^{-1}. The inclusion of the DFM resulted in increased in vitro dry matter disappearance (IVDMD) after 24 and 48 hrs for four common roughage sources. In vitro neutral detergent fiber digestibility (IVNDFD increased at 24 and 48 hrs after the addition of DFM in five of the forages. Overall IVDMD and IVNDFD increased when forages were sorted by quality (Pan et al., 2022). Additionally, in vitro starch digestion was greater after inoculating vessels containing cereal grain samples with the DFM. Leading these authors to conclude that the *Bacillus* based DFM improved in vitro DM, fiber, and starch digestibility of a variety of forages, dairy TMR, and cereal grain feedstuffs.

A study using a *Bacillus* based DFM, *Bacillus licheniformis* and *Bacillus subtilis*, to determine the performance and ruminal effects on mid lactation (182 ± 50 DIM) Holstein dairy cows, which were fitted with rumen cannulas (Lamontagne et al., 2023). There was a reported increase, close to 10-fold, of *Bacillus subtilis* relative concentrations in the rumen of DFM fed animals, when compared to controls. Treatment did not affect lactation performance, acetate, propionate, or butyrate concentrations, pH, and ammonia nitrogen from rumen samples. The DFM animals did have a tendency to show increased ruminal concentrations of isobutyrate and isovalerate. The authors stated that the milk branched-chain fatty acid composition is sensitive to changes of the ruminal microbiota (Lamontagne et al., 2023). Indicating that the modifications that were seen in this study might potentially lead to changes in the branched-chained fatty acid composition of milk from DFM supplemented animals. Oyebade et al. (2023) reported that

supplementing multiparous dairy cows (41 ± 7 days in milk) with a mixture of *Lactobacillus animalis* and *Propionibacterium freudenreichii* at 3×10^9 cfu·day^{-1} or *Lactobacillus animalis, Propionibacterium freudenreichii, Bacillus subtilis,* and *B. licheniformis* at 11.8×10^9 cfu·day^{-1} improved dietary crude fat digestibility and certain immune parameter measurements compared to non-supplemented controls.

2.40 Summary

The beef industry is a complex system of operations that are constantly changing and adapting to fit consumer demands. As the consumer looks for a beef product containing less antibiotics and hormones the use of direct-fed microbials increases. Direct-fed microbials have been used in livestock production systems for many years. These products have shown that they have the ability to benefit the intestinal microorganisms of cattle, while impacting immune function and improving performance of feedlot cattle. The combination of different strains of bacteria has been reported to have additional benefits to cattle, impacting more than just one system within the animals. Therefore, the objective of the subsequent study was to investigate the impact of combination bacterial direct-fed microbial product, BOVAMINE DEFEND® Plus, on growth performance, carcass characteristics, estimated dry matter digestibility, and immune parameters in finishing beef cattle.

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CHAPTER 3

INFLUENCE OF BOVAMINE DEFEND® PLUS ON GROWTH PERFORMANCE, CARCASS CHARACTERISTICS, ESTIMATED DRY MATTER DIGESTIBILITY, RUMEN FERMENTATION CHARACTERISTICS, AND IMMUNE FUNCTION IN FINISHING BEEF STEERS.[1,2]

Summary:

One hundred and eighty crossbred beef steers (406.0 ± 2.2 kg) were used to determine the impact of a novel direct-fed microbial (DFM) on growth performance, carcass characteristics, rumen fermentation characteristics, and immune response in finishing beef cattle. Steers were blocked by body weight and randomly assigned, within block, to 1 of 2 treatments (3 replicates/treatment: 30 steers/replicate). Treatments included: 1) no DFM (control) and 2) DFM supplementation at 50 mg·animal^{-1}·d^{-1} (BOVAMINE DEFEND® Plus). All steers were fed a high-concentrate finishing diet and individual feed intake was recorded daily via the GrowSafe® system. Body weights were collected every 28 d. On d 55, 10 steers per pen were injected with ovalbumin (OVA). Jugular blood samples were collected from each steer on d 0, 7, 14, and 21 post-injection. On d 112, the same steers were injected again with OVA and intramuscularly with a pig red blood cell solution. Jugular blood samples were collected from each steer on d 0, 7, 14, and 21 post-injection. On d 124 rumen fluid was collected from 3 steers per treatment and used to estimate in vitro rumen fermentation characteristics. Equal numbers of steers per treatment

