EARLY FUNCTIONAL AND COMMUNITY DEVELOPMENT OF THE EQUINE HINDGUT MICROBIOME IN SEMI-FERAL- AND DOMESTIC CONVENTIONALLY-MANAGED FOALS INCLUDING CASES OF FOAL DIARRHEA

by

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ABSTRACT

The early development of the gut microbiome has been proven to be an essential part in maintaining a healthy neonate in both humans and animals. During this time, the foal is more susceptible to gut dysbiosis. As a result, gastrointestinal abnormalities such as foal heat diarrhea can occur. Foal diarrhea is a common non-infectious digestive issue that usually occurs in the foal in their first few weeks of life. Currently, no studies have been conducted analyzing foal diarrhea in non-conventionally managed foals. In the first study conducted, ten domestic conventionally managed (DCM) Standardbred and ten semi-feral managed (SFM) Shetland-type pony foals and dams were studied for analysis of the early development of their hindgut microbiomes to determine changes as the foals aged as well as the effects of different management techniques. In the second study conducted, seven diarrheic foals and seven age- and domestication management-matched (semi-feral- or domestic conventionally-managed) healthy foals were sampled for analysis of their hindgut microbiome to determine the effects of foal diarrhea. In both studies, rectal swab microbial communities were determined using next generation sequencing and a total of 25 different phyla were found with the most abundant phylum present being Firmicutes followed by Bacteroidetes in both foals and dams.

Dams were found to have a significantly higher mean diversity than foals (PD whole tree, nonparametric t-test, $p<0.001$). When comparing foals by week of age, week 1 foals had a significantly lower mean diversity than week 2, week 3, week 4, week 5 and week 6 foals (PD whole tree, nonparametric t-test, $p<0.01$). Significant

differences were also found between semi-feral managed and domestic conventionally managed foals (ANOSIM, $p<0.01$, PERMANOVA, $p<0.05$, $n_{samples}=116$, $n_{groups}=2$) as well as well as between different foal ages (ANOSIM, PERMANOVA, $p<0.001$, $n_{samples}=116$, $n_{groups}=6$), showing that management type and age have notable effects on the horse at an early stage in their life. *Lactobacillus spp*. and Lactobacillaceae gen., bacteria associated with lactic acid production and starch-induced laminitis, were found to be enriched only in DCM foals, specifically during their second and third week of life (Kruskal-Wallis, LDA score>2.0, $p<0.05$). Predicted function of the microbiome was also analyzed and SFM foals were found to have a significantly higher mean sequence count in the OTUs contributing to lipid, general carbohydrate, complex carbohydrate, simple carbohydrate and protein digestion ($p<0.001$, Kruskal-Wallis). DCM foals were also found to have a microbiome more similar to their dams in their fifth and sixth week of life than were SFM foals to their dams in their sixth week of life.

There were significant differences found between diarrheic and non-diarrheic foals in specific OTUs but not when analyzing their gut communities as a whole. This may suggest that the bacteria found to be significantly different are those contributing to mild diarrhea, but these changes are not drastic enough to cause serious illness in the foal. *Bacteroides fragilis, Prevotella spp., Veillonella spp.*, Lachnospiraceae gen. and Clostridiales fam. are some of the taxa found to be significantly higher in diarrheic foals. Ruminococcaceae gen., *Bacteroides spp., Butyricimonas spp., Odoribacter spp., Oscillospira spp., Fusobacterium spp.* and *Escherichia coli* were some of the taxa found to be significantly higher in non-diarrheic foals. The function of the microbiome was also analyzed and non-diarrheic foals were found to have a significantly higher abundance of the OTUs responsible for starch digestion, general carbohydrate

digestion, simple carbohydrate digestion, complex carbohydrate digestion and protein digestion when compared to diarrheic foals ($p<0.05$, Kruskal-Wallis).

These studies provide insight into how management and foal diarrhea can affect the foal's hindgut microbiome as well as gives a detailed description of the function and community of this microbial community in the foal's first six weeks of life.

Chapter 1

INTRODUCTION

Overview of the Equine Digestive System

The horse (*Equus caballus*) is a monogastric/nonruminant herbivore and its symbiotic relationship with the microbial population present in its stomach, small intestine and large intestine is imperative for survival. This microbial population is commonly known as the gut microbiota or gut microbiome and is made up of Bacteria, Archaea and Eukaryotes. The gut microbiota allows the horse to digest and utilize feedstuffs that its digestive enzymes cannot by the process of fermentation. The fermentation of plant material by bacteria is essential for the horse because its diet is mainly composed of fibrous vegetation.

The equine gastrointestinal tract can be broken into two major compartments: the foregut and hindgut [1]. The foregut is made up of the stomach and small intestine and is mainly responsible for the digestion of soluble carbohydrates, starch, proteins and fats. The hindgut is composed of the large colon, cecum and small colon and is mainly responsible for the fermentation of insoluble carbohydrates. The majority of the volume, approximately two-thirds, of the horse's digestive tract is made up of the hindgut and most of the bacterial digestion occurs here [2]. In the equine stomach, the majority of bacterial digestion occurring is the fermentation of soluble carbohydrates and starch. This digestion is mainly completed by acid-tolerant lactic acid producing bacteria [3]. As carbohydrates are fermented by these bacteria and lactic acid is produced, the pH of the digesta decreases to approximately 2.6.

After passing through the stomach, digesta travels through the small intestine where enzymatic breakdown and absorption occurs, and the pH of the digesta neutralizes to approximately 7.2 [4, 5]. Also in the small intestine, fats are emulsified and converted into fatty acids and glycerol by bile secreted from the horse's liver [6]. Proteins are digested here as well by proteases and are converted to amino acids. Enzymes in the small intestine, specifically in the duodenum and proximal jejunum, are the primary digesters of starch. There are also many different bacteria that are able to produce the enzymes responsible for starch breakdown, such as the *Streptococcus* and *Lactobacillus* genera [7]. Amylolytic bacteria are able to break down the alpha-glycosidic linkages found in starch and convert it into lactic acid.

The horse has adapted to a fiber-rich diet with approximately 35-60% of their diet being composed of cell wall carbohydrates [7]. The hindgut is the major compartment responsible for breaking down and absorbing the nutrients from these dietary plant fibers. This is because the horse's digestive enzymes are unable to break the beta-glycosidic linkages found in cellulose, hemicellulose and pectin, which are the major components of fibrous plant material. The cecum and colon have a pH of 6.0, which provides the ideal environmental condition for anaerobic microorganisms to participate in digestion [8]. In the hindgut, anaerobic microorganisms, such as the *Ruminococcus* and *Fibrobacter* genera, adhere to plant cell walls and this close association allows concentrated microbial enzymes to reach the substrates [7]. These enzymes hydrolyze plant fibers into soluble sugars, which are then converted into short chain fatty acids, also known as volatile fatty acids, by the process of fermentation. Short chain fatty acids, such as propionate, butyrate and acetate, provide the horse with approximately 60-70% of their energy [9, 10].

The Equine Gut Microbiome

The horse's digestive system bacterial community is very diverse, whereas its Archaea population consists of methanogens exclusively and its Eukaryotic population is made up of fungi and protozoa. Protozoa and fungi are found to be present only in the hindgut with abundances of approximately $5X10^4$ to $1.5X10^5$/mL contents and $2X10^2$ to $2.5X10^3$ fungal units/g content, respectively [11]. The amount of bacteria reported in the large intestine was approximately $5X10^8$ to $5X10^9$ bacteria/g content. The microbiome in the horse's gut is of major interest to researchers because it is the main component that allows the horse to digest complex carbohydrates such as cellulose and hemicellulose. It is important to understand both the differences in function as well as the microbial populations in each part of the horse's digestive system. Analyzing a core microbiome for each gastrointestinal tract compartment would allow researchers to understand and interpret changes in the horse during a particular disease state or under certain management conditions.

There have been several studies using next generation sequencing to characterize the microbial communities present in the different compartments of the horse's digestive tract. In regards to the colon microbiome, there was a study conducted in which five slaughtered horses with a grass-based diet were sampled for their colonic wall tissue and colonic lumen contents [12]. They sampled from four different segments of the colon, including the caecum, proximal colon, mid colon and distal colon. From these samples, they found 272 sequences that were classified into 168 OTUs (Operational Taxonomic Units). They discovered that 72% of their total sequences belonged to a low %G+C Gram-positive phylum, 20% belonged to a Cytophaga-Flexibacter-Bacteroides phylum and 37% belonged to a clostridial group. At the time of this study, only 5% of their sequences were matched to known

sequences available in public databases. Therefore, it is difficult to draw conclusions from this study in regards to the core microbiome of the horse's large intestine.

Another study used 8 horses to analyze the microbiome and metabolome of samples from the right dorsal colon, cecum and feces [13]. They used 16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) and quantitative PCR (q-PCR) to compare the microbial communities and used Fourier transform infrared (FT-IR) spectroscopy to analyze the metabolites in the samples. They found that the cecum was the most unique when compared to samples from the colon and feces in both its metabolome and microbiome and that alpha diversity was higher in the colon and feces than in the cecum. They found no significant differences between the microbiomes of fecal and colon samples but did find significant differences when they compared 3 pony (n=5) and horse (n=3) samples. However, this comparison between horse and pony microbiomes needs to be further investigated with a larger sample size. They also reported that the right dorsal colon had the highest amount of total volatile fatty acids, followed by the cecum and feces. They did not report on any specific bacteria present in each compartment and were only able to analyze T-RFLP peaks and a combination of Manhattan distances and unweighted pair group method with arithmetic mean (UPGMA). Their major conclusion was that fecal samples were successful in representing the microbiome and metabolome of the right dorsal colon but not of the cecum.

Fecal samples and, in some cases, rectal swabs are the most noninvasive methods currently used to sample the gut microbiome. However, questions have been raised as to whether fecal samples can accurately represent the horse's gut microbiome as well as which parts of the horse's digestive tract they are most similar to. There are a few studies that have looked at the sufficiency of fecal samples in representing the

horse's gut as a whole. A more descriptive study investigated the differences in microbial composition in the stomach, duodenum, ileum, cecum, pelvic flexure, pelvic flexure mucosa, small colon, rectum and feces of 11 horses [14]. They found that bacteria in the Firmicutes phylum were most abundant in all compartments of the horse's GI tract. *Lactobacillus spp.* and *Sarcina spp.* were found to be enriched in the stomach while *Streptococcus spp.* was enriched in the duodenum. In the ileum, *Actinobacillus* and *Clostridium sensu stricto* were most abundant and the genus *'5 genus incertae sedis'* was the most abundant in the large colon and feces. They also saw a trend that diversity increased towards the distal gut. In contrast to the previous study, they determined that the microbial communities from the cecum to feces were very similar. Therefore, they concluded that fecal samples may be useful in representing the distal hindgut, including the cecum.

There was also a study conducted by Reed et al. in which they used generation sequencing to analyze samples from 6 pony yearlings' cecum, ventral colon, dorsal colon and feces [15]. They found that the fecal microbial community is very similar to the dorsal colon microbiome and that the microbiome of the cecum was very similar to that of the ventral colon. They also demonstrated that the Bacteroidetes phylum was the most abundant in all compartments and that the Verrucomicrobia phylum increased in abundance in the dorsal colon and feces. These findings provide researchers with the confidence that fecal samples are sufficient in representing certain parts of the horse's hindgut microbiome, however, it is still important to understand the differences between each compartment of the horse's complex digestive tract.

Common Management Practices and Their Effects on the Horse

Management is a very important aspect of horse ownership and includes regulating the horse's diet, exercise, social interaction and housing. It can be a major causal factor of many different diseases and behavioral abnormalities in the horse such as laminitis and stereotypic behaviors. Laminitis, a major persisting issue in the horse industry, can be triggered by overfeeding, high intake of soluble carbohydrates and severe concussion trauma to the laminae due to overworking. With the increased use of supplemental feeds in the domestic horse, overfeeding of carbohydrates, specifically starch and sugar, is becoming more common. Management practices can also greatly affect the horse's gut microbiome. For example, there have been studies addressing how factors such as weaning method in the foal, diet, exercise and general management influence the horse's gut microbiota [16, 17, 18, 19, 20].

Effects of Diet on the Gut Microbiome of Carnivores, Herbivores and Omnivores

Diet is known to be a very important contributing factor to an animal's gut microbiome and, in turn, their health. There have been studies that compare the gut microbiota of different vertebrate animals in order to determine whether diet, phylogeny or other factors most greatly influence the gut microbiome. The main conclusion drawn from these studies is that there is a clear separation between the gut microbial diversity in herbivores, carnivores and omnivores [21, 22, 23]. They found that herbivores have the most diverse microbiota when compared to omnivorous and carnivorous vertebrates. Muegge et al. also found that diet affects the animal's gut microbiota more than host phylogeny in that it influences the taxa present in the gut [23].

Another study analyzed the gut microbiome of different types of herbivores, specifically 25 hindgut fermenting and 16 ruminant herbivores as well as 2

monogastric omnivores [24]. The hindgut fermenters were comprised of four different animal species whereas the ruminants were comprised of five different animal species. The two monogastric omnivores were pigs and were used as a small comparison group. All of the ruminant subjects had a diet of grass only while 11 of the hindgut fermenters were fed commercial feed and the rest had a grass diet. They extracted total bacterial genomic DNA from each animal's feces and sequenced the V4 region of the 16S rRNA gene using 454 Titanium sequencing, a type of 16S rRNA gene amplicon pyrosequencing. From this study, they were able to make a few conclusions about how digestion type can affect the gut microbiome. Firstly, they found that the ruminant fecal microbiota has a greater alpha diversity than the hindgut fermenter microbiota, but also showed that the donkey (hindgut fermenter) has the most diverse gut community and the rabbit (hindgut fermenter) has the least diverse. A PCoA (Principal Coordinate Analysis) plot based on weighted UniFrac distances was also used to show the gut microbial community composition and abundance groupings of the different types of herbivores and omnivore subjects. Ruminants and hindgut fermenters are clearly separated in the PCoA plot while the omnivore subjects somewhat overlap with the monogastric hindgut fermenting herbivores, which are more physiologically similar to them than ruminants. These groupings could be attributed to the significantly higher abundance of the Firmicutes phylum, including the predominant taxa of the Clostridia class, Clostridiales order and Ruminococcaceae family ($p \leq .05$), in ruminants when compared to hindgut fermenters. This study, as well as those comparing carnivores, herbivores and omnivores, shows that diet, digestive physiology and digestion type significantly affect the gut microbiome.

Effects of Diet on the Horse Gut Microbiome

Horses are naturally adapted to be continuous grazers, however, due to the inability to meet their dietary needs on the forage available, domesticated horses are commonly supplemented with grain-based feed. It is possible and, in most cases, more ideal for the horse to survive and thrive on a forage-only diet when the horse is able to freely graze on sufficient forage. Horses that are able to continuously graze are constantly secreting saliva, which buffers the acidity of their stomach contents. This acidity is caused by the fermentation of non-structural carbohydrates with lactic acid as a byproduct [3]. The emergence of concentrate feeds came about due to the higher nutritional and energy requirements of exercise horses, the convenience of concentrate feeds to horse owners and limited access to sufficient natural forage and land space.

Currently, there are many different feed options that are commonly marketed towards certain life stages and working statuses of the horse, including maintenance, growing, working, lactating, pregnant and geriatric. Concentrate feeds can be helpful for horse owners when trying to achieve a balanced diet for their horse. However, when these feeds are administered inappropriately, there can be an increased risk for physiological issues such as laminitis and colic. Laminitis occurs when there is weakened adhesion between the distal phalynx and lamellae of the inner hoof wall. This inflammatory lesion can eventually cause complete detachment and rotation of the coffin bone as well as extreme pain for the horse. Concentrate feeds normally fed to the horse two to three times a day can be high in starch and fructans. An excess of starch is thought to be a contributing factor to dietary laminitis by way of the fermented components released by bacteria into the bloodstream during lactic acidosis, however, the specific processes involved are still unknown [25, 26].

The effects of diet on the horse's gut microbiome have been researched using both culture methods and next generation sequencing. A recent study aimed to determine the effects of the transition from a fiber-rich diet to a starch-rich diet on the horse's microbiome [16]. They used six fistulated horses and collected samples from their cecum, right ventral colon and feces and analyzed the metabolites and basic bacterial composition in the samples. While transitioning from a high fiber to a high starch diet, they saw an increase in total anaerobic, amylolytic and lactate-utilizing bacteria and a decrease in cellulolytic bacteria in the cecum and colon. They also saw an increase in lactate and lipopolysaccharides after the transition. Since this study did not use next generation sequencing to characterize all the bacteria present during a diet change, it would be interesting to do this in the future.

Another study utilized 16S rRNA gene amplification and sequencing to determine the differences between the microbiomes of four horses fed either a timothy hay diet or a timothy hay and whole oats diet [27]. They found that horses fed the oats/hay diet had lower acetate levels, higher propionate levels and a higher abundance of bacteria in the Bacteroidetes and Proteobacteria phyla, like Porphyromonadaceae, Veillonellaceae, Oxalobacteraceae and Succinivibrionaceae. Horses fed the hay diet had a higher abundance of several taxa in the Firmicutes phylum, such as Catabacteriaceae, Clostridiaceae, Lachnospiraceae and Ruminococcaceae. They also discovered that subjects fed the hay diet had a significantly more diverse microbiome when compared to subjects fed the oats/hay diet. These studies show that diet has a large impact on the horse's hindgut microbial community and, therefore, horse owners should carefully monitor their feeding practices.

Gut bacteria and diet are known to have a large role in laminitis. Bacteria in the hindgut are responsible for breaking down undigested sugar and starch. When

there is a sudden increase in dietary starch, it can cause an excess of lactic acid bacteria in the hindgut. This can lead to lactate accumulation, gut acidity and the release of bacterial toxins into the bloodstream, which can trigger systemic inflammation. When comparing 10 healthy horses and 8 chronic laminitic horses using 454 pyrosequencing, chronic laminitic horses were found to have higher Chao1 bacterial diversity than control healthy horses [28]. They also found that the most abundant phylum in both groups was Firmicutes followed by Verrucomicrobia. They did find a significantly higher abundance of the Clostridiaceae family and two Clostridiales OTUs in healthy horses, however, when using Jacknifed weighted and unweighted UniFrac distance measures, they did not find any significant differences in the two groups' communities as a whole. This finding may suggest that there are not drastic differences between the gut microbiomes of healthy and chronic laminitic horses, however, more studies should be conducted using different and a higher number of subjects.

In order to replicate the events that occur during starch-induced laminitis, researchers created an *in vitro* laminitis model enriched with starch and/or lactate [29]. They used microcosms made up of fecal samples from three adult horses to determine the community and short chain fatty acid composition differences during this rapid increase in starch or starch/lactate. They found that following starch induction, there was an increase in Streptococcaceae and a decrease in Ruminococcaceae and Lachnospiraceae. Then, they detected an increase in Lactobacillaceae when the Streptococcaceae levels decreased. They also found that a taxa closely related to *Megasphaera elsdenii* was high in abundance in starch and starch/lactate cultures in which lactate levels increased and then decreased, but was low in abundance in cultures in which lactate stayed at an increased level. Therefore, they hypothesized

that *Megasphaera elsdenii* may have a role in the reduction of lactate. They also found that a taxa closely related to *Veillonella montpellierensis* was high in abundance in control and lactate cultures, but was not in starch and starch/lactate cultures. Another major conclusion from this project was that the Veillonellaceae family was highly abundant in the starch-enriched model and that these bacteria had the ability to decrease lactate levels. This study shows the changes that occur during the induction of laminitis and gives us insight into possible new treatment options.

