The Influence of Probiotic Supplements on Microbial Diversity in the Gastrointestinal Microbiome of Healthy Horses

Thesis

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ABSTRACT

Bacteria play a crucial role in the gastrointestinal health of many animals, including horses. Stress, illness, medication and diet can easily alter microbial populations in the equine gastrointestinal tract, leading to decreased gut health and poor performance. Probiotics are often utilized therapeutically to re-establish homeostasis of gut bacteria following gastrointestinal upset in horses. Recently there has been increased interest in the prophylactic use of probiotic supplements in healthy horses. Further research is warranted to identify effective probiotic strains for horses, and optimize the dose administered and duration of supplementation needed to maximize health and performance. Therefore, the objective of this research was to evaluate the influence of probiotic supplements in three separate studies on the microbial diversity of the gastrointestinal microbiome of healthy horses. In the first study, seven Quarter Horse mares (10.0 ± 2.0 yr) were randomly assigned to one of two treatments in a crossover design. The 49 d experimental periods consisted of a 14 d adaptation period followed by a 35 d sampling period. All horses received a control diet of 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL) or the control diet supplemented with 140 g of a human probiotic supplement containing 10⁹ cfu per dose of Lactobacillus acidophilus (TRT) for the 49 d experimental period. Fecal samples were collected on d 0 before supplementation and on d 14, 21, 28, 35, 42, and 49 of the
experimental periods. In the second study, 12 Quarter Horses (4 geldings, 8 mares; 1.5 ± 0.5 yr) were randomly assigned to one of two treatments for a period of 77 d. All horses received a control diet of 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL) or the control diet supplemented with 10 g of a commercial livestock probiotic supplement containing 10^7 cfu per dose of *Lactobacillus acidophilus* (TRT). Fecal samples were collected on d 0 before supplementation and then every 7 d throughout the study. In the third study, six Miniature Horse geldings (7.5 ± 3.5 yr) were used in a replicated 3 x 3 Latin Square design. Each horse received a control diet of 1.5% BW of a mixed grass hay and water ad libitum and was randomly assigned to one of three treatment groups during each 14 d period: control diet only (CTL), control diet supplemented with 0.11 g/kg of BW of a commercial equine probiotic containing 2.0 x 10^7 cfu/g of *Bacillus subtilis* (LO) or control diet supplemented with 0.22 g/kg of BW of the commercial equine probiotic (HI). Fecal samples were collected on d 0 before supplementation and then on d 3, 7, 10 and 14 of each period. For each study, fecal samples were pooled by treatment group and time. DNA was extracted and subjected to PCR-DGGE with primers specific to 16S rRNA gene sequences to evaluate changes in bacterial diversity. PCR-DGGE images were analyzed with BioNumerics software to generate dendrogram comparisons based on the position and number of bands with further evaluation using Principal Coordinate Analysis (PCA). Band counts were analyzed using the LOESS procedure of SAS and a p-value of ≤ 0.05 was considered statistically significant. In the first study in which Quarter Horse mares received a human probiotic, no differences in band counts were observed in any of the bacterial populations.
evaluated. However, PCA and dendrogram analyses revealed clusters by treatment indicating changes in the microbial profile of *Lactobacillus* species. In the second study with young Quarter Horses, the probiotic supplement intended for livestock species altered the microbial diversity of starch-utilizing bacteria. PCA and dendrogram analyses revealed clusters by treatment in both *Lactobacillus* and *Streptococcus* species. In the third study with Miniature Horses, the equine probiotic supplement altered the diversity of the microbial profiles at the end of the 14 d sampling period. The number of bands representing total bacteria was greater in the CTL group suggesting less variation the microbial profiles of horses receiving the HI probiotic dose on d 14 of the sampling period. Dendrograms representing *Bacillus* spp. showed clustering by treatment on d 7, 10 and 14 of the sampling period in horses receiving the HI probiotic dose suggesting that the equine probiotic supplement containing *Bacillus* bacteria was able to alter the microbial profile of bacteria and that the dose given can influence the gastrointestinal microflora. Together, these studies suggest that probiotic supplements can change the microbial diversity in the gastrointestinal tracts of healthy horses. In addition, the type of probiotic given, the dose administered and the duration of supplementation can influence bacteria in the gastrointestinal microbiome. Probiotics may have prophylactic implications to optimize horse health and performance.
DEDICATION

Dedicated to all of the animals in my life, past and present, that inspired me to pursue a career in Animal Sciences.
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Chapter 1
Literature Review
1.1 Introduction

Horses are hindgut fermenters and consume a diet largely consisting of fibrous plant material which is mostly digested in the hindgut (Julliand, 2005). The horse’s gastrointestinal microbiome is a complex community with microorganisms that are mainly responsible for protecting the host against pathogens and providing a barrier against food and other antigens (Lu and Walker, 2001; Costa and Weese, 2012). The microbial populations in the horse’s gastrointestinal tract are crucial for the health and performance since horses do not have the mammalian enzymes needed to breakdown cellulose and utilize the nutrients from the plant materials they consume (Argenzio et al., 1974). Stress, illness, medication and diet can shift microbial populations in the hindgut of horses leading to decreased gut health (Rolfe, 2000). Issues related to gastrointestinal upset have been identified as the leading cause of death in horses (Costa and Weese, 2012). The therapeutic use of probiotics in horses is well documented although studies have reported mixed results (Parraga et al., 1997; Kim et al., 2001; Desrochers et al., 2005). However, there has been a recent interest in the prophylactic use of probiotics for horses to optimize gut health (Ishizaka et al., 2014; Tanabe et al., 2014). Therefore, the establishment of a dynamic yet balanced microbiome is critical for the health and performance of the horse.

1.2 Gastrointestinal Microbiome

The gastrointestinal microbiome of the horse contains numerous microorganisms consisting of bacteria, protozoa, fungi and archaea that possess important qualities for the
health and performance of the horse. Feedstuffs consumed by the horse play a factor in what microorganisms are present in the gut. If the horse consumes a diet largely composed of forage, the microbes needed to digest the forage have been found in higher concentrations (Willing et al., 2009). The gastrointestinal tract houses dynamic populations of microorganisms that have multiple roles in nutrient breakdown and utilization and are located in different parts of the tract (Julliand, 2005). Microbes in the foregut of the horse are involved in the breakdown of non-structural carbohydrates including starch during lactic acid fermentation as well as proteins and lipids (Pilliner, 1999). Microbes found in the hindgut of the horse also have many roles including cellulose digestion, lactate utilization, protein hydrolysis and amylase digestion (Kern et al., 1974; Julliand, 2005; Frape, 2010). These hindgut bacteria mainly belong to the phylum Firmicutes (46-72%) followed by Bacteroidetes (20%) (Kern et al., 1973; Daly et al., 2001; Shepherd et al., 2012; Dougal et al., 2013). Although many digestive processes occur in the equine hindgut, it is the primary site for cellulose and hemicellulose digestion (Bonhomme-Florentin, 1988; Pilliner, 1999). The complex carbohydrates are digested and absorbed as volatile fatty acids (VFAs), mainly acetate, propionate and butyrate, to be utilized as a source of energy (Cunha, 1991).

1.2.1 Proteolytic Bacteria

The main function of proteolysis is to hydrolyze protein into various peptides and amino acids. The small peptides can be converted into VFAs for energy, while larger peptides are utilized as microbial crude protein (Wright and Hungate, 1967; Maczulak et
Although little information is known about the microbial digestion of protein in horses compared to ruminant species (Wallace, 1996), proteolytic bacteria have been isolated in various parts of the gastrointestinal tract, including the small intestine and cecum (Frape, 2010). Common proteolytic bacteria in the horse’s gastrointestinal tract include *Streptococcus lutetiensis* and *Bacteriodes* spp. (Kern *et al*., 1973; Julliand, 2005).

### 1.2.2 Starch-Utilizing Bacteria

Starch-utilizing bacteria include species that produce lactic acid from starch digestion and species that can utilize the lactic acid produced. Starch-utilizing bacteria are predominately found in the foregut, but are also in the hindgut of the horse (de Fombeille *et al*., 2003; Julliand, 2005). Several species of starch-utilizing bacteria have been identified in the gastrointestinal tract of the horse and include *Streptococcus* spp. and *Lactobacillus* spp. (Bailey *et al*., 2003; Al Jassim *et al*., 2005; Julliand, 2005).

*Streptococcus* bacteria are efficient starch-utilizing bacteria and are major producers of lactic acid (Varloud *et al*., 2007). These bacteria can tolerate the harsh conditions in the gastrointestinal tract which allow them to proliferate in multiple segments of the tract (Hungate, 1966; Latham *et al*., 1971). The most predominant *Streptococcus* species isolated from the gastrointestinal tract of the horse is *Streptococcus lutetiensis* (Milinovich *et al*., 2008). An increase in the concentration of *Streptococcus* spp. has been observed when starch was added to the diet of horses (Garner *et al*., 1978; Milinovich *et al*., 2006; Milinovich *et al*., 2007; Milinovich *et al*., 2008).
Lactobacillus spp. ferment starch and simple sugars that the horse ingests and represent a major starch-utilizing species in the equine gastrointestinal tract (Bailey et al., 2003; Al Jassim et al., 2005). Lactobacillus spp. have been observed at concentrations of $10^5$ to $10^7$ bacteria per g of feces in the horse (Endo et al., 2009; Willing et al., 2009). The most prevalent species of Lactobacillus isolated from feces of horses is Lactobacillus equi (Morotomi et al., 2002; Endo et al., 2007). The production of lactic acid by Lactobacillus spp. is utilized as a substrate by lactate-utilizing bacteria.

Bacteria that utilize lactic acid in the hindgut environment are crucial for a horse’s gastrointestinal health by buffering a drop in pH that could result in disrupting homeostasis of hindgut microbes (Julliand, 2005). Most of the research on the equine microbiome has focused on microbial populations that produce or utilize lactic acid due to the implications these populations of bacteria have on the hindgut microflora. Excess production of lactic acid is associated with conditions such as laminitis, acidosis and various metabolic disorders (Al Jassim et al., 2005). The two main species of lactate-utilizers identified in the equine hindgut are Megasphaera and Veillonella (Baruc et al., 1983; Maczulak et al., 1985; Julliand, 2005). Although lactate-utilizing bacteria have been found at an average concentration of $10^7$ cfu/mL in cecal and colon contents in horses, the concentration of these lactate-utilizers is often higher in the colon than the cecum (Julliand et al., 2001; Medina, 2002; de Fombelle et al., 2003).
1.2.3 Fiber-Utilizing Bacteria

Fiber-utilizing bacteria are responsible for fiber digestion and the utilization of structural carbohydrates including cellulose, hemicellulose and pectin. Fiber-digesting species fill an essential niche for the horse as the horse cannot breakdown and utilize fiber by its own enzymatic production. Fiber-utilizing bacteria include fibrolytic species that assist in the degradation of polysaccharides in the cell wall as well as cellulolytic species that hydrolyze the cellulose portion of the fiber cell wall to produce compounds such as VFAs which are absorbed into the blood stream (Mackie and Wilkins, 1988; Cheng et al., 1991).

