EFFECT OF PROBIOTIC ADMINISTRATION ON DIARRHEA INCIDENCE, SEVERITY, AND THE FECAL MICROBIOME IN NEONATAL FOALS

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ABSTRACT

An experiment was conducted to investigate the effects of probiotic supplementation on diarrhea incidence and severity in neonatal foals, and to characterize the bacterial population in the neonatal gut related to "foal heat" diarrhea. Twenty-four stock-type newborn foals were used in a completely randomized design. Foals were enrolled in the trial within 24 h of age and randomly assigned to treatment; control (C) or probiotic (P). Animals assigned to P received 6 g of Pro Biostatin® containing 500 $\times 10^6$ cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 orally twice daily for 14 d. The trial began in February and ended in June, 2016, with each foal enrolled from birth to 28 d of age. Fecal samples were collected from foals at d 0, 7, 14, 21, and 28 and a corresponding mare fecal sample was collected on d 28 by the consulting veterinarian or trained farm staff. Blood was collected on d 0, 7, 14, 21, and 28 and analyzed using IDEXX ProCyte Dx® Hematology Analyzer (IDEXX Laboratories, Westbrook, Maine) to determine complete blood count variables. Additionally, health observations were made daily and foal feces were scored using a 3-point system (0 to 2) based on severity. Fecal samples were sent to the University of Arkansas Animal Science lab for DNA sequencing and microbiome analysis.

Diarrhea was observed in 95% of foals throughout the study. There was a trend for a main effect of treatment on the incidence of diarrhea in neonatal foals ($P = 0.105$). A

numerical difference was observed in the length of diarrhea bouts, which decreased with subsequent diarrhea events. There was no effect of probiotic treatment on diarrhea severity in neonatal foals (no diarrhea, $P = 0.522$; mild diarrhea, $P = 0.267$; and severe diarrhea, $P = 0.333$). However, foals developed severe diarrhea within the first 14 d postpartum. There was a treatment × day interaction for certain CBC variables; RBC ($P = 0.024$), and hemoglobin ($P = 0.049$) concentration, hematocrit percentage ($P = 0.037$), and platelet volume ($P = 0.012$). Additionally, there was a main effect of d on all red blood cells (RBC, $P = <.0001$) and all white blood cells (WBC, $P = 0.0053$) except lymphocyte concentration. The predominant bacteria phylum identified in the feces was *Firmicutes* and *Bacteroidetes*. The trend was similar for all animals. As foals matured, bacterial community structure and diversity also became more enriched and began to approach a similar community structure as their dam. Results from this study indicate that administration of a probiotic containing 500×10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 had a tendency to decrease diarrhea incidence but did not significantly affect diarrhea severity in neonatal foals. Future research is needed to further elucidate the role of the intestinal microbiome in neonatal foal heat diarrhea and potentially identify novel bacteria candidates that may have beneficial probiotic effects.

Key Words: foal heat diarrhea, *Kluyveromyces fragilis*, microbiome, probiotic, *Saccharomyces cerevisiae*

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CHAPTER I

INTRODUCTION

Preventive measures to treat foals that develop diarrhea in their first few months of life are currently limited and innovative approaches are needed. In foals up to 6 mo of age, diarrhea is a serious concern for owners and veterinarians. Almost all foals develop transient diarrhea within the first weeks of life (John et al., 2015). Diarrhea can lead to many deteriorating conditions such as dehydration, electrolyte imbalances and potentially fatal infections. Diagnosing the cause of diarrhea in foals can be difficult because of the myriad of potential factors. Effective diagnosis depends on knowledge of the most common pathogens, the availability, specificity and sensitivity of diagnostic tests, and interpretation of test results, which can be time consuming (Dunkel and Wilkis, 2004). Neonatal foals can develop sepsis, bacteremia, septic joints, and the gastrointestinal barrier can be compromised (Beard, 2009). The intestinal microflora is a crucial line of resistance against colonization by exogenous microbes, therefore the problem of tissue invasion by pathogens is highly relevant to prevent (John et al., 2015).

The use of probiotics for farm animals has increased considerably over the last 15 yr (Chaucheyras-Durand and Durand, 2010). The most significant effects of probiotics have been reported when they have been included in the diet of animals during particularly stressful periods for the gut microbiome and the animal (Chaucheyras-

Durand and Durand, 2010). Probiotics are potentially of therapeutic benefit to animals to help facilitate the bacterial population of the gut via competitive exclusion or direct effects of harmful pathogenic species and minimize the length of diarrhea in young foals. To further understand the dense microbial community within neonatal foals, researchers must create a clear picture of the equine microbiome. Culture-based techniques are limited by only being able to cultivate 1% of the organisms in an environment (Rastogi and Sani, 2011). Therefore, molecular approaches are required in order to analyze bacterial diversity in fecal samples (Costa et al., 2012). Because the pathogenesis of diarrhea can be extensive and prevention measures are limited, probiotic administration is a proposed option for the prevention of neonatal foal diarrhea (Schoster et al., 2014).

CHAPTER II

REVIEW OF LITERATURE

Neonatal Diarrhea

Diarrhea in man and animals has been studied for hundreds of years, yet there is much to be learned about how to control it, and little has been learned about how to control it, or what causes it. Infectious diarrhea of neonatal animals is one of the most common and economically devastating conditions encountered in the animal industry (Holland, 1990). At birth, the neonate can respond to a large number of antigens, but lacks the specific immune function to combat various infections, such as enterocolitis also known as diarrhea (Holland, 1990). Diarrhea associated with infectious agents leads to the deteriorating condition of neonates and will directly hinder the overall performance of the animal early in their life (Yadav, 2013). An array of infectious agent's cause diarrhea in food animal species, many of which lead to severe intestinal lesions, alterations in enzyme activity, alterations in nutrient transport mechanisms, or a combination of these effects (Holland, 1990). Typically, diarrhea remains undiagnosed in neonates and culture methods are currently the most useful tool for identification of the specific agents suspected to be associated with the cause. In calves under 3 wk of age, 10 infectious agents have been identified, with enterotoxigenic *E. coli* being the primary culprit (Yadav, 2013). In 2007, the National Animal Health Monitoring System for U.S.

dairy reported half of the deaths among unweaned calves was attributed to diarrhea (Yong-il and Kyoung-Jin, 2014). Similarly, neonatal lambs and goats are also affected by enterotoxigenic *E. coli* and Rotavirus, as well as *Cryptosporidium*. Agents commonly affecting young piglets are enterotoxigenic *E. coli*, Coronavirus, Rotavirus and *Isospora suis.* In young foals, diarrhea may be associated with Rotavirus, *Cryptosporidium* and *Strongyloides* (Yadav, 2013). Neonatal diarrhea triggering dehydration, metabolic acidosis, and electrolyte depletion is an important cause of morbidity and mortality in neonatal farm animals (Yadav, 2013).

Diarrhea affecting neonates can be designated as infectious (viral, bacterial, fungal, or protozoal) or non-infectious mechanisms. Non-infectious diarrhea is often mild, transient, and non-contagious, and may be a result of dietary intolerances, ingestion of sand, over feeding, or unsanitary housing conditions (McCue, 2009a). The greatest loss and economic impact comes from infectious diarrhea, which can be life-threating (Dunkel and Wilkins, 2004). Bacteria can produce enterotoxins that influence the crypt cells to hyper-secrete, invading the intestinal mucosa and eliciting an inflammatory response that mediates hyper-secretion through prostaglandins (Holland, 1990). Rotavirus, the most common viral cause of diarrhea in calves and lambs, replicates in the mature absorptive and enzyme-producing enterocytes on the villi of the small intestine. This leads to rupture and sloughing of the enterocytes with release of the virus to infect adjacent cells (Gruenberg, 2014).

Pathogenesis of Diarrhea in Foals

Diarrhea is a major concern beginning just after birth and lasting up to 5 mo of

age in young foals. Diarrhea can be relatively harmless in some cases, but it can also become fatal without intervention. Horses are monogastric herbivores whose digestive system is anatomically developed for them to ingest small amounts of forage in a continuous manner (Hutton et al., 2003). The digestive system of the horse is quite unique. The system has a relatively small but efficient stomach for grain utilization, and a large cecum and colon for roughage utilization. The purpose of the digestive system of the horse is to process feedstuffs into their component nutrients for absorption and utilization by the body. The horse has the largest and most complex large intestine of any of the domestic animals (Hutton et al., 2003). The equine diet of grasses necessitates the assistance of microbes for digestion of celluloses, but unlike ruminants, the horses' digestive system defers this fermentation until ingested food reaches the cecum (Frandson et al., 2006). The majority of microbial activity takes place in the large intestine, which constitutes approximately 60 % of the gastrointestinal tract (GIT; Dicks et al., 2014). In addition to digestion and absorption, the intestinal barrier also acts as the first line of defense against a vast amount of food allergens, exogenous antigens, and commensal bacteria (Tanabe et al., 2014).

Foals are born with a sterile gut and are not yet hindgut fermenters (Julliand et al., 1996, and Corley and Hollis, 2009). The transition timeline of bacterial population of the GIT is unclear, but must occur sometime between birth and weaning (Earing et al., 2012). Julliand et al. (1996) suggested rapid colonization of the GIT by the diverse maternal and environmental organisms the foal is exposed to after birth, and noted the total fecal microbial population was 10^8 colony forming unit (cfu)/g in the first 24 h.

Foals often experience diarrhea concurrent with the mares first postpartum estrus or “foal heat”. Mares usually first come into estrus (heat) by 6 to 8 d postpartum (McCue, 2009b). The term ‘foal heat diarrhea’ is sometimes used to refer to diarrhea associated with foal heat, although ‘foal heat diarrhea’ is a misnomer according to Magdesian, (2005). This early-onset diarrhea was initially believed to be caused by alterations in the composition of milk during the mare’s first estrous period after parturition, hence the term “foal heat” (Magdesian, 2005). However, Kuhl et al. (2011) characterized the pathogenesis of diarrhea in mares and foals for 6 wk after birth. Mares were teased daily for estrous behavior and further signs of estrus were checked by rectal palpation and use of ultrasonography of the ovaries. Additionally, fecal samples were taken for bacteriological analysis of mare and foal pairs, as well as blood samples for plasma and serum analysis. The authors determined neonatal diarrhea in foals is not linked to postpartum estrus in the dam, but is observed at a time when fluctuations in fecal bacterial flora changes from a neonatal to a postnatal pattern (Kuhl et al., 2011).

Diarrhea is defined as an increase in the fluidity of the stool, ranging from soft to watery liquid of the feces, accompanied by an increase in stool frequency (Xie et al., 1997). Diarrhea can result in severe dehydration, metabolic acidosis, electrolyte derangements, and bacteremia (Sanchez, 2012). Pieszka et al. (2015) reported that almost 80% of all suckling foals exhibited diarrhea symptoms within the first mo of life. In addition, Sanchez (2012) stated up to 50% of diarrheic foals less than 30 d of age are considered bacteremic, (bacteria circulating in the blood). Common infectious causes of equine neonatal diarrhea include *Salmonella* spp., *E. coli*, *Clostridium* spp.,

Cryptosporidium, Rhodococcus, Rotavirus, and parasites (Dunkel and Wilkins, 2004; Frederick et al., 2009; McCue, 2009a; Sanchez, 2012).

Current literature offers several different hypotheses for the etiology of foal heat diarrhea, particularly those of non-infectious origins. Masri et al. (1986) suggested that foal heat diarrhea is caused by hypersecretions of Na, K and Cl ions in the small intestine mucosa, and an immature colon may be overwhelmed and unable to compensate. Sgorbini et al. (2008) evaluated whether a yeast flora was present in the intestine of healthy foals and whether yeast overgrowth might be associated with foal heat diarrhea. Sterile fecal swabs were taken from the (n = 92) foals included in the study at birth, and at 2, 5, 8, and 12 d of life for bacterial cultural. After analysis, a correlation between the onset of diarrhea and presence of yeast failed to be assessed, only 32 of 460 samples (7%) were positive for yeasts (Sgorbini et al., 2008). This demonstrated that the equine gastrointestinal tract did not appear to be commonly colonized by yeasts during the first 15-d of life, and yeasts are not involved in foal heat diarrhea (Sgorbini et al., 2008). Kuhl et al. (2011) suggested that in foals, maturation of the intestinal tract occurs during a comparable time period and may in part be linked to foal heat diarrhea. These changes in bacterial flora in many foals can be accomplished without clinical signs of diarrhea (Kuhl et al., 2011). John and others (2015) also concluded in their research that diarrhea might be a part of normal physiological development of the intestinal microflora. While studying the effects of *Bacillus cerus* var. *toyoi* supplementation, diarrhea occurred in up to 90% of the foals for at least 1 d between d 8 to 16 of life (John et al., 2015). The authors alluded that a reduction of DMO in feces of foals may be due to increasing

digestion of solid nutrients like crude fiber. In which case, hemicellulose and cellulose could be responsible for a higher water-binding capacity and a reduction in intestinal passage (John et al., 2015).

Tanabe et al. (2014) stated the pathogenesis of diarrhea is affected by physiological and mental stress, and results in intestinal inflammation in neonatal foals. These authors studied the regulative effects of 5 bacterial strains on splenocyctes and intestinal epithelial cells. They conducted a 2-part experiment in which anti-inflammatory and intestinal barrier-protective activities were tested using bacterial cultural techniques. Additionally, a probiotic containing *L. ruminis* KK14, *L. equi* KK 15, *L. reuteri* KK18, *L. johnsonii* KK21, and *B. boum* HU, (LacFi^{TM}) was administered to treatment foals (n = 101) and compared to control (n = 29). In the first study, tight junction barrier impairment was induced by TNF-α in the human epithelial Caco-2 cells and the protective effects of probiotic (LacFi $^{TM)}$ were evaluated by measuring the transepithelial resistance, which represents tight junction permeability. Results reported that all strains exhibited intestinal barrier protective activity, although further investigations are necessary to clarify the results. In the second study, Tanabe et al. (2014) evaluated the incidence of diarrhea in neonatal Thoroughbreds after administration of probiotic containing 5 strains of *Lactobacillus*. Researchers concluded diarrhea prevention by the probiotic product might be attributed to the improvement in cytokine balance and to the enforcement of the tight junction barrier and subsequent maintenance of intestinal integrity.

Pieszka et al. (2015) hypothesized that foal heat diarrhea may be linked to

osmotic changes within the intestinal tract at this specific age in foals. After testing a kaolin-clay intestinal absorbent in 76 foals at 2 different stud farms in Poland, diarrhea morbidity rate was determined (Pieszka et al., 2015). The beneficial effect of kaolin clay observed in the study suggested that foal heat diarrhea can be caused by disturbances in the intestinal osmotic balance.

History of Diarrhea Treatments

In the last 100 years, treatment of diarrhea has evolved. Treatments have included opium, use of oral glucose and electrolyte solutions, a variety of chemotherapeutic agents, intestinal absorbents, and more recently probiotics have been researched (Berschneider and Argenzio, 1982). Additionally, Chinese veterinary medicine has used herbal remedies for more than a thousand years. Veterinarians in China use either an herbal tea or powdered herb concoctions based on generations of clinical experience (Xie et al., 1997). However, McCue (2009a) stated that the best means of prevention and treatment is good hygiene and cleanliness of stall environments.

Treatment can be dependent on the pathogenesis of the diarrhea, and a particular case may have more than one cause. The cause of death in a diarrheic animal is usually dehydration and cardiovascular collapse (Berschneider and Argenzio, 1982). Sodium-hydrogen exchange mechanisms present in the intestine are directly affected by the toxins or destruction of the cells in the intestine. The high concentration of H ions in the extracellular fluid cause H to diffuse into cells forcing K ions out. The K imbalance inhibits cellular repolarization causing muscular weakness and eventually failure of the cardiac muscles ultimately leading to animal fatality (Berschneider and Argenzio, 1982).

