THESIS

EFFECTS OF DIRECT-FED MICROBIALS ON WEIGHT GAIN AND GASTROINTESTINAL BACTERIA MICROBIOME COMPOSITION IN WEANED HEIFERS

Submitted by

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ABSTRACT

EFFECTS OF DIRECT-FED MICROBIALS ON WEIGHT GAIN AND GASTRO-INTESTINAL BACTERIA MICROBIOME COMPOSITION IN WEANED HEIFERS

Weight gain and fecal analysis on 179 heifer from two breeds of cattle, Angus and Salers, were analyzed after being treated with a direct fed microbial (DFM) supplement. Heifers were split into one of three treatment groups with the control being drenched with water, a second group being drenched with 1/2oz of the DFM, and a third group being drenched with 1oz of the DFM. Heifers were monitored over a four week period and drenched on day 0 and day 28 to measure changes in weight gain and microbiome composition. Fecal samples were taken rectally from the fecal group (FG) weekly during the duration of the trial. Overall, the final model ANOVA resulted in a p-value of .9689, concluding no significant difference between the 3 treatment levels for weight gain. Time proved to be the predominant driver of gastro-intestinal microbial composition and probiotic supplementation did not lead to significant changes to the microbiome community structure. Chao1 and ACE models ran on fecal samples indicate significant impact of probiotic treatment on microbial richness and indicate that supplementation leads to greater diversity and, in turn, may mean a more robust microbiome.

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DEDICATION

I would like to dedicate this manuscript to my mom, Sherry, who never let me forget it needed to be finished.

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CHAPTER I

INTRODUCTION

The purpose of this study was to evaluate the effects of direct-fed microbials (DFM) on weight gain (WG) and gastrointestinal bacterial microbiome composition (GBMC) when administered to weaned beef heifers. Weaning is an especially stressful time where calves typically have little desire to eat and gain poorly. Weaned cattle typically have high stress levels associated with separation anxiety and undergo a reduction in weight caused by reduced feed intake. Bagley (1997) found that the production loss and death loss of calves at weaning is second only to the losses at calving. Dust, heat, dehydration, feed change, and processing including vaccinations and deworming are some of the stressors newly weaned beef cattle experience (Bagley, 1997). These stresses make decreased performance and increased morbidity quite frequent in these cattle with potentially high death loss (Krehbiel et al., 2003).

Pathogens, stress, digestive and metabolic upset, and the use of antimicrobials can change the balance of intestinal bacteria which may impair digestion and make the animal more susceptible to disease (Quigley, 2011). By adding beneficial bacteria to the diet, animal health and performance may improve. These beneficial bacteria that come from direct fed microbial (DFM) have also been reported to promote the development of the immune system (both structure and function) in young animals by signaling the immune system to produce immunoglobulins and other components to maintain the competence of the immune system (Quigley, 2011). Direct-fed microbials have been employed in ruminant production for over 30 years (McAllister et al., 2011).

Cattle drenched with the DFM can show a shift in the total population of bacteria that is directly correlated to weight gain. The DFM could increase rumen function and efficiency, allowing the cattle to get on feed faster and reduce weight loss. A correlation between a shift in the total population of bacteria and weight gain could represent a beneficial impact of drenching with the DFM.

CHAPTER II

LITERATURE REVIEW

Direct fed microbials (DFM) and probiotics are two terms that are often used interchangeably (Quigley, 2011) when being discussed in a production agriculture setting. "Probiotic" was more specifically defined by Fuller in 1989 as well as Heyman and Ménard (2002) to be "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." Direct-fed microbial's are living organisms, and loosely spoken, probiotics can be used in the same breath. However, the term probiotics has been used to reference viable microbial cultures, culture extracts, enzyme preparations, and often a combination of all three (Yoon and Stern., 1995). The Food and Drug Administration therefore redefined DFM's due to the clarity concerns as a source of live (viable) naturally-occurring microorganisms (Yoon and Stern, 1995; Krehbiel et al., 2003).

Yeast

There has been significant research with DFM's implementing yeast cultures into the diet. Wiedmeier et al. (1987), Harrison et al. (1988), and Newbold et al. (1992) all concluded that the population of microorganisms in the rumen can be influenced by the addition of fungal culture supplements to ruminant diets. Yoon and Stern conducted a review on DFM's in 1995 which concluded that the addition of fungal cultures to the diet of ruminants was seen to: stimulate microbial growth, stabilize rumen pH, change the pattern of rumen microbe fermentation, increase the digestibility of nutrients that were ingested, allow for a greater nutrient flow to the small intestine, allow for more nutrient retention, and alleviate stress.

To be classed as a DFM's however, the supplement must be a live and viable organism. Regulatory requirements have limited the microbial species within DFM products to organisms that are generally recognized as safe, such as lactic acid-producing bacteria, fungi, or yeast (McAllister et al., 2011). Now, some sources list yeast as a non-living organism (Quigley, 2011); but Eckles and Williams published a report in 1925 on the benefits of yeast supplementation for lactating cows and since then, brewers yeast has been successfully used as a protein source in ruminant diets (Steckley et al., 1979). In fact, active yeast cultures were proven to increase milk yield by 1.1 kg/d by Renz in 1954. However, yeast cannot be considered a probiotic because stationary phase cells are nongrowing and are already arrested at the same point in the cell cycle (Hartwell, 1973).

The major fermentation product of saccharomyces cerevisiae (SC), the common yeast culture, is ethanol and this can lead to a toxicity problem when high levels of live yeast are used in the diet (Yoon and Stern, 1995). This toxicity can easily be avoided by using dead and dried yeast (Bruning and Yokoyama, 1988). Therefore, the Association of American Feed Control Officials (AAFCO) released a publication in 1991 to define a yeast culture as, "a dry product composed of yeast and the media on which it was grown, dried in such a manner as to preserve the fermenting capacity of the yeast." As a result, yeast cultures must be considered a prebiotic and not DFM's. Direct-fed microbials of rumen origin, involving lactate-utilizing species and plant cell wall-degrading isolates have also been explored, but have not been commercially used (McAllister et al., 2011).

Health

Pathogens, stress, metabolic upset, and the use of antimicrobials can upset the balance of intestinal bacteria which may impair digestion and make the animal more susceptible to disease

(Quigley, 2011). Originally, DFM's were used primarily in young ruminants to accelerate establishment of the intestinal microflora involved in feed digestion and to promote gut health but advancements have led to more sophisticated mixtures of DFM's that are targeted at improving fiber digestion and preventing ruminal acidosis in mature cattle (McAllister et al., 2011). By adding beneficial bacteria to the diet, animal health and performance may improve. Beneficial bacteria that come from DFM's are also seen to promote the development of the immune system (both structure and function) in young animals by signaling the immune system to produce immunoglobulins and other components to maintain the competence of the immune system (Quigley, 2011). Oral administration of lactobacilli generally resulted in an augmentation of innate immune responses as well as an elevated production of immunoglobulin (Krehbiel et al., 2003). Salimen et al. (1996) and Holzapfel et al. (1998) also concluded that DFM's modulated immune function. More recently, there has been an emphasis on the development of DFM that exhibit activity in cattle against potentially zoonotic pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* (McAllister et al., 2011).

The Bovine Alliance on Management and Nutrition claims that commensal (beneficial) bacteria: can ferment carbohydrates and produce short-chain fatty acids thus reducing pH and the growth of pathogens by out competing for the same source of nutrients as the pathogens, while promoting intestinal cell growth (Quigley, 2011). Jones and Rutter (1972) suggested that attachment to the intestinal wall was important for enterotoxin-producing strains of E. coli to induce diarrhea and therefore bacterial competition from DFM's with pathogens for sites of adherence on the intestinal surface would be advantageous. Salimen et al. (1996) and Holzapfel et al. (1998) found that bacterial DFM's modify the balance of intestinal microorganisms, adhere to intestinal mucosa, prevent pathogen adherence and influence gut permeability. Through

outcomes on fiber digestion and rumen health, second-generation DFM have also resulted in improvements in milk yield, growth and feed efficiency of cattle, but results have been inconsistent (McAllister et al., 2011).

Young Calves

An animal's gastrointestinal tract is constantly being challenged by large numbers of bacteria, viruses, and protozoa found in feed, bedding, and the environment (Quigley, 2011). Sound management begins the moment the calf is born. In fact, McGuirk and Ruegg (2011) of the University of Wisconsin claim the highest morbidity and mortality rates generally occur in baby calves prior to weaning and cite The National Animal Health Monitoring System (NAHMS) in estimating preweaning mortality of U.S. dairy calves to be 10.8%. Snodgrass et al. (1986) conducted a study analyzing diarrhea in calves. From 32 farms, fecal samples from 351 calves were collected and 128 were found to be suffering from diarrhea.

This neonatal time in a ruminant's life is a very stressful period. Constant interactions and exposure to foreign objects and materials make susceptibility to disease and sickness very probable. In stressed calves, the microbial population is in transition and extremely sensitive; abrupt changes in diet or the environment can cause alterations in microbial populations in the gastrointestinal tract stated Krehbiel et al. (2003) that stress leads to an increase in diarrhea and is directly associated with a decrease in the population of Lactobacillus in the gut. Since diarrhea is known to be the number one cause of death in young calves (McGuirk and Ruegg, 2011), the use of DFM's in young calves has been studied extensively.

Supplementing calves with a DFM serves for rapid adaptation to solid feed by accelerating the establishment of ruminal and intestinal microorganisms and avoiding the establishment of enteropathogens, which often result in diarrhea, is the primary goal (Krehbiel et

al., 2003). Starting in 1977, Bechman et al. discovered that feeding calves viable cultures of Lactobacillus decreased the incidence of diarrhea. In 1980, Gilliland et al. reported that this decreased incidence of diarrhea was associated with a consistently increased shedding of Lactobacillus and decreased shedding of coliforms in the feces in response to the supplementation of the DFM. Shedding of coliforms is greatly reduced when the animal has normal stool and no diarrhea, which is often seen to be related to animals not experiencing intestinal disorders (Gilliland et al., 1980). Jenny et al. conducted a review in 1991 and found that in studies on calves where no advantage of feeding a DFM was found, the calves were not typically experiencing any health problems.