Effects of Exercise on the Horse and Their Microbiome

The effects of exercise on the horse's gut microbial community have recently been analyzed [17]. Before this study in 2016, there were no others using next generation sequencing to demonstrate how exercise affects their gut. They used 16S rRNA gene sequencing to characterize the fecal microbiomes of eight horses acting as their exercise trained subjects and four control horses over a three month period. They found significant differences in the abundances of the Bacteroidetes, Proteobacteria and Spirochaetes phyla in the exercise trained group over the three months while there were no significant differences found in the control group throughout the study period. At the genus level, *Dysgonomonas spp.* and *Treponema spp.* showed significant changes in abundance during the training period. Their main conclusion was that exercise greatly affects the horse's gut microbiota, especially when training initially begins. This study shows again that how you manage a horse can have drastic effects on their gut and, in turn, their body as a whole.

There was another recent study that used nine fillies to determine the effects of acute exercise and aerobic conditioning on the gut microbiome [18]. Within this treatment group, there were three horses supplemented with L-carnitine and another three supplemented with chromium. After acute exercise, they noticed a significant

decrease in the phylum Chlamydiae and in the genus *Mycobacterium*. They also observed a decrease in plasma pH and a significant increase in lactate before and after fatigue. Using a PCoA plot, they visualized a clustering of samples collected before and after the exercise period of 42 days. This analysis suggested that the subjects adapted to the aerobic conditioning after 42 days of this program and their microbiome stabilized. It is also important to note that significant changes were observed in the microbiomes of the control group during the study period. These changes was suggested to be due to the fillies assigned to the control group were ones that already had an altered microbiota or because of an adjustment in their diet during the study period. It may be helpful to conduct future studies on the effects of exercise on the horse's gut microbiome using an increased number of subjects as well as different horse breeds.

Management Practice Effects on the Foal Gut Microbiome

In the beginning of the horse's life, foals can be much more susceptible to disease and gut dysbiosis than a healthy adult horse. This is why management practices during this time are so imperative in order to raise a healthy foal. The gut plays a major role for the foal in that it helps in attaining its nutrient requirements and fighting off pathogens. Currently, studies on the effects of management on the foal's gut microbiome have consisted of how weaning method and probiotic supplementation can affect their gut microbial population [19, 30].

A group of researchers [19] were interested in determining the effect of different weaning methods on the foal gut microbiome. They divided 9 foals into two different treatment groups, which were abrupt (n=5) and gradual (n=4) weaning. They then collected fecal samples from these foals on the day before weaning, the day of weaning and on days 1, 2, 3, 4 and 7-post weaning. They were also interested in

attempting to characterize the stress of weaning on these foals. They collected blood samples to measure cortisol levels, including on the day before weaning, the day of weaning and days 1, 2 and 4-post weaning. They also recorded each foal's heart rate on the day of weaning at 10-minute intervals starting one hour before weaning up until two hours post-weaning as well as for one hour starting at 24 hours post weaning. When analyzing fecal microbial data, they found no differences in species diversity or community membership between gradual and abrupt weaning samples or between before and after weaning samples. They did, however, see significantly increased cortisol levels in the abrupt weaning group on day 1 post weaning and increased heart rate for 50 minutes after weaning on the day of weaning ($p<0.05$). The researchers hypothesized that the foal's fecal microbiota matures fully prior to weaning and, therefore, weaning was not able to cause significant changes in its composition and diversity. They based this hypothesis on a previous study they had conducted in which they found that the foal's microbiome was not significantly different than their dams beginning at 1 month of age [19].

There was also a recent study on the effects of diet supplementation on the foal's fecal microbiome investigating probiotic administration [30]. They also investigated the effects of a *K. fragilis* and *S.cerevisiae* probiotic on the incidence of foal heat diarrhea and used twenty-four newborn foals to do this. The most abundant phyla were Firmicutes and Bacteroidetes and noticed the trend that as the foals aged, their bacterial community structure and diversity became more similar to their dams. They also observed that the probiotic did not significantly affect diarrhea severity but did tend to decrease diarrhea incidence. This study was only published as an abstract, so no details were given on the taxa present in samples.

The Feral Horse Gut Microbiome

Currently, there are only two studies that have reported on the feral horse gut microbiome. In both of these studies, the horse's diets consisted of only naturally found forage. In the first study, researchers evaluated the stomach microbiome of ten feral Australian Brumby horses [3]. They were able to sample the stomach microbiome using a government-accredited culling program and by collecting their stomach contents. Using 16S rRNA genome sequence amplification and construction of a 16S rRNA gene clone library, 26 OTUs were identified with 23 of these OTUs belonging to the Firmicutes phylum and the remaining 3 OTUs belonging to the Proteobacteria phylum. This is a low number of OTUs found when compared to horse studies using fecal samples to represent the gut microbiomes in which tens of thousands of OTUs are usually observed. All of the Firmicutes OTUs were composed of bacteria in the Lactobacillaceae, Streptococcaceae and Veillonellaceae families. The researchers did not find any bacteria from the Bacteroidetes phylum in the feral horses' stomachs, which is a phylum that has been associated with xylan digestion. They also discovered that 49.7% of the clones found in the feral horses were related to equine hindgut bacterial species and 44.5% were related to horse (and other mammals) mouth or throat bacterial species. They noted that the absence of fibrolytic groups in the Brumby horse stomach supports the common understanding that the equine stomach is primarily responsible for regulating the passage of food into the hindgut rather than for plant fiber digestion. A functional analysis of this data using a program such as PICRUSt would enable a deeper understanding of the roles of the bacteria found in the Brumby horse stomach.

In the more recent feral horse study, the researchers aimed to determine how domestication affects the horse gut microbiome by sampling from 44 Przewalski's

horses and 28 domestic horses living in adjacent grasslands in Mongolia [20]. Using 16SrRNA amplicon sequencing of fecal-extracted DNA samples, they found that Przewalski's horses have a significantly more diverse fecal microbiome than domesticated horses and that the two groups differed in microbiome composition. They also discovered that the Przewalski's horse group had significantly lower inter-individual variation than the domesticated horse group. The major differences found in taxonomic composition were that feral horses had a higher abundance of the orders Clostridiales, Bacteroidales and Erysipelotrichales while the domestic horses had a higher abundance of Spirochaetales. They also showed that age had a significant effect on the fecal microbiome in the Przewalski's horses. Przewalski's horses less than a year of age had a less diverse and more compositionally distinct microbial community than those older than 1 year old.

Their population of Przewalski's horses was composed of subjects born in three different locations. Through analysis of these different birth locations, horses born in zoos and transferred to the reserve in 2011 (n=4) had a distinct and less diverse microbial community than horses born on the reserve (n=20) or horses transferred from 2004 to 2005 from a reserve in France (n=15). This observation may suggest that a domestic lifestyle can have a lasting impact on a horse's gut microbiome. They were also able to characterize each horse's diet using amplicon sequencing of the P6 loop of the chloroplast *trn*L intron from fecal samples. They found that the plant diversity in the feral and domesticated horse fecal samples was similar but that they differed in plant taxa composition, which shows that the two groups chose different plants to meet their dietary needs.

The major findings from this study prove that there is a difference in the gut microbiome diversity and composition between these particular feral and domesticated

horse populations. Studies using different feral and domestic horses and with more subjects should be conducted to provide a better understanding of the specific differences between their gut microbial communities. This study also only contained one-time samples from five Przewalski's foals, so there is a need for more in-depth research into how a conventional domestic lifestyle affects the foal in the early stages of their life.

Development of the Human Gut Microbiome

Research on the human gut microbiome can be more advanced than that on the animal gut microbiome, so these studies can provide guidance for future research on the animal's gut. The gut microbiome has been a major area of recent interest that researchers use to more deeply understand potential causal aspects of many different diseases and syndromes. Bacterial communities inhabiting the digestive system have been proven to significantly affect not only the gut but also other parts of the human body. Researchers have studied how this gut community can affect the immune response, gastrointestinal tract, endocrine system, behavior, overall health and even cognitive function in both humans and animals [31, 32, 33, 34, 35, 36]. Many studies have also specifically focused on how all of these systems contribute to the gut-brain axis [37, 38, 39]. In humans, gut dysbiosis has been linked to many disorders, including obesity, autism spectrum disorders, diabetes, colorectal cancer, inflammatory bowel diseases and other diseases caused by pathogenic bacteria [40, 41, 42, 43].

The early development of the gut microbiome has been proven to be an essential part in maintaining a healthy neonate as well [44, 45]. Therefore, maintaining an appropriate gastrointestinal microbial environment is imperative for host health,

especially at the neonatal stage when the individual is more susceptible to disease. The development of the human neonatal gut microbiome has been of interest to microbial researchers. By studying the fetal meconium, they found that colonization of the gut begins even before delivery [46]. They also determined that the neonate's microbiome in their first week of life is majorly composed of the Actinobacteria, Proteobacteria and Bacteroidetes phyla as well as the Firmicutes phylum at a much lower level. Another study followed fourteen healthy full-term infants throughout their first year of life and used rDNA microarray technology to characterize their gut microbiomes [47]. It was found that the phylum level diversity for all samples was mainly composed of the *Flexibacter-Cytophaga-Bacteroides* phylum, Proteobacteria phylum and Firmicutes and Actinobacteria phylum. They also noticed that genera such as *Staphylococcus, Streptococcus* and *Enterobacteria* appeared earlier in the infant's life whereas *Eubacteria* and *Clostridium* appeared later. There was another study that analyzed the effects of weaning infants from breast milk or formula and introducing solid foods on the neonatal gut microbiome [47]. They found that this transition caused an increased abundance of the genera *Bacteroides, Clostridium* and *Streptococcus* as well as a decreased abundance of the genus *Bifidobacterium*. It has also been discovered that the infant's microbiome goes through a rapid change in their first year of life and then stabilizes to that of an adult by 3 years of age [44].

The human neonatal gut microbiome is very susceptible to dysbiosis in both healthy full-term infants and, even more so, in pre-term infants. This susceptibility to external factors due to their underdeveloped immune system can affect the neonate both short- and long-term [44]. Necrotizing entercolitis is a significant issue in neonatal intensive care units and is believed to be caused by a general disturbance of the gut's normal colonization instead of the overgrowth of a single pathogenic type of

bacteria [48]. There have been conflicted findings on the differences in taxa between healthy and necrotizing enterocolitis affected infants. One study recorded an increase in the Proteobacteria phylum and a decrease in the Firmicutes phylum during necrotizing enterocolitis [49]. Another study found no differences in the microbiota between necrotizing enterocolitis affected and healthy infants [50]. Therefore, there are not always obvious changes in the gut microbiome during gastrointestinal disease or dysbiosis.

The Development of the Horse Gut Microbiome

Research in the area of the foal gut microbiome has recently consisted of short-term studies on how factors like diarrhea, *R. equi* pneumonia, weaning and probiotic supplementation can affect the foal's gut microbial community. Those on the more general early development of the foal gut and its microbiome with frequent sampling are somewhat limited. In addition, there have been few studies using next generation sequencing to characterize the gut microbiome.

Long-term studies on the development of the foal's gut microbiome and those with relatively large sample sizes are limited. Most researchers in this area have taken a more fragmented sampling approach to analyze events such as when the foal's microbiome stabilizes to that of an adult and how weaning affects their microbiome. A study conducted by Jacquay et al. used 9 mare-foal pairs to understand the development of the foal's microbiome and the similarity to their dams' microbiome and milk composition [19]. They collected samples from mare milk, mare feces and foal feces when the foal was 0, 2 and 7 days old as well as 1, 2, 3 and 4 months old. Amplification of the V4 region of the 16S rRNA gene was completed and samples were sequenced using Illumina Miseq. After analyzing the microbes present in each of

the different types of samples, they found that newborn foal meconium was highly similar in species diversity and composition to mare milk, consisting mainly of the genera *Enterococcus, Bacillus, Pseudomonas and Lactococcus*. They also determined that the foal's gut microbial community was not significantly different to their dam's at the phylum level beginning at 1 month old.

Another study took a more longitudinal approach and sampled the feces of 11 mare-foal pairs. In order to characterize the foal gut microbiome, they collected samples on day 1 (n=9) and then grouped the remaining samples collected into increased ranges of days 2-30 (n=12), 31-60 (n=8), 61-120 (n=6), 121-180 (n=21), 181-240 (n=8) and 241-270 (n=7) [51]. They then extracted DNA from the samples, amplified the V4 region of the 16S rRNA gene and sequenced using Illumina Miseq. It is also important to note that five samples from five different foals were treated with antibiotics during the study and these samples were still used in the microbiome analysis. This could be an issue because antimicrobial administration has been shown to cause significant changes in the horse's gut microbiome [52]. From their sequences, they were able to classify 29 different phyla with a median of 1,519 observed OTUs per sample.

Some of their major findings on significant differences in taxa when comparing foals to mares included that newborn foals had a higher abundance of Acidobacteria than adult mares, foals aged 121-180 days and 181-240 days had a higher abundance of Fibrobacteres and Spirochaetes than mares and mares had a lower abundance of Chlamydiae than foals aged 31-60 days. They also found that 40% of the reads taken from foals aged 2-30 days were from the genus *Akkermansia*. These researchers found that the foal's microbiome structure and membership tended to remain stable at 60 days of age until 9 months of age. From 60 days old to 9 months

old, their microbiome was most similar to their dams', but there were still differences in community membership.

The Foal Immune System and Gastrointestinal Diseases

Gut dysbiosis in the foal can be very detrimental because of the foal's vulnerability to pathogens at this age. The foal has innate immunity at birth, but several adaptive immune responses can take up to a year to develop to that of an adult horse. The correct development of the foal's immune system is very important in protecting from microbial pathogens as well as gastrointestinal disease [53].

The horse has epitheliochorial placentation, which prevents the transfer of immunoglobulins from the mare to the fetus in utero. Therefore, ingestion of colostrum by the foal is imperative before the foal is no longer able to absorb the immunoglobulins, maternal immune cells and cytokines and before the colostrum transitions to milk. Ingestion of colostrum should occur approximately 12-24 hours after parturition, so this time period is a very important one for the successful management of foals [53]. Colostrum is also very nutrient rich and decreases in nutrients throughout lactation. The average nutrient composition of colostrum/milk during the first week of lactation found was 2.07% fat, 2.64% protein, 40,640 somatic cells/mL, 6.15% lactose and 23.16% milk urea nitrogen [54]. It then decreases in nutrient content gradually to 1.65% fat, 1.65% protein, 26,500 somatic cells/mL, 6.81% lactose and 23.88% milk urea nitrogen by the twelfth week of lactation.

Some innate immune responses are thought to be as functional as an adult horse at birth; however, several adaptive immune responses can take a year to fully develop. At birth, the foal already has the ability to start adaptive immune responses involving the production of IgG1, IgG3, IgG5 and IgA antibodies and these responses

reach the level of an adult horse at around 3 months of age [53]. Immune responses involving antibodies like IgG4, IgG7 and IgE can take the foal's entire first year of life to develop to the level of an adult horse. This slower type of development is also true for the production of interferon-gamma by Th1 (T helper 1) cells and cytotoxic T cells because this type of response also can take a year to fully develop. Another important immune response component is the production of IL-4 (interleukin-4) by Th2 (T helper 2) cells and this type of response has been found to be undetectable in the foal's first 3 months of life. Therefore, in the first 3 months and even the first year of the foal's life, they are highly susceptible to disease.

Gut dysbiosis is a common occurrence in the foal's life and it has been found that diarrhea affects up to 60% of foals in their first 6 months [55]. This type of diarrhea, also referred to as foal heat diarrhea, is a transient, non-infectious type. This condition is mild and usually does not require any veterinary treatment such as fluid administration or antibiotic treatment. However, during foal diarrhea, the foal can experience discomfort on a minimal level, including a slight electrolyte imbalance, dehydration and lethargy [56]. In rare cases, the foal's immune system can be compromised and their mild diarrhea can turn into a more life-threatening infection.

This type of transient foal diarrhea is also known as foal heat diarrhea because it has been connected to the dam undergoing her first estrous cycle after parturition. Its cause has yet to be definitively determined, however, it is not thought that their dam's hormonal changes are a factor. Researchers studied the occurrence of diarrhea in the foal in accordance with their dam's estrus cycle and found that the mare's first postpartum estrus had no impact on the onset or duration of foal diarrhea [57]. More widely accepted causes of foal heat diarrhea are dysbiosis in the foal's gut microbial

community as well as changes in diet such as increased access to forage, access to grain and coprophagy [58].

Microbial changes in the gut during foal diarrhea, especially using next generation sequencing, have yet to be extensively researched. Most studies in this area have used real-time PCR and culture methods to isolate specific hypothesized infectious agents, including viruses, bacteria and bacterial toxins [59, 60, 61]. In the study conducted by Slovis et al., they analyzed the fecal samples of 88 Thoroughbred foals. They found that the prevalence of any tested infectious agents in the gastrointestinal-diseased group was 63.2% while the prevalence in the healthy group was 43.2%. They also found that coinfections were significantly more frequent in diseased foals and that equine coronavirus, *Clostridium difficile* toxins A and B, *Neorickettsia risticii, Clostridium perfringens* alpha toxin, *Lawsonia intracellularis, Cryptosporidium* spp. and *Salmonella* spp. were prevalent in the diseased foals.

Another study using PCR looked specifically at foals with diarrhea [60]. They examined fecal samples of 233 foals for *Salmonella* spp., viruses, *Clostridium difficile* toxins, *Clostridium perfringens* and its enterotoxin, *Cryptosporidium* spp. and metazoan parasites. In 122 of the foals, at least one infectious agent was found and Rotavirus (55%) was the most frequently detected followed by *C. perfringens* (18%) and *Salmonella* spp. (12%). They also noted that the type of infectious agent found did not affect the survival of the foal. Using next generation sequencing for these types of studies would allow the researchers to characterize each foal's gut microbiome during GI-disease as a whole instead of only testing for specific pathogens and their toxins.

A recent study characterized the foal's gut microbiome during foal diarrhea using high throughput sequencing [55]. They sampled from nine foals with diarrhea and eleven healthy foals at two different time points of 1-14 and 15-28 days old. They

found that non-diarrheic foals during the 15-28 day age range had a significantly higher Chao richness index when compared to healthy foals during the 1-14 day age range and to the diarrheic foals. They also identified 117 enriched taxa in healthy foals at the 15-28 day age range, including many from the Lachnospiraceae and Ruminococcaceae families. Their major finding was that the effect of diarrhea on the foal's gut microbiome was inconsistent while age sampled had more of a consistent effect. Therefore, there is a need for more studies on foal diarrhea using next generation sequencing.

Next Generation Sequencing

Many equine studies on the gut microbiome have used culture-based procedures to characterize the bacteria present. However, it is known that a significant amount of organisms present in the gut are unculturable using standard culture methods. Culture techniques can be helpful when trying to identify specific bacteria that cause disease or when trying to briefly analyze the microbiome as a whole. There are still many challenges in using culturing to analyze the microbiome because it can provide researchers with an inaccurate depiction of the microbial community. Therefore, the emergence of next generation sequencing techniques have been helpful in achieving a deeper understanding of the gut microbiome in horses as well as other animals.