Cellulolytic bacteria have been observed at concentrations of $10^4$ to $10^7$ bacteria/mL of intestinal contents in the horse (Julliand et al., 1999; Medina et al., 2002; de Fombelle et al., 2003). Cellulolytic bacteria are often found in higher concentrations in the cecum compared to the colon since microbial fermentation of fiber mainly occurs in the cecum (Frape, 2010). The predominant cellulolytic bacteria isolated in the cecum are *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes* (Julliand et al., 1999; Drougal et al., 2000). Although these species are regarded as the primary cellulolytic bacteria in the horse, there are other bacterial species that participate in cellulose hydrolysis. Daly and colleagues (2001) observed that *Clostridium* spp., *Butyrivibrio* spp., and *Eubacterium* spp. also aid in cellulose degradation.
1.2.4 Lipid-Utilizing Bacteria

Most of the fat ingested by the horse is digested enzymatically in the small intestine (Swinney et al., 1995). A few studies have shown that lipid-utilizing bacteria, such as *Butyrivibrio* species, assist in the breakdown of fat into glycerol and fatty acids (Brown and Moore, 1960; Pagan, 1998); however, there are no reports characterizing these bacteria in the horse. The majority of research related to lipid digestion in the horse has focused on the influence of dietary fat on fiber digestibility (Julen et al., 1995).

1.2.5 Protozoa, Fungi, and Archaea

The gastrointestinal microbiome of the horse contains important microorganisms in addition to bacteria. Archaea, fungi and protozoal species have also been isolated from the equine hindgut and are thought to play a role in the utilization of fiber (Julliand, 2005). Protozoa are also thought to be involved in the hydrolysis of pectin and hemicellulose in the horse’s cecum (Frape, 2010). The protozoal species *Cycloposthium* and *Belpharocorys* are able to utilize polygalacturonase, pectin lyase, and pectinesterase to break down pectin (Bonhomme-Florentin, 1988). Archaea have been detected in small numbers, at approximately 3.5% of total microbial cell numbers in the horse, but little is known about their function (Yamano et al., 2008).

1.3 Factors Influencing the Gastrointestinal Microbiome

The gastrointestinal microbiome is a complex and dynamic environment which houses a large population of indigenous microbes, both commensal and pathogenic. It is
thought that these pathogenic microbes are not harmful to the host when kept in balance and only become harmful when the microbiome is disrupted and pathogenic microbes are able to proliferate and dominate the microbiome (Costa and Weese, 2012; Steelman et al., 2012). There are many factors that can disrupt the homeostasis of the normal flora including stress, illness, medication and diet.

1.3.1 Stress

Stress is considered a stimulus to the body that elicits a reaction from the disruption of a physiological equilibrium (Rostagno, 2009). When horses undergo stressful events and cortisol is not limited, disruption of homeostasis of the gut microbes can occur causing the indigenous pathogenic bacteria to proliferate and become harmful to the host (Blum et al., 2002). Events that can cause stress to the horse include long periods of transport, weaning and illness (Goodson et al., 1988; de Fombelle et al., 2001; Fazio and Ferlazzo, 2003).

It is common for horses to be frequently transported for recreation, sport and health assessment at a veterinary clinic (Faubladier et al., 2013). Transport stress can be heightened by various stimuli including change in temperature and vibrations (Waran et al., 1995; Broom, 2005). Transportation for both long and short periods of time has been shown to elicit a stress response in the horse and changes in bacterial profiles (Boensma et al., 2006; Stull et al., 2008). Six ponies were transported for 3.5 hrs to evaluate the effects of stress on the bacterial populations in their gastrointestinal microbiome. It was concluded that stress from transportation did not affect fecal pH or consistency, but did
alter the microbial profiles of these ponies as observed by characterization and microbial profiling analyses (Boensma et al., 2006).

Weaning has been reported to induce stress in horses (McCall et al., 1987). Research by Apter and Householder (1996) noted that plasma cortisol concentrations were higher in foals following weaning compared to pre-weaning foals. Although there is limited information in horses regarding the effect of weaning stress on the hindgut microbial community (Swanson, 2002), weaning stress in other monogastric animals has been shown to impact bacterial populations. Research by Konstantinov and colleagues (2004) noted the occurrence of gastrointestinal disorders in piglets due to changes in enteric bacteria following weaning.

Illness and gastrointestinal disorders are commonly observed following the incidence of stress. Many pathogens in the environment can be picked up and affect an animal's gastrointestinal microflora, causing illness. If the gastrointestinal microbiome of the horse is dynamic and homeostatic, the indigenous microbes can prevent pathogens such as Salmonella from proliferating. Otherwise, Salmonella infection can occur, requiring antibiotic therapy and even hospitalization. Parraga and colleagues (1997) reported expenses between $10,000 and $420,000 per outbreak of Salmonella that resulted in hospitalization. The occurrence of Salmonellosis may cause fatal colic (Guardabassi et al., 2008; Frape, 2010).

Another pathogen that can be detrimental to horse health is Clostridium difficile. This bacterium has been associated with colic in horses as well as isolated in foals with diarrhea (Magdesian et al., 2002; Arroyo et al., 2007). This bacterium has the ability to
survive in feces up to four yrs which can lead to environmental exposure (Baverud et al., 2003). *Clostridium perfringens* has been shown to cause enterocolitis in foals due to the endotoxin produced (Weese et al., 2001; Albini et al., 2008; Frape, 2010). These fluctuations of intestinal microflora have been associated with health complications such as colic and laminitis (Milinovich et al., 2007; Durham, 2008).

### 1.3.2 Medication

Antibiotics are commonly administered to horses for various bacterial infections. Treatment with antibiotic medications can disrupt the indigenous microbial populations within the gastrointestinal tract of horses as well as the targeted bacteria causing illness (Guardabassi et al., 2008; Gronvold et al., 2010). This shift in microbial populations of the hindgut can lead to the incidence of undesired effects such as diarrhea and colonization by pathogens. Research by White and Prior (1982) noted that when horses were given oxytetracycline, there was a significant increase in the concentrations of coliform bacteria. However, there were no changes observed in coliform bacteria concentrations following the administration of trimethoprim-sulphadiazine. A study by Frape (2010) reported that the overuse of oxytetracycline was linked to chronic diarrhea and the onset of salmonellosis in horses.

In addition to antibiotics, other medications such as pain relievers commonly administered to horses following an injury have been shown to influence a horse’s gastrointestinal microflora. Phenylbutazone (8.8 mg/kg) was administered to six healthy adult horses for 21 d. Two of the horses developed acute enterocolitis on d 7 and 10 of
phenylbutazone administration and had to be hospitalized for supportive care. Additionally, horses receiving the phenylbutazone treatment had increased concentrations of acetic acid which can alter microbial populations due to a change in pH (McConnico et al., 2008).

### 1.3.3 Diet

Changing the main type of feedstuff in the diet can alter microbial populations in horses. Six mature geldings were used in a crossover design with two 29 d periods to evaluate the changes in fecal bacteria when concentrate or forage only diets were fed. Lower counts of lactic acid bacteria were observed when forage only diets were fed. *Lactobacillus ruminis* was recovered from horses receiving the concentrate diet, but not when horses were consuming the forage diet suggesting the type of feedstuffs plays a factor on microbial populations found in the horse (Willing et al., 2009).

Abrupt changes in the horse’s diet have also been shown to change both the diversity and abundance of microbial populations (Kern et al., 1973; de Fombelle et al., 2001; Drougal et al., 2001; Bailey et al., 2003; Berg et al., 2005). Research by de Fombelle and others (2001) fed different starch diets to three mature male ponies in a 3x3 Latin square design with one-month periods. One diet consisted of 100% hay, a second diet was 70% hay and 30% barley while a third diet was 50% hay and 50% barley. The researchers observed that total counts of anaerobic bacteria in the large intestine increased 29 hrs following the diet change from 100% hay to the 70% hay and 30% barley diet while total counts of *Streptococcus* spp. increased in the cecum five hrs after
the diet change. When the diet was changed from 100% hay to 50% hay and 50% barley, the concentration of aerobic bacteria rapidly increased five hrs after the diet change. A rapid increase was also observed in the total counts of *Lactobacillus* and *Streptococcus* spp. five hrs after the diet was changed. The abrupt incorporation of barley at two different levels altered the microbial communities in the hindgut of the ponies, regardless of the level of inclusion.

When horses consume a large quantity of readily digestible carbohydrates, the starch that is unfermented from the small intestine will carry over into the hindgut of horses and shift microbial populations (Bailey *et al.*, 2003; Willing *et al.*, 2009). The fermentation of starch to lactic acid creates acidic conditions and the release of endotoxins as certain bacteria are lysed due to the unfavorable pH (Mungall *et al.*, 2001; Milinovich *et al.*, 2005; Frape, 2010). Previous research suggests when the pH of the hindgut reaches levels near or below 6, cellulolytic and protozoal microorganisms are inhibited, while lactic acid bacteria such as *Streptococcus* and *Lactobacillus* are favored (Al Jassim and Rowe, 1999). Even lactate utilizing bacteria become overwhelmed with the large influx of lactic acid and may die (Frape, 2010). The release of endotoxins and change in microbial profiles has been associated with laminitis in the horse (Garner *et al.*, 1977).

### 1.4 Probiotics

Probiotics are live microorganisms that provide beneficial effects to the host when administered in an adequate dose (Chaucheyras-Durand and Durand, 2010). Stimulating
beneficial microbial populations in the gastrointestinal microbiome can promote health and performance of the animal, which may prevent the occurrence of gastrointestinal upset. Probiotics are commonly administered to recover homeostasis of microbial populations within the gastrointestinal microbiome following gastrointestinal upset.  

There are a few potential mechanisms in which indigenous bacteria may competitively exclude other bacteria. The first is through nutrient utilization. Indigenous bacteria consume the nutrients present in the microbiome leaving no available nutrients for exogenous microbes (Montes and Pugh, 1993). A second mechanism is through competition for binding sites to the gut epithelium. It is thought that commensal bacteria have the ability to competitively exclude potentially harmful bacteria by creating a barrier to the gut mucosa and epithelium (Salminen et al., 1996; Tuomala et al., 1999). *Lactobacillus* species are able to compete with pathogenic bacteria for binding sites by binding to the gastrointestinal epithelium, which prevents the pathogens from adhering (Montes and Pugh, 1993). Another proposed mechanism is due to production of substances that inhibit pathogenic bacteria, including the production of bacteriocins (Steer et al., 2000; Fooks and Gibson, 2002). Lactic acid bacteria are known to produce various antimicrobial compounds such as hydrogen peroxide, which can lower the redox potential of some pathogenic, aerobic bacteria. Previous research also describes the ability of *Lactobacillus acidophilus* to produce carbon dioxide, which is capable of decreasing the luminal pH (Naidu et al., 1999).  

