EVALUATION OF DIRECT FED MICROBIALS TO IMPROVE FERMENTATION EFFICIENCY AND PATHOGEN EXCLUSION USING AN *IN VITRO* MODEL

by

KATHERINE PATRICIA FELDMANN

(Under the Direction of Todd R. Callaway)

ABSTRACT

The objective of this research was to evaluate DFM strain efficacy and modes of action utilizing *in vitro* fermentation models. Three experiments were conducted; the first focused on selecting a DFM strain for inhibition of *Salmonella* Typhimurium utilizing co-culture and mixed microorganism *in vitro*, which demonstrated *L. lactis* has antagonistic activity against *Salmonella* Typhimurium and potentially inhibits in the lower gastrointestinal tract. The second study compared DFM effects on fermentation parameters in rumen and fecal mixed microorganism *in vitro* models and found while DFM increased total gas production in fecal *in vitro*, there were no clear differences in DFM treatment affecting fermentation patterns. The third study evaluated DFM effects on rumen and fecal microbiome *in vitro* analyzing alpha diversity indices and genera abundances and found many differences caused by DFM in which *L. lactis* showed the most promise for further work *in vivo*.

INDEX WORDS: Direct Fed Microbials, Pathogen Inhibition, Fermentation, Microbiome, *in vitro*

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DEDICATION

I would like to dedicate this thesis to the Feldmanns, the Foleys, and the whole family tree. This life would not be possible without generational love and support, and I will continue to carry you all with me.

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CHAPTER 1

INTRODUCTION

The world population is projected to reach over 9 billion by 2050, meaning crop and livestock production systems must increase the supply of food produced dramatically (FAO, 2009). The use of antimicrobials in livestock feed began as early as the 1950s and was an effective method to increase animal growth and feed efficiency to produce more pounds of animal-derived protein for consumers (Hardy, 2002). However, by the early 2000s the extent of a growing global challenge of antimicrobial resistance to medically important drugs, became recognized and brought added public scrutiny over the prophylactic use of antimicrobial compounds in livestock production (Smith and Coast, 2002). The establishment of the "One Health" approach advanced addressing and unifying responses to antimicrobial resistance across human, livestock, and environmental sectors (Tarazona et al., 2020). These growing concerns resonated with consumers who voiced opposition to purchasing animal-derived products that had been fed antimicrobials (Consumer Reports: Meat On Drugs, 2012). These changes in industry and consumer attitudes surrounding antimicrobial usage for livestock production resulted in exploration into a variety of alternative strategies to improve animal performance.

"Alternatives to antibiotics" (**ATA**) encompasses an assortment of substances that can be substituted for therapeutic drugs which have become increasing ineffective in the face of antimicrobial resistance (Seal et al., 2013; Callaway et al., 2021). Compounds that have shown promise as ATA for use in food animals include: vaccines, eubiotics, prebiotics, antibodies, bacteriophages, peptides, and phytochemicals (Seal et al., 2013). One of these approaches that

has garnered much attention is probiotics, as they have been long discussed as having beneficial effects on health (Silva et al., 2020; Hosono, 1992; Metchnikoff, 1907). In the livestock industry, probiotics are often referred to as direct fed microbials (**DFM**) which encompasses live microorganisms which when fed in adequate amounts confer a health benefit to the host (FAO/WHO, 2006; Fuller, 1989). In recent years, the use of DFM has been widely adopted by the cattle industry.

The goal of feeding DFM is to improve rumen and gastrointestinal tract (**GIT**) health and alter the ruminal microbial ecosystem composition thereby improving growth and feed efficiency (McAllister et al., 2011). The primary modes of action that have been investigated using live animal research include pathogen inhibition, GIT immunomodulation, and microbial fermentation end product alteration (Krehbiel et al., 2003). Pathogen inhibition studies have often focused on using DFM as a pre-harvest strategy to reduce *Escherichia coli* O157:H7 and *Salmonella* serovar prevalence in cattle to increase food safety and potentially animal health (Brashears et al., 2003; Peterson et al., 2007; Stephens et al., 2007). Improving ruminal fermentation efficiency using DFM has addressed a wide variety of issues such as methane reduction, incidence and severity reduction of subacute ruminal acidosis, and improved overall animal productivity and health (Michalet-Doreau and Morand, 1996; Nocek and Kautz, 2006; Jeyanathan et al., 2014; Kelsey and Colpoys, 2018). Studies examining DFM effects on GIT immunomodulation have evaluated changes to the rumen and GIT microbiome as well as regulation of adaptive and innate immune system activity (Adjei-Fremah et al., 2018; Ogunade et al., 2020). Direct fed microbials remain a rapidly evolving area of research but due to their complex nature it can be difficult to ascertain direct effects on the host. Additionally, fewer

studies have focused on utilizing *in vitro* models to form a foundation of potential direct DFM effects on the microbial population of the gut.

In order to better understand the relationship between DFM and potential impact on the host animal, we performed a series of *in vitro* mixed microorganism fermentations using ruminal or fecal populations to understand specific modes of action of five different potential DFM/probiotic strains. The objective of the present research was to distinguish differences between candidate DFM cultures in a mixed microorganism model and to use *in vitro* methodology to better understand strain characteristics and as selection criteria for use in future investigations *in vivo*. The first experiment evaluated the direct anti-pathogenic activity against pure cultures of *S.* Typhimurium, to determine if a candidate DFM strain could inhibit a common pathogenic *Salmonella* serovar and play a role as a pre-harvest foodborne pathogen reduction strategy. The second experiment examined various end products produced by mixed rumen and fecal microorganism fermentation to determine if a candidate DFM strain altered the fermentation patterns that benefit production metrics, including reduced methane emissions and more energy-containing volatile fatty acid profiles. The third experiment examined DFM candidate strain impacts on the microbiome from rumen and fecal *in vitro* mixed microorganism fermentations, to determine if microbial communities were influenced by DFM feeding and if microorganism shifts impacted dietary energy harvest or immunological factors. We hypothesized that there would be differences between DFM candidate strains *in vitro* and these *in vitro* mixed microorganism fermentations would demonstrate which strains would be: most capable of pathogen inhibition, altering fermentation patterns, and/or shifting microbial communities in the rumen and lower GIT.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Ruminant animals have a unique relationship with their native microbial population of the rumen, or pre-gastric fermentation chamber (Hungate, 1966). The presence of the rumen microbial population enables ruminant animals to degrade feedstuffs that other animals are unable to utilize (e.g., cellulose). The degradation of feedstuffs by rumen microbial fermentation produces volatile fatty acids (**VFA**) and microbial cells, which provide energy and protein to the ruminant animal (Wolin, 1960; Owens and Basalan, 2016). However, ruminal fermentation is not perfectly efficient, and end products are produced that are not utilized by the host (e.g., methane, ammonia) which reduces the feed efficiency of cattle (Satter and Slyter, 1974; Russell and Hespell, 1981).

Cattle feed efficiency ranges from 6 to 2 pounds of feed for a pound of gain (or milk), compared to the much more efficient production in monogastrics which ranges from 1.5 to 4 pounds of feed per pound of gain (Koch et al., 1963; Becker et al. 1963). The wasteful end products of ruminal fermentation represent a loss of carbon, energy, and nitrogen that the animal could otherwise utilize, and some of the wasteful end products are harmful to the environment and can play a role in global climate change (Capper and Bauman, 2013). It has long been a goal of researchers and producers to improve the feed efficiency of ruminant animals, and manipulation of ruminal and hindgut fermentation has been an avenue examined by many.

However, knowledge development on the interactions between feedstuffs, rumen physiology, and the microbial community has been limited until more recently.

Within the past 15 years, advancements in culture-independent techniques such as 16S rRNA sequencing and metagenomics have greatly furthered understanding of complex rumen and gastrointestinal interactions (Kim et al., 2017; Seshadri et al., 2018). These methods have revealed how microbial communities can be associated with factors such as diet and host phenotypes such as feed efficiency (Henderson et al., 2015; Myer et al., 2015). As knowledge continues to increase on feedstuff-microbiota-host interactions, new ways to successfully manipulate rumen and gastrointestinal fermentation for the improvement of cattle production are likely to be discovered.

Direct Fed Microbials Use as Alternatives to Antimicrobials

Since the 1950s, antibiotics were widely used in cattle production to improve commercially impactful production parameters such as feed efficiency and exclusion of pathogenic microorganisms (CAST, 1981). The primary consumer of antibiotics in the beef cattle industry was the feedlot sector. In 2011, 49% of cattle in large-scale feedlots were fed antibiotics for growth promotion (Sneeringer et al., 2015). In the dairy industry, metaphylactic antibiotic use predominantly occurs in the development of dairy heifer replacement females. Approximately 20% of dairy heifer operations used antibiotics as growth promotants in 2007 (Sneeringer et al., 2015).

Over time, there has been a dramatic increase in consumer concerns surrounding medically important antimicrobial resistance in bacteria, which has driven a modern reexamination of antibiotic usage in the livestock industry (Kirchhelle, 2018; Sneeringer et al., 2019). This follows more than 20 years of a ban on the use of antibiotics as growth promotants in

the European Union (Casewell et al., 2003). In 2012, a consumer report found 72% of respondents were very/extremely concerned that widespread antibiotic use in livestock would lead to bacterial resistance to drugs used in human medicine (Consumer Reports: Meat On Drugs, 2012). Beginning in 2017, the United States Food and Drug Administration (**FDA**) implemented the Veterinary Feed Directive, which prohibited the sub-therapeutic use of antibiotics deemed medically important, with their use for the treatment of disease in livestock requiring veterinarian approval and tracking of antimicrobial use (FDA GFI#213, 2017).

Antibiotic resistance concerns have further led to renewed interest in discovering and implementing alternative strategies. In 2012 the World Health Organization organized the first international symposium in Paris, France to address this topic in animal production systems (Seal et al., 2013). This led to the development of the term "alternatives to antibiotics" (**ATA**). Alternatives to antibiotics are defined as any substance that can be substituted for therapeutic drugs that are increasingly becoming ineffective against pathogenic bacteria, viruses, or parasites (Seal et al., 2013; Callaway et al., 2021). Some of the ATA that have been utilized in experimental settings include: innate immune molecules with antimicrobial capabilities, naturally occurring antibacterial lytic enzymes, bioactive phytochemicals, and other novel biotherapeutic alternatives pre-, post-, and pro- biotics (Callaway et al., 2021). Many of these have been classified together recently as "eubiotics", meaning a product that impacts (positively) the native microbial population (El Jeni et al., 2023).

One of particular interest due to its already existing prevalence in the food production system is probiotics. In 1907, Metchnikoff proposed the idea of useful microbes found in food products that could be isolated and consumed to promote health, which we now understand as probiotics. Since then, there have been several attempts to further expand this idea and apply it to

livestock production (Vanbelle et al., 1990; Gaggìa et al., 2010; Cheng et al., 2014; El Jeni et al., 2023). While the definition of probiotics has undergone many revisions, they have been most recently defined as “live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host” (FAO/WHO, 2006). In the United States, probiotics are regulated by the Food and Drug Administration-Center for Veterinary Medicine (**FDA CVM**) as a feed additive and must obtain Generally Recognized as Safe (**GRAS**) status which is achieved through either an established history of use in food prior to 1958 or consensus among qualified experts (FDA 21 CFR§182,184,186, 1977). The global probiotic market size is expected to increase from 5.1 billion USD in 2022 to 10.7 billion USD by 2032 with the United States as one of the countries at the forefront of implementation of these products (Paraskevakos; Spherical Insights LLP, 2023). As the probiotic market continues to increase in size, understanding how probiotics can be utilized in livestock production is imperative, as well as understanding how they work in some situations and not others.

DFM Product Types

What was once referred to as probiotics in the livestock industry has been more clearly defined by The Office of Regulatory Affairs of the FDA and The Association of American Feed Control Officials as direct fed microbials (**DFM**). Direct fed microbial products must contain live microorganisms (McAllister et al., 2011; Yoon and Stern, 1995). It is important to acknowledge that defining DFM-based products remains a dynamic and evolving process within the livestock industry as they may also additionally contain pre-, post-, or eubiotic approaches (Figure 2.1); however, for the purpose of this review, DFM will be used to refer to the use of live microorganisms solely.

Direct fed microbials can be categorized by differentiating bacterial from fungal DFM initially, after which bacterial DFM can be further delineated using metabolic, morphological, and taxonomic classification systems (EFSA, 2017; Yoon and Stern, 1995). The largest category is lactic acid bacteria (**LAB**) which are characterized by the ability to produce lactic acid as a major end product of fermentation (Bouchard et al., 2016). Bacterial genera that fall under this category include: *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, and *Enterococcus*. In the cattle industry, *Lactobacillus* strains are the predominant LAB genera used in DFM supplements. The next category of DFM amongst non-LAB bacteria is high G+C content gram-positive bacteria, which are characterized by having a high proportion of guanine and cytosine nucleotides in their chromosomal DNA, which is typically greater than 60% (Ventura et al., 2006). Direct fed microbials in this group come from the *Actinobacteria* phylum, which includes *Bifidobacterium* and *Propionibacterium* bacterial strains. Another group that is commonly used are *Bacillus spp*., which are sporulating gram-positive bacteria that can form endospores in response to adverse conditions, allowing them to better maintain viability in long term storage in comparison to non-spore forming DFM (Mingmongkolchai and Panbangred, 2018). The last category of non-LAB bacteria DFM are gram-negative bacteria. *Prevotella bryantii* is the sole DFM species that we will discuss that is gram-negative, but other potential DFM gram-negative species exist; however, its use in cattle has, to date, been limited to research use only (Chiquette et al., 2008; Chiquette et al., 2012). Additionally, yeast cultures have been routinely used in ruminant DFM supplements and include *Aspergillus*, *Kluyveromyces*, *Saccharomyces*, and *Trichoderma* genera (Vohra et al., 2016). These different categories of DFM are routinely combined to make multi-strain DFM based on the concept that strains may demonstrate synergistic activity enhancing potential probiotic effects (Lambo et al., 2021).

Direct fed microbials are primarily administered orally to cattle and can be used in both solid and liquid forms. Bolus capsules can be given several times to the animal in a specific period, often during stressful events such as at birth or weaning; additionally, drenches or gels/pastes are another option that can be utilized during these times (BAMN, 2011). The most common modes of DFM administration are powders, pellets or capsules mixed into feed or water to deliver daily supplementation to cattle, but special considerations must be taken with water or pellet supplementation as these delivery methods have aspects that affect strain survival and effectiveness (BAMN, 2011; Branco-Lopes et al., 2023). Due to the wide variety of DFM products available, a comprehensive cost-benefit analysis of individual DFM remains challenging and is largely determined by producer criteria and needs as well as farm-specific factors such as type of cattle, environmental constraints, and goals (Brown and Nagaraja, 2009).

Modes of Action

The diversity of DFM products used in cattle production highlights the need to understand the underlying fundamentals of impact to the host and allows for targeting specific DFM to the proper phase of production and species (beef vs dairy). The rumen and gastrointestinal tract (**GIT**) in ruminant animals are dynamic microbial environments which are susceptible to diet changes and stressful events and factors such as weaning, transportation, overcrowding, feed deprivation, and extreme weather (Freestone and Lyte, 2010; Verbrugghe et al., 2012; Ort et al., 2018). The physiological response to these stressors can result in gut dysbiosis, suppression of immunological factors, and a general decrease in fitness of the animal (Freestone and Lyte, 2010; Rostagno, 2009). This negative impact on production has resulted in investigations using DFM to counter stressful events and maintain rumen and GIT homeostasis. While this remains a rapidly evolving area of research, DFM are thought to impact the rumen

and GIT through three broadly characterized modes of action: pathogen inhibition, GIT immunomodulation, and microbial fermentation end product alteration (Krehbiel et al., 2003).

Pathogen Inhibition

At several stages throughout production, cattle can become colonized by and carry foodborne pathogens (e.g., *Escherichia coli* O157:H7, *Salmonella* serovars, *Campylobacter* spp.) which is thought to primarily occur in the lower GIT (Wallis et al., 1995; Grauke et al., 2002; Naylor et al., 2003; Inglis et al., 2005). An important criterion for GIT microbial survival of pathogens is the ability to colonize the mucosal epithelium (Savage, 1977). Host cells in the mucosal epithelium have various cell surface receptors that can be exploited by microbial pathogens, which possess molecular and macromolecular structures known as adhesins that facilitate the first stage of disease, tissue colonization and attachment (Finlay and Cossart, 1997). After pathogenic microbes penetrate the mucosal layer and attach to the epithelium, biofilm formation can begin which increases pathogen resistance to antimicrobial substances and the likelihood for chronic infection in the host (Parsek and Singh, 2003; Motta et al., 2021). The prevention of pathogen adhesion and biofilm formation may be achieved through the use of DFM. Fuller and Gibson (1997) proposed that certain DFM may have the ability to compete with pathogens for adhesion receptors, and thus competitively exclude them from colonizing the GIT. *Lactobacillus* species and *E. coli* strain Nissile 1917 have demonstrated potential to inhibit pathogen adhesion and outcompete pathogen biofilm formation (Lee et al., 2003; Hancock et al., 2010; Woo and Ahn, 2013). Several LAB strains have been investigated as potential candidates for the competitive exclusion of *E. coli* O157:H7 in cattle, especially *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* mixtures (Brashears et al., 2003; Peterson et al., 2007).

Despite this, understanding the exact mechanisms behind certain microbial species being able to inhibit the attachment of pathogens to the intestinal epithelium is lacking.

Some of the DFM cultures used in the animal feeding industry may be able to inhibit GIT pathogen colonization through the production of antimicrobial compounds, such as bacteriocins. Bacteriocins are a heterogenous assortment of antimicrobial peptides produced by gram-positive bacteria that target other gram-positive bacteria occupying a similar ecological niche (Jack et al., 1995; Cotter et al., 2013). Of particular interest are an antimicrobial category produced by some LAB, which belong to class I bacteriocins known as lantibiotics. The most extensively researched lantibiotic is nisin, produced by the bacterial species *Lactococcus lactis* (Perez et al., 2014). Lantibiotics form pores in the cell membranes of target bacteria (often competitors in the same ecological niche), disrupting the proton motive force and ion gradients, including leakage of ATP, thus causing cellular death (Pérez-Ramos et al., 2021). Bacteriocins produced by LAB typically inhibit gram-positive pathogens (Umu et al., 2016; Darbandi et al., 2022), and the inclusion of bacteriocins or bacteriocin-producing bacterial strains in cattle rations may alter the rumen ecology to exclude bacterial species such as *Streptococcus bovis* and pathogenic bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* (Russell and Mantovani, 2002; Wells et al., 1997).

Probiotic bacteria species also produce antimicrobial compounds including organic acids and hydrogen peroxide (Malik et al., 2021). The production of organic acids (i.e. lactic acid, acetic acid, citric acid, etc.) can result in their uptake by pathogenic bacteria (Russell and Diez-Gonzalez, 1997). Inside the cell, organic acid toxicity (anion accumulation) can alter intracellular pH and disrupt membrane potential, thereby depleting cellular energy for essential metabolic functions (Ricke, 2003). Organic acids have demonstrated potential to alter the gastrointestinal

pH and inhibit pathogens such as *Salmonella* in poultry (Broom, 2015; Dittoe et al., 2018); however, the impact of organic acids on GIT pathogen inhibition in cattle remains relatively unexplored, primarily due to the large ruminal volume and high concentration of VFA present naturally in the rumen which are toxic to bacteria (Wolin, 1969).

Hydrogen peroxide is a powerful oxidizing agent that causes cellular DNA damage and can result in microbial cell death (Juven and Pierson, 1996). Production of hydrogen peroxide by lactic acid bacteria in conjunction with other antimicrobials such as lactic acid can enhance pathogen inactivation (Atassi and Servin, 2010); however, one caveat of hydrogen peroxide production by LAB is the presence of oxygen, and thus it may be of limited importance as a factor of pathogen inactivation in the highly anaerobic GIT. Nevertheless, the production of several antimicrobial compounds by microorganisms remains a key factor in their potential use as DFM for pathogen inhibition.

GIT Immunomodulation

The GIT is not only responsible for the digestion of foods and absorption of nutrients, but also plays an important role in functional response of the immune system (Furness et al., 2013; Ashraf and Shah, 2014; Ma et al., 2018). The first line of host animal defense is the physical barrier of intestinal epithelial cells (**IEC**) (Goto and Ivanov, 2013). Intestinal epithelial cells are generated from stem cells located at the base of intestinal crypts, which differentiate into different IEC such as enterocytes which secrete immunoglobulins, Goblet cells which are responsible for mucosal production, and Paneth cells that produce antimicrobial peptides and proteins (Kong et al., 2018). These IEC work in conjunction to prevent microbial invasion past the GIT epithelium. Additionally, tight junctions attach these IEC together in close proximity which allows for the diffusion of ions but restricts the passage of small molecules such as

pathogenic microorganisms (Balda and Matter, 2008). Underneath the basal face of IEC lies gut-associated lymphoid tissue (**GALT**), which comprises up to 70 percent of the host immune system (Langkamp-Henken et al., 1992). The GALT is made up of two components: 1) inductive sites of organized lymphoid tissue such as Peyer's patches, caecal and appendix patches, and 2) isolated lymphoid follicles and effector sites such as the lamina propria that are more loosely organized (Scott et al., 1993; Mason et al., 2008; Donaldson et al., 2015; Abo-Shaban et al., 2023). Peyer's patches participate in immunosurveillance through antigen sampling by microfold cells, also known as M cells (Heel et al., 1997). In cattle, two predominant Peyer's patches exist, with the ileal patch playing an important role as the main lymphoid organ for B-cell development and the jejunal patch functioning as secondary lymphoid tissue as part of the mucosal immune system (Yasudaa et al., 2006). Additionally, the caecum also possesses patches which participate in immunosurveillance in the lower GIT (Mowat and Agace, 2014). Isolated lymphoid follicles develop in response to changes in the GIT and have a role in promoting the antibody IgA (Knoop and Newberry, 2012). As an effector site, the lamina propria contains a diverse array of immune cells from both innate (i.e. macrophages) and adaptive (i.e. B and T cells) immunity branches that are primed in the event of microbial invasion past IEC (Schenk and Mueller, 2008; Nagler-Anderson, 2001). The complex interplay between different GIT immune system components allows for the maintenance of intestinal homeostasis, thereby preventing inflammation and infection.

As early as birth, the colonization of microbes can dictate the development of the immune system. In germ-free mice, the inductive sites of the GALT remain poorly developed in comparison to specific-pathogen-free mice; however, upon the introduction of commensal microbiota this effect was reversed (Chinen and Rudensky, 2012; Kabat et al., 2014).

Additionally, conventionalization of germ-free mice resulted in the stimulation and development of innate and adaptive immune functions through increased transcription of innate immune factors and activation of T cells (El Aidy et al., 2012). In newborn calves, the rumen and hindgut are rapidly colonized by bacterial phyla with *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* being the most abundant (Jami et al., 2013; Rey et al., 2014; Alipour et al., 2018; Song et al., 2018). Concurrently, the permeability of the gut in calves remains high for the first 24 h of life to assist the oral transfer of immunoglobins in colostrum but leaves the calf vulnerable to pathogenic infections (Araujo et al., 2015). *Firmicutes* have been associated with the production of the volatile fatty acid (**VFA**) butyrate (Louis et al., 2010; Postler and Ghosh, 2017). Butyrate is vital for maintaining the integrity of the intestinal epithelium and has been implicated in promoting the assembly of tight junctions, especially occludins and zonula occludens, through increased activation of activated protein kinase (Peng et al., 2009; Camilleri, 2019; Siddiqui and Cresci, 2021; Usuda et al., 2021). *Proteobacteria* have demonstrated a role in priming IgA response (Mirpuri et al., 2013), and in the phyla *Bacteroidetes*, some members of the *Bacteroides* genus may play a role in developing the immune system through bacterial polysaccharide production and modulating inflammation by regulating lymphocyte and cytokine expression (Mazmanian et al., 2005; Tan et al., 2019). Furthermore, the colonization of newborn GIT by these early microbial species can be influenced by factors such as maternal diet, mode of delivery (vaginal vs cesarean), and newborn nutrition (colostrum vs milk replacer), and in human studies have demonstrated potential negative outcomes of immune development such as allergies, autoimmune diseases, or obesity (Martin et al., 2010; Zhang et al., 2021). Such findings indicate the potential to identify relevant microbiota and metabolite production in the development of the calf immune system as well as factors that positively impact this process,

which could provide insight on strategies to mitigate negative outcomes (i.e. disease and mortality) during calf maturation.