16S rRNA gene sequencing is based on non-enriched PCR products and allows for a more reliable analysis of the microbiome. The 16S rRNA gene sequences are used to study bacteria because of its presence in virtually all bacteria, its function has been preserved over time and its size of 1,500 base pairs makes it large enough for informatics/analytics purposes [62]. This type of next generation sequencing is very

helpful in characterizing a microbial community in both its diversity and member abundance. In most cases, 16S rRNA gene sequencing is able to provide genus level identification (over 90%) and, in some cases, species level identification (65-83%) [62].

Programs such as QIIME (Quantitative Insights Into Microbial Ecology) allow researchers to process their sequence data and analyze the microbial composition. It includes many different software tools like FLASh (Fast Length Adjustment of SHort reads), FastQC, UCLUST, PyNAST and FastTree [63, 64, 65, 66, 67, 68]. In the current studies, a specific workflow was used to extract DNA from samples, sequence the DNA, process the sequence data and analyze the microbial data (Figure 1).

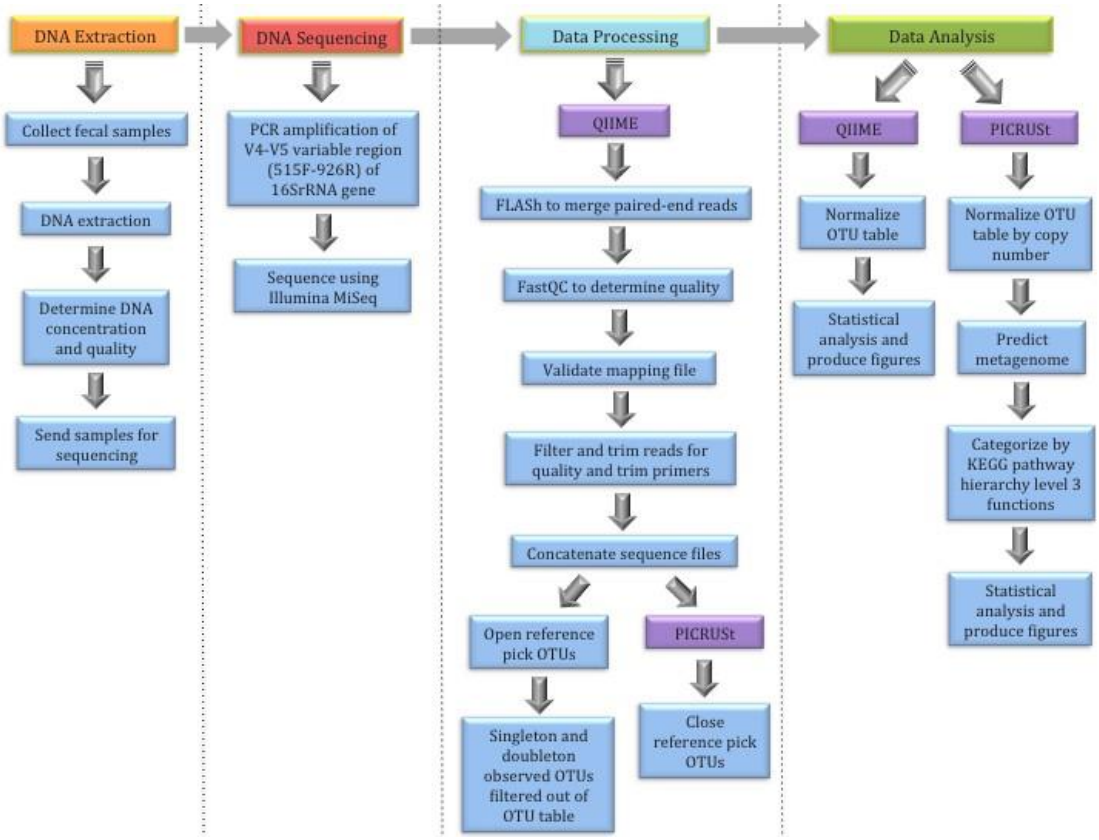

Figure 1. Flow chart of work flow used in both studies to extract, sequence and analyze gut bacterial community in horses.

For sample processing, a fecal DNA isolation kit was used to extract DNA from samples, the DNA concentration was determined fluorometrically, the quality of samples were determine using a spectrophometer and ethanol precipitation was used for low quality or quantity samples. Then, the microbial community present in the samples was determined using amplification of the V4-V5 variable region of the 16S rRNA gene as well as sequencing using Illumina MiSeq. Data received from sequencing was processed using QIIME and the included programs, which involved merging paired-end reads, determining the quality of reads, filtering sequence reads,

trimming sequences for quality and to remove primers, open-reference picking OTUs, removing singleton and doubleton OTUs from the OTU table and normalizing the OTU table using CSS (Cumulative Sum Scaling). The online Galaxy version 1.1.1 of a program called PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was also used in both studies in order to predict the function of the microbiota [69].

It can be difficult to assign individual bacteria to specific functions manually, so programs like PICRUSt can be convenient for researchers to determine the function of their microbial community. Functional tools like this are also helpful when transcriptomic data is not available. However, there are still some limitations in predicting the function using extracted DNA rather than RNA. Developers of PICRUSt found that there was high overall accuracy in assigning bacteria to specific functions, but there may be some incorrect predictions in gene families or pathways that have highly variable distribution throughout the tree of life. They also noted that this program could be improved if the habitat information of the community being analyzed was provided. PICRUSt is only able to predict the functions of bacteria and archaea, so the functions of viruses and eukaryotes in the metagenome cannot be predicted computationally. Even with these limitations, PICRUSt is still a useful tool for researchers when they do not have the resources to collect transcriptomic data or to add a layer of functional understanding to their genomic study.

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Chapter 2

ASSESSMENT OF EARLY FUNCTIONAL AND COMMUNITY DEVELOPMENT OF THE EQUINE HINDGUT MICROBIOME IN SEMI-FERAL- AND DOMESTIC CONVENTIONALLY-MANAGED FOALS

Abstract

The early development of the gut microbiome has been proven to be an essential part in maintaining a healthy neonate in both humans and animals. In the current study, ten domestic conventionally managed (DCM) Standardbred and ten semi-feral managed (SFM) Shetland-type pony foals and dams were studied for analysis of the early development of their hindgut microbiomes to determine changes as the foals aged as well as the effects of different management techniques. Rectal swab microbial communities were determined using next generation sequencing and a total of 25 different phyla were found with the most abundant phylum present being Firmicutes followed by Bacteroidetes in both foals and dams. Dams were found to have a significantly higher mean diversity than foals (PD whole tree, nonparametric t-test, $p<0.001$). When comparing foals by week of age, week 1 foals had a significantly lower mean diversity than week 2, week 3, week 4, week 5 and week 6 foals (PD whole tree, nonparametric t-test, $p<0.01$).

Significant differences were also found between semi-feral managed and domestic conventionally managed foals (ANOSIM, $p<0.01$, PERMANOVA, $p<0.05$, $n_{samples}=116$, $n_{groups}=2$) as well as well as between different foal ages (ANOSIM,

PERMANOVA, $p<0.001$, $n_{samples}=116$, $n_{groups}=6$), showing that management type and age have notable effects on the horse at an early stage in their life. *Lactobacillus spp.* and Lactobacillaceae gen., bacteria associated with lactic acid production and starch-induced laminitis in adult horses, were found to be enriched only in DCM foals, specifically during their second and third week of life (Kruskal-Wallis, LDA score>2.0, $p<0.05$). The predicted function of the microbiome was also estimated computationally and SFM foals were found to have a significantly higher mean sequence count in the OTUs contributing to lipid, general carbohydrate, complex carbohydrate, simple carbohydrate and protein digestion ($p<0.001$, Kruskal-Wallis). DCM foals were also found to have a microbiome more similar to their dams in their fifth and sixth week of life than were SFM foals to their dams in their sixth week of life. This study provides insight into how management can affect the foal's hindgut microbiome as well as gives a detailed description of the function and composition of this microbial community in the foal's first 6 weeks of life.

Introduction

The gut microbiome has been a major topic of interest in recent years because of its influence on many different body systems. Researchers have studied how this gut community can affect the immune response, gastrointestinal tract, endocrine system, behavior, overall health and even cognitive function in both humans and animals [1, 2, 3, 4, 5, 6]. The early development of the gut microbiome has been proven to be an essential part in maintaining a healthy neonate as well [7, 8]. In foals, the development of their microbiome has not yet been extensively researched. This is an important topic to explore because gut dysbiosis during this immunologically sensitive period can cause serious impact on the foal.

There are many different gastrointestinal disorders common in the horse that have been associated with gut dysbiosis, including starch-induced laminitis, colitis, diarrhea and gastric ulcers [9, 10, 11, 12, 13]. These abnormalities have been proven to be correlated with differences in microbial diversity and abundances when compared to healthy horses. In humans, gut dysbiosis has been linked to many disorders, including obesity, autism spectrum disorders, diabetes, colorectal cancer, inflammatory bowel diseases and other diseases caused by pathogenic bacteria [14, 15, 16, 17]. Therefore, maintaining an appropriate gastrointestinal microbial environment is imperative for host health, especially at the neonatal stage when the individual is more susceptible to disease.

The establishment of a stable, adult gut microbiome is important and can implicate the correct development and health status of an individual. Studies specifically focusing on the early development of the equine gut have found that the foal's bacterial community stabilizes to that similar to an adult horse at approximately 1 to 2 months old [18, 19]. More detailed studies on the early development of the foal's microbiome in which sampling is more frequent during their first weeks of life are lacking. Those using 16S rRNA amplification and sequencing to characterize this community are also limited.

Major research in the area of the foal gut microbiota has recently consisted of short-term studies on how factors like diarrhea, *Rhodococcus equi* pneumonia vaccination, weaning and probiotic supplementation can affect their gut community [20, 21, 18]. At this time, there have been few studies using next generation sequencing to characterize the gut microbiome. Most researchers in this area of study have used PCR or culture methods to identify certain pathogenic bacteria or to determine the basic diversity of a foal's microbiome. Next generation sequencing

allows for a deeper understanding of the diversity and exact abundances of bacteria present in the gut and can allow researchers to make more detailed conclusions from their data.

In the horse industry, management practices have been a popular topic of interest. Management practices can have significant effects on the horse's health and behavior. Domesticated horses and ponies are thought to be more prone to major health issues, such as laminitis and gastric ulcers, than feral horses because of the way in which they are managed [22]. Factors such as grazing access, exercise, social interaction and diet have been proven to be contributing factors to a horse's health. One study analyzed the feral fecal microbiome in horses and found that Przewalski's horses had a distinct and more diverse bacterial community when compared to domestic horses living in adjacent grasslands [23]. The major differences found in taxonomic composition were that feral horses had a higher abundance of the orders Clostridiales, Bacteroidales and Erysipelotrichales while the domestic horses had a higher abundance of Spirochaetales. They also showed that age had a significant effect on the fecal microbiome in the Przewalski's horses because horses less than a year of age had a less diverse and more compositionally distinct microbial community than those older than 1 year old. The samples from horses less than 1 year old were one-time samples from five different Przewalski's foals, so there is a need for more in-depth research into how a conventional domestic lifestyle affects the foal in the early stages of their life. This was the major aim of the current study and could provide insight into how management can affect the foal's microbiota during a time in which this bacterial community is still rapidly changing.

Methods

Subjects

In the current study, ten domestic conventionally managed (DCM) Standardbred and ten semi-feral managed (SFM) Shetland-type pony foals and dams were studied. There were seven males and three females in the SFM group of foals and five males and five females in the DCM group of foals. All foals and dams included in this study were healthy at birth with no serious gastrointestinal problems and no administration of antimicrobials, anti-inflammatories or supplemental products such as probiotics or digestion supplements at any stage during sampling. Factors such as gender, housing condition, access to grazing and diarrhea occurrences were also recorded and assessed for all subjects.

The ten DCM foals were born and maintained on Winbak Farm, a Standardbred breeding farm located in Chesapeake City, Maryland. Each DCM foal was born in a stall and was kept with their dam in a stall during their first week of life. The DCM foals and dams then made the transition to a small paddock for approximately eight hours per day until they reached 45 days of age. In most instances, there were two foal-dam pairs per paddock. During the rest of the day, each foal-dam pair were enclosed in a stall. After their first 45 days of life, the foals were permanently located in a large pasture with their dam as well as other foal-dam pairs. These DCM foals had access to their dam's feed (Table 1) all throughout the study period and had access to grass at the beginning of their second week of life.

Table 1. Guaranteed analysis of DCM dam's feed (Winbak Original 14 Custom Cube, McCauley Bros., Versailles, KY), which the foal had access to throughout the study period.

Crude Protein, min	14.0%
Crude Fat, min	3.5%
Crude Fiber, max	12.0%
Calcium, min	1.0%
Calcium, max	1.5%
Phosphorus, min	0.75%
Copper, min	30 ppm
Selenium, min	0.4 ppm
Zinc, min	100 ppm
Vitamin A, min	4000 IU/lb
Vitamin D, min	800 IU/lb
Vitamin E, min	100 IU/lb

The Shetland-type pony foals were born into a semi-feral herd maintained since 1994 at the University of Pennsylvania School of Veterinary Medicine in Kennett Square, Pennsylvania. DNA-based parentage is confirmed for all offspring (Gluck Equine Parentage Testing Laboratory, University of Kentucky, Lexington, KY). The herd consists of 11 harem groups and one bachelor band with a total of 105 animals. The ponies have had no history of laminitis or major gastrointestinal diseases. Handling by humans in the semi-feral herd is limited to required preventative health care (daily observation, annual vaccinations and deworming when necessary) completed by highly skilled technicians experienced with these procedures using positive reinforcement. In addition, each SFM foal received a 30-minute "gentling"

experience of positive reinforcement-based acclimation to human interaction with 21 specific compliance goals including touch all over the body, simulated veterinary examination and routine health care procedures, introduction of a halter, and introduction to leading if time allowed when they were between the age of two and four weeks old. The environment of the semi-feral herd consisted of a 40-acre enclosure with natural forages and water sources as well as natural shelters such as hedges and light forest.

Sampling Protocol

Rectal swab samples were taken from foals once a week until the foal was either 5 or 6 weeks old. All ten SFM foals were sampled until week 6 while six DCM foals were sampled until week 6 and the remaining 4 foals were sampled until week 5 due to the inability to access them for sampling during their sixth week of life. Each dam was sampled once throughout the study period to result in a total of 10 dam samples. Swab samples were collected in triplicate using cotton-tipped swabs inserted approximately 2 to 3 inches into the foal's/dam's rectum and rotated circumferentially twice before retraction. While taking the swab sample, the handler used positive reinforcement of scratching of the foal's/dam's neck, shoulder, or rump. For most sampling, two handlers were necessary (one handler to take the swab sample and the other to restrain the foal/dam).

Each swab tip was cut off from the swab handle with scissors into a plastic bag and stored on ice for no more than an hour and until there was access to a freezer. When back at the lab, each swab tip was placed in a bead tube containing 750 microliters of bead solution. The tubes were then stored in a freezer at -20 degrees C until ready for extraction.

DNA Extraction and Sequencing

Genomic DNA was extracted from each swab sample using MO BIO Laboratories PowerFecal DNA Isolation Kit®. All provided protocol steps in the commercial kit were followed except 50 µL of solution C6 was used during the last step instead of 100 µL and this solution was left to sit for 5 minutes in the spin filter before the final centrifugation. Total DNA concentration in each sample was determined using a Qubit® fluorometer and sample quality was determined using a Nanodrop® spectrophotometer. One sample from each triplicate set with the highest DNA concentration and best absorbance ratio (260/280=1.8) was sequenced. Triplicate sample sets with all low DNA quantity and quality were ethanol precipitated using the following protocol: pool samples and determine volume, add ½ volume of 5M ammonium acetate, leave overnight at -20°C, add 2.5 volumes of 100% cold ethanol, centrifuge for 30 minutes at 15,000 rpm, remove supernatant, wash with 100µL of 70% cold ethanol, centrifuge for 15 minutes at 15,000 rpm, remove supernatant, air dry in a laminar flow hood and re-suspend in elution buffer. Microbial communities were determined using amplification of the V4-V5 variable region of the 16S rRNA gene (515yF GTGYCAGCMGCCGCGGTAA- 926pfR CCGYCAATTYMTTTRAGTTT) and sequenced using Illumina MiSeq (RTL Genomics, Lubbock, TX).

Bioinformatics Analysis

QIIME (Quantitative Insights Into Microbial Ecology) was used for microbial data processing and statistical analysis [24]. FLASh (Fast Length Adjustment of SHort reads) was used at its default parameters to merge paired-end reads generated from sequencing and FastQC was used to determine the quality of reads [25, 26]. A mapping file was then created and sequence reads were filtered and trimmed for

quality and to remove primers.

All sequence files were then concatenated into one file and OTUs were picked. OTUs were open reference picked with UCLUST as the picking method for reference and de novo steps against the Greengenes version 13_8 database [27, 28]. OTUs observed only once or twice were filtered out of the OTU table and the OTU table was normalized using CSS (cumulative sum scaling). A core set of QIIME diversity analyses were run on the samples, which produced alpha diversity (PD whole tree, Chao richness index, nonparametric t-test), alpha rarefaction (PD whole tree, Chao richness index), beta diversity (ANOSIM, PERMANOVA) and group significance (Kruskal-Wallis) results. Enriched taxa in different study groups were also analyzed using LEfSe (Linear Discriminant Analysis Effect Size) [29].

RStudio was used for data visualization and analysis [30]. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to analyze the functional components of each sample's gut bacterial community [31]. OTUs were closed reference picked against the Greengenes version 13_5 database in QIIME to use for PICRUSt analysis [28]. The Galaxy Version 1.1.1 of PICRUSt was used with the following workflow: the 'Normalize by Copy Number' command was run to correct the OTU table for multiple 16S copy number, the 'Predict Metagenome' command was run on the normalized OTU table to obtain metagenome predictions and the 'Categorize by Function' command was run on the 'Predict Metagenome' output to obtain specific KEGG functions at pathway hierarchy level 3, the most specific level.

Effect of Breed on Horse's Hindgut Microbiome

As part of the current study, an analysis of breed effects on the horses gut microbiome was conducted in order to justify the comparison of Standardbred foals

and dams to Shetland-type pony foals and dams. When comparing 8 adult ponies to 15 Standardbred adult horses, their microbiomes were found to be significantly different (ANSOIM, PERMANOVA, $p<0.05$). These subjects had a multitude of different diets and were managed in different ways. When analyzing the significantly different taxa at the family level, the Mogibacteraceae family was found to be different in both comparisons of SFM versus DCM dams and Standardbred versus pony adults (Kruskal-Wallis, $p<0.05$). Mogibacteraceae was enriched in SFM dams as well as Standardbred adults.

Results

Microbial Composition Summary

A total of 136 samples were taken from 20 foals ($n_{samples}=116$) and 20 dams ($n_{samples}=20$) and were then analyzed. There was a total of 81,365 observed OTUs from all samples and a total of 3,887,277 sequence counts (mean±s.d= 28,582.92±16,448.23; range= 3,469-69,307; median= 26,783.5). There was a total of 25 different phyla into which the OTUs were classified. The most abundant phylum present was Firmicutes followed by Bacteroidetes in both foals and dams. The average abundance of Firmicutes in foals and dams was 43.82% and 38.3% and the average abundance of Bacteroidetes in foals and dams was 30.4% and 32.38%, respectively.