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were transported to a commercial abattoir on d 145, 167, and 185 of the experiment, harvested, and carcass data collected. Initial body weight (BW) was similar across treatments. On d 28 and 55, steers receiving DFM had heavier BW ($P < 0.01$) compared to controls. Average daily gain was greater in DFM-supplemented steers from d 0 to 28 ($P < 0.01$) and d 0 to 55 ($P < 0.01$) of the experiment compared to controls. Overall dry matter intake was greater ($P < 0.04$) and overall feed efficiency was similar in DFM-supplemented steers compared to controls. Dressing percentage ($P < 0.02$) was greater in steers receiving DFM compared to controls. Antibody titers to injected antigens were similar across treatments. However, red blood cell superoxide dismutase activity was greater ($P < 0.05$) in DFM-supplemented steers compared to controls. In vitro molar proportions of isobutyric and butyric acid were greater ($P < 0.01$) and dry matter (DM) digestibility tended ($P < 0.07$) to be greater in rumen fluid obtained from steers supplemented with DFM. These data suggest that BOVAMINE DEFEND® Plus supplementation improves growth performance during the initial period of the finishing phase, increases overall dry matter intake and dressing percentage, and may impact antioxidant status in beef cattle.

Keywords: cattle, digestibility, in vitro, fermentation

Introduction:

Direct-fed microbials (DFM) have been used to aid livestock production systems for over twenty years (LeJeune and Wetzel, 2007), and are defined, by The office of Regulatory Affairs of the Food and Drug Administration (FDA), as products fed to livestock that contain live microorganisms from naturally occurring sources (Brashears et al., 2005). Bacterial DFM products have the potential to positively benefit the balance of intestinal microorganisms while preventing pathogen adherence, modulating immune function, and influencing permeability of

gut tissues (Krehbiel et al., 2003). Furthermore, published data would suggest that cattle supplemented with DFM have improved feed conversion efficiency and average daily gains (ADGs; Krehbiel et al., 2003, Cull et al., 2015).

BOVAMINE DEFEND® Plus, a DFM, is a combination of live bacterial cultures with the aim of improving normal functions of the gastrointestinal (GI) tract such as digestion, absorption, immune function, and barrier function. Research conducted by Silva et al. (2022) and Preedy et al. (2023), has demonstrated positive benefits of DFM supplementation on several production variables of finishing feedlot cattle. BOVAMINE DEFEND® Plus (*Lactobacillus animalis* 506, *Propionibacterium freudenreichii* 507, *Bacillus licheniformis* 809, and *Bacillus subtilis* 597) supplementation has been reported to inhibit *Clostridium perfringens* types A and C growth in vitro and reduced the proportion of newborn beef calves with abnormal diarrhea after being challenged with *C. perfringens* type A (Guimaraes et al., 2023). We hypothesized that BOVAMINE DEFEND® Plus would increase growth performance while improving carcass characteristics and immune function in finishing beef steers. Therefore, the objective of the current experiment was to investigate the impact of BOVAMINE DEFEND® Plus on growth performance, carcass characteristics, estimated dry matter (DM) digestibility, and immune parameters in finishing beef cattle.

Materials and Methods:

Prior to the initiation of this experiment all animal care, handling, and procedures were approved by the Colorado State University Animal Care and Use Committee (Approval #: 2453).

Cattle Processing: Two hundred and twenty-nine crossbred beef steers (BW = 415.9 ± 2.3 kg) were transported to the Colorado State University Agriculture, Research, Development,

and Education Center (ARDEC), in Fort Collins, CO. Upon arrival, steers were handled, processed, and allotted to treatments, according to our standard animal processing procedures (Caldera et al., 2017; Budde et al., 2019). Briefly, steers were individually weighed, identified with an unique ear tag, vaccinated with Presponse® (*Pasteurella multocida* Bacterial Extract-*Mannheimia haemolytica* Toxoid, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) and Pyramid 2 plus Type II BVD (Infectious Bovine Rhinotracheitis Virus and Bovine Viral Diarrhea (Types I and II), Boehringer Ingelheim Vetmedica, Inc.) respiratory vaccines, injected with Promectin® (Ivermectin, Vedco, Inc.), drenched with Synanthic® (Oxfendazole, Boehringer Ingelheim Vetmedica, Inc.) for parasite control, and implanted with Revalor – XS® (200 mg trenbolone acetate and 40 mg estradiol, Merck Animal Health, DeSoto, KS). Following initial weighing, steers were housed together in groups of approximately 40 animals per pen with ad libitum access to long-stem grass hay and water overnight.