Research by Desrochers and colleagues (2005) evaluated the effects of probiotic supplementation on the duration and severity of diarrhea in 14 horses with enterocolitis.
The horses were randomly assigned to two treatment groups: placebo or probiotic. The horses in the probiotic treatment received cultures of *Saccharomyces boulardii* for 10 d. Following the probiotic therapy the researchers concluded that the severity and duration of diarrhea was decreased due to probiotic supplementation. Additional research by Ward and colleagues (2004) investigated the effects of supplementing probiotics to horses with *Salmonella* infection. The probiotic included *L. acidophilus*, *L. casei*, *L. plantarum*, and *E. faecium*. The findings of the study indicated that probiotic administration decreased *Salmonella* infection by nearly 65% in hospitalized horses.

*Bacillus* spp. are commonly utilized in commercial probiotics for their spore forming qualities. These properties give probiotics with *Bacillus* a longer shelf life and, in the case of *Bacillus subtilis*, are not harmful to the host unlike some *Bacillus* species. There is limited research on the use of *Bacillus* spp. as probiotics in horses. A study by de Vaux and Julliand (1994) supplemented $10^{10}$ spores of *Bacillus cereus* to three cannulated ponies for five wks. The results from this study indicated that probiotic supplementation resulted in an increase of total anaerobes and proteolytic bacteria were observed in the cecum. Although there is limited information in horses, probiotics containing *Bacillus* spp. have been more extensively studied in other species. Previous research by Alexopoulus and others (2004) studied the effects of feeding a probiotic supplement containing cultures of *B. subtilis* to weaned pigs. Following supplementation, pigs receiving the probiotic had a decreased incidence of diarrhea associated with *E. coli* compared to pigs not receiving the probiotic. Maruta and colleagues (1996) observed that supplementing *B. subtilis* to pigs had a greater influence on microbial populations in gilts.
than in sows. Probiotic supplementation increased the abundance of *Lactobacillus* and *Bifidobacterium*, while decreasing *Streptococcus* spp. in gilts.

Although many studies support the use of probiotics, there are also conflicting studies. In one study, two different commercial probiotic supplements were administered for 7 d to 186 horses suffering from *Salmonella* infection and colic. The first probiotic given contained strains of *L. acidophilus*, *L. casei*, *L. plantarum*, and *S. faecium* administered in a 30g dose containing $1 \times 10^7$ cfu/g while the second probiotic contained *B. thermophilum*, *B. longum*, *acidophilus*, and *S. faecium* and was administered as a 15g dose containing $2.75 \times 10^8$ cfu/g. It was concluded that neither probiotic had any effect on *Salmonella* shedding (Parraga *et al.*, 1997). A study by Weese and Rousseau (2005) investigated the effects of supplementing 153 foals at 18 to 36 hours of age with a probiotic containing $2 \times 10^{11}$ cfu of *L. pentosus* WE7 daily for 7 d. Clinical signs of diarrhea were observed for 14 d. The results of this study suggested probiotic supplementation was associated with a higher incidence of diarrhea in supplemented foals. These studies support the idea that the species and dosage of probiotics play a large factor in the efficacy of probiotic supplementation and more research is needed to determine the optimum guidelines for probiotic supplementation.

Probiotic supplements may have beneficial effects when administered prophylactically in horses. Probiotic supplementation with cultures containing *Lactobacillus acidophilus* was studied in ten healthy adult horses for 28 d. The researchers concluded that probiotic supplement improved fecal condition and increased lactic acid bacterial populations in the hindgut (Ishizaka *et al.*, 2014). Another study
evaluated the effects of supplementing probiotics containing lactic acid bacteria to thoroughbred foals. Foals receiving probiotic supplementation had an increased rate of gain and a lower occurrence of diarrhea (Yuyama, 2004). An additional study by Tanabe and others (2014) also found a decreased incidence of diarrhea when Thoroughbred foals were supplemented with multiple strains of *Lactobacillus* and *Bifidobacterium* spp. from birth to 20 wks of age. However, Weese and colleagues (2003) studied healthy adult horses that were fed a probiotic supplement containing *Lactobacillus rhamnosus* LGG, which was of human origin. The supplement was provided for a period of five d at three different levels of probiotic, $1 \times 10^9$, $1 \times 10^{10}$, and $5 \times 10^{10}$ cfu/50 kg BW, respectively. It was concluded that supplementation failed to achieve a dose response due to the addition of the probiotic.

### 1.5 Summary

The health and well-being of horses is largely dependent on a dynamic yet homeostatic population of microorganisms. Lactic acid bacteria appear to play a vital role in this homeostatic relationship as many gastrointestinal illnesses are characterized by a shift in lactic acid bacterial populations. Although limited information is known about the gastrointestinal microbiome of horses as compared to other species, a better understanding of this complex environment is needed to optimize gut health and performance. The microbial profile of the microbiome of horses is highly variable between individuals and the effects from various probiotic supplements differ between subjects (Mackie *et al.*, 1999). Probiotic supplements may have great value
prophylactically, preventing gastrointestinal upset and disease. Therefore, the objective of this research is to evaluate the influence of probiotic supplements on the microbial populations in the gastrointestinal tracts of healthy horses.
Chapter 2

The Influence of Probiotic Supplementation on the Microbial Diversity in the Gastrointestinal Microbiome of Mature Quarter Horses


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ABSTRACT

Bacteria play a crucial role in the gastrointestinal health of many animals, including horses. Stress, illness, medication and diet can easily disrupt microbial populations in the equine gastrointestinal tract leading to decreased gut health and poor performance. Probiotics have been utilized in horses and other species to re-establish homeostasis of the gut bacteria. Probiotics may also have prophylactic implications, preventing gastrointestinal upset from occurring. However, there is limited research investigating the ability of probiotics to influence a stable gastrointestinal environment. The objective of this research was to evaluate the influence of a probiotic supplement containing *Lactobacillus acidophilus* on the microbial diversity of healthy mature horses. Seven mature Quarter Horse mares (10.0 ± 2.0 yr) were randomly assigned to one of two treatments in a crossover design of two 49 d experimental periods. An initial adaptation period of 14 d was followed by a 35 d sampling period. All horses received a control diet (CTL) of 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay *ad libitum* or the control diet supplemented with 140 g of a probiotic supplement containing $10^9$ cfu of *Lactobacillus acidophilus* (TRT) for a period of 49 d. Fecal samples were collected on d 0 before supplementation and on d 14, 21, 28, 35, 42, and 49 of both experimental periods and pooled by treatment group and time. DNA was extracted from fecal samples and subjected to PCR-DGGE using primers specific to 16S rRNA gene
sequences to investigate changes in diversity of total bacteria, *Lactobacillus* and *Streptococcus* species. PCR-DGGE images were analyzed with BioNumerics software to generate dendrogram comparisons based on the position and number of bands with further evaluation using Principal Coordinate Analysis (PCA). Band counts were calculated based using the LOESS procedure of SAS and a p-value of ≤ 0.05 was considered statistically significant. There were no differences observed in band counts representing total bacteria and some starch-utilizing bacteria. There were also no differences observed in total bacterial diversity using dendrograms or PCA. However, PCA and dendrograms analyses revealed clustering by treatment group in *Lactobacillus* species. PCA with *Streptococcus* spp. revealed no clustering by treatment group suggesting this type of starch-utilizing bacteria was not influenced by probiotic supplementation. Probiotic supplementation with *Lactobacillus acidophilus* influenced the microbial diversity of *Lactobacillus* spp., but not total bacterial diversity or the diversity of *Streptococcus* species. These results suggest the bacterial species in the probiotic supplement may play a factor in what bacteria are influenced in the horse’s gastrointestinal tract.
INTRODUCTION

Microbial populations in the gastrointestinal microbiome are complex and important to the horse’s health and well-being. A complex and dynamic gastrointestinal microbiome is essential for the utilization of feedstuffs (Mackie and Wilkins, 1988; de Fombelle et al., 2003). Stress, illness, medication and diet can easily alter microbial populations in the equine hindgut and lead to poor health and performance. It is important to maintain homeostasis of the gastrointestinal microbiome and to recover homeostasis if gastrointestinal upset has occurred.

Probiotics are live microorganisms that elicit beneficial effects to the host (Garrett et al., 2010). Most probiotic research in horses has focused on the use of lactic acid bacteria, specifically Lactobacillus species since many lactic acid bacteria are able to tolerate the harsh acidic conditions of the gastrointestinal microbiome and resist activity of intestinal lysozyme (Frape, 2010). In addition, most of the research has focused on the ability of probiotics to re-establish homeostasis following gastrointestinal upset. The ability of probiotics to reduce the severity and duration of diarrhea was studied in horses with enterocolitis. The supplement contained $10 \times 10^9$ yeast cells of Saccharomyces boulardii and was administered every 12 hours for 14 days. Horses receiving the probiotic had significantly reduced severity and duration of diarrhea than horses
receiving the placebo (Desrochers et al., 2005). However, research by Parraga and others (1997) evaluated the use of two commercially available probiotics supplemented to 186 horses with Salmonella infection. One of the probiotics contained $1 \times 10^7$ cfu/g of lactic acid bacteria, while the other contained $2.75 \times 10^8$ cfu/g of lactic acid bacteria. The probiotic therapy was administered for seven days and it was determined that the probiotics had no effect on Salmonella shedding and the incidence of diarrhea.

Probiotics may have prophylactic benefits to help prevent gastrointestinal upset from occurring. Although the importance of homeostasis in the equine gastrointestinal microbiome is noted, there is limited published literature regarding probiotic supplementation in healthy horses. In a study evaluating the use of Lactobacillus in healthy adult horses, the probiotic had low microbial colonization. It was determined that the probiotic was ineffective in adult horses due to its inability to colonize the gastrointestinal tract (Weese et al., 2003). Thoroughbred foals whose diet was supplemented with lactic acid bacteria had an increased rate of gain and a lower occurrence of diarrhea (Yuyama, 2004). Additionally, a study by Ishizaka and others (2014) found that supplementing 10 adult geldings with probiotics containing Lactobacillus cultures for 28 days had increased lactic acid bacterial populations and improved fecal condition due to probiotic administration.

Since there is limited information concerning the prophylactic use of probiotics and healthy horses, there is significant need for further knowledge. Therefore, the objective of this study was to evaluate the influence of a commercial supplement
containing *Lactobacillus acidophilus* on the microbial diversity in the gastrointestinal tracts of healthy mature Quarter Horse mares.
MATERIALS AND METHODS

Experimental Design

Seven mature Quarter Horse mares (10.0 ± 2.0 yr; 545.4 ± 55.6 kg) were randomly assigned to one of two treatments in a crossover design of two 49 d experimental periods. An initial 14 d adaptation period was followed by a 35 d sampling period. All horses received a control diet (CTL) of 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum or the control diet supplemented with 140 g of a probiotic supplement containing $10^9$ cfu per dose of Lactobacillus acidophilus (TRT) for a period of 49 d. Throughout the study, the horses were fed individually and housed in outdoor paddocks with access to shelter at all times.