The core microbiomes for SFM and DCM foals and dams were analyzed for OTUs present in 95% of samples in each group and these OTUs were grouped together based on taxonomy (Table 2). The SFM foals had a more diverse core microbiome than DCM foals and the only shared OTU between SFM and DCM foals

was Bacteroides spp. The SFM and DCM dams had a much higher number of OTUs making up their core microbiomes. The SFM dam core microbiome was also more diverse than the DCM dam core microbiome. Interestingly, *Bacteroides spp.* was not found in either the DCM or SFM dam core microbiomes, which was an OTU shared by both DCM and SFM foals. Most of the SFM foal core microbiome members were not found in either of the dam groups, including *Bacteroides fragilis*, Enterobacteriaceae gen. and Erysipelotrichaceae gen. Rikenellaceaea gen., which was found in the DCM foal core microbiome, was also not found in either of the dam core microbiomes.

Table 2. Core microbiome of SFM and DCM foals and dams consisting of grouped OTUs based on taxonomy present in 95% of samples in each group.

	Semi-feral Managed			**Domestic Conventionally Managed**		
	FOALS	DAMS		FOALS	DAMS	
Shared	*Bacteroides spp.*	Bacteroidales fam.	*Methanocorpusculum spp.*	*Bacteroides spp.*	Bacteroidales fam.	*Methanocorpusculum spp.*
	Fusobacterium spp.	*Mogibacterium spp.*	Lachnospiraceae gen.		*Fusobacterium spp.*	Lachnospiraceae gen.
		Mogibacteriaceae gen.	BS11 gen.		*Mogibacterium spp.*	BS11 gen.
		Clostridiales fam.	RFP12 gen.		Mogibacteriaceae gen.	RFP12 gen.
		Finegoldia spp.	RF16 gen.		Clostridiales fam.	RF16 gen.
		Ruminococcaceae gen.	*Prevotella spp.*		*Finegoldia spp.*	*Prevotella spp.*
		Clostridiaceae gen.	*BF311 spp.*		Ruminococcaceae gen.	*BF311 spp.*
		Streptococcus spp.	*Porphyromonas spp.*		Clostridiaceae gen.	*Porphyromonas spp.*
		Sphaerochaeta spp.	Alphaproteobacteria ord.		*Streptococcus spp.*	Alphaproteobacteria ord.
		Unassigned	*Anaerococcus spp.*		*Sphaerochaeta spp.*	Unassigned
		Arcanobacterium spp.			*Arcanobacterium spp.*	*Anaerococcus spp.*
Unique	*Bacteroides fragilis*	Tremblayales fam.	*Akkermansia spp.*	Rikenellaceae gen.	*Porphyromonas endodontalis*	*Helcococcus spp.*
	Enterobacteriaceae gen.	*Oscillospira spp.*	*RFN20 spp.*			
	Erysipelotrichaceae gen.	*vadinCA11 spp.*	*p-75-a5 spp.*			

Table 2 cont.

		YS2 fam.	*CF231 spp.*			
		Christensenellaceae gen.	*Phascolarctobacterium spp.*			
		RF39 fam.	*Eubacterium spp.*			
		Synergistaceae gen.	Pirellulaceae gen.			
		Paludibacter spp.	*YRC22 spp.*			
		Desulfovibrionaceae gen.	*Suterella spp.*			
		Paraprevotellaceae gen.	*Peptoniphilus spp.*			

Community Statistical Analysis of Foal and Dam Hindgut Microbiome

Foal samples were grouped into six different age groups determined by the foal's age in weeks during the time of sampling. Foals were also grouped by DCM or SFM, gender, access to grazing (access or no access) and where they were housed during the week of sampling (field, stall, or both).

Alpha diversity was analyzed for all different foal and dam groups. Mean diversity between SFM and DCM groups was not significantly different when comparing dams, foals and all foal and dam samples. When comparing foals and dams, dams had a significantly higher mean diversity than foals (PD whole tree, nonparametric t-test, $p<0.001$). When comparing the six different age groups among foals, week 1 foals had a significantly lower mean diversity than week 2 foals, week 3 foals, week 4 foals, week 5 foals and week 6 foals (PD whole tree, nonparametric t-test, $p<0.01$).

Differences amongst foals were statistically analyzed between and within the DCM and SFM groups. Significant differences were found between DCM and SFM foals, age, foals, grazing access and housing as well as within each domestication group between age groups (Table 3). These findings show that, in this specific study group, both age and management type affected the foal's hindgut microbiome. Significant differences were also found between dams and foals and between SFM and DCM when comparing all dam and foal samples. When analyzing dams only, significant differences were found between SFM and DCM dams. This shows that management can affect not only foals, but adult horses as well.

Pairwise comparisons of ages amongst SFM and DCM foals were also analyzed (Table 3). When comparing all ages in the DCM foals, significant differences were found between all ages except for week 2 vs. 3, 3 vs. 4, 3 vs. 5, 3 vs. 6, 4 vs. 5, 4 vs. 6 and 5 vs. 6 foals. When comparing all ages in the SFM foals, significant differences were found between all ages except for week 3 vs. 4, 4 vs. 5, 4 vs. 6 and 5 vs. 6 foals. Because of the higher amount of differences found between ages in DCM foals, this may indicate that the SFM foals had a more consistent microbiome throughout the study period than DCM foals. Significant differences were found between 6-week-old SFM foals and SFM dams as well as between 6-week-old DCM foals and DCM dams. Therefore, it is clear that these foal's gut microbiomes have not yet stabilized to that of an adult at 6 weeks of age.

Table 3. Statistical analysis of different foal and dam groups using ANOSIM and PERMANOVA tests.

	Group Comparison	Number of Groups Compared	Number of Subjects Compared	ANOSIM Significance Level
Foals	SFM vs. DCM	2	116	$p<0.01$
	Individual Foals	20	116	$p<0.001$
	Weeks of Age	6	116	$p<0.001$
	Grazing Access	2	116	$p<0.05$
	Housing	3	116	$p<0.01$
	DCM Age Week 1 vs. 2	2	20	$p<0.05$
	DCM Age Week 1 vs. 3	2	20	$p<0.01$
	DCM Age Week 1 vs. 4	2	20	$p<0.01$
	DCM Age Week 1 vs. 5	2	20	$p<0.01$
	DCM Age Week 1 vs. 6	2	16	$p<0.01$
	DCM Age Week 2 vs. 4	2	20	$p<0.01$
	DCM Age Week 2 vs. 5	2	20	$p<0.01$
	DCM Age Week 2 vs. 6	2	16	$p<0.01$
	SFM Age Week 1 vs. 2	2	20	$p<0.01$
	SFM Age Week 1 vs. 3	2	20	$p<0.01$
	SFM Age Week 1 vs. 4	2	20	$p<0.01$

Table 3 cont.

	SFM Age Week 1 vs. 5	2	20	p<0.01
	SFM Age Week 1 vs. 6	2	20	p<0.01
	SFM Age Week 2 vs. 3	2	20	p<0.05
	SFM Age Week 2 vs. 4	2	20	p<0.05
	SFM Age Week 2 vs. 5	2	20	p<0.01
	SFM Age Week 2 vs. 6	2	20	p<0.01
	SFM Age Week 3 vs. 5	2	20	p<0.05
	SFM Age Week 3 vs. 6	2	20	p<0.01
Dams	SFM vs. DCM Dams	2	20	p<0.01
Foals/Dams	Foals vs. Dams	2	136	p<0.001
	SFM vs. DCM All	2	136	p<0.05

There were also many significantly different OTUs between SFM and DCM foals at different ages as well as SFM and DCM dams. The most highly significant taxa belonging to the Firmicutes and Bacteroidetes phyla were plotted (Figures 2, 3 and 4).

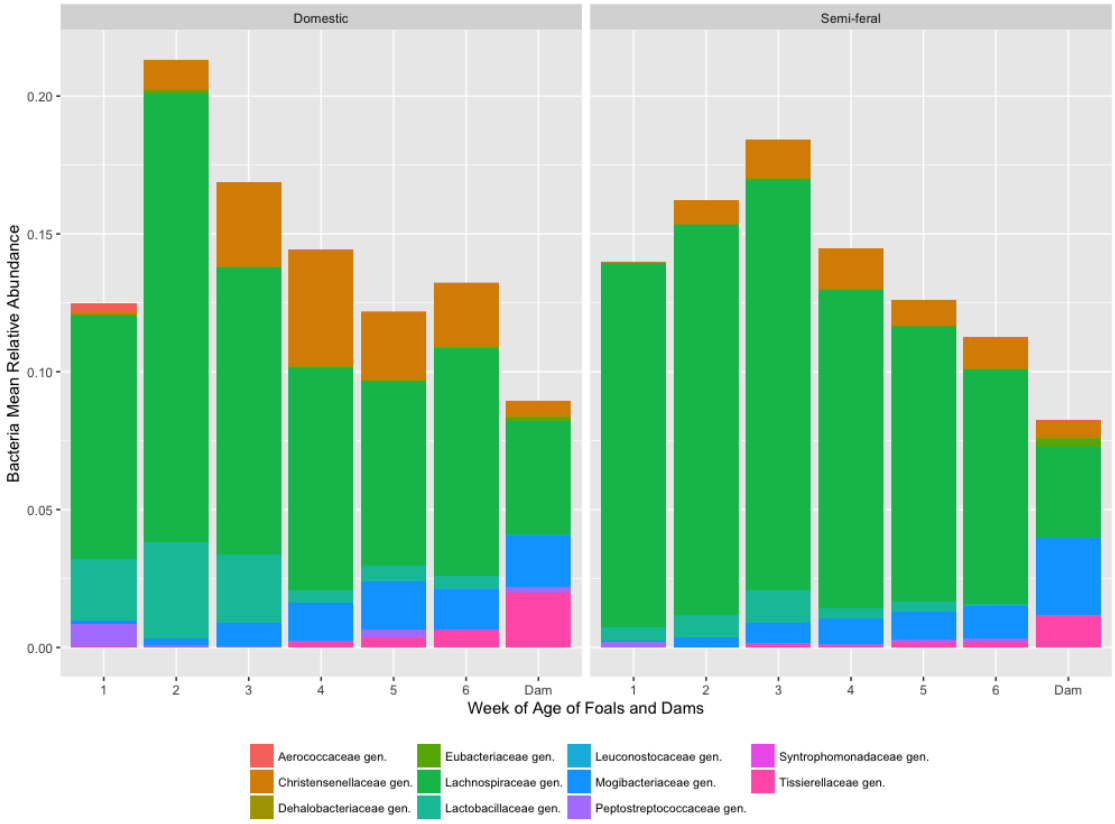

Figure 2. Highly significant ($p<0.01$) Firmicutes at the family level between semi-feral and domestic foals at different age groups as well as semi-feral and domestic dams.

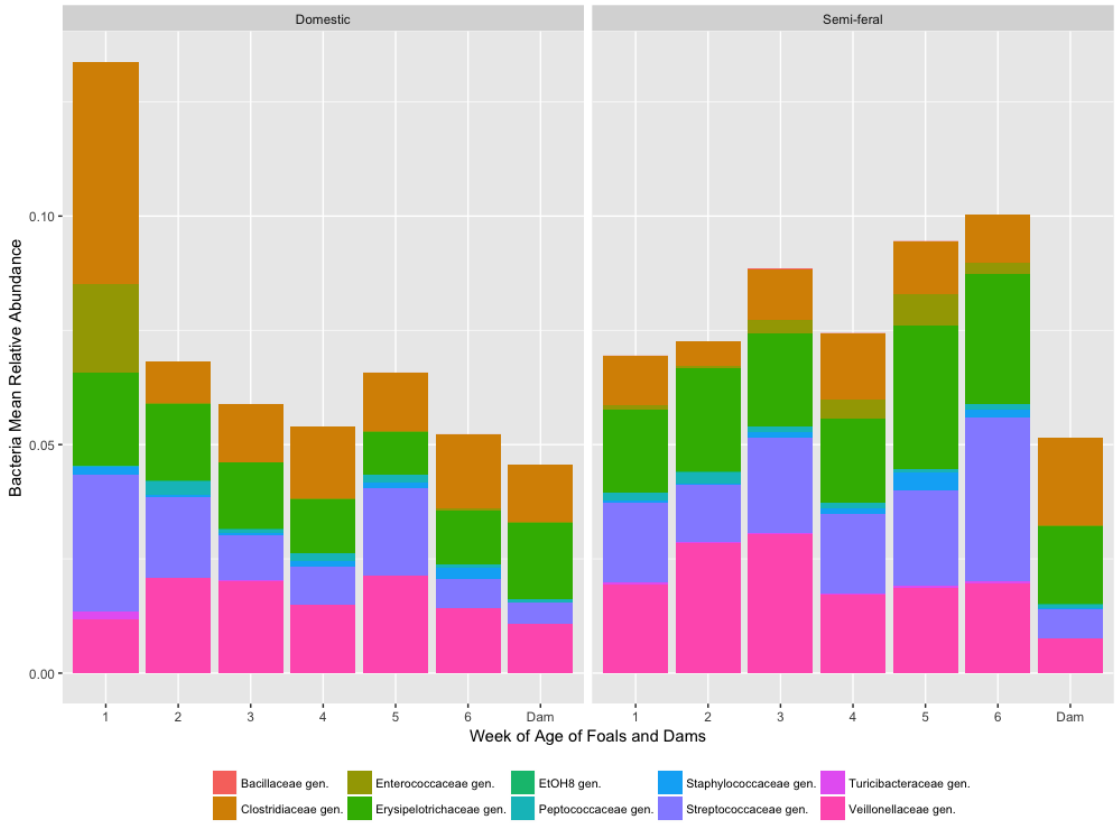

Figure 3. Highly significant ($p<0.01$) Firmicutes at the family level between semi-feral and domestic foals at different age groups as well as semi-feral and domestic dams.

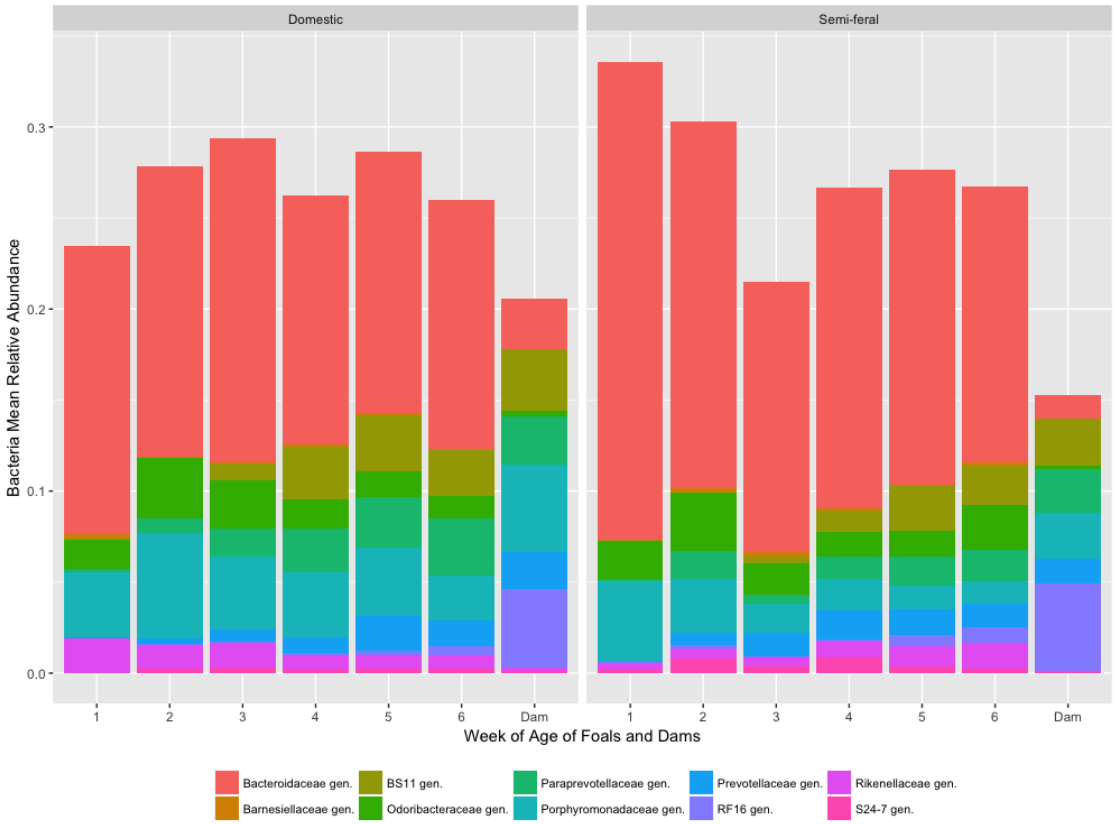

Figure 4. Highly significant ($p<0.01$) Bacteroidetes at the family level between semi-feral and domestic foals at different age groups as well as semi-feral and domestic dams.

When analyzing only semi-feral foals versus domestic foals, Lactobacillaceae gen. was found to be significantly more abundant in DCM foals than in SFM foals and semi-feral and domestic dams (Table 4). This is interesting because it is a family that contains many lactic acid producing bacteria and is more prevalent in starch-induced laminitis [32].

Table 4. Highly significantly different taxa at the family level between SFM and DCM foals (Kruskal-Wallis, $p<0.01$). Taxa are shown in the group in which they were enriched.

Semi-feral Managed Foals	Domestic Conventionally Managed Foals
Erysipelotrichaceae gen.	Aerococcaceae gen.
Chlamydiaceae gen.	Lactobacillaceae gen.
Rhodocyclaceae gen.	Porphyromonadaceae gen.
Pasteurellaceae gen.	Corynebacteriaceae gen.
Anaeroplasmataceae gen.	Pseudomonadaceae gen.
S24-7 gen.	Turicibacteraceae gen.
Alcaligenaceae gen.	Sphingomonadaceae gen.
	Clostridiaceae gen.
	Moraxellaceae gen.
	Victivallaceae gen.
	Eubacteriaceae gen.
	Tissierellaceae gen.

Enriched taxa were also analyzed using LEfSe (Linear Discriminant Analysis Effect Size). DCM and SFM foals were analyzed separately for each of their 6 age groups (Tables 5 and 6). 182 taxa were found to be significantly enriched in the different age groups in DCM foals and 151 taxa were found to be significantly enriched in the different ages in SFM foals ($p<0.05$, Kruskal-Wallis, LDA score>2.0). Week 5 SFM foals and week 4 DCM foals were found to have *Methanobrevibacter spp.* and Methanobacteriaceae gen. enriched in their microbiomes, which are taxa associated with the digestion of complex carbohydrates and methane production. *Fibrobacter spp.* and Fibrobacteraceae gen. are also associated with complex plant carbohydrate digestion and were found to be enriched in week 4 SFM foals. *Lactobacillus spp.* and Lactobacillaceae gen. were found to be enriched in DCM foals aged 2 and 3 weeks, which reinforces this same finding using a Kruskal-Wallis test stated previously.

Table 5. Significantly enriched taxa at the family, genus and species level found in SFM foals from ages 1 to 6 weeks ($p<0.05$, Kruskal-Wallis, LDA score>2.0).