After initial colonization, the composition of the rumen microbial community undergoes rapid changes. The proportion of *Proteobacteria* decreases as the proportion of *Bacteroidetes* increases through 2 months of age, along with archaeal and fungal communities establishing themselves. Then, from 2 months through 2 years of age, there is a convergence towards a mature microbial consortium predominantly composed of *Bacteroidetes* and *Firmicutes* with a more diverse, though likely restricted array of genera (Jami et al., 2013; Rey et al., 2014; Dill-Mcfarland et al., 2017). As the ruminant becomes fully functional, the composition of the microbial population begins to stabilize as it reaches maturation. This mature microbial community plays an essential role in maintaining intestinal homeostasis via cross talk, a mechanism utilized by microbiota to interact with the intestinal epithelium and mucosal immune system through the production of different immunomodulatory metabolites that upregulate IEC immune transcription factors against pathogenic bacteria and increase mucosal production (Clark and Coopersmith, 2007; Shastry and Rekha, 2020). Volatile fatty acids, also commonly referred to as short chain fatty acids are the major source of energy for ruminants and are produced by the fermentation of protein, fiber, and other carbohydrates by commensal gastrointestinal microorganisms (Bergman, 1990; Alarcon et al., 2018). Not only do VFA such as acetate, propionate, and butyrate play a role in the maintenance of rumen and intestinal epithelium integrity, but they have also been linked to modulating innate inflammatory responses by inducing pro-inflammatory effects via neutrophil recruitment that are vital for defense mechanisms against pathogen invasion (Brestoff and Artis, 2013; Alarcon et al., 2018). Secondary bile acids produced by the metabolization of primary bile acids in the liver by

commensal microbiota possess antimicrobial capabilities such as inactivation of endotoxins and act as signaling molecules for inflammatory pathways (Bertók, 2004; Begley et al., 2005; Chen et al., 2019). Additionally, the microbial population of the rumen is unique in its ability to synthesize adequate amounts of dietary vitamin K and B, especially that of B_{12} which is not found in the GIT of other organisms (Bechdel et al., 1928; Scott, 1967; Seshadri et al., 2018). While the exact mechanisms are unknown, these vitamins have been noted to have an anti-inflammatory effect, and their synthesis within the GIT may play a role in immunomodulation (Yoshii et al., 2019; Lai et al., 2022).

Probiotic bacterial species contain microorganism-associated molecular patterns (**MAMPs**) that are recognized by pattern recognition receptors (**PRRs**) expressed by innate immune cells, which in turn can prime the innate immune system for efficient response to pathogen exposure (Mackey and McFall, 2006; Bron et al., 2012; Llewellyn and Foey, 2017; Negi et al., 2019). Metabolites produced by probiotic species such as VFA, indole, and secreted proteins can enhance protection of the intestinal epithelial barrier (Liu et al., 2020). Supplementation of probiotic strains have also been linked to increasing production of IgA and induce development of regulatory T-cells, which play a vital role in preventing autoimmunity (Delcenserie et al., 2008). The administration of probiotic species in cattle can impact expression of genes involved in immunity and homeostasis through MAMPs that activate Toll-like receptor and Wingless signaling pathways (Adjei-Fremah et al., 2018). In cattle supplemented with yeast-based DFM, an increase in gene expression involved in MAMPs detection and anti-inflammatory pathways, as well as T-cell differentiation and anti-inflammatory metabolites was observed (Adeyemi et al., 2019; Idowu et al., 2022). Similarly, a study utilizing a LAB DFM saw an alleviation of the inflammatory response (Chida et al., 2021) while a different DFM blend

resulted in increased activation of immune cells such granulocytes and T-cells as part of the response against invading pathogens (Oyebade et al., 2023). However, a meta-analysis of DFM use on cattle immunity evaluating inflammatory biomarkers such as plasma and serum antibodies, acute phase proteins, and blood metabolites found no significant association between probiotics and immunomodulatory effects (Barreto et al., 2021). A challenge of DFM supplementation is there is no "one size fits all" approach, as the complexity of the GIT immune system and factors such as diet, age, physiological state, genetics, and the commensal microbial population represent confounding variables. Greater understanding on the mechanisms behind strain-specific probiotic impact on cattle rumen and GIT immunomodulation in conjunction with other modes of action is needed before developing DFM as a targeted approach to improving cattle health and immunity.

Microbial Fermentation

Cattle have the advantage of the catabolic activity of a foregut fermentation within the reticulorumen, which is a highly reduced, anaerobic environment that is selective for a population of strict anaerobic and facultative anaerobic microorganisms. In addition to the bacteria, the reticulorumen also is home to many archaea, fungi, and protozoa species. Collectively, this microbial consortium functions in a niche-based manner to systematically degrade fiber into components that can be used for metabolic processes by microbes to benefit the animal (Hoover and Miller, 1991). A major product of this microbial fermentation are VFA, of which acetate, propionate, and butyrate are the most abundant with lower concentrations of isobutyrate, valerate, and isovalerate produced (Dijkstra, 1994). Volatile fatty acids are absorbed through the rumen wall and enter the bloodstream; however, the majority of butyrate is utilized for epithelial maintenance (Hoover and Miller, 1991). Acetate can account for over 40% of VFA

produced and is a precursor for fatty acid synthesis and is linked to backfat and milkfat synthesis (Baldwin and Smith, 1971; Bergman, 1990). Propionate is the only gluconeogenic VFA and is therefore of great importance in energy capture and plays an important role in carcass marbling (Baldwin and Smith, 1971; Smith and Grouse, 1989). Because of the role of these VFA, nutritionists often utilize the acetate: propionate ratio (**A:P**) as a "rule of thumb" to estimate the energetic efficiency of the fermentation. Diet has a dramatic impact on the ruminal A:P ratio, with high forage diets having a greater A:P ratio and high concentrate diets having a lower A:P ratio, representing a shift in energy availability to the animal based on VFA metabolism (Balch and Rowland, 1957).

Starch from high concentrate (grain) diets is rapidly fermented by the microbial population of the rumen, which results in sharp increases of VFA and lactate production causing greater reductions in pH and lower overall pH compared to forage-based diets (Russell, 1998). Acidification of ruminal fluid to reach a pH below 6.0 results in the loss of fibrolytic microorganisms and an overabundance of starch and lactate fermenters, further exacerbating pH fluctuations (Erfle et al., 1982; Russell and Rychlik, 2001). Persistent declines in pH below 5.6 to 5.0 result in sub-acute ruminal acidosis (**SARA**) and below 5.0 is considered acute acidosis, both metabolic diseases resulting in impaired feed efficiency and health of cattle (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2012). Traditionally acidosis has been controlled by the use of antimicrobials (Nagaraja et al., 1981; Nagaraja et al., 1985; Butaye et al., 2003). More recently, DFM have been investigated as an alternative method to mitigate SARA and chronic/acute acidosis. An *Enterococcus faecium* and *Saccharomyces cerevisiae* DFM raised minimum ruminal pH and decreased pH fluctuations (Chiquette, 2009; Chiquette et al., 2012). Additionally, DFM containing cultures of the yeast *S. cerevisiae* or bacterial strain *Megasphaera*

elsdenii increased mean ruminal pH and reduced pH fluctuations respectively (Michalet-Doreau and Morand, 1996; Aikman et al., 2011). While these studies found no effect of DFM treatment on ruminal VFA concentrations, Zhang et al. (2022) observed feeding a *S. cerevisiae* DFM strain increased mean ruminal pH, lowered lactate concentrations, and reduced the A:P ratio. Goto et al. (2016) reported including a bacterial probiotic blend in cattle rations resulted in a higher mean ruminal pH and lower lactate concentration but found no change in VFA concentrations. While the impact of DFM on VFA and lactate concentrations remains unclear, the ability to positively influence ruminal pH suggests DFM may be a viable strategy in treating SARA in cattle.

Methane Production

The domain *Archaea* is a small fraction of microbiota in the rumen, around 0.3 to 3.0 percent, but plays a vital role in the microbial fermentation (Janssen and Kirs, 2008). Disposal of hydrogen produced during fermentation is necessary in order to regenerate NAD+ for the oxidation of sugars by microbiota (Bryant et al., 1967; Thiele and Zeikus, 1988). Ruminal members of *Archaea* are predominantly methanogens, which can utilize this hydrogen along with carbon dioxide to form methane (Janssen and Kirs, 2008; McAllister and Newbold, 2008). While this production of methane is essential to continuing microbial fermentation, it is a waste (non-beneficial) by-product for cattle, which on average, lose 6 – 12% of digestible energy to eructated methane (Johnson and Johnson, 1995). Additionally, methane is a greenhouse gas that has been identified as a contributing factor to global warming (Moss et al., 2000; Dong et al., 2006; US EPA, 2006; Opio et al., 2012).

The livestock sector contributes to approximately 29% of total global methane emissions, of which 90% is enteric methane primarily produced by cattle (Beauchemin et al., 2008; Ragnauth, 2010; Capper and Bauman, 2013). Therefore, strategies that reduce enteric methane

production from cattle are necessary from both a feed efficiency and environmental standpoint, which can dramatically impact cattle producer profitability (Bowen et al., 2020; Lalman et al., 2022). Methane reduction strategies can focus on either inhibiting methanogenesis by redirecting hydrogen to alternative products, decreasing the production of hydrogen, or providing alternative sinks for hydrogen and electron disposal in the rumen (McAllister and Newbold, 2008). It has been proposed that DFM may be able to reduce enteric methane by providing an alternate electron sink; however, few studies have focused on methane mitigation and of those, results remain inconclusive (Mwenya et al., 2004; Jeyanathan et al., 2016; Astuti et al., 2018; Jeyanathan et al., 2019). Future research into DFM methane mitigation and its viability as a methane reduction strategy in cattle is imperative.

Feed Efficiency and Productivity

Direct fed microbials have improved overall feed efficiency and productivity in cattle. The utilization of multi-strain DFM for use in dairy cattle has long focused on improving milk yield and composition, along with dry matter intake (**DMI**) and apparent total tract digestibility (**ATTD**). DFM that were solely composed of LAB did not produce any differences in performance or digestibility (Raeth-Knight et al., 2007; Xu et al., 2017); however, the addition of yeast-based DFM had a positive impact on milk production. Nocek and Kautz (2006) tested a DFM utilizing two strains of *E. faecium* and a yeast strain and found an increase in milk production and DMI postpartum, but no effect on milk fat percentage. A similar multi-species DFM containing three *E. faecium* strains and *S. cerevisiae* yielded no changes in DMI or milk yield but did find ATTD for starch was increased (AlZahal et al., 2014). Another DFM utilizing LAB, *Bacillus spp*., and yeasts increased DMI as well as milk yield and its components (Merati and Towhidi, 2022). While these results of DFM implementation are variable, the use of multiple

bacterial strains with the inclusion of yeast have the potential to positively impact dairy cattle milk production. Use of multi-strain DFM for beef cattle has focused on improving feed efficiency and ruminal fermentation parameters. Multi-strain DFM have demonstrated the ability to improve average daily gain (**ADG**) and feed efficiency (gain: feed) in certain production settings (Kelsey and Colpoys, 2018; Moreira et al., 2019). Dias et al. (2022) demonstrated that these more complex DFM decreased the ruminal A:P ratio and ammonia concentrations, similarly to Ogunade et al. (2020) which observed differences in several ruminal VFA and reduced ruminal ammonia concentrations.

For both dairy and beef cattle, a key component in understanding DFM impact on feed efficiency and productivity has been understanding how a specific DFM influences the composition of the native ruminal microbial community. Ghorbani et al. (2002) investigated a bacterial DFM and found no impact on DMI or ruminal pH but found increased ammonia-N levels associated with higher protozoa and lower amylolytic bacteria relative abundances. In contrast, other DFM caused microbial community shifts that reduce ammonia-N concentrations. Ogunade et al. (2020) noted lower *Prevotella* genera relative abundances and greater populations of *Succinivibrio*, *Ruminococcus*, and *Succiniclasticum* when feeding a multi-bacterial and yeast strain DFM, whereas Pinloche et al. (2013) investigating a yeast DFM also found lower *Prevotella* and higher *Ruminococcus* abundances, along with other foundational bacterial genera such as *Megasphaera*, *Eubacterium*, and *Selenomonas*. Another DFM with LAB found decreases in ammonia-N concentrations might be associated with decreases in proteolytic or proteolytic-associated microbial populations (Monteiro et al., 2022). Collectively, data suggests DFM alter the native rumen microbial community composition and thus shift fermentation end product patterns that impact the host animal energy and protein supply. Furthermore, DFM may also

change the fecal microbiome through increasing concentrations of ruminal microbes, and therefore increase hindgut fermentation rates (Xu et al., 2017; Mansilla et al., 2022). Understanding how DFM elicit changes in the native gastrointestinal microbial communities and consequently alter fermentation patterns is imperative to developing targeted DFM for improving performance and productivity in dairy and beef cattle.

Use of *In Vitro* Models

While *in vivo* fermentation models have been widely used and provide a great value in unraveling biological impacts, limitations exist such as being more time and resource intensive as well as ethical considerations in the use of research animals. *In vitro* fermentation models can be a viable alternative to *in vivo* studies due to being more cost effective, easily replicable, and creating a more controlled experimental environment to isolate specific factors impacting the complex microbial consortium. Given the complex nature of interactions between DFM and the rumen and gastrointestinal system, some pitfalls of probiotic research include strain selection criteria, teasing out single strain versus multi-strain effects, dosage levels, and specific GIT compartment targets (Shanahan, 2003). Further, the impact of DFM is not just felt at the microbial, immune, or host physiology level, but profoundly impacts all three levels; therefore, it is important to be able to study each type of interaction individually in isolation.

In vitro models can be effective in addressing some of these pitfalls. We can evaluate several potential DFM strains both individually and collectively at variable doses in a single study, all while limiting confounding variables and conditions. Additionally, *in vitro* fermentation models can be created that simulate different components of the GIT (e.g., rumen- or colon-specific fermentations) to understand anatomically specific microbial impacts. A major issue, however, is the lack of "standardized" *in vitro* fermentation models for addressing specific

modes of DFM action which can potentially create incongruencies between *in vitro* and *in vivo* trials. Consequently, several *in vitro* fermentation methodologies exist for investigating DFM modes of action in cattle.

In Vitro Model Selection Criteria

While many of the probiotic strains are classified as GRAS, not all microbes are ideal for DFM use in ruminants. Selection criteria for evaluating the probiotic potential of microbial species include: ability to survive the GIT environment, adhesive properties, and antimicrobial susceptibility (Morelli, 2000; de Melo Pereira et al., 2018). The ability of a potential probiotic organism to survive gastrointestinal environmental stressors is important, therefore tolerance assays examined include: acid tolerance, bile salt tolerance, GIT juice tolerance, and digestive enzyme tolerance tests. Surface adhesion properties may be tested utilizing auto aggregation and hydrophobicity assays which can confirm whether microorganisms can adhere to the intestinal epithelium and have potential to outcompete opportunistic pathogen attachment. Safety assessments for livestock DFM often center around antimicrobial/antibiotic resistance, which may be investigated through the detection of antimicrobial resistance genes utilizing disk diffusion, Etest, and other diffusion assays to test antimicrobial susceptibility. Several studies have utilized these methodologies for developing cattle DFM (Brashears et al., 2003; Nader-Macías et al., 2008; Puphan et al., 2015; Maldonado et al., 2018; Lin et al., 2020). If microorganisms pass these selection criteria and demonstrate reasonable evidence of being able to thrive in the GIT, then further *in vitro* investigations can be conducted regarding modes of action.

Pathogen Inhibition *In Vitro* Models

The detection of inhibition of specific pathogens using *in vitro* fermentation model systems typically focus on examining either direct or indirect effects of DFM supplementation. Direct inhibitory fermentation models include utilizing agar spot or well diffusion assays in pure culture, as well as co-culture studies (Oelschlaeger, 2010). The agar spot test consists of placing drops of the potential probiotic strain onto an agar plate, which is then incubated to form colonies before soft agar containing a population of the selected pathogen is overlayed onto the plate and undergoes another incubation period (Wells et al., 1997). After this, pathogen inhibition is measured as clearing zones in the agar overlay around the DFM strain. This methodology, as originally described by (Schillinger and Lucke, 1989) can be modified to support growth conditions of the test strain and pathogen being used. The well diffusion assay was developed by Magaldi et al. (2004) for antifungal susceptibility and has become a popular method for testing direct inhibition of pathogens by probiotic microbial species. In the well diffusion assay, agar containing the pathogen is allowed to form a lawn, wells are cut in the agar to which liquid containing the potential probiotic strains can be added, after which plates are incubated. Following incubation, inhibition zones quantify antagonistic activity of the probiotic strains against pathogens. Co-culture relies on inoculating a broth culture anaerobically with both probiotic and pathogen strains simultaneously which are incubated and serially diluted and plated to determine the remaining CFU/mL of pathogen (Grilli et al., 2015; Callaway et al., 2008). These direct *in vitro* models for pathogen inhibition show a clear effect of a test probiotic strain on a target pathogen; however, these results are not directly translatable to the GIT environment because all other microbe-microbe and microbe-host interactions are eliminated from this model.

Conversely, indirect *in vitro* models, while making it difficult to ascertain the exact cause of effect on pathogens, more closely simulate the complex consortium effects of the GIT. These

models include the use of gastrointestinal tract sources in which the potential probiotic strain and pathogen are inoculated into, which are incubated before performing serial dilutions and plating to determine surviving pathogen concentrations. In cattle, both rumen fluid and fecal fluid can be used to simulate rumen and lower GIT environments (Grilli et al., 2015; Jung et al., 2021; Callaway et al., 2008). Utilizing a two-pronged approach to determine the potential of a probiotic culture to inhibit pathogens *in vitro* which utilizes both direct and indirect models is a mechanism to align *in vitro* results more closely to predicted *in vivo* outcomes.

Mixed Gastrointestinal Content Fermentation *In Vitro* Models

The alteration of fermentation end products can be investigated utilizing *in vitro* models that rely on rumen and fecal fluid collected from donor animals such as cannulated cattle. Two dynamic models can be used to determine fermentation parameters such as gas production, pH, VFA, ammonia-N, digestibility (e.g. NDF and ADF disappearance), as well as changes to the native microbiome composition. Mixed microorganism batch culture was refined and perfected by Tilley and Terry (1963) and then further improved by Goering and Van Soest (1970). This batch model consists of collecting rumen or fecal fluid from an animal, diluting it with an anoxic buffered media solution (typically 33% v/v), inoculating anoxic serum bottles with a test substrate, and then anaerobically sealing the bottles. As each fermentation timepoint is reached, the bottles are "killed" via unsealing and the fermentation fluid can be collected for analysis of different fermentation end products. One drawback with the use of batch cultures is that they fail to capture movement of digesta from rumination. The development of the Ankom Daisy® Incubator was designed to compensate for some of these shortcomings by mechanically rotating jars during incubation (Tassone et al., 2020). Advantages of the use of batch culture include being able to test many treatments at one time as well as being cost and time efficient. A major

drawback of the batch culture model, however, is the inability to evaluate long term fermentation effects due to the buildup of gas and fermentation end products, which can alter end products through end product inhibition, so that these results may not be directly comparable to *in vivo* studies.

Continuous flow cultures can address this issue through a constantly maintained flow of rumen fluid and removal of fermentation end product buildup. Continuous culture models are either single-flow (**SFCC)** or dual-flow (**DFCC**). The rumen simulation technique (**RUSITEC**) developed by Czerkawski and Breckenridge (1977) and perfected by Frumholtz et al. (1989) is a SFCC in which the rumen fluid mixture has one entrance and one exit and continuous flow is maintained by sparging and agitators that keep the fluid and substrate contained in nylon bags moving in addition to buffered media being infused from below the vessel. The DFCC was developed by Hoover et al. (1976) in which the liquid and solid fractions are separated at two different flow rates to mimic more closely what occurs in the rumen. Due to this design, carbon and energy sources are added directly into the vessel by a pump system instead of being contained in a nylon bag. While these systems may be a more accurate mimic of the rumen environment, their cost and maintenance required have limited their popularity in comparison to batch culture studies. Several modifications to batch and culture systems exist, which have been described in previous reviews (Yáñez-Ruiz et al., 2016; Tassone et al., 2020; Deitmers et al., 2022; Vinyard and Faciola, 2022). Both batch and continuous culture *in vitro* models have great value in providing insight on how ruminal fermentation can be altered and remain the most extensively used models for research on DFM modes of action in cattle (Lynch and Martin, 2002; Yang et al., 2004; Doto and Liu, 2011; Ellis et al., 2016; Sun et al., 2016; Monteiro et al., 2022; Pan et al., 2022; Cappellozza et al., 2023; Jeyanathan et al., 2016).

Immunomodulation *In Vitro* Models

Understanding probiotic impact on the immune system using an *in vitro* model relies on utilizing cell cultures. One type of model is a 2-D cell culture, in which IEC are arranged in a single layer. As IEC are one component of the GIT immune system, they can be combined with immune cell types such as dendritic cells to mimic the immune response more closely to microorganism exposure (Bermudez-Brito et al., 2013). There have been several successful attempts in developing bovine IEC cultures to better understand probiotic immunomodulatory effects (Chiba et al., 2012; Takanashi et al., 2013; Zhan et al., 2017; Raabis et al., 2019); however, these 2-D models lack the ability to capture more intricate features of the GIT immune system such as multicellular interactions, native microbiota, and the natural flow of biochemical processes (Barrila et al., 2018). Thus, there have been efforts to create 3-D models for better understanding these types of exchanges, such as an enteroid or "mini gut" that is developed from intestinal crypt or stem cells (Zachos et al., 2016). The successful establishment of bovine enteroid models holds great promise for furthering our understanding of DFM impact on the GIT immune system (Hamilton et al., 2018; Lee et al., 2021; Kawasaki et al., 2023; Nishihara et al., 2023).

Challenges and Concerns

Despite the promise of DFM as a viable ATA in livestock production, there are several hurdles to their widespread inclusion in the industry. Currently, the regulation of DFM is as a feed additive by FDA-CVM if the producers have made no structural or functional claims, otherwise they must undergo a new animal drug application (CPG Sec. 689.100 Direct-Fed Microbial Products). New animal drug applications can be a very costly and extraordinarily time-consuming endeavor; therefore, most products do not make any health claims or refer to DFM

vaguely as “beneficial”, making it difficult to ascertain how DFM products supposedly impact animal health. Additionally, while a minimum level of viable microorganism populations is required to get an effect, there is no requirement to determine the actual efficacy of the listed microorganism concentration (BAMN, 2011). Furthermore, it is important to consider factors such as storage and feeding conditions (e.g., aerobic and dry conditions), type of feed used, and processing methods (e.g., pelleting) which may impact the stability and viability of DFM. Though DFM cultures are GRAS, they cannot be assessed as zero risk due to host individuality and the potential for side effects which can generate some caution regarding their usage (Zommiti et al., 2020). Another type of caution for DFM is the issue of antimicrobial resistance. DFM strains for use in cattle have been documented to possess antimicrobial resistance to medically important antimicrobials (Amachawadi et al., 2018). Such findings place emphasis on using methodologies that carefully examine DFM antimicrobial resistance and their potential to transmit resistance to other microorganisms. Future regulations and research should seek to address these issues regarding DFM usage in the livestock industry.

Conclusion

Direct fed microbials are a promising alternative to antibiotics in the livestock industry, and their increasing popularity highlights a need for continued research on their role in animal production. Further, the use of DFM is seen by many consumers as “green” or environmentally friendly. The current understanding of DFM action is that they possess the potential to positively impact cattle production metrics, such as improving food safety through direct and indirect inhibition of foodborne pathogenic bacteria, improving GIT immunomodulation and therefore improving animal health, enhancing feed efficiency and growth metrics via ruminal fermentation end product alteration, and reducing negative environmental impacts. While *in vivo*

investigations are valuable, there can be great difficulty in unraveling the exact mode of action by any particular microbial DFM species. Therefore, the investigation of DFM impacts relies on the use of many different *in vitro* fermentation models, but the lack of a standard approach with these fermentation models can create incongruencies between *in vitro* and *in vivo* results. Further research is needed to create a standardized *in vitro* procedure for investigating DFM modes of action in the gastrointestinal tract of cattle. Additionally, there are also several concerns and challenges surrounding DFM implementation in the livestock industry that must be addressed for future usage to replace antimicrobials.