The importance of bacterial DFM (primarily Lactobacillus species) fed to young and/or stressed calves has been to establish and maintain normal intestinal microorganisms, rather than as a production stimulant (Krehbiel et al., 2003). Producers should not be looking for added growth or weight increases but more so overall health and well-being. Nakanishi et al. (1993) studied Holstein calves given a DFM to stimulate rumen development, which did prove to occur, but did not find any performance benefits. Two studies, one by Abu-Tarboush (1996) on 24 Holstein bull calves and one by Morrill et al. (1977) on 143 Holstein calves, both found no improvement in average daily gain by feeding a DFM.

There are several studies however, that go against these principals. Timmerman et al. (2005) fed two different direct-fed microbial formulations to 1- to 2-week old veal calves in four different experiments. The first two experiments administered daily probiotic supplementation for 15 days and the second two experiments administered daily supplementation for 56 days using a multispecies probiotic (Timmerman et al., 2005). Results from all four experiments suggested that direct-fed microbials increased growth and feed efficiency in calves during the

first two weeks. This appeared to be especially true when calves were stressed and disease incidence was significant (Timmerman et al., 2005). Bechman et al. (1997) reported improved rates of gain and Beeman (1985) used 52 Holstein steers with a history of diarrhea in a trial that yielded results with increased average daily gain for cattle being treated with a DFM. Cruywagen et al. (1996) reported no significant health effect of adding Lactobacillus acidophilus to young milk-fed calves.

This variation is likely the result of differences in diet, pathogen type and stress to name a few. Variations from one gram of a DFM to several ounces can play a major role as well as the specific strain or strains of the DFM. But, performance response is not near as important in the early stages of a ruminant's life when enteric disease is most prevalent and improved health and reduction in the incidence and severity of diarrhea is a more important response (Krehbiel et al., 2003). Under stressed conditions, direct-fed microbials may reduce the risk or severity of scours caused by an upset in the normal intestinal flora of calves (Quigley, 2011).

Dairy Cattle

Krehbiel et al. (2003) claims that relative to beef cattle, little research has been done evaluating the efficacy of bacterial DFM's for lactating dairy cows. Raeth-Knight et al. (2007) did however, conduct a trial on Holstein cows during midlactation studying the effect of feeding a DFM on: performance, nutrient digestibility, and rumen fermentation. These month-long trials yielded very similar results as the feedlot trials conducted by Yang et al. in 2003. Dry matter intake was similar in cows across all treatments with no difference in total tract digestibility of dry matter, neutral detergent fiber, crude protein, or starch (Raeth-Knight et al., 2007). In addition, rumen pH, VFA's, and ammonia did not differ significantly across treatments (Raeth-Knight et al., 2007).

What is interesting to note though, is that Raeth-Knight et al. (2007) found no difference in milk yield between cows treated with a DFM and cows that were not, whereas Krehbiel et al. (2003) claims increased milk yield for cattle given a DFM to be a consistent response. Krehbiel et al. (2003) cites three different trials that show drastic differences in milk yield, and increases, in dairy cattle treated with a DFM versus those that were not. Milk composition changes were seen to be variable by Krehbiel et al. (2003) with no change seen by Raeth-Knight et al. (2007).

Feedlot Cattle

Ruminants continue to be challenged well beyond weaning though, and DFM's have been used extensively in feedlot cattle during the finishing phase. Overwhelming data has been collected on DFM's fed daily to demonstrate that adding a lactate-producing or utilizing bacteria to the diet of finishing cattle improves feed efficiency and daily gain (Swinney-Floyd et al., 1999; Galyean et al. 2000; Rust et al., 2000; Ware et al., 1988). In 2000, Huck et al. use DFM's in a phase feeding protocol across 126 finishing experiments with results that illustrated a 2.5% to 5% increase in daily gain and a 25 increase in feed efficiency for cattle fed a DFM versus those that were not.

Though originally thought to only benefit post-ruminal activities, there is indication that DFM's may also benefit the rumen and help to prevent ruminal acidosis (Krehbiel et al., 2003). Significant health and performance problems are common with ruminants that experience acidosis including: reduction in feed intake, reduced daily gain and reduced feed efficiency (Owens et al., 1998). Limiting acidosis is a crucial step in the improved production and performance of feedlot cattle. Because DFM's have been shown to reduce the incidence of diarrhea and number of intestinal coliforms, a surge in research has been conducted which shows some indication that certain bacterial DFM's have beneficial effects on rumen health. This is

largely due to supplementing the rumen with lactic acid producing and/or utilizing bacteria enhances the ability of the rumen ecosystem to moderate excessive lactic acid production (Yang et al., 2003). Studies conducted by Robinson et al. (1992), Kung and Hession (1995), and Ghorbani et al. (2002) use different strains of DFM's in their respective experiements but all yield comparable results showing: reduction or prevention in lactate accumulation, higher ruminal pH, and even increased concentrations of acetate in ruminal fluid thus leading to a reduced risk of metabolic acidosis.

A study was conducted by Yang et al. (2003) in Lethbridge, Alberta at the Agriculture and Agri-Food Canada Research Center on cattle in a finishing yard to test whether the addition of a DFM to the diet had any effect on ruminal pH, fermentation, bacterial populations, digestion, or microbial protein synthesis. The overall goal was to see if this addition and the effects would limit or decrease the occurrence of acidosis. The results from this study were in unanimous opposition to any benefits of DFM's being added in the diet with no effect on the prevention of sub-clinical acidosis (Yang et al., 2003). There were no effects on fermentation, bacterial populations, microbial protein synthesis or digestion and s researchers concluded that there is little benefit in providing DFM's that produce or utilize lactic acid when the rumen microflora are adapted to a high grain diet (Yang et al., 2003).

Weaned Calves

Attempting to maintain animal health and performance at weaning has long been one of, if not the biggest, challenge facing producers. This is especially crucial with calves coming straight off the cow who were not weaned prior to entering the feedyard. These cattle often undergo a variety of stresses such as: recent weaning, transport, fasting, assembly, vaccination, castration, and even dehorning (Krehbiel et al., 2003), making decreased performance quite

frequent in these cattle with high death loss. Typically, these stresses and observed outcomes are a result of altered microorganisms in the rumen and lower gut (Williams and Maloney, 1984).

A review composed by Krehbiel et al. (2003) analyzed different research to better understand the effects of DFM's. Seven trials occurring in the eighties were largely in favor of DFM use showing significant benefits. Across these trials, feeding a DFM at processing, throughout the receiving period, or both, resulted in a 13.2% increase in daily gain, a 2.5% increase in feed consumption, 6.3% improvement in feed to gain and a 27.7% reduction in morbidity (Krehbiel et al., 2003). These numbers show tremendous benefit from DFM usage. Gill et al. (1987) conducted a research trial to study the effect of probiotic feeding on health and performance of newly-arrived stocker calves during a 28-day receiving period. This study yielded similar results with a 9.3% increase in daily gain, a 9.5% improvement in feed efficiency and a 10.9% reduction on morbidity (Gill et al., 1987).

However, another 5 studies concluded that the use of DFM's did not increase performance, weight gain, or decrease morbidity in newly weaned or newly received calves (Krehbiel et al., 2003). In fact, Krehbiel et al. (2001) conducted a study using DFM's on 466 newly received calves finding that daily gain did not differ among the group receiving the DFM and the control group. What the 2001 study did discover though, was that calves given the DFM during the first antimicrobial treatment were less likely to be treated a second time within 96 hours and that the number of calves treated twice tended to be lower for calves administered a DFM than calves that were not (Krehbiel et al., 2001).

The results of these 12 trials show similar outcomes to those gathered in young, preweaned calves. Weight gain and feed efficiency is extremely variable and may be affected by many factors. But, overall health and immune response is largely in favor of cattle being treated

with a DFM. Like the neonatal calf, response to the use of a DFM might be greater when newly weaned or receiving cattle are more prone to health problems (Krehbiel et al., 2003). Outliers do still exist however, with extremely sick and extremely healthy calves being unlikely to respond to DFM use (Gill et al., 1987).

Challenges

Development of DFM that are effective over a wide range of ruminant production systems remains challenging because comprehensive knowledge of microbial ecology is lacking with few studies employing molecular techniques to study the interaction of DFM with native microbial communities and therefore advancements in the metagenomics of microbial communities and the genomics of microbial-host interactions may enable DFM to be formulated to improve production and promote health, responses that are presently often achieved through the use of antimicrobials in cattle (McAllister et al., 2011).

Microbiome Research

The bacterial populations that reside in the gut of animals are diverse and numerous with the majority of these bacteria being vital to the maintenance of an animal's health and even minor perturbations in these populations may cause dramatic shifts that can affect livestock productivity (Dowd et al., 2008). This understanding has pushed researchers to uncover whether the microbiome composition in an animal can alter things like weight gain or feed intake. The use of probiotics, prebiotics and competitive exclusion products have been used to try and establish a healthy gastrointestinal flora in animals that can improve animal performance or prevent colonization of the animal with pathogens because these beneficial health effects relate to the ability of these intestinal bacterial populations to supply vital nutrients, convert metabolites and beneficially interact with host cells (Dowd et al., 2008).