	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
Firmicutes	Peptostreptococcaceae gen. 2	*Holdemania spp.*	*Veillonella dispar*		*Coprobacillus spp. 2*	*Selenomonas noxia*
	Clostridium spp. 1		*Veillonella spp.*			*Mogibacterium spp.*
	Ruminococcus gnavus		Christensenellaceae gen.			Mogibacteriaceae gen.
	Ruminococcus spp.		Lachnospiraceae gen. 2			Mogibacteriaceae gen. 2
Bacteroidetes		*Odoribacter spp.*		S24_7 gen.		*YRC22 spp.*
		CF231 spp.		*Prevotella spp. 2*		Rikenellaceae gen. 2
		Paraprevotellaceae gen.		Prevotellaceae gen.		
				Prevotella spp. 1		
				Prevotella copri		
				Prevotella spp.		
				Paraprevotellaceae gen. 2		
Proteobacteria	Aeromonadaceae gen.	*Desulfovibrio spp. 2*			Methylobacteriaceae gen.	*Campylobacter spp.*
					Helicobacter spp.	Campylobacteraceae gen.
Euryarchaeota			Dehalobacteriaceae gen.		*Methanobrevibacter spp.*	*vadinCA11 spp.*
					Methanobacteriaceae gen.	Methanomassiliicoccaceae gen.

Table 5 cont.

				Methanocorpusculaceae gen.
				Methanocorpusculum spp.
Actinobacteria	Coriobacteriaceae gen. 2			
Fibrobacteres		*Fibrobacter spp.*		
		Fibrobacteraceae gen.		
		Fibrobacter succinogenes		
Spirochaetes			*Treponema spp.*	
Planctomycetes				Pirellulaceae gen.
Chlamydiae				*Chlamydia spp.*
Verrucomicrobia				RFP12 gen.

Table 6. Significantly enriched taxa at the family, genus and species level found in DCM foals from ages 1 to 6 weeks ($p<0.05$, Kruskal-Wallis, LDA score>2.0).

	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
Firmicutes	*Butyricicoccus spp.*	*Lactobacillus reuteri*	*Selenomonas ruminantium*	Mogibacteriaceae gen. 1	Veillonellaceae gen. 2	*Sarcina spp.*
	Butyricicoccus pullicaecorum	Peptococcaeae gen. 2	Selenomonas gen.	*Ruminococcus flavefaciens*	*Peptococcus spp.*	*Peptoniphilus spp.*
	Clostridium perfringens	*Holdemania spp.*	*Lactobacillus spp.*	*Ruminococcus spp.*	*Coprobacillus spp. 2*	*Rummeliibacillus spp.*
	Clostridium spp.	*Anaerotruncus spp.*	*Lactobacillus spp. 1*	*RFN20 spp.*	Mogibacteriaceae gen. 2	*Pseudobutyrivibrio spp.*
	Clostridium spp. 1	*rc4_4 spp.*	*Coprococcus spp. 2*			*Mogibacterium spp.*
	Peptostreptococcaceae gen. 2	Peptococcaceae gen.	*Clostridium spp. 2*			Mogibacteriaceae gen.
	Turicibacter spp.	*Blautia spp. 2*	*Phascolarctobacterium spp.*			Leuconostocaceae gen.
	Enterococcaceae gen. 1	*Roseburia spp. 2*			*Finegoldia spp.*	
	Enterococcus spp. 1		Lachnospiraceae gen.			Tissierellaceae gen.
	Enterococcus spp. 2		Lachnospiraceae gen. 1			
	Enterococcus casseliflavus	Lachnospiraceae gen. 2				
	Enterococcus spp.		*Lactobacillus spp. 2*			
	Enterococcaceae gen.		Lactobacillaceae gen.			
	Vagococcus spp.		*Dorea spp. 2*			
	Blautia spp.					
	Blautia producta					

Table 6 cont.

	Ruminococcus spp. 2					
	Eubacterium spp.					
	Eubacterium dolichum					
	Oscillospira spp.					
Bacteroidetes		*Bacteroides ovatus*	*Butyricimonas spp.*	*5_7N15 spp.*	*Bacteroides plebeius*	RF16 gen.
			Odoribacteraceae gen.	Prevotellaceae gen. 2	CF231 spp.	*YRC22 spp.*
			Porphyromonadaceae gen.	*BF311 spp.*	*Prevotella copri*	*Prevotella spp.*
				Paludibacter spp.	*Prevotella spp.*	Paraprevotellaceae gen.
				Paraprevotellaceae gen. 2	*Prevotella spp. 2*	
					Prevotallaceae gen.	
					BS11 gen.	
Proteobacteria	Enterobacteriaceae gen. 2	*Actinobacillus spp.*				Oxalobacteraceae gen. 1
	Enterobacteriaceae gen. 1	Pasteurellaceae gen.				
	Enterobacteriaceae gen.					
	Erwinia dispersa					
	Erwinia spp.					
	Erwinia spp. 1					
	Erwinia spp. 2					
	Citrobacter spp.					
	Proteus spp.					

Table 6 cont.

	Escherichia spp.				
	Escherichia coli				
	Sphingomonas spp.				
	Morganella spp.				
	Klebsiella spp.				
Actinobacteria	*Eggerthella spp.*		*Actinomyces spp. 2*	Actinomycetaceae gen.	*Corynebacterium spp. 2*
	Eggerthella lenta				Corynebacteriaceae gen.
Verrucomicrobia		*Akkermansia muciniphila*		*Akkermansia spp.*	R4_41B gen.
					RFP12 gen.
Euryarchaeota			Dehalobacteriaceae gen.		Methanimicrococcus spp.
			Methanobrevibacter spp.		Methanosarcinaceae gen.
			Methanobacteriaceae gen.		Methanomassiliicoccaceae gen.
					vadinCA11 spp.
					Methanocorpusculaceae gen.
					Methanocorpusculum spp.
Spirochaetes			*Treponema spp.*	Spirochaetaceae gen.	
Chlamydiae					*Chlamydia spp.*
Planctomycetes				Pirellulaceae gen.	
Synergistetes				Synergistaceae gen. 2	

Table 6 cont.

Cyanobacteria	Synechococcaceae gen.	
		Synechococcus spp.

Using an MDS/PCoA (Multidimensional Scaling/Principal Coordinate Analysis) plot, the Bray-Curtis Dissimilarity between 1-week-old foals, 5 or 6-week-old foals and dams was plotted, which takes into account OTU abundance as well as presence/absence of an OTU (Figures 5 and 6). The domestic dams and their 5/6 weeks old foals were clustered tighter than the semi-feral dams and their 6-week-old foals. This is shown in the higher amount of overlap in the ellipsoids of the domestic dams and their 5/6-week-old foals. It is apparent that as the foals age, their microbiome becomes more similar to that of their dams.

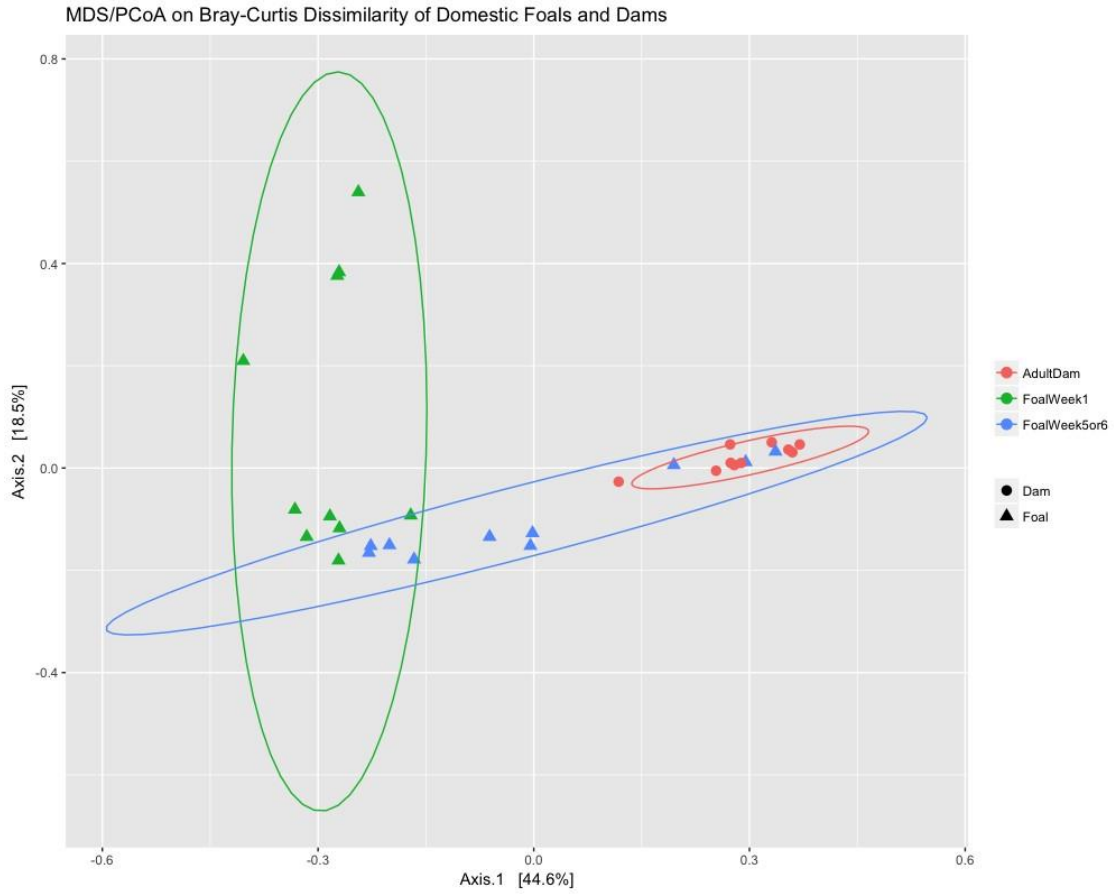

Figure 5. MDS/PCoA plot of the relationship between 1-week-old and 5/6-week-old domestic foals as well as domestic dams using Bray-Curtis Dissimilarity. Ellipsoids representing a 95% confidence interval were used to surround each dam or foal group.

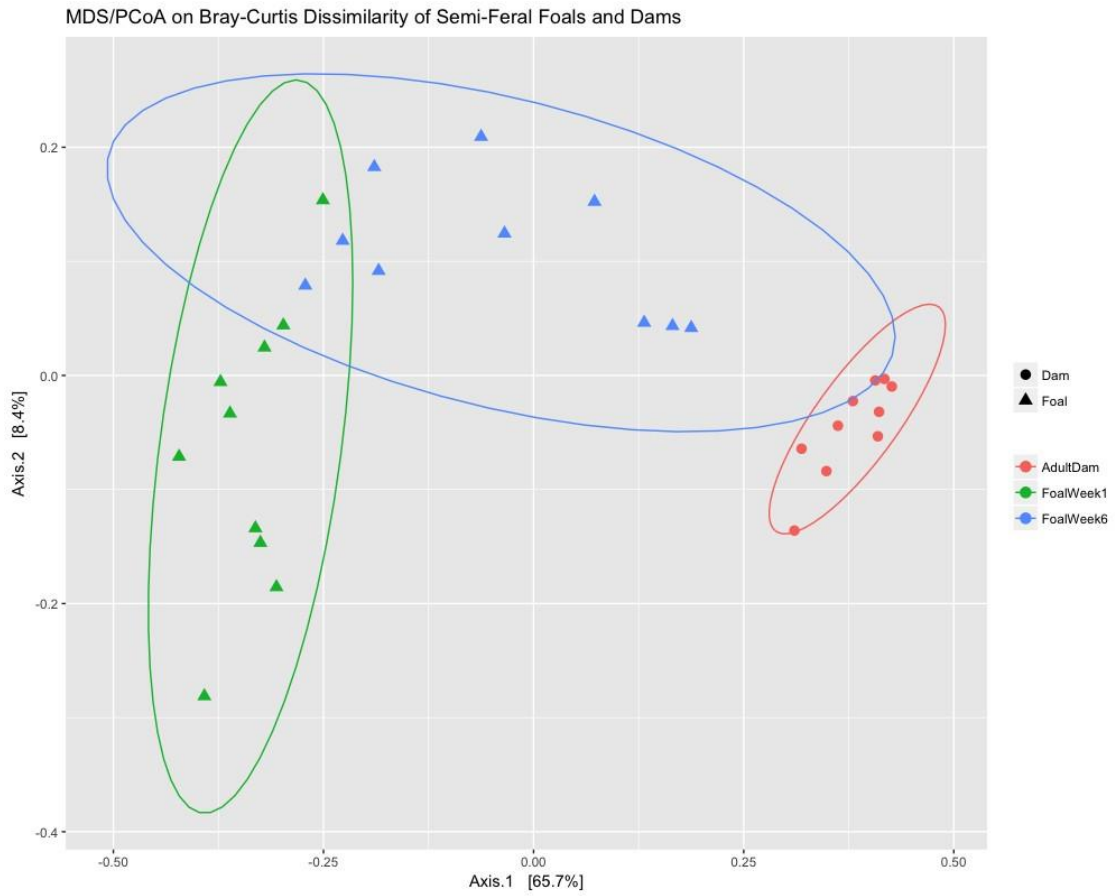

Figure 6. MDS/PCoA plot of the relationship between 1-week-old and 6-week-old semi-feral foals as well as semi-feral dams using Bray-Curtis Dissimilarity. Ellipsoids representing a 95% confidence interval were used to surround each dam or foal group.

Predicted Functional Analysis of Foal and Dam Hindgut Microbiome

For predicted functional analysis, PICRUSt was used and OTUs were categorized into different KEGG functions. Appropriate Level 3 KEGG predictions were then sorted into six different digestion related categories (Table 7).

Table 7. Appropriate KEGG functions categorized into six different types of digestion.

Type of Digestion	**KEGG functions**
General carbohydrate	Carbohydrate digestion and absorption
	Carbohydrate metabolism
Complex carbohydrate	Propanoate metabolism
	Butanoate metabolism
	Glycan biosynthesis and metabolism
	Glycosaminoglycan degradation
	Other glycan degradation
Simple carbohydrate	Fructose and mannose metabolism
	Galactose metabolism
Starch	Starch and sucrose metabolism
Protein	Protein digestion and absorption
	Amino acid metabolism
	Alanine, aspartate and glutamate metabolism
	Glycine, serine and threonine metabolism
	Cysteine and methionine metabolism
	Valine, leucine and isoleucine degradation
	Lysine degradation
	Arginine and proline metabolism
	Histidine metabolism
	Tyrosine metabolism
	Phenylalanine metabolism
	Tryptophan metabolism
Lipid	Glycerolipid metabolism
	Glycerophospholipid metabolism
	Lipid metabolism
	Sphingolipid metabolism
	Ether lipid metabolism
	Fat digestion and absorption
	Fatty acid metabolism

Significant differences were found between the 6 different age groups in both semi-feral and domestic foals in all types of digestion: general carbohydrate, lipid, protein, complex carbohydrate, starch and simple carbohydrate ($p<0.05$, Kruskal-Wallis). Week 1 semi-feral and domestic foals had the greatest amount of general carbohydrate-, lipid-, protein-, complex carbohydrate-, starch- and simple carbohydrate-digesting bacteria when compared to the rest of the age groups, including dams. This finding is most likely due to nutrient-rich colostrum and mare's milk during the foal's first week of life and the gradual decrease in nutrient content as time progressed. As the foals aged, it was apparent that the abundance of the OTUs contributing to each digestion type gradually decreased to reach levels similar to those of their dams (Figure 7). Both SFM and DCM foals at every age group were found to have significantly higher levels in all types of digestion than SFM and DCM dams ($p<0.05$, Kruskal-Wallis). Significant differences were also found between SFM and DCM foals with SFM foals having a significantly higher mean sequence count in the OTUs contributing to each type of digestion ($p<0.001$, Kruskal-Wallis). No significant differences were found in the digestion types between SFM and DCM dams, which may indicate that SFM and DCM adult microbiomes are functionally similar.

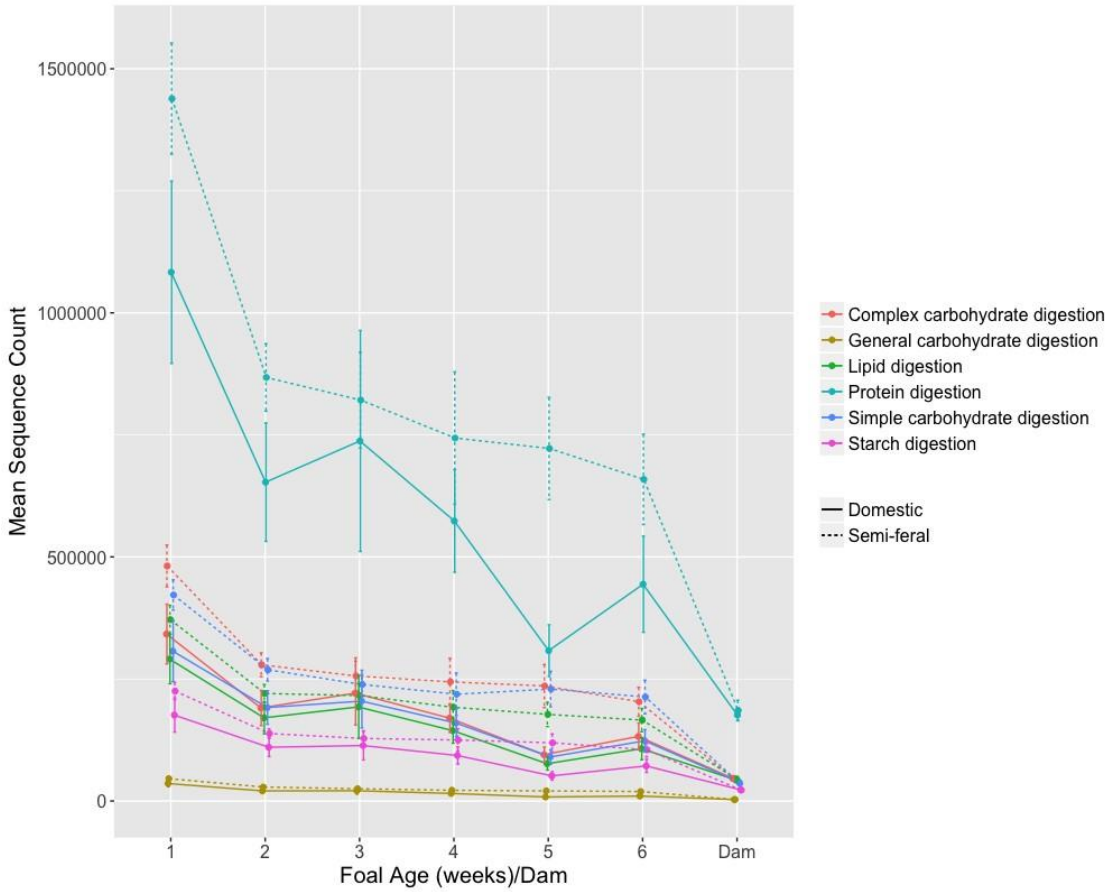

Figure 7. Mean sequence counts of the taxa responsible for the major digestion functions of semi-feral and domestic foals from week 1 to week 6 of life and semi-feral and domestic dams. Standard error lines are also included.

Discussion

In this specific study group, there were significant effects of management type and age on the hindgut microbiome in foals and dams. There were not only significant differences in specific OTUs between SFM and DCM foals but also when their hindgut microbial communities were compared as a whole. Firmicutes was the predominant phylum in both foals and dams, which was a similar trend found in other horse studies [19, 11]. It was also interesting to find that DCM foals in their fifth and sixth week of life had more overlap with their dams than SFM six-week-old foals had to their dams in the PCoA plots. This may indicate that DCM foals possess a microbiome more similar to that of an adult at an earlier age than SFM foals.