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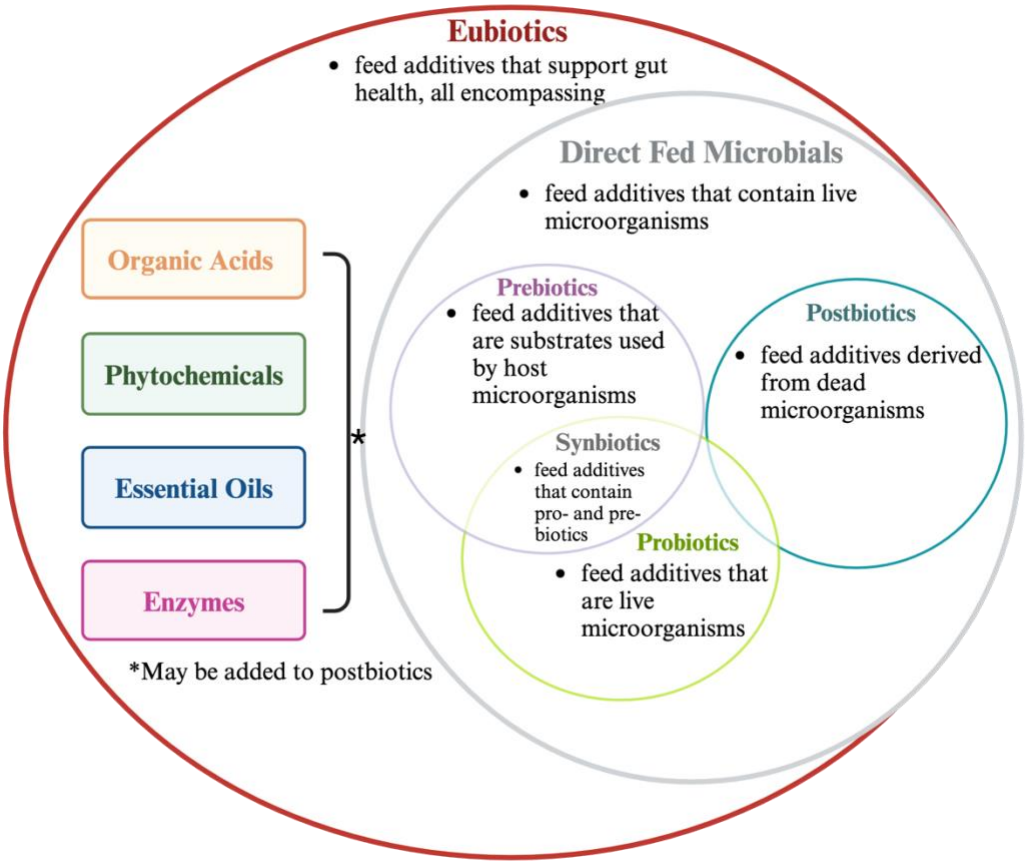

Figure 2.1 Current terminology and definitions used in the livestock industry to describe feed additives targeted towards improving gastrointestinal health and animal performance.

CHAPTER 3

SELECTION OF *LACTOCOCCUS LACTIS* STRAIN WITH ANTI-PATHOGENIC ACTIVITY AGAINST *S.* TYPHIMURIUM IN CO-CULTURE AND IN MIXED RUMINAL AND FECAL MICROORGANISM *IN VITRO* FERMENTATIONS

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ABSTRACT

The risk of foodborne pathogen contamination in meat products is a serious concern for livestock producers and consumers alike. While post-harvest mitigation strategies effectively reduce foodborne pathogen transmission, reducing *Salmonella* in the food supply remains challenging because of its ability to reside in lymph nodes. Therefore, pre-harvest pathogen reduction strategies have focused on reducing *Salmonella* prevalence in live cattle thereby reducing overall contamination of beef products. The use of direct fed microbials (DFM) or probiotics can impact microbial populations of the gut; however, limited research exists examining the mode of action of DFM *in vitro* for the reduction of *Salmonella*. This study aimed to determine if co-culture methods and mixed ruminal microorganism *in vitro* fermentations could be used to select a candidate DFM strain with anti-*Salmonella* activity. Experiment 1 consisted of a 5×2 factorial design using pathogen and candidate DFM co-cultures with three replicates of five treatments at 4 and 24 h. Cultures were grown in anaerobic tryptic soy broth inoculated with both *S.* Typhimurium and candidate DFM strains: *Lactococcus lactis*, *Enterococcus faecium*, *L. diolivorans*, or *B. subtilis* at 10^9 and 10^6 CFU/mL doses compared with controls (**CON**). At both 4 and 24 h, there were Treatment × Dose interactions ($P < 0.001$) with *L. lactis* and *E. faecium* at 10^9 CFU/mL having the greatest reduction in *S.* Typhimurium at 4 h ($P < 0.027$) compared to CON while at 24 h *L. lactis* at 10^9 CFU/mL had the greatest reduction in *S.* Typhimurium compared to CON ($P < 0.001$). *L. lactis* at 10^9 CFU/mL reduced *S.* Typhimurium by at least 1-log more than other treatments. *L. lactis* at 10^9 CFU/mL impacts were evaluated in a ruminal and fecal mixed microorganism *in vitro* model. DFM treatment did not impact *S.* Typhimurium levels in mixed rumen microorganism fermentations ($P > 0.322$), but in

the fecal fermentations *L. lactis* reduced *S.* Typhimurium at both 4 and 24 h ($P < 0.001$). Collectively, results suggest *L. lactis* has the potential to reduce *Salmonella* carriage in cattle.

Keywords: direct fed microbials, *Lactococcus lactis*, *Salmonella**,** in vitro*, cattle

Introduction

In the United States, *Salmonella* infections cause more than 1 million illnesses per year and is one of the leading cause of hospitalizations and deaths from bacterial foodborne pathogens (Scallan et al., 2011). Salmonellosis cases are typically associated with poultry and fresh produce products but have been linked to a wide variety of foods (IFSAC, 2023). Because *Salmonella* predominantly resides in the gastrointestinal tract (**GIT**) of animal species, cattle can be reservoirs and contribute to foodborne illness outbreaks (Laufer et al., 2015; Gutema et al., 2019). Beef and dairy products are thought to be responsible for 10% of confirmed outbreak cases and are an important target for on-farm *Salmonella* mitigation strategies (IFSAC, 2023).

Pathogen reduction strategies on carcasses include hide interventions such as dehairing and washing systems and carcass interventions such as steam vacuuming, organic acids, and oxidizer antimicrobials (Koohmaraie et al., 2005; Wheeler et al., 2014). While these post-harvest strategies work well for surface contamination, *Salmonella* can be located in peripheral lymph nodes (**PLN**) that render these strategies ineffective (Arthur et al., 2008; Li et al., 2015; Edrington et al., 2016; Porwollik et al., 2018). Because the lymph nodes provide a degree of protection against post-harvest mitigation strategies, pre-harvest strategies may be effective in reducing *Salmonella* contamination of beef products, and these strategies include the use of vaccines, phages, antimicrobials, and eubiotic approaches, including the use of direct fed microbials (**DFM**) (Callaway et al., 2013; El Jeni et al., 2023).

Direct fed microbials have been used to reduce *Salmonella* shedding and prevalence in lymph nodes in feedlot cattle (Stephens et al., 2007; Vipham et al., 2015; Brown et al., 2020). Tabe et al. (2008) reported a *L. acidophilus* and *P. freudenreichii* DFM at 10^9 CFU/mL did not reduce *Salmonella* fecal shedding but did reduce the probability of new *Salmonella* infections

among DFM treated feedlot steers. Similarly, Stephens et al. (2007) also utilized a *L. acidophilus* and *P. freudenreichii* DFM at 10^9 CFU/mL; however, *Salmonella* shedding in feces was reduced compared to control. Studies further utilized this same DFM blend to address *Salmonella* PLN prevalence in addition to fecal shedding and found a reduction in *Salmonella* fecal shedding compared to CON. but did not find a reduction in *Salmonella* PLN prevalence (Flach et al., 2022). Vipham et al., (2015) used the same *L. acidophilus* and *P. freudenreichii* DFM blend and reported a reduction of subiliac lymph node *Salmonella* prevalence; however, a similar study utilizing a different DFM blend only reported a reduction in *Salmonella* prevalence in the inguinal lymph nodes (Brown et al., 2020).

Because of the difficulties in analyzing the efficacy of DFM *in vivo*, *in vitro* mixed microorganism models have also been used to investigate DFM anti-pathogenic activity against *Salmonella*. Habib et al. (2022) used a *M. eldensii* DFM utilizing a mixed rumen and fecal fluid mixed microorganism *in vitro* model and reported no reduction in rumen microorganism fermentations but found a 1-$\log_{10}$ CFU reduction in DFM treated fecal fermentations compared to CON. To our knowledge no studies have utilized both co-culture and mixed rumen and fecal fluid microorganism *in vitro* models to investigate anti-pathogen activity of DFM against *Salmonella*. The objective of this study was to determine if these *in vitro* fermentation models could be used to select a candidate DFM strain with anti-*Salmonella* activity that would provide evidence to support *in vivo* cattle feeding trials.

Material and Methods

Bacterial Strains and Culture Conditions

Four DFM strains were obtained from Provita Supplements Inc. (Minnetonka, MN). *Lactococcus lactis* (***L. lactis***), *Enterococcus faecium* (***E. faecium***), *Lactobacillus diolivorans* (***L.***

diolivorans), and *Bacillus subtilis (**B. subtilis**)* spp. were grown separately in anoxic tryptic soy broth (**ATSB**) (Remel, Lenexa, Kansas). Tryptic soy broth was prepared according to manufacturer's directions, and after autoclaving was cooled under an O_2-free CO_2 atmosphere. Subsequently, ATSB was anoxically aliquoted into Balch tubes (Bellco Glass, Vineland, NJ; 9 mL per tube), sealed with butyl rubber stoppers and aluminum crimps, and then autoclaved again. The *Salmonella* Typhimurium (***S.* Typhimurium**) used in this study was originally obtained from the culture collection of the Food and Feed Safety Research Unit, Agricultural Research Service-USDA, College Station, TX and was naturally resistant to novobiocin (NO) and made resistant to nalidixic acid (NA) through successive transfers in ATSB containing up to 20 μg/mL NA.

Experiment 1: Co-culture

Salmonella Typhimurium was incubated overnight at 39°C before being serially diluted (10-fold increments) and added to each ATSB experimental tube at approximately 10^4 CFU/mL at time 0. Initial *S.* Typhimurium concentration was confirmed utilizing serial dilution and plate counts performed in duplicate. Each candidate DFM strain was individually incubated overnight at 39°C and then added to experimental tubes (10 mL) to achieve final concentrations of 10^9 CFU/mL or 10^6 CFU/mL. Each treatment consisted of each individual DFM strain at each of the two doses. Tubes containing *S.* Typhimurium in pure culture served as control (**CON**). Each treatment was performed in triplicate (n = 3) for each timepoint. All tubes were incubated at 39°C and were removed at 4 and 24 h of incubation. After removal, *S.* Typhimurium concentrations were determined using serial dilutions (10-fold increments) in 0.9% sterile saline solution (pH 7.3) and were subsequently plated on brilliant green agar (Becton-Dickinson Difco

Inc, Mississauga, ON) supplemented with 25 μg/mL NO and 20 μg/mL NA) which were incubated overnight at 39°C for direct counting.

Experiment 2

Based upon results from experiment 1, the *L. lactis* strain was chosen for further testing and added to tubes containing *S*. Typhimurium (10^2 and 10^4 CFU/mL) at an inoculation level of 10^9 CFU/mL. The same co-culture model was utilized to confirm *L. lactis* anti-pathogenic activity against *S.* Typhimurium. Each treatment was performed in quintuplicate (n = 5) for each timepoint.

Experiment 3: Mixed Microorganism in vitro

The *L. lactis* strain at a dose level of 10^9 CFU/mL was investigated further utilizing rumen and fecal fluid mixed microorganism fermentation *in vitro* models. Animals in the present study were cared for following guidelines approved by the University of Georgia's Animal Care and Use Committee. Approximately two hours after feeding, ruminal and fecal contents were collected from two 1000-kg cannulated Holstein dairy steers fed a 60% concentrate: 40% forage total mixed ration diet. Contents from each steer were strained through a fine mesh paint strainer into separate thermoses to remove undigested feedstuffs while maintaining minimal aerobic headspace. Thermoses were transported to the laboratory where contents were emptied and strained a second time, then maintained anaerobically under bubbling O_2-free CO_2 for 30 min allowing feed particles to be buoyed to the surface by gas production. Once stratified, particle-free rumen and fecal fluid were separately pooled and pipetted anaerobically (33% vol/vol) to anoxic medium (Cotta and Russell, 1982; Callaway and Martin, 1997). Rumen fluid and fecal media mixtures (9 mL) were anaerobically transferred to anoxic 26-mL Balch tubes containing *L. lactis* at 10^9 CFU/mL and non-DFM supplemented CON. Tubes were flushed with O_2-free

CO_2 and then sealed with rubber stoppers and crimps. Subsequently (within 5 min), *S.* Typhimurium was inoculated via syringe into all experimental tubes at 10^4 CFU/mL and was confirmed with plate counts performed in duplicate. Each treatment was performed in quintuplicate ($n = 5$) for each timepoint. After 4 and 24 h incubation at 39°C, *S.* Typhimurium concentrations were determined using the same serial dilution and plating methodology as experiment 1.

Statistical Analysis

For all experiments, analyses were performed using tube as the experimental unit. For experiment 1, the data were analyzed as completely randomized with a 5 x 2 factorial arrangement. Treatment (**TRT**), Dose, and their interaction served as fixed effects. Analysis of models were done using the one-way ANOVA procedure of RStudio 2023.03.0+386 (RStudio, PBC, Boston, MA) for each timepoint. Pairwise comparisons between treatment and factor level were computed using the Tukey-HSD test. Statistical significance was declared at $P \leq 0.05$. For experiments 2 and 3, the data were analyzed as completely randomized with TRT as the fixed effect. Analysis of the model were done using the Two Sample t-test procedure of RStudio 2023.03.0+386 (RStudio, PBC, Boston, MA) for each timepoint. Statistical significance was determined at $P \leq 0.05$ for all experiments.

Results

Experiment 1

Several DFM candidate strains had impacts on *S.* Typhimurium populations, and a TRT × Dose interaction was observed after 4 h of incubation ($P < 0.001$, Figure 3.1, Table 3.1). The co-culture containing *Lactobacillus diolivorans* at 10^6 CFU/mL had greater *S.* Typhimurium concentrations than did CON, *E. faecium* and *L. lactis* at 10^6 and 10^9 CFU/mL and *B. subtilis*

inoculated at 10^9 CFU/mL ($P < 0.009$); however, it did not differ from its own 10^9 CFU/mL dose or *B. subtilis* at 10^6 CFU/mL ($P > 0.217$). *L. diolivorans* at 10^9 CFU/mL had higher *S.* Typhimurium populations than did *B. subtilis*, *E. faecium* at 10^9 CFU/mL and *L. lactis* at both DFM doses ($P < 0.018$) but did not differ from CON or *E. faecium* at 10^6 CFU/mL ($P > 0.516$). *Lactococcus lactis* at 10^6 CFU/mL did not differ from CON or *B. subtilis* at 10^9 CFU/mL ($P > 0.350$), but *B. subtilis* at 10^9 CFU/mL had lower counts compared to CON ($P < 0.002$). *Enterococcus* faecium and *L. lactis* at 10^9 CFU/mL did not differ from each other ($P = 0.618$) but had the lowest *S.* Typhimurium concentrations compared to the other treatment groups ($P < 0.027$).

After 24 h, TRT × Dose interactions were also observed ($P < 0.001$, Figure 3.1, Table 3.1). CON had greater *S.* Typhimurium counts compared to *E. faecium*, *B. subtilis*, and *L. lactis* at both dose levels and *L. diolivorans* at 10^9 CFU/mL ($P < 0.012$) but did not differ from *L. diolivorans* at 10^6 CFU/mL ($P = 0.999$). *L. diolivorans* at 10^9 CFU/mL had greater counts than *E. faecium*, *B. subtilis*, and *L. lactis* at both dose levels ($P < 0.001$) but did not differ from its 10^6 CFU/mL dose ($P = 0.086$). *Enterococcus faecium* at 10^6 CFU/mL had higher *S.* Typhimurium populations compared to both *L. lactis* doses ($P < 0.012$) and did not differ from its 10^9 CFU/mL dose or either *B. subtilis* dose levels ($P > 0.248$). *Enterococcus faecium* added at10^9 CFU/mL had greater *S.* Typhimurium concentrations compared to *L. lactis* at 10^9 CFU/mL ($P < 0.001$) but did not differ from *L. lactis* at 10^6 CFU/mL ($P = 0.775$). *Lactococcus lactis* added at 10^9 CFU/mL had the lowest *S.* Typhimurium concentration compared to all other treatment groups ($P < 0.001$).

Experiment 2

When *S.* Typhimurium was inoculated into tubes at 10^2 CFU/mL, there was an effect of TRT at both 4 and 24 h ($P < 0.001$, Figure 3.2a). *L. lactis* at 10^9 CFU/mL had lower *S. Typhimurium* counts compared to CON. When *S.* Typhimurium was inoculated at 10^4 CFU/mL, TRT decreased *S.* Typhimurium populations at both 4 and 24 h compared to CON ($P < 0.001$, Figure 3.2b).

Experiment 3

In the rumen fluid mixed microorganism *in vitro* model, there was no effect of TRT at either 4 or 24 h ($P > 0.322$, Figure 3.3); however, in the fecal fluid mixed microorganism *in vitro* model, there was an effect of TRT at 4 and 24 h ($P < 0.001$, Figure 3.4). *L. lactis* addition at 10^9 CFU/mL had lower *S.* Typhimurium counts compared to CON.

Discussion

Direct fed microbials can inhibit pathogenic bacteria through the production of bacteriocins, antimicrobial compounds, or other competitive exclusion characteristics (Krehbiel et al., 2003; McAllister et al., 2011; Ban and Guan, 2021). Bacteriocins are antimicrobial peptides that target microorganisms often occupying the same ecological niche (Chikindas et al., 2018). Bacteriocin production from lactic acid bacteria has been well documented, such as the production of the bacteriocin nisin by *Lactococcus lactis* (Perez et al., 2014); however, they typically inhibit gram-positive pathogens (e.g., *Listeria monocytogenes*) and not gram-negative pathogens like *Salmonella* serovars (Umu et al., 2016; Darbandi et al., 2022). The production of antimicrobial compounds such as organic acids may also have anti-pathogenic effects (Malik et al., 2021). Additionally, DFM may be able to outcompete pathogens for adhesion receptors on the intestinal epithelium and thus physically exclude them from the GIT (Fuller, 1997). These direct anti-pathogen characteristics can be investigated by utilizing a co-culture *in vitro* model,

which is beneficial to evaluate direct interactions between populations, improve culturing success for a certain population, or establish genetic interactions between populations (Goers et al., 2014). The co-culture model utilized established direct interactions between the DFM and *S.* Typhimurium.

In experiment 1, after 4 h of incubation *B. subtilis*, *E. faecium*, and *L. lactis* at 10^9 CFU/mL demonstrated lower *S.* Typhimurium concentrations compared to CON; however, while all were statistically significant only *L. lactis* and *E. faecium* at 10^9 CFU/mL demonstrated practical differences with over 1-$\log_{10}$ (90%) reduction in *S.* Typhimurium concentrations. At 24 h incubation, all strains at both dose levels except *L. diolivorans* at 10^6 CFU/mL were statistically different from the CON. Strains of *L. lactis*, *B. subtilis* and *E. faecium* all achieved at least 1-$\log_{10}$ reductions compared to CON, but *L. lactis* at 10^9 CFU/mL outperformed all other probiotic strains and its 10^6 CFU/mL dose by achieving over a 2.5-$\log_{10}$ (>99.0%) reduction in *S.* Typhimurium compared to CON. These results agree with previous research that higher CFU/mL will tend to perform better compared to a lower dose (Younts-Dahl et al., 2004). The clear difference between *L. lactis* at 10^9 CFU/mL and other DFM suggested strong anti-pathogenic activity against *S.* Typhimurium that warranted further investigation.

In experiment 2, *L. lactis* at the chosen dose level was challenged in the same co-culture *in vitro* model as experiment 1 with varying inoculation doses of *S.* Typhimurium to demonstrate a response curve and confirm results seen in the previous study. Similar to results from the first experiment, at 4 h incubation *L. lactis* demonstrated almost a 1-$\log_{10}$ reduction in *S.* Typhimurium CFU/mL compared to CON and at 24 h over a 3-$\log_{10}$ reduction (99.9%) in *S.* Typhimurium concentration compared to CON. When *S.* Typhimurium was inoculated at 10^2 CFU/mL, *L. lactis* still demonstrated a 1-$\log_{10}$ reduction at 4 h but had greater reduction of *S.*

Typhimurium at 24 h with a 4-$\log_{10}$ (99.99%) difference compared to CON, demonstrating a greater ability to inhibit lower initial concentrations of *S.* Typhimurium.

These results align broadly with other studies investigating *L. lactis* anti-pathogenic activity against *Salmonella* and potential pathogen inhibition modes of action. Abdollahi et al. (2018) utilized a co-culture model of *L. lactis* and *Salmonella enterica* Paratyphi A and reported *L. lactis* had greater production of mature and active nisin when exposed to *Salmonella enterica* Paratyphi A; however, as nisin is predominantly effective against gram-positive bacteria, it is unlikely pathogen reduction was due to this mode of action. More likely, the production of antimicrobial compounds such as organic acids could explain the anti-pathogenic activity observed (Sabo et al., 2020). Another explanation could be the limitation of *S.* Typhimurium mobility. Nakamura et al. (2015) investigated *S.* Typhimurium response to *L. lactis* metabolites and found lactose fermentation by *L. lactis* to acetate impairs the flagellar motility of *S.* Typhimurium and decreases intracellular pH, which may have a negative impact on *S.* Typhimurium survival. Additionally, Gómez et al. (2016) looked at *L. lactis* effects on pathogen biofilm formation and reported *L. lactis* effectively reduced biofilm formation of *S.* Typhimurium. It is plausible that a combination of these factors explains the anti-pathogenic activity demonstrated by *L. lactis* towards *S.* Typhimurium in our co-culture *in vitro* models.

To simulate the GIT environment of cattle more accurately, rumen and fecal fluid mixed microorganism *in vitro* models were utilized to investigate *L. lactis* anti-pathogenic activity against *S.* Typhimurium in experiment 3. There was no reduction in *S.* Typhimurium populations at 4 h or 24 h in the rumen fluid microorganism *in vitro*; however, in the fecal fluid mixed microorganism *in vitro* there was a 0.86-$\log_{10}$ reduction at 4 h and at 24 h a 0.91-$\log_{10}$ difference between *L. lactis* and CON was observed. These results are somewhat consistent with what

Habib et al. (2022) observed utilizing a *M. elsdenii* strain, though the greatest difference between the probiotic species and *Salmonella* in fecal fluid occurred at 72 h incubation with a 0.87-$\log_{10}$ reduction, whereas 24 h incubation saw no differences and at 48 h there was only a 0.17-$\log_{10}$ difference. Had longer incubation timepoints been utilized in the current study, *S.* Typhimurium concentrations in the fecal fluid may have been further reduced by *L. lactis*. Additionally, it remains unclear why rumen and fecal fluid results differed though commensal microbiota, VFA production, and other secondary metabolites may be contributing factors (Mattila et al., 1988; Costa et al., 2012).

Conclusion

Collectively, the present results suggest *L. lactis* has anti-pathogenic activity potential against *S.* Typhimurium. The *in vitro* co-culture models demonstrated direct anti-pathogenic activity of *L. lactis* against *S.* Typhimurium with consistent population reductions at 4 and 24 h of incubation. Moreover, utilizing mixed rumen and mixed fecal fluid microorganism fermentation models demonstrated that *S.* Typhimurium was reduced in hindgut fermentations but not in ruminal fermentations. While the basis of these differences remains uncertain, it is clear *L. lactis* may have the ability to reduce *Salmonella* in an *in vivo* trial setting.