After initial weighing, steers were ranked by body weight (BW), and individuals that were beyond ± 2 SD from the mean BW were eliminated from further consideration for the experiment as described by Caldera et al. (2017). Briefly, the remaining steers were assigned a random number from 1 to 1000 using the random number function in Excel 2007® (Microsoft Corporation, Redmond, WA). Steers with the lowest random numbers were eliminated from the experiment reducing the number of remaining steers to 180. The 180 eligible steers were ranked by weight and divided into 3 weight block replicates, each one consisting of 60 steers. Within each weight block replicate, steers were ranked by weight and randomly assigned to one of 2 pens. This randomization schedule resulted in 3 weight block replicates, each containing 2 pens with 30 steers per pen with similar BW distribution for a total of 6 pens. Replicates within a weight block were randomly assigned to treatments. Treatments consisted of: 1) no DFM

(control) and 2) DFM supplementation at 50 mg·animal^{-1}·d^{-1} (BOVAMINE DEFEND® Plus). The following day, steers were weighed prior to feeding and visual ear tags identifying each animal were applied. Steers were then sorted into their respective treatment pens and the experiment initiated. The initial BW used for the experiment was the average of the 2 full BW obtained on d -1 and 0. Each pen (15 × 43 m) housing 30 animals was equipped with 4 GrowSafe® units (in order to determine individual animal feed intake for the duration of the experiment), an automatic waterer, concrete bunk pad, and a metal roof (15 × 3 m) covering the GrowSafe® units and approximately 7% of the entire pen.

Pens were checked daily to ensure that cattle were in the appropriate pens, had ad libitum access to water, and that all GrowSafe® units had enough feed to supply all cattle in the pen with 24 h of feed. Furthermore, all cattle were monitored for health and locomotion daily. Steers exhibiting symptoms of respiratory disease were removed from the pen and rectal body temperatures recorded. Steers with body temperatures greater than 39.4°C were considered to have clinical disease. All clinically ill steers were treated according to the appropriate treatment protocol and immediately returned to their original pen.

Diets: All steers were fed a steam-flaked corn-based high-energy finishing diet. Steers were adjusted to the finishing diet using a series of step-up diets where the roughage portion of the diet was replaced with corn during each step-up diet. Diet changes during the step-up program were simultaneous (every 5 to 7 days) for both treatments and cattle reached the finishing diet by approximately d 24 of the experiment. The finishing diet (Table 1) was formulated to meet or exceed NASEM (2016) requirements for growing and finishing beef cattle. Nutrient target values were: 13.1% crude protein (CP) with 3.5% CP equivalent from non-protein nitrogen, 0.7% calcium, 0.36% phosphorus, 0.6% potassium, 0.25% magnesium, 0.15 mg Co/kg

dry matter (DM); 0.3 mg Se/kg DM, 30 g per metric ton of monensin (Rumensin®, Elanco Animal Health, Greenfield, IN), and 10 g per metric ton of tylosin on a DM basis. Vitamins A and E were included in the diets at 2,200 and 9.4 IU/kg of DM, respectively, and were administered in the liquid supplement and macro – and micro minerals were added as inorganic sources to meet the targeted values of the total mixed ration (TMR). DFM supplement was added to the diet fresh daily from pre-weighed packages. The DFM supplement (BOVAMINE DEFEND® Plus; Chr. Hansen A/S, Hørsholm, Denmark) contained a combination of *Lactobacillus animalis* 506, *Propionibacterium freudenreichii* 507, *Bacillus licheniformis* 809, and *Bacillus subtilis* 597. A beta agonist (ractopamine hydrochloride; Optaflexx®; 400 $mg \cdot animal^{-1} \cdot d^{-1}$) was fed to all cattle for the last 29 days on feed. Diets were delivered once daily in the morning (0800h) in amounts to allow all steers ad libitum access to feed over a 24-h period.

Weighing, sampling, and carcass data collection: Steers were individually weighed on d -1, 0, approximately every 28 d, and on two consecutive days at the end of the experiment. At the time of slaughter, control and DFM supplemented steers from the same BW block (n = 3 BW blocks) were transported to a commercial abattoir and slaughtered. Weight blocks were harvested after receiving the finishing diet for 145, 167, or 185 d. Following harvest, hot carcass weight was determined, and liver abscesses were scored, as described by Elanco (2019). Carcasses were allowed to chill for approximately 24 to 36 h before additional carcass data was obtained. Carcass data were collected by trained professionals. Carcass data collected included dressing percentage, longissimus muscle area (LMA), subcutaneous adipose tissue thickness, adjusted subcutaneous adipose tissue thickness (USDA, 1989), kidney, pelvic, and heart fat (KPH), marbling score, quality grade, and yield grade (calculated).