Microbial Analysis

Fecal samples were collected on d 0 before supplementation and on d 14, 21, 28, 35, 42, and 49 of both experimental periods to evaluate changes in diversity of the gastrointestinal microbiome. Fecal samples were stored at -20°C until further analysis. One gram from each fecal sample from horses in the same treatment group was homogenized and pooled by day. Bacterial DNA was extracted from the pooled fecal samples following a protocol of repeated bead beating plus column (RBB+C; Yu and Morrison, 2004) with a modified protocol for elution of 50 uL of AE instead of 200 uL.
The DNA quality was confirmed by 1.0 % agarose gel electrophoresis for 1 hr at 80 V, and DNA concentrations were determined using Quant-iT™ Broad-Range DNA Assay Kit (Invitrogen; Carlsbad, CA). The modified protocol included 50uL of working solution, instead of 200 µL, 2.5 µL of DNA and standards were utilized instead of 10 µL. The primers utilized for PCR-DGGE were specific to the V2-V3 regions of the 16S rRNA gene to evaluate total bacteria (HDA1, HDA2), and lactic acid bacteria including Lactobacillus (Lac1, Lac2) and Streptococcus (Lac3, Lac2) (Table 1). The reaction mixture for the universal PCR contained 0.25 µL of each 100 uM primer, 0.25 µL Taq polymerase, 1.0 µL of BSA, 1.75 µL of 50 mM MgCl₂, 0.40 µL of 100 mM dNTP and 5.0 µL of buffer. Fifty ng of DNA was added to each reaction and distilled H₂O (Life technologies Corp.; Eugene, OR) was added to reach a total reaction volume of 50 µL.

**DGGE Analysis**

PCR products were subjected to subsequent denaturing gradient gel electrophoresis analyses. Before samples were used for DGGE, 4.0 µL of each PCR product were exposed to 1.0% agarose gel electrophoresis to confirm successful amplification of the V2-V3 region. Then, 8.0 µL aliquots of PCR product were resolved in a 7.5% polyacrylamide gel containing a 40%-60% gradient of denaturants (formamide and urea) for universal amplified PCR products and a 30%-70% gradient for all other PCR products. The DGGE gel was subjected to 0.5% TAE at 60° C and 82 V for 16 h using INGENY phorU-2 (Ingeny; Leiden, The Netherlands). The images were captured using AlphaImager HP® (ProteinSimple; Santa Clara, California).
Qualitative and Statistical Analysis

Microbial diversity from PCR-DGGE was evaluated with BioNumerics (Applied Maths NV; Sint-Martens-Latem, Belgium). The microbial DNA bands in each lane were detected on the gel manually and using the automatic band search function. Gels were normalized using internal reference bands and external reference markers (Invitrogen Inc., Carlsbad, CA). A 1.0% position tolerance was set for band matching to help correct minimal migratory variation. The DGGE banding patterns were transformed into a binary (presence or absence of bands) correlation matrix in BioNumerics. Dendrograms were created based on the unweighted pair-group method with arithmetic average (UPGMA) and the Jaccard function. Similarity was represented with a similarity coefficient derived from the pairwise comparison of DGGE banding patterns between two samples. Bacterial diversity richness was evaluated by the number of DGGE-bands present using the LOESS procedure of SAS v 9.3 (SAS Institute Inc.; Cary, NC) and a two-tailed t-test in Microsoft Excel, with significance at p-value ≤ 0.05. Principle component analysis (PCA) was performed using a combination of BioNumerics and Microsoft Excel to provide visual assessment of similarity between treatment groups from the DGGE profiles.
RESULTS

Animal Performance

No adverse effects due to probiotic administration were observed during the study. BW averaged 545.4 ± 55.6 kg at the start of the study compared to 574.9 ± 51.0 kg at the end of the study.

Total Bacteria

There were no differences in the number of bands representing total bacteria observed in microbial profiles due to probiotic supplementation (Figure b.1 a). Similarity coefficients averaged 86 % throughout the study and d 35 of the sampling period with 72% similarity (Table 2). This data suggests that the microbial profiles of horses receiving the probiotic supplement were 28% different due to probiotic supplementation on d 35.

Dendrograms and PCA showed no differences in the microbial populations of total bacterial profiles due to probiotic supplementation (Figure b.2 a). However, PCA revealed clusters by time (Figure b.2 b). Samples collected on d 0 to 21 are represented in one cluster, while samples collected on d 29 to 49 (except d 35) are represented in a second cluster. The first principal component (PC1) axis accounted for 32.69% of the variation. The second principal component (PC2) accounted for 22.84% the variation.
**Lactobacillus Spp.**

There were no differences in band counts representing *Lactobacillus* species between treatment groups (Figure b.1 b). Similarity coefficients averaged 58% throughout the study (Table 2). Fecal samples collected on d 21 appeared to have the most variation at 49% similarity between banding patterns of horses supplemented with probiotic compared to those no receiving supplementation. Microbial profiles were approximately 51% different due to probiotic supplementation on d 21.

Dendrograms showed clustering by treatment in the microbial profiles of *Lactobacillus* species (Figure b.3 a). PCA also revealed clustering due to treatment (Figure b.3 b). Samples collected from CTL horses on d 0, 21, 28 and 35 are represented in one cluster while all samples collected from TRT horses and CTL samples on d 28 to 49 are represented in another cluster. The first principal component (PC1) axis accounted for 55.86% of the variation. The second principal component (PC2) accounted for 20.01% the variation.

**Streptococcus Spp.**

There were no differences in the number of bands of total bacteria between treatment groups (Figure b.1 c). Similarity coefficients averaged 67% throughout the study (Table 2). Fecal samples collected on d 28 were 56% similar between banding patterns of horses supplemented with the probiotic compared to those not receiving supplementation. Microbial profiles were approximately 44% different due to probiotic
supplementation on d 28 of the sampling period suggesting the probiotic was able to influence microbial diversity.

Dendrograms analyses showed some differences in *Streptococcus* spp. profiles due to probiotic supplementation with fecal samples collected from TRT horses on d 21, 28 and 42 were included in a cluster (Figure b.4 a). However, PCA revealed no differences due to probiotic supplementation (Figure b.4 b). The first principal component (PC1) axis accounted for 61.09% of the variation. The second principal component (PC2) accounted for 19.31% the variation.

**DISCUSSION**

Probiotics may provide prophylactic benefits to horses to help prevent gastrointestinal upset and poor health and performance. Most probiotic research in horses has focused on the use of lactic acid bacteria since many lactic acid bacteria are able to tolerate the harsh acidic conditions of the gastrointestinal tract (Frape, 2010). However, most studies have focused on the ability of the probiotic to re-establish normal gut bacterial populations following gastrointestinal upset rather than its effectiveness in a stable gastrointestinal environment.

In the present study, a probiotic supplement containing *Lactobacillus acidophilus* was administered to healthy mature Quarter Horse mares. This probiotic supplement is commonly administered to humans. PCA and dendrogram analyses revealed microbial
diversity of *Lactobacillus* populations was clustered together due to probiotic supplementation with *Lactobacillus acidophilus*. Similarly, a study by Ishizaka and others (2014) found that supplementing 10 healthy adult geldings with probiotics containing $5.6 \times 10^8$ cfu/g of *Lactobacillus acidophilus* for 28 days had increased lactic acid bacterial populations and improved fecal condition due to probiotic administration.

In addition to *Lactobacillus* populations, the present study observed some influence in the microbial profiles of *Streptococcus* spp. due to the probiotic administration. Dendrograms revealed one cluster including samples from TRT horses on d 21, 28 and 42. However, PCA revealed no differences between treatment groups. Since this probiotic containing *Lactobacillus acidophilus* is commonly supplemented to humans, more of a response in *Streptococcus* species may have been observed if the probiotic given was specific for use in horses.

Probiotic supplementation may be advantageous as a preventative therapy for healthy horses. Prophylactic use of probiotics may help combat the occurrence of illness before it occurs, instead of simply using probiotics as treatment (Kopp-Hoolihan, 2001). However, the efficacy of probiotic use remains poor without a better understanding of the equine microbiome. Further research is needed to determine the prophylactic benefits of probiotic supplementation to healthy horses.
Chapter 3

The Influence of Probiotic Supplementation on the Microbial Diversity in the Gastrointestinal Microbiome of Young Quarter Horses


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ABSTRACT

Bacteria play a crucial role in the gastrointestinal health of many animals, including horses. Stress, illness, medication and diet can easily disrupt microbial populations in the equine gastrointestinal tract leading to decreased gut health and poor performance. Probiotics have been utilized in horses and other species to re-establish homeostasis of the gut bacteria. Probiotics could also have prophylactic implications, preventing gastrointestinal upset from occurring. However, there is limited research investigating the ability of probiotics to influence a stable gastrointestinal environment. The objective of this research was to evaluate the influence of a probiotic supplement on the microbial diversity of the gastrointestinal microbiome of young, healthy horses. Twelve Quarter Horses (4 geldings, 8 mares; 1.5 ± 0.5 yr) were randomly assigned to one of two treatments. All horses received a control diet (CTL) of 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum or the control diet supplemented with 10 g of a commercial probiotic supplement containing 10^7 cfu per dose of Lactobacillus acidophilus (TRT) for a period of 77 d. Fecal samples were collected every 7 d and pooled by treatment group and time. DNA was extracted from fecal samples and subjected to PCR-DGGE using primers specific to 16S rRNA gene sequences to investigate changes in diversity of total bacteria, Lactobacillus and Streptococcus species. PCR-DGGE images were analyzed with BioNumerics software to
generate dendrogram comparisons based on the position and number of bands with further evaluation using Principal Coordinate Analysis (PCA). Band counts were analyzed using the LOESS procedure of SAS and a p-value of $\leq 0.05$ was considered statistically significant. No differences were observed in the number of bands between treatment groups in total bacteria and Streptococcus bacteria. However, in Lactobacillus spp. the TRT horses had a greater number of bands on d 70 and 77 than horses in the CTL group. There were no differences observed in microbial diversity in total bacteria using dendrogram and PCA analyses. Evaluation with PCA and dendrograms revealed clustering by treatment group in Streptococcus bacterial populations. Overall, these results suggest probiotic supplementation was able to influence the microbial profiles of starch-utilizing bacteria including Lactobacillus and Streptococcus populations.
INTRODUCTION

The gastrointestinal microbiome is complex and dynamic, and it is necessary that the microbial populations remain in homeostasis for overall health and performance. Stress, illness, medication and diet can easily disrupt microbial populations in the equine hindgut and lead to poor health and performance. If gastrointestinal upset occurs, it is crucial that homeostasis of the microbiome is re-established.

Probiotics are live microorganisms that elicit beneficial effects to the host (Garrett et al., 2010). Stimulating indigenous microbial populations through the use of probiotics can prove quite beneficial for the host to maintain homeostasis of the gastrointestinal microbiome for optimum health and performance. Probiotics for horses commonly include bacterial species such as Lactobacillus and Bifidobacterium (Weese, 2002). Most probiotic research in horses has focused on the use of lactic acid bacteria, specifically Lactobacillus species since many lactic acid bacteria are able to tolerate the harsh acidic conditions of the gastrointestinal tract (Frape, 2010).