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Table 3.1 *S.* Typhimurium populations in a co-culture model in anaerobic Tryptic Soy Broth when challenged with DFM strains at time 0. Samples were collected after fermentation at 4 and 24 h[a]

		S. Typhimurium count (log10 CFU/mL) at incubation time[b]	
Treatment[c]	Dose (CFU/mL)	4 h	24 h
LL	10^9	3.622[e]	6.143[e]
	10^6	4.649[cd]	7.487[d]
EF	10^9	3.856[e]	7.661[cd]
	10^6	4.947[b]	7.896[c]
LD	10^9	5.121[ab]	8.442[b]
	10^6	5.437[a]	8.726[ab]
BS	10^9	4.291[d]	7.790[c]
	10^6	5.396[a]	7.770[c]
CON		4.870[bc]	8.806[a]

[a]*S.* Typhimurium at 0 h was 4.448 log10 CFU/mL
[b]Within each timepoint, means with different superscripts differ ($P < 0.05$)
[c]$n = 3$ experimental units/treatment mean

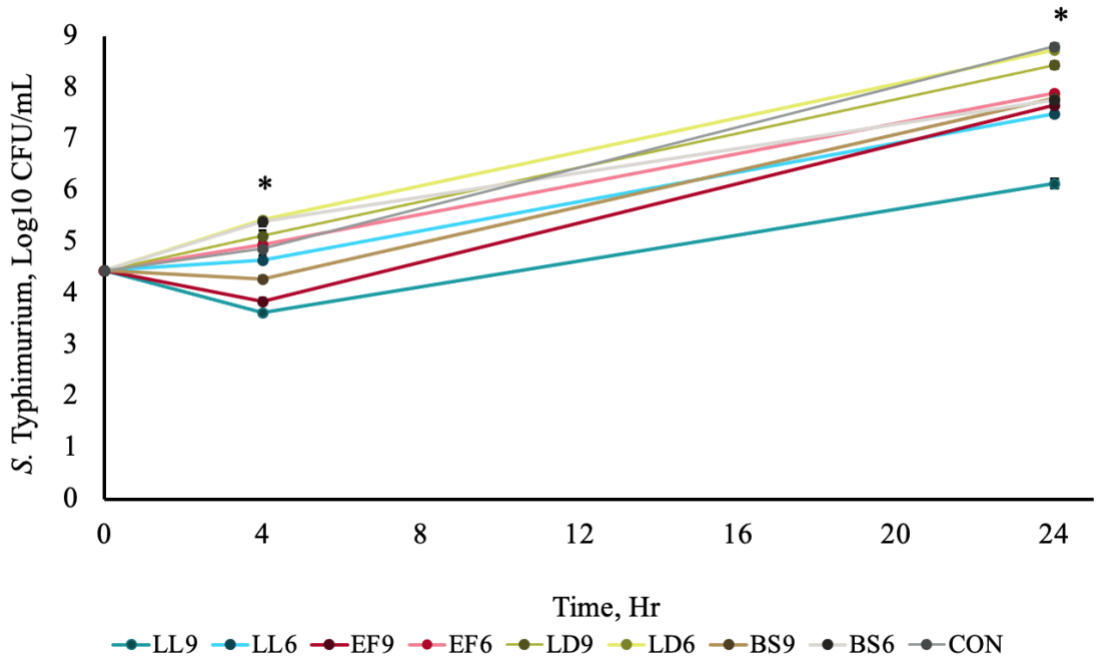

Figure 3.1 Effect of DFM strain and dose level (n = 3) on *S.* Typhimurium concentration at 4 and 24 h in co-culture *in vitro*. Error bars indicate standard error and may be smaller than the symbol. *indicates TRT × Dose interactions at timepoint ($P < 0.05$).

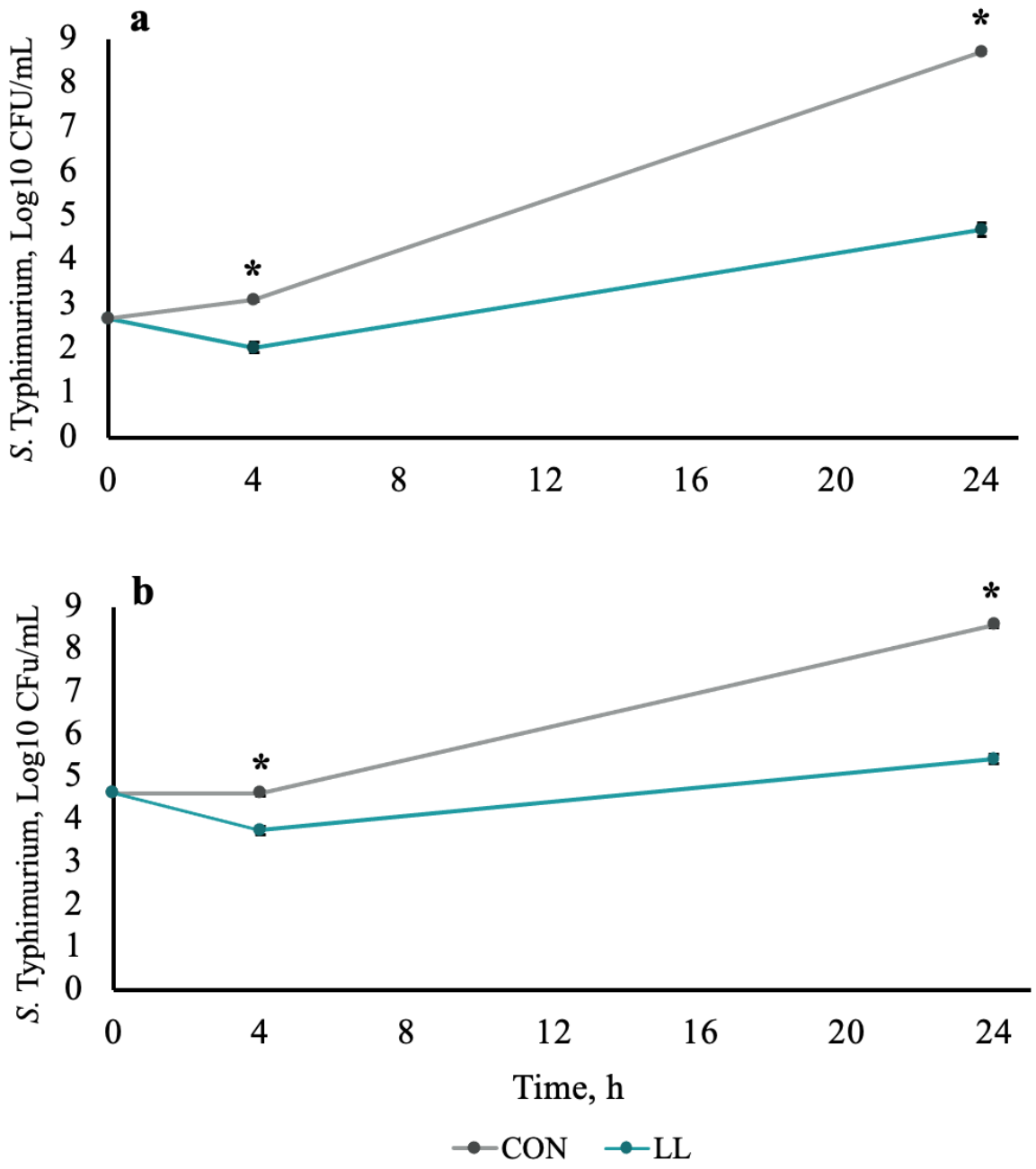

Figure 3.2 *Lactococcus lactis* effect on *S.* Typhimurium concentration when inoculated (**a**) at 10^2 CFU/mL (**b**) and at 10^4 CFU/mL in a co-culture *in vitro* at 4 and 24 h (n = 5). Error bars indicate standard error and may be smaller than the symbol. *indicates TRT effect at timepoint ($P < 0.05$).

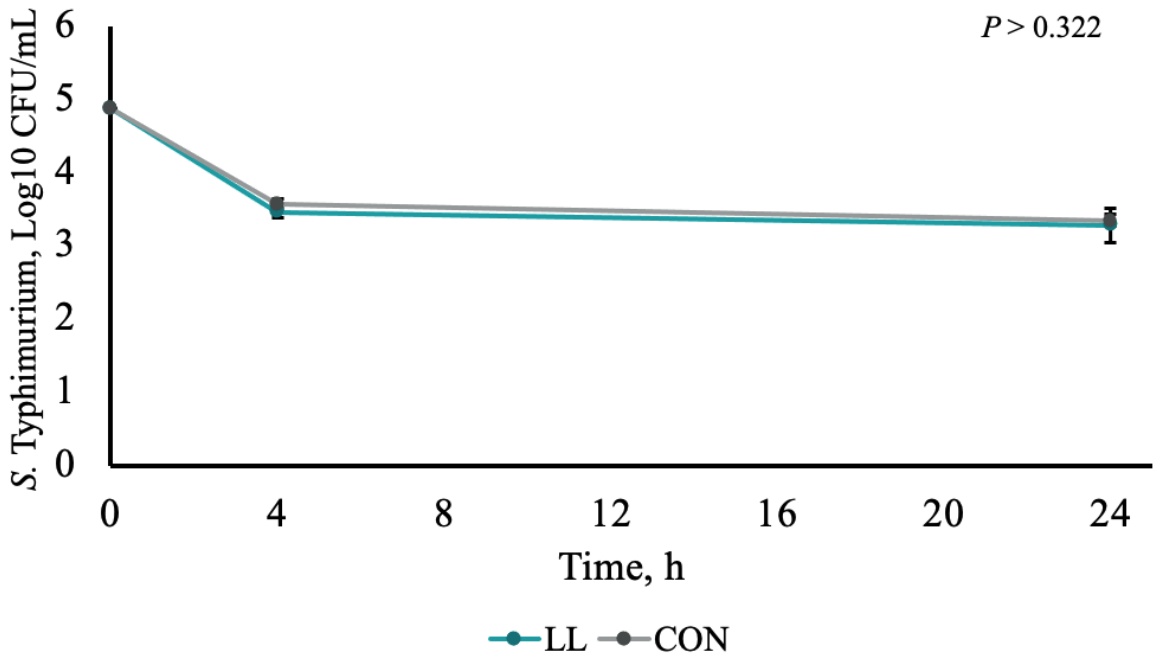

Figure 3.3 *Lactococcus lactis* effect on *S.* Typhimurium concentration in a rumen mixed microorganism *in vitro* at 4 and 24 h (n = 5). Error bars indicate standard error and may be smaller than the symbol.

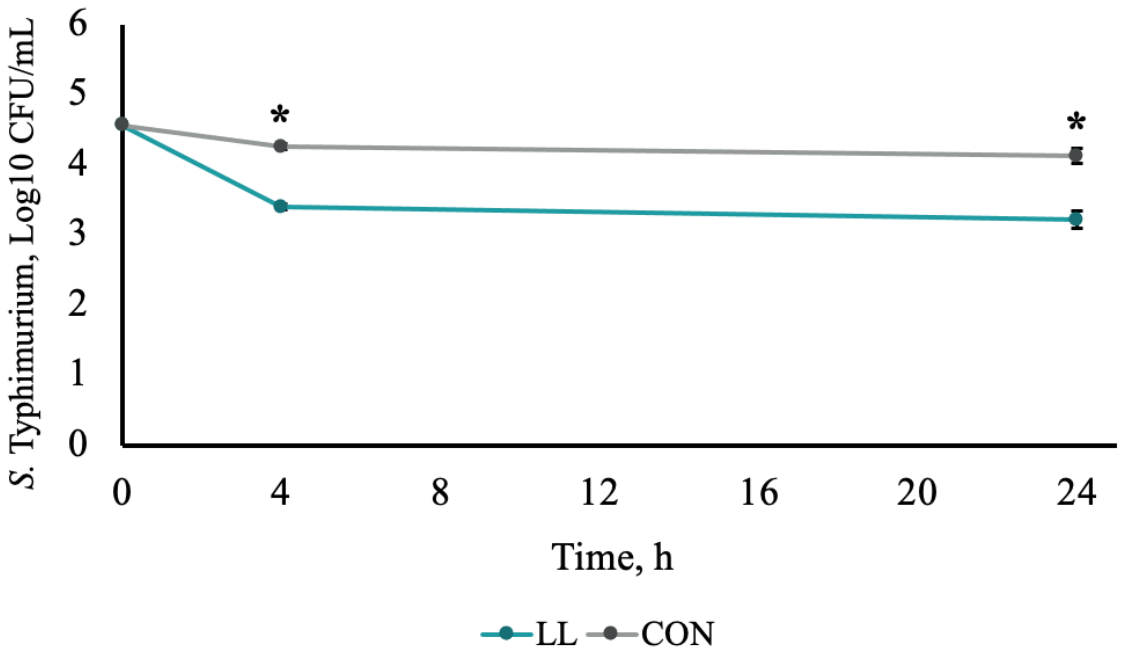

Figure 3.4 *Lactococcus lactis* effect on *S.* Typhimurium concentration in a fecal mixed microorganism *in vitro* at 4 and 24 h (n = 5). Error bars indicate standard error and may be smaller than the symbol. *indicates TRT effect at timepoint ($P < 0.05$).

CHAPTER 4

EVALUATING DIRECT FED MICROBIAL IMPACT ON FERMENTATION PARAMETERS UTILIZING RUMINAL AND FECAL MIXED MICROORGANISM BATCH CULTURE *IN VITRO*

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ABSTRACT

The beef industry long relied upon antimicrobial compounds to improve production metrics by changing the gastrointestinal tract microbial population. However, antimicrobial resistance concerns have driven increasing interest in alternatives to antibiotics (**ATA**). This includes the use of probiotics or direct fed microbials (**DFM**), which alter fermentation end-products to improve production metrics *in vivo* as well as *in vitro*, but no standardized methods exist for comparing treatment impacts. The objective of this study was to utilize rumen and fecal mixed microorganism batch culture *in vitro* models to investigate DFM effects on fermentation end products. Rumen and fecal fluid were collected from cannulated dairy steers (n =2) fed a total mixed ration (**TMR**) diet and gastrointestinal fluid was diluted (33% v/v) in anoxic media. Individual 120 mL serum bottles for each *in vitro* fermentation source were assigned using a completely randomized block design with a 6 × 3 factorial arrangement, consisting of two replicates of 6 treatments (**TRT**) (DFM strains *L. lactis*, *E. faecium*, *P. fruedenreichii*, *L. diolivorans*, and *B. subtilis* at 10^9 CFU/mL for each treatment) and bottles containing fluid and TMR only served as control (**CON**) at three timepoints (2, 4, and 24 h) with day as a blocking and random factor and was repeated twice. At each timepoint, pH, total gas production (mL), methane, hydrogen, volatile fatty acids (**VFA**), and ammonia concentrations, as well as dry matter (**DM**) digestibility were determined. There were no TRT × Time interactions for all parameters except total gas production in the fecal fermentations in which all DFM strains increased total production compared to CON. TRT effects were observed for *Propionibacterium freudenreichii* cultures which had lower propionate compared to CON in rumen *in vitro* and had a lower A:P ratio compared to CON in fecal *in vitro*. Results suggest DFM strains had little

impact on *in vitro* rumen and fecal mixed microorganism fermentation parameters, though further research into *P. freudenreichii* may be warranted.

Keywords: Direct fed microbials, *in vitro*, batch culture, rumen, fecal

Introduction

The widespread use of antimicrobials in beef cattle production improved cattle growth and efficiency by selectively inhibiting ruminal microorganisms (Visek, 1978; Cameron and McAllister, 2016) estimated global livestock antimicrobial consumption at 57,000 tons annually and projected a 67% total increase by 2030; however, prophylactic antimicrobial use has driven increasing consumer concerns over antimicrobial resistance (Consumer Reports: Meat On Drugs, 2012). Nonetheless, eliminating antimicrobial use in the beef industry is an economic and sustainability challenge. In the absence of antimicrobial usage, respiratory diseases, lameness, and liver abscess incidences cost beef producers $66 to $96 median net revenue losses per head (Lhermie et al., 2020).

Eubiotics are an "alternative to antibiotics" (**ATA**) which are substitutes for therapeutic antimicrobials (Seal et al., 2013; Callaway et al., 2021). In the livestock industry, eubiotics include direct fed microbials (**DFM**) or probiotics, which are live microorganisms used as feed additives to beneficially affect host animals by altering gastrointestinal tract (**GIT**) microflora (AFRC, 1989). Direct fed microbials have multiple modes of action such as inhibiting pathogenic bacteria, promoting GIT immunomodulation, and altering microbial fermentation end products which has been of particular interest to the cattle industry (Krehbiel et al., 2003, El Jeni et al., 2023). Direct fed microbials impact on the microbial population may improve fermentation efficiency, reduce enteric methane emissions, and alleviate sub-acute ruminal acidosis in cattle (Yoon and Stern, 1995; McAllister et al., 2011; Jeyanathan et al., 2014); however, exploring DFM impacts vary widely based upon microorganisms utilized and host animal factors (Reuben et al., 2022).

In vitro mixed microorganism fermentation models have been used to evaluate DFM impacts (Martin and Nisbet, 1992). Microbial fermentation measurements such as gas production, ammonia concentration, pH, volatile fatty acid (**VFA**) profiles, and dry matter (**DM**) disappearance are indicators of changes to ruminal microbial activity (Owens and Basalan, 2016). Alterations to fermentation patterns can indicate DFM ability to modulate ruminal pH changes, reduce total gas and methane production, increase feedstuff digestibility, and shift energy and nitrogen availability to the host animal based on ammonia and VFA profiles (Yang et al., 2004; Jeyanathan et al., 2016; Sun et al., 2016; Monteiro et al., 2022; Pan et al., 2022; Cappellozza et al., 2023); however, there is no standardized *in vitro* model to evaluate changes in fermentation parameters. The objective of the present study was to utilize mixed ruminal and fecal microorganism batch culture *in vitro* models to investigate DFM impact on fermentation end-product metrics.

Material and Methods

DFM Strains and Culture Conditions

Five DFM strains were obtained from Provita Supplements Inc. (Minnetonka, MN). *Lactococcus lactis* (***L. lactis***), *Propionibacterium freudenreichii* (***P. freudenreichii***), *Lactobacillus diolivorans* (***L. diolivorans***), *Enterococcus faecium* (***E. faecium***), and *Bacillus subtilis* (***B. subtilis***) were cultured in tryptic soy broth (Remel, Lenexa, Kansas). All strains were aerobically incubated at 37°C for 24h and were maintained by weekly subculture.

In Vitro Fermentation

Animals used in this study followed guidelines approved by the University of Georgia's Animal Care and Use Committee. Approximately two hours after feeding, ruminal contents were collected from two 1000-kg cannulated Holstein dairy steers fed a total mixed ration (**TMR**) diet

(Table 4.1). Ruminal and fecal contents from each steer were strained through a nylon paint strainer into separate thermoses for each animal to remove undigested feedstuffs while maintaining minimal aerobic headspace. The thermoses were left undisturbed at 39°C for 30 min to allow feed particles to rise due to gas production. After stratification, the middle layer of particle-free gastrointestinal fluid was pipetted anaerobically (33% vol/vol) to anoxic medium prepared according to the methodology described by Cotta and Russell (1982) and Callaway and Martin (1997). Gastrointestinal fluid and media mixture (45 mL) was anaerobically transferred to 120 mL serum bottles [(WHEATON, Millville, New Jersey), $N = 36$] containing TMR [0.5 ± 0.05 g (2 mm screen dry matter particles)] Probiotic cultures in TSB tubes ($n = 5$, 10mL each) grown to 10^9 CFU/mL were centrifuged at 4,000 rpm for 10 minutes. The resulting supernatant was removed (9 mL) before resuspension of cell cultures ($n = 5$, 1 mL each) in serum bottles containing TSB (45 mL) to achieve 10^{10} CFU/mL, which was confirmed utilizing serial dilutions. Probiotic strains were then added (5mL) to treatment serum bottles to achieve final concentrations of 10^9 CFU/mL and 50 mL for final volume. Treatments consisted of each strain and tubes containing rumen fluid and TMR were control (**CON**) at three time points 2, 4, and 24 hours. Each treatment was done in duplicate ($n = 2$) at each timepoint, and the experiment was repeated. Tubes were flushed with CO_2, sealed with butyl rubber stoppers and aluminum crimps, and were incubated at 39°C in a CO_2 incubator (VWR Scientific, Radnor, Pennsylvania) for 2, 4, or 24 h. At each timepoint, individual bottles were removed and allowed to equilibrate to room temperature before gas measurements were collected. Afterwards, the crimp and butyl rubber stoppers were removed, and fluid was poured into cups for pH analysis before being frozen at -80 °C for further analyses.

Gas Analysis

After 2, 4, and 24 h of incubation, aluminum crimps were removed from the serum bottles. For total gas, lubricated 60 mL syringes were inserted into the butyl rubber stopper and measured as mL of plunger displacement. For hydrogen (**H_2**) and methane (**CH_4**), a 5-mL syringe was used to collect a 5mL gas sample from each tube. Gas was injected into a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a Porapak Q column [GL Sciences Inc. USA, Rolling Hills Estates, California (60°C, 20 mL/min of N2 carrier gas)] and analyzed for H_2 and CH_4. Gas concentrations (mM) were calculated by measuring peak heights compared to controls.

VFA Analysis

Volatile fatty acid concentrations were determined in a water-based solution using ethyl acetate extraction. Samples (2 mL) were pipetted into microcentrifuge tubes and centrifuged for 10 min at 10,000 × *g*. Fluid supernatant (1 mL) was vortexed with 0.2 mL of 25% meta-phosphoric acid for 10 seconds. Samples were frozen overnight in a -20°C freezer, thawed to room temperature, and centrifuged at 10,000 x *g* for 10 min. Supernatant fluid (0.75mL) was transferred into vials and 0.15 mL VFA internal standard was added. The solution was mixed with 1.8-mL ethyl acetate to achieve a 2:1 ratio. Vials were vortexed for 10 s and allowed to settle for 5 min. The resulting supernatant was pipetted, transferred to a GC vial, and analyzed by gas chromatography (Agilent 7820A GC; Agilent Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m × 0.53 mm, Varian CP7767; Varian Inc.). Sample injection volume was 1.0µL and the carrier gas was hydrogen. Column temperature was maintained at 110°C, and injector and detector temperatures were 200 and 220°C, respectively. The acetate: propionate (**A:P**) ratio was calculated from acetate and propionate

concentrations observed, and total VFA concentration was the sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and caproate concentrations.

Ammonia Analysis

Samples were analyzed using colorimetric determination (Chaney and Marbach, 1962). Tubes were incubated at 39 °C for 20 minutes before concentration determination at 630 nm.

Dry Matter Digestibility Analysis

The TMR ration was dried in a force-air oven at 55 °C for 24 h before being ground to 2 mm particles in a Wiley mill (Thomas Scientific, Swedesboro, NJ). Dry matter basis of TMR was conducted weighing duplicate 0.50 g samples that were subsequently dried in a force-air oven at 100 °C for 24 h. Dry matter basis for the ground TMR was calculated as a percentage of remaining weight over initial weight and as an average of duplicates. 0.50 ± 0.05 g TMR were weighed into pre-weighed F57 filter bags (ANKOM Technology, Macedon, NY) and values were adjusted to a DM basis. F57 filter bags were sealed and placed inside serum bottles before addition of fluid media and treatments. Post time point incubations, bags were removed from serum bottles and dried in a force-air oven at 55 °C for 48 h before determining remaining weight. Values were adjusted for residual fecal and rumen particulate using the average of duplicate blank F57 filter bags for each timepoint and mass of the F57 filter bags was subtracted to achieve the final weight. Dry matter digestibility was calculated as a percentage of initial weight minus final weight over initial weight of TMR on a DM basis.

Statistical Analysis

All analyses were performed using duplicate average of serum bottles on same day as the experimental unit. Day was utilized as a blocking factor and was considered a random effect. All measurements were analyzed as a completely randomized block design with a 6 × 3 factorial

arrangement. Treatment (**TRT**), Time, and their interaction served as fixed effects. Analysis of models were done using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC). Pairwise comparisons between treatment and factor level were computed using the Tukey-Kramer test. Statistical significance was declared at $P \leq 0.05$.

Results

pH

For all mixed rumen microorganism fermentations, there were no TRT × Time interactions or TRT effects ($P > 0.869$), but there were Time effects ($P < 0.001$, Table 4.2). Between 2 and 4 h the pH did not differ ($P = 0.523$) but both timepoints had higher pH values compared to 24 h ($P < 0.012$), which was expected.