Titanium dioxide (TiO_2) was added to the diet on d 98 of the experiment as described by others (Titgemeyer, et al., 2001; Ebert et al., 2016). Fecal samples were collected directly from the rectum and collections were conducted from the same 10 randomly selected animals per pen, once daily on d 112, 113, 114, 121, 122, 123. For every 24 h period, time of collection was advanced 2 h to minimize effects of diurnal variation (Titgemeyer et al., 2001; Ebert et al., 2016). The fecal samples with TiO_2 were used to estimate dry matter digestibility. Titanium dioxide was determined as described by Myers et al. (2004).

Immune parameters: On days 0, 55 and 112, all cattle were bled via jugular venipuncture to assess immune parameters. Blood was collected into three separate 7-ml vacutainer tubes. Two non-heparinized vacutainer tubes for serum collection, and the other vacutainer tube was a heparinized trace-mineral-free vacutainer tube for red blood cells and plasma (Becton Dickinson Co., Franklin Lakes, NJ). Total serum immunoglobulin G (IgG) concentrations were determined using a radial immunodiffusion assay kit (Kent Laboratories, Bellinghan, WA). Superoxide dismutase enzyme activity and interferon gamma concentrations were determined using a SOD 525™ Assay Kit (Biotech® 21010; Oxis Health Products, Inc., Portland, OR) and ELISA assay (Biosource KBC1231, Biosource International, Inc., Camarillo, CA), respectively.

On d 55 of the experiment, 10 steers from each pen (n = 60 steers total) were randomly selected and injected with ovalbumin (OVA). Briefly, as described by Dorton (2005), two milliliters of a solution containing 160 mg of OVA (Sigma A5503), 60 mL Freund's Incomplete Adjuvant (FIA; Sigma F-5506), and 60 mL of sterile phosphate buffered saline (PBS) were injected subcutaneously and one milliliter was injected intradermally to give a total injection of 4000 μg of OVA/animal. Blood samples were collected via jugular venipuncture in non-

heparinized vacutainer tubes (Becton Dickenson Co., Franklin Lakes, NJ) prior to injection and 7, 14, and 21 d post injection.

On d 112 the same subset of cattle was then injected with OVA (as described previously) and with 5 ml of a 25% purified pig red blood cell (PRBC; i.m. in the neck muscle) solution as described by Kuhlman et al. (1988). Blood samples were collected from these animals immediately prior to ovalbumin and PRBC injection on d 112, and again on d 119, 126, and 133. All serum samples were analyzed for antibody titers specific to ovalbumin using an ELISA procedure described by Engvall and Perlmann (1972). Antibody titers specific for PRBC were measured using a microtiter hemagglutination assay to determine total immunoglobulin (Ig), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations specific for PRBC (Ferket and Qureshi, 1992).

In vitro fermentation: Rumen fluid was collected, on d 124, using a stomach tube as described by Engle and Spears (2000), from three control and three DFM supplemented steers from the same weight block replicate and used as inoculum for in vitro analysis of diet DM digestibility. A composite rumen fluid sample was made from the three steers per treatment. The rumen fluid composite for each treatment was mixed with McDougall's solution and incubated for 0, 6, 12 and 24 h (in quadruplicate) for in vitro analysis. The total mixed ration for each treatment was used as the fermentation substrate for the rumen fluid obtained from steers on the same treatments. A modified McDougall's buffer solution (39.20 g $NaHCO_3$, 14.80 g Na_2HPO_4, 2.28 g KCl, 1.88 g NaCl, 0.48 $MgSO_4*7H_2O$ per 2 L H_2O) was mixed with rumen fluid at a 1:1 ratio, simulating saliva production during rumination (Tilley and Terry, 1963). Short chain fatty acids (SCFA), and in vitro dry matter disappearance (IVDMD), were determined as described by Levenson et al. (2022). Briefly, to simulate rumen motility, vaccine bottles were gently swirled

every 4-h. Samples were removed at each time point and centrifuged at 1,000 × *g* for 30 min (Beckman Model TJ-6; Beckman Coulter, Indianapolis, IN). A 2.0-ml aliquot of the supernatant was extracted from the in vitro vessel post centrifugation, acidified with meta-phosphoric acid, and frozen at -80°C until analyzed for SCFA concentrations. The remaining supernatant was aspirated, and the indigestible residue dried in a forced air-drying oven at 60°C for 120 h to determine IVDMD.