A study by Dowd et al. (2008) analyzed fecal samples from a group of dairy cattle that displayed 274 different bacterial species and 142 separate genera, a very high diversity of both. It has been indicated that the microbial population of lower intestinal bacteria of cattle are dominated by strict anaerobes such as *Bacteroides* spp., *Clostridium* spp., and *Bifidobacterium* spp while facultative anaerobes, such as the enterobacteriaceae, are typically reported to occur in numbers at least 100-fold lower than the strict anaerobes (Drasar and Barrow, 1985). Dowd et al. (2008) supported findings in which the predominant genera found in each of the samples were *Clostridium*, *Bacteroides, Porphyromonas, Ruminococcus, Alistipes, Lachnospiraceae, Prevotella, Lachnospira, Bacteroidales, Akkermansia*, and *Enterococcus* spp and that *Clostridium*, *Porphyromonas*, *Bacteroides*, *Ruminococcus*, *Alistipes*, *Lachnospira*, and *Prevotella* spp were consistently very prevalent and found in all of the cattle samples. A study conducted by Malmuthuge et al., (2014) on preweaned bull calves found the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* predominating in the gastroinstestinal tract.

Myer et al. (2015) conducted a study to characterize the microbiome of the cattle rumen among steers differing in feed efficiency and the association of the microbial populations with ADG and ADFI were analyzed in order to determine whether microbial populations differed by low vs. high ADG, low vs. high ADFI, or their interaction. No significant changes in diversity or richness were indicated, and UniFrac principal coordinate analysis did not show any separation of microbial communities within the rumen but, the abundances of relative microbial populations and operational taxonomic units did reveal significant differences with reference to feed efficiency groups (Myer et al., 2015). Bacteroidetes and Firmicutes were the dominant phyla in all ruminal groups, with significant population shifts in relevant ruminal taxa, including phyla

Firmicutes and Lentisphaerae, as well as genera *Succiniclasticum*, *Lactobacillus*, *Ruminococcus*, and *Prevotella* (Myer et al., 2015).

Microbiome of preweaned calves

Bacterial colonization in the gastrointestinal tracts of preweaned calves is very important because it can influence early development, postweaning performance, health and that the gastrointestinal tracts of newborns contain a less diverse microbiome than those of adults, and progressive colonization over time increases this diversity (Malmuthuge et. al, 2014). As a result, Malmuthuge et. al (2014) conducted a study to investigated the composition of the bacteria along the gastrointestinal tract preweaned bull calves using pyrosequencing to understand the segregation of bacteria between the mucosal surface and digesta and reveal that a total of 83 genera belonging to 13 phyla were distributed throughout the gastrointestinal tract of preweaned calves with rumens containing the most diverse bacterial population, consisting of 47 genera, including 16 rumen-specific genera, followed by the large intestine and then the small intestine. The majority of bacteria found on the rumen epithelial surface and within the small intestine could not be identified due to a lack of known genus-level information and thus, future studies will be required to fully characterize the microbiome during the development of the rumens and the mucosal immune systems of newborn calves (Malmuthuge et al., 2014).

Molecular methodologies developed over the past decade now enable researchers to examine the diversity of the gut microflora independent of cultural methods (Dowd et al., 2008). Malmuthuge et al. (2014) conducted quantitative PCR analysis of selected abundant bacterial genera (*Prevotella*, *Bacteroides*, *Lactobacillus*, and *Faecalibacterium*) and revealed that their prevalence was significantly different among the gastrointestinal tract regions and between mucosa- and digesta-associated communities. The new method of bTEFAP is not limited to

detecting organisms via culture methods, and can be used to define what constitutes a healthy or an unhealthy microbiome profile by correlating populations of bacterial species with dietary energy and protein utilization, host growth rate and efficiency, host gene expression, and host immune function (Dowd et. al, 2008). Bacterial 16S rRNA gene amplicons were sequenced from the harvested bovine rumen fluid samples using next-generation sequencing technology in the study conducted by Myer et al. (2015) to suggest the involvement of the rumen microbiome as a component influencing the efficiency of weight gain at the 16S level, which can be utilized to better understand variations in microbial ecology as well as host factors that will improve feed efficiency.

CHAPTER III

MATERIALS AND METHODS

Animals

Heifers utilized in the trial were an accumulation of 2 breeds, Salers and Angus cattle. The heifers within each breed were not full sisters, but were of similar mating and pedigrees. Their breed difference was therefore generalized.

Trial set-up

The trial utilized 179 heifers from the MJB Ranch in Lodge Grass, MT. The heifers were a mixture of Angus and Salers females that had grown on native rangeland while at the side of the cow. Co-mingling took place in a heifer development lot with all heifers being fed the same ration, having availability of water, with general management practices consistent. All heifers received the same series of processing shots and antibiotics prior to being weaned. The shots given were Vision 7 somnus and Vista with a Dectomax pour-on. The heifers were grown and developed on the same ranch with similar forage availability. The heifers were all weaned within a 24-hour period and hauled to the heifer development yard at the MJB Ranch. Cows were left on the original pasture for complete separation. Ad libitum brome grass hay and water was provided to all heifers for the duration of the 4-week trial.

The heifers were spilt into three separate groups to receive: the control, Group A, and Group B. The control group was drenched with 1oz of water. Group A was drenched with 1oz of the DFM and Group B was drenched with 1/2oz of the DFM. The DFM used was a product called Jackpot designed by Bio S.I. Jackpot is a diverse probiotic blend of soil-borne microbes

found naturally in the soil that are digested during grazing. The Jackpot is a brown liquid supplement with an earthy odor and a pH of 7.0 that should be stored away from the sun and at room temperature. Groups were sorted and assigned to treatment in a systematic manner; the first heifer down the chute received the drench with water and every third heifer after that was drenched the same. The second heifer down the chute was drenched with 1oz of the DFM and then every third heifer after that got the same. The third heifer was drenched with 1/2oz of the DFM and then every third heifer after that was also drenched with 1/2oz of the DFM. Heifers received their initial drench on Day 0.

Within the three groups, there were two subgroups split based on breed composition. 26 Angus heifers were split in to the control, Group A, and Group B. 19 Salers heifers were also then split in to one of the 3 groups. The group of 26 Angus heifers and 19 Salers heifers underwent the same systematic method of selection for their drench. This group of 45 heifers was the fecal collection group (FG). The FG was tagged with a numerical yellow tag.

The FG was chosen based on age. All heifers in the FG were born within 2 weeks of one another. This was consistent for both the Angus and Salers group. The 26 selected Angus heifers were sorted from the group of 179 head and processed first. The Angus FG group was worked down the chute and drenched using the systematic sample sorting technique listed above. Each heifer in the Angus FG group was tagged, drenched, weighed, and had a fecal sample collected. The Salers FG group was then sorted and processed systematically being tagged, drenched, weighted and had a fecal sample collected.

The fecal samples were collected with a standard, plastic AI glove straight from the rectum of each heifer. Fecal samples and weights were taken on the same day every week and within a 3 hour window in the morning. Heifer identification, breed, and date were recorded for

each fecal sample. Fecal samples were collected and then place immediately in a cooler with dry ice for quick freezing. Fecal samples were transported in the cooler with dry ice from the ranch in Montana to ARDEC at Colorado State University where they were stored in deep chest freezers. All fecal samples were shipped with wet ice. All fecal matter was tested at the same lab. Health issues were treated with vaccine designed for each illness, regardless of treatment group.

During the study, researchers were unable to collect fecal samples from 3 Angus heifers and 1 Salers heifer. These 4 heifers continued to get weighed every 7 days with the fecal group and had visual fecal inspection done, but no fecal samples were collected during the trial on those 4.

Three heifers in the non-FG group were shipped and sold prior to the completion of the trial due to a ranch management decision. The data from those 3 heifers was removed. The remaining 134 heifers in the non-FG were then processed down the chute. Each heifer was systematically drenched and an initial weight was recorded. A final head count of 172 heifer calves was the number used in the statistical analysis.

Limitations

Heifers were developed on a free choice, grass hay ration during the trial. Total consumption was not controlled, nor monitored. Grass hay intake prior to each weight and fecal collection was not monitored. Water was available ad libitum. Daily water consumption and water consumption prior to each weight and fecal collection was not monitored. Variability in age did exist with heifers ranging from 5 to 8 months old.

Weeks 1 & 2

An Angus heifer in the Angus FG group was found dead on day 6. Her data was removed from the trial. Only heifers in the FG were processed on day 7. Both the Angus FG and the

Salers FG were worked through the chute, weighed, and had a fecal sample taken that was froze on dry ice. Researchers were unable to locate 1 Angus FG heifer and her data from the first collection was eliminated. Fecal samples were collected on 22 Angus heifers and 18 Salers Heifers. Weights were collected on 44 heifers.

Day 14

On day 14, the 44 heifers from the FG were processed in down the chute receiving the same drench they had received on day 0. The weights were collected on all 44. Fecal samples were collected from 22 Angus heifers and 18 Salers heifers that were frozen in dry ice. Visual evaluation of the fecal sample was assessed on day 14 as heifers from the FG group where processed through the chute. FG heifers were deemed to have solid or loose visual fecal matter. All 44 heifers in the FG were evaluated.

The 134 heifers in the non-FG were also reprocessed down the chute and drenched with the same drench they received on day 0. Weights were taken and recorded on each heifer in the non-FG. No visual fecal matter evaluation was performed on the non-FG group on day 14.

Day 21

The FG was worked on day 21. Fecal samples were collected on 22 Angus heifers and 18 Salers heifers. The 40 fecal samples were then froze in the dry ice. Weights were taken and recorded on all 44 heifers in the FG.

Day 28

The FG was worked first on day 28. Fecal samples were collected on 22 Angus heifers and 18 Salers heifers. The 40 fecal samples were then froze in the dry ice. Weights were taken and recorded on all 44 heifers in the FG. Visual fecal evaluation was once again performed on day 28 as heifers from the FG where processed through the chute. All 44 heifers in the FG were

evaluated for loose or solid fecal matter. The 134 heifers in the non-FG were worked down the chute and weighed.