The first step in treatment is to prevent further infection in the neonate, and any preventative measures should aim to enhance the animal's natural defense of immunity. Immunity often involves the generation of inflammation, the removal and destruction of pathogens, the development of memory specifically against the antigenic insult and tissue repair (Felippe, 2016). Once infection occurs many veterinarians prefer to use chemical agents directed against the invading organism or toxin. Antimicrobials such as penicillin or trimethoprim-sulphonamide combinations are available with an endless variety of specific actions and side effects (Corley and Hollis, 2009). Antitoxins are another route of treatment, including activated charcoal, kaolin and various clays (Berschneider and Argenzio, 1982).

Kaolin clay treatment has been demonstrated to significantly influence the duration of foal heat diarrhea (Pieszka et al., 2015). These researchers administered treatment on the first day of diarrhea and for 3 d following, and diarrhea severity was scored on a 3-point scale. Kaolin clay–treated foals had significantly shorter duration of diarrhea (3.26 d) as compared to control foals (4.30 d). The authors concluded that treatment with a kaolin clay paste reduced the duration of foal heat diarrhea and alleviated its severity (Pieszka et al., 2015).

Antimicrobial therapy has been employed in infectious diarrheic cases. However, successful antimicrobial therapy depends on a number of different factors including appropriate dosage and selection of appropriate compounds (Corley and Hollis, 2009). Penicillin is an effective broad-spectrum option for treatment against gram-positive and anaerobic infection. Additionally, aminoglycosides, such as Amikacin, have a synergistic

effect with penicillin against certain organisms, particularly gram-negative and some gram-positive bacteria (Corley and Hollis, 2009). These authors also suggested an oral metronidazole therapy for foals with severe diarrhea. Although some antimicrobial treatments may be beneficial; others can exacerbate diarrhea particularly in adult horses. In humans, antimicrobial agents are reported to disrupt the normal gastrointestinal microbiota and opportunistic pathogens can then occupy available niches and produce toxins (Harlow et al., 2013). A similar hypothesis in horses led Harlow et al. (2015) to explore the common aliment among adult horses, Antimicrobial Associated Diarrhea (AAD), which occurs due to a disruption of the normal enteric microflora by antibiotics.

The study by Harlow et al. (2013) evaluated the effects of common antibiotics on cellulolytic bacteria, *lactobacilli*, and AAD-associated pathogens in the feces of healthy horses. Fifteen Thoroughbreds were given an antimicrobial challenge. Researchers used a control group, trimethoprim-sulfadiazine, and ceftiofur as treatments. After a 3 wk dietary adaption period, treatment was applied for 1 wk and then animals went through an additional 1 wk withdrawal period. Cellulolytic bacteria decreased by more than 99% during administration of either antibiotic ($P < 0.0001$) and were still less than controls at the end of the withdrawal period ($P < 0.0001$). Fecal samples from horses challenged with ceftiofur had 75% fewer lactobacilli than those from control horses at the end of the antibiotic challenge period ($P < 0.05$). The results indicated that antimicrobials can disrupt the normal gastrointestinal microbiota and allow proliferation of *Salmonella* spp. and *C. difficile* (Harlow et al., 2013). The authors stated that antimicrobial treatment may actually exacerbate diarrhea in foals.

Other methods of diarrhea treatment include cell metabolism moderators, antispasmodics and motility control, and fluid therapy (Berschnedier and Argenzio, 1982). More recently the use of probiotics has been investigated to help mitigate the effects of diarrhea as a control measure, but there are conflicting results. Some researchers have reported unsuccessful attempts to mitigate or control diarrhea (Weese and Rousseau, 2005 and Schoster et al., 2015a, 2015b), others (Yuyama et al., 2004 and Tanabe et al., 2014) reported administration of probiotic products decreased the incidence of diarrhea. Weese and Rousseau (2005) conducted a randomized controlled clinical trial on 153 foals to evaluate the efficacy of *Lactobacillus pentosus* WE7 as a probiotic. However, probiotic administration was significantly associated with development of signs of depression, anorexia, and colic or the need for veterinary examination and treatment (Weese and Rousseau, 2005). Similarly, Schoster et al. (2015b) reported animals treated with a combination probiotic containing 4 *Lactobacillus* species and *Bifidobacterium animalis lactis* were more likely to develop diarrhea requiring veterinary intervention. Yet, Yuyama et al. (2004) reported the incidence of diarrhea was significantly lower (14.8 %) in the probiotic treated foals than in the control animals (51.9%) after administering a probiotic mixture containing 5 strains of *Lactobacilli* isolated from horses. Additionally, Tanabe et al. (2014) observed a reduction in incidence of diarrhea when a *Lactobacilli* based probiotic was administered, decreasing incidence from 75.9 % (control) to 30.7% (probiotic). With limited research and multiple formulations of probiotics available, further research is warranted to determine if probiotics can be a beneficial treatment option for neonatal diarrhea in horses.

Bacteria of the Equine Gastrointestinal Tract

The role of intestinal microflora in animal health and disease is of particular interest to researchers and veterinarians. Gut microbiota dysbiosis, a shift in the bacterial balance, as a whole can cause a wide range of diseases in the horse including colitis, grass sickness, laminitis, and diarrhea (Schoster el al., 2014). Probiotics can potentially improve intestinal balance, enhance growth, and prevent and treat diarrhea.

The major constituent of the microbiota in the gastrointestinal tract of the horse is lactic acid bacteria, they play a role in digestion, but also are a key player in stimulation of the immune system (Dicks et al., 2014). *Lactobacillus* are the most prevalent microbes in the stomach, whereas the large and small intestine are dominated by proteolytic bacteria such as *Streptococcus* spp. and *Bacteroides* spp. (Dicks et al., 2014). Approximately 50 species belonging to 30 genera of ciliates have been identified within the various hindgut compartments. The complexity of the equine intestinal microbiome is indicated by Costa et al. (2012) after he and fellow researchers characterized the fecal microbiome of healthy horses and compared them to that of horses with undifferentiated colitis. After DNA extraction and 16S rRNA gene PCR and sequencing of equine feces, researchers concluded that the intestinal tract houses one of the richest and most complex microbial populations on the planet, and plays a critical role in health (Costa et al., 2012).

The hindgut microbiome is characterized by the diversity and structure of its microbes and their functions. The hindgut of the horse is a very unique and complex environment. Horses receive a vast majority of their energy requirements from the VFA that are produced during fermentation. Each section of the hindgut may contain varying

populations and species of bacteria that interact with the digesta. Julliand and Grimm (2016) stated that the hindgut can be considered each their own ecosystem compartment divided into; the cecum, large ventral colon, large dorsal colon, small colon, and rectum.

The fecal bacterial flora represents a combination of shed mucosal bacteria and non-adherent luminal bacterial (Kuhl et al., 2011). By 4 wk of age, bacterial flora in foals resembled an adult pattern (Kuhl et al., 2011). Earing et al. (2012) used PCR-DGGE profiles of mare and foal pairs. After comparison, researchers reported that by d 14, the foals and mares had similar numbers of bands of bacteria. This provides further evidence that colonization proceeds rapidly and a mature microbial community is present in the first few weeks of life (Earing et al., 2012).

Many studies to date access the hindgut microbiota composition through feces, eliminating the need for cannulation or euthanasia (Julliand and Grimm, 2016). In 1951, Adam conducted a study on fecal representativeness, comparing the abundance and diversity of protozoa within the hindgut. Adam concluded that ciliate populations in the feces were similar to those found in the distal region of the hindgut, but different from the proximal part (Adam, 1951). In a recent review, Julliand and Grimm (2016) agreed with Adam, and stated that the fecal bacterial microbiome shows similarities with the distal part of the hindgut as evidence by the combination of research data over the last 10 yr, which utilized culture-independent techniques (Julliand and Grimm, 2016). Earing et al. (2012) sampled 9 mare and foal pairs from birth to 12 wk of age, and analyzed samples by PCR using amplification of the 16S rRNA gene. Their work along with Costa et al. (2015b), who characterized the bacterial colonization of the intestinal tract of healthy

foals until 9 mo of age using next generation sequencing, have laid the ground work for researchers. However, there is still much to learn about the microbiome of the equine gastrointestinal tract, particularly during diarrhea. The establishment of intestinal microflora and maturation of the gastrointestinal mucosa are some of the suspected reasons for diarrhea in this period of life (John et al., 2015).

Next Generation Sequencing Technology for Microbial Community Analysis

Non-culture based laboratory techniques of feces can help researchers to establish an etiology of foal heat diarrhea. The DNA sequencing is a laboratory method used to determine the sequence of a DNA molecule, a method developed by Frederick Sanger in 1975 (Nature Education, 2014). Culture-based methods are important in investigating the microbial ecology of environments, but they are biased in their evolution of microbial genetic diversity by selecting a particular population of microorganisms (Rastogi and Sani, 2011). The major limitation of culture-based techniques is that over 99% of the microorganisms in any environment observed through a microscope are not cultivable by standard culturing techniques (Hugenholtz, 2002). Costa et al. (2015a) characterized and compared the bacterial structures found in the different equine GIT compartments using high-throughput sequencing. Eleven mature horses donated for euthanasia for reasons unrelated to GIT disease were utilized. Samples from nine compartments of the GIT were collected and DNA was extracted for 16S rRNA gene PCR and sequencing. Additionally, researchers sequenced data with an Illumina MiSeq for 250 cycles and bioinformatics analysis was performed using the software mothur (v1.32; Schloss et al, 2009). The total number of phylotypes per compartment were also reported (x = 113 phylotypes per

compartment). With the use of next-generation sequencing researchers were able to reveal the variation in microbial populations between the compartments of the equine GIT (Costa et al., 2015a). Marked differences among compartments even at high taxonomic levels were found, with *Firmicutes* comprising the main bacterial phylum in all compartments. Bacterial communities from feces were not significantly different from the ones found in the large colon. The bacterial population comprising the equine intestinal tract varies greatly among compartments and fecal samples may be useful as representative of changes occurring in the distal compartments (Costa et al., 2015a).

Further work by Costa et al. (2015b) characterized the bacterial colonization in the intestinal tract of healthy foals and the development of the microbiota until 9 mo of age using next generation sequencing. Eleven pregnant mares from 1 farm were enrolled and fecal samples were collected longitudinally from mares and foals during their first day postpartum and again periodically until foals were 9 mo of age (Costa et al., 2015b). The V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina MiSeq platform (Costa et al., 2015b). Researchers reported newborn foals had a rich and diverse bacterial community, which was mainly comprised of the *Firmicutes* phylum with several low abundant genera being unique at this age. Foals aged 2 to 30 d had significantly decreased diversity compared to older animals, with the majority of organisms classified as *Akkermansia* spp. *Firmicutes*, *Verrucomicrobia,* and bacteria unclassified at the phylum level comprised almost 90% of bacteria found in feces of foals older than 60 d. The *Firmicutes* phylum comprises a diverse group of bacterial species, and tends to predominate in the intestinal microbiota of most animal species. The initial

gut colonization was very dynamic and changed significantly after the first day of life, but foals had a relatively stable intestinal microbiota after 60 d of life (Costa et al., 2015b). Sequencing and phylogenetic analysis of 16S rRNA genes provided the foundation for modern study of microbial communities (Zhou et al., 2015).

Probiotics

The global probiotics market exceeded $35 billion in 2015 (Grand View Research, 2016). The health effects attributed to the use of probiotics are numerous. Schrezenmeir and de Vrese (2001), stated the following outcomes are well documented: 1) probiotics lower frequency and duration of diarrhea associated with antibiotics, rotavirus infection, and chemotherapy; 2) probiotics stimulate humoral and cellular immunity; and 3) probiotics decrease unfavorable metabolites. Organisms of the probiotic groups are added to all sorts of foods, particularly yogurts, but are also found in drinks and other common food supplements such as pills, or powders (Floch, 2013). Different strains of probiotic bacteria may exert different effects based on specific capabilities and enzymatic activities (Schrezenmeir and de Vrese, 2001). The most common probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* family (NCCIH-NIH, 2015).

Probiotics are a potential method to therapeutically modify the intestinal microbiome and prevent disease. There is limited research on probiotics and their use in horses. Probiotics are defined as a live microorganism that delivers a healthy benefit to the host, when administered in adequate amounts, providing a beneficial effect beyond that of their nutritional value (Ishizka et al., 2014 and Schoster et al., 2014). Probiotics

must be: 1) able to survive acid and bile dominated environments, 2) survive intestinal transit, 3) colonize the intestinal tract for a short period of time, 4) aero-tolerant (organisms that cannot use oxygen for growth) to survive processing and storage, and 5) must possess other properties enabling them to produce a beneficial effect (Weese, 2002). The main targets for probiotic use in horses are gestating mares, race horses, and foals, and other classes of horses that experience periods of stress or diet change (Chaucheyras-Durand and Durand, 2010).

Probiotics are also known as 'direct-fed microbials'; and can be classified as a drug and need to be approved by the Food and Drug Authority (FDA). Currently there are no approved probiotics for medicinal uses in horses, however probiotics can also be classified as a feed supplement and 'generally regarded as safe' (GRAS) and do not need to go through drug approval by FDA (Schoster et al., 2014). The supplement labels may make claims about how the product affects the structure or function of the body without FDA approval, but they cannot make health claims (claims that the product reduces the risk of disease) without FDA consent (NCCIH-NIH, 2015).

There are 4 primary mechanisms of action by which probiotics prevent colonization of the digestive tract by pathogenic strains and prevent disease: 1) modulation of the host innate and acquired immune system, 2) antimicrobial production, 3) competitive exclusion, and 4) inhibition or inactivation of bacterial toxins (Schoster et al., 2014). Probiotics can influence the host's immune system as live or dead bacteria through their metabolites, cell wall components, or DNA. Intestinal epithelial cell receptors recognize probiotics, which modulate tight junctions and enhance mucin

production. Additionally, certain probiotic strains can produce various substances that are effective against microbes, such as fatty acids, lactic acid and acetic acid. Probiotic strains adhere to epithelial cells in the host and interfere with pathogen adherence by blocking receptors or by increasing mucin production. However, this mechanism of competitive exclusion appears to be specific to certain probiotic strains (Thomas and Versalovic, 2010). Some probiotics also block the effects of enteropathogenic bacteria by having an anti-toxin effect and inactivating toxins through metabolic mechanisms (Schoster et al., 2014).

Probiotics have diverse effects on the immune system that can be stimulatory or inhibitory depending on the biological features of probiotic strains used (Schoster et al., 2014). Some probiotic strains can produce fatty acids, lactic acid, and acetic acid which are effective against microbes. Additionally, probiotics adhere to epithelial cells in the host and interfere with pathogen adherence by blocking receptors or by increasing mucin production. Some probiotics can also block the effects of enteropathogenic bacteria (Schoster et al., 2014). Probiotics provide beneficial effects to the host by enhancing intestinal epithelial cell function, protecting against physiological stress, modulating cytokine secretion profiles, influencing T-lymphocyte populations, and enhancing antibody secretion (Thomas and Versalovic, 2010).

Most commercially available probiotics are composed of non-spore-forming lactic acid bacteria such as *lactobacilli, bifidobacteria,* and *enterococci* (Weese, 2002). *Lactobacillus equi., L. crispaus, L. johnsonii, L. reuteri* and *L. salivarius* have been administered to horses and proven to be beneficial to the host with regard to growth

promotion and resistance to diarrhea (Yuyama et al., 2004). Live yeasts have also been evaluated for their probiotic properties, and *Kluyveromyces fragilis* is a strain marketed for use in horses, which claims to survive passage through the stomach and have various effects on the gastrointestinal tract (GIT; Weese, 2002).

Stercova et al. (2016) evaluated the effects of *Saccharomyces cerevisiae* CNCM I-4407 on nutrient digestibility and fecal microflora in dogs. The study included 24 young beagle dogs allocated to control or live yeast groups. After an adaption period, dogs received capsuled *Saccharomyces cerevisiae* at 2.9×10^8 cfu/g. Dogs that received the live yeast treatment had greater weight gain, improved apparent digestibility of NDF and had reduced *E. coli* and fecal enterococci counts in feces (Stercova et al., 2016).