After thawing at room temperature, the samples designated for SCFA analysis were centrifuged at 28,000 × *g* at 5°C for 15 min and the supernatant removed and placed into a 1.5 ml gas chromatography vials and analyzed for SCFA (Levenson et al., 2022). The SCFA concentrations were determined via gas chromatography (Agilent 6890N, Santa Clara, CA, USA) fitted with a fused silica capillary column (30 m × 0.25 µm × 0.25 µm) and a flame ionization detector as described by Gifford et al. (2021).

Dry matter disappearance (DMD) was determined for all samples by weighing the 50 ml conical tubes prior to dispensing the vaccine bottle rumen contents into the tube and after drying in the forced air-drying oven at 60°C for 120 h. The IVDMD was calculated as follows: IVDMD, % = ((initial substrate DM mass - (undigested DM mass-microbial DM residue mass)) / (initial substrate DM mass) × 100.

Statistics: Feedlot performance, immune parameters, carcass characteristics, in vivo dry matter apparent digestibility, and in vitro fermentation data were analyzed on an individual animal or digestion vessel basis for a randomized complete block design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Treatment and where appropriate, time and the interactions of treatment × time were included in the model as a fixed classification effect, and weight block was included in the model as a random effect. Covariates of initial BW were used in the analysis

of all performance and carcass response variables. Outlier tests were performed on all data. Data points exceeding 3 standard deviations above or below the mean were removed from the data set prior to analysis. A type three ANOVA table was constructed using the Kenward-Roger method of computing denominator degrees of freedom. Backwards elimination with AIC criteria was used to remove nonsignificant ($P \geq 0.05$) covariates from the model. The main effect of treatment, time, and the treatment × time interactions (where appropriate) were determined significant at $P < 0.05$. For the appropriate response variables, if the treatment × time interaction was not significant, overall treatment means were reported. Treatment means were separated ($P \leq 0.05$) using the PDIFF option of the LSMEANS statement of SAS (SAS Inst. Inc., Cary, NC, USA). Categorical data were evaluated using PROC GLIMMIX of SAS assuming a binomial distribution. The Link = Logit option was included in the model and the LSMEANS and SEM were calculated from the output statement. Significance was determined at $P \leq 0.05$ for all response variables.

Results

A total of 5 steers were removed from the experiment due to lameness associated with severe foot rot. Two steers were removed within the first 28 d of the experiment and euthanized (Control; n=90, Treatment; n=88). Three additional steers were removed from the experiment approximately 28 d prior to slaughter. These three animals were slaughtered but their carcass data were not included in the statistical analysis (Control; n=90, Treatment; n=85). All five steers were from the DFM treatment.

The impacts of the DFM on growth performance of feedlot steers are presented in Table 2. Initial BW were similar across treatments ($P = 0.88$), but on d 28 and 55 of the experiment, steers receiving DFM had heavier BW ($P < 0.01$) compared to controls. Average daily gain

(ADG) was greater in DFM-supplemented steers from d 0 to 28 ($P < 0.01$) and d 0 to 55 ($P < 0.01$) of the experiment compared to controls. Overall, dry matter intake (DMI) was greater ($P < 0.04$) in DFM-supplemented steers when compared to controls. However, final BW, overall ADG, and feed efficiency were similar across treatments ($P \geq 0.1$).

The influence of DFM on carcass data is presented in Table 3. Dressing percentage ($P < 0.02$) and United States Department of Agriculture (USDA) yield grade ($P < 0.05$) were greater, and 12th rib subcutaneous fat depth tended ($P < 0.10$) to be greater in DFM supplemented steers when compared to controls. Hot carcass weights, longissimus muscle area, marbling score, USDA quality grade, calculated yield grade, and percent liver abscesses were similar across treatments ($P \geq 0.1$).

There was no treatment by time interactions for any of the in vitro rumen fermentation characteristics measured, therefore only the main effects are reported in Table 4. Isobutyric and butyric acid concentrations (mM) were greater ($P < 0.01$) and DM disappearance tended ($P < 0.07$) to be greater for digestion vessels containing inoculum obtained from DFM-supplemented steers compared to controls. Acetic and propionic acid concentrations (mM) and total SCFA concentrations (mM) were similar across treatments ($P \geq 0.1$). Furthermore, in vivo estimates of dry matter digestibility (using TiO_2 as an indigestible marker) were similar across treatments (78.3% and 83.8% ± 2.9 for Control and DFM treatments, respectively; data not shown).