Most research evaluating the use of probiotics in horses has focused on therapeutic use. However, this research has been met with mixed results. Previous research with horses hospitalized for Salmonella infection were supplemented with a probiotic containing cultures of Lactobacillus and Enterococcus species for five days. No
differences were observed in clinical signs or the incidence of *Salmonella* shedding in horses receiving the probiotic compared to horses receiving the placebo (Kim *et al.*, 2001). Desrochers and colleagues (2005) supplemented horses with acute enterocolitis with a probiotic containing *Saccharomyces*. The probiotic therapy was supplemented every 12 hours for 14 days and it was concluded the therapy reduced the symptoms of diarrhea and gastrointestinal upset.

There is limited published literature regarding probiotic supplementation in healthy horses. Research by Ishizaka and colleagues (2014) supplemented probiotics containing lactic acid bacteria to 10 mature healthy geldings for 28 days. The horse’s receiving the probiotic appeared to have improved fecal condition and increased populations of lactic acid bacteria than those horses not receiving the probiotic supplement.

Probiotics may have prophylactic implications and be able to prevent gastrointestinal upset from occurring (Kopp-Hoolihan, 2001). The ability to use probiotics as prevention before gastrointestinal upset occurs would be very beneficial to the health and performance of the horse. Since there is limited information concerning the use of probiotics and healthy horses there is significant need for further knowledge. Therefore, the objective of this study was to evaluate the influence of a probiotic supplement containing *Lactobacillus acidophilus* on the gastrointestinal microbiome of healthy young Quarter Horses.
MATERIALS AND METHODS

**Experimental Design**

Twelve Quarter Horses (4 geldings, 8 mares; 1.5 ± 0.5 yr; 406.6 ± 108.2 kg) were randomly assigned to a control diet (CTL) consisting of 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay *ad libitum* or the control diet supplemented with 10 g of a commercially available probiotic supplement containing $10^7$ cfu per dose of *Lactobacillus acidophilus* (TRT) for a period of 77 d. Throughout the study, horses were fed individually and housed in two outdoor paddocks with access to shelter at all times.

**Microbial Analysis**

Fecal samples were collected on d 0 prior to supplementation and then every 7 d throughout the 77 d sampling period to evaluate changes in microbial diversity of the gastrointestinal microbiome. Fecal samples were stored at -20°C until further analysis. One gram from each fecal sample from horses in the same treatment group was homogenized and pooled by day to form a composite sample. Bacterial DNA was extracted from the pooled fecal samples following a protocol of repeated bead beating plus column (RBB+C; Yu and Morrison, 2004) with a modified protocol for elution of 50 uL of AE instead of 200 uL. The DNA extraction was confirmed by 1.0 % agarose gel.
electrophoresis for 1 hr at 80 V, and DNA concentrations were determined using a modified protocol of the Quant-iT™ Broad-Range DNA Assay Kit (Invitrogen; Carlsbad, CA). The modified protocol included 50uL of working solution, instead of 200 µL, 2.5 µL of DNA and standards were utilized instead of 10 µL. The primers utilized for PCR-DGGE were specific to the V2-V3 regions of the 16S rRNA gene to evaluate total bacteria (HDA1, HDA2), and lactic acid bacteria including *Lactobacillus* (Lac1, Lac2) and *Streptococcus* (Lac3, Lac2) (Table 1). The reaction mixture for the universal PCR contained 0.25 µL of each 100 uM primer, 0.25 µL Taq polymerase, 1.0 µL of BSA, 1.75 µL of 50 mM MgCl$_2$, 0.40 µL of 100 mM dNTP and 5.0 µL of buffer. Fifty ng of DNA was added to each reaction and distilled H$_2$O (Life technologies Corp., Eugene, OR) was added to reach a total reaction volume of 50 µL.

**DGGE Analysis**

PCR products were subjected to subsequent denaturing gradient gel electrophoresis analyses. Before samples were used for DGGE, 4.0 µL of each PCR product were exposed to 1.0% agarose gel electrophoresis to confirm successful amplification of the V2-V3 region. Then, 8.0 µL aliquots of PCR product were resolved in a 7.5% polyacrylamide gel containing a 40%-60% gradient of denaturants (formamide and urea) for universal amplified PCR products and a 30%-70% gradient for all other PCR products. The DGGE gel was subjected to 0.5% TAE at 60° C and 82 V for 16 h using INGENY phorU-2 (Ingeny; Leiden, The Netherlands). The images were captured using AlphaImager HP® (ProteinSimple; Santa Clara, California).
**Qualitative and Statistical Analysis**

Microbial diversity from PCR-DGGE was evaluated with BioNumerics (Applied Maths NV; Sint-Martens-Latem, Belgium). The microbial DNA bands in each lane were detected on the gel manually and using the automatic band search function. Gels were normalized using internal reference bands and external reference markers (Invitrogen Inc., Carlsbad, CA). A 1.0% position tolerance was set for band matching to help correct minimal migratory variation. The DGGE banding patterns were transformed into a binary (presence or absence of bands) correlation matrix in BioNumerics. Dendrograms were created based on the unweighted pair-group method with arithmetic average (UPGMA) and the Jaccard function. Similarity was represented with a similarity coefficient derived from the pairwise comparison of DGGE banding patterns between two samples. The number of DGGE-bands present evaluated bacterial diversity richness using the LOESS procedure of SAS v 9.3 (SAS Institute Inc., Cary, NC) and a two-tailed t-test in Microsoft Excel and significance at p-value $\leq 0.05$. Principle component analysis (PCA) was performed using a combination of BioNumerics and Microsoft Excel to provide visual assessment of similarity between treatment groups from the DGGE profiles.
RESULTS

Animal Performance

No adverse effects due to probiotic administration were observed during the study. BW averaged 406.6 ± 108.2 kg at the start of the study compared to 424.3 ± 104.1 kg at the end of the study.

Total Bacteria

There were no differences in the number of bands representing total bacteria observed in microbial profiles due to probiotic supplementation (Figure b.5 a). Similarity coefficients averaged 83 % throughout the study. Fecal samples collected on d 35 had 63% similarity between banding patterns of horses supplemented with probiotic compared to those no receiving supplementation (Table 3). This data suggests microbial profiles were 37% different due to probiotic supplementation on d 35.

Dendrograms showed no differences in the microbial populations of total bacterial profiles due to probiotic supplementation (Figure b.6 a). PCA reveals clustering by time (Figure b.6 b). Samples collected earlier in the study regardless of treatment are in one cluster, while samples collected in the middle of the sampling period are in a second cluster. Lastly, a third cluster contains the samples collected at the end of the sampling period.
period. The first principal component (PC1) axis accounted for 40.69% of the variation. The second principal component (PC2) accounted for 14.13% the variation.

*Lactobacillus Spp.*

There were no differences in band counts representing *Lactobacillus* species between treatment groups prior to d 70. Band counts were higher in TRT horses on d 70 and 77 of the sampling period compared to the CTL horses (Figure b.5 b). Similarity coefficients averaged 34% throughout the study (Table 3). Fecal samples on d 35 appeared to have 21% similarity between banding patterns of horses supplemented with probiotic compared to those no receiving supplementation, suggesting microbial profiles were 79% different due to probiotic supplementation on d 35.

Dendrograms revealed clustering by treatment in the microbial profiles of *Lactobacillus* spp. due to probiotic supplementation (Figure b.7 a). PCA also revealed clustering due to probiotic supplementation (Figure b.7 b). Fecal samples collected from CTL horses on d 0 to 49 are included in one cluster, while samples collected from TRT horses d 28 to 77 are included in a second cluster. Interestingly, a third cluster contained the remaining CTL and TRT samples. The first principal component (PC1) axis accounted for 51.50% of the variation. The second principal component (PC2) accounted for 26.89% the variation.
Streptococcus Spp.

No differences in the number of bands representing *Streptococcus* species were observed (Figure b.5 c). Similarity coefficients averaged throughout the study were 65% similar. Fecal samples collected on d 49 of the sampling period had a similarity coefficient of 54% due to probiotic supplementation.

Dendrograms showed clustering in the microbial populations of *Streptococcus* spp. due to probiotic supplementation (Figure b.8 a). PCA also revealed clustering due to treatment (Figure b.8 b). Samples collected from CTL horses on d 7, 14, 28, 35, 42, 56 and 63 were included in one cluster, while an additional cluster contained TRT samples collected on d 7 to 42. The first principal component (PC1) axis accounted for 46.33% of the variation. The second principal component (PC2) accounted for 17.38% the variation.

DISCUSSION

Probiotic supplements for horses commonly include bacteria species such as *Lactobacillus* and *Bifidobacterium* because of their ability to survive the harsh conditions of the gastrointestinal tract and influence beneficial bacteria (Weese, 2002; Frape, 2010). Often times, research evaluating the use of probiotics is conducted in horses with traditional culture techniques. The use of molecular techniques may provide additional information about the diversity and complexity of the gastrointestinal microbiome. The methodologies utilized in the present study investigate the influence on probiotic administration on the microbial profile of healthy horses. Primer selection is also
important to consider since it appears that the probiotic organism may influence similar bacterial species in the horse.

The probiotic administered in this present study containing *Lactobacillus acidophilus* is commonly used in all livestock species including horses. PCA and dendrogram analyses revealed microbial diversity of *Lactobacillus* and *Streptococcus* populations was influenced due to probiotic supplementation with *Lactobacillus acidophilus*. Microbial profiles of both species of starch-utilizing bacteria were clustered by treatment. The results of this current study are supported by a similar study in horses. Medina and colleagues (2002) observed that supplementing probiotics to horses influenced both *Lactobacillus* and *Streptococcus* populations. Another study found that supplementing 10 healthy adult geldings with probiotics containing $5.6 \times 10^8$ cfu/g of *Lactobacillus acidophilus* for 28 days observed increased lactic acid bacterial populations due to probiotic administration (Ishizaka *et al.*, 2014).

In the current study, the number of bands representing *Lactobacillus* bacteria was higher in the TRT group on d 70 and 77 as compared to horses in the CTL group. Based on these results the probiotic supplemented in this study appeared to take 70 d for the microbial diversity to be influenced. It is important to note that the probiotic supplemented in the present study was designed for all livestock species. It is possible a probiotic designed specifically for horses may have observed an influence of microbial diversity sooner than 70 d.