Processing the Fecal Samples

Fecal samples were collected form the FG on day 0, 7, 14, 21, and 28. All fecal samples were frozen immediately in dry ice, transported to ARDEC at CSU and stored in deep chest freezers. After the trial, the fecal samples were shipped with wet ice to the University of North Texas Health Science Center in Fort Worth, TX. Metagenomics analysis of the fecal samples was conducted at the University of North Texas by Yan Zhang and Michael Allen.

DNA Isolation

Whole genomic DNA was extracted from approximately 100 mg fecal material using a MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) with modifications [1]. Briefly, fecal slurries (1:1 feces/water) were centrifuged at 16,000 x g for 10 minutes and the supernatant was removed. The pellet was transferred to the MO BIO bead beating tube with buffer, vortexed to resuspend the pellet, and heat-treated at 65 °C for 10 minutes and then 95 °C for 10 minutes. Bead beating was performed on the MO BIO Vortex-Genie 2 for 10 min, then the standard protocol in the manufacturer's instructions was followed for the remaining DNA extraction procedure. DNA was eluted with 50 µl C6 buffer.

16S rRNA gene Amplicon Library Preparation and Illumina MiSeq sequencing

The 16S rRNA gene was amplified using universal bacterial primers targeting the V4 hypervariable region as previously described. The primers were modified to contain paired-end Illumina adapter region for sequencing on the Illumina MiSeq platform. Each sample was prepared in duplicate 25 µl PCR reactions containing AccuPrime™ PCR Buffer II, 200µM dNTPs, 1 U AccuPrime™ Taq DNA Polymerase High Fidelity (Life Technologies, Carlsbad,

CA), 0.2 μM each of forward and reverse primers, and ~10 ng genomic DNA. PCR program steps are: denaturation at 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 20 sec, annealing at 52 °C for 40 sec, extension at 68 °C for 40 sec; and a final extension at 68 °C for 5 min. PCR products were examined following electrophoresis on a 1.5% agarose gel and remaining volumes of duplicate PCR were combined and purified using the Agencourt AMPure according to respective manufacturers' instructions (Beckman Coulter Inc, Brea, CA). Index PCR were performed with AccuPrime™ Taq DNA Polymerase and Nextera® XT Index Kit v2 (Illumina, San Diego, CA) to ligate specific barcodes to each sample as per the Illumina protocol. The amplified libraries were purified using the Agencourt AMPure magnetic beads and quantified using the Quant-iT™ PicoGreen® dsDNA Assay (Thermo Fisher Scientific Inc). Equal amounts of each amplicon libraries were pooled together and quantified using Qubit® high-sensitivity assay (Thermo Fisher Scientific, Inc). The pooled amplicon library was then diluted to 4 nM, denatured and further diluted to 10 pM following the MiSeq loading protocol. The final library was spiked with 5% Phi X control library, loaded into a MiSeq v2 cartridge and sequenced on Illumina MiSeq instrument (Illumina, San Diego, CA).

Sequence Analysis

Sequences generated from the MiSeq were processed using MiSeq SOP through mothur v.1.32.1 as previously described. Operational taxonomic units (OTUs) were assigned with the average neighbor clustering algorithm based on 97% sequence similarity. Taxonomic classification was conducted using the Greengenes database with a minimum of 80% confidence. Sequences classified as mitochondria, chloroplast, archaea and eukaryote, as well as unknown sequences, were removed from the data set.

To investigate differences in microbial community diversity among sampling locations, both α- diversity and β-diversity were calculated. Diversity indices (Shannon diversity and evenness) and richness (Chao1 and abundance coverage-based estimator (ACE) estimators were generated based on OTU grouped at 97% sequences similarity for species-level classification. β-diversity in different gastrointestinal tract locations was investigated using UniFrac distances and principle coordinate analysis (PCoA) [8]. Diversity estimators, UniFrac, and Principal Coordinate Analysis (PCoA) were performed using mothur.

Permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were performed to determine whether the effects of time changes or probiotic treatment on bacterial and community composition were statistically significant. PERMANOVA and ANOSIM were performed on Bray-Curtis similarity matrices constructed using family level relative abundance data through PAST program. ANOSIM generates a value of R measuring how separate groups are reviewed by Clark. $R = 1$ means significantly different; $R = 0$ means no difference; $R > 0.75$ suggests good separation; $R > 0.5$ indicates differences with some overlapping; and $R < 0.25$ means almost no differences.

General Health

14 heifers were treated during the duration of the trial for non-trial related illnesses. 12 heifers contracted respiratory illness signs and were treated with NuFlor Gold. One heifer contracted a lump on her shoulder. The lump was lanced, drained, and disinfected. The heifer was then given a dose of LA 200. Penicillin was used to treat a heifer that had signs of a bacterial pneumonia.

Three of the 14 heifers that were treated for illness were in the Salers FG group. One Salers FG heifer from the control and 1 Salers FG heifer from Group B were each treated with

NuFlor Gold for signs of a respiratory illness. The 3rd Salers FG heifer was from Group A and was the heifer that received the penicillin.

Animal Welfare

All cattle used in this study were handled as described in the CSU Animal Care and Use Protocol. Cattle were handled slowly and with care.

Statistical Analysis

A mixed procedure model in SAS was used for the statistical analysis. The dependent variable was set as total weight gain. The degrees of freedom method was residual. There were 2 class levels: one for the three treatment groups and 1 for the 2 breeds. Treatment group was broken into 3 levels set at values of 0, 0.5, and 1. Treatment level with a value of 0 represented the treatment group that was drenched with water. Treatment level with a value of 0.5 represented the treatment group that was drenched with ½ an ounce of the DFM. Treatment level with a value of 1 represented the treatment group that was drenched with an entire ounce of the DFM. Breed was broken down in to 2 levels represented by an "a" for heifers that were Angus and an "s" for heifer that were Salers.

A type 3 tests of fixed effects was run to establish an ANOVA table for the full model. The type 3 tests of fixed effects ANOVA table ran p-values for treatment group, breed, and also for the interaction between treatment and breed. Degrees of freedom was set at 2 for treatment, 1 for breed, and 2 for the interaction between treatment and breed. Alpha values were set at .05 establishing a 95% confidence interval.

A second type 3 test of fixed effects ran an ANOVA table that was used as the final model to analyze the p values of the 2 classes' treatment and breed. Degrees of freedom for the treatment class was set at 2. Degrees of freedom for the breed class was set at 1. Least squares

means established a model based mean estimate. Using the mean estimate, the upper and lower limits established a confidence interval.

CHAPTER IV

RESULTS

The analysis variable for total gain with an N of 172 heifers yielded a mean of 25 with a standard deviation of 20.93 having a minimum of -35 and a maximum of 77 (table 1). Across all treatment levels and between the 2 breeds, the average weight gain was 11.34 kilograms (kg) over the course of the study.

Table 1:

Analysis Variable : Totalgain

N	Mean	Std Dev	Minimum	Maximum
172	25.0058140	20.9336175	-35.0000000	77.0000000

Results shown from the full model ANOVA, which included the interaction between treatment and breed, resulted in a p-value of .649 showing an interaction that is not significant (table 2). The final model ANOVA resulted in a p-value of .9689, concluding no significant difference between the 3 treatment levels (table 3). The p-value in the final model ANOVA table from breed had a p-value of .0574. The p-value of .0574 for breed shows that a difference between breeds is approaching significance.

Table 2:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	166	0.01	0.9858
Breed	1	166	3.63	0.0585
Treatment*Breed	2	166	0.43	0.6490

Table 3:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	168	0.03	0.9689
Breed	1	168	3.66	0.0574

Treatment 0 had a model based mean estimate of 24.6166 with a confidence interval of 19.1318 to 30.1014 (table 4) and the p-value for treatment 0 was .8972 (table 5). Treatment 0.5 had a model based mean estimate of 25.1258 with a confidence interval of 19.6966 to 30.5551 (table 4) and the p-value for treatment 0.5 was .8017 (table 5). Treatment 1 had a model based mean estimate of 25.6071 with a confidence interval of 20.0970 to 31.1173 (table 4) and the p-value for treatment 1 was .9024. The p-values for treatment 0, treatment 0.5, and treatment 1 show that the difference between the means is not significant.

Breed “a” had a model based mean estimate of 22.0215 with a confidence interval of 17.6123 to 26.4301 (table 5). Breed “s” had a model based mean estimate of 28.2115 with a

confidence interval of 23.6562 to 32.7668 (table 4). The p-value for breed "a" and "s" was .0574 meaning that the difference between breeds is approaching significant (table 5).

Table 4:

Least Squares Means

Effect	Breed	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
Treatment		**0**	24.6166	2.7783	168	8.86	<.0001	0.05	19.1318	30.1014
Treatment		**0.5**	25.1258	2.7501	168	9.14	<.0001	0.05	19.6966	30.5551
Treatment		**1**	25.6071	2.7911	168	9.17	<.0001	0.05	20.0970	31.1173
Breed	**a**		22.0215	2.2334	168	9.86	<.0001	0.05	17.6123	26.4307
Breed	**s**		28.2115	2.3074	168	12.23	<.0001	0.05	23.6562	32.7668

Table 5:

Differences of Least Squares Means

Effect	B	T	B	T	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
Treatment	**0**		**0.5**		-0.5093	3.9360	168	-0.13	0.8972	0.05	-8.2796	7.2611
Treatment	**0**		**1**		-0.9906	3.9381	168	-0.25	0.8017	0.05	-8.7652	6.7841
Treatment	**0.5**		**1**		-0.4813	3.9183	168	-0.12	0.9024	0.05	-8.2168	7.2542
Breed	**a**		**s**		-6.1900	3.2348	168	-1.91	0.0574	0.05	-12.5761	0.1961

Overview of sequencing results

After filtering the short low quality sequences and chimera sequences, Illumina MiSeq generated 7,543,814 high-quality sequences with an average length of 252 bp (mean ± standard error

53,125 ± 12,292 per individual sample; n = 142). Total 25 bacterial phyla were identified, of which 91.0% of sequences were assigned to three bacterial phyla: Firmicutes (71.2%), Bacteroidetes (13.3%), and Tenericutes (6.4%). Actinobacteria, Proteobacteria and other rare phyla with relative abundance less than 1% accounted for 1.5%, 1.3%, and 1.7% of total sequences. 4.5% sequences were not able to be assigned to known bacterial phyla. At deeper phylogenetic levels, 61.8% and 26.1% were classifiable to known bacterial families and genera. Gastrointestinal microbiome taxonomic composition at different phylogenetic levels were shown in Figs. 1-4.