The influence of DFM supplementation on immune parameters measured in this experiment is presented in Table 5. There were no treatment by time interactions for any of the immune parameters measured; therefore, only treatment main effects are presented. Total serum IgG concentrations, IgG and IgM antibodies specific to ovalbumin, and total immunoglobulins specific for PRBC were similar across treatments ($P \geq 0.1$). However, treatment was a significant

source of variation for plasma interferon-gamma concentrations and superoxide dismutase activity. Interferon-gamma concentrations ($P < 0.04$) and superoxide dismutase activity ($P < 0.02$) were greater in DFM-supplemented steers compared to control steers.

Discussion

The DFM (BOVAMINE DEFEND® Plus) used in this study contained a combination of *Lactobacillus animalis* 506, *Propionibacterium freudenreichii* 507, *Bacillus licheniformis* 809, and *Bacillus subtilis* 597. *Lactobacillus animalis* used in the current experiment, is a lactic acid-producing bacteria that has barrier function capabilities within the rumen environment and *Propionibacterium freudenreichii*, a gram-positive, lactic acid-utilizing bacteria that has been reported to increase propionate production in the rumen (Brashears et al., 2005; McAllister et al., 2011). *Bacillus licheniformis* is a potent competitor of potentially harmful bacteria within the rumen, while *Bacillus subtilis* is a growth inhibitor of potentially harmful bacteria within the GI tract of cattle (Mingmongkolchai and Panbangred, 2018). Initial research has demonstrated that the aforementioned combination tested improved the health of newborn male beef calves inoculated with *C. perfringens* type A (Guimaraes et al., 2023). Based on our data, BOVAMINE DEFEND® Plus improves growth performance during initial periods of the finishing phase, increases overall dry matter intake, may impact antioxidant status, and increases dressing percentage.

Numerous experiments have been conducted investigating the impacts of individual or combinations of DFM bacteria on cattle performance, pathogenic challenge growth, and animal health. Results of these experiments have varied. In a pooled summary of eight feedlot experiments, Ware et al. (1988) reported that steers receiving *Lactobacillus acidophilus* (1×10^8 cfu·steer^{-1}·d^{-1}) had improved ADG and feed efficiency compared to non-supplemented controls.

Inclusion of *Lactobacillus animalis* as a DFM in the diet of finishing feedlot steers has also been reported to improve weight gains, gain to feed ratio, and hot carcass weights (Hanford et al., 2011; Cull et al., 2012 and 2015). Supplementing *Propionibacterium freudenreichii* at 1×10^9 cfu·animal^{-1}·day^{-1} dose decreased shedding of *E. coli* in feces of mixed breed beef steers fed a high concentrate finishing diet (Elam et al., 2003) and reduced total CH_4 production as a result of increasing propionate production (Meale et al., 2014). Reid and Burton (2002) reported that DFM containing *Propionibacterium freudenreichii* can prevent *E. coli O157* infections, and therefore shedding, by inhibiting penetration of *E. coli O157* into the intestinal mucosal layer. *Propionibacterium* strains have also been reported to increase ruminal propionate production therefore reducing enteric methane emissions from forage fed cattle (Jeyanathan et al. 2014). It is difficult to determine the specific mode of action that DFM has on animal performance due to the variety of bacteria included in different DFM products. However, it appears that the bacteria mentioned previously can improve rumen fermentation characteristics and reduce gastrointestinal tract growth of pathogens which may help explain the improvement in animal performance.

Previous studies have investigated the impact of supplementing a combination of *Lactobacillus animalis* and *Propionibacterium freudenreichii* on cattle performance and disease resistance. Supplementing *Lactobacillus animalis* and *Propionibacterium freudenreichii,* at a combined dose of 4×10^9 cfu·animal^{-1}·day^{-1}, to lactating (120 d in milk) dairy cows, improved milk yield, protein yield and energy-corrected milk by 7.6, 6.9, and 6.0 percent, respectively, compared to non-supplemented cows (Boyd et al., 2011). Furthermore, DM digestibility was increased by three percent in DFM-supplemented dairy cows compared to controls. In feedlot cattle consuming a high concentrate diet, Galyean et al. (2000) investigated the inclusion of $1 \times$

10^6 cfu·animal^{-1}·day^{-1} or 1×10^9 cfu·animal^{-1}·day^{-1} *Lactobacillus animalis* with 1×10^9 cfu·animal^{-1}·day^{-1} *Propionibacterium freudenreichii.* The researchers reported heavier final body weights and greater average daily gains when cattle were supplemented with *Lactobacillus animalis* and *Propionibacterium freudenreichii*, irrespective of dose of *Lactobacillus animalis*.