In farm animals, researchers used a piglet model to investigate the protective effects of probiotic administration against diarrhea in weanlings (Shu et al., 2001). Seventeen piglets were allocated to 2 groups; the test group was administered *Bifidobacterium lactis* HN019 at 1×10^9 cfu/d. When compared to controls, piglets that received *B. lactis* HN019 had a lower severity of weanling diarrhea and maintained greater feed conversion efficiency (Shu et al., 2001). Additionally, the authors also reported protective effects of probiotic feeding associated with reduced concentrations of fecal Rotavirus and *E. coli*, and greater gastrointestinal tract pathogen-specific antibody titers (Shu et al., 2001).

In cattle, *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 was previously evaluated for efficacy in neonatal dairy heifer calves (Clymer et al., 2015). A total of 770 individually housed Holstein, Jersey and cross-bred heifers at a commercial

calf-rearing facility were used. Two direct-fed microbial (DFM) products were compared. An established DFM containing *Propionibaterium freudenreichii* NP24 and *Lactobacillus acidophilus* NP51 (Bovamine, Nutrition Physiology Company LLC, Guymon, OK) and a newly developed DFM containing *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 (ProBiostatin, GLC Direct LLC, Paris, KY) were blended with milk and administered for 60 d at a tapering dose rate. Researchers reported improved performance and growth rate during the first 10 to 28 d of age (Clymer et al., 2015). Additionally, a numerical reduction in overall disease morbidity was also evident in the ProBiostatin group.

Use of Probiotics in Horses

In horses, probiotics target the cecum and colon during periods of stress or diet change (Chaucheyras-Durand and Durand, 2010). In gestating and lactating mares, probiotics are targeted at increasing diet digestibility and improving milk quantity and quality. In race horses, probiotics are targeted at avoiding hindgut disorders, increasing digestibility of the diet, and limiting impacts of stress from transportation. However, the scope of the current literature on equine probiotic use has focused mainly on gastrointestinal disease application (Schoster et al., 2014).

Faubladier et al. (2013a) evaluated fecal bacterial communities and activity in horses with or without live yeast supplementation. Horses were supplemented with $2x10^{10}$ cfu/d of *Saccharomyces cerevisiae* CNCM I-1077 for a 19 d in a crossover design with a 23-d washout period. Supplementation with the live yeast was observed to help reduce the negative impact of transportation on the fecal bacterial ecosystem (Faubladier

et al., 2013a). Additionally, Ishizaka et al. (2014) conducted a randomized double-blinded, placebo-controlled crossover study to evaluate the ability of probiotics to improve intestinal conditions in adult horses. A product containing 5.6 $x10^8$ cfu/g of *L. acidophilus* and 2.6 x 10^4 cfu/g of yeast with *S. cerevisiae* and *S. boulardii* yielded proliferation of probiotic bacteria in the equine intestine, suggesting oral administration of probiotics may have the ability to improve the intestinal environment without the risk of adverse effects (Ishizaka et al., 2014).

In foals, probiotics target limiting diarrhea and promoting growth. Yuyama et al. (2004) utilized a host-specific *Lactobacillus* probiotic on 54 neonatal foals administered daily for 7 d. The probiotic lead to a significant increase in BW in 1-mo old foals and a significantly lower incidence of diarrhea at 3 wk of age. These researchers observed BW changes at d 0, 7, 14, 21, and 30. Additionally, fecal characteristics were tracked up to 30 d of age utilizing a scoring scale of 0 to3. Samples were analyzed for intestinal microflora and short-chain fatty acids. Hematological and biochemical studies were performed on blood samples collected at d 1, 7, and 14, as well as mo 1, 2, and 3 of age. Researchers reported no statistically significant effects between control and treated groups (Yuyama et al., 2004).

Schoster et al. (2015a) conducted a randomized, placebo-controlled field trial to evaluate the effect of a newly designed probiotic on the incidence of foal diarrhea. Overall incidence of diarrhea was 59% and did not differ between treatment groups; there was no benefit observed from administering a 3 wk course of probiotics (Schoster et al., 2015a). In the same year, Schoster and colleagues also tested a newly designed

probiotic and evaluated the effect on the incidence of foal diarrhea in a randomized field trial (Schoster et al., 2015b). The probiotic contained *Lactobacillus rhamnosus* SP1, *L. rhamnosus* LHR19, *Lactobacillus plantarum* LPAL, *L. plantarum* BG112, and *Bifidobacterium animalis lactis* BLC1 and was administered once daily for 21 d. Yet again, foals treated with probiotics did not benefit and were more likely to develop diarrhea requiring veterinary intervention (Schoster et al., 2015b). With conflicting reports on probiotic use in diarrheic foals, further research is warranted to investigate the potential effects of different probiotic strains on mitigating the effects of neonatal foal heat diarrhea.

Statement of the Problem and Justification

The pathogenesis of foal heat diarrhea has yet to be pinpointed, but this ailment is clearly multifaceted and requires further investigations by researchers. Foals that develop diarrhea in the first few wk of life are at risk for dehydration, septic joints, and fatal infection. There are limited therapies available to treat severe diarrhea in neonates and some antibiotics can exacerbate changes in gut flora resulting in a more severe clinical condition than without antimicrobial treatment. Therefore, providing a supplemental probiotic during the first 28 d of neonatal life to mitigate effects of foal heat diarrhea is potentially of clinical benefit to the equine community. Probiotics may modify the intestinal microbiota and prevent disease (Schoster et al., 2014). The objectives of the current study are to: 1) determine the effects of probiotic supplementation with *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 on diarrhea incidence and severity in neonatal foals, and 2) to characterize the bacterial population changes in the neonatal gut in the first 28 d of life.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Animals

In the spring of 2016, 24 stock-type newborn foals were used in a completely randomized design experiment to evaluate the effects of probiotic supplementation on diarrhea incidence and severity, as well as immune responses. The experimental trial began in February 2016 and ended in June 2016. Foals from 2 farm locations in the Amarillo, TX area were used: local ranch (Amarillo, TX) and veterinary hospital (Canyon, TX). Mares at the local ranch (n = 18) received a diet of alfalfa hay twice daily and water *ad libitum*. Mares located at veterinary hospital (n = 5) also received alfalfa hay 2x/d and a rice bran-ration of Equine MoJo (Agritrition, Amarillo, TX) top dressed over hay once a day. Mares were housed individually in 7.5 × 11 m foaling stalls at least 2 wk prior to, and until d-28 postpartum.

Foals were enrolled in the trial within 24 h of age and randomly assigned to treatment; control (C) or probiotic (P). All foals that were born by natural delivery and considered clinically normal by the consulting veterinarian at the time of enrollment were eligible for inclusion. Foals at both locations were managed according to standard and

accepted procedures as outlined by the veterinary hospital. Post-partum management included administering tetanus toxoid vaccine, vitamin A, D, and E, and a naval iodine dip. Additionally, the consulting veterinarian drew blood via jugular venipuncture for complete blood count (CBC), chemistry analysis, and IgG SNAP test (IDEXX Laboratories, Westbrook, Maine).

Foals enrolled in the trial at both locations received standard care in case of illness (failure of passive transfer, contracted tendons, or diarrhea) according to the consulting veterinarian. Foals with an IgG blood concentration less than 600 mg/dL (according to SNAP Test; IDEXX Laboratories, Westbrook, Maine) received 400 mL of donor-colostrum. Foals exhibiting contracted tendons were administered a single dose of oxytetracycline (Bio-Mycin® 200, Boehringer Ingelheim, St. Joseph, MO) at 200 mg/mL, and legs were wrapped with cotton bandages until tendons relaxed. Additionally, foals exhibiting signs or symptoms of severe diarrhea within the first 4-d post-parturition were immediately administered a combination of penicillin (VetriPen™ G, Vet ONE, Boise, ID) at 30,000 IU/kg BW, amikacin sulfate (25 mg/kg BW), and flunixin meglumine (Prevail, Vet ONE, Boise, ID) at 0.55mg/kg BW for 7 d. The consulting veterinarian monitored foals with diarrhea via complete blood count (CBC), serum chemistry, and fibrinogen analyses as described subsequently. Mineral oil was applied to the foals' tail and perianal area as needed to prevent scalding. Any mares with retained placentas were treated with standard procedures including oxytocin injection (Bimeda-MTC Animal Health Inc., Cambridge Ontario, Canada) at 20 IU/dose, and a weighted palpation glove was tied on to the placenta. All animals enrolled in the trial were under strict observation by a consulting veterinarian and farm or hospital personnel.

Treatments

Foals were randomly assigned to treatments, regardless of diarrhea morbidity. Treatments were identified as: control (C, no probiotic supplementation), and treatment (P, probiotic supplementation of Pro Biostatin® (GLC Direct LLC, Lexington, KY)). Animals assigned to P received 6 g of probiotic paste orally twice daily containing 500×10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026. The day foals were born was classified as d 0. All foals began treatment during the following 24-h cycle (d 1). Probiotic paste (P) was administered orally, twice daily between 0630 to 0730 and between 1630 to1730 beginning on d 1. Treatment continued twice daily for 14 d. Foals assigned to C were subjected to the same physical handling by farm or hospital personnel at the same rate and frequency as foals assigned to P. Foaling dates and times differed for each mare.

Sample Collection

Blood

Blood was collected by the attending veterinarian or farm personnel from foals via jugular venipuncture on d 0, 7, 14, 21, and 28 using a 3 mL syringe (Covidien, Mansfield, MA), and dispensed into a 1 mL EDTA tube (IDEXX VetCollect Tubes, Westbrook, MA). Upon collection tubes were inverted 4 to 6 times to thoroughly mix EDTA to prevent unwanted blood clotting. Blood samples were then transported immediately at ambient temperature to the veterinary hospital for d 0 IgG, complete blood count analysis and chemistry analysis.

Feces

Fecal samples were collected from the rectum via grab sample using a sterile glove at d 0, 7, 14, 21, and 28 and placed in a 50 mL conical tube by the consulting veterinarian or trained farm staff. A mare fecal sample was also collected on d 28 via a grab sample from fresh feces on the ground and placed into a 50 mL conical tube (UltraCruz® Centrifuge tube, Santa Cruz Biotechnology, Santa Cruz, CA). All samples were immediately frozen at -80 C° until submitted for analysis.

Additionally, foal fecal score was determined daily using a 3-point system (0 to 2) adapted and modified from previous equine studies (Yuyama et al., 2004 and Pieszka et al. 2015) at both am and pm treatment times. The fecal scoring scale used was defined as follows; 0 = normal (no diarrhea, clean rear, solid to soft feces, color and texture resembles that of an adult equine), 1 = mild diarrhea (wet "messy" tail and perianal area, loose and wet feces, discoloration or slight odor), and 2 = severe diarrhea (extremely wet, perianal area, croup and tail have fecal staining, projectile feces with a strong odor).

Laboratory Analysis

Blood

Blood samples for d 0, 7, 14, 21, and 28 were analyzed for complete blood count (CBC) using IDEXX ProCyte Dx® Hematology Analyzer (IDEXX Laboratories, Westbrook, Maine). Day 0 blood samples were also analyzed using Catalyst Dx® Chemistry Analyzer with an Equine 15 CLIP and IgG SNAP test (IDEXX Laboratories, Westbrook, Maine).

Feces

Fecal samples were shipped overnight on ice to University of Arkansas for bacterial DNA sequencing and microbiome analysis. The University of Arkansas Animal Science Laboratory in collaboration with the Arkansas High Performance Computing Center analyzed samples in an open-format, using high-throughput sequencing. The term "open-format" refers to technologies whose potential experimental results cannot be anticipated prior to performing the analysis (Zhou et al., 2015). High-throughput sequencing also known as "next generation sequencing" is defined as sequencing techniques like (Illumina, Inc.) that are capable of sequencing multiple DNA molecules in parallel, enabling hundreds of millions of DNA molecules to be sequenced at a time (Churko et al., 2013). The DNA was extracted from individual fecal samples using PowerFecal^{TM} DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to manufacturer's instructions, and was stored at -80°C before further analysis. After high-quality DNA was obtained, targeted genes were amplified with conserved primers (Zhou et al., 2015). Then after non-target DNA fragments were removed by gel electrophoresis, target DNA was quantified, sequenced and analyzed using bioinformatics approaches (Zhou et al., 2015). Sequencing was performed using Illumina Miseq (Illumina, Inc. San Diego, CA) and analyzed by using the mothur v. 1.38.0 software package (Schloss et al., 2009) at the University of Arkansas Animal Science laboratory. The mothur software aims to be a comprehensive software package that allows users to use a single piece of software to analyze community sequence data (Schloss et al., 2009). Mothur is designed as an open-source, platform- independent, community-supported software for describing,

and comparing microbial communities allowing a user to analyze more than 222,000 sequences in less than 2 h with a laptop computer (Schloss et al., 2009).

Statistical Analysis

Hematological parameters were analyzed as a linear mixed model with one-way treatment structure in a completely randomized design with repeated measures (PROC MIXED; SAS 9.4 Institute Inc., Cary, NC), with animal serving as the experimental unit. The class statement included treatment, day, location, and gender. The model statement included day, treatment, and their interaction. Means separation and *P*-values were determined using LSMEANS with PDIFF option. Orthogonal contrasts were used to determine linear, quadratic, and cubic effects of day on hematology variables. Differences are discussed when $P \leq 0.05$; tendencies are discussed when $P > 0.05$ and < 0.15. Duration of diarrhea bouts duration were also analyzed as a linear mixed model with repeated measures, with animal serving as the experimental unit. The class statement included id, gender, antibiotics, location, treatment and bout. The model statement included treatment, bout, and their interaction. Diarrhea prevalence and severity were analyzed using Fisher's exact test (PROC FREQ). Microbiome data was not statistically analyzed and results reported reflect observations of the author.

CHAPTER IV

RESULTS AND DISCUSSION

One animal was removed from the study due to circumstances unrelated to experimental treatment. There were 8 foals at both locations (n = 6; local ranch, and n = 2; veterinary hospital) treated with the previously outlined antibiotic protocol for severe diarrhea by the consulting veterinarian. Additionally, foals with contracted tendons (n = 2) were treated according to the previously mentioned described regimen.

Complete Blood Count

Hematology analysis for C vs. P treated neonatal foals are shown in Table 1. Individual values for hematology variables are shown in Appendix Tables A-1, A-2, A-3, A-4, A-5, A-6, A-7, A-8, A-9, and A-10. All hematology variable values were within normal range for foals, with the exception of the neutrophil to lymphocyte ratio (UC Davis, 2001). There are important and significant differences in normal hematologic parameters between neonatal foals and mature horses (Barton, 2015). During gestation, dynamic changes occur in the hemostatic system of the foal (Barton et al., 1995). Over the 28-d period of this study, a decrease in red blood cell (RBC) parameters was observed in foals. The observed decrease in foal RBC is in agreement with Barton (2015). There was a treatment × day interaction on RBC concentration ($P = 0.02$), hemoglobin concentration ($P = 0.05$), hematocrit percentage ($P = 0.04$), and platelet concentration

(P = 0.01). Although, the interaction between treatment and day takes statistical precedence, there was also an apparent effect of treatment on lymphocyte concentration (P = 0.044). However, foals are more likely to experience stress when handled for venipuncture and physiologic lymphocytosis subsequent to catecholamine release can result in the rapid increase in the total lymphocyte count (Barton, 2015).

The effects of day on hematology variables during the first 28-d postpartum are shown in Table 2. There was a main effect of d on RBC (P = <.0001) and WBC (P = 0.0053). White blood cells were greatest on d 7 and decreased with time (cubic, P = 0.024). Red blood cells were greatest on d 0 and also decreased with time (cubic, P = 0.005). For all blood leukocyte percentages except neutrophils, there was an increase overtime with d 28 being the greatest; lymphocytes (cubic, P = 0.026), monocytes (cubic, P < 0.001), eosinophils (cubic, P = 0.025), and basophils (cubic, P = 0.076). Whereas neutrophil concentration was greatest on d 0 and decreased with time (quadratic, P < 0.001). A high neutrophil to lymphocyte ratio on d 0, which then decreased over time, was also observed in the hematology variables of neonatal foals. Barton (2015) suggests an increased neutrophil to lymphocyte ratio on d 0 may be due in part to the endogenous release of cortisol at parturition. Blood erythrocyte's, RBC count (cubic, P = 0.005) and hematocrit percentage (cubic, P = 0.017) were greatest on d 0 and decreased over time. However, platelets were lowest on d 7 and generally increased over time (cubic, P = 0.001). Aoki and Ishii (2012) suggested changes in hematology variables over time in neonatal foals may be related to; dehydration at birth, intake of colostrum, beginning of urination, functional change of hematopoiesis, change in liver metabolism, and change in milk composition.

Diarrhea Incidence and Severity

Diarrhea was observed in 95% of foals throughout the study. This is in agreement with John et al. (2015) who reported diarrhea occurred in up to 90% of the foals for at least 1 d. However, these authors reported a greater incidence of diarrhea between d 8 and 16, whereas the 95% reported in the current study reflects the overall diarrhea incidence for the 28-d period. Schoster et al. (2015a) reported a 59% incidence of diarrhea in foals, Kuhl et al. (2011) reported 86%, and John et al. (2015) reported 88%. The greater incidence of diarrhea observed in the current study may be due in part to the small number of animals used (n=23), or a potential pathogenic source of contamination in the housing environment that was not identified. Cohen (1994) conducted a prospective study to describe the causes of, and farm management factors associated with, disease and death in a population of foals in Texas. Morbidity associated with diarrhea was reported (21.1%), and mortality was reported (2.6%) among the 2,468 foals included in the survey (Cohen, 1994). The author noted a significant trend in decreasing risk of death and disease with increasing age. The authors also reported the principal cause of death in foals less than 7 d of age was septicemia and diarrhea (Cohen, 1994).

The effect of treatment on incidence of diarrhea in neonatal foals can be found in Table 3a. and 3b. There was a trend for an effect of treatment ($P = 0.105$) on the incidence of diarrhea in neonatal foals. A mean incidence of 2.91 in C and 1.83 in P was observed. During the 28-d period foals were affected by diarrhea a maximum of 6 times, with only one foal never displaying diarrhea. The duration of diarrhea bouts can be found in Figure 1. The duration of diarrhea bouts in this study tended to decrease as the

incidence increased, which agreed with Cohen (1994). There was a significant bout effect (P = 0.001) observed with each proceeding bout of diarrhea decreasing in length. The effect of gender, location, and antibiotic administration on incidence of diarrhea in neonatal foals can be found in Tables 4a, 4b, 5a, 5b, 6a, and 6b. There was no effect of gender (P = 0.821) on diarrhea incidence (Colts, 2.29; Fillies, 2.44). Additionally, there was no effect of location (P = 0.242) on diarrhea incidence. Foals located at the local ranch had a mean of 2.56 diarrhea bouts; whereas foals located at the veterinary hospital had a mean 1.6 diarrhea bouts. A numerical difference was noted between the two locations with foals located at the veterinary hospital having fewer bouts of diarrhea. This difference may due to the small number of animals (n = 5) observed at the veterinary hospital, but there may have been differences in management between facilities as well e.g. (frequency of stall cleaning, diet, or differences in farm personnel management). Furthermore, there was no effect of antibiotic regimen (P = 0.633) on diarrhea incidence (Antibiotics, 2.13; No Antibiotics, 2.47). The antibiotic regimen administered by the consulting veterinarian was not part of the current study design however, the data was still statistically analyzed to provide further insight into diarrhea incidence. While there appeared to be no statistical significance in antibiotic treatment, diarrhea was observed by the consulting veterinarian to improve with 72 h after antibiotic administration.

The effect of treatment on diarrhea severity can be found in Table 7. There was no effect of treatment on diarrhea severity in neonatal foals (no diarrhea, P = 0.522; mild diarrhea, P = 0.267; and severe diarrhea, P = 0.333). Although foals tended to only develop severe diarrhea within the first 14 d postpartum. Overall 65% (15 of 23) of foals

developed mild diarrhea, and 30% (7 of 23) of foals developed severe diarrhea. This is in agreement with Weese and Rousseau (2005). These authors conducted a similar study to evaluate the efficacy of *Lactobacillus pentosus* WE7 as a probiotic for prevention of neonatal diarrhea. A subjective assessment of fecal consistency was made daily (firm, normal, soft, or diarrheic). Researchers reported overall 51% of foals developed soft feces and 29% developed diarrhea (Weese and Rousseau, 2005). The effect of gender, location, and antibiotic administration on diarrhea severity for this study can be found in Tables. 8, 9, and 10. There was no effect of gender on diarrhea severity (no diarrhea, P = 0.609; mild diarrhea, P = 0.221; and severe diarrhea, P = 0.294). Additionally, there was no effect of location on diarrhea severity (no diarrhea, P = 0.217; mild diarrhea, P = 0.325; and severe diarrhea, P = 0.130). There was no effect of antibiotics on diarrhea severity (no diarrhea, P = 0.652; mild diarrhea, P = 0.195; and severe diarrhea, P = 0.130).

Daily observational evaluations on each foal were made regarding temperament, behavior, and signs of illness. Many of these assessments may provide further insight to the etiology of foal-heat diarrhea. Behaviors observed included: licking or chewing stall walls; chewing or eating mare's mane and tail; nursing; drinking water; eating hay and shavings; and bucking, kicking and playing. Additionally, coprophagy was observed in more than 70% of foals at least once, beginning as early as d 2. Coprophagy of the dam's feces by the foal is a normal behavior. Mullen et al. (2016) suggested an evolutionary adaptation for efficient inoculation of the GIT and considered the behavior part of normal development in the horse. The investigative nature of young foals, licking and chewing

surfaces, may also lead to ingestion of bacteria potentially triggering an immune response associated with mild diarrhea 'foal heat diarrhea'. Foals exhibiting mild diarrhea during the study remained bright, alert, and continued to nurse with normal vigor and did not require veterinary intervention. Mild diarrhea was managed by farm personnel with mineral oil application as needed to prevent skin scalding. Foals exhibiting severe diarrhea less than 7 d of age also displayed signs of depression or lethargy (n = 2), difficulty nursing (n = 2), shivering (n = 1) and blood in fecal material (n = 1). Indicating these foals may have developed diarrhea from an infectious agent. Foal 20 also had an umbilical cord infection known as omphalitis, in addition to severe diarrhea presenting on d 0.

Fecal Bacteria Microbiome

One hundred and thirty-nine fecal samples were analyzed, with 5 samples from each foal, and 1 sample from each dam submitted for analysis. Samples from d 21, 28 for foal 3 and mare 3 were not analyzed, and d 28 for mare 11 and foal 19 were not included. The overall fecal community composition by bacterial phylum within neonatal foals paired with their dam in the first 28-d postpartum is presented in Figure 2. The overall fecal community composition by bacterial genus level within neonatal foals paired with their dam in the first 28-d postpartum is presented in Figure 3. A visual assessment of each figure illustrates the predominant bacteria phylum to be *Firmicutes* and *Bacteroidetes*, which is in agreement with Julliand and Grimm (2016). However, Costa et al. (2015a) reported *Verrucomicrobia* rather than *Bacteroidetes*. These researchers characterized and compared the bacterial structures observed in the different

gastrointestinal compartments using high throughput sequencing. The authors reported marked differences among compartments even at high taxonomic levels, but *Firmicutes* comprised the main bacterial phylum in all compartments. Many members of this phylum are related to the breakdown of fiber, and specific components such as *Lachnospiraceae* and *Ruminococcaceae* are getting increasing attention as potentially important determinants of gut health. However, this phylum also contains various pathogens such as *Clostridium difficile* and *Clostridium perfringens* (Costa et al., 2015b). Within the feces 8 core phylotypes were identified, the most abundant being *Firmicutes, Verrucomicrobia, Spirochaetes, Fibrobacteres, Bacteroidetes,* and *Proteobacteria* (Costa et al., 2015a). The bacterial profiles found at higher taxonomic levels revealed that fecal samples can be representative of changes occurring in the distal compartments of the equine gastrointestinal tract (Costa et al., 2015a).

At the bacteria phylum level, there appeared to be changes over time in the foal fecal community. On d 0, *Proteobacteria* appear to be dominant, whereas on d 7 an apparent shift and increase in *Bacterioidetes* occurs. Additionally, in several foals around d 0 to d 7, an apparent increase in the *Verrucomicrobia* was observed. This is in agreement with Costa et al. (2015b), who also reported a dynamic shift in which several low abundant phyla virtually disappear after the first day of life. This is also consistent in newborn calves and dogs (Mayer et al., 2012; and Suchodolski et al., 2013). *Proteobacteria* include harmless symbionts and pathogens such as Salmonella and *E. coli*. Whereas, *Verrucomicrobia* consist of non-motile bacteria that are typically abundant in the environment particularly in soil and feces (Costa et al., 2015a). Finally, the

Actinobacteria phylum remained 10% abundance throughout the 28-d period. Schoster et al. (2014) reported healthy horses have greater abundance of *Actinobacteria* and *Spirochetes* whereas diarrheic horses have a greater abundance of *Fusobacteria*. *Actinobacteria* are known as a group of secondary metabolite producers that are useful to human medicine.

At the genus level *Ruminococcaceae* appears to be the predominant bacteria at approximately 15% abundance, in addition to a large population of unclassified bacteria. When visually accessing the genus level of the foal fecal communities there are also changes over time observed within the community. At d 0 a large population of *Enterobacteriaceae* were observed, these bacteria are members of the *Proteobacteria* phylum. After d 0 *Enterobacteriaceae* disappear and there was an apparent increase in the unclassified bacteria population after d 7 and continuing until d 28. This is in agreement with Costa et al. (2015b) that reported the presence of several low abundant genera that virtually disappear after the first day of life.

The alpha diversity plots for Shannon index, Chao index, and OTU's are presented in Figures 4, 5, and 6. The alpha diversity demonstrates the bacterial community richness and evenness within a sample. In each of the alpha diversity plots, there is an increase in community diversity over time. The neonatal foal's gut microbiota is developing over the 28-d period and becoming more like the dam's microbial community. The beta diversity plots for Braycurtis OTU and Jclass OTU calculations are presented in Figures 7 and 8. The beta diversity accounts for abundance and accesses structure of bacterial communities between samples. In each of these plots, the mare

samples on d 28 all appear clustered together, depicting the similarities in bacterial community structure. Additionally, the foal samples are all dispersed throughout the plot, representing the large variation within the microbiome community within the first 28-d postpartum. When samples are separated into individual beta diversity plots (Figure 9) the transition taking place in the gut microbiome over the 28-d period was more clearly elucidated. The trend was similar for all animals, as foals matured bacterial community structure also increased and began to approach a similar community structure as the dam, which is in agreement with Faubladier et al., 2013b.

Faubladier et al. (2013b) monitored age-related changes in the bacterial community structure in foal feces at pre- and post-weaning using Automated Ribosomal Intergenic Spacer Analysis. The results suggested that establishment of the intestinal bacterial community in foals is a sequential process, which reaches its climax state at around 1 mo of age (Faubladier et al., 2013b). Costa et al. (2015b) also characterized the intestinal bacterial colonization of newborn foals from birth to 9 mo. The authors further validated that newborn foals rapidly develop a rich and diverse fecal microbiota that undergoes change over time. The greater community richness after birth may result from environmental exposure, as the foal encounters a wide range of maternal and environmental bacteria that are transient organisms. These researchers determined that foals had a relatively stable intestinal microbiota after 60 d of life (Costa et al., 2015b).

CHAPTER V

CONCLUSIONS AND IMPLICATIONS

Results from this study indicate that administration of a probiotic containing 500 x 10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026 provides limited improvements on diarrhea incidence but, did not reduce severity in neonatal foals during the first 28-d postpartum. There was a significant Trt ×Day interaction on RBC volume, hemoglobin volume, hematocrit percentage and platelet volume. There was a main effect of Day on RBC, WBC, and leukocytes. Diarrhea was observed in 95% of foals; no main effect of treatment, gender, location, or antibiotics was observed on incidence and severity of diarrhea in neonatal foals.

During the first wk postpartum neonatal foals undergo many changes while developing their immune system and gut microbiome. Diarrhea during this time period can cause a major economic impact on owners and require veterinary intervention. Observations of the relative abundance of bacterial communities in foal's fecal microbiome, were observed to change over time and approached resemblance to the dam by d 28. The predominant bacteria phylum was identified to be *Firmicutes* and the predominant genus *Ruminococcaceae*. Future research is needed to further elucidate the role of the intestinal microbiome in neonatal foal-heat diarrhea and to potentially identify novel bacteria with beneficial probiotic effects.

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Table 1. Effect of probiotic administration on hematology parameters of neonatal foals.

	Treatment [1]			*P*-value		
Item	Control	Probiotic	SEM	Treatment	Day	Trt × Day
White blood cells, $\times 10^3/\mu L$	10.8	9.99	0.48	0.25	0.0053	0.67
Neutrophils, $\times 10^3/\mu L$	7.05	6.61	0.74	0.42	<0.001	0.69
Neutrophils, %	63.5	64.8	4.4	0.52	<0.001	0.89
Lymphocytes, $\times 10^3/\mu L$	3.34	2.97	0.18	0.044	<0.001	0.18
Lymphocytes, %	30.7	29.2	3.9	0.44	<0.001	0.92
Neutrophil: lymphocyte	2.44	2.49	0.41	0.79	<0.001	0.62
Monocytes, $\times 10^3/\mu L$	0.58	0.53	0.026	0.17	<0.001	0.92
Monocytes, %	5.46	5.31	0.33	0.62	<0.001	0.92
Eosinophils, $\times 10^3/\mu L$	0.04	0.06	0.012	0.30	<0.001	0.11
Eosinophils, %	0.43	0.66	0.13	0.21	<0.001	0.32
Basophils, $\times 10^3/\mu L$	0.03	0.04	0.005	0.18	0.70	0.75
Basophils, %	0.31	0.31	0.041	0.85	0.021	0.67
Red blood cells, $\times 10^6/\mu L$	9.49	8.85	0.58	0.15	<0.001	0.024
Hemoglobin, g/100 mL	12.7	11.6	1.2	0.13	<0.001	0.049
Hematocrit, %	33.5	30.2	3.2	0.13	<0.001	0.037
Platelets, $\times 10^3/\mu L$	218	209	8.8	0.45	<0.001	0.012

[1] Control = no treatment administration and Probiotic = administration of Pro Biostatin® (GLC Direct LLC, Lexington, KY). Animals assigned to P received 6g orally twice daily for fourteen days of Pro Biostatin®, containing 500 x 10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026.

Table 2. Effect of sampling day on hematology parameters of neonatal foals on d 0, 7, 14, 21, and 28.

	Day						Contrast, *P*-value		
Item	0	7	14	21	28	SEM	Linear	Quadratic	Cubic
White blood cells, $\times 10^3/\mu L$	10.1	11.5	10.1	10.6	9.67	0.660	0.23	0.21	0.024
Neutrophils, $\times 10^3/\mu L$	7.90	8.09	6.25	6.46	5.45	0.842	0.82	<.0001	0.026
Neutrophils, %	76.7	68.7	61.0	59.8	54.6	4.34	<.0001	<.0001	0.15
Lymphocytes, $\times 10^3/\mu L$	2.10	2.95	3.33	3.67	3.72	0.194	<.0001	<.0001	0.78
Lymphocytes, %	20.7	25.4	32.1	33.6	37.8	3.79	<.0001	<.0001	0.026
Neutrophil: lymphocyte	3.89	2.86	2.03	1.92	1.63	0.477	0.0013	<.0001	0.45
Monocytes, $\times 10^3/\mu L$	0.28	0.62	0.65	0.62	0.60	0.031	<.0001	<.0001	<.0001
Monocytes, %	2.73	2.96	6.42	5.90	6.27	0.415	<.0001	<.0001	<.0001
Eosinophils, $\times 10^3/\mu L$	0.004	0.03	0.044	0.066	0.12	0.032	0.0007	<.0001	0.042
Eosinophils, %	0.05	0.29	0.45	0.66	1.29	0.329	0.0009	<.0001	0.025
Basophils, $x10^3/\mu L$	0.03	0.036	0.031	0.031	0.029	0.013	0.68	0.77	0.45
Basophils, %	0.18	0.33	0.32	0.40	0.33	0.089	0.0027	0.037	0.076
Red blood cells, $\times 10^6/\mu L$	10.7	9.15	8.82	8.72	8.45	0.557	<.0001	<.0001	0.005
Hemoglobin, g/100 mL	14.6	12.4	11.6	11.3	10.8	1.15	<.0001	<.0001	0.11
Hematocrit, %	41.5	32.3	30.0	28.6	26.8	3.17	<.0001	<.0001	0.017
Platelets, $\times 10^3/\mu L$	211	144	230	239	243	12.8	0.0036	<.0001	0.0001

Table 3a. Effect of treatment on the average number of diarrhea bouts in neonatal foals during the first 28-d post-partum ($P = 0.105$).

Item	Incidence of Diarrhea	SEM
Control [1]	2.91	0.459
Probiotic [2]	1.83	0.439

[1] Control = no probiotic treatment administered

[2] Probiotic = administration of Pro Biostatin® (GLC Direct LLC, Lexington, KY). Animals assigned to P received 6 g orally 2x daily for 14 d of Pro Biostatin®, containing 500×10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026.

Table 3b. Effect of treatment on the duration of diarrhea bouts in neonatal foals during the first 28-d post-partum.

	Length of diarrhea in days		*P*- value		
Bout	Control [1]	Probiotic [2]	Treatment	Bout	Treatment × Bout
1st	2.32	2.79	0.631	0.001	0.6034
2nd	0.82	0.83			
3rd	0.77	0.29			
4th	0.45	0.25			
5th	0.23	0.04			
6th	0.18	0.00			

[1] Control = no probiotic treatment administered

[2] Probiotic = administration of Pro Biostatin® (GLC Direct LLC, Lexington, KY). Animals assigned to P received 6 g orally 2x daily for 14 d of Pro Biostatin®, containing 500×10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026.

Table 4a. Effect of gender on mean incidence of diarrhea in neonatal foals during the first 28-d postpartum ($P = 0.821$).

Item	Incidence of Diarrhea	SEM
Colts	2.29	0.433
Fillies	2.44	0.540

Table 4b. Effect of gender on the duration of diarrhea bouts in neonatal foals during the first 28-d postpartum.

	Duration of diarrhea, (d)		*P*- value		
Bout	Colts [1]	Fillies [2]	Gender	Bout	Gender × Bout
1st	2.11	3.28	0.251	0.001	0.742
2nd	0.82	0.83			
3rd	0.57	0.44			
4th	0.25	0.50			
5th	0.04	0.28			
6th	0.00	0.22			

Table 5a. Effect of location on mean incidence of diarrhea in neonatal foals during the first 28-d postpartum ($P = 0.242$).

Item	Incidence of Diarrhea	SEM
Ranch [1]	2.56	0.370
Hospital [2]	1.60	0.701

[1] Ranch = local ranch (Amarillo TX)
[2] Hospital = veterinary hospital (Canyon, TX).

Table 5b. Effect of location on the duration of diarrhea bouts in neonatal foals during the first 28-d postpartum.

	Duration of diarrhea, (d)		*P*- value		
Bout	Ranch [1]	Hospital [2]	Location	Bout	Location × Bout
1st	3.06	0.80	0.013	0.003	0.282
2nd	0.78	1.00			
3rd	0.64	0.10			
4th	0.44	0.00			
5th	0.16	0.00			
6th	0.11	0.00			

[1] Ranch = local ranch (Amarillo TX)
[2] Hospital = veterinary hospital (Canyon, TX).

Table 6a. Effect of antibiotic administration on mean incidence of diarrhea in neonatal foals during the first 28-d postpartum (P = 0.633).

Item	Incidence of Diarrhea	SEM
No Antibiotics [1]	2.47	0.416
Antibiotics [2]	2.13	0.570

[1] No antibiotics = foal did not experience severe diarrhea (severity score = 2), therefore was not administered additional veterinary intervention

[2] Antibiotics = foals exhibiting signs or symptoms of severe diarrhea within the first 4 d post-parturition were immediately administered a combination of Penicillin (30,000 i.u./kg bwt), Amikasin (25 mg/kg bwt), and Flunixin Meglumine (.55mg/kg bwt) for 7 d.

Table 6b. Effect of antibiotic administration on duration of diarrhea bouts in neonatal foals during the first 28-d postpartum.

	Duration of diarrhea, (d)		*P*- value		
Bout	No Antibiotics [1]	Antibiotics [2]	Antibiotics	Bout	Antibiotics × Bout
1st	2.63	2.44	0.451	0.003	0.508
2nd	0.93	0.63			
3rd	0.67	0.25			
4th	0.47	0.13			
5th	0.10	0.19			
6th	0.00	0.25			

[1] No antibiotics = foal did not experience severe diarrhea (severity score = 2), therefore was not administered additional veterinary intervention

[2] Antibiotics = foals exhibiting signs or symptoms of severe diarrhea within the first 4 d post-parturition were immediately administered a combination of Penicillin (30,000 i.u./kg bwt), Amikasin (25 mg/kg bwt), and Flunixin Meglumine (.55mg/kg bwt) for 7 d

Table 7. Effect of treatment on observed diarrhea severity score.

	Treatment [1], %, n		
Severity Score [2]	Control	Probiotic	*P*-value [3]
0	0.0% (0)	8.3% (1)	0.522
1	72.7% (8)	58.3% (7)	0.267
2	27.3% (3)	33.3% (4)	0.333

[1] Control = no treatment administration, Probiotic = administration of Pro Biostatin® (GLC Direct LLC, Lexington, KY). Animals assigned to P received 6 g orally 2x daily for 14 d of Pro Biostatin®, containing 500 × 10^6 cfu/g *Kluyveromyces fragilis* B0399 and *Saccharomyces cerevisiae* 1026.

[2] 0 = normal/ no diarrhea (clean rear, solid to soft feces, color and texture resembles that of an adult equine), 1 = mild diarrhea (wet "messy" tail and perianal area, loose and wet feces, discoloration or slight odor), 2 = severe diarrhea (extremely wet rear end, perianal area, croup and tail have fecal staining, squirting projectile feces with a strong odor)

[3]Difference of treatment means within severity score were determined using Fisher's exact test.

Table 8. Effect of gender on observed diarrhea severity score.

	Gender, %, n		
Severity Score [1]	Colts	Fillies	*P*-value [2]
0	7.1% (1)	0.0% (0)	0.609
1	57.1% (8)	77.8% (7)	0.221
2	35.7% (5)	22.2% (2)	0.294

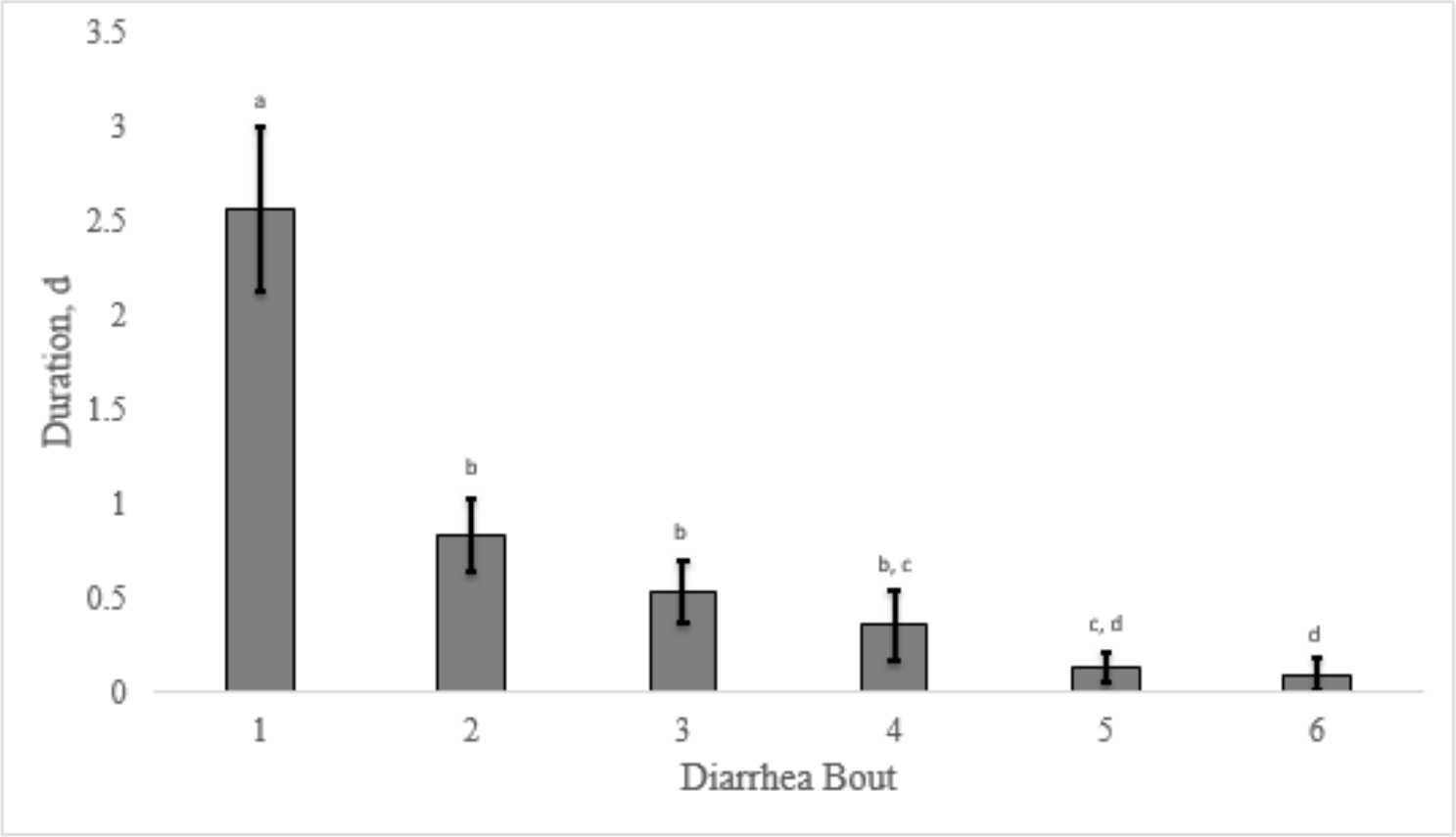
[1] 0 = normal/ no diarrhea (clean rear, solid to soft feces, color and texture resembles that of an adult equine), 1 = mild diarrhea (wet "messy" tail and perianal area, loose and wet feces, discoloration or slight odor), 2 = severe diarrhea (extremely wet rear end, perianal area, croup and tail have fecal staining, squirting projectile feces with a strong odor)[2]Difference of treatment means within severity score were determined using Fisher's exact test.

Table 9. Effect of location on observed diarrhea severity score

	Location [1], %, n		
Severity Score [2]	Ranch	Hospital	*P*-value [3]
0	0.0% (0)	20.0% (1)	0.217
1	61.1% (11)	80.0% (4)	0.325
2	38.9% (7)	0.0% (0)	0.130

[1] Ranch = local ranch (Amarillo TX), Hospital = veterinary hospital (Canyon, TX).[2] 0 = normal/ no diarrhea (clean rear, solid to soft feces, color and texture resembles that of an adult equine), 1 = mild diarrhea (wet "messy" tail and perianal area, loose and wet feces, discoloration or slight odor), 2 = severe diarrhea (extremely wet rear end, perianal area, croup and tail have fecal staining, squirting projectile feces with a strong odor)
[3]Difference of treatment means within severity score were determined using Fisher's exact test.

Figure 1. Duration (d) of diarrhea bouts in neonatal foals during the first 28-d postpartum



Bouts with unlike superscript letters differ $P \leq 0.014$

Figure 2. Bacteria phylum community composition of fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

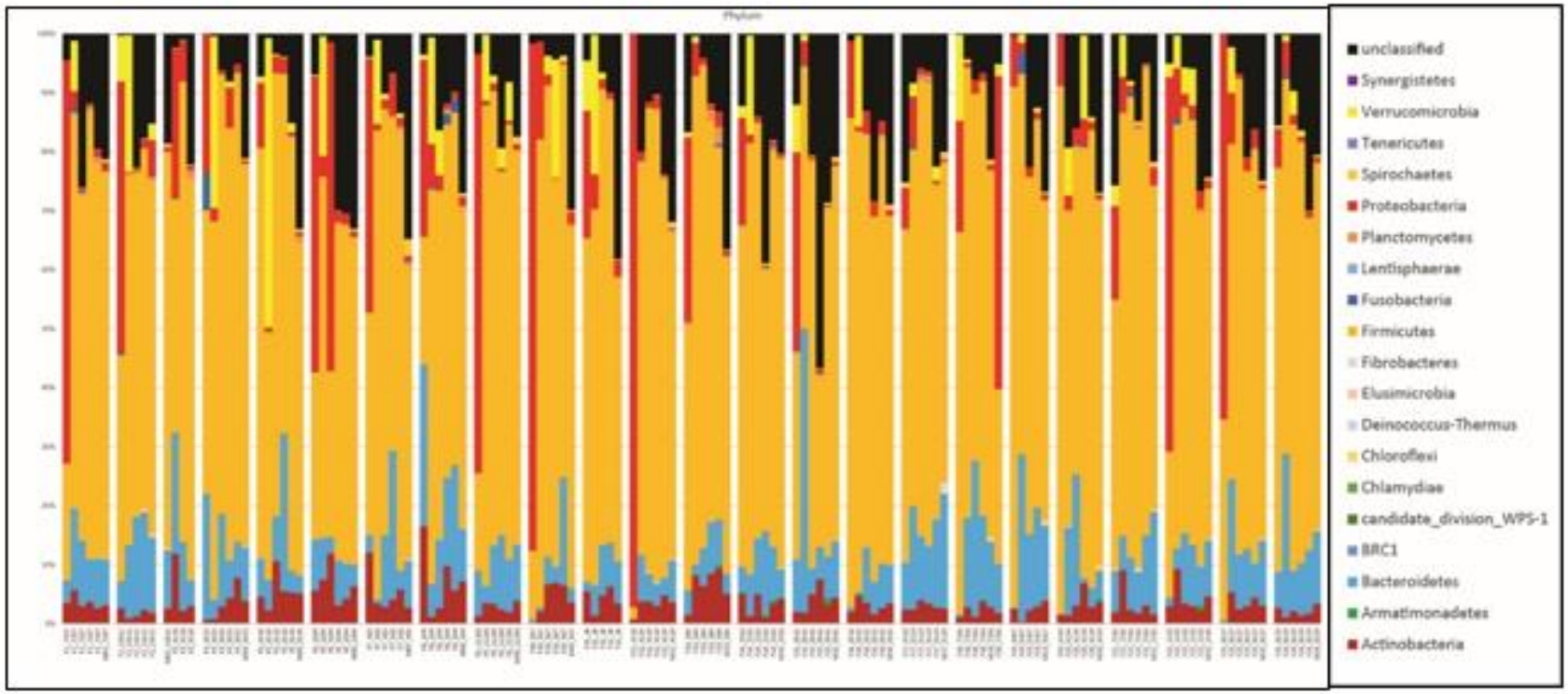


Figure 3. Bacteria genus community composition of fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

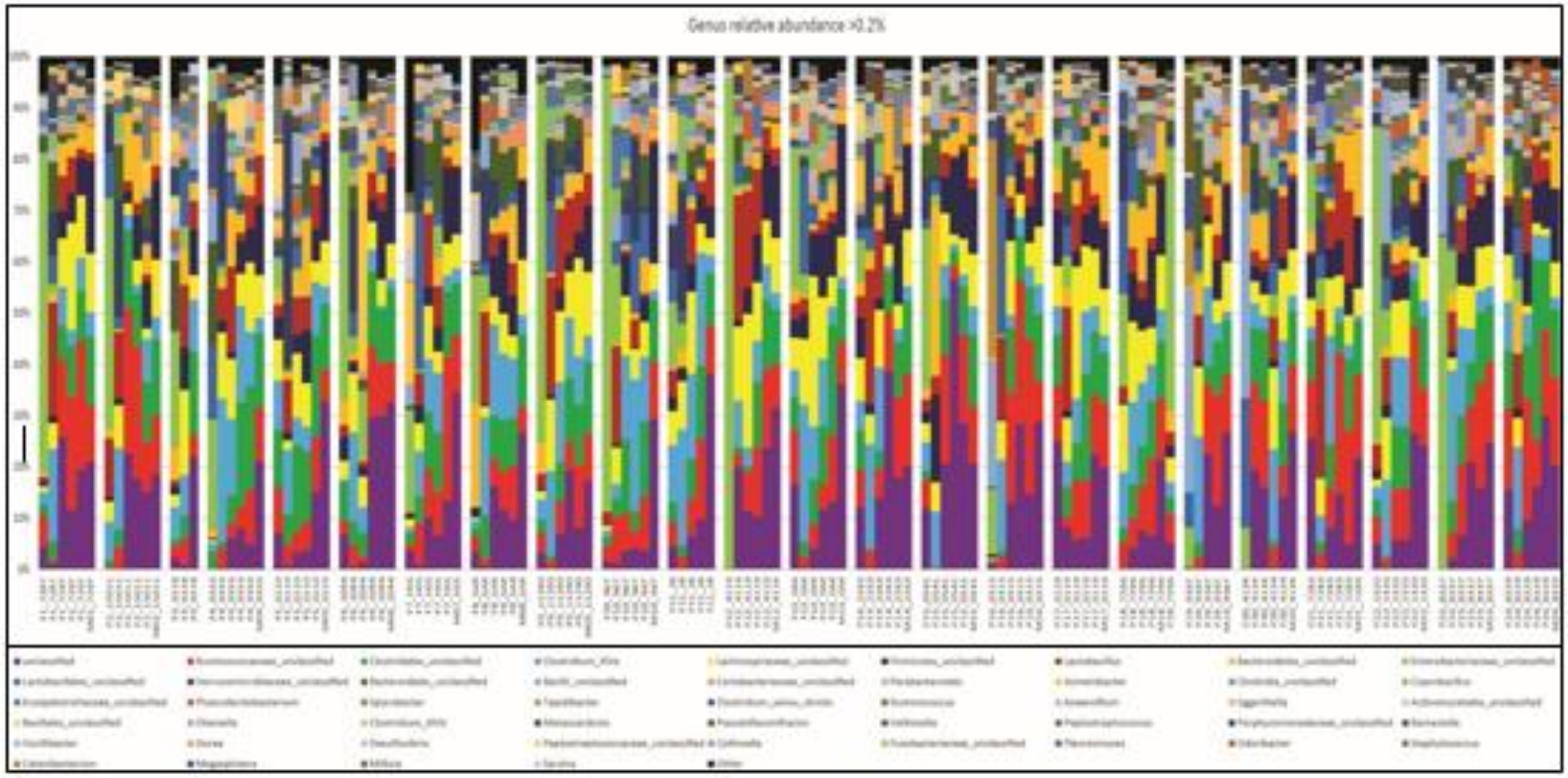


Figure 4. Shannon alpha diversity plot for fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

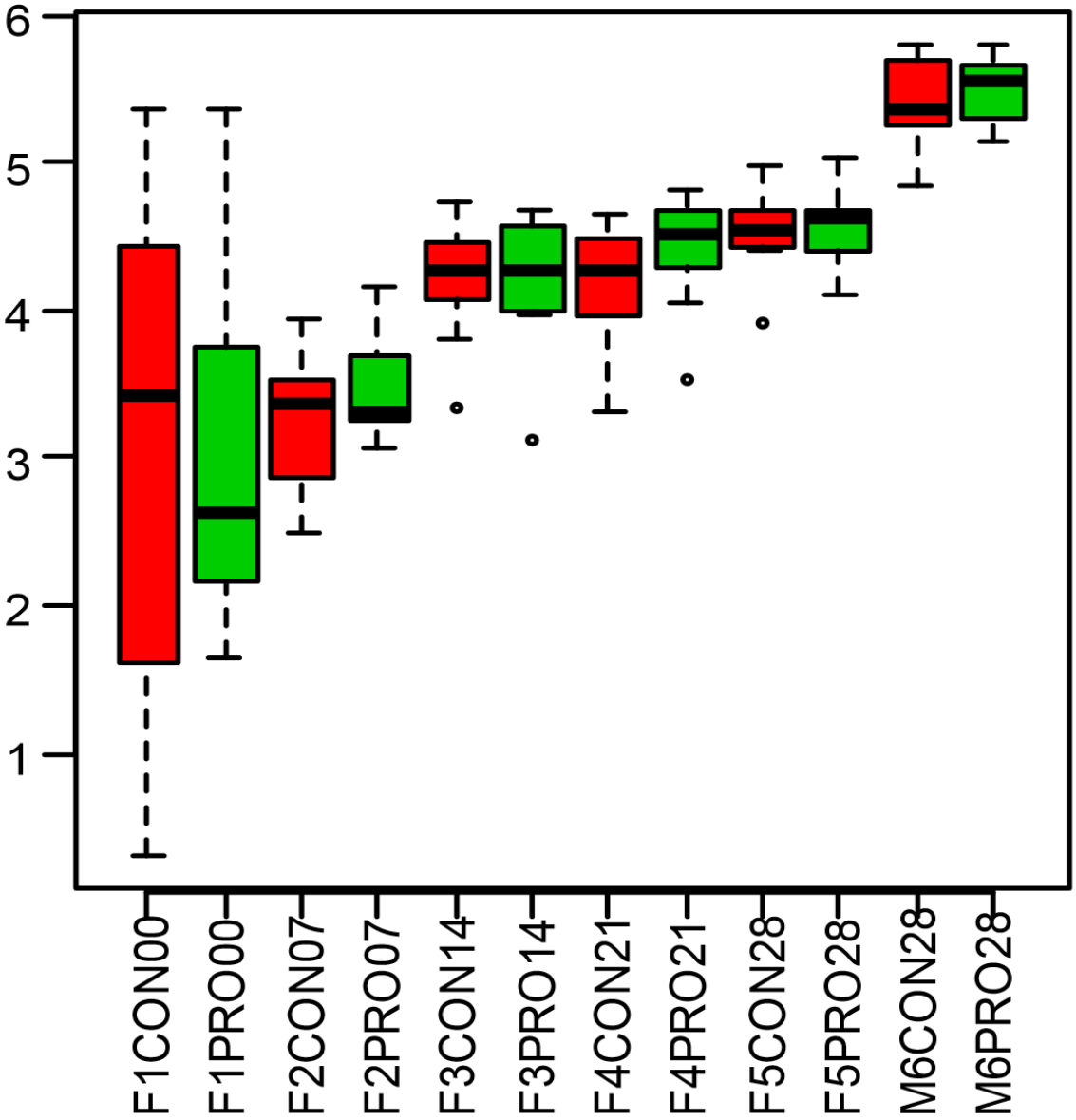


Figure 5. Observed OTUs alpha diversity plot for fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

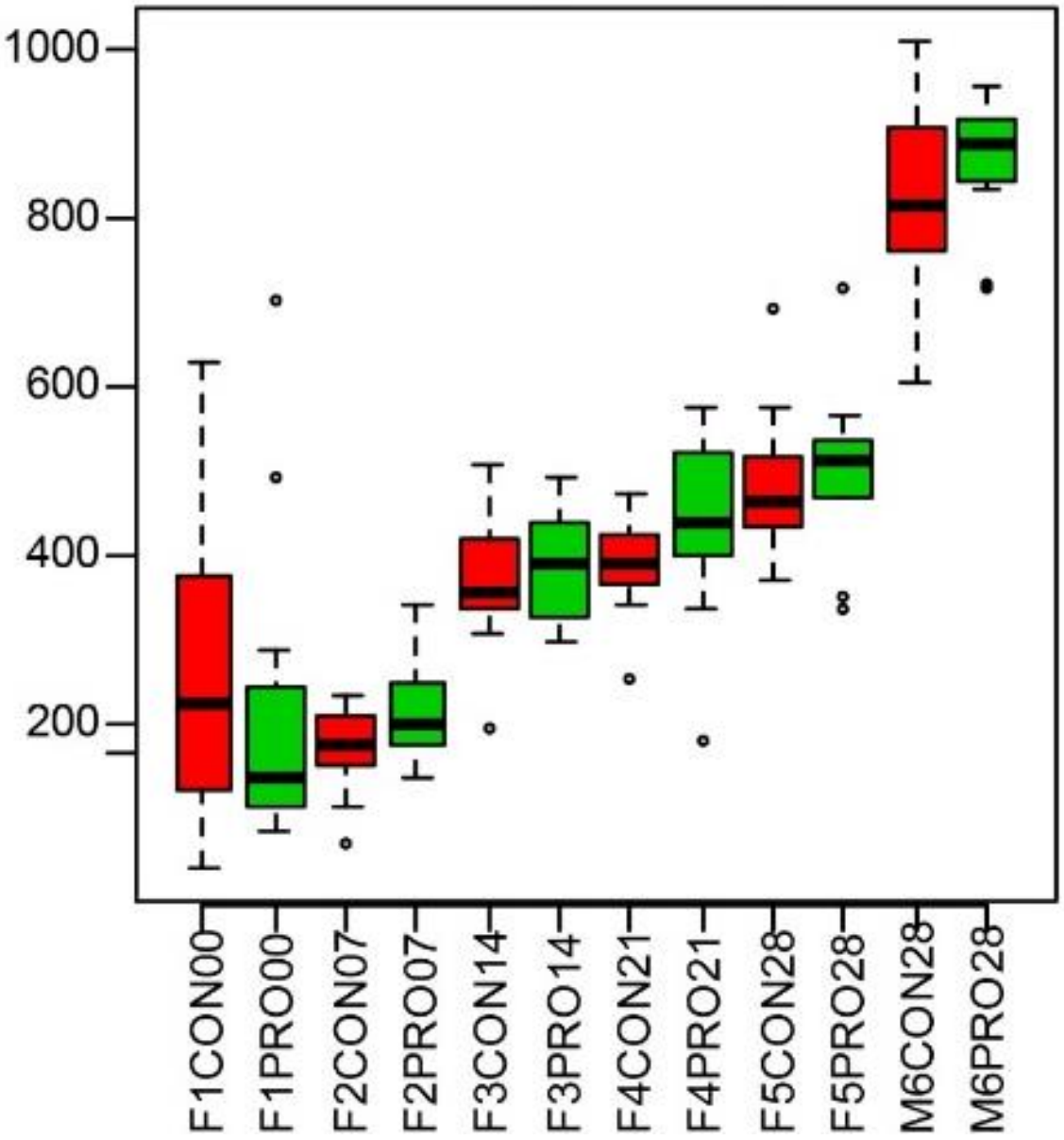


Figure 6. Chao Index alpha diversity plot for fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

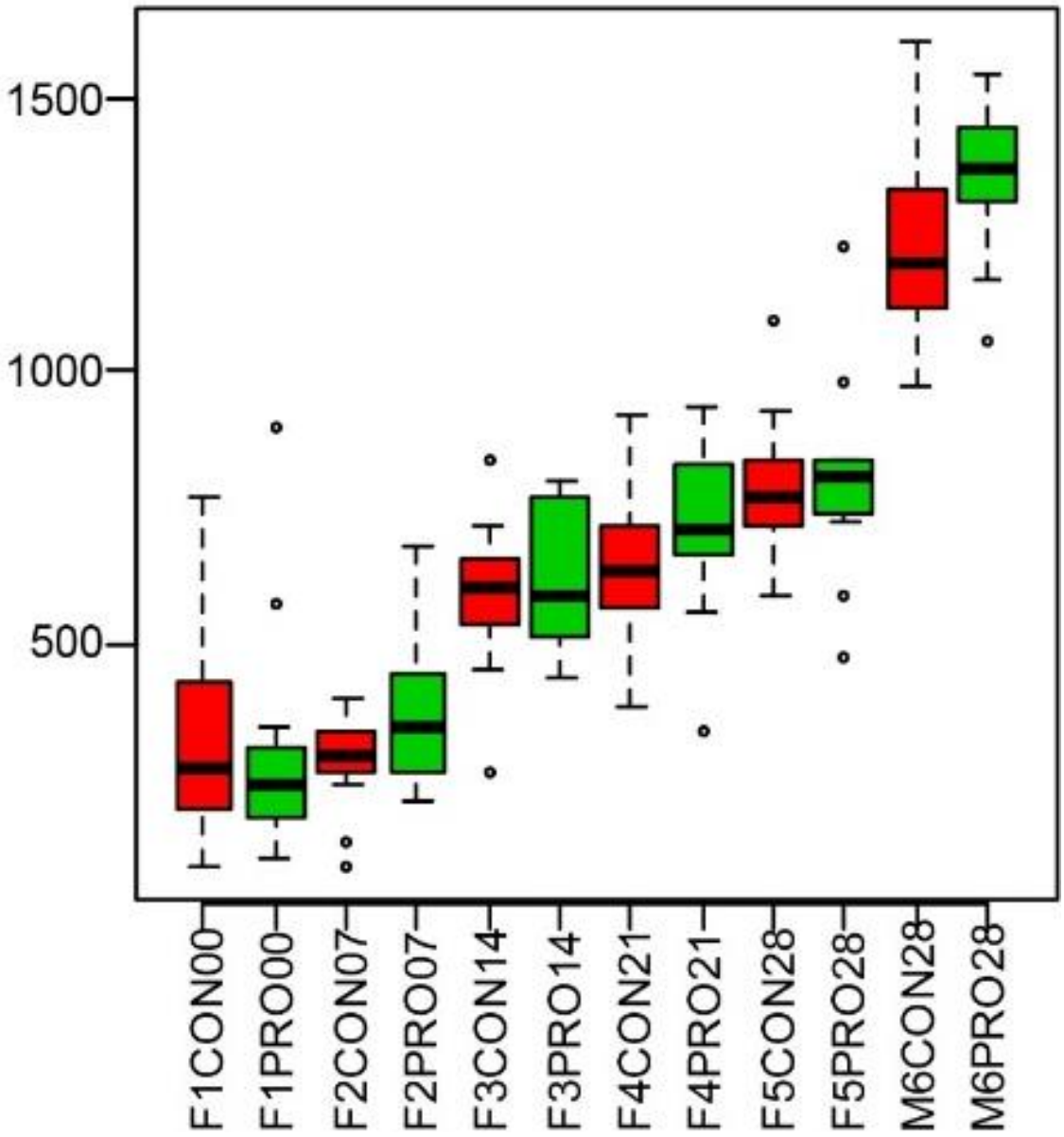


Figure 7. Braycurtis OTU beta diversity plot for fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

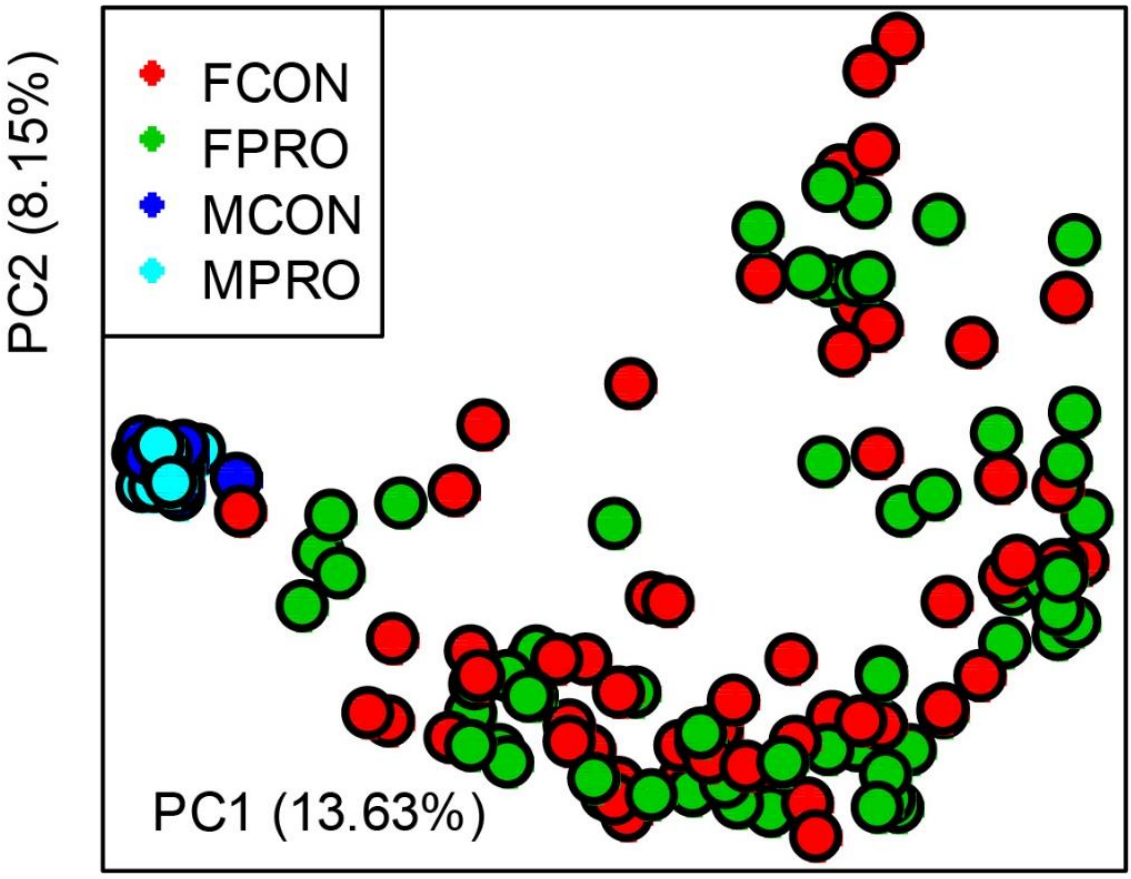


Figure 8. Jclass OTU beta diversity plot for fecal samples from neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.

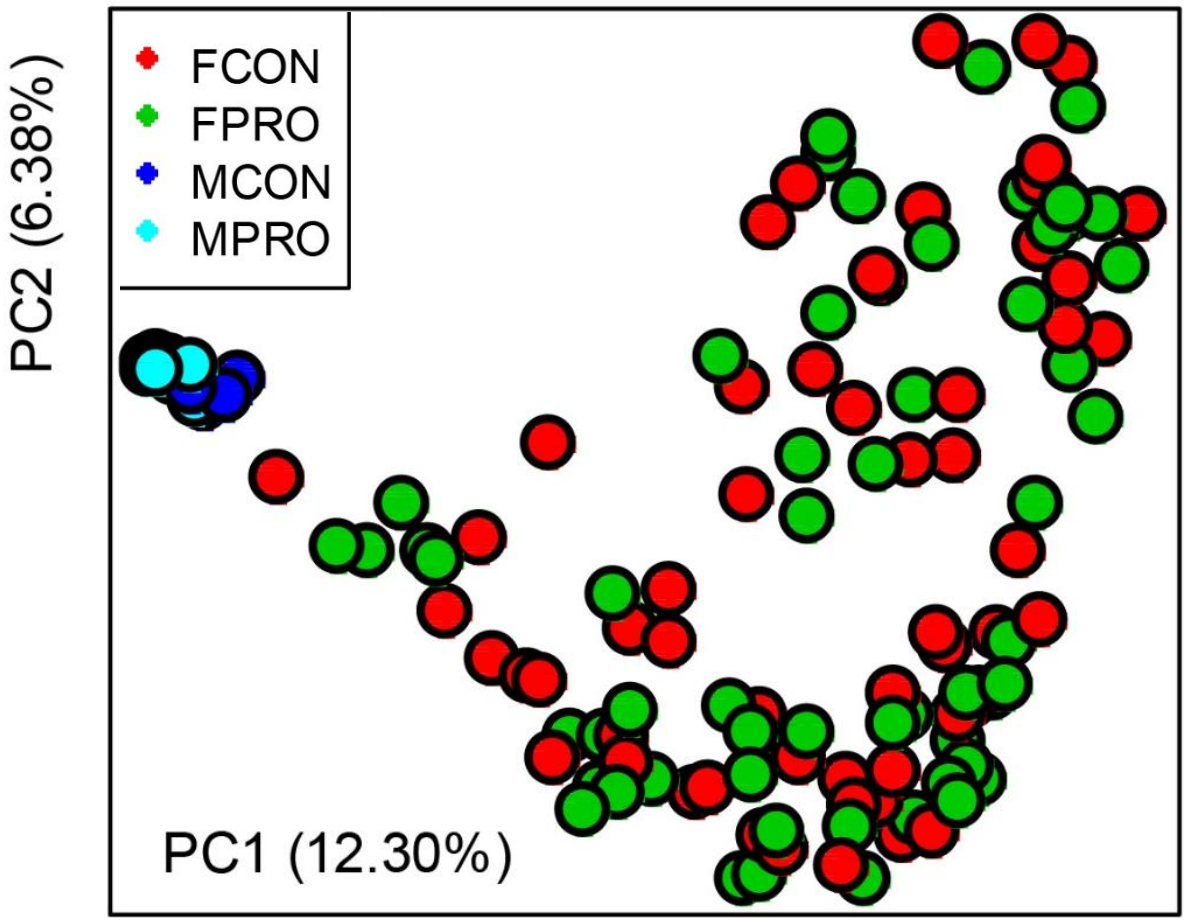
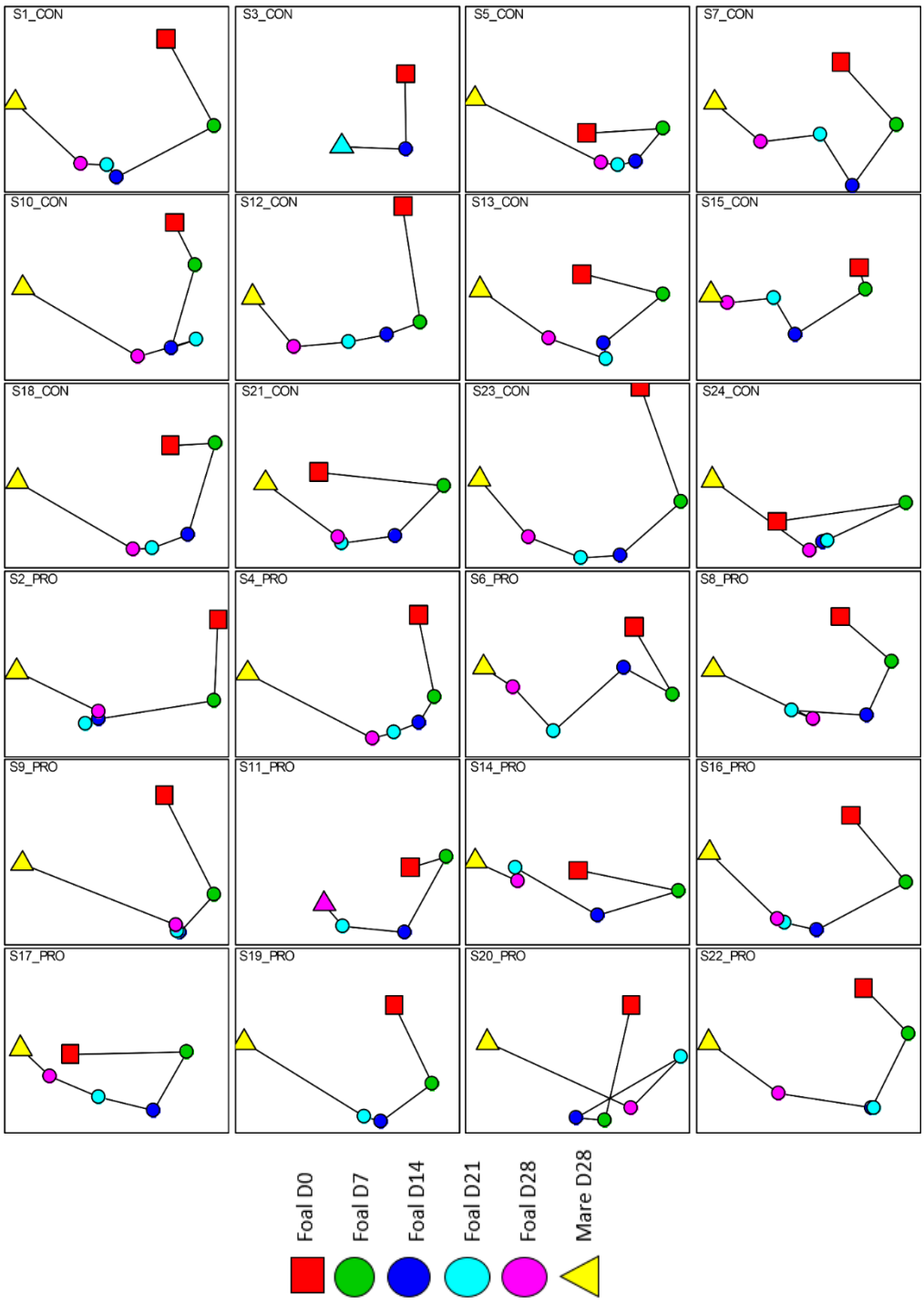


Figure 9. Individual animal beta diversity plots for neonatal foals receiving one of two treatments (Control or Probiotic) paired with their dam over the course of 28 d sampled on d 0, 7, 14, 21, and 28.



APPENDIX TABLES

Table A-1. Individual white blood cells ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
Control					
1	10.07	12.73	10.52	9.31	6.95
5	4.07	7.03	9.67	10.60	8.11
7	12.45	11.40	10.55	12.06	11.07
10	8.66	11.72	8.34	10.53	10.52
12	13.73	21.28	12.37	10.27	9.97
13	7.84	10.95	11.34	11.50	12.10
15	15.94	12.51	9.56	12.52	13.24
18	6.99	7.79	7.05	8.03	6.73
21	12.95	11.43	10.07	12.02	13.03
23	12.98	13.86	11.69	13.45	13.86
24	10.65	9.39	10.16	10.33	8.23
Probiotic					
2	10.75	13.07	8.33	8.65	8.39
4	5.76	10.06	9.40	8.61	7.78
6	9.03	9.29	9.29	6.94	7.98
8	9.28	8.33	14.31	11.70	8.74
9	14.53	12.80	12.86	13.95	11.69
11	6.10	11.69	9.51	11.96	7.31
14	9.65	10.03	9.68	10.23	7.73
16	7.33	8.86	8.39	9.98	9.59
17	10.99	9.83	7.00	7.80	7.18
19	12.28	14.52	12.25	12.51	10.92
20	8.12	10.95	8.99	8.79	9.53
22	10.47	14.38	9.83	12.15	11.06

Table A-2. Individual neutrophils ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

Horse	Day				
	0	7	14	21	28
Control					
1	7.08	8.46	6.55	4.93	3.01
5	2.36	3.45	5.25	5.45	4.13
7	9.44	7.31	6.22	6.80	5.25
10	6.64	8.65	5.20	6.39	6.37
12	11.32	17.03	8.38	6.62	6.62
13	5.76	7.52	6.17	6.55	6.93
15	13.25	9.00	6.27	7.11	7.52
18	4.56	4.15	2.89	3.36	2.35
21	10.51	7.52	5.68	6.77	6.86
23	10.08	10.09	6.64	8.03	7.86
24	6.88	5.60	5.81	5.88	3.82
Probiotic					
2	8.41	9.49	4.80	4.90	4.02
4	4.19	6.00	5.04	4.62	3.71
6	6.99	5.02	5.02	3.03	3.55
8	7.24	4.75	9.41	7.48	4.59
9	10.61	9.15	9.21	9.96	8.19
11	3.69	8.71	5.55	7.77	2.84
14	7.40	6.96	6.63	6.48	3.70
16	5.83	6.09	5.28	6.67	6.29
17	8.74	7.21	4.27	5.01	4.39
19	9.03	9.64	6.50	7.01	5.49
20	6.14	7.47	5.00	4.61	5.02
22	8.51	10.03	5.40	6.72	5.95

Table A-3. Individual lymphocytes ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
			Control		
1	2.64	3.22	3.32	3.84	3.24
5	1.58	2.86	3.50	4.31	3.29
7	2.71	3.45	3.59	4.44	4.80
10	1.65	2.50	2.56	3.42	3.47
12	1.61	3.19	3.13	2.87	2.68
13	1.89	2.88	4.41	4.21	4.44
15	2.42	2.95	2.76	4.44	5.00
18	2.32	3.19	3.64	3.99	3.78
21	1.99	3.26	3.50	4.46	5.33
23	2.32	3.03	4.09	4.59	5.00
24	3.59	3.17	3.61	3.95	3.90
			Probiotic		
2	2.00	2.66	2.89	3.07	3.40
4	1.41	3.36	3.80	3.42	3.51
6	1.82	3.36	3.36	3.21	3.55
8	1.78	2.86	4.05	3.52	3.47
9	3.39	3.00	2.89	3.10	2.86
11	2.21	2.49	3.30	3.32	3.68
14	2.06	2.54	2.54	3.12	3.46
16	1.35	2.22	2.65	2.72	2.69
17	2.08	2.13	2.25	2.31	2.37
19	2.83	4.05	4.70	4.56	4.10
20	1.70	2.72	3.17	3.59	3.73
22	1.69	3.63	3.71	4.70	4.32

Table A-4. Individual monocytes ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
			Control		
1	0.33	1.01	0.56	0.48	0.53
5	0.13	0.66	0.86	0.70	0.61
7	0.29	0.60	0.65	0.75	0.92
10	0.36	0.50	0.50	0.61	0.59
12	0.76	0.93	0.78	0.67	0.60
13	0.19	0.49	0.68	0.65	0.64
15	0.22	0.50	0.47	0.87	0.68
18	0.10	0.40	0.46	0.60	0.51
21	0.42	0.59	0.83	0.71	0.74
23	0.56	0.69	0.89	0.71	0.67
24	0.17	0.57	0.67	0.46	0.40
			Probiotic		
2	0.15	0.61	0.50	0.46	0.44
4	0.19	0.80	0.80	0.55	0.49
6	0.24	0.69	0.76	0.63	0.55
8	0.48	0.58	0.69	0.76	0.58
9	0.18	0.45	0.59	0.80	0.70
11	0.18	0.47	0.45	0.58	0.53
14	0.13	0.51	0.41	0.53	0.57
16	0.15	0.41	0.39	0.40	0.35
17	0.35	0.70	0.95	0.75	0.67
19	0.26	0.70	0.73	0.52	0.67
20	0.24	0.65	0.68	0.64	0.74
22	0.33	1.01	0.56	0.48	0.53

Table A-5. Individual eosinophils ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
			Control		
1	0.00	0.02	0.05	0.06	0.15
5	0.00	0.03	0.03	0.06	0.05
7	0.00	0.03	0.06	0.05	0.09
10	0.00	0.02	0.03	0.06	0.05
12	0.00	0.05	0.06	0.07	0.05
13	0.00	0.03	0.05	0.07	0.03
15	0.01	0.04	0.04	0.06	0.03
18	0.00	0.03	0.04	0.07	0.06
21	0.00	0.03	0.05	0.06	0.09
23	0.00	0.03	0.04	0.09	0.27
24	0.00	0.02	0.04	0.03	0.05
			Probiotic		
2	0.01	0.04	0.03	0.07	0.09
4	0.00	0.08	0.08	0.13	0.37
6	0.01	0.02	0.03	0.05	0.12
8	0.00	0.03	0.03	0.06	0.03
9	0.01	0.02	0.04	0.05	0.06
11	0.00	0.02	0.02	0.03	0.02
14	0.01	0.01	0.03	0.04	0.01
16	0.01	0.02	0.04	0.04	0.03
17	0.01	0.04	0.07	0.16	0.61
19	0.01	0.04	0.05	0.05	0.08
20	0.00	0.03	0.03	0.04	0.03
22	0.00	0.02	0.05	0.06	0.15

Table A-6. Individual basophils ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
			Control		
1	0.02	0.02	0.04	0.00	0.02
5	0.00	0.03	0.03	0.08	0.03
7	0.01	0.01	0.03	0.02	0.01
10	0.01	0.05	0.05	0.05	0.04
12	0.04	0.08	0.02	0.04	0.02
13	0.00	0.03	0.03	0.02	0.06
15	0.04	0.02	0.02	0.04	0.01
18	0.01	0.02	0.02	0.01	0.03
21	0.03	0.03	0.01	0.02	0.01
23	0.02	0.02	0.03	0.03	0.03
24	0.01	0.03	0.03	0.01	0.06
			Probiotic		
2	0.00	0.05	0.03	0.04	0.03
4	0.03	0.03	0.03	0.02	0.02
6	0.01	0.01	0.06	0.02	0.01
8	0.30	0.04	0.04	0.07	0.03
9	0.01	0.02	0.03	0.02	0.03
11	0.01	0.04	0.04	0.02	0.02
14	0.01	0.03	0.02	0.02	0.03
16	0.01	0.06	0.05	0.04	0.04
17	0.06	0.09	0.03	0.03	0.05
19	0.01	0.02	0.04	0.02	0.03
20	0.03	0.04	0.01	0.05	0.02
22	0.02	0.02	0.04	0.00	0.02

Table A-7. Individual red blood cells ($\times 10^6/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
			Control		
1	10.97	10.42	9.57	9.75	9.60
5	12.24	11.84	11.29	9.99	9.64
7	9.13	7.67	7.81	8.15	8.58
10	11.03	9.25	7.72	8.47	9.00
12	11.42	8.77	7.57	7.74	7.98
13	10.78	8.82	8.84	8.69	9.08
15	11.17	9.27	9.01	8.92	8.96
18	12.55	10.24	9.71	8.98	8.90
21	12.37	11.70	9.63	9.53	9.60
23	12.72	9.89	10.43	11.05	10.56
24	11.24	9.40	9.04	9.50	9.17
			Probiotic		
2	10.05	7.98	8.13	8.05	7.08
4	8.68	7.05	7.93	8.36	7.11
6	11.49	9.64	9.64	9.30	10.15
8	9.35	7.97	8.49	7.97	6.84
9	11.51	8.74	8.18	8.68	7.77
11	8.52	7.88	7.97	7.80	7.08
14	10.08	8.45	8.29	7.73	7.42
16	12.51	12.83	11.79	11.62	11.61
17	9.83	8.02	8.06	7.42	6.20
19	9.89	8.28	8.11	7.93	7.67
20	12.46	10.56	10.44	9.81	9.83
22	10.88	10.39	9.91	9.90	9.05

Table A-8. Individual hemoglobin (g/100 mL) in neonatal foals on d 0, 7, 14, 21, 28.

Horse	Day				
	0	7	14	21	28
Control					
1	15.40	14.30	13.00	13.30	12.60
5	16.90	16.80	15.20	13.00	12.50
7	12.80	10.70	10.80	10.80	11.10
10	16.40	13.90	11.10	12.20	12.70
12	15.90	12.10	9.90	9.80	9.90
13	15.50	12.40	12.50	11.80	12.10
15	15.10	12.80	12.00	11.90	11.60
18	16.90	13.90	12.60	11.60	11.50
21	17.80	16.70	13.60	13.40	13.20
23	17.40	13.60	14.00	14.40	13.60
24	15.40	12.90	12.10	12.20	12.10
Probiotic					
2	13.80	11.00	11.00	10.80	9.60
4	16.90	13.60	13.60	12.50	13.40
6	12.40	10.60	11.10	10.20	9.10
8	16.00	12.20	11.00	11.20	10.10
9	9.80	9.30	9.30	9.30	8.60
11	14.30	12.00	11.30	10.20	9.50
14	17.20	17.80	15.90	15.50	15.00
16	12.70	10.40	10.20	9.50	8.20
17	13.10	10.90	10.40	10.40	9.70
19	18.10	15.30	14.80	13.70	13.30
20	14.30	13.90	12.60	12.60	11.60
22	15.40	14.30	13.00	13.30	12.60

Table A-9. Individual hematocrit (%) in neonatal foals on d 0, 7, 14, 21, 28.

Horse	Day				
	0	7	14	21	28
Control					
1	44.30	39.40	34.80	34.80	33.70
5	46.30	41.60	38.70	32.00	29.90
7	35.90	27.40	26.20	26.20	27.20
10	46.60	35.80	29.20	31.20	32.50
12	47.40	32.40	26.00	24.90	24.00
13	45.80	34.10	33.00	31.30	32.00
15	44.10	33.60	31.70	31.00	30.60
18	49.80	37.10	33.90	29.90	28.80
21	49.70	44.00	34.60	34.20	33.80
23	48.90	34.30	36.10	37.50	34.40
24	43.00	33.10	30.70	31.20	29.10
Probiotic					
2	38.40	27.60	26.90	25.60	21.40
4	46.00	35.10	35.10	32.00	34.50
6	33.50	25.70	27.40	24.20	19.50
8	45.30	30.70	27.50	28.20	23.80
9	25.50	22.50	22.50	21.60	19.00
11	39.70	30.40	28.50	25.20	23.20
14	51.40	49.30	43.30	41.30	40.20
16	38.40	27.90	26.50	23.50	18.30
17	38.00	29.40	27.20	25.40	23.60
19	52.80	40.60	40.20	36.50	36.00
20	39.90	36.40	33.40	32.60	28.50
22	44.30	39.40	34.80	34.80	33.70

Table A-10. Individual platelets ($\times 10^3/\mu L$) in neonatal foals on d 0, 7, 14, 21, 28.

	Day				
Horse	0	7	14	21	28
Control					
1	216.00	148.00	251.00	303.00	319.00
5	198.00	88.00	117.00	169.00	191.00
7	165.00	121.00	223.00	235.00	296.00
10	248.00	85.00	183.00	198.00	219.00
12	300.00	168.00	255.00	314.00	254.00
13	143.00	105.00	244.00	263.00	294.00
15	226.00	109.00	193.00	199.00	278.00
18	248.00	183.00	259.00	239.00	237.00
21	253.00	38.00	241.00	255.00	237.00
23	299.00	242.00	290.00	318.00	297.00
24	243.00	116.00	218.00	237.00	243.00
Probiotic					
2	108.00	158.00	198.00	205.00	146.00
4	237.00	248.00	248.00	272.00	314.00
6	207.00	207.00	272.00	196.00	206.00
8	242.00	143.00	171.00	225.00	190.00
9	175.00	214.00	257.00	250.00	282.00
11	276.00	85.00	199.00	228.00	228.00
14	192.00	168.00	270.00	184.00	220.00
16	244.00	107.00	219.00	195.00	209.00
17	100.00	90.00	216.00	235.00	227.00
19	88.00	148.00	257.00	235.00	250.00
20	238.00	128.00	221.00	258.00	240.00
22	216.00	148.00	251.00	303.00	319.00

Table A-11. Individual diarrhea incidence by treatment in neonatal foals

Horse	Incidence	Length of Diarrhea Bout (d)					
		1st	2nd	3rd	4th	5th	6th
		Control					
1	3	6.5	1.0	2.5	0.0	0.0	0.0
5	3	2.5	1.0	0.5	0.0	0.0	0.0
7	1	3.5	0.0	0.0	0.0	0.0	0.0
10	2	3.5	1.5	0.0	0.0	0.0	0.0
12	1	1.0	0.0	0.0	0.0	0.0	0.0
13	2	2.0	1.0	0.0	0.0	0.0	0.0
15	2	0.5	2.0	0.0	0.0	0.0	0.0
18	3	0.5	1.0	1.0	0.0	0.0	0.0
21	6	2.0	0.5	1.5	1.0	1.5	2.0
23	5	1.5	0.5	2.0	3.5	1.0	0.0
24	4	2.0	0.5	1.0	0.5	0.0	0.0
		Probiotic					
2	4	3.5	1.5	2.0	1.0	0.0	0.0
4	1	4.0	0.0	0.0	0.0	0.0	0.0
6	2	1.0	3.5	0.0	0.0	0.0	0.0
8	1	4.0	0.0	0.0	0.0	0.0	0.0
9	2	0.5	1.5	0.0	0.0	0.0	0.0
11	1	2.0	0.0	0.0	0.0	0.0	0.0
14	3	2.0	1.5	0.5	0.0	0.0	0.0
16	5	1.5	2.0	1.0	2.0	0.5	0.0
17	0	0.0	0.0	0.0	0.0	0.0	0.0
19	1	9.0	0.0	0.0	0.0	0.0	0.0
20	1	3.0	0.0	0.0	0.0	0.0	0.0
22	1	3.0	0.0	0.0	0.0	0.0	0.0

Table A-12. Overall frequency of diarrhea severity scores in neonatal foals by day.

	Number of Animals		
	0	1	2
D0	19	1	3
D1-AM	19	2	2
D1-PM	19	4	0
D2-AM	19	2	2
D2-PM	20	3	0
D3-AM	21	1	1
D3-PM	22	0	1
D4-AM	22	0	1
D4-PM	21	2	0
D5-AM	22	1	0
D5-PM	21	2	0
D6-AM	19	4	0
D6-PM	19	4	0
D7-AM	20	3	0
D7-PM	20	3	0
D8-AM	20	3	0
D8-PM	18	5	0
D9-AM	17	6	0
D9-PM	19	4	0
D10-AM	19	4	0
D10-PM	18	4	1
D11-AM	18	5	0
D11-PM	19	4	0
D12-AM	20	3	0
D12-PM	19	4	0
D13-AM	18	5	0
D13-PM	20	3	0
D14-AM	19	4	0
D14-PM	19	4	0
D15-AM	17	6	0
D15-PM	17	5	1
D16-AM	18	5	0
D16-PM	19	4	0
D17-AM	18	5	0
D17-PM	17	6	0
D18-AM	19	4	0
D18-PM	20	3	0
D19-AM	18	5	0
D19-PM	16	7	0
D20-AM	19	4	0
D20-PM	20	3	0
D21-AM	19	4	0
D21-PM	18	5	0
D22-AM	19	4	0
D22-PM	20	3	0
D23-AM	22	1	0
D23-PM	21	2	0
D24-AM	20	3	0
D24-PM	21	2	0
D25-AM	19	4	0
D25-PM	19	4	0
D26-AM	20	3	0
D26-PM	21	2	0
D27-AM	20	3	0
D27-PM	22	1	0
D28-AM	21	2	0
D28-PM	21	2	0

0=No Diarrhea, 1=Mild Diarrhea, & 2= Severe Diarrhea

APPENDIX FIGURES

Figure A-1. Foal Linear Discriminate Analysis plot

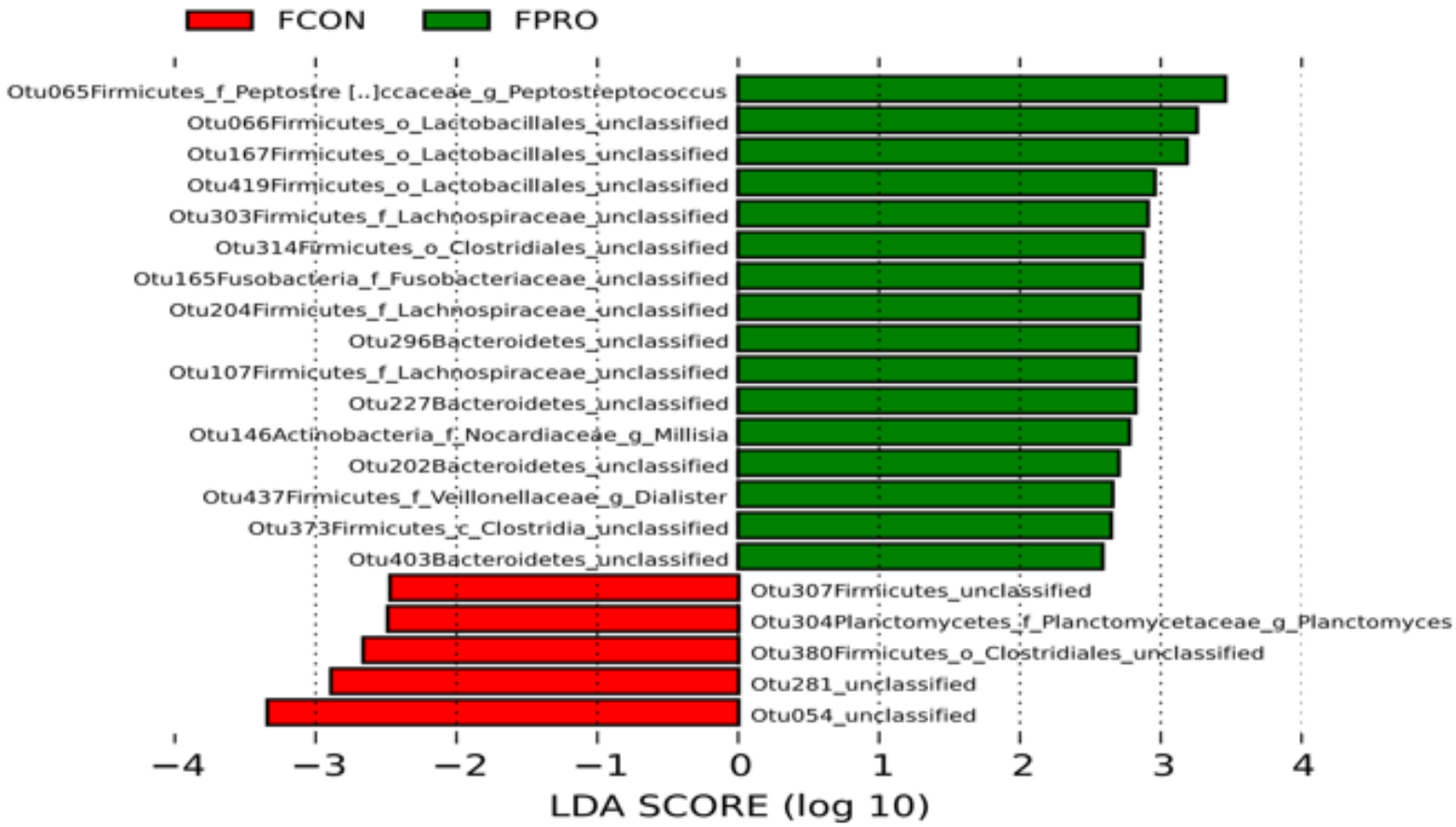


Figure A-2. Mare Linear Discriminate Analysis plot