Probiotics may provide prophylactic benefits by buffering gastrointestinal upset and reducing the incidence of poor health and performance (Kopp-Hoolihan, 2001).
However, there is limited research on the use of probiotics supplemented to healthy horses. Further research is needed to determine if probiotic supplementation with the probiotic in this present study may be advantageous as preventative therapy for young healthy horses, since it was able to influence the microbial profile of two types of starch-utilizing bacteria in young healthy Quarter Horses.
Chapter 4

The Influence of Probiotic Supplementation on the Microbial Diversity in the Gastrointestinal Microbiome of Miniature Horses


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ABSTRACT

Bacteria play a crucial role in the gastrointestinal health of many animals, including horses. Stress, illness, medication and diet can easily disrupt microbial populations in the equine gastrointestinal tract leading to decreased gut health and poor performance. Probiotics have been utilized in horses and other species to re-establish homeostasis of the gut bacteria. Probiotics may also have prophylactic implications, preventing gastrointestinal upset from occurring. However, there is limited research investigating the ability of probiotics to influence a stable gastrointestinal environment. Therefore, the objective of this study was to evaluate the influence of a probiotic supplement on microbial diversity of mature healthy horses. Six Miniature Horse geldings (7.5 ± 3.5 yr) were used in a replicated 3 x 3 Latin Square design. Each horse received a control diet of 1.5% BW of a mixed-grass hay and water ad libitum and was randomly assigned to one of three treatment groups during each 14 d period: control diet only (CTL), control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI). Fecal samples were collected on d 0 before supplementation and then on d 3, 7, 10 and 14 of each period. Fecal samples were pooled by treatment group and time. DNA was extracted from fecal samples and subjected to PCR-DGGE using primers specific to 16S rRNA gene sequences to
investigate changes in diversity of total bacteria, *Lactobacillus* and *Bacillus* species. PCR-DGGE images were analyzed using BioNumerics software to generate dendrogram comparisons based on the position and number of bands with further evaluation using Principal Coordinate Analysis (PCA). Band counts were calculated using the LOESS procedure of SAS and a p-value of ≤ 0.05 was considered statistically significant. No differences in band counts were observed in any of the microbial populations evaluated, except in total bacteria in which CTL had a greater number of bands compared to the HI treatment group at d 14. Dendrograms generated for universal, *Lactobacillus* and *Bacillus* spp. primers showed some clustering by treatment group. However, further investigation with Principal Coordinate Analysis (PCA) revealed no differences in microbial diversity due to probiotic supplementation. Overall, the results from this study indicate the probiotic was able to influence the microbial profile towards the end of the sampling period on d 14. The dose of the probiotic and the primer selected to evaluate changes in the diversity of the gastrointestinal microbiome seem to play a factor in the response observed.
INTRODUCTION

Bacteria play a crucial role in the gastrointestinal health of many animals, including horses. The gastrointestinal microbiome is complex and dynamic and is necessary for hindgut fermentation and utilization of feedstuffs. Stress, illness, medication and diet can easily disrupt microbial populations in the equine hindgut and lead to decreased gut health overall performance.

Probiotics are live microorganisms that elicit beneficial effects to the host (Garrett et al., 2010). Probiotics for horses commonly include bacterial species such as Lactobacillus, Bifidobacterium and Enterococcus (Weese, 2002). However, de Vaux and Julliand (1994) supplemented $10^{10}$ spores of Bacillus cereus to three cannulated ponies for five weeks and observed an increase of total anaerobes and proteolytic bacteria in the cecum. Supplementation with probiotic organisms containing spores, such as Bacillus subtilis, is beneficial to the host due to the spores’ ability to survive processing and product storage (Hong et al., 2005).

The majority of research evaluating the therapeutic usage of probiotics in horses and other species with a disrupted gastrointestinal microbiome has produced mixed results (Parraga et al., 1997; Ward et al., 2004; Desrochers, 2005; Naylor and Dunked, 2009; Milinovich et al., 2010). Supplementation with probiotics containing Saccharomyces boulardii to horses with acute enterocolitis reduced the length of time
and severity of compromised gut health (Desrochers et al., 2005). However, research by Kim and others (2001) found conflicting results when investigating if probiotic supplementation could reduce the incidence of *Salmonella* shedding in hospitalized horses when supplemented with $5 \times 10^9$ cfu each of *Lactobacillus lactic* and *Enterococcus faecium* and $10^8$ live yeast cells for five days. No differences were observed in clinical signs or *Salmonella* shedding in horses receiving the probiotic compared to horses receiving the placebo.

The importance of homeostasis in the equine gastrointestinal tract is noted and recently interest has developed regarding the prophylactic use of probiotic supplements in healthy horses. In a study evaluating the use of *Lactobacillus* in healthy adult horses, the probiotic was administered to adult horses at doses of $1 \times 10^9$ cfu/50kg BW per day, $1 \times 10^{10}$ cfu/50kg BW per day and $5 \times 10^{10}$ cfu/50kg BW per day for 5 d. The researchers determined the probiotic was ineffective due to its inability to colonize the gastrointestinal tract (Weese et al., 2003). Recent research by Ishizaka and colleagues (2014) demonstrated that supplementing a healthy horse’s diet with lactic acid bacterial cultures for 28 days appeared to improve fecal condition and increase populations of lactic acid bacteria.

Since there is limited information concerning the use of probiotics containing *Bacillus* spp. and horses, there is significant need for further knowledge. Therefore, the objective of this study was to evaluate the influence of a probiotic supplement containing *Bacillus subtilis* on the gastrointestinal microbiome of healthy Miniature Horse geldings.
MATERIALS AND METHODS

Experimental Design

Six mature Miniature Horse geldings (7.5 ± 3.5 yr; 134.5 ± 39.5 kg) were used in a replicated 3 x 3 Latin Square design. Each horse received 1.5% BW of a mixed-grass hay (control diet) and was randomly assigned to one of three treatment groups during each 14 d period: control diet only (CTL), control diet supplemented with 0.11 g/kg BW of a commercially available probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or control diet supplemented with 0.22 g/kg BW of the probiotic (HI). Throughout the study, horses were housed and fed individually in 1.5 x 2.0 m stalls and had access to water and a trace mineral salt block *ad libitum*. Horses were given free access to exercise for 1 hr/d in a 10 m x 10 m sand paddock.

Microbial Analysis

Fecal samples were collected on d 0 prior to supplementation and on d 3, 7, 10 and 14 of each period to evaluate changes in microbial diversity of the gastrointestinal microbiome. Fecal samples were stored at -20°C until further analysis. One gram from each fecal sample from horses in the same treatment group was homogenized and pooled by day for a composite sample. Bacterial DNA was extracted from the pooled fecal samples following a protocol of repeated bead beating plus column (RBB+C; Yu and
Morrison, 2004) with a modified protocol for elution of 50 uL of AE instead of 200 uL. The DNA product was confirmed by 1% agarose gel electrophoresis for 1 hr at 80 V, and DNA concentrations were determined using a modified protocol of the Quant-iT™ Broad-Range DNA Assay Kit (Invitrogen; Carlsbad, CA). The modified protocol included 50uL of working solution, instead of 200 µL, 2.5 µL of DNA and standards were utilized instead of 10 µL. The extracted DNA was purified and microbial diversity was investigated using PCR-DGGE. The primers utilized were specific to the V2-V3 regions of the 16S rRNA gene to evaluate total bacteria (HDA1, HDA2), and lactic acid bacteria including *Lactobacillus* (Lac1, Lac2) and *Bacillus* genera (Banff, Bar) (Table 1). The reaction mixture for PCR contained 0.25 µL of each 100 uM primer, 0.25 µL Taq polymerase, 1.0 µL of BSA, 1.75 µL of 50 mM MgCl₂, 0.40 µL of 100 mM dNTP and 5.0 µL of buffer. Fifty ng of DNA was added to universal and *Lactobacillus* reaction and distilled H₂O (Life technologies Corp; Eugene, OR) was added to reach a total reaction volume of 50 µL. One ng of DNA was added to the *Bacillus* PCR-DGGE reaction and distilled H₂O (Life technologies Corp; Eugene, OR) was added to reach a total reaction volume of 50 µL.

**DGGE Analysis**

PCR products were subjected to subsequent denaturing gradient gel electrophoresis (DGGE) analysis. Before samples were used for DGGE, 4.0 µL of each PCR product were exposed to 1.0% agarose gel electrophoresis to confirm successful amplification of the V2-V3 region. Then, 8.0 µL aliquots of PCR product were resolved
in a 7.5% polyacrylamide gel containing a 40%-60% gradient of denaturants (formamide and urea) for universal amplified PCR products and a 30%-70% gradient for all other PCR products. The DGGE gel was subjected to 0.5% TAE at 60° C and 82 V for 16 h using INGENY phorU-2 (Ingeny; Leiden, The Netherlands). The images were captured using AlphaImager HP® (ProteinSimple; Santa Clara, California).

**Qualitative and Statistical Analysis**

Microbial diversity from DGGE was evaluated with BioNumerics (Applied Maths NV; Sint-Martens-Latem, Belgium). The microbial DNA bands in each lane were manually detected on the gel and using the automatic band search function. Gels were normalized using internal reference bands and external reference markers (Invitrogen Inc.; Carlsbad, CA). A 1.0 % position tolerance was set for band matching to help correct minimal migratory variation. The DGGE banding patterns were transformed into a binary (presence or absence of bands) correlation matrix in BioNumerics. Dendrograms were created based on the unweighted pair-group method with arithmetic average (UPGMA) and the Jaccard function. Similarity was represented with a similarity coefficient derived from the pairwise comparison of DGGE banding patterns between two samples. Bacterial diversity richness was evaluated by the number of DGGE-bands present using the LOESS procedure of SAS v 9.3 (SAS Institute Inc.; Cary, NC) and a two-tailed t-test in Microsoft Excel and significance with a p-value ≤ 0.05. Principle component analysis (PCA) was performed using a combination of BioNumerics and Microsoft Excel to provide visual assessment of similarity between treatment groups from DGGE profiles.
RESULTS

Animal Performance

No adverse effects due to probiotic administration were observed during the study. BW averaged 134.5 ± 39.5 kg at the start of the study compared to 123.4 ± 28.1 kg at the end of the study.

Total Bacteria

No differences in band counts representing total bacteria observed for samples collected on d 0, 3, 7 and 10. On d 14 the control diet (CTL) had an increased number of bands compared to horses receiving the high dose of the probiotic (HI) with a p-value ≤ 0.05 (Figure b.9 a). Similarity coefficients averaged by treatment were 72%, 71%, 69%, comparing control to low, control to high and low to high, respectively (Table 4). Fecal samples collected on d 14 had similarity coefficients with an average of 51% similarity between banding patterns of horses supplemented with probiotic compared to those no receiving supplementation. This data suggests microbial profiles were altered 49% due to probiotic supplementation.

Dendrogram and Principal Coordinate Analysis (PCA) of the total bacterial profiles showed that horses receiving the probiotic supplement (LO and HI) exhibited greater variation in microbial diversity over the 14 d period (Figures b.10 a and b). The
first principal component (PC1) axis accounted for 39.3% of the variation and the second principal component (PC2) accounted for 22.1% the variation.

**Lactobacillus Spp.**

There were no differences in band counts representing *Lactobacillus* spp. due to probiotic supplementation (Figure b.9 b). Similarity coefficients averaged by treatment were 58%, 59%, 64%, comparing control to low, control to high and low to high, respectively (Table 5). Fecal samples collected on d 14 appeared to have the most variation of similarity coefficients with an average of 49% similarity between banding patterns of horses supplemented with probiotic compared to those not receiving supplementation, suggesting microbial profiles were altered 51% due to probiotic supplementation.