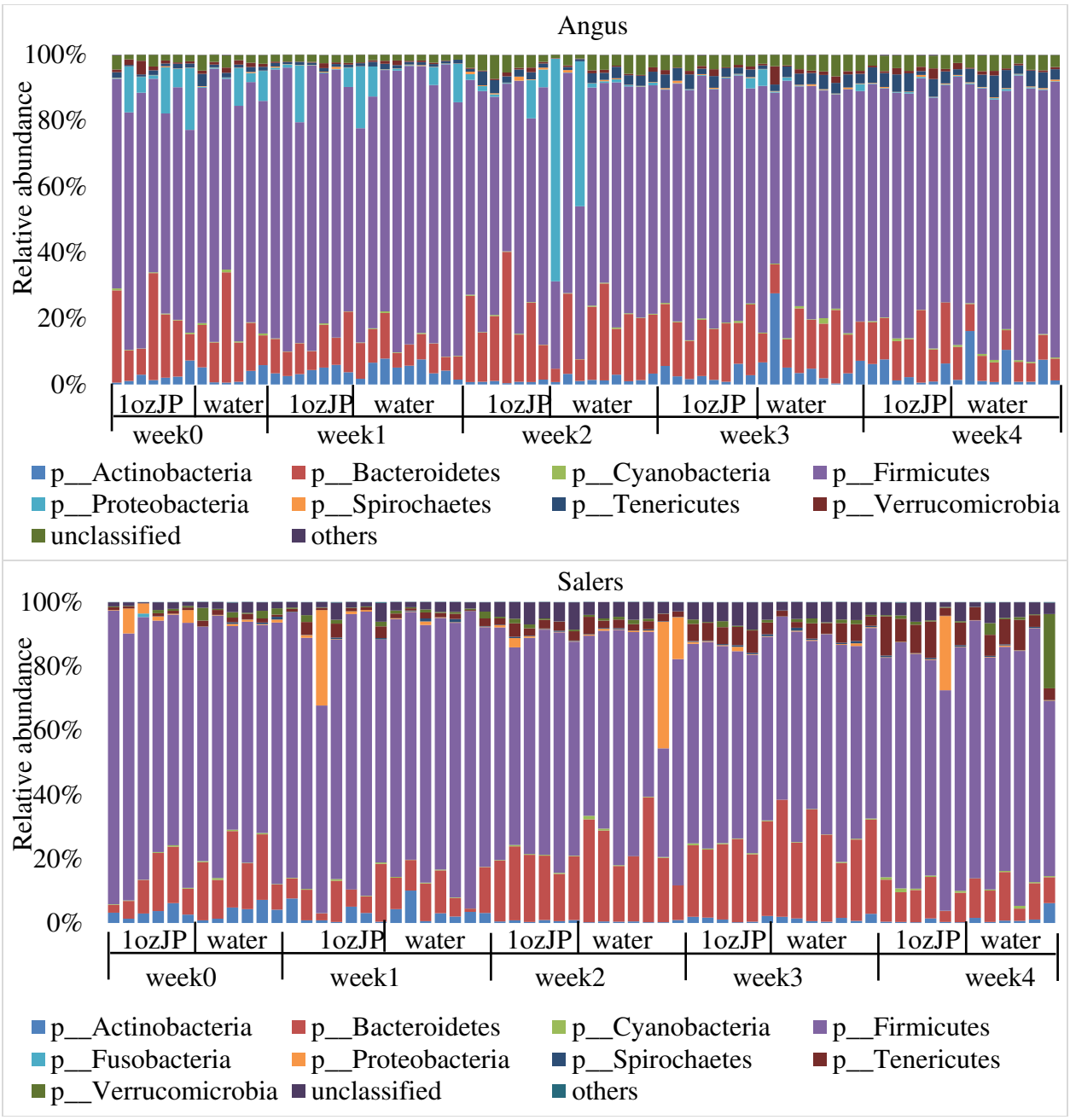


Fig. 1. Phylum-level microbial community changes. Only phyla with >1% relative abundance are shown.

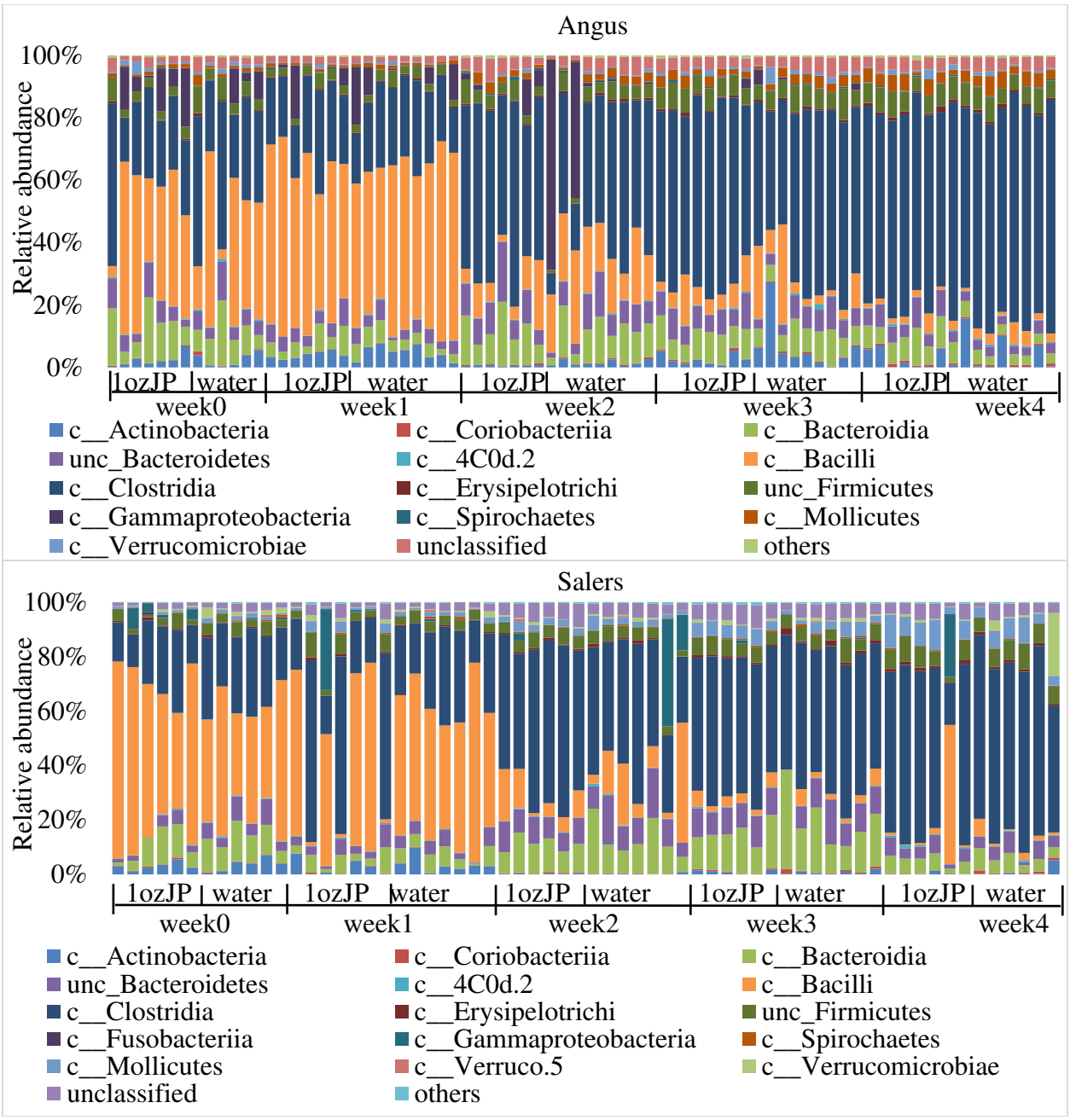


Fig. 2. Class-level microbial community changes. Only classes with >1% relative abundance are shown.