Fecal microorganism fermentations did not have TRT x Time interactions ($P = 0.1913$) but did have TRT and Time effects ($P < 0.012$), Table 4.4). *Lactococcus lactis* treated fermentations had higher pH compared to *B. subtilis* treated fermentations ($P < 0.012$) but did not differ from other treatments or CON ($P > 0.1568$). Other treatments did not differ from each other or CON ($P > 0.089$). The pH values were highest at 2 h compared to 4 and 24 h ($P < 0.001$), and lowest at 24 h compared to 2 and 4 h ($P < 0.001$).

Gas Analysis

For rumen microorganism fermentations, there were no TRT × Time interactions or TRT effects for total gas, methane, and hydrogen ($P > 0.633$, Table 4.2); however, there were Time effects for all three gas analyses ($P < 0.005$). For total gas, the lowest volume was at 2 h compared to 4 and 24 h ($P < 0.003$). Total gas increased at 4 h compared to 2 h but was less than 24 h ($P < 0.003$), and at 24 h was at greatest volume ($P < 0.001$). Hydrogen concentrations at 2 h

were lower than 24 h ($P < 0.004$), but at 4 h did not differ from 2 or 24 h ($P > 0.211$). Methane concentrations were highest at 24 h ($P < 0.001$) but did not differ between 2 and 4 h ($P = 0.225$).

For fecal microorganism fermentations, there were TRT × Time interactions for total gas ($P < 0.001$, Table 4.4). At 24 h, *L. lactis*, *E. faecium*, *L. diolivorans*, and *B. subtilis* treated fermentations had the greater total gas volume compared to CON at 24 h and the other TRT × Time groups ($P < 0.020$) but did not differ from each other or *P. freudenreichii* treated fermentations at 24 h incubation ($P > 0.9457$). *Propionibacterium freudenreichii* treated fermentations at 24 h had greater total gas volume compared to treatments at 2 and 4 h ($P < 0.001$) but did not differ from CON at 24 h ($P = 0.109$). At 4 h, *P. freudenreichii* and *E. faecium* produced more gas volume compared 2 h treatments ($P < 0.044$) but did not differ from each other or other DFM treatments at 4 h ($P > 0.3445$). *Bacillus subtilis* treated fermentations at 4 h had greater gas volume compared to *L. diolivorans* treated fermentations at 2 h ($P < 0.031$) but not at 2 or 4 h ($P > 0.062$). *Lactococcus lactis* treated fermentations at 4 h did not differ from any treatments at 4 or 2 h ($P > 0.051$). Hydrogen had no TRT × Time interactions nor TRT or Time effects ($P > 0.300$). Methane had no TRT × Time interactions or TRT effects ($P > 0.066$) but did have Time effects ($P < 0.001$). Methane concentrations did not differ between 2 and 4 h ($P = 0.639$); however, both were lower compared to 24 h ($P < 0.001$).

Volatile Fatty Acid Analysis

Rumen fluid fermentation concentrations of acetate, isobutyrate, butyrate, isovalerate, valerate, and caproate had no TRT × Time interactions or TRT effects ($P > 0.1384$) but all exhibited Time effects ($P < 0.001$, Figure 4.1, Table 4.3). For these VFA, 2 and 4 h concentrations did not differ ($P > 0.0790$) but both timepoints were lower than 24 h ($P < 0.001$). Propionate concentrations demonstrated no TRT × Time interactions ($P > 0.632$) but did have TRT and Time effects ($P <$

0.0375). *Propionibacterium freudenreichii* and *E. faecium* containing fermentations had lower propionate concentrations compared to CON ($P = 0.050$) but did not differ from other treatments ($P > 0.902$). Other treatments did not differ from CON or each other ($P > 0.916$). Propionate concentrations were lowest at 2 h compared to 4 and 24 h ($P < 0.010$) and greatest at 24 h compared to 2 and 4 h ($P < 0.001$). The A:P ratio and total VFA had no TRT × Time interactions or TRT effects ($P > 0.1705$) but did have Time effects ($P < 0.001$). The A:P ratio did not differ between 2 and 4 h ($P = 0.799$) however both timepoints had greater A:P ratios than at 24 h ($P < 0.001$). The total VFA did not differ between 2 and 4 h ($P = 0.0726$) but both timepoints were less than total VFA at 24 h ($P < 0.001$).

Fecal microorganism fermentation concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate demonstrated no TRT × Time interactions or TRT effects ($P > 0.124$) but did have Time effects ($P < 0.001$, Figure 4.2, Table 4.5). Acetate, propionate, isobutyrate, isovalerate, valerate concentration timepoints all differed ($P < 0.001$) with 2 h being the lowest and 24 h having the highest concentrations. Butyrate concentrations did not differ between 2 and 4 h ($P = 0.080$) but both timepoints were lower than at 24 h ($P < 0.001$). The VFA caproate had no TRT × Time interactions nor TRT or Time effects ($P > 0.296$). The A:P ratio did not have TRT × Time interactions ($P = 0.301$) but did have TRT and Time effects ($P < 0.001$). *Propionibacterium freudenreichii* treated fermentations had lower A:P ratios compared to CON and other treatments ($P < 0.019$). Other treatments did not differ from each other or from CON ($P > 0.3631$). Total VFA did not have TRT × Time interactions or TRT effects ($P > 0.2632$) but did have Time effects ($P < 0.001$). Total VFA differed at each timepoint ($P < 0.001$) with the lowest concentration at 2 h having the lowest and 24 h being the highest total VFA concentration.

Ammonia Analysis

Rumen microorganism fermentations did not have TRT × Time interactions or TRT effects ($P > 0.2309$) but did have Time effects ($P < 0.001$, Table 4.2). Ammonia concentrations were lowest at 2 h compared to 4 and 24 h ($P < 0.029$). Ammonia concentrations at 24 h were greater than 2 and 4 h ($P < 0.001$).

Fecal microorganism fermentations did not exhibit TRT × Time interactions or TRT effects ($P > 0.8911$) but did have Time effects ($P < 0.001$, Table 4.4). Ammonia concentrations for 2, 4, and 24 h were all statistically different from each other ($P < 0.001$) with 2 h exhibiting the lowest and 24 h containing the greatest concentration.

Dry Matter Digestibility Analysis

For rumen microorganism fermentations there were no TRT × Time interactions or TRT effects ($P > 0.6183$); however, there were Time effects ($P < 0.001$, Table 4.2). Dry matter digestibility did not differ between 2 and 4 h ($P = 0.7758$) but both timepoints had lower digestibility compared to 24 h ($P < 0.001$).

Fecal microorganism fermentations had no TRT × Time interactions or TRT effects ($P > 0.290$) but did have Time effects ($P < 0.001$, Table 4.4). Dry matter digestibility did not differ between 2 and 4 h ($P = 0.070$) but both timepoints had lower digestibility compared to 24 h ($P < 0.001$).

Discussion

Producers seek to alter microbial fermentation end products to maximize production efficiency in ruminants (Yoon and Stern, 1995). Ruminal feedstuff fermentation, especially diets that contain high concentrate values can result in undesirable pH fluctuations (Russell, 1998). Sub-acute ruminal acidosis (SARA) is a metabolic disease that occurs when ruminal microbial

communities and anatomy (i.e. papillae) are not adapted to handle an influx of lactic acid production from high concentrate diets (Kleen et al., 2003). The overproduction of lactic acid results in a decrease in ruminal pH, and consistent fluctuations of pH below 5.6 to 5.0 result in impaired feed efficiency and health of cattle (Nagaraja and Titgemeyer, 2007). One strategy that has been shown to stabilize ruminal pH and mitigate SARA impacts is the addition of DFM that can utilize lactic acid and therefore prevent accumulation that results in pH declines (Calsamiglia et al., 2012). The DFM strains in the present study demonstrated no TRT effects or TRT × Time interactions for ruminal fermentation pH. These results agree with Monteiro et al. (2020) who examined *Lactobacillus* strains and *P. freudenreichii* in an *in vitro* ruminal fermentation system and utilized a TMR ration. One consideration for these results is the use of a TMR diet, which contains lower concentrate values compared to what is observed in a feedlot ration and thus does not simulate conditions that result in lactic acid overproduction and dramatic pH fluctuations (González et al., 2012). Another is that bacterial DFM are typically lactic acid producers instead of utilizers, which would not result in pH stabilization. This is supported by studies that have demonstrated bacterial DFM, unless paired with yeast cultures, do not stabilize ruminal pH (Michalet-Doreau and Morand, 1996; Chiquette, 2009; Aikman et al., 2011; Chiquette et al., 2012).

Total gas production is a predictor of organic matter (**OM**) degradation and increases with increased degradation (Dijkstra et al., 2005). By-products of gas production by ruminants, such as methane, are targets for improving feed efficiency and sustainability as the production of enteric methane by cattle results in six to twelve percent energy loss on average and is a contributing factor to global greenhouse gas emissions (Johnson and Johnson, 1995; Meale et al., 2012; Capper and Bauman, 2013; Bačėninaitė et al., 2022). All DFM strains had no TRT effects

or TRT × Time interactions for total gas production and methane concentrations in the mixed microorganism ruminal fermentation *in vitro*. This agrees with Ellis et al. (2016) who utilized similar *L. lactis* and *E. faecium* as well as *Lactobacillus* DFM strains and found no effect on cumulative gas production or methane concentrations; however other *Lactobacillus* strains along with *P. freudenreichii* and *B. subtilis* DFM have produced conflicting results. Monteiro et al. (2020) found *Lactobacillus* strains and *P. freudenreichii* reduced total gas production *in vitro*. Similarly, Jeyanathan et al. (2016) demonstrated *Lactobacillus* and *P. freudenreichii* strains capable of reducing the methane to total gas production ratio. Cappellozza et al. (2023) found *Bacillus* DFM increased total gas production. Differences in results may be attributed to different diet compositions used in *the vitro* models, as diet can largely drive total gas and methane production in the rumen (Jentsch et al., 2007; Zicarelli et al., 2011). While these studies solely utilized rumen *in vitro* models, this study also utilized a fecal *in vitro* model. No TRT effects or TRT × Time interactions were observed for methane; however, there were TRT effects and TRT × Time interactions observed for total gas production. All DFM strains increased total gas production compared to CON by at least 17 percent. Though 2 and 4 h measurements did not differ much, at 24 h all DFM except *P. freudenreichii* had greater total gas production compared to CON. While OM was not a parameter directly investigated in the present study, results suggest some DFM addition may increase OM digestibility in hindgut fermentation and warrant further investigation.

Volatile fatty acids (e.g., acetate, propionate, and butyrate) are critical to ruminant metabolism as they can constitute 70% of metabolizable energy (Siciliano-Jones and Murphy, 1989). Propionate is the main VFA contributor to gluconeogenesis and also contributes to carcass marbling (Young, 1977; Smith and Grouse, 1989), acetate can be utilized primarily for

milk fat synthesis (Folley and French, 1950), and their ratio serves as a rough representation of energy availability to the animal (Balch and Rowland, 1957). Butyrate is absorbed by the gastrointestinal epithelium and promotes epithelial integrity (Sakata and Tamate, 1978). Additionally, minor VFA such as isoacids valerate, isobutyrate, and isovalerate can positively impact cellulolytic microbial fermentation and milk production parameters such as milk fat and milk yield in dairy cattle (Andries et al., 1987; Copelin et al., 2021). The DFM strains in the present study did not have any TRT effects or TRT × Time interactions for the majority of VFA investigated; however, in rumen fluid fermentations there were TRT effects for propionate and in fecal fluid a TRT effect for the A:P ratio. *Propionibacterium freudenreichii* and *E. faecium* treated fermentations had on average approximately 9% lower concentrations of propionate in rumen fluid compared to CON, though they did not differ from other treatment groups. In fecal fluid, *Propionibacterium freudenreichii* A:P ratio was approximately 15% lower compared to CON and all other DFM strains. *Propionibacterium freudenreichii* strains have been found to have varying effect on VFA profiles. Yang et al. (2004) found *Propionibacterium* DFM decrease caproate concentrations; however, contrasting results were reported by Chen et al. (2020) comparing multiple *P. freudenreichii* strains in which some affected VFA profiles and others had no effect. Varying impact by DFM strain used is likely dependent on strain specific characteristics of probiotic species. The present VFA results of this study suggests DFM strains *L. lactis*, *E. faecium*, *L. diolivorans*, and *B. subtilis* addition to fermentations have negligible impacts on altering VFA profiles while *P. freudenreichii* appears to have some effect on propionate and A:P ratio in rumen and fecal fermentations, respectively.

Ammonia generated from microbial degradation of nitrogenous compounds (e.g., amino acid deamination) or the hydrolysis of urea, and its conversion to microbial protein is important

as more than 80% of microbial protein is utilized by the animal as a protein source (Abdoun et al., 2006); however, microorganisms often generate more ammonia than what can be utilized for microbial protein synthesis and results in accumulation in the blood stream and eventually conversion to urea and excretion as a waste product (Russell et al., 1992). Excess ammonia production and disposal is an energetic drain on the animal but is also an important point source of N pollution (Hristov et al., 2011). Therefore, ammonia concentrations must be at levels which mitigate nitrogen waste without negatively impacting microbial protein synthesis. In this study, DFM strains had no impact on ammonia concentrations in rumen or fecal *in vitro* fermentations. These results agreed with Yang et al. (2004) who found DFM strains *Propionibacterium* and *E. faecium* no impact on ammonia concentration or nitrogen metabolism; however, Sun et al. (2016) reported a *Bacillus subtilis natto* DFM increased ammonia concentrations and microbial crude protein synthesis. Whether DFM can influence ammonia and microbial protein synthesis likely depends on metabolic activity and rates for producing microbial protein and ammonia during fermentation.

Dry matter digestibility is an indication of microbial efficiency in converting the nutritional value of feedstuffs into energy for the animal (Tilley and Terry, 1963). In this study, there were no TRT effects or TRT × Time interactions for dry matter digestibility in the rumen or fecal fluid *in vitro* model. These results somewhat agree with previous literature. Yang et al. (2004) found *Propionibacterium* and *E. faecium* strains had no impact on DM digestibility i*n vitro*; however, two studies that utilized *Bacillus* DFM found consistent improvements in DM digestibility across a wide variety of feedstuffs and diet formulations (Pan et al., 2022; Cappellozza et al., 2023). Differences from the present study may be attributed to the use of monoculture versus multi-culture DFM, as these previous studies utilized two *Bacillus* strains

instead of the monoculture approach of the present study and Yang et al. (2004). The use of multi-strain DFM can produce a synergistic effect to positively impact production parameters and may work to improve DM digestibility over single strain DFM (Lambo et al., 2021).

Conclusion

Some major challenges in characterizing DFM impact on ruminal and fecal fermentation end products include the variable nature of DFM strains, multi- or mono- culture usage, feedstuff substrates, and *in vitro* methodology used. These challenges create difficulties in comparing literature to form a consensus on DFM *in vitro*. The results of this study suggests DFM strains do not impact pH, methane, ammonia, or dry matter digestibility in short-term rumen or fecal *in vitro* models. While DFM did not impact total gas production in the rumen *in vitro* model, all DFM increased total gas production in fecal fluid *in vitro*. Most VFA parameters were not impacted by DFM, however, *P. freudenreichii* decreased propionate in rumen fluid and the A:P ratio in fecal fluid *in vitro.* Overall, the use of rumen and fecal mixed microorganism batch culture *in vitro* models did not demonstrate potential fermentation parameter manipulation by DFM addition, though differences caused by *P. freudenreichii* in fecal fermentations may warrant further investigation.

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Table 4.1 Total mixed ration (TMR) added to *in vitro* mixed microorganism fermentation ingredient composition on a DM basis

Ingredient	% in diet DM
UGA Wheatlage 9.22	42.00
Corn, Ground Shelled	30.66
Corn, Gluten Feed	9.11
Dry Soybean Meal 48%	8.11
9.16.22 UGA Min Prebatch#1	6.11
Soybean Hulls	4.00

Table 4.2 Effect of DFM strains on *in vitro* mixed ruminal microorganism fermentation end products.

		Treatments						SEM[1]	*P*-value[2]		
Parameter	Time, h	*L. lactis*	*P. freudenreichii*	*E. faecium*	*L. diolivorans*	*B. subtilis*	CON		TRT	Time	TRT × Time
Total Gas, mL								7.795	0.633	<0.001	0.899
	2	9.063	8.875	7.563	8.563	8.125	10.250				
	4	16.750	18.500	18.125	17.750	17.438	18.125				
	24	68.13	75.563	70.750	70.563	78.625	79.813				
Methane, mM								7.795	0.633	<0.001	0.899
	2	1.367	1.123	1.212	1.166	0.981	1.322				
	4	2.628	2.662	2.428	1.997	2.384	3.041				
	24	13.711	16.607	14.536	16.801	16.445	16.914				
Hydrogen, mM								0.083	0.789	0.005	0.709
	2	0.156	0.162	0.104	0.202	0.294	0.096				
	4	0.074	0.119	0.067	0.128	0.090	0.118				
	24	0.297	0.297	0.285	0.249	0.170	0.146				
pH								0.136	0.947	<0.001	0.869
	2	6.675	6.710	6.688	6.730	6.703	6.710				
	4	6.685	6.673	6.660	6.690	6.658	6.680				
	24	6.478	6.488	6.445	6.388	6.393	6.360				
Ammonia, mM								0.654	0.591	<0.001	0.231
	2	4.622	4.175	4.632	4.487	4.572	4.616				
	4	5.479	5.523	5.796	5.240	5.074	5.201				
	24	11.869	14.135	13.141	13.195	14.798	13.890				
DM Digestibility, %								3.230	0.618	<0.001	0.999
	2	22.033	22.438	20.682	21.185	20.916	20.988				
	4	23.313	22.031	21.065	22.458	21.328	21.907				
	24	47.323	46.414	45.006	46.452	46.049	43.833				

[1]$n = 4$ experimental units/treatment mean
[2]significance declared at $P < 0.05$

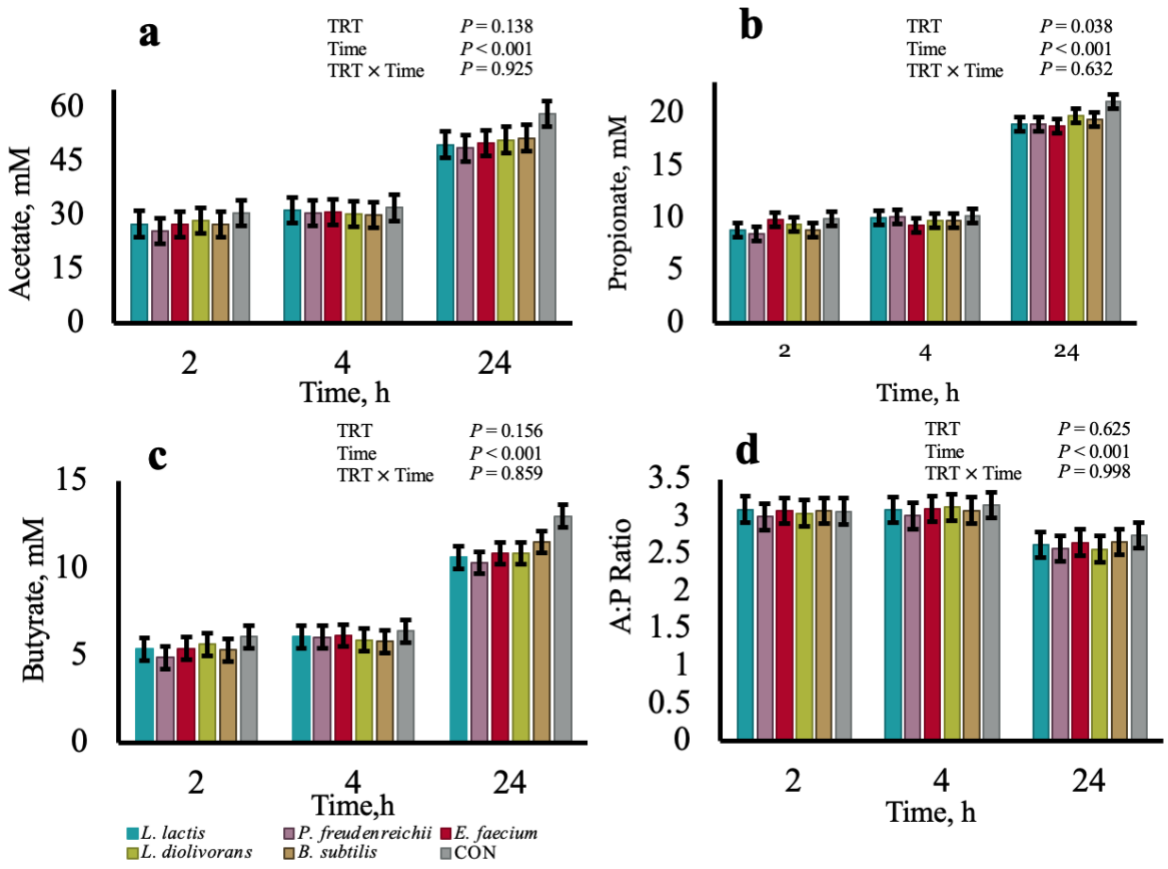

Figure 4.1 Direct fed microbial strain effect (n = 4) on (**a**) acetate, (**b**)propionate, (**c**) butyrate, and (**d**) A:P ratio in rumen *in vitro*. Error bars indicate standard error of the mean. Significance declared at $P < 0.05$.

Table 4.3 Effect of DFM strains on individual VFA and total VFA[3] concentration in *in vitro* ruminal mixed microorganism fermentations.

		Treatments						SEM[1]	*P*-value[2]		
Parameter	Time, h	*L. lactis*	*P. freudenreichii*	*E. faecium*	*L. diolivorans*	*B. subtilis*	CON		TRT	Time	TRT × Time
Isobutyrate, mM								0.057	0.997	<0.001	0.982
	2	0.444	0.414	0.450	0.472	0.446	0.502				
	4	0.506	0.513	0.512	0.496	0.494	0.522				
	24	1.358	1.352	1.366	1.357	1.416	1.312				
Valerate, mM								0.086	0.943	<0.001	0.514
	2	0.613	0.620	0.627	0.635	0.597	0.693				
	4	0.723	0.727	0.754	0.691	0.673	0.749				
	24	2.442	2.341	2.476	2.437	2.539	2.244				
Isovalerate, mM								0.128	0.996	<0.001	0.985
	2	0.877	0.786	0.870	0.916	0.844	0.965				
	4	0.997	1.012	1.029	0.995	0.952	1.005				
	24	2.762	2.712	2.716	2.701	2.891	2.694				
Caproate, mM								0.088	0.616	<0.001	0.624
	2	0.332	0.307	0.344	0.226	0.212	0.240				
	4	0.463	0.341	0.304	0.359	0.213	0.241				
	24	0.523	0.492	0.517	0.500	0.580	0.579				
Total VFA, mM								4.824	0.171	<0.001	0.925
	2	44.164	41.235	44.124	45.886	43.775	49.129				
	4	50.270	49.559	49.631	48.609	48.109	51.314				
	24	86.472	84.919	86.972	88.777	89.946	99.323				

[1]$n = 4$ experimental units/treatment mean
[2]significance declared at $P < 0.05$
[3]Total VFA is sum of propionate, acetate, butyrate, isobutyrate, valerate, isovalerate, and caproate

Table 4.4 Effect of DFM strains on *in vitro* mixed fecal microorganism fermentation end products.