However, other researchers have reported no impact of single bacterial strains of *Lactobacillus animalis* or *Propionibacterium freudenreichii* or combinations of *Lactobacillus animalis* and *Propionibacterium freudenreichii*, on feedlot cattle growth performance and carcass characteristics (Vasconcelos et al., 2008; Luebbe et al., 2013; Thompson et al., 2020; Cull et al., 2022). The reason for the variable impacts of DFM supplementation on beef cattle growth and carcass characteristics is unknown. There are many factors that can impact an animal's response to DFM supplementation such as: 1) species and strain supplemented, 2) dose and duration of DFM supplementation, 3) stage of growth, and 4) environmental stressors.

Bacillus licheniformis and *Bacillus subtilis* are both spore forming bacteria that are stable in the digestive tract of mammals. They can function as competitors of pathogens and improve nutrient digestion (Mingmongkolchai and Panbangred, 2018; Su et al., 2020). The majority of research investigating the addition of *Bacillus licheniformis* and *Bacillus subtilis* to livestock diets has been conducted in non-ruminant species with little published research in cattle. Other authors have reported that the inclusion of *Bacillus licheniformis* and *Bacillus subtilis*, at a combined dose of 3.2×10^9 cfu·g^{-1}, improved in vitro DM, fiber, and starch digestibility of different forage-based substrates, dairy TMR, and high-starch feedstuffs (Pan et al., 2022; Cappellozza et al., 2023). Oyebade et al. (2023) reported that supplementing multiparous dairy cows (41 ± 7 days in milk) with a mixture of *Lactobacillus animalis* and *Propionibacterium freudenreichii* at 3×10^9 cfu·day^{-1} or *Lactobacillus animalis, Propionibacterium freudenreichii,*

Bacillus subtilis, and *B. licheniformis* at 11.8×10^9 cfu·day^{-1} improved dietary crude fat digestibility and certain immune parameter measurements compared to non-supplemented controls. Other researchers have reported improvements in milk production, milk components, and fermentation characteristics in dairy cows supplemented with *Bacillus subtilis* (Sun et al., 2013) and improved health in newly received feedlot cattle beef cattle (Colombo et al., 2021). These data indicate that certain bacterial DFM alone or in combination can impact cattle performance and health and are in agreement with the improvement in cattle performance and greater red blood cell superoxide dismutase activity observed in the DFM-supplemented cattle compared to controls in the current experiment.

Conclusion

To our knowledge, the current experiment is the first to examine the impact of the DFM BOVAMINE DEFEND® Plus containing *Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus licheniformis*, and *Bacillus subtilis* on feedlot cattle performance, carcass characteristics, immune function, and rumen fermentation characteristics. These data suggest that BOVAMINE DEFEND® Plus supplementation improves growth performance during initial periods of the finishing phase, increases overall dry matter intake and dressing percentage, and may impact antioxidant status in beef cattle. Future research examining the impact of DFM on feed intake and disease resistance is warranted.

Table 1. Dry matter ingredient composition of the basal finishing diet[a].

Ingredient	%
Steam-flaked corn	60.3
Distillers grains	14.3
Corn silage	11.0
Liquid supplement [b]	6.8
Wheat straw	4.8
Limestone	1.6
Rumensin/Tylan supplement [c]	0.9
White Salt	0.3
Chemical composition	
DM, % as fed	71.3
CP, %	13.0
Acid Detergent Fiber, %	9.9
Neutral Detergent Fiber, %	17.7
Ether extract, %	4.2
NEg, Mcal/kg	1.54
NEm, Mcal/kg	2.2
Calcium, %	0.68
Phosphorus, %	0.32
Magnesium, %	0.22
Potassium, %	0.64
Sulfur, %	0.21
Copper, mg/kg	15.2
Selenium, mg/kg	0.24
Manganese, mg/kg	36.7
Zinc, mg/kg	54.3
Cobalt, mg/kg	0.16
Iron, mg/kg	81.2

[a] Optaflexx^{TM} was included in the diet at a rate of 400 mg·animal^{-1}·day^{-1}.
[b] Liquid supplement provided in a molasses base included: 3.72% NPN (Urea), 0.61% Ca ($CaCO_3$), 0.26% Salt (NaCl), 0.05% K (KCl), 2,343 IU/kg Vitamin A, 9.4 IU/kg Vitamin E
[c] Formulated to provide 30.0 g of Rumensin/metric ton and 10.0 g of Tylan/metric ton.

Table 2. Influence of BOVAMINE DEFEND® Plus on growth performance of feedlot steers.

Item	Treatment[a]		SEM	P-value
	Control	DFM		
Initial n=	90	90	---	---
Body weight, kg				
Initial	405.7	406.2	2.2	0.88
Day 28	457.9	468.4	4.9	0.01
Day 55	521.1	528.8	5.7	0.01
Day 84	578.7	582.0	5.4	0.34
Day 112	626.6	634.0	6.8	0.10
Day 144	668.6	672.9	8.6	0.44
Day 168	685.1	692.2	9.4	0.36
Day 185	700.7	705.0	12.5	0.50
Average daily gain, kg·animal^{-1}·day^{-1},[b]				
Day 0-28	1.86	2.22	0.10	0.01
Day 0-55	2.10	2.23	0.07	0.01
Day 0-84	2.05	2.10	0.06	0.33
Day 0-112	1.97	2.04	0.06	0.11
Day 0-144	1.82	1.85	0.06	0.44
Day 0-168	1.73	1.78	0.07	0.33
Day 0-185	1.79	1.83	0.04	0.52
Overall dry matter intake, kg·animal^{-1}·day^{-1}	10.37	10.74	0.12	0.04
Overall feed efficiency (gain:feed)	0.173	0.170	0.003	0.41

[a]CON: control diet, DFM: Direct-Fed Microbial Treatment BOVAMINE DEFEND® Plus fed at 50 mg·animal^{-1}·day^{-1}.
[b]Weight blocks were slaughtered on d 145, 167, and 185

Table 3. Influence of BOVAMINE DEFEND® Plus on carcass characteristics of feedlot steers.

	Treatment[a]			
Item	CON	DFM	SEM	P-value
Hot carcass weight, kg	418.4	424.0	2.97	0.18
Dressing Percentage[b]	62.2	63.0	0.22	0.02
12th rib subcutaneous fat depth, cm	1.36	1.45	0.04	0.10
Longissimus muscle area, cm^2	91.6	92.7	0.84	0.37
Marbling Score[c]	629.0	627.5	11.77	0.92
USDA Yield Grade	2.79	2.95	0.06	0.05
USDA Quality Grade	5.80	5.79	0.12	0.95
Calculated Yield Grade	3.19	3.33	0.06	0.11
Liver Abscess, % (n/total)	42.2 (38/90)	53.4 (47/88)	5.41	0.44

[a]CON: control diet, DFM: Direct-Fed Microbial Treatment BOVAMINE DEFEND® Plus fed at 50 mg·animal^{-1}·day^{-1}.

[b]Final live body weight pencil-shrunk by 4% prior to dressing percentage calculation.

[c]Slightly Abundant=800, Moderate=700, Modest=600, Small=500, Slight=400.

Table 4. Influence of ruminal inoculum from control and BOVAMINE DEFEND® Plus supplemented steers on in vitro fermentation characteristics.

	Treatment[a]			
Item	CON	DFM	SEM	P-value
Dry matter digestion, %	50.49	58.13	2.89	0.07
Acetic acid, mM	39.65	39.39	1.32	0.89
Propionic acid, mM	25.38	25.09	0.76	0.79
Isobutyric acid, mM	0.83	1.12	0.07	0.01
Butyric acid, mM	9.83	11.61	0.46	0.01
Total SCFA[b], mM	75.01	77.90	2.97	0.55

[a]CON: control diet; DFM: Direct-Fed Microbial Treatment BOVAMINE DEFEND® Plus fed at 50 mg·animal^{-1}·day^{-1}.
[b]Short chain fatty acids.

Table 5. Influence of BOVAMINE DEFEND® Plus on blood immune parameters in feedlot steers.

Item	Treatment[a]		SEM	P-value
	Control	DFM		Treatment
Total serum Immunoglobulin G, mg/ml	2565.9	2586.0	28.9	0.63
Ovalbumin IgG Titers, $\log_{10}$	0.011	0.010	0.001	0.64
Ovalbumin IgM Titers, $\log_{10}$	0.053	0.055	0.002	0.20
Total PRBC Titers, $\log_2$	1.321	1.363	0.094	0.76
Superoxide dismutase, U/mg hemoglobin	0.344	0.372	0.020	0.02
Interferon gamma, $\log_{10}$	0.133	0.148	0.005	0.04

[a]CON: control diet, DFM: DirectFed Microbial Treatment using BOVAMINE DEFEND® Plus fed at 50 mg·animal^{-1}·day$^{-1.}$

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