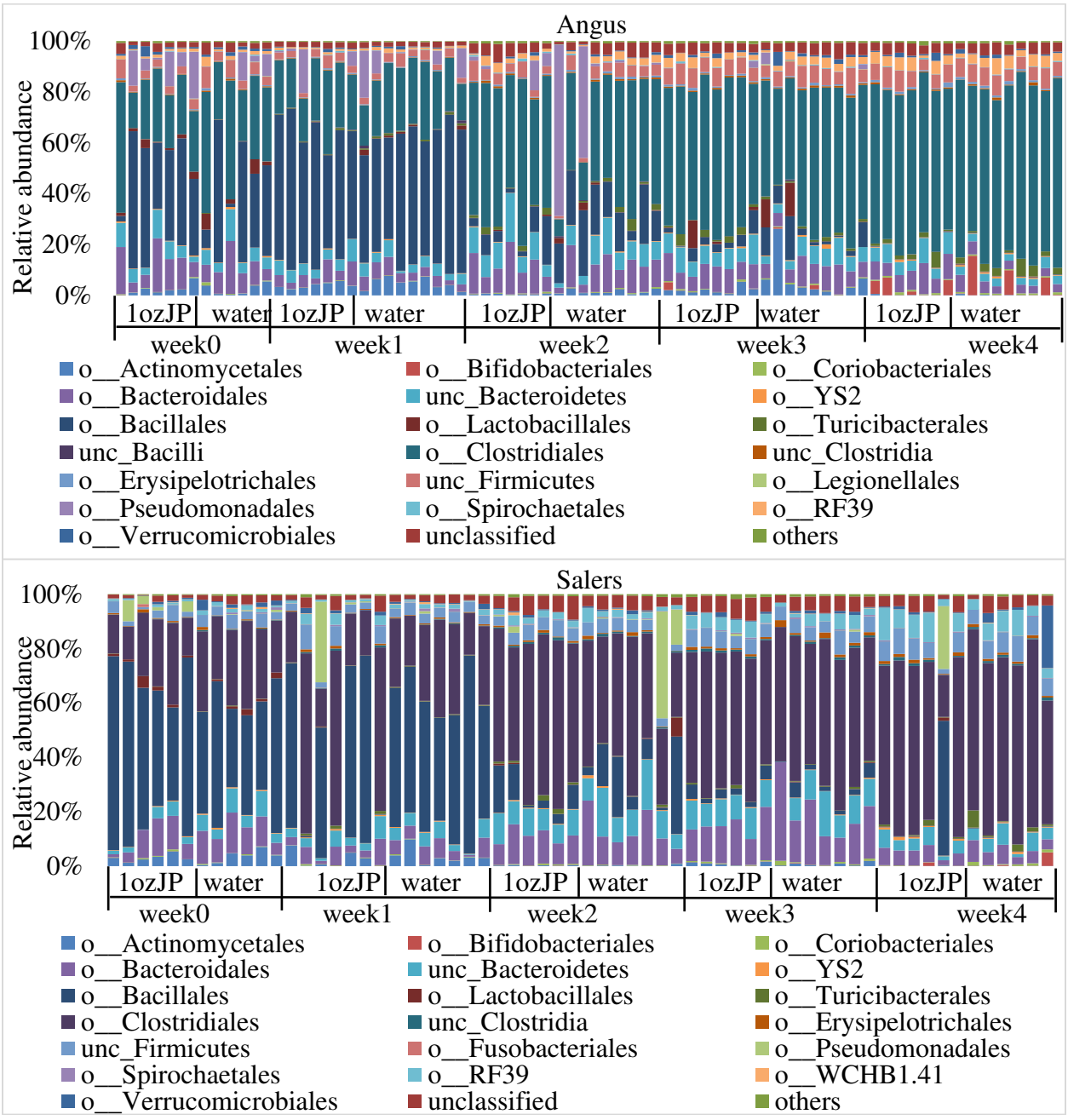


Fig. 3. Order-level microbial community changes. Only orders with >1% relative abundance are shown.

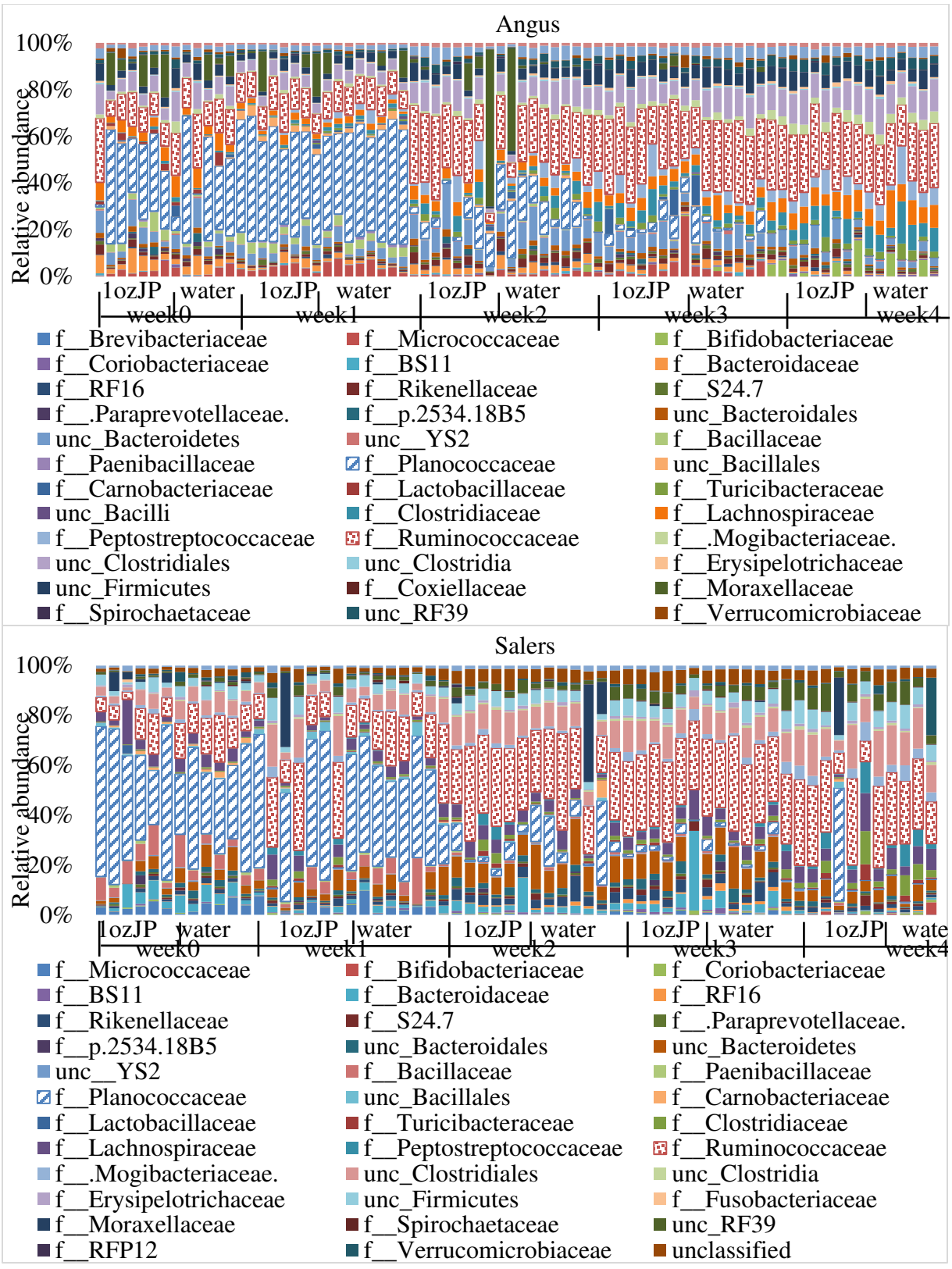


Fig. 4. Family-level microbial community changes. Only families with >1% relative abundance are shown.

Effect of time and probiotic treatment on cattle gastrointestinal microbial diversity

Two-way PERMANOVA analyses suggested that probiotic treatments did not have significant changes in microbial diversity; however, time changes had significant effects on microbial diversity (Table 6) for both Angus and Salers cattle. Both probiotic treatments and time changes showed significant changes in microbial richness of Angus cattle. For Salers cattle, time and interactions of time and treatment showed significant effects on microbial richness. The microbial diversity and richness changes were shown in Fig. 5.

Table 6: Two-way PERMANOVA of gut microbial diversity and richness associated with time and probiotic treatment (p-value based on 9999 permutations). Bold text indicates a statistically significant result ($p <= 0.05$).

		Shannon		ACE		Chao1	
		F	p	F	p	F	p
Angus	treatment	0.95631	0.3003	3.6455	**0.0459**	5.7064	**0.0116**
	time	9.8307	**0.0001**	7.6807	**0.0001**	6.3003	**0.0001**
	Interaction	-1.3695	0.5517	0.51517	**0.0416**	0.68554	**0.0276**
Salers	treatment	0.067902	0.7966	0.86424	0.3505	2.2897	0.1207
	time	9.0902	**0.0001**	13.443	**0.0001**	16.336	**0.0001**
	Interaction	0.74998	0.1472	1.5057	**0.0487**	2.3093	**0.0129**