Dendrogram and PCA analyses representing *Lactobacillus* populations showed that the microbial profile of horses receiving the probiotic supplement (LO and HI) were clustered together on d 14 of the sampling period (Figures b.11 a and b). The first principal component (PC1) axis accounted for 35.03% of the variation. The second principal component (PC2) accounted for 26.18% the variation.

**Bacillus Spp.**

No differences in the number of bands representing *Bacillus* species were observed due to probiotic supplementation (Figure b.9 c). Similarity coefficients
averaged by treatment were 78%, 74%, 70%, comparing control to low, control to high and low to high, respectively (Table 6). There was no difference by day in similarity coefficients due to probiotic supplementation.

The dendrogram of the *Bacillus* bacterial profiles showed that horses receiving the high dose (HI) of the probiotic supplement had similar microbial profiles with clustering observed on d 7, 10, and 14 compared to the horses receiving the control diet (CTL) and low dose of the probiotic (LO) (Figure b.12 a). However, PCA revealed no clustering due to treatment (Figure b.12 b). The first principal component (PC1) axis accounted for 62.33% of the variation. The second principal component (PC2) accounted for 24.65% the variation.

**DISCUSSION**

Probiotics for horses commonly include bacterial species such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Weese, 2002). de Vaux and Julliand (1994) found that supplementing $10^{10}$ spores of *Bacillus cereus* daily to three ponies for five weeks had no effect on the cellulolytic activity of microbes, but did observe an increase in anaerobic and proteolytic bacteria in the cecum using traditional culture techniques. Additionally, there was an increase in nitrogen availability to the host. The study by de Vaux and Julliand (1994) supplemented the ponies for a much longer period than the current study. In the present study, Miniature Horses were supplemented a probiotic containing *Bacillus subtilis* for 14 d. The probiotic supplemented in this study was targeted for use in horses. The results of this study indicated that the probiotic began to change microbial diversity
in total bacteria and starch-utilizing bacteria around d 14 of the sampling period. Greater influence of microbial profiles might have been observed if the probiotic was supplemented for a longer period.

When administering probiotics, dosing is very important for desired results. Horses in the present study received the probiotic supplement containing $10^7$ cfu/g of *Bacillus* cultures at two different doses and did not experience adverse effects. PCA and dendrogram analyses representing total bacteria revealed the control samples had more similar microbial profiles than horses receiving the probiotic supplement (LO and HI). This suggests probiotic administration (LO and HI) increased the variation of the microbial profile of total bacteria in horses receiving the probiotic. After 7 d of probiotic supplementation, horses receiving the high dose (HI) had more similar microbial profiles in *Bacillus* spp. indicating the probiotic containing *Bacillus* spp. was able to influence the microbial diversity of the gastrointestinal tract. A study by Ishizaka and others (2014) found that supplementing 10 mature geldings with probiotics containing lactic acid bacteria for 28 days had increased lactic acid bacterial populations due to probiotic administration.

Often times, research investigating the influence of probiotic supplements on the bacterial populations of horses and other species is done using traditional bacterial culture techniques. The use of molecular techniques could provide information about the diversity and complexity of the gastrointestinal microbiome. The methodologies utilized in this study are evaluating the diversity of microbial profiles using molecular techniques based solely on the number of bands or both the number and position of the bands.
Primer selection also affects the influence due to probiotic supplementation observed. Primers should be selected based on the bacterial species in the probiotic being administered as well as other populations the probiotic could be affecting.

Probiotic supplementation may be advantageous as a preventative therapy against gastrointestinal upset for healthy horses. Prophylactic use of probiotics may help combat the incidence of illness before it occurs, instead of simply using probiotics as a treatment (Kopp-Hoolihan, 2001). However, the efficacy of probiotic use remains poor without a better understanding of the equine microbiome and standard protocols for the use of probiotics to optimize horse health and performance.
Chapter 5
General Discussion

The findings from this present research support the idea that strain selection, dose administered and the duration of probiotic administration are important to achieve a response in the microbial diversity of healthy horses. Most research with probiotics and horses is evaluated using traditional culture techniques. However, the present research utilized molecular techniques to investigate changes in microbial diversity in healthy horses following probiotic administration. When using molecular techniques to investigate changes in microbial diversity primer selection is important. The current research chose primers to investigate changes in the microbial profile of total bacteria and starch-utilizing bacteria since the probiotic supplements administered in the three separate studies contained starch-utilizing bacteria.

Future research is needed to evaluate changes in fiber-utilizing bacteria and species-specific starch-utilizing bacteria. Additional methodologies including sequencing may provide insight into specific changes in microbial diversity due to probiotic supplementation. Since the present research observed changes in the microbial diversity of healthy horses due to probiotic supplementation, further study is needed to determine the prophylactic use of probiotics for horses.
The use of probiotic supplements prophylactically may have a great impact in the equine industry. Probiotic supplements can alter microbial diversity, which may optimize the health and performance of horses. Gastrointestinal-associated disease is the leading cause of morbidity and mortality in horses and the prophylactic use of probiotics may buffer the disturbance of microbial populations when stress, illness, medication and change in diet occur.
REFERENCES


and glycoside hydrolase activities in horses fed a high-fiber or high-starch diet. J. Anim. Sci. 87:2844-2852.


Appendix A

Tables
Table 1. Primer sequences used for PCR-DGGE. Bolded sequences correspond to the GC clamp.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Target Bacteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA1</td>
<td>CGCCCGGGGCGCGCCCCCGG CGGGCGGGGGGCACCGGGG - GACTCCTACGGGAGGCAGCAGT</td>
<td>Universal</td>
<td>Walter et al., 2000</td>
</tr>
<tr>
<td>HDA2</td>
<td>GTATTACCGCGGCTGCTGGGAC</td>
<td>Universal</td>
<td>Walter et al., 2000</td>
</tr>
<tr>
<td>Lac1</td>
<td>AGCAGTAGGGAATCTTTCCA</td>
<td>Lactobacillus Weissella Pediococcus Leuconostoc</td>
<td>Endo et al., 2007</td>
</tr>
<tr>
<td>Lac2</td>
<td>CGCCCGGGGCGCGCCCCGGG CGGCCCGGGGGGCACCGGGG - ATTYCACCGCTACACATG</td>
<td>Lactic Acid Bacteria</td>
<td>Endo et al., 2007</td>
</tr>
<tr>
<td>Lac3</td>
<td>AGCAGTAGGGAATCTTTCCG</td>
<td>Streptococcus Enterococcus Tetracenococcus Vagococcus Lactococcus</td>
<td>Endo et al., 2007</td>
</tr>
<tr>
<td>BacF</td>
<td>ATGGCTGTGTCGTCAGCT</td>
<td>Bacillus</td>
<td>Chen et al., 2012</td>
</tr>
<tr>
<td>BacR</td>
<td>CGCCCGGCGCGCGCCCGCGCC CGTCCCCCGCGCCCCCGCCC - GACGGGCGGTGTGTGAC</td>
<td>Bacillus</td>
<td>Chen et al., 2012</td>
</tr>
</tbody>
</table>
Table 2. Similarity coefficients (%) calculated based on the similarity of PCR-DGGE profiles of pooled fecal samples from Quarter Horse mares (n=7) generated with universal primers (HDA1, HDA2), *Lactobacillus* primers (Lac1, Lac2), and *Streptococcus* primers (Lac3, Lac2).

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>HDA1, HDA2</th>
<th>Lac1, Lac2</th>
<th>Lac3, Lac2</th>
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<tbody>
<tr>
<td>Treatment Comparison</td>
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<td>Day</td>
<td>CTL - TRT</td>
<td>CTL - TRT</td>
<td>CTL - TRT</td>
</tr>
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<td>14</td>
<td>88</td>
<td>67</td>
<td>74</td>
</tr>
<tr>
<td>21</td>
<td>85</td>
<td>49</td>
<td>71</td>
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<tr>
<td>28</td>
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<td>91</td>
<td>57</td>
<td>65</td>
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<tr>
<td>49</td>
<td>90</td>
<td>64</td>
<td>78</td>
</tr>
</tbody>
</table>

All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL) or the control diet supplemented with 140 g of a probiotic containing 10⁹ cfu of *Lactobacillus acidophilus* (TRT). Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment.
Table 3. Similarity coefficients (%) calculated based on the similarity of PCR-DGGE profiles of pooled fecal samples from young Quarter Horses (n=12) generated with universal primers (HDA1, HDA2), *Lactobacillus* primers (Lac1, Lac2), and *Streptococcus* primers (Lac3, Lac2).

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>HDA1, HDA2</th>
<th>Lac1, Lac2</th>
<th>Lac3, Lac2</th>
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<tr>
<td><strong>Treatment Comparison</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day</strong></td>
<td><strong>CTL - TRT</strong></td>
<td><strong>CTL - TRT</strong></td>
<td><strong>CTL - TRT</strong></td>
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<td>0</td>
<td>86</td>
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<td>56</td>
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<td>77</td>
<td>90</td>
<td>23</td>
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All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL) or the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus* (TRT). Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d.
Table 4. Similarity coefficients (%) calculated based on the similarity of PCR-DGGE profiles of pooled fecal samples from Miniature Horses (n=6) generated with universal primers (HDA1, HDA2).

<table>
<thead>
<tr>
<th>Day</th>
<th>CTL - LO</th>
<th>CTL - HI</th>
<th>LO - HI</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>75</td>
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<tr>
<td>14</td>
<td>50</td>
<td>47</td>
<td>56</td>
</tr>
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</table>

All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14.
Table 5. Similarity coefficients (%) calculated based on the similarity of PCR-DGGE profiles of pooled fecal samples from Miniature Horses (n=6) generated with *Lactobacillus* primers (Lac1, Lac2).

<table>
<thead>
<tr>
<th>Day</th>
<th>CTL - LO</th>
<th>CTL - HI</th>
<th>LO - HI</th>
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<td>14</td>
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</table>

All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14.
Table 6. Similarity coefficients (%) calculated based on the similarity of PCR-DGGE profiles of pooled fecal samples from Miniature Horses (n=6) generated with Bacillus primers (BacF, BacR).