		Treatments						SEM[1]	*P*-value[2]		
Parameter	Time, h	*L. lactis*	*P. freudenreichii*	*E. faecium*	*L. diolivorans*	*B. subtilis*	CON		TRT	Time	TRT × Time
Total Gas, mL[3]								1.790	0.003	<0.001	0.019
	2	1.150^{de}	1.125^{de}	1.125^{de}	0.625^{e}	1.063^{de}	1.125^{de}				
	4	6.750^{cde}	9.250^{c}	7.375^{c}	6.375^{cde}	7.063^{cd}	4.563^{cde}				
	24	54.438^{a}	52.625^{ab}	55.562^{a}	55.438^{a}	53.688^{a}	47.000^{b}				
Methane, mM								1.109	0.095	<0.001	0.066
	2	0.182	0.273	0.182	0.212	0.121	0.137				
	4	0.804	0.607	0.698	0.592	0.743	0.561				
	24	6.439	6.682	1.673	2.323	6.361	4.325				
Hydrogen, mM								0.435	0.300	0.352	0.972
	2	0.139	0.429	0.162	0.366	0.378	0.699				
	4	0.501	0.778	0.502	0.355	0.162	1.043				
	24	0.561	0.627	0.414	0.534	0.608	0.717				
pH								0.032	0.012	<0.001	0.191
	2	6.820	6.818	6.833	6.860	6.778	6.828				
	4	6.770	6.743	6.720	6.743	6.708	6.733				
	24	6.540	6.525	6.528	6.490	6.478	6.453				
Ammonia, mM								0.561	0.911	<0.001	0.891
	2	3.769	3.798	3.726	3.602	3.873	3.856				
	4	4.907	4.680	4.720	5.153	4.623	5.039				
	24	11.463	10.969	11.837	11.123	10.936	11.090				
DM Digestibility, %								1.302	0.290	<0.001	0.896
	2	20.182	19.854	19.233	18.689	19.045	19.950				
	4	21.160	20.738	20.908	20.812	19.589	19.923				
	24	33.634	32.303	31.287	31.650	32.229	31.909				

[1]$n = 4$ experimental units/treatment mean
[2]significance declared at $P < 0.05$
[3]TRT × Time with different letters differ ($P < 0.05$)

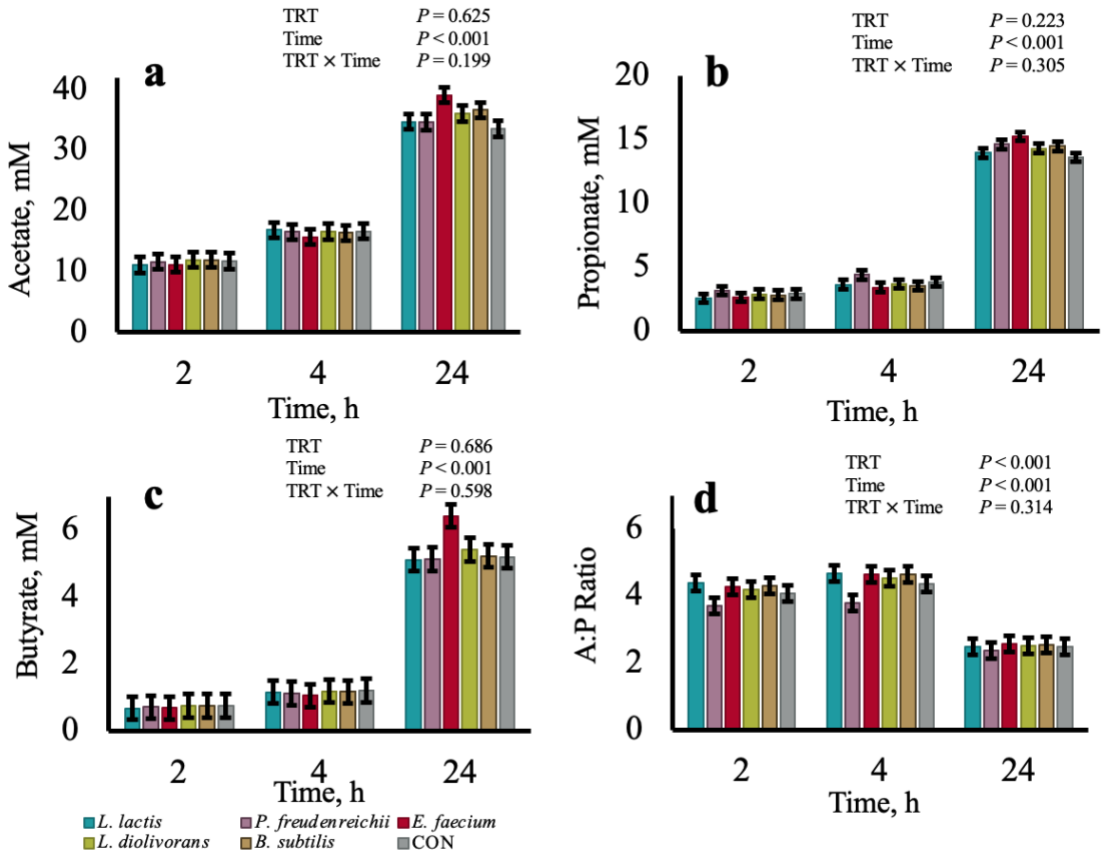

Figure 4.2 Direct fed microbial strain effect (n = 4) on (**a**) acetate, (**b**) propionate, (**c**) butyrate, and (**d**) A:P ratio in fecal *in vitro*. Error bars indicate standard error of the mean. Significance declared at $P < 0.05$.

Table 4.5 Effect of DFM strains individual VFA and total VFA[3] concentration in *in vitro* fecal mixed microorganism fermentations.

		Treatments						SEM[1]	*P*-value[2]		
Parameter	Time, h	*L. lactis*	*P. freudenreichii*	*E. faecium*	*L. diolivorans*	*B. subtilis*	CON		TRT	Time	TRT × Time
Isobutyrate, mM								0.022	0.124	<0.001	0.130
	2	0.184	0.193	0.195	0.208	0.205	0.197				
	4	0.237	0.234	0.230	0.239	0.240	0.237				
	24	1.041	0.987	1.121	1.067	1.076	1.003				
Valerate, mM								0.059	0.981	<0.001	0.155
	2	0.175	0.291	0.178	0.196	0.192	0.227				
	4	0.392	0.391	0.328	0.398	0.384	0.445				
	24	1.826	1.785	1.986	1.834	1.861	1.784				
Isovalerate, mM								0.056	0.394	<0.001	0.201
	2	0.201	0.212	0.205	0.221	0.218	0.219				
	4	0.298	0.291	0.281	0.304	0.301	0.306				
	24	1.659	1.603	1.886	1.683	1.672	1.583				
Caproate, mM								0.084	0.41	0.296	0.630
	2	0.000	0.148	0.103	0.008	0.000	0.017				
	4	0.166	0.194	0.131	0.017	0.017	0.015				
	24	0.079	0.037	0.184	0.098	0.136	0.125				
Total VFA, mM								2.064	0.728	<0.001	0.263
	2	14.943	16.391	15.196	16.237	16.145	16.091				
	4	22.737	23.173	21.158	22.450	22.059	22.719				
	24	58.407	58.840	65.966	60.504	61.080	56.910				

[1]$n = 4$ experimental units/treatment mean
[2]significance declared at $P < 0.05$
[3]Total VFA is sum of propionate, acetate, butyrate, isobutyrate, valerate, isovalerate, and caproate

CHAPTER 5

EVALUATING DIRECT FED MICROBIAL IMPACT ON THE MICROBIOME OF RUMINAL AND FECAL MIXED MICROORGANISM BATCH CULTURE *IN VITRO*

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ABSTRACT

The bovine gastrointestinal tract microbiome has been evaluated using culture-independent methods such as 16s rRNA sequencing and metagenomics. Results have increased our understanding on the complex role the microbiome plays in influencing host metabolic and immunological functions. Direct fed microbials (**DFM**) are a feed additive that influences microbial consortia dynamics, but little research has investigated DFM impact on the microbiome. The objective of this study was to evaluate DFM impact on rumen and fecal microbiome dynamics utilizing an *in vitro* mixed microorganism model. Rumen and fecal fluid were collected from cannulated dairy steers (n = 2) fed a total mixed ration (**TMR**) diet and diluted in anoxic media (33% v/v). Individual serum bottles for fecal and ruminal fermentations containing TMR were assigned to a completely randomized block design with a 7 × 3 factorial arrangement, consisting of two replicates of 7 treatments (**TRT**): untreated control plus feed (CONF), untreated control (CON), or DFM strains *L. lactis*, *E. faecium*, *P. fruedenreichii*, *L. diolivorans*, and *B. subtilis* at 10^9 CFU/mL collected at: 2, 4, and 24 hours with day as a blocking and random factor. The experiment was performed in replicate on 2 separate days. Samples were collected after which DNA extraction and 16s rRNA sequencing was performed. Alpha diversity indices observed features, Peilou's evenness, and Shannon's diversity as well as genera above 0.5% abundance were determined. There were TRT × Time interactions for rumen and fecal alpha diversity indices. *L. lactis* had reduced ($P < 0.05$) evenness and diversity compared to control groups in fecal *in vitro*. At the genera level, common microbiome results indicated microbial genera more susceptible to substrate changes with affected genera overlapping between rumen and fecal samples. Additionally, distinct genera for rumen and fecal microbial communities appeared to be impacted differentially by individual DFM treatments. Collectively,

results suggest *in vitro* models are effective in evaluating DFM impacts on composition of the rumen and fecal microbiome and *L. lactis* effects warrant further investigation in a live animal trial.

Keywords: direct fed microbials, rumen, fecal, microbiome, *in vitro*

Introduction

In recent years, the gastrointestinal tract (**GIT**) of food animals has become more widely recognized as hosting a microbial consortium that can dramatically impact metabolic and immunologic functions that critically affect host physiology (Shreiner et al., 2015). Ruminants are unique in having a foregut fermentation that occurs in the rumen, which plays host to a diverse population of microorganisms essential to the breakdown and degradation of forages to energy for the animal (Osorio-Doblado et al., 2023). With advancements in culture-independent analyses such as 16S rRNA sequencing and metagenomics, our understanding of the complexity and diversity of the rumen microbiome has increased along with understanding that certain environmental/dietary factors influence microbiota dynamics (Kim et al., 2017; Seshadri et al., 2018).

One factor proven to influence the rumen microbiome is diet (Henderson et al., 2015). In order to improve ruminant feed efficiency, the industry has sought methods to alter the ruminal microbial population, and this includes the use of a variety of feed additives (Clemmons et al., 2019). Most notably, the addition of antimicrobials into feed rations has been widely used in the cattle industry to improve commercially impactful production parameters such as feed efficiency and growth through the selective inhibition of microorganisms (Cameron and McAllister, 2016). A dramatic increase in consumer and industry concerns regarding medically important antimicrobial resistance in bacteria has driven a reexamination of antimicrobial usage in the cattle industry along with increased regulation (Kirchhelle, 2018; Sneeringer, 2019).

Alternatives to antibiotics (**ATA**), which are compounds that can be substituted for therapeutic antimicrobials that are increasingly becoming ineffective against pathogenic bacteria, viruses, or parasites (Seal et al., 2013; Callaway et al., 2021). One ATA of particular interest is

the use of probiotics, known as direct fed microbials (**DFM**) in the livestock industry, which are live microorganisms used as feed additives to beneficially affect host animals by improving gastrointestinal tract (**GIT**) microflora (Fuller, 1989). Direct fed microbials influence the rumen microbiome composition, though understanding the impact (and consistency) of these influences remains unclear (Yáñez-Ruiz et al., 2015; Ban and Guan, 2021). Additionally, little research exists on utilizing an *in vitro* model to investigate impacts of DFM solely on the microbial population (Monteiro et al., 2022). The objective of this study was to utilize mixed ruminal and fecal *in vitro* fermentations to isolate DFM impact on the microbial population of the gastrointestinal tract.

Material and Methods

DFM Strains and Culture Conditions

Five DFM strains were obtained from Provita Supplements Inc. (Minnetonka, MN). *Lactococcus lactis* (***L. lactis***), *Propionibacterium freudenreichii* (***P. freudenreichii***), *Lactobacillus diolivorans* (***L. diolivorans***), *Enterococcus faecium* (***E. faecium***), and *Bacillus subtilis* (***B. subtilis***) were cultured in tryptic soy broth (TSB; Remel, Lenexa, Kansas). All strains were aerobically incubated at 37°C for 24h and were maintained for continued experimental use through weekly subculture in TSB media.

In Vitro Fermentation

Cattle husbandry practices adhered to the guidelines approved by the University of Georgia's Animal Care and Use Committee. Approximately two hours after morning feeding, ruminal contents were collected from two 1000-kg cannulated Holstein dairy steers fed a total mixed ration (**TMR**) diet. Ruminal and fecal contents from each steer were removed by hand and strained through a nylon paint strainer into separate thermoses to remove undigested feedstuffs

while maintaining minimal aerobic headspace. The thermoses were left to sediment at 39°C for 30 min, allowing feed particles to be buoyed to the surface by gas production. Once stratified, particle-free rumen or fecal fluid was pipetted anaerobically (33% vol/vol) to anoxic medium described by Cotta and Russell (1982) and Callaway and Martin (1997). Rumen fluid or fecal fluid media (45 mL) was anaerobically transferred to 120 mL serum bottles [(WHEATON, Millville, New Jersey), $N = 42$] containing TMR [0.5 ± 0.05 g (2 mm screen dry matter particles)] or no TMR. Probiotic cultures in TSB tubes ($n = 5$, 10 mL each) grown to 10^9 CFU/mL were centrifuged at 4,000 rpm for 10 minutes. The resulting supernatant was removed (9 mL) before resuspension of cell cultures ($n = 5$, 1mL each) in serum bottles containing TSB (45 mL) to achieve 10^{10} CFU/mL, which was confirmed utilizing serial dilutions. Probiotic strains were then added (5mL) to treatment serum bottles to achieve final concentrations of 10^9 CFU/mL and 50 mL for final volume. Treatments consisted of each DFM strain and tubes containing rumen fluid and TMR were control plus feed (**CONF**) to represent a fed state and tubes containing only rumen fluid were control (**CON**) to represent an endogenous state. Fermentation end products were collected at three time points: 2, 4, and 24 hours. Each treatment was performed in duplicate ($n = 2$) for each timepoint, and the experiment was repeated on sequential days. Tubes were flushed with CO_2, sealed with butyl rubber stoppers and aluminum crimps, and were incubated at 39°C in a CO_2 incubator (VWR Scientific, Radnor, Pennsylvania) for 2, 4, or 24 h. The same methodology was repeated for fecal fluid *in vitro*. Fermentations at each time point were removed and allowed to come to room temperature before the crimp and butyl rubber stoppers were removed and fluid was collected and frozen at -80 °C for further analysis.

DNA Extraction

DNA extraction for all samples was performed using a hybrid DNA extraction protocol with both mechanical and enzymatic method as previously described by Williamson et al. (2022) with slight modifications. Both sample sources were pipetted (350 μL) into 2 mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA) in which 1 mL InhibitEx Buffer (QIAGEN, Venlo, Netherlands) was added for enzymatic inhibition. Samples were then homogenized using a QIAGEN vortex adapter (QIAGEN, Venlo, Netherlands) for cellular disruption. Samples were placed in a 95 °C water bath for five minutes before being vortexed and centrifuged. Afterwards, 15 μL Proteinase K (QIAGEN, Venlo, Netherlands) was added to a new microcentrifuge tube before subsequently adding 200 μL sample supernatant and 200 μL Buffer AL (QIAGEN, Venlo, Netherlands). Tubes were vortexed and placed in a water bath at 70 °C for ten minutes. Post water bath, tubes were centrifuged before adding 200 μL of ethanol and centrifuged again. Then, 600 μL of content were transferred to a QIAamp spin column (QIAGEN, Venlo, Netherlands) and DNA elution and purification was carried out using a series of columns and specialized buffers according to manufacturer's specifications (QIAamp Fast DNA Stool Mini Kit; QIAGEN Venlo, Netherlands). Calculation of DNA concentration in the resulting eluate was performed spectrophotometrically using the Qubit 4 Fluorometer (Therno Fischer Scientific, Waltham, MA). Samples with a minimum volume of 100 μL and concentrations of at least 4 ng/μL were stored at -80°C until sequencing analysis.

16S rRNA Sequencing

Following DNA extraction, samples were transported on ice to Kelly Products Inc. LLC (Covington, GA) for library preparation and 16s ribosomal ribonucleic acid (**rRNA**) gene sequencing. The library preparation step included polymerase chain reaction (**PCR**) with the forward: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse: S-D-Bact-

0785-a-A-21 (5′- GACTACHVGGGTATCTAATCC-3′) primer pairs (Klindworth et al., 2013), followed by PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). A second PCR step was then performed to attach Illumina's indices and sequencing adapters (Nextera XT Index Kit; Illumina Inc., San Diego, CA, USA), and subsequently another PCR clean-up step using AMPure XP beads was performed. After this final library clean up, the library was quantified using qPCR, and nucleotides were sequenced using an Illumina MiSeq instrument and a MiSeq v3 reagent kit (Illumina Inc., San Diego, CA, USA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for the sequencing runs.

Bioinformatics Analysis

The sequencing data was demultiplexed and converted into FASTQ files which were imported into QIIME 2 (Boylen et al., 2019). Non-biological nucleotides and sequences were denoised, dereplicated, and chimera-filtered using DADA2 (Callahan et al., 2016). A pre-trained Naïve Bayes classifier trained with the Greengenes2 data base was used to assign taxonomies with reads classified by taxon using the fitted classifier (McDonald et al., 2023). Sequencing depth was set at 7,385 sequences per sample.

Statistical Analysis

Analyses were performed for alpha diversity indices (e.g., observed features, Peilou's evenness, and Shannon's diversity index) in addition to the genus taxonomic level for taxon above an average 0.5% abundance for each sample type. Analysis was performed using duplicate average of serum bottles on same day as the experimental unit. Day was utilized as a blocking factor and was considered a random effect. All measurements were analyzed as a completely randomized block design with a 7×3 factorial arrangement. Treatment (**TRT**), Time, and their

interaction served as fixed effects. Analysis of models were done using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC). Pairwise comparisons between treatment and factor level were computed using the Tukey-Kramer test. Statistical significance was declared at $P \leq 0.050$ using post-hoc analysis *p*-values. F test *p*-values are not reported in consideration after controlling for family error rate, some discrepancies exist between the F-test and Tukey-Kramer. The authors chose to proceed with *p*-values resulting from the more conservative Tukey-Kramer test.

Results

Rumen Microbial Alpha Diversity

There was no TRT × Time interactions for observed features or TRT effects ($P > 0.098$), but there were Time effects ($P = 0.001$, Figure 5.1A). Observed features for 2 and 4 h were greater compared to 24 h ($P = 0.001$) but did not differ from each other ($P = 0.999$). For Peilou's evenness, there were TRT × Time interactions ($P < 0.047$, Figure 5.1B). Treatments CONF, *L. diolivorans*, and *E. faecium* at 2 h and *E. faecium* and *P. freudenreichii* at 4 h had greater evenness compared to *L. diolivorans* and *E. faecium*, at 24 h, but was not different from each other or from other TRT × Time groups ($P > 0.199$). Treatments *L. diolivorans* and CON at 4 h and CON at 2 h were more even compared to *L. diolivorans* at 24 h ($P < 0.047$) but did not differ from each other or from other TRT × Time groups ($P > 0.092$). Other TRT × Time groups did not differ ($P > 0.110$). For Shannon's diversity index there were TRT × Time interactions ($P < 0.047$, Figure 5.1C). Treatments CONF and *P. freudenreichii* at 2 h and CONF at 4 h had greater diversity compared to *E. faecium* and *L. diolivorans* at 24 h but did not differ from each other or other TRT × Time groups ($P > 0.125$). Other TRT × Time groups did not differ ($P > 0.142$).

Fecal Microbial Alpha Diversity

There were no TRT × Time interactions nor TRT or Time effects for observed features ($P > 0.5271$, Figure 5.2A). For Peilou's evenness, there were TRT × Time interactions ($P < 0.050$, Figure 5.2B). Treatments *L. diolivorans* at 2 h and CON at 4 h had greater evenness compared to *L. lactis*, *B. subtilis,* and *E. faecium* at 24 and *L. lactis* at 2 h ($P < 0.0500$) but did not differ from each other or other TRT × Time groups ($P > 0.124$). Treatments CON at 24 h, *B. subtilis*, *E. faecium*, *P. freudenreichii*, and CONF at 2h, and *P. freudenreichii*, CONF, and *L. diolivorans*, at 4 h were greater than *L. lactis*, *B. subtilis*, and *E. faecium* at 24 h ($P < 0.011$) but did not differ from each other or treatments CON and *L. lactis* at 2 h, *B. subtilis*, *E. faecium*, and *L. lactis* at 4 h, and *L. diolivorans*, *P. freudenreichii*, and CONF at 24 h ($P > 0.113$). Treatments CON and *L. lactis* at 2 h, *B. subtilis*, *E. faecium*, and *L. lactis* at 4 h, and *L. diolivorans*, *P. freudenreichii*, and CONF were greater than *B. subtilis* and *E. faecium* at 24 h ($P < 0.0209$) but did not differ from each other or L. lactis at 24 h ($P > 0.058$). Treatment *L. lactis* at 24 h was greater than *E. faecium* at 24 h ($P = 0.0110$) but did not differ from *B. subtilis* at 24 h ($P = 0.700$). Treatments *B. subtilis* and *E. faecium* at 24 h did not differ from each other ($P = 0.9465$). For Shannon's diversity, there were no TRT × Time interactions ($P > 0.057$) but there were TRT and Time effects ($P < 0.0273$, Figure 5.2C). Treatments CON and CONF had greater diversity compared to *L. lactis* ($P < 0.0273$) but did not differ from each other or from other TRT groups ($P > 0.0546$). All DFM strains did not differ from each other ($P > 0.0841$). For Time there was greater diversity at 2 and 4 h compared to 24 h ($P < 0.017$) but they did not differ from each other ($P = 0.946$).

Rumen Bacterial Genera

There were 39 rumen genera present at > 0.5% average abundance. Of these, 32 genera had no TRT × Time interactions nor TRT or Time effects ($P > 0.197$, Figure 5.3). There were five genera: *Cryptobacteroides* (6.685%), unidentified *Lachnospiraceae* genus (3.178 %), *Treponema_D* (0.711 %), *Streptococcus* (0.571 %), and unidentified *Bacteroidales* genus (0.557 %) that had TRT × Time interactions ($P < 0.047$, Figure 5.4). For *Cryptobacteroides*, CON at 24 h had greater abundance compared to *P. freudenreichii*, *E. faecium*, *B. subtilis*, and CON at 2 h, *B. subtilis*, *P. freudenreichii*, and *L. diolivorans* at 4 h, and *L. lactis*, *P. freudenreichii*, and *B. subtilis* at 24 h ($P < 0.047$) but did not differ from other TRT × Time groups ($P > 0.053$). Other TRT × Time groups did not differ ($P > 0.529$). For the unidentified *Lachnospiraceae* genus, treatment CON at 24 h had greater abundance compared to *B. subtilis*, *L. lactis*, *E. faecium*, *L. diolivorans*, and *P. freudenreichii* at 2 h, *B. subtilis*, *L. diolivorans*, and CON at 4 h, and *P. freudenreichii* and *L. diolivorans* at 24 h ($P < 0.040$) but did not differ from other TRT × Time groups ($P > 0.102$). Treatments *E. faecium* at 24 h and *P. freudenreichii* at 4 h had greater abundance compared to *P. freudenreichii* at 2 h ($P < 0.040$) but did not differ from each other or other TRT × Time groups ($P > 0.188$). Other TRT × Time groups did not differ ($P > 0.0824$). For *Treponema_D*, CON at 2 h, *L. diolivorans*, *P. freudenreichii*, and *B. subtilis* at 4 h, and *E. faecium* at 24 h had greater abundance compared to *L. diolivorans* at 24 h ($P < 0.027$) but did not differ from each other or other TRT × Time groups ($P > 0.080$). Other TRT × Time groups did not differ ($P > 0.1720$). For *Streptococcus*, *E. faecium* at 24 h had greater abundance compared to CONF, *L. lactis*, *P. freudenreichii*, and *L. diolivorans* at 2 h, *L. diolivorans* at 4 h, and *L. lactis*, *L. diolivorans*, and CON at 24 h ($P < 0.037$), but did not differ from other TRT × Time groups ($P > 0.098$). Other TRT × Time groups did not differ ($P > 0.2601$). For the unidentified *Bacteroidales* genus, *L. lactis* at 24 h had greater abundance than *P. freudenreichii* at 2 h, CON,

B. subtilis, and *L. lactis* at 4 h, and *P. freudenreichii*, and *L. diolivorans* at 24 h ($P < 0.040$) but did not differ from other TRT × Time groups ($P > 0.0548$). Treatment with *E. faecium* at 24 h had a higher abundance than *B. subtilis* and *L. lactis* at 4 h and *L. diolivorans* at 24 h ($P < 0.038$) but did not differ from other TRT × Time groups ($P > 0.054$). Treatment CONF at 4 h had greater abundance compared to *L. lactis* at 4 h ($P < 0.026$) but did not differ from other TRT × Time groups ($P > 0.052$). Other TRT × Time groups did not differ ($P > 0.1045$). Two genera: *Fibrobacter* (0.868%) and *Succinivibrio* (0.721%) had no TRT × Time interactions or TRT effects ($P > 0.189$) but Time effects were observed ($P < 0.039$, Figure 5.5). *Fibrobacter* abundance at 2 h was greater than 4 h ($P = 0.039$) but did not differ from 24 h ($P = 0.198$). Abundances at 4 and 24 did not differ ($P = 0.724$). *Succinivibrio* abundance at 4 h was greater than 2 and 24 h ($P < 0.008$) while 2 and 24 h abundance did not differ ($P = 0.479$).

Fecal Bacterial Genera

There were 40 fecal genera present at > 0.5% average abundance. Of these, 33 genera had no TRT × Time interactions nor TRT or Time effects ($P > 0.069$, Figure 5.6). Two genera: *Fibrobacter* (0.780%) and an unidentified *Muribaculaceae* genus (0.645%) had TRT × Time interactions ($P < 0.048$, Figure 5.7). For *Fibrobacter*, treatment with *P. freudenreichii* at 4 h had greater abundance compared to treatments with *L. lactis*, *E. faecium*, *CON*, and *B. subtilis* at 4 h and *L. diolivorans, P. freudenreichii*, and CONF at 24 h ($P < 0.0234$), but did not differ from other TRT × Time groups ($P > 0.0989$). Treatment with *L. diolivorans* at 4 h had greater abundance compared to *E. faecium*, CON, and *B. subtilis* at 4 h and *P.freudenreichii* and CONF at 24 h ($P < 0.048$), but did not differ from other TRT × Time groups ($P > 0.0637$). Other TRT × Time groups did not differ ($P > 0.1311$). For the unidentified *Muribaculaceae* genus, CONF at 24 h had greater abundance than *L. lactis*, CONF, *P. freudenreichii*, *E. faecium*, and CON at 2 h,

all treatments at 4 h, and *E. faecium*, CON, *B. subtilis*, and *L. lactis* at 24 h ($P < 0.042$) but did not differ from *B. subtilis* and *L. diolivorans* at 2 h and *L. diolivorans* and *P. fruedenreichii* at 24 h ($P > 0.081$). The treatment *L. diolivorans* at 24 h had greater abundance compared to *B. subtilis* and CONF at 4 h ($P < 0.045$) but did not differ from other TRT × Time groups ($P > 0.094$). Other TRT × Time groups did not differ ($P > 0.658$). One genus: *Lactococcus_A_346120* (0.789%) had no TRT × Time interactions or Time effects but did have TRT effects ($P < 0.037$, Figure 5.8). Treatments *P. freudenreichii* and *L. lactis* had greater abundance compared to other TRT groups ($P < 0.037$) but did not differ from each other ($P = 0.846$). Other TRT groups did not differ ($P = 1.000$). Four genera: *Cryptobacteroides* (6.054%), an unidentified *Lachnospiraceae* genus (3.525%), *Treponema_D* (1.454%), and *Succinivibrio* (0.803%) had no TRT × Time interactions or TRT effects ($P > 0.1384$), but did have Time effects ($P < 0.047$, Figure 5.9). *Cryptobacteroides* and the unidentified *Lachnospiraceae* genus had higher abundances at 24 h compared to 4 h ($P < 0.043$) but did not differ from 2 h ($P > 0.165$). Abundances at 2 and 4 h did not differ ($P > 0.150$). For *Treponema_D*, 4 h were at higher abundances compared to 2 h ($P = 0.004$) but did not differ from populations at 24 h ($P = 0.284$). Additionally, *Treponema_D* abundances at 2 and 24 h did not differ ($P = 0.175$). For *Succinovibrio*, abundances were higher at 4 and 24 h compared to 2 h ($P < 0.002$) but did not differ from each other ($P = 0.296$).

Discussion

Microbial alpha diversity indices examined in this study include parameters such as richness, evenness, and overall diversity of microbial communities within a sample (Kim et al., 2017). Alpha diversity is influenced by animal factors such as diet, body size and composition, and gut physiology (Reese and Dunn, 2018; Pinart et al., 2022). Research has suggested that

low-diversity microbial communities can increase stability within the gut and can have a beneficial impact on production metrics such as rumen feed efficiency in cattle (Coyte et al., 2015; Shabat et al., 2016). In the present study, rumen microbial population observed features, Peliou's evenness, and Shannon diversity demonstrated similar temporal relationships, with indices not differing between 2 and 4 h but were decreased by 24 h. One DFM strain, *E. faecium*, followed this pattern when examining Peilou's evenness; however, despite TRT × Time interactions there was no overall TRT effects for any of the DFM candidate strains compared to control groups. Fecal observed features had no differences, but Peilou's evenness and Shannon's diversity demonstrated differential DFM effects. For Peilou's evenness, *L. lactis* and *E. faecium* treatments led to lower evenness compared to both controls and *P. fruedenreichii* and *L. diolivorans* treatments but were similar to *B. subtilis* treatment evenness. *B. subtilis* treatments had lower evenness than CON but was no different than CONF, whereas *L. diolivorans* and *P. freudenreichii* treatments did not differ from each other or controls. When examining TRT × Time interactions, 24 h had the greatest decreases in evenness, with *E. faecium* having the lowest evenness compared to all other treatment groups except *B. subtilis*. Though Shannon's diversity had no TRT × Time interactions, another treatment effect emerged. *L. lactis* treatments had lower diversity compared to both control groups but did not differ from other DFM candidate strains. None of the other candidate DFM strains differed from control groups. These results suggest that the DFM strains examined may not impact rumen microbial alpha diversity to a significant degree but can impact fecal microbial community composition. DFM *L. lactis* and *E. faecium* demonstrated the ability to lower evenness and diversity, which agrees with Monteiro et al., (2022) that DFM treatment can lower diversity indices. While these differences reflect short-

term changes, these results suggest further exploration is warranted for long-term DFM feeding impact on microbial communities and whether lower diversity confers a benefit to the host.

Research suggests the existence of a core microbiome that is universal across a host species, though there is difficulty in establishing what microorganisms are core members (Henderson et al., 2015; Neu et al., 2021). For this study, a common microbiome was evaluated at the genera level as any genus that averaged a relative abundance of 0.5% or greater for each sample type. This resulted in 39 rumen and 40 fecal genera composing a common microbiome from the cannulated Holstein dairy steers. For each sample type, seven genera emerged with TRT and/or Time effects and/or TRT × Time interactions. Of these, five genera: *Cryptobacteroides*, unidentified *Lachnospiraceae* genus, *Treponema_D, Fibrobacter,* and *Succinivibrio* overlapped between sample types. *Cyrptobacteroides* in rumen fluid appeared to be influenced by energy state (fed vs. fasted) more so than by DFM treatment (Furman et al., 2020; Chai et al., 2024). The CON treatment at 24 h had greater *Cryptobacteroides* abundance compared to 2 h, though 4 h abundance did not differ from either timepoints. Additionally, at 24 h CON had greater abundance compared to *L. lactis*, *P. freudenreichii*, and *B. subtilis* treatments and although it was not significantly different from other treatments, CON abundance was numerically greater. In fecal fluid, *Cryptobacteroides* abundances fluctuated over time with 24 h abundance greater than 4 h but 2 h abundance did not differ from either timepoint. The unidentified *Lachnospiraceae* genus was influenced by *L. diolivorans* treatments, which had lower abundance compared to both control groups but did not differ from other DFM strains. In fecal fluid, the unidentified *Lachnospiraceae* genus experienced similar Time effects as did *Cryptobacteroides*. The genus *Treponema_D* in rumen fermentations had TRT × Time interactions and its abundance was greatest at 4 h compared to 2 and 24 h, but no apparent patterns existed for changes resulting

from treatments. In fecal fluid, abundances were greater at 4 h compared to 2 h but did not differ from 24 h. *Fibrobacter* populations in rumen fermentations had Time effects with the greatest abundance detected at 2 h compared to 4 h, but no difference was observed between 2 and 24 h. In fecal fermentations, *Fibrobacter* had differences between treatment groups at 24 h. Treatment CONF had greater abundance compared to *L. lactis*, *E. faecium*, *B. subtilis* treatments, and CON but DFM *P.freudenreichii* and *L. diolivorans* treatments did not differ. *Succinivibrio* demonstrated Time effects for both rumen and fecal fluid fermentations. Numerical bacterial abundances across timepoints followed a similar pattern, though significant differences varied as rumen fermentation abundances were greater at 4 h compared to 2 and 24 h and fecal fermentation abundances were greater at 4 and 24 h compared to 2 h.

Risely (2020) suggested the composition of the core microbiome might be driven by a combination of factors, such as abundance, stability, keystone taxa, and impact on host biological demands. These results demonstrated microbial members that remained relatively stable to dietary influence and ones that were more susceptible to change. Furthermore, microbial members susceptible to being changed by DFM treatment have been linked to impacting host physiology, such as *Lachnospiraceae* members both playing a role in VFA production but also certain metabolic diseases (Paz et al., 2018; Vacca et al., 2020). *Treponema* from the gut has been identified as a causative agent of bovine digital dermatitis (Zinicola et al., 2015). *Fibrobacter* members are involved in cellulose degradation and *Succinivibrio* members are typically thought to be involved in glucose/starch metabolism; and both have been linked to greater prevalence in high feed efficiency cattle (Petri et al., 2013; Abbas et al., 2020; Auffret et al., 2020). These susceptible genera may be influenced by DFM to impact host physiology, though further work is needed to fully elucidate effects.

Genera that did not overlap in gastrointestinal sample type but were significant including: *Streptococcus* and an unidentified *Bacteroidales* genus in rumen fermentations and unidentified *Muribaculaceae* genus and *Lactococcus_A_346120* in fecal fermentations. At 24 h, *Streptococcus* abundances varied with *L. lactis*, *L. diolivorans*, and CON treatments having lower abundance compared to *E. faecium*. Overall, *E. faecium* had greater abundance of *Streptococcus* compared to *L. lactis* and *L. diolirovans* treatments but did not differ from other treatments. *L. lactis* and *L. diolivorans* treatments also did not differ from each other or other treatments. *Streptococcus* genera proliferate during metabolic diseases such as sub-acute ruminal acidosis (Khafipour et al., 2009). Lower abundances, therefore, may indicate DFM *L. lactis* and *L. diolivorans* treatments could contribute to rumen stability while DFM such as *E. faecium* could contribute to *Streptococcus* proliferation that increases instability. For *Bacteroidales* at 24 h, *P. freudenreichii* and *L. diolivorans* treatments had lower abundance compared to *L. lactis* but did not differ from other treatment groups. *L. lactis* treatments did not differ in any of the major population metrics from other treatment groups either. Without further taxonomic classification it is difficult to ascertain what role the unidentified *Bacteroidales* may play, as this order has been shown to have positive and negative attributes (Paz et al., 2018). In fecal fermentations, the unidentified *Muribaculaceae* genus also experienced changes at 24 h. Treatment CONF had greater abundance compared to *L. lactis*, *E. faecium*, *B. sutbilis*, and CON but did not differ from *P. freudenreichii* or *L. diolivorans*. Evolving research suggests *Muribaculaceae* members have roles in regulating immune functions and energy metabolism (Taiwo et al., 2024). Recently, studies have found *Muribaculaceae* (formerly the S24-7 genera) to be positively correlated with marbling in cattle (Krause et al., 2020). While no DFM increased relative abundance of this genera in this short-term *in vitro* model, results further emphasize that this genera's abundance

has the potential to be influenced in a way that positively impacts several economically critical production metrics (e.g., beef quality grade). The *Lactococcus_A_346120* genus demonstrated clear treatment effects, because only two DFM treatments (*L. lactis* and *P.freudenreichii*) were maintained at similar abundances *in vitro*. In contrast, by 2 h, all other DFM treatments had allowed *Lactococcus_A_346120* abundances to decrease to 0% of the population. *Lactococcus* species have often demonstrated significant probiotic potential in vivo, as well as the ability to regulate immune responses (Saleena et al., 2023). The ability to maintain certain native genera by some DFM strains but not others suggest DFM can have both synergistic and antagonistic effects on native microorganisms, and while it does not appear these effects translate to shifts in fermentation patterns, however, further research with an *in vivo* model may elucidate potential immunomodulatory effects.

Conclusion

Comparatively little research has examined DFM impact specifically on the rumen and fecal microbiome composition in cattle. The present study utilized an *in vitro* mixed ruminal and fecal microorganism fermentation as a model to determine if microbial community changes catalyzed by DFM treatment could be evaluated before conducting an *in vivo* trial. While DFM did not appear to have much impact on rumen microbial alpha diversity in this short-term *in vitro* model, the DFM candidate *L. lactis* reduced evenness and diversity compared to control groups in fecal microorganism fermentations, though whether this is beneficial to the host animal remains unclear. Results from examining a common microbiome at the genera level demonstrated that certain microbial members are more reflective of dietary change than others, and that the same reflective microbial members often overlap in part between rumen and fecal microbial communities. Genera that are susceptible to change, but are distinctly related to sample type

(e.g., ruminal or fecal) communities appear especially affected by DFM treatment. One genus, *Lactococcus_A_346120*, was maintained in fecal *in vitro* by *L. lactis* and *P. freudenreichii* only. DFM impact on the microbiome was successfully evaluated utilizing an *in vitro* model. These results suggest *L. lactis* is of interest for further research in an *in vivo* setting, as changes to fecal alpha diversity and genera in combination with demonstrated anti-pathogenic activity indicate the most potential of DFM strains evaluated to affect the GIT microbiome in cattle.

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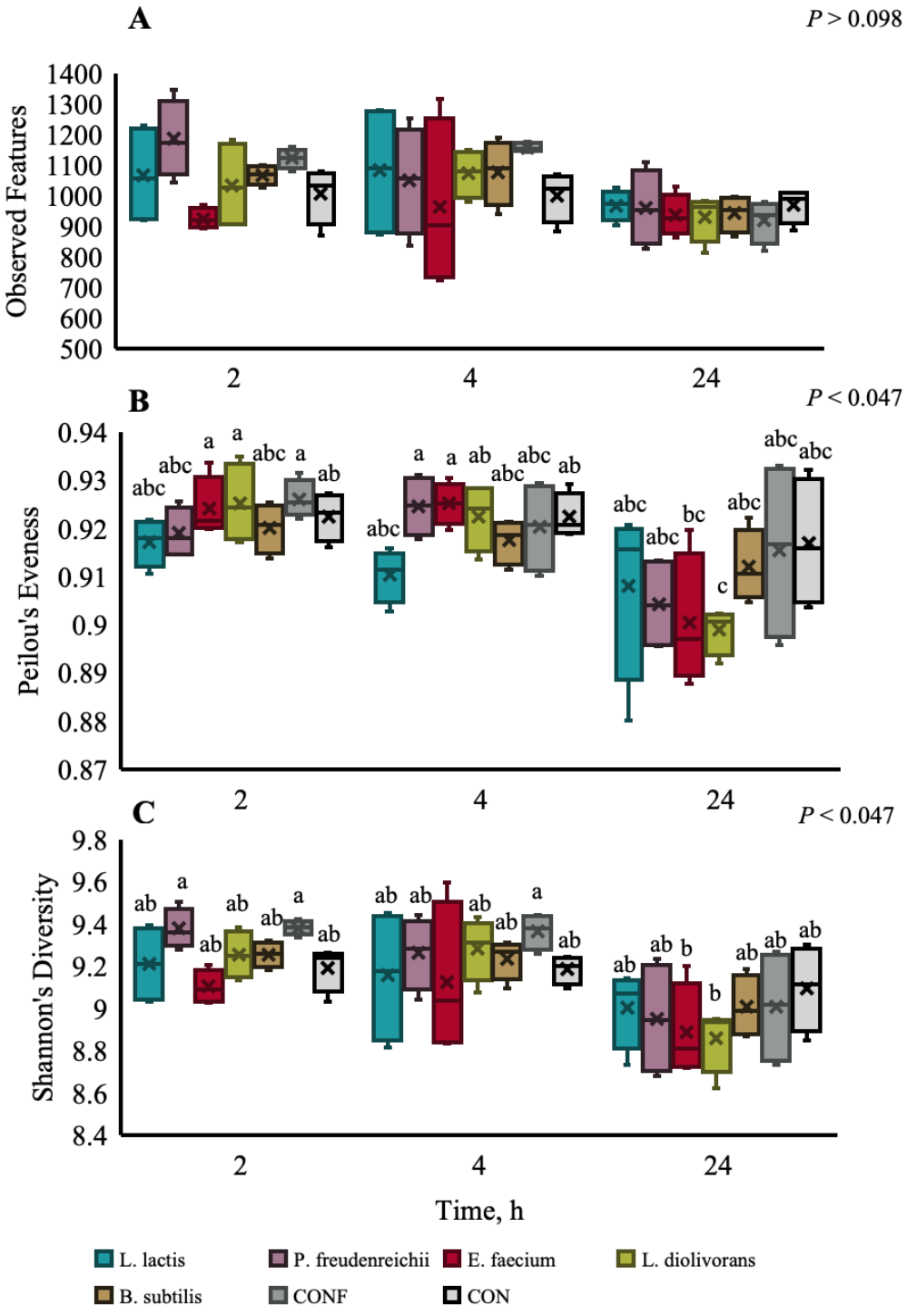

Figure 5.1 Boxplots of alpha diversity indices for (**A**) observed features, (**B**) Peilou's evenness, and (**C**) Shannon's diversity for in vitro mixed rumen microorganism fermentation microbiome TRT × Time groups (n = 4). All DFM were dosed at 10^9 CFU/mL. The line inside each box represents the median value and the x represents the mean value. The P-value represents TRT × Time interactions and groups with differing lowercase letters differ ($P < 0.05$).

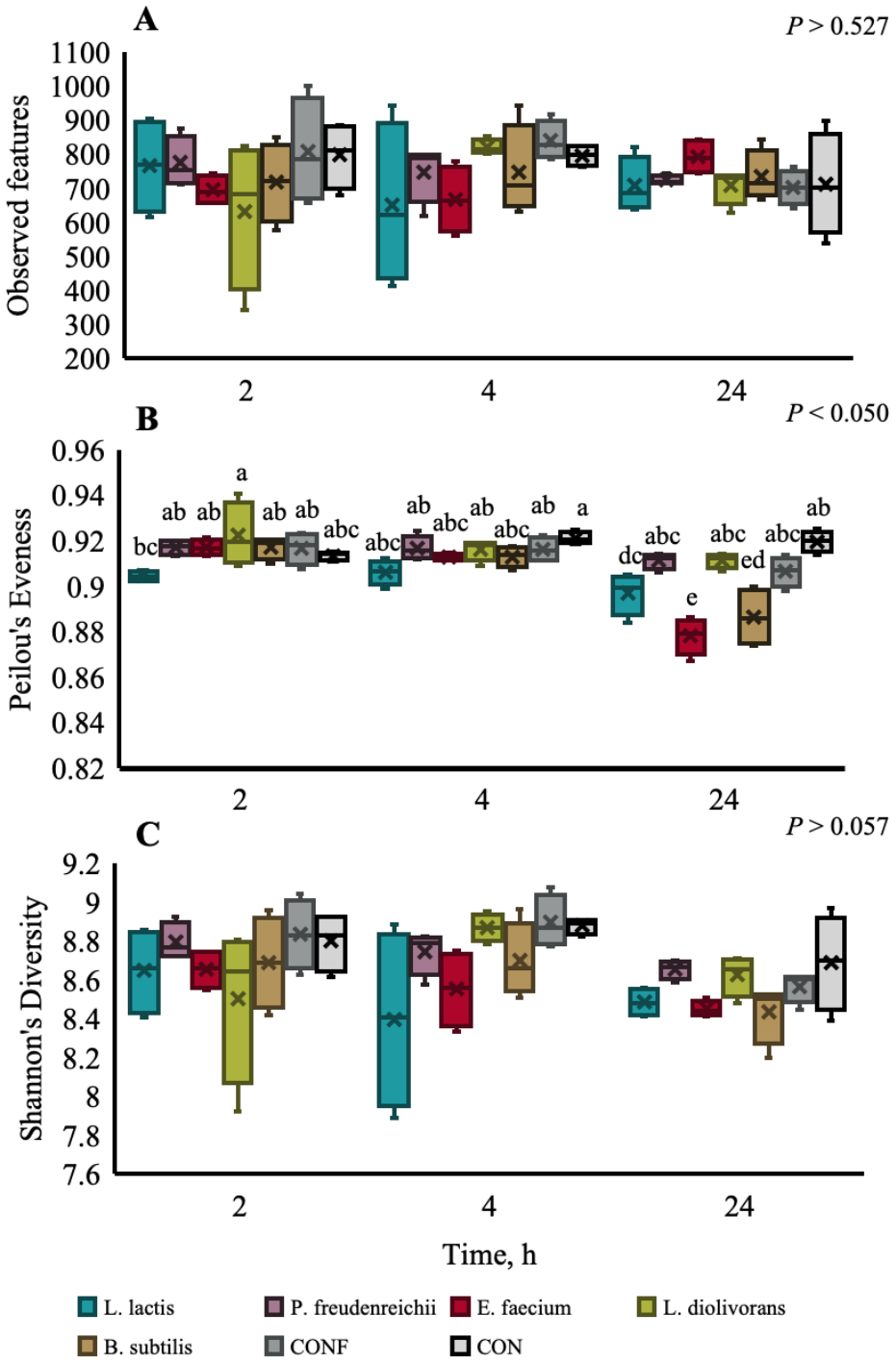

Figure 5.2 Boxplots of alpha diversity indices for (**A**) observed features, (**B**) Peilou's evenness, and (**C**) Shannon's diversity for different fecal *in vitro* TRT × Time groups (n = 4). All DFM were dosed at 10^9 CFU/mL. The line inside each box represents the median value and the x represents the mean value. The P-value represents TRT × Time interactions and groups with differing lowercase letters differ ($P < 0.05$).

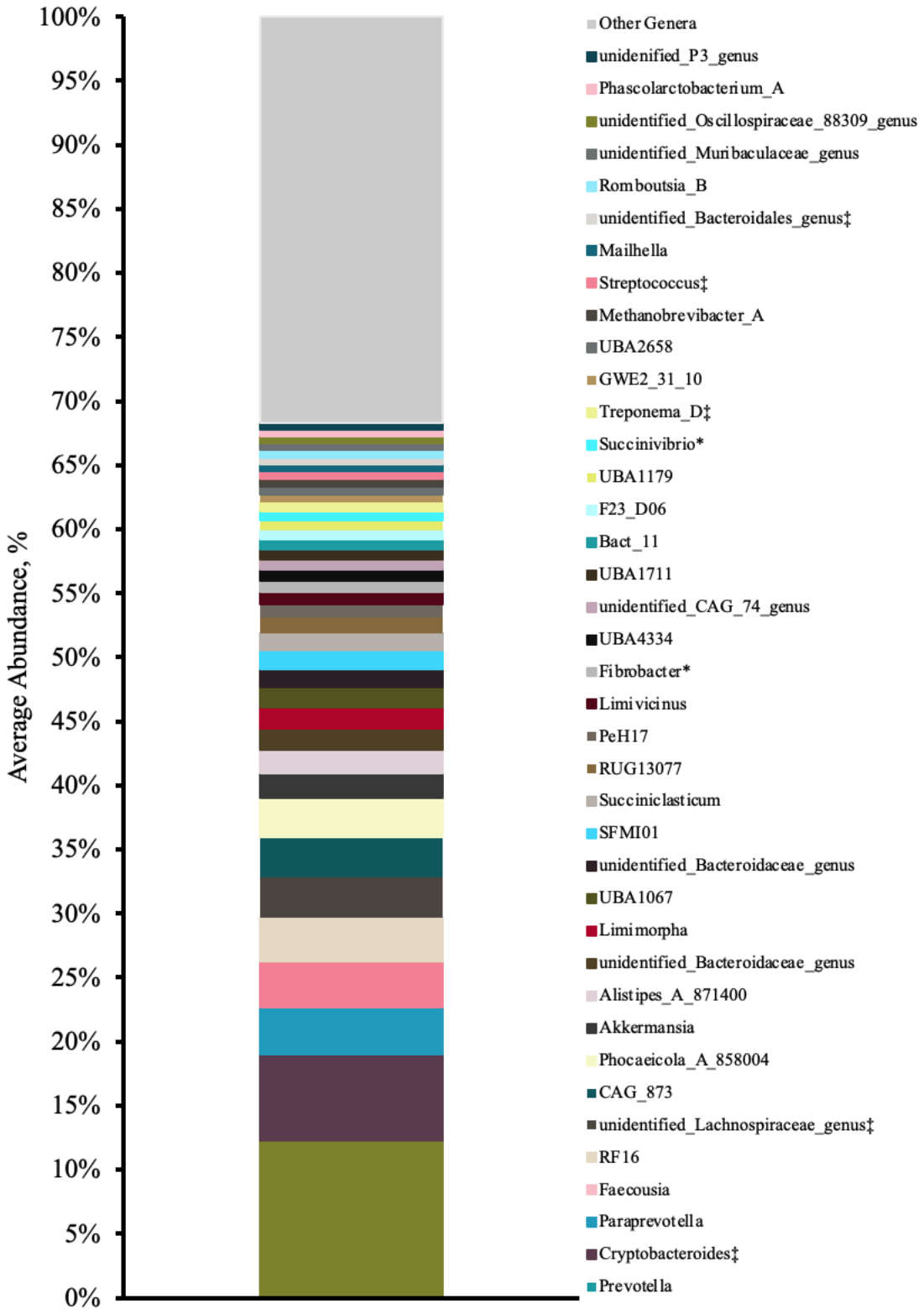

Figure 5.3 Average abundance of rumen genera above 0.5% for all samples (n = 88). ‡ indicates TRT × Time interactions, † indicates TRT effects, and * indicates TRT effects ($P < 0.05$).

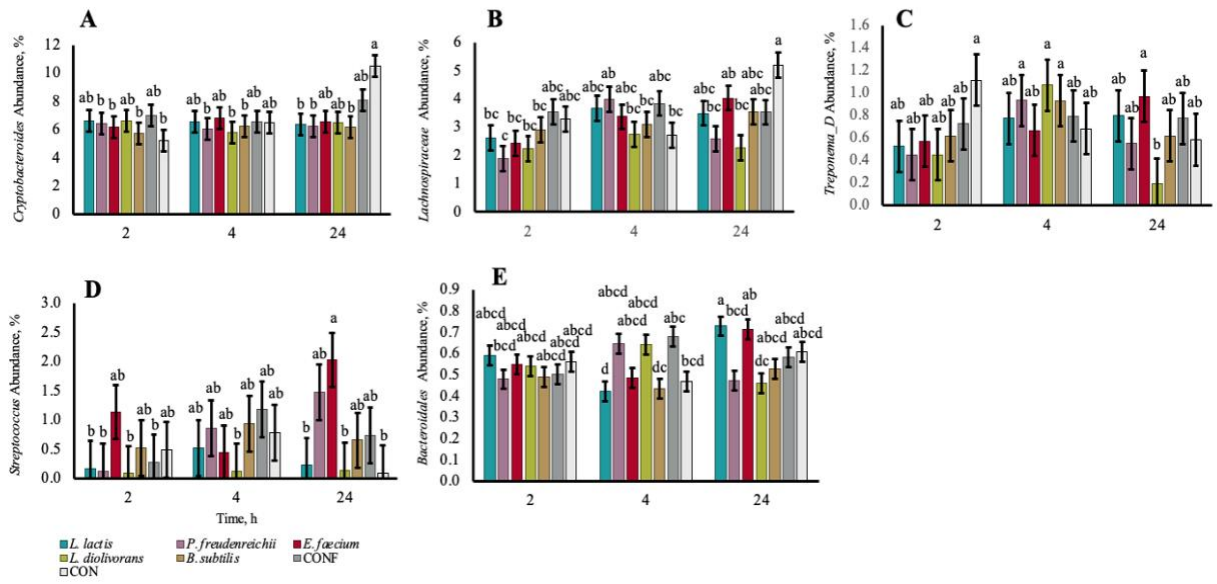

Figure 5.4 Rumen genera (**A**) Cryptobacteroides, (**B**) unidentified *Lachnospiraceae* genus, (**C**) *Treponema_D*, (**D**) *Streptococcus*, and (**E**) unidentified *Bacteriodales* genus with TRT × Time interactions (n = 4). All DFM were dosed at 10^9 CFU/mL. Groups with different lowercase letters differ ($P < 0.05$).

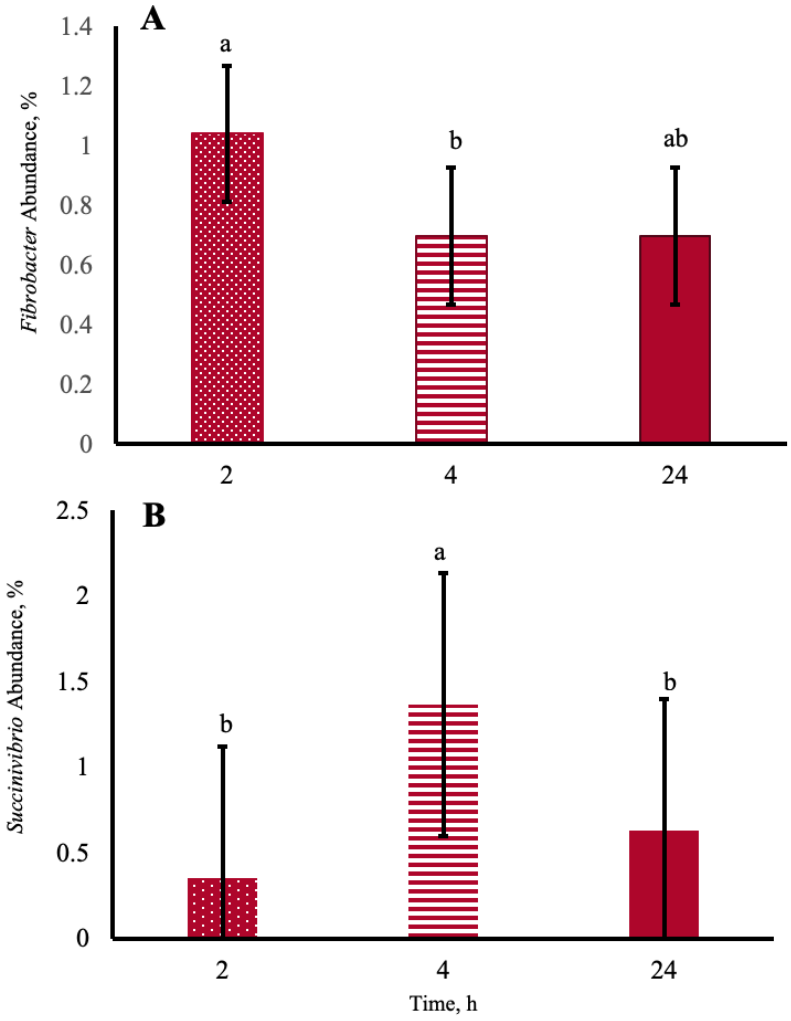

Figure 5.5 Rumen genera (**A**) *Fibrobacter* and (**B**) *Succinivibrio* with Time effects (n = 28). Timepoints with different lowercase letters differ ($P < 0.05$).

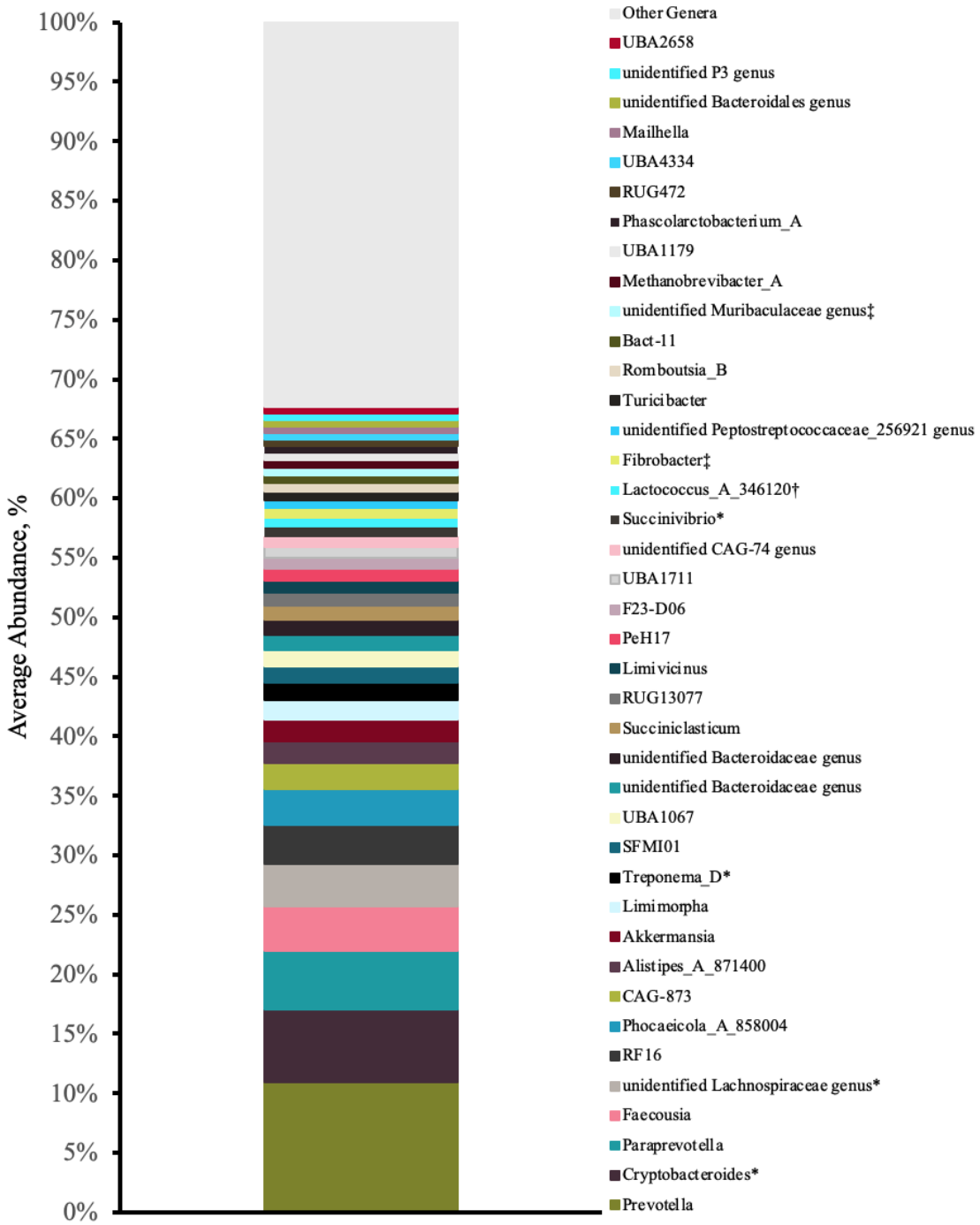

Figure 5.6 Average abundance of fecal genera above 0.5% for all samples (n = 88). ‡ indicates TRT × Time interactions, † indicates TRT effects, and * indicates TRT effects ($P < 0.05$).

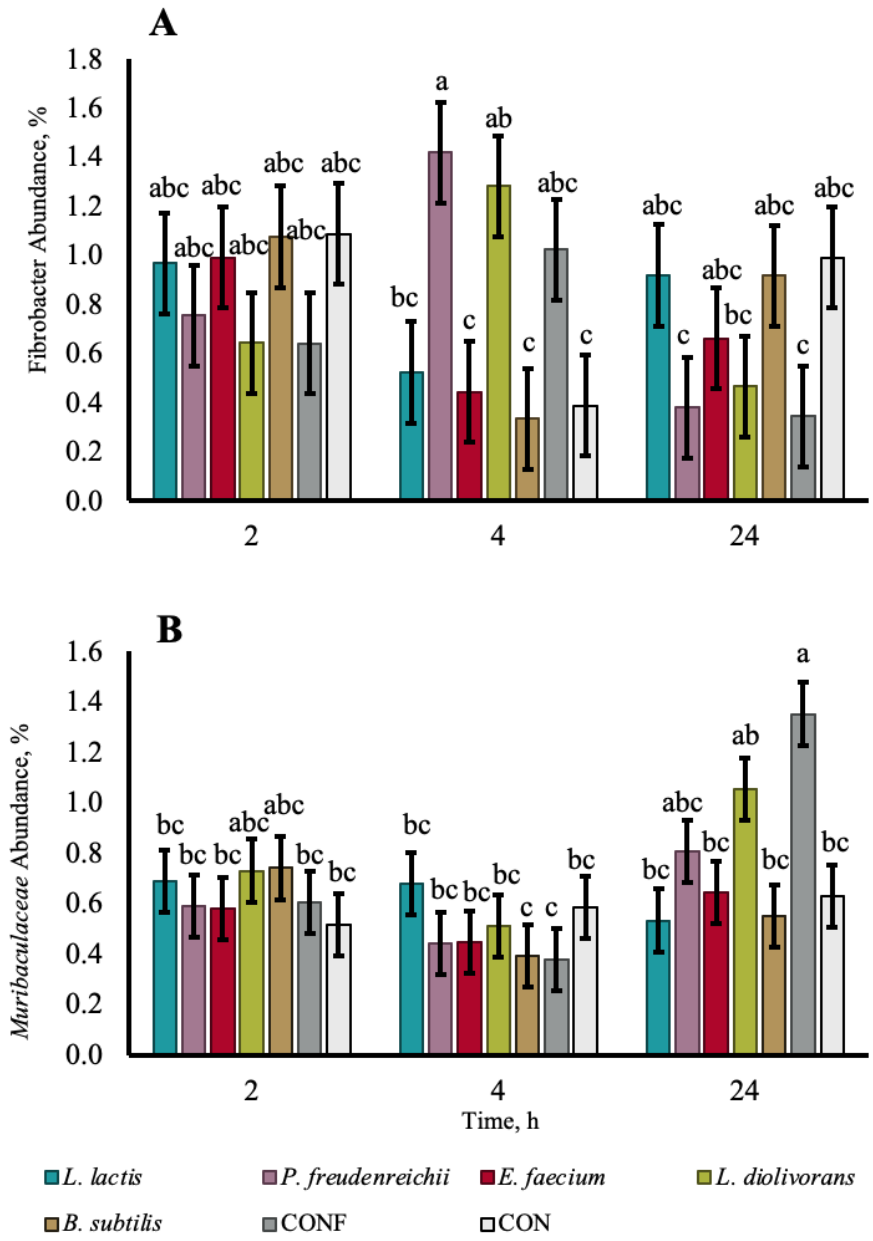

Figure 5.7 Fecal genera (**A**) *Fibrobacter* and (**B**) unidentified *Muribaculaceae* genus with TRT × Time interactions (n = 4). All DFM were dosed at 10^9 CFU/mL. Groups with different lowercase letters differ ($P < 0.05$).

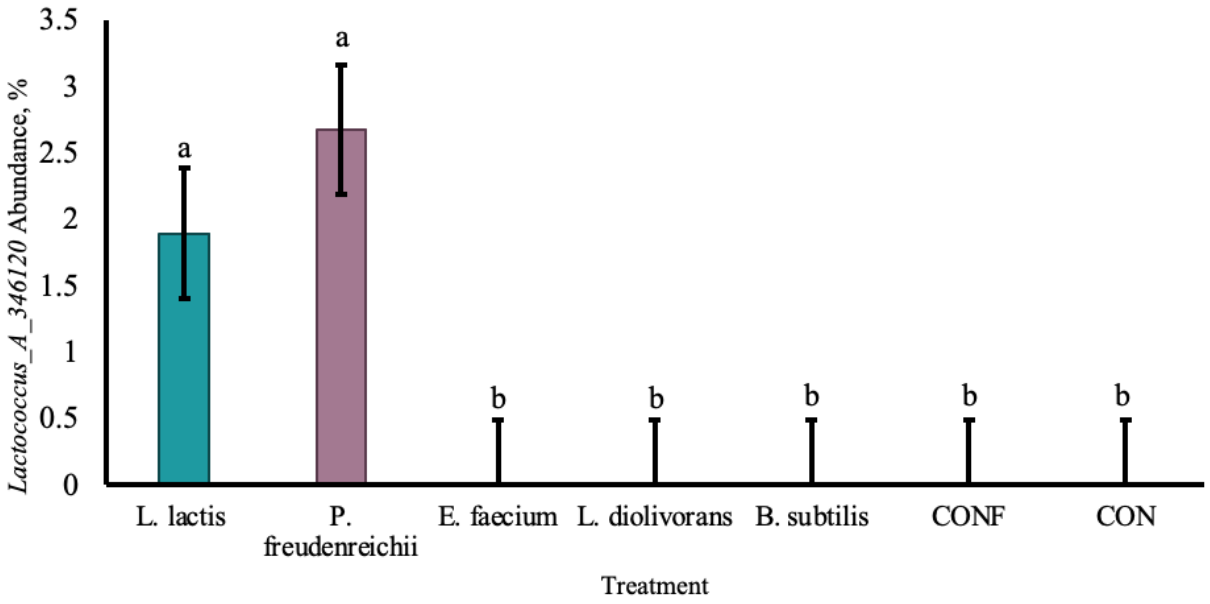

Figure 5.8 Fecal genus *Lactococcus_A_346120* with TRT effects (n = 12). All DFM were dosed at 10^9 CFU/mL. Treatments with different lowercase letters differ ($P < 0.05$).

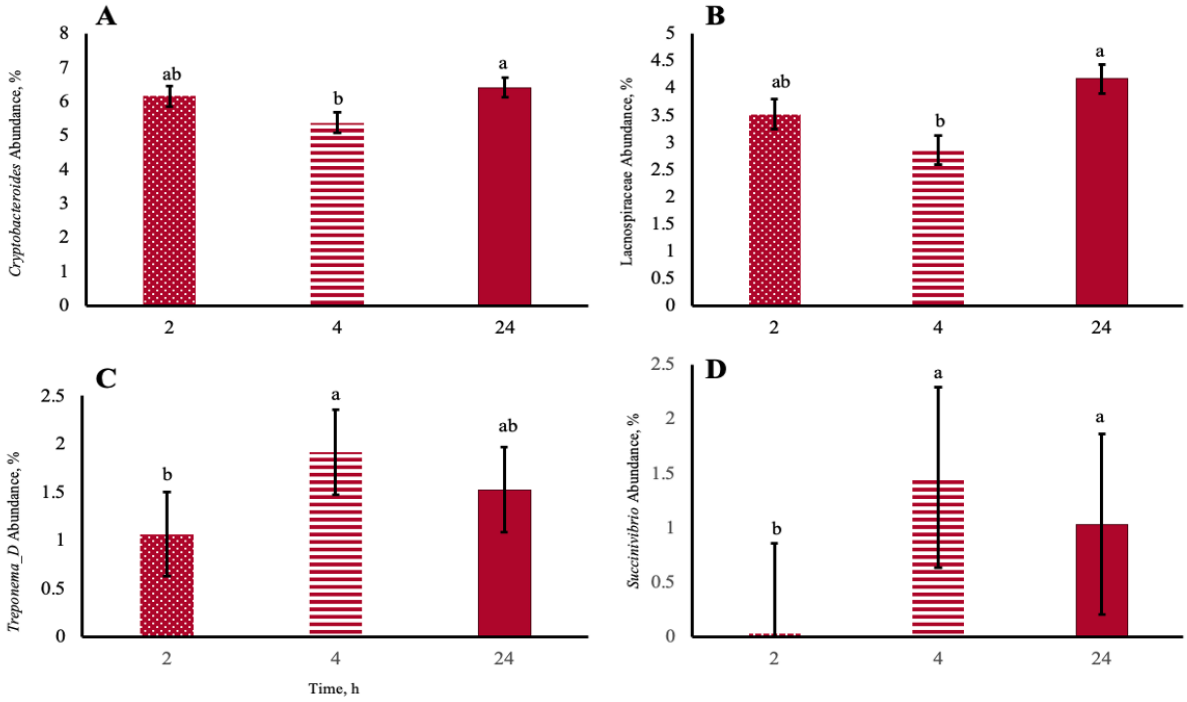

Figure 5.9 Fecal genera (**A**) *Cryptobacteroides*, (**B**) unidentified *Lachnospiraceae* genus, (**C**) *Treponema_D*, and (**D**) *Succinivibrio* with Time effects (n = 28). Timepoints with different lowercase letters differ ($P < 0.05$).

CHAPTER 6

CONCLUSION

After evaluating the potential of five candidate DFM strains, the results indicate the efficacy of some strains over others. The direct fed microbial candidates appeared to either cause no obvious changes/only minor effects or had observable differences across the experiments conducted. Interestingly, bacterial DFM used in this study did not demonstrate clear evidence of the ability to alter ruminal or fecal fermentation patterns *in vitro*, which aligns with previous research. However, using these DFM candidates holds promise to inhibit *Salmonella* populations and manipulation of the native microbiome. More potent or promising DFM candidates should show the ability to operate via more than one mode of action simultaneously. Furthermore, *in vitro* mixed microorganism fermentations are a useful avenue for investigating DFM potential prior to initiating live animal trials. The creation of a standardized model with which to compare DFM can provide valuable insight into how DFM may affect the animal and microbial populations of the gastrointestinal tract before commiting to a complicated and expensive feeding trial.

In the first study, results indicated that a *Lactococcus lactis* DFM had the potential to inhibit *S.* Typhimurium populations and could function as a pre-harvest pathogen mitigation strategy. Selection from a co-culture *in vitro* model demonstrated *Lactococus lactis* consistently had over 99% reduction of *S.* Typhimurium populations. When added to subsequent mixed gastrointestinal microorganism *in vitro* fermentations, *Lactoccus lactis* did not inhibit *S.* Typhimurium in rumen fluid, but achieved a 90% reduction of S. Typhimurium populations in

fecal fluid. These results suggest *Lactococcus lactis* exerts an inhibitory effect against *S.* Typhimurium in the lower gastrointestinal tract, and could reduce fecal shedding as well as lymph node prevalence of this important foodborne pathogen, potentially reducing human illnesses.

In the second study, the candidate DFM strains did not alter the *in vitro* mixed ruminal or fecal microorganism fermentation patterns. All DFM candidate strains increased total gas production in the mixed fecal microorganism fermentation *in vitro*. Increased gas production indicates the ability to increase organic matter digestibility in the lower gastrointestinal tract. The candidate DFM strain *P. freudenreichii* did alter volatile fatty acid concentrations, such as decreasing propionate concentration in mixed rumen microorganism fermentations, and reduced the A:P ratio in mixed fecal microorganism fermentations *in vitro*. However, with no impact on pH, methane, hydrogen, ammonia, or dry matter digestibility in rumen or fecal *in vitro* models, it is inconclusive that any of the present DFM candidates impact gastrointestinal fermentations.

In the third study, mixed rumen and fecal microorganism fermentation *in vitro* microbiome alpha diversity indices such as observed features, Peilou's evenness, and Shannon's diversity as well an established 0.5% abundance or greater common microbiome at the genera level were evaluated following DFM treatment. Rumen alpha diversity indices demonstrated no DFM treatment effects, but in mixed fecal microorganism fermentations treatment with *Lactococcus lactis* lowered both Peilou's evenness and Shannon's diversity compared to untreated control groups. Investigating at the genera level revealed that certain members of the ruminal and fecal mixed microorganism fermentation microbiota appear to be more influenced by dietary inclusion compared to others. Microbiota that appear to be less stable include *Cryptobacteroides*, unidentified *Lachnospiraceae* genus, *Treponema_D*, *Fibrobacter*, and

Succinivibrio genera. Futhermore, genera distinct to each source of microorgansims that were affected by DFM strains can have negative impacts on host physiology such as with the *Streptococcus* genus, or positive effects such as with the important *Muribaculaceae* genus. Interestingly, one genus in the fecal mixed microorganism *in vitro* fermentation, *Lactococcus_A_346120*, was only maintained beyond 2 h in the presence of two DFM, *Lactococcus lactis* and *Propionibacterium freudenreichii*. Overall, the results indicated *Lactococcus lactis* had the greatest potential to alter the mixed microorganism fermentation microbiome, especially when comparing alpha diversity and genera changes in fecal fluid.

Collectively, these results suggest that *Lactococcus lactis* holds the most promise as a potential DFM for use in cattle *in vivo*. *Lactococcus lactis* demonstrated anti-pathogenic activity against *S.* Typhiumurium and altered to the fecal microbiome in in vitro fermentations. The inhbitory activity demonstrated by *Lactoccus lactis* against *S.* Typhiumurium in fecal fluid is likely due to direct effects (e.g., antimicrobial production) as demonstrated in both the co-culture *in vitro* model and by indirect effects stemming from changes to the fecal microbiome composition. Additional research is required to determine long term effects of *Lactococcus lactis* on the host animal as well as examining GIT immunomodulation in addition to pathogen inhibition and microbiome changes. The use of *Lactococcus lactis* as a DFM can potentially improve cattle production and food safety metrics, and it may be a potent tool in the alternatives to antimicrobials arsenal.