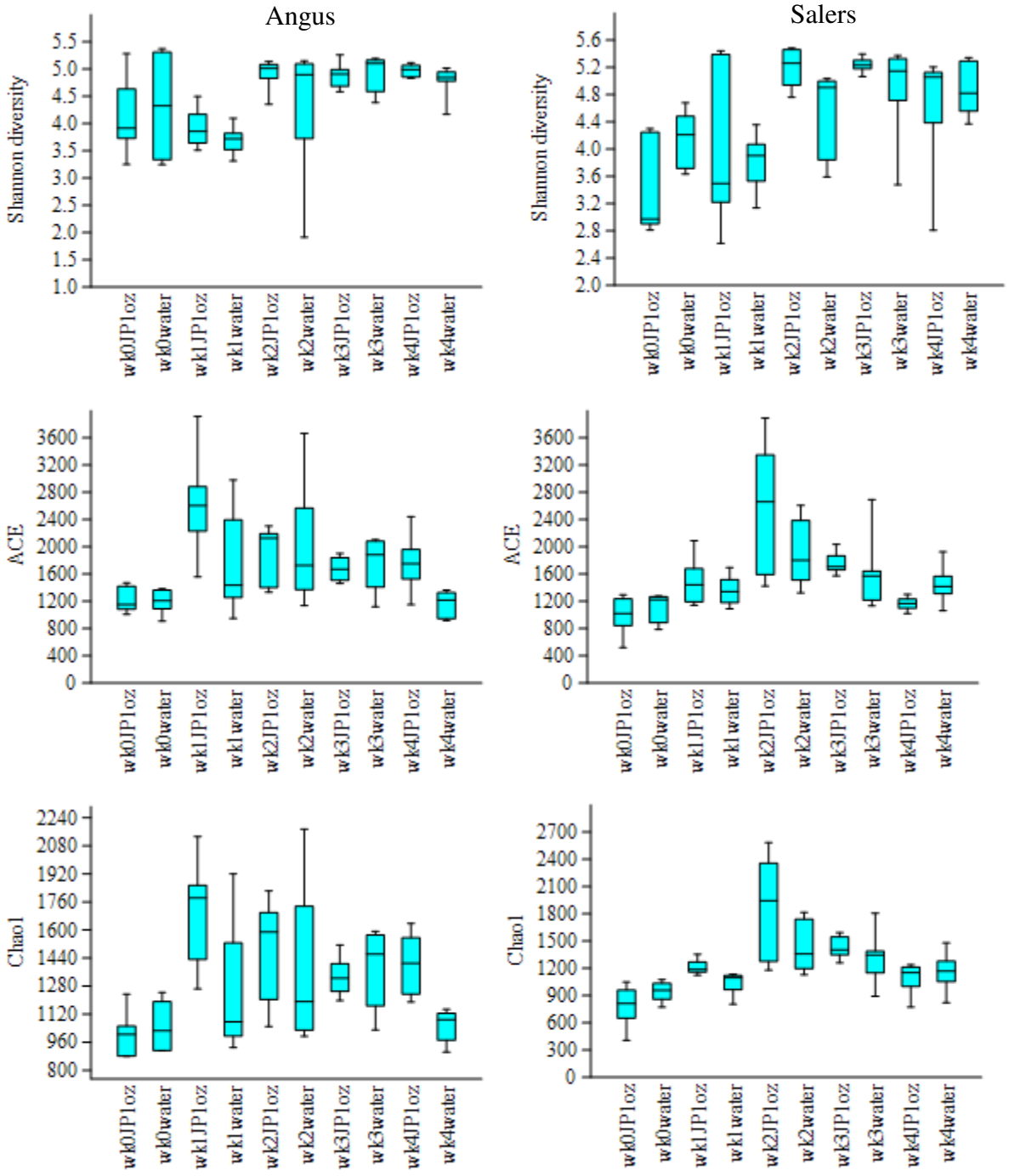
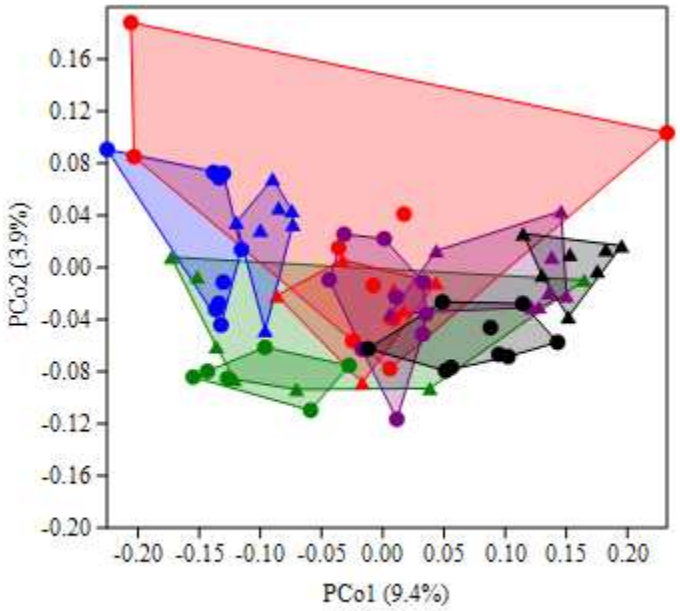


Fig. 5. Changes in microbial diversity and richness.

Effect of time and probiotic treatment on cattle gastrointestinal microbial community composition

PCoA analyses based on unweighted UniFrac distances showed that gut microbial communities grouped by time rather than probiotic treatment (Fig. 6). The probiotic treatment samples barely separated from the control at each time point except for the Angus samples at week4. Samples in week1 clearly separated from week3 and week4 in Angus. Samples in week0 and week1 grouped together and clearly separated from week3 and week4 in Salers cattle. PCoA analyses based on weighted UniFrac distances also revealed that gut microbial community composition shifted by time rather than probiotic treatment (Fig. 7). Family-level PERMANOVA and ANOSIM analyses suggested that microbial communities significantly changed over time. However, probiotic treatments did not lead to significant microbial community shifts (table 7). Pairwise comparisons of microbial community similarity are summarized in Table 8.

Angus



Salers

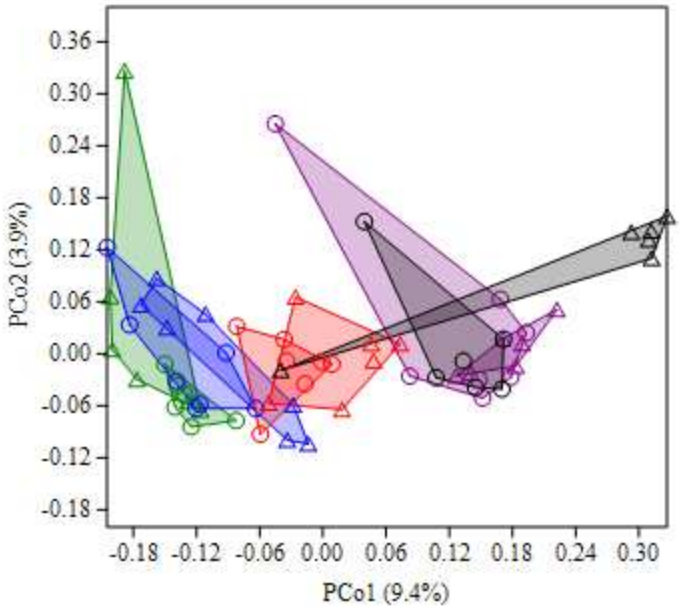


Fig. 6. Principal coordinate analysis (PCoA) based on unweighted UniFrac distances showed that gut microbial community differences associated with time and probiotic treatment. Triangles and dots represent treated and control groups, respectively. Green, blue, red, purple, and black represent week0, week1, week2, week3, and week4, respectively.

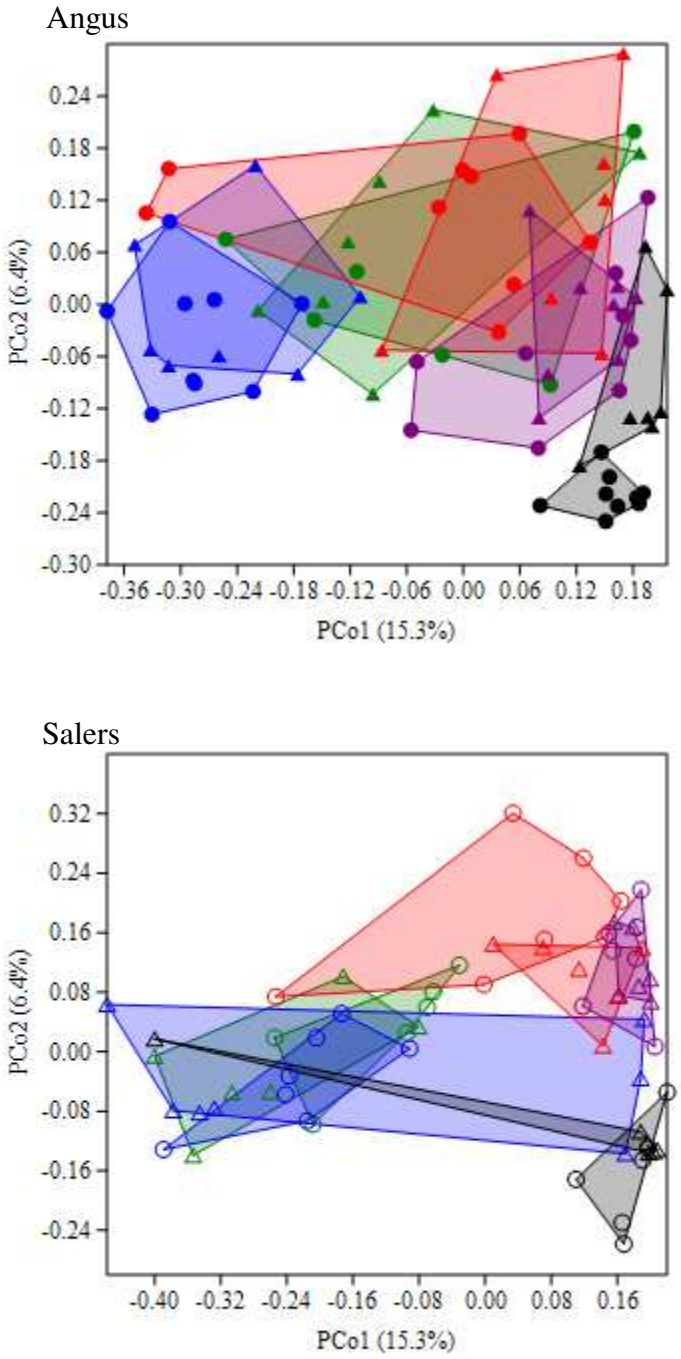


Fig. 7. Principal coordinate analysis (PCoA) based on weighted UniFrac distances showed that gut microbial community differences associated with time and probiotic treatment. Triangles and dots represent treated and control groups, respectively. Green, blue, red, purple, and black represent week0, week1, week2, week3, and week4, respectively.

Table 7: Summary of PERMANOVA and ANOSIM. Bold text indicates a statistically significant result (p <= 0.05).