<table>
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<th>Day</th>
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<th>LO - HI</th>
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<td>3</td>
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<td>14</td>
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</table>

All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11 g/kg of BW of a commercial probiotic containing $2 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14.
Appendix B

Figures
Figure b.1. Influence of probiotic supplementation on the gastrointestinal microbiome of Quarter Horse mares (n=7) as shown through band counts. All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. DGGE images were uploaded to BioNumerics software (Applied Maths; Austin, TX). Band counts were generated using primers specific to 16S rRNA sequences a) total bacteria a) total bacteria (HDA1, HDA2), b) *Lactobacillus* species (Lac1, Lac 2), and c) *Streptococcus* species (Lac3, Lac2). Band counts were analyzed with PROC LOESS of SAS v. 9.3. Significant differences were declared at P $\leq$ 0.05 (*).
Figure b.2. Relatedness of PCR-DGGE profiles representing total bacteria (HDA1, HDA2) from pooled fecal samples from Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.3. Relatedness of PCR-DGGE profiles representing *Lactobacillus* species (Lac1, Lac 2) from pooled fecal samples from Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.4. Relatedness of PCR-DGGE profiles representing *Streptococcus* species (Lac3, Lac2) from pooled fecal samples from Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.5. Influence of probiotic supplementation on the gastrointestinal microbiome of young Quarter Horses (n=12) as shown through band counts. All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Band counts were generated using primers specific to 16S rRNA sequences a) total bacteria (HDA1, HDA2), b) *Lactobacillus* species (Lac1, Lac 2), and c) *Streptococcus* species (Lac3, Lac2). Band counts were analyzed with PROC LOESS of SAS v. 9.3. Significant differences were declared at $P \leq 0.05$ (*).
Figure b.6. Relatedness of PCR-DGGE profiles representing total bacteria (HDA1, HDA2) from pooled fecal samples from young Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.7. Relatedness of PCR-DGGE profiles representing *Lactobacillus* species (Lac1, Lac 2) from pooled fecal samples from young Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing 10⁷ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.8. Relatedness of PCR-DGGE profiles representing *Streptococcus* species (Lac3, Lac2) from pooled fecal samples from young Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.9. Influence of probiotic supplementation on the gastrointestinal microbiome of Miniature Horses (n=6) as shown through band counts. All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing 2.0 x 10^7 cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Band counts were generated using primers specific to 16S rRNA sequences a) total bacteria (HDA1, HDA2), b) *Lactobacillus* species (Lac1, Lac 2), and c) *Bacillus* species (BacF, BacR) Band counts were analyzed with PROC LOESS of SAS v. 9.3. Significant differences were declared at $ P \leq 0.05 $ (*).
Figure b.10. Relatedness of PCR-DGGE profiles representing total bacteria (HDA1, HDA2) from pooled fecal samples from Miniature Horses (n=6). All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard coefficient and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.11. Relatedness of PCR-DGGE profiles representing *Lactobacillus* species (Lac1, Lac 2) from pooled fecal samples from Miniature Horses (n=6). All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Figure b.12. Relatedness of PCR-DGGE profiles representing *Bacillus* species (BacF, BacR) from pooled fecal samples from Miniature Horses (n=6). All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11 g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Dendrograms (a) were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions. PCA (b) was generated from a binary similarity matrix to assess the similarity between treatments.
Appendix C

Supporting Figures
Figure c.1. PCR amplification of microbial DNA from probiotic supplement containing *Lactobacillus acidophilus* (Lac1, Lac 2) on a 1% agarose gel. Lane 1 is a 100 base pair ladder. Lane 2 is the probiotic supplement.
Figure c.2. PCR amplification of microbial DNA from probiotic supplement containing *Lactobacillus acidophilus* (Lac1, Lac 2) on a 1% agarose gel. Lane 1 is a 100 base pair ladder. Lane 2 is the probiotic supplement.
Figure c.3. PCR amplification of microbial DNA from probiotic supplement containing *Bacillus subtilis* (BacF, BacR) on a 1% agarose gel. Lane 1 is a 100 base pair ladder. Lane 2 is the probiotic supplement.
Figure c.4. PCR-DGGE banding pattern of total bacteria in the gastrointestinal microbiome of Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with universal primers (HDA1, HDA2) amplification on an 8% polyacrylamide gel. Lane 1 = probiotic, lane 2 = d0, lane 3 = d14 TRT, lane 4 = d14 CTL, lane 5 = d21 TRT, lane 6 = d21 CTL, lane 7 = d28 TRT, lane 8 = d28 CTL, lane 9 = d35 TRT, lane 10 = d35 CTL, lane 11 = d42 TRT, lane 12 = d42 CTL, lane 13 = d49 TRT, lane 14 = d49 CTL.
Figure c.5. PCR-DGGE banding pattern of *Lactobacillus* species in the gastrointestinal microbiome of Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing 10^9 cfu of *Lactobacillus acidophilus*. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with *Lactobacillus* primers (Lac1, Lac2) amplification on an 8% polyacrylamide gel. Lane 1 = probiotic, lane 2 = d0, lane 3 = d14 CTL, lane 4 = d21 CTL, lane 5 = d28 CTL, lane 6 = d35 CTL, lane 7 = d42 CTL, lane 8 = d49 CTL, lane 9 = d14 TRT, lane 10 = d21 TRT, lane 11 = d28 TRT, lane 12 = d35 TRT, lane 13 = d42 TRT, lane 14 = d49 TRT.
Figure c.6. PCR-DGGE banding pattern of Streptococcus species in the gastrointestinal microbiome of Quarter Horse mares (n=7). All horses received a control diet of received 0.5% BW of 12% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 140 g of a probiotic containing $10^9$ cfu of Lactobacillus acidophilus. Fecal samples were collected on d 0, 14, 21, 28, 35, 42, and 49 and pooled by treatment. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with Streptococcus primer (Lac3, Lac2) amplification on an 8% polyacrylamide gel. Lane 1 = probiotic, lane 2 = d0, lane 3 = d14 TRT, lane 4 = d14 CTL, lane 5 = d21 TRT, lane 6 = d21 CTL, lane 7 = d28 TRT, lane 8 = d28 CTL, lane 9 = d35 TRT, lane 10 = d35 CTL, lane 11 = d42 TRT, lane 12 = d42 CTL, lane 13 = d49 TRT, lane 14 = d49 CTL.
Figure c.7. PCR-DGGE banding pattern of total bacteria in the gastrointestinal microbiome of Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with universal primers (HDA1, HDA2) amplification on an 8% polyacrylamide gel. Lane 1 = d0 CTL, lane 2 = d0 PRO, lane 3 = d7 CTL, lane 4 = d7 TRT, lane 5 = d14 CTL, lane 6 = d14 TRT, lane 7 = d21 CTL, lane 8 = d21 TRT, lane 9 = d28 CTL, lane 10 = d28 TRT, lane 11 = d35 CTL, lane 12 = d35 TRT, lane 13 = d42 CTL, lane 14 = d42 TRT, lane 15 = d49 CTL, lane 16 = d49 TRT, lane 17 = d56 CTL, lane 18 = d56 TRT, lane 19 = d63 CTL, lane 20 = d63 TRT, lane 21 = d70 CTL, lane 22 = d 70 TRT, lane 23 = d77 CTL, lane 24 = d77 TRT, and lane 25 = probiotic.
Figure c.8. PCR-DGGE banding pattern of *Lactobacillus* species in the gastrointestinal microbiome of Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with *Lactobacillus* primers (Lac1, Lac 2) amplification on an 8% polyacrylamide gel. Lane 1 = probiotic, lane 2 = d0 CTL, lane 3 = d7 CTL, lane 4 = d14 CTL, lane 5 = d21 CTL, lane 6 = d28 CTL, lane 7 = d35 CTL, lane 8 = d42 CTL, lane 9 = d49 CTL, lane 10 = d56 CTL, lane 11 = d63 CTL, lane 12 = d70 CTL, lane 13 = d77 CTL, lane 14 = d0 TRT, lane 15 = d7 TRT, lane 16 = d14 TRT, lane 17 = d21 TRT, lane 18 = d28 TRT, lane 19 = d35 TRT, lane 20 = d42 TRT, lane 21 = d49 TRT, lane 22 = d56 CTL, lane 23 = d63 TRT, lane 45 = d70 TRT, and lane 25 = d77 TRT.
Figure c.9. PCR-DGGE banding pattern of *Streptococcus* species in the gastrointestinal microbiome of Quarter Horses (n=12). All horses received a control diet of received 0.5% BW of 14% CP pelleted concentrate with water and mixed grass hay ad libitum (CTL). Horses in the treatment (TRT) group received the control diet supplemented with 10 g of a probiotic containing $10^7$ cfu of *Lactobacillus acidophilus*, while horses in the control (CTL) group received the control diet. Fecal samples were collected and pooled by treatment every 7 d for a period of 77 d. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with *Streptococcus* primers (Lac3, Lac2) amplification on an 8% polyacrylamide gel. Lane 1 = probiotic, lane 2 = d0 CTL, lane 3 = d7 CTL, lane 4 = d14 CTL, lane 5 = d21 CTL, lane 6 = d28 CTL, lane 7 = d35 CTL, lane 8 = d42 CTL, lane 9 = d49 CTL, lane 10 = d56 CTL, lane 11 = d63 CTL, lane 12 = d70 CTL, lane 13 = d77 CTL, lane 14 = d0 TRT, lane 15 = d7 TRT, lane 16 = d14 TRT, lane 17 = d21 TRT, lane 18 = d28 TRT, lane 19 = d35 TRT, lane 20 = d42 TRT, lane 21 = d49 TRT, lane 22 = d56 CTL, lane 23 = d63 TRT, lane 45 = d70 TRT, and lane 25 = d77 TRT.
Figure c-10. PCR-DGGE banding pattern of total bacteria in the gastrointestinal microbiome of Miniature Horses (n=6). All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Analysis of V2-V3 region of the 16S rRNA gene of pooled fecal samples is represented with universal primer (HDA1, HDA2) amplification on an 8% polyacrylamide gel. Lane1 = probiotic, lane 2 = d0 CTL, lane 3 = d3 CTL, lane 4 = d7 CTL, lane 5 = d10 CTL and lane 6 = lane d14 CTL. Lane 7 = d3 LO, lane 8 = d7 LO, lane 9 =d10 LO and lane 10 = d14 LO. Lane 11 = d3 HI, lane 12 = d7 HI, lane 13 = d10 HI and lane 14 = d14 HI.
Figure c.11. PCR-DGGE banding pattern of *Lactobacillus* species in the gastrointestinal microbiome of Miniature Horses (n=6). All Miniature Horses were fed a control diet of 1.5% BW mixed grass hay (CTL), the control diet supplemented with 0.11g/kg of BW of a commercial probiotic containing $2.0 \times 10^7$ cfu/g of *Bacillus subtilis* (LO) or the control diet supplemented with 0.22 g/kg of BW of the commercial probiotic (HI) in a 3x3 Latin square design. Fecal samples were collected and pooled by treatment on d 0, 3, 7, 10 and 14. Analysis of V2-V3 region of the 16S rRNA gene of fecal samples is represented with *Lactobacillus* primer (Lac1, Lac 2) amplification on an 8% polyacrylamide gel. Lane 1 = commercial probiotic. Lane 2 = d0 CTL, lane 3 = d3 CTL, lane 4 = d7 CTL, lane 5 = d10 CTL and lane 6 = lane d14 CTL. Lane 7 = d3 LO, lane 8 = d7 LO, lane 9 = d10 LO and lane 10 = d14 LO. Lane 11 = d3 HI, lane 12 = d7 HI, lane 13 = d10 HI and lane 14 = d14 HI.
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