In order to definitively determine the stabilization period of the SFM and DCM foal microbiomes, it would be necessary to follow these subjects for a longer period of time. In previous studies, researchers found that domestic conventionally managed foals had a stable, adult-like microbiome at 1 to 2 months old [18, 19]. In the current study, both SFM and DCM foals had a highly significantly different microbiome in their fifth and sixth weeks of life than their dams (ANOSIM, PERMANOVA, $p<0.01$). Week 5 and 6 DCM foals and week 6 SFM foals were found to have significantly higher levels in all types of digestion than their dams as well. Therefore, these foals did not have an adult-like microbiome in regards to both composition and function during this study period but may have established a stable one in the subsequent weeks after sampling had ended.

It can be difficult to assign individual bacteria to specific functions manually, so programs like PICRUSt can be convenient for researchers to determine the function of their microbial community. Functional tools like this are also helpful when

transcriptomic data is not available. After analyzing the major digestion functions of foals, week 1 foals were found to have the greatest amount of general carbohydrate-, lipid-, protein-, complex carbohydrate-, starch- and simple carbohydrate-digesting bacteria. The most abundant type of digestion in foals was protein digestion followed by complex carbohydrate, simple carbohydrate, lipid, starch and general carbohydrate digestion. Their levels of each type of digestion gradually decreased and became more similar to that of their dams as the foal aged. In a study conducted on the nutrient composition of mare milk, milk in their first week of lactation was found to be composed of approximately 2.64% protein, 2.07% fat, 6.15% lactose, 23.16% milk urea nitrogen and a somatic cell count of 40,640 cells/mL [33]. Both fat content and protein decreased in the milk as the lactation weeks progressed, which may explain why both protein digestion and lipid digestion bacterial sequence counts were found to have decreased as the foals aged in the current study.

With the relatively small number of foals and dams in this study (n_{foals}=20, n_{dams}=20), it is difficult to make drastic inferences on how management affects the horse hindgut microbiome. However, in this sampling of foals and mares, there were clear differences between semi-feral managed and domestic conventionally managed subjects. Diet most likely played a major role in the differences seen in their microbiomes because DCM foals had access to their dam's concentrate feed as well as hay while SFM foals only had access to natural forage. DCM foals also had much more limited access to natural forage than SFM foals when they were able to graze in their first few weeks of life. There were major changes in both DCM and SFM foals' microbiomes as they aged as well. The DCM foals had a changing diet throughout the study period. In their first week, they had no access to grazing and then gained access for the remaining weeks for approximately 8 hours per day. These changes in diet may

contribute to the differences found between ages in DCM foals.

Another important aspect of this study was the breed comparison between Standardbreds and ponies in their gut microbiomes. There has been a study that investigated breed differences using fecal samples from 184 horses and they found that there was no significant effect of breed on the gut microbiome [34]. When comparing 8 ponies to 15 Standardbred adult horses, their microbiomes were found to be significantly different. However, when analyzing the significantly different taxa at the family level, the only overlap between the comparisons of SFM versus DCM dams and Standardbred versus pony adults was Mogibacteraceae. This family was enriched in SFM dams as well as Standardbred adults, so the predicted trend that this family would be enriched in the same breeds was not true. This may indicate that differences in breed may not explain the distinct gut microbiomes in SFM and DCM foals and dams, but rather management has a more significant effect. There is, however, still a need for further analysis on breed effects on the horse's gut microbiota.

This study provides insight into how management in the horse industry can affect the horse's microbiome even at an early age. At this immunologically sensitive time in the horse's life, they are more prone to gut dysbiosis. Therefore, characterizing the foal's gut bacterial community is important in order to identify a normal microbiome and to then take the first step in correlating dysbiosis with different conditions or disease states. Since SFM and DCM dams also had distinct microbiomes from one another, it is apparent that management factors such as diet, socialization and housing can affect horses in their adult life as well. Some of the differences in management between the semi-feral- and domestic conventionally-managed subjects were that SFM foals and dams had a higher amount of social interaction and grazing

access than DCM foals and dams as well as these groups having differences in environmental exposure to pathogens and stress levels. Mainly due to the higher starch content commonly found in the domestic horse's diet, there is a higher prevalence of diseases like starch-induced laminitis and gastric ulcers [22]. Horses are adapted to be continuous grazers, which can be difficult to achieve in the domestic setting. Since diet and the microbiome are so interconnected, the gut microbial community and functionality may also be contributing factors to the higher prevalence of gastrointestinal-related disease in domesticated horses.

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Chapter 3

ANALYSIS OF HINDGUT MICROBIOME FUNCTIONAL AND COMMUNITY DIFFERENCES BETWEEN DIARRHEIC AND NON-DIARRHEIC SEMI-FERAL- AND DOMESTIC CONVENTIONALLY-MANAGED FOALS

Abstract

Foal heat diarrhea is a common non-infectious digestive issue that usually occurs in the foal in their first few weeks of life. Currently, no studies have been conducted analyzing foal diarrhea in non-conventionally managed foals. In the present study, seven diarrheic foals and seven age- and domestication management-matched (semi-feral- or domestic conventionally-managed) healthy foals were sampled for analysis of their hindgut microbiome. Rectal swab microbial communities were determined using next generation sequencing and a total of 25 different phyla were found with the most abundant phylum present being Firmicutes followed by Bacteroidetes in both foals and dams.

There were significant differences found between diarrheic and non-diarrheic foals in specific OTUs but not when analyzing their gut communities as a whole. This may suggest that the bacteria found to be significantly different are those contributing to mild diarrhea, but these changes are not drastic enough to cause serious illness in the foal. *Bacteroides fragilis, Prevotella spp., Veillonella spp*., Lachnospiraceae gen. and Clostridiales fam. are some of the taxa found to be significantly higher in diarrheic

foals. Both *Prevotella spp.* and *Veillonella spp.* were found to have an increased abundance in human children with IBS (Irritable Bowel Syndrome) when compared to healthy children [1].

Ruminococcaceae gen., *Bacteroides spp., Butyricimonas spp., Odoribacter spp., Oscillospira spp., Fusobacterium spp.* and *Escherichia coli* were some of the taxa found to be significantly higher in non-diarrheic foals. Higher abundances of Ruminococcaceae gen. and *Oscillospira spp.* have also been found in healthy human children when compared to children with Crohn's disease [2]. Probiotic supplementation of these taxa may be a treatment option for immunologically compromised foals that may not have the ability to efficiently recover from foal diarrhea. The predicted function of the microbiome was also computationally analyzed and non-diarrheic foals were found to have a significantly higher abundance of the OTUs responsible for starch digestion, general carbohydrate digestion, simple carbohydrate digestion, complex carbohydrate digestion and protein digestion when compared to diarrheic foals ($p<0.05$, Kruskal-Wallis). This study provides insight into how the foal is affected at a community and functional level in their hindgut microbiome during foal diarrhea.

Introduction

Foal diarrhea, also referred to as foal heat diarrhea, is a transient, non-infectious type of diarrhea and is very common in the foal during their first few weeks of life. This condition is mild and usually does not require any veterinary treatment such as fluid administration or antibiotic treatment. However, during foal diarrhea, the foal can experience discomfort on a minimal level, including a slight electrolyte

imbalance, dehydration and lethargy [3]. In rare cases, the foal's immune system can be compromised and their mild diarrhea can turn into a more life-threatening infection.

This type of transient foal diarrhea is also known as foal heat diarrhea because it has been connected to the dam undergoing her first estrous cycle after parturition. Its cause has yet to be definitively determined, however, it is not thought that their dam's hormonal changes are a factor. Researchers studied the occurrence of diarrhea in the foal in accordance with their dam's estrus cycle and found that the mare's first postpartum estrus had no impact on the onset or duration of foal diarrhea [4]. More widely accepted causes of foal heat diarrhea are dysbiosis in the foal's gut microbial community as well as changes in diet such as increased access to forage, access to grain and coprophagy [5]. There are many different gastrointestinal disorders common in the adult horse that have been associated with gut dysbiosis, including starch-induced laminitis, colitis, diarrhea and gastric ulcers [6, 7, 8, 9, 10]. These abnormalities have been proven to cause or to be caused by differences in microbial diversity and abundances when compared to healthy horses.

Microbial changes in the gut during foal diarrhea, especially using next generation sequencing, have yet to be extensively researched. Most studies in this area have used real-time PCR and culture methods to isolate specific hypothesized infectious agents, including viruses, bacteria and bacterial toxins [11, 12, 13]. A recent study characterized the foals gut microbiome during foal diarrhea at both 2 and 4 weeks old using high throughput sequencing [14]. They found that foals with diarrhea had a significantly lower richness index when compared to non-diarrheic foals. However, their major findings were that the effect of diarrhea on the foal's gut microbiome was inconsistent while age sampled had more of a consistent effect.

Currently, no studies have been conducted analyzing foal diarrhea in non-conventionally managed foals. In the present study, the aim was to compare foal diarrhea cases in conventionally managed domestic foals and semi-feral managed foals. Using next generation sequencing and bioinformatics tools, we were able to complete an in-depth analysis on their microbiomes and predict the digestion functionality of them.

Methods

Subjects

In the current study, seven diarrheic foals and seven age- and domestication management-matched healthy foals were sampled for analysis of their hindgut microbiome. Eight of these subjects were domestic conventionally managed (DCM) Standardbred foals and six were semi-feral managed (SFM) Shetland-type pony foals. Of the diarrheic foals, three were SFM foals aged 4 (n=1) and 5 (n=2) weeks old and four domestic foals aged 2 (n=2), 4 (n=1) and 5 (n=1) weeks old. All foals included in this study were healthy at birth with no serious gastrointestinal problems and no administration of antimicrobials, anti-inflammatories or supplemental products such as probiotics or gastrointestinal supplements at any stage during sampling.

The eight domesticated foals were born and maintained on Winbak Farm, a Standardbred breeding farm located in Chesapeake City, Maryland. Each domestic foal was born in a stall and was kept with their dam in a stall during their first week of life. The domestic foals and dams then made the transition to a small paddock for approximately eight hours per day until they reached 45 days of age. In most instances, there were two foal-dam pairs per paddock. During the rest of the day, each

foal-dam pair was enclosed in a stall. After their first 45 days of life, the foals were permanently relocated to a large pasture with their dam as well as other foal-dam pairs. These domestic foals had access to their dam's feed (Table 1 found on page 39) all throughout the study period and had access to grass at the beginning of their second week of life.

The six Shetland-type pony foals were born into a semi-feral herd maintained since 1994 at the University of Pennsylvania School of Veterinary Medicine in Kennett Square, Pennsylvania. DNA-based parentage is confirmed for all offspring (Gluck Equine Parentage Testing Laboratory, University of Kentucky, Lexington, KY). The herd consists of 11 harem groups and one bachelor band with a total of 105 animals. The ponies have no history of laminitis or major gastrointestinal diseases. Handling by humans in the semi-feral herd is limited to required preventative health care (daily observation, annual vaccinations and deworming when necessary) completed by highly skilled technicians experienced with these procedures using positive reinforcement. In addition, each SFM foal received a 30-minute "gentling" experience of positive reinforcement-based acclimation to human interaction with 21 specific compliance goals including touch all over the body, simulated veterinary examination and routine health care procedures, introduction of a halter, and introduction to leading if time allows when they are between the age of two and four weeks old. The environment of the semi-feral herd consisted of a 40-acre enclosure with natural forages and water sources as well as natural shelters such as hedges and light forest.

Sampling Protocol

Rectal swab samples were used to sample each foal's hindgut microbial community. Samples were taken for a separate study from foals once a week until the foal was either 5 or 6 weeks old. The samples taken in the weeks before and after diarrhea occurrence were used to compare to the diarrhea sample and determine major shifts in microbial composition and function. Swab samples were collected in triplicate using cotton-tipped swabs inserted approximately 2 to 3 inches into the foal's rectum and rotated circumferentially twice before retraction. While taking the swab sample, the handler used positive reinforcement of scratching of the foal's neck, shoulder, or rump. For most sampling, two handlers were necessary (one handler to take the swab sample and the other to restrain the foal). Each swab tip was cut off from the swab handle with scissors into a plastic bag and stored on ice for no more than an hour and until there was access to a freezer. When back at the lab, each swab tip was placed in a bead tube containing 750 microliters of bead solution. The tubes were then stored in a freezer at -20 degrees C until ready for extraction.

Microbial DNA Extraction

Genomic DNA was extracted from each swab sample using MO BIO Laboratories PowerFecal DNA Isolation Kit®. All provided protocol steps in the commercial kit were followed except 50 µL of solution C6 was used during the last step instead of 100 µL and this solution was left to sit for 5 minutes in the spin filter before the final centrifugation. Total DNA concentration in each sample was determined using a Qubit® fluorometer and sample quality was determined using a Nanodrop® spectrophotometer. One sample from each triplicate set with the highest DNA concentration and best absorbance ratio (260/280=1.8) was sequenced. Triplicate sample sets with all low DNA quantity and quality were ethanol precipitated using the following protocol: pool samples and determine volume, add ½ volume of 5M ammonium acetate, leave overnight at -20°C, add 2.5 volumes of

100% cold ethanol, centrifuge for 30 minutes at 15,000 rpm, remove supernatant, wash with 100µL of 70% cold ethanol, centrifuge for 15 minutes at 15,000 rpm, remove supernatant, air dry in a laminar flow hood and re-suspend in elution buffer. Microbial communities were determined using amplification of the V4-V5 variable region of the 16S rRNA gene and sequenced using Illumina MiSeq (515F-926R))(RTL Genomics, Lubbock, TX).

Bioinformatics Analysis

QIIME (Quantitative Insights Into Microbial Ecology) was used for microbial data processing and statistical analysis [15]. FLASh (Fast Length Adjustment of SHort reads) was used at its default parameters to merge paired-end reads generated from sequencing and FastQC was used to determine the quality of reads [16, 17]. A mapping file was then created and sequence reads were filtered and trimmed for quality and to remove primers.

All sequence files were then concatenated into one file and OTUs were picked. OTUs were open reference picked with UCLUST as the picking method for reference and de novo steps against the Greengenes version 13_8 database [18, 19]. OTUs observed only once or twice were filtered out of the OTU table and the OTU table was normalized using CSS (cumulative sum scaling). A core set of QIIME diversity analyses were run on the samples, which produced alpha diversity (PD whole tree, Chao richness index, nonparametric t-test), alpha rarefaction (PD whole tree, Chao richness index), beta diversity (ANOSIM, PERMANOVA) and group significance (Kruskal-Wallis) results. Enriched taxa in different study groups were also analyzed using LEfSe (Linear Discriminant Analysis Effect Size) [20].

RStudio was used for data visualization and analysis [21]. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to analyze the functional components of each sample's gut bacterial community [22]. OTUs were closed reference picked against the Greengenes version

13_5 database in QIIME to use for PICRUSt analysis [19]. The Galaxy Version 1.1.1 of PICRUSt was used with the following workflow: the 'Normalize by Copy Number' command was run to correct the OTU table for multiple 16S copy number, the 'Predict Metagenome' command was run on the normalized OTU table to obtain metagenome predictions and the 'Categorize by Function' command was run on the 'Predict Metagenome' output to obtain specific KEGG functions at pathway hierarchy level 3, the most specific level.

Results

Microbial Composition Summary

A total of 39 samples were collected from 14 different foals and were then analyzed. These samples were composed of 7 'during diarrhea', 7 'before diarrhea', 6 'after diarrhea', 7 'during no diarrhea', 7 'before no diarrhea' and 7 'after no diarrhea' samples (Table 8). One of the samples was missing from the 'after diarrhea' group because one of the 'during diarrhea' samples was the last sample taken and no 'after diarrhea' sample was available for analysis.

Table 8. Ages of foals in difference diarrheic and non-diarrheic groups, including average ages.

	Number of 1-Week-Old Foals	Number of 2-Week-Old Foals	Number of 3-Week-Old Foals	Number of 4-Week-Old Foals	Number of 5-Week-Old Foals	Number of 6-Week-Old Foals	Average Age (Weeks)
'During Diarrhea'	0	2	2	3	0	0	3.86
'Before Diarrhea'	2	0	2	3	0	0	2.86
'After Diarrhea'	0	0	2	0	2	2	4.67
'During No Diarrhea'	0	2	0	2	3	0	3.86
'Before No Diarrhea'	2	0	2	3	0	0	2.86
'After No Diarrhea'	0	0	2	0	2	3	4.86

There were a total of 81,365 observed OTUs from all samples and a total of 1,106,539 sequence counts (mean±s.d= 28,372.8±15,126.75; range= 3,469-67,531; median= 26,975). There was a total of 25 different phyla into which the OTUs were classified. The most abundant phylum present was Bacteroidetes followed by Firmicutes in both diarrheic and non-diarrheic foals. Diarrheic foals had an average of 38.3% Firmicutes and 40.1% Bacteroidetes while non-diarrheic foals had an average of 37% Firmicutes and 41.4% Bacteroidetes. The core microbiome of diarrheic and non-diarrheic foals was plotted for comparison (Figure 8).

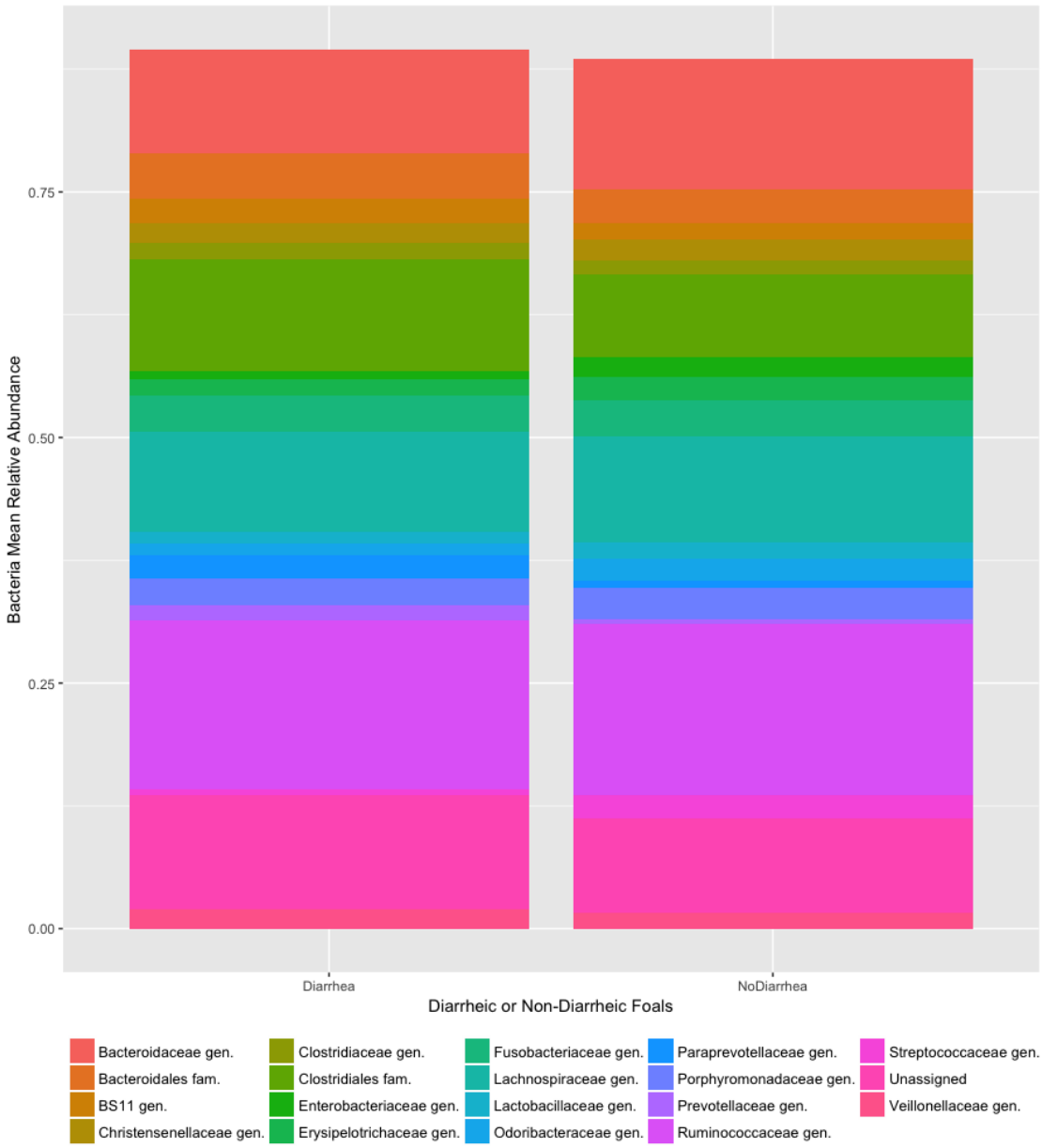

Figure 8. Core microbiome taxa at the family level with a relative abundance greater than 0.01 in at least one of the 2 groups (diarrheic or non-diarrheic).

Community Statistical Analysis of Foal

Alpha diversity was analyzed between diarrheic and non-diarrheic foals. There were no significant differences found in the mean diversity between diarrheic and non-diarrheic foals (PD whole tree, nonparametric t-test, $p=0.345$).

Significant differences were found in specific OTUs when comparing diarrheic foals to non-diarrheic foals and these OTUs were grouped by taxa ($p<0.05$, Kruskal-Wallis) (Figures 9 and 10). However, when comparing their entire microbial communities and not specific OTUs using ANOSIM and PERMANOVA statistical tests, no significant difference was found. There were also no significant differences between the 'before diarrhea', 'during diarrhea', 'after diarrhea', 'before no diarrhea', 'during no diarrhea' and 'after no diarrhea' groups when analyzing their communities as a whole using ANOSIM and PERMANOVA statistical tests. Interestingly, non-diarrheic foals were found to have a significantly higher abundance of *Escherichia coli* than found in diarrheic foals, however, this specific OTU may be a harmless strain of *E. coli*. Ruminococcaceae gen. was found to be significantly higher in non-diarrheic foals, which is a type of bacteria responsible for complex carbohydrate digestion. *Bacteroides fragilis, Prevotella spp., Veillonella spp.*, Lachnospiraceae gen, RF39 fam., Bacteroidales fam., RF16 gen. and Clostridiales fam. were found to be significantly higher in diarrheic foals while Ruminococcaceae gen., *Bacteroides spp., Butyricimonas spp., Odoribacter spp., Oscillospira spp., Fusobacterium spp., Parabacteroides spp.* and Unassigned were found to be significantly higher in non-diarrheic foals.

Enriched taxa were also analyzed using LEfSe (Linear Discriminant Analysis Effect Size). *Prevotella spp.* and *Veillonella spp.* were found to be enriched in diarrheic foals while *Ruminococcus spp.* was enriched in non-diarrheic foals ($p<0.05$,

Kruskal-Wallis, LDA score>2.0). These are similar findings to those stated previously using a Kruskal-Wallis test.

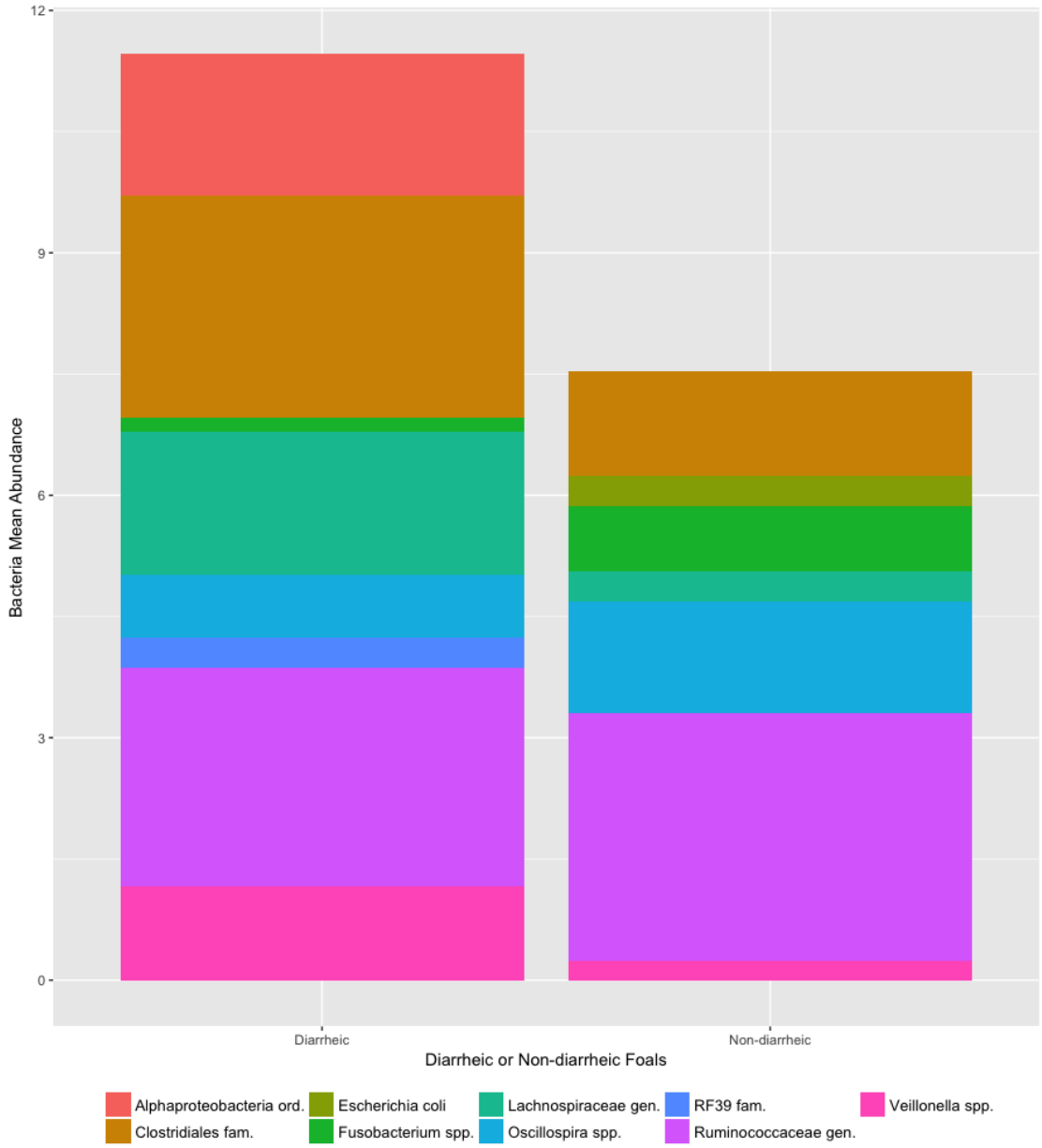

Figure 9. OTUs belonging to the Firmicutes, Tenericutes and Proteobacteria phyla significantly different between diarrheic and non-diarrheic foals (Kruskal-Wallis, $p<0.05$).

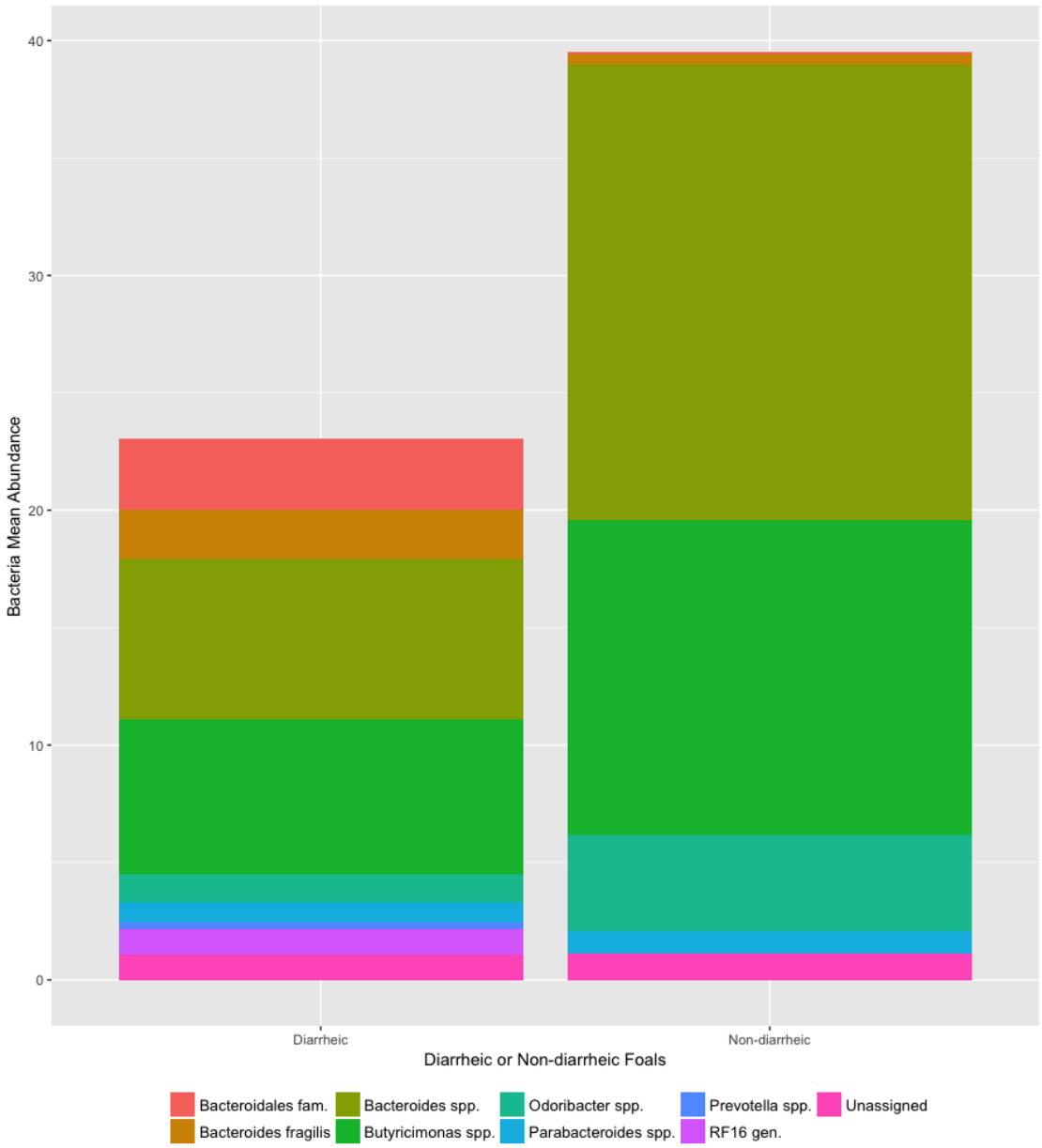

Figure 10. OTUs belonging to the Bacteroidetes phylum and unassigned taxa significantly different between diarrheic and non-diarrheic foals (Kruskal-Wallis, $p<0.05$).

Using an MDS/PCoA (Multidimensional Scaling/Principal Coordinate Analysis) plot, the Bray-Curtis Dissimilarity between ‘before diarrhea’, ‘during diarrhea’, ‘after diarrhea’, ‘before no diarrhea’, ‘during no diarrhea’ and ‘after no diarrhea’ groups was plotted, which takes into account OTU abundance as well as presence/absence of an OTU (Figure 11). There was a high amount of overlap in the ellipsoids of all groups, including between the ‘during diarrhea’ and ‘during no diarrhea’ groups. This reinforces the finding that there was no significant difference found between diarrheic and non-diarrheic foals when analyzing their microbiomes as a whole.

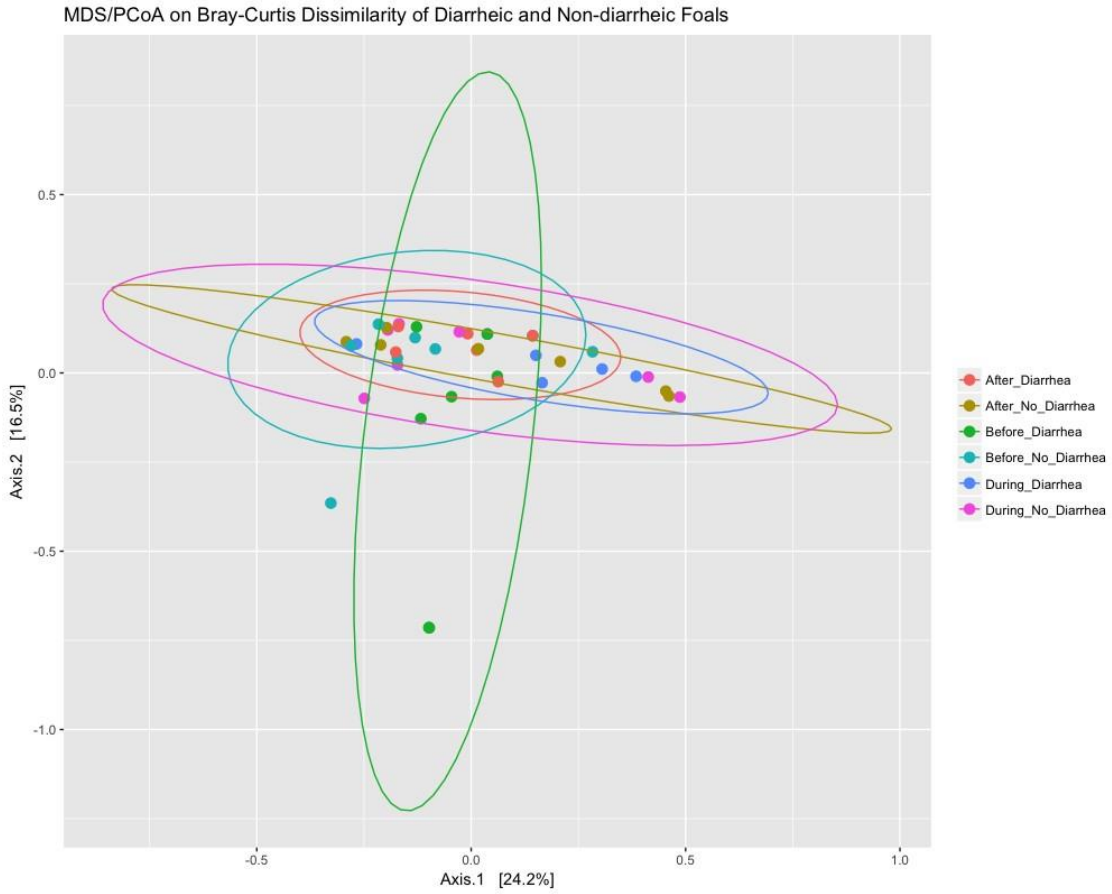

Figure 11. MDS/PCoA plot of the relationship between 'before diarrhea', 'during diarrhea', 'after diarrhea', 'before no diarrhea', 'during no diarrhea' and 'after no diarrhea' groups using Bray-Curtis Dissimilarity. Ellipsoids representing a 95% confidence interval were used to surround each group.

Functional Analysis of Foal Hindgut Microbiome

For functional analysis, PICRUSt was used and OTUs were categorized into different KEGG functions. Appropriate Level 3 KEGG predictions were then sorted into six different digestion related categories (Table 7 found on page 66).

Non-diarrheic foals were found to have a significantly higher abundance of the OTUs responsible for starch digestion, general carbohydrate digestion, simple carbohydrate digestion, complex carbohydrate digestion and protein digestion when compared to diarrheic foals ($p<0.05$, Kruskal-Wallis). This shows that non-diarrheic foals had a higher capability of these major digestion functions than diarrheic foals. The 6 different before/during/after diarrhea/non-diarrhea groups were also significantly different in each of the digestion types ($p<0.05$, Kruskal-Wallis) (Figure 12). There was a trend in the digestion data in which every 'before diarrhea' group had the highest level of digestion when comparing to every other group. The 'before no diarrhea' group also had a higher level of all of the digestion types than the 'during no diarrhea' and 'after no diarrhea' groups. This could be due to the fact that the 'before diarrhea' and 'before no diarrhea' groups consisted of foals with the lowest average age when compared to the rest of the groups. In a previous study using the same foals, it was found that all of the digestion types were significantly affected by age and that the digestion levels were highest when the foals were 1 week old and decreased as the foals aged.

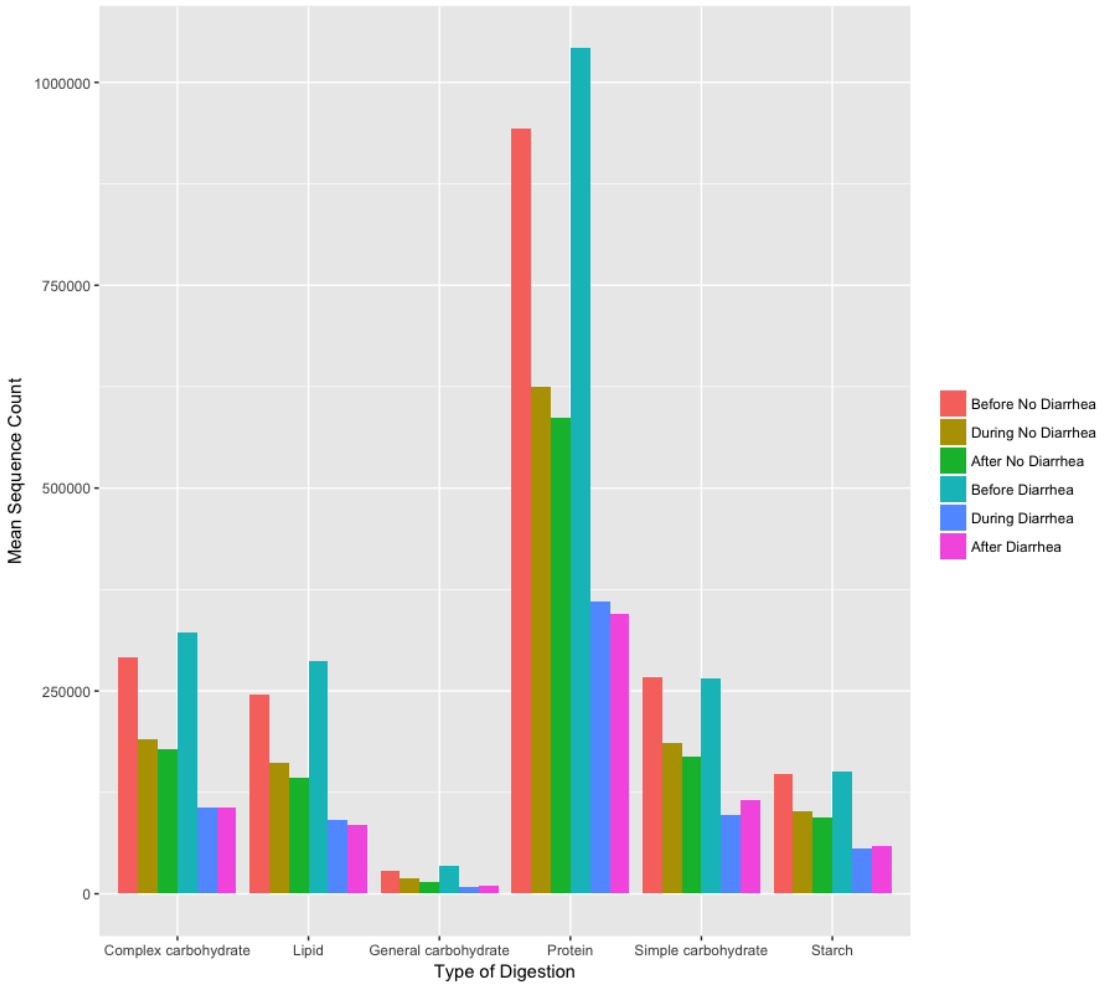

Figure 12. Levels of complex carbohydrate, lipid, general carbohydrate, protein, simple carbohydrate and starch digestion in the 6 different groups: 'before no diarrhea', 'during no diarrhea', 'after no diarrhea', 'before diarrhea', 'during diarrhea' and 'after diarrhea'.

Discussion

There were many significant differences found between diarrheic and non-diarrheic foals in specific OTUs. Even though there was not a difference between these two groups when analyzing their hindgut communities as a whole, the differences in specific taxa can help explain the events occurring in their gut during foal diarrhea. This type of foal diarrhea is usually not very serious and the foal is commonly able to recover from it without veterinary attention such as fluid administration or antibiotic therapy. Therefore, it is not surprising that there were no significant differences found in the two groups' microbiomes as a whole. It may be that only the significantly different OTUs found between diarrheic and non-diarrheic foals are those responsible for causing mild diarrhea.

Both *Prevotella spp.* and *Veillonella spp.* were found to be enriched in diarrheic foals, which is interesting because these two taxa were also discovered to have an increased abundance in human children with IBS (Irritable Bowel Syndrome) when compared to healthy children [1, 23, 24, 25]. Members of *Veillonella spp.* are known to ferment lactate produced by other bacteria while members of *Prevotella spp.* are well known as dietary fiber fermenters [23]. These strong correlations between *Prevotella spp.* and *Veillonella spp.* and gut issues is an important finding and should be further studied in diarrheic horses. Ruminococcaceae gen. and *Oscillospira spp.* were found to be significantly higher in non-diarrheic foals when compared to diarrheic foals. This was another common finding in healthy human children when compared to children with Crohn's disease [2]. Probiotic supplementation of these taxa may be a treatment option for immunologically compromised foals that may not have the ability to efficiently recover from foal diarrhea.

It is also important to note that there were significant differences found in starch digestion, general carbohydrate digestion, simple carbohydrate digestion, complex carbohydrate digestion and protein digestion. Non-diarrheic foals were found to have higher capabilities in each of these types of digestion when compared to diarrheic foals. This may implicate that the dysbiosis occurring in the gut of foals with foal diarrhea is causing diarrhea as well as lower digestion efficiency. Also found in the digestion data, the 'before diarrhea' and 'before no diarrhea' groups had the highest levels of digestion when compared to their respective 'during diarrhea', 'after diarrhea', 'during no diarrhea' and 'after no diarrhea' groups. In a previously conducted study using the same group of foals, it was concluded that 1-week-old foals had the highest capability in all digestion types. These levels decreased over time, so the 'before' groups may have had the highest abundances of bacteria responsible for each type of digestion because these groups were made up of foals with the lowest average age. It can be difficult to assign individual bacteria to specific functions manually, so programs like PICRUSt can be convenient for researchers to determine the function of their microbial community. Functional tools like this are also helpful when transcriptomic data is not available. It would be interesting to conduct a future study using transcriptomic data to determine the function of the microbiome. This would allow for a deeper understanding of the events occurring in the gut during foal diarrhea.

In determining the effects of foal diarrhea on the gut microbiome, it is difficult to separate the effects of age from diarrhea. This is because this type of foal diarrhea usually occurs early in the foal's life when their microbiome is still drastically changing and is not yet stable to that of an adult. In a previously conducted study using the same foals, it was found that the foal's gut microbiome was significantly

affected by both age and management type. In the current study, the diarrheic foal group consisted of two 2-week-old foals, two 4-week-old foals and three 5-week-old foals. In order to address this, foals were age and management type-matched with a non-diarrheic foal. Therefore, we were confident in our comparisons between diarrheic and non-diarrheic foals, however, it was still difficult to make definitive inferences about the changes in their gut microbiomes between the 'before', 'during' and 'after' groups because of the effects of age.

With the unique sample group available for this study of both domestic conventionally managed foals and semi-feral managed foals, we were able to characterize a new aspect of the hindgut microbiome during foal diarrhea. Foal diarrhea was found to be a common condition in foals in both domestic and feral type management settings. This shows that domestication may not have an influence on the occurrence of foal diarrhea and that it is a natural process in some foals. There is also a lack of studies on the microbiome during foal diarrhea using next generation sequencing. The definitive cause of foal diarrhea has still not yet been determined, so more studies characterizing the bacterial community present during this time may be helpful.

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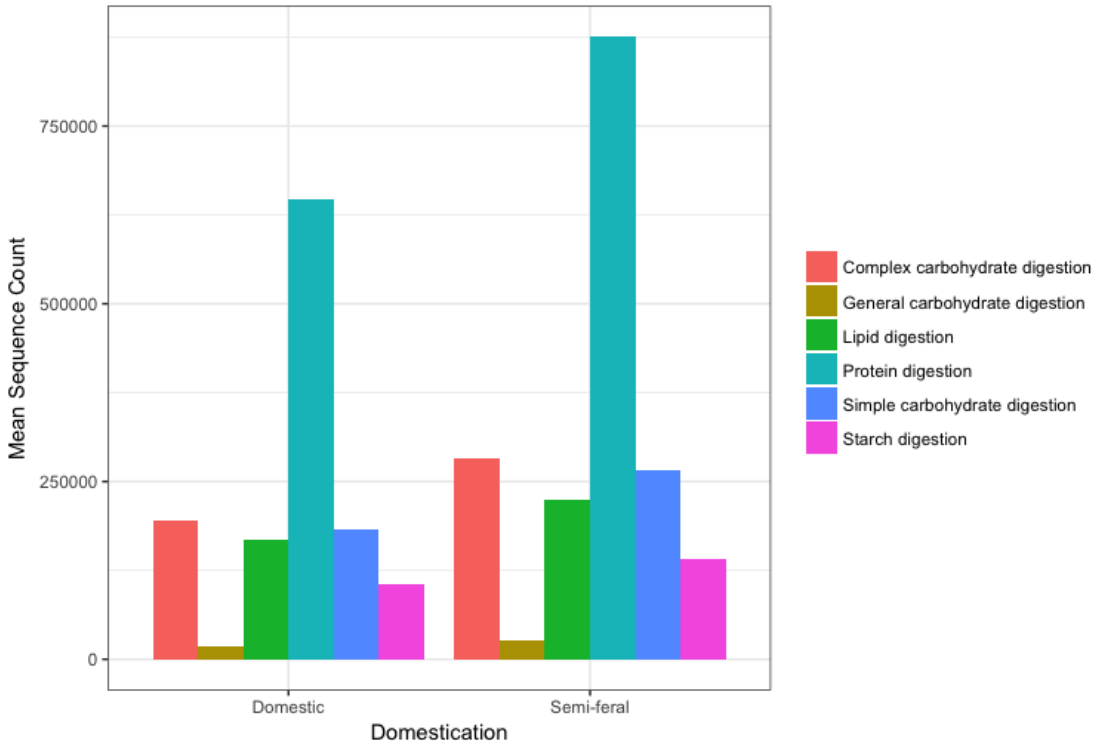
Appendix A

MAJOR DIGESTION FUNCTIONS OF SFM AND DCM FOALS



Appendix B

FULL LIST OF SIGNIFICANTLY ENRICHED TAXA FOUND IN SFM FOALS

Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Peptostreptococcaceae gen. 2	*Holdemania spp.*	Dehalobacteriaceae gen.	RF3 ord.	Methanobacteriales fam.	*vadinCA11 spp.*
Clostridium spp. 1	*Desulfovibrio spp. 2*	Coriobacteriaceae gen. 2	ML615J_28 fam.	*Methanobrevibacter spp.*	Methanomassiliicoccaceae gen.
Aeromonadaceae gen.	Clostridiales fam. 1	Synergistia ord.	*Prevotella spp. 1*	Methanobacteria ord.	Thermoplasmata ord.
Ruminococcus gnavus	*CF231 spp.*	Synergistetes class	Alphaproteobacteria ord.	Methanobacteriaceae gen.	E2 fam.
Ruminococcus spp.	Paraprevotellaceae gen.	Synergistales fam.	Fibrobacteria ord.	4COd_2 ord.	Planctomycetia ord.
Bacteroidales fam.	*Odoribacter spp.*	RF32 fam.	Fibrobacteres class	YS2 fam.	*Campylobacter spp.*
		Veillonella dispar	*Fibrobacter spp.*	Rhizobiales fam.	Pirellulales fam.
		Veillonella spp.	Fibrobacteraceae gen.	*Coprobacillus spp. 2*	Pirellulaceae gen.
		Christensenellaceae gen.	*Fibrobacter succinogenes*	Methylobacteriaceae gen.	Campylobacteraceae gen.
		RF39 fam.	Fibrobacterales fam.	*Treponema spp.*	Planctomycetes class
		Tenericutes class	*Prevotella copri*	Spirochaetales fam.	*Selenomonas noxia*
		Lachnospiraceae gen. 2	*Prevotella spp.*	*Helicobacter spp.*	Chlamydiia ord.
		Clostridiales fam. 2	Paraprevotellaceae gen. 2	Epsilonproteobacteria ord.	Chlamydiales fam.
			MVP_15 ord.	Campylobacterales fam.	Chlamydiae class

Appendix B cont.

FULL LIST OF SIGNIFICANTLY ENRICHED TAXA FOUND IN SFM FOALS

	PL_11B10 fam.	*Chlamydia spp.*
	Spirochaetes class	*YRC22 spp.*
	S24_7 gen.	*Mogibacterium spp.*
	Bacteroidales fam. 2	Mogibacteriaceae gen.
	Prevotella spp. 2	Mogibacteriaceae gen. 2
	Prevotellaceae gen.	Methanocorpusculaceae gen.
		Methanocorpusculum spp.
		Methanomicrobia ord.
		Coriobacteriia ord.
		Coriobacterales fam.
		Euryarchaeota class
		Archaea phyl.
		RFP12 gen.
		Verruco_5 ord.
		Actinobacteria class
		Rikenellaceae gen. 2

Appendix C

FULL LIST OF SIGNIFICANTLY ENRICHED TAXA FOUND IF DCM FOALS

Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Citrobacter spp.	*Lactobacillus reuteri*	*Selenomonas ruminantium*	Dehalobacteriaceae gen.	Veillonellaceae gen. 2	Spirochaetaceae gen.
Butyricicoccus spp.	Peptococcaceae gen. 2	Selenomonas gen.	Mogibacteriaceae gen. 1	*Bacteroides plebeius*	*Methanimicrococcus spp.*
Butyricicoccus pullicaecorum	*Haldemania spp.*	*Lactobacillus spp.*	*Methanobrevibacter spp.*	*Peptococcus spp.*	Methanosarcinaceae gen.
Pseudomonadales fam.	*Eggerthella spp.*	*Lactobacillus spp. 1*	Methanobacteriaceae gen.	*Coprobacillus spp. 2*	Methanosarcinales fam.
Lactobacillales fam. 2	*Eggerthella lenta*	*Coprococcus spp. 2*	Methanobacteria ord.	*CF231 spp.*	Oxalobacteraceae gen. 1
Sphingomonadales fam.	Bifidobacteriales fam.	*Clostridium spp. 2*	Methanobacteriales fam.	*Prevotella copri*	Methanomassiliicoccaceae gen.
Turibacterales fam.	*Anaerotruncus spp.*	RF39 fam.	*5_7N15 spp.*	*Prevotella spp.*	E2 fam.
Turibacteraceae gen.	*rc4_4 spp.*	Tenericutes class	Prevotellaceae gen. 2	WCHB1_41 fam.	Thermoplasmata ord.
Turicibacter spp.	Peptococcaceae gen.		*Ruminococcus flavefaciens*	Actinomycetaceae gen.	*vadinCA11 spp.*
Moraxellaceae	*Actinobacillus spp.*		RF3 ord.	Mogibacteriaceae gen. 2	Chlamydiales fam.
Enterococcus spp. 1	Alphaproteobacteria ord.		ML615J_28 fam.	Actinobacteria class	Chlamydiia ord.
Proteus spp.	Pasteurellaceae gen.		*Ruminococcus spp.*	*Akkermansia spp.*	Chlamydiae class
Escherichia spp.	Pasteurellales fam.		Coriobacteriia ord.	Unassigned	Elusimicrobia ord.
Escherichia colie	*Bacteroides ovatus*		Coriobacteriales fam.	*Prevotella spp. 2*	Elusimicrobia class
Clostridium spp. 1	*Blautia spp. 2*		*Actinomyces spp. 2*	Prevotallaceae gen.	*Sarcina spp.*
Erwinia spp. 2	*Phascolarctobacterium spp.*		*BF311 spp.*	BS11 gen.	R4_41B gen.
Sphingomonas spp.	*Roseburia spp. 2*		*Paludibacter spp.*		Pedosphaerae ord.

Appendix C cont.

FULL LIST OF SIGNIFICANTLY ENRICHED TAXA FOUND IN DCM FOALS

Lactobacillales fam. 1	Lachnospiraceae gen. 1	*RFN20 spp.*	Pedosphaerales fam.
Vagococcus spp.	*Lactobacillus spp. 2*	Clostridiales fam. 1	*Chlamydia spp.*
Erwinia dispersa	Lactobacillaceae gen.	Paraprevotellaceae gen. 2	Armatimonadetes class
Erwinia spp.	Lachnospiraceae gen. 2	*Treponema spp.*	RB046 fam.
Morganella spp.	*Dorea spp. 2*	Spirochaetales fam.	SJA_176 ord.
Enterococcaceae gen. 1	*Akkermansia muciniphila*	Spirochaetes class	Pirellulacea gen.
Klebsiella spp.	*Butyricimonas spp.*	Clostridiales fam. 2	Pirellulales fam.
Enterococcus spp. 2	Lachnospiraceae gen.		Planctomycetia ord.
Enterobacteriaceae gen.	Odoribacteraceae gen.		Synergistaceae gen. 2
Blautia spp.	Porphyromonadaceae gen.		Planctomycetes class
Blautia producta			*Peptoniphilus spp.*
Peptostreptococcaceae gen. 2			Synergistia ord.
Enterobacteriaceae gen. 1			Synergistetes class
Eubacterium spp.			Synergistales fam.
Eubacterium dolichum			4C0d_2 ord.
Enterococcus casseliflavus			YS2 fam.
Enterococcus spp.			*Rummeliibacillus spp.*
Enterococcaceae gen.			*Pseudobutyrivibrio spp.*
Erwinia spp. 1			Tremblayales fam.
Ruminococcus spp. 2			*Mogibacterium spp.*

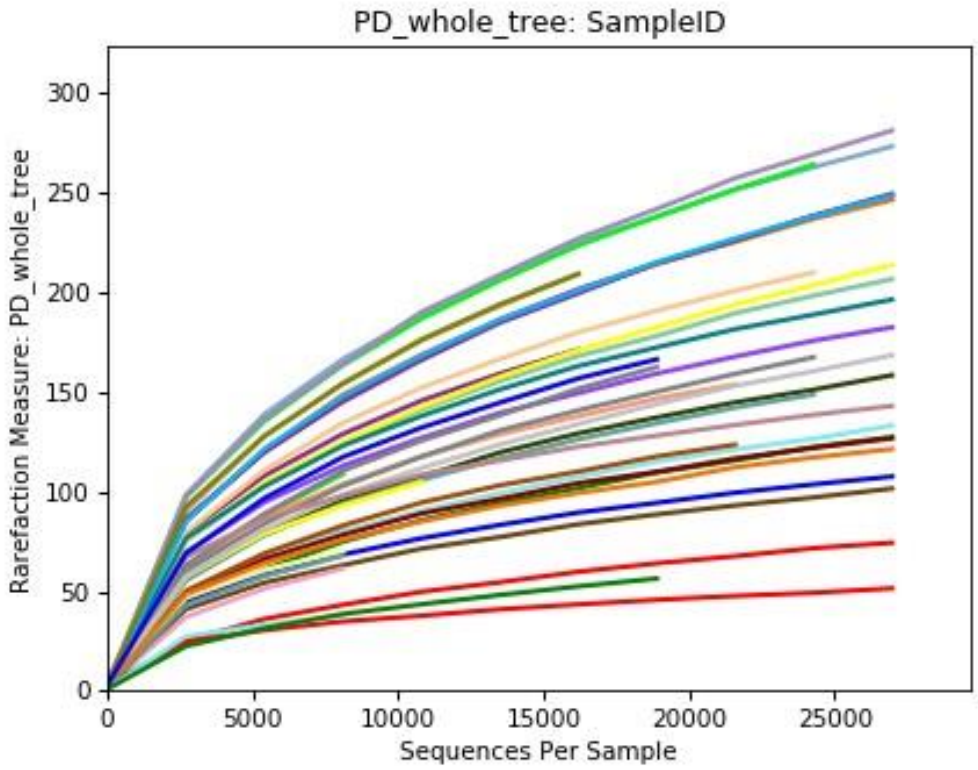
Appendix C cont.

FULL LIST OF SIGNIFICANTLY ENRICHED TAXA FOUND IN DCM FOALS

Oscillospira spp.	*Corynebacterium spp. 2*
Clostridium perfringens	Corynebacteriaceae gen.
Clostridium spp.	Mogibacteriaceae gen.
Lactobacillales fam.	RF16 gen.
Bacteria phyl.	Synechococcales fam.
Enterobacteriaceae gen. 2	Synechococcaceae gen.
Enterobacteriales fam.	*Synechococcus spp.*
Gammaproteobacteria ord.	Leuconostocaceae gen.
	Synechococcophycideae ord.
	YRC22 spp.
	Finegoldia spp.
	Tissierellaceae gen.
	RFP12 gen.
	Verruco_5 ord.
	Methanocorpusculaceae gen.
	Methanocorpusculum spp.
	Methanomicrobia ord.
	Euryarchaeota class
	Archaea phy.
	Prevotella spp.
	Paraprevotellaceae gen.
	Bacteroidales fam. 2

Appendix D

ALPHA RAREFACTION PLOT OF DIARRHEIC AND NON-DIARRHEIC FOAL STUDY SAMPLES USING PD WHOLE TREE



Appendix E

ALPHA RAREFACTION PLOT OF DOMESTIC CONVENTIONALLY- AND SEMI-FERAL- MANAGED FOAL AND DAM STUDY SAMPLES USING PD WHOLE TREE