		Angus		Salers	
Two-way PERMANOVA		F	p	F	p
	treatment	2.0551	0.0849	0.28793	0.8285
	time	22.65	**0.0001**	13.677	**0.0001**
	Interaction	-0.75342	0.1545	0.31856	0.2193
Two-way ANOSIM		R	p	R	p
	treatment	0.044002	0.1371	0.076494	0.0553
	time	0.62086	**0.0001**	0.4924	**0.0001**

Table 8. Pairwise comparison of microbial community similarity. Bold text indicates a statistically significant result (Bonferroni corrected p <= 0.05).

Angus									
R-value\p-value	0JP1oz	0water	1JP1oz	1water	2JP1oz	2water	3JP1oz	3water	4JP1oz	4water
wk0JP1oz		1	0.4995	0.072	0.153	0.4185	**0.0225**	**0.0135**	**0.0045**	**0.0045**
wk0water	-0.07011		0.972	0.1125	0.279	1	**0.045**	0.135	**0.036**	**0.0315**
wk1JP1oz	0.2459	0.2381		1	**0.0225**	**0.0225**	**0.0135**	**0.009**	**0.036**	**0.0135**
wk1water	0.3918	0.3922	-0.0298		**0.0045**	**0.0045**	**0.0135**	**0.0045**	**0.009**	**0.009**
wk2JP1oz	0.6093	0.5132	0.967	0.9827		1	1	1	0.216	**0.0135**
wk2water	0.3189	0.2643	0.5383	0.6461	0.1523		**0.036**	0.1665	**0.027**	**0.0045**
wk3JP1oz	0.7573	0.6657	0.9993	1	0.03936	0.3186		1	1	**0.009**
wk3water	0.7483	0.5352	0.9688	0.9901	0.1133	0.2322	-0.01259		0.8595	**0.009**
wk4JP1oz	0.8183	0.7011	1	1	0.3411	0.4893	0.1676	0.2153		1
wk4water	0.9109	0.8264	1	1	0.677	0.6409	0.5447	0.5295	0.1802	
Salers										
R-value\p-value	0JP1oz	0water	1JP1oz	1water	2JP1oz	2water	3JP1oz	3water	4JP1oz	4water
wk0JP1oz		1	1	1	0.1125	0.0765	0.108	**0.045**	0.2385	0.081
wk0water	0.1741		1	1	0.099	0.108	0.108	**0.0315**	0.4005	0.162
wk1JP1oz	0.1257	0.1601		1	1	1	1	1	1	1
wk1water	0.2513	0.1376	0.2002		**0.0315**	0.054	**0.018**	**0.045**	0.072	**0.0495**
wk2JP1oz	0.9352	0.9685	0.1825	0.9775		1	1	1	0.1935	0.072
wk2water	0.7354	0.5357	0.1905	0.6589	0.04894		1	1	0.5805	**0.0225**
wk3JP1oz	0.9611	0.9963	0.3042	0.9974	0.2111	0.2156		1	0.0765	0.117
wk3water	0.9405	0.8664	0.2886	0.9291	0.0291	0.09232	-0.03704		0.2205	0.1035
wk4JP1oz	0.7722	0.6556	0.1362	0.7354	0.4278	0.3016	0.5759	0.3135		1
wk4water	0.9981	0.9963	0.2817	0.9921	0.4407	0.4775	0.4889	0.3717	-0.0037	

CHAPTER V

DISCUSSION

Conclusions

Overall, time proved to be the predominant driver of GI microbial composition. Probiotic supplementation, regardless of treatment, did not lead to significant changes in the microbiome community structure during the course of these experiments. A wide variety of extenuating circumstances could have caused the lack of response. The change in diet from grazing on pasture to being fed hay, as well as altering the environment that the heifers were living in may have been factors. Simple changes in the gut microbiome due to development could also have played a role in minimizing the effect of probiotic treatment.

Family-level PERMANOVA and ANOSIM analyses may have shown that probiotic treatments did not lead to significant microbial community shifts but, estimates based on Chao1 and ACE models indicate significant impact of probiotic treatment on microbial richness. This indicates that supplementation leads to greater diversity and, in turn, may mean a more robust microbiome resistant to dysbiosis. The results from this study may be have been influenced if the treatments would have been administered more frequently, such as once a week or once every day. Because probiotics are not established in the gut, continual supply to the animal would increase the opportunity for benefit. There is potential that females treated with 1oz of the DFM daily may have seen a greater weight gain that those in the control. Further analyses would be required to fully address this issue. Other expanse on this study could include giving the heifers their first treatment dose at the time they were given their preweaning vaccinations to elongate

the amount of they were on the DFM and to increase the overall dose of the DFM. If heifers had already been receiving the beneficial bacteria prior to being weaned, there may have been a more significant change in weight gain. Additionally, it should be noted that 2-way PERMANOVA results of treatment in Salers cattle indicated a p-value of 0.0553, which is just outside of the $p < 0.05$ cutoff usually applied as a test of significance. This value is sufficiently close to warrant additional investigation to further clarify the impact of product addition in these cattle.

Visual analysis of fecal matter was collected on the FG heifers on collection 3 and 5 and is displayed in tables 9 and 10 respectively. Unfortunately, the identification number of each heifer was not collected to coincide with the difference between clean/solid fecal samples versus dirty/loose fecal samples. Since records were not kept on heifer identification in relation to visual fecal matter display, no statistical analysis was able to be conducted on the data that was collected. This is regrettable because we were not able to demonstrate whether cattle treated with a DFM recovered from diarrhea more quickly and/or had less incidence of diarrhea to begin with. It was noted that as a group, cattle treated with the DFM had less diarrhea on fecal collection days than did cattle from the control group but, additional research would need to be conducted to track this on a per animal basis.

Table 9:

Fecal Collection #3 10/6/2015

Treatment	Clean/Solid Fecal	Dirty/Loose Fecal
Water - 16 head	4	12
1/2 oz - 14 head	8	6
1 oz - 14 head	12	2

Table 10:

Jackpot Trial Fecal Collection #5 10/20/15

Treatment	Clean/Solid Fecal	Dirty/Loose Fecal
Water - 16 head	3	13
1/2 oz - 14 head	7	7
1 oz - 14 head	13	1

Implications

If the most significant impact is indeed the duration of time that the DFM needs to be administered, with longer being favored, than producers could shift to the more traditional methods of application such as having the DFM in the free choice drinking water or top dressing on the feed. There is a chance that drenching the cattle with the DFM will never provide a steady enough supply of beneficial bacteria to the gastrointestinal tract. Further research would need to be conducted with this product to understand if the outcome would have been altered if the product was offered ad libitum. If a steady supply did indeed allow for greater weight gain and a more resilient microbiome, producers would have an option to ease the weaning process.

The indication of greater diversity and a more robust microbiome in heifers treated with the DFM could have a major industry impact with further study. Recognizing that stress and environmental shifts often lead to at least a temporary lack of beneficial bacteria in the gastrointestinal tract means that there is opportunity for a DFM to positively impact the microbiome if given over a longer period of time or in a larger dose. If producers had a product that would allow their weaned cattle to recover more quickly or be less susceptible to dysbiosis, the results would be incredible. Especially when considering that there is also potential for a DFM to decrease the incidence of diarrhea. Inevitably, when an animal has severe diarrhea that only adds to the stress load of that animal and further amplifies a negative gastrointestinal balance. By eliminating diarrhea all together, or at least decreasing its severity, there would be a

greater chance at maintaining a stable and vigorous microbiome. In turn, this could lead to weight gain, decrease in sickness, faster/shorter weaning period, or a quicker transition to a weaned diet. All very beneficial options for producers.

Probiotic supplementation to beef and dairy cattle with a DFM is quite variable. Little common ground exists in the research results which makes any definitive conclusion on the effectiveness of these DFM products very difficult. Further research needs to be conducted to account for the variability in organism type, diet of the animal, age, overall health and stress. The one thing that does appear to be constant over the research that has been done however, is that there does not appear to be any negative effects from the use of DFM's.

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APPENDIX

SUPPLEMENTAL TABLES AND FIGURE

Table 11:

Class Level Information

Class	Levels	Values
Treatment	3	0 0.5 1
Breed	2	a s

Figure 8:

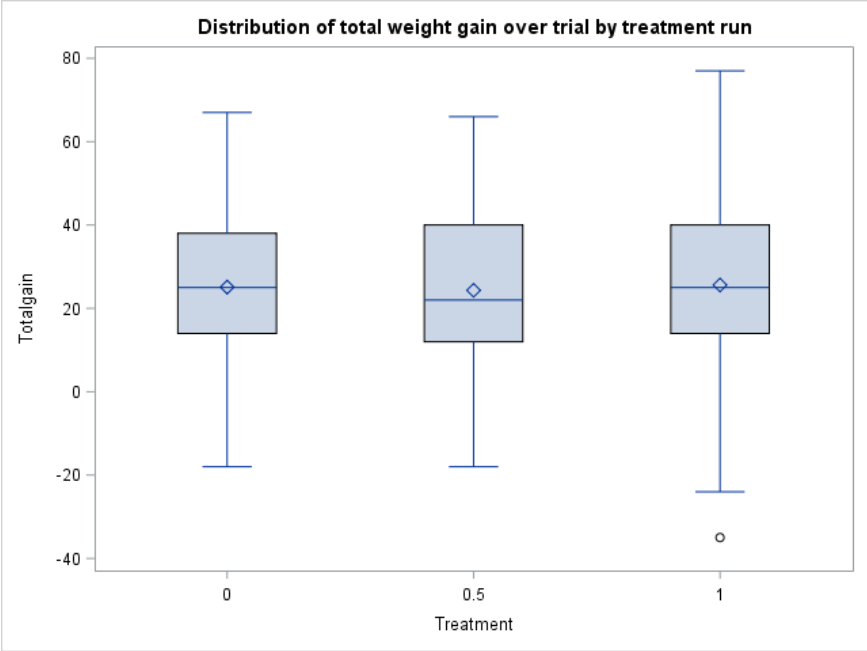

Figure 9: