Development of the Equine Hindgut Microbiome in Semi-feral and Domestic Conventionally-Managed Foals

Meredith K. Tavenner[a], Sue M. McDonnell[b], Amy Biddle[a]

[a]Department of Animal and Food Sciences, University of Delaware, College of Agriculture and Natural Resources, Newark, DE 19711

[b]Havemeyer Equine Behavior Laboratory, University of Pennsylvania School of Veterinary Medicine, New Bolton Center, Kennett Square, PA 19348

Corresponding author: asbiddle@udel.edu

Abstract

Background

Early development of the gut microbiome is an essential part of neonate health in animals. It is unclear whether the acquisition of gut microbes is different between domesticated animals and their wild counterparts. In this study, fecal samples from ten domestic conventionally managed (DCM) Standardbred and ten semi-feral managed (SFM) Shetland-type pony foals and dams were compared using 16S rRNA sequencing to identify differences in the development of the foal hindgut microbiome related to time and management.

Results

Gut microbiome diversity of dams was higher than foals overall, and foals from both groups at Week 1 had less diverse gut microbiomes than subsequent weeks. The core microbiomes of SFM dams and foals had more taxa overall, and greater numbers of taxa within species groups when compared to DCM dams and foals. The gut microbiomes of SFM foals demonstrated enhanced diversity of key groups: Verrucomicrobia (RFP12), Ruminococcaceae, *Fusobacterium* spp., and *Bacteroides* spp., based on age and management. Lactic acid bacteria *Lactobacillus spp.* and Lactobacillaceae gen. were enriched only in DCM foals, specifically during their second and third week of life. Predicted microbiome functions estimated computationally suggested that SFM foals had higher mean sequence counts for taxa contributing to the digestion of lipids, simple and complex carbohydrates, and protein. DCM foal microbiomes were more similar to their dams in week five and six than were SFM foals at the same age.

Conclusions

This study demonstrates the impact of management on the development of the foal gut microbiome in the first 6 weeks of life. The higher numbers of taxa within and between bacterial groups found in SFM dams and foals suggests more diversity and functional redundancy in their gut microbiomes, which could lend greater stability and resiliency to these communities. The colonization of lactic acid bacteria in the early life of DCM foals suggests enrichment in response to the availability of dams' feed. Thus, management type is an important driver of gut microbiome establishment on horses, and we may look to semi-feral horses for guidance in defining a healthy gut microbiome for domestic horses.

Keywords: microbiome, gut, horse, foal, management, development

Background

The gut microbiome is important for immune response, gastrointestinal tract health, endocrine system functioning, behavior, and even cognitive function in both humans and animals [1-6]. In humans, gut dysbiosis has been linked to many conditions, including obesity, autism spectrum disorders, diabetes, colorectal cancer, inflammatory bowel diseases as well as diseases caused by pathogenic bacteria [7-10]. In the horse, common gastrointestinal disorders have been associated with gut dysbiosis, including starch-induced laminitis, colitis, diarrhea and gastric ulcers [11-15]. These abnormalities have been correlated with differences in microbial diversity and abundances when compared to healthy horses.

The early development of the gut microbiome is an essential part of immune system training and maintenance of a healthy neonate. Failure to establish healthy commensal interactions in early development can result in chronic inflammation and autoimmune issues [16,17]. 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 of age [18,19]. A comparison of the gut microbiomes of 11 mare-foal pairs showed a higher abundance of Acidobacteria in newborn foals than mares, a higher abundance of Fibrobacteres and Spirochaetes in foals aged 121-240 days than mares and a lower abundance of Chlamydiae in mares than foals aged 31-60 days [19]. Another study using 16S rDNA sequencing to characterize the microbiomes of foals in the first 10 days of life and their respective Standardbred dams, reported that the initial colonization of foals' gut microbiota (from the meconium) reflected bacteria found in the dams' milk, including *Enterococcus* spp. and Enterobacteriaceae [20]. By day three, the foals' gut bacterial communities were similar to

78 that of their dams', with the acquisition of fiber fermenting microorganisms. The impact of
79 management, and specific drivers on the early development of the foal microbiome are unclear
80 Short-term studies of the foal gut microbiota have focused on effects of diarrhea,
81 *Rhodococcus equi* pneumonia vaccination, weaning, and probiotic supplementation, identifying
82 specific pathogenic bacteria or determining changes in the diversity of the foals' microbiomes
83 [18, 21, 22]. Development of the foal microbiome is suggested to be established prior to
84 weaning since no difference in gut microbiome species diversity or community membership
85 were found between foals experiencing gradual and abrupt weaning [18], and foals'
86 microbiomes were not significantly different than their dams' beginning at 1 month of age [19].
87 In this study, we surveyed the gut microbiome of normal foals with respect to their dams for the
88 first 6 weeks of life in order to map the acquisition of bacterial community members and inferred
89 functions.
90 Due to differences in diet and feeding practices, horses and ponies managed domestically
91 are thought to be more prone to health issues such as laminitis and gastric ulcers than feral
92 horses [23]. Factors such as grazing access, exercise, social interaction and diet contribute to
93 equine health. Horses are naturally adapted to be continuous grazers, however grain-based feed
94 is often added to the diets of domestic horses to meet their energy or other nutritional
95 requirements, and domestically managed horses often experience intermittent fasting. Horses
96 that are able to continuously graze secrete more saliva, which buffers the acidity of their stomach
97 contents. This acidity is caused by the secretion of gastric fluid as well as fermentation of non-
98 structural carbohydrates by lactic acid bacteria in the stomach [3].
99 Comparisons of the gut microbiomes of domestic and feral or semi-feral horses have
100 shown differences in diversity and community structure. When compared to domestic horses

living in adjacent grassland, feral Przewalski's horses had a distinct and more diverse bacterial community [24]. Feral Przewalski's horses had a higher abundance of the orders Clostridiales, Bacteroidales and Erysipelotrichales, while domestic horses had a higher abundance of Spirochaetales. Additionally, the feral horses less than a year of age had a less diverse and more compositionally distinct microbial community than those older than 1 year old [24]. Bacterial 16S rDNA surveys of fecal samples from Hokkaido native horses and light horses observed that native horses had a more diverse microbiome than light horses as well as a higher abundance of *Fibrobacter succinogenes* [25]. A specific cluster of bacteria related to cellulolytic bacteria were only found in native horses while one related to soluble sugar-utilizing species were only found in light horses [25]. In the current study, we report differences between semi-feral and domestic management in the development of the foal hindgut microbiome. Understanding the impact of management on the development, structure, and inferred function of the equine gut microbiome points to practices such as access to pasture, grain, and/or other horses with the potential to impact microbiome development at the earliest ages of life.

Results

Microbial Composition Summary

Samples were taken weekly for the first six weeks of life from 20 foals ($n_{samples}$=116) and 20 dams ($n_{samples}$=20) for a total of 136. There were a total of 81,365 observed operational taxonomic units (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). OTUs were classified into 19 phyla (Figure 1). The most abundant phylum present was Bacteroidetes followed by Firmicutes in both foals and dams. The average abundance of Bacteroidetes in foals and dams was 55.2%

and 48.3% and the average abundance of Firmicutes in foals and dams was 22.5% and 23.7%, respectively.

Seven Firmicutes families were found to be significantly different between DCM and SFM dams and foals across the time course: Mogibacteriaceae, Streptococcaceae, Ruminococcaceae and Erysipelotrichaceae were enriched in SFM groups, while Christensenellaceae, Lactobacillaceae, and Peptostreptococcaceae were more abundant in DCM groups (Figure 2). Four Bacteroidetes families were found to be significantly different between DCM and SFM dams and foals across the time course: Bacteroidaceae was enriched in SFM groups, while Paraprevotellaceae, Porphyromonadaceae, and Rikenellaceae were more abundant in DCM groups (Figure 3). Six families in other phyla were found to be significantly different between DCM and SFM dams and foals across the time course: Fusobacteriaceae and a family of Tenericutes (RF39) were enriched in SFM foals, a family of Verrucomicrobia (RFP12) and an Alphaproteobacteria family were more abundant in SFM dams, while Methanocorpusculaceae and a family of Spirochaetes were more abundant in DCM groups (Figure 4).

Effect of Breed on Horse's Hindgut Microbiome

When comparing adult ponies to Standardbred adult horses from this study with horses from the Equine Microbiome database [26], no microbiome differences were found to be significant with respect to breed (Kruskal-Wallis, corrected $p > 0.05$), however clustering of samples by principle coordinate analysis (PCoA) of Bray-Curtis Dissimilarity point to significant community differences based on management and study (Figure 5).

Core Microbiomes of SFM and DCM Dams and Foals

The core microbiomes of SFM and DCM foals and dams, defined as OTUs present in 95% or more of samples in each group, were different in terms of composition and numbers of

OTUs comprising each taxon (Figure 6). Overall, SFM foals and dams had higher numbers of taxa in their core microbiomes, and more OTUs in almost every group. The core microbiome of SFM foals was comprised of five taxa, only one of which, *Bacteroides* spp., was shared with DCM foals (Figure 6A). For this shared taxa, the SFM core microbiome featured five OTUs, while the DCM core microbiome had one. Besides *Bacteroides* spp., the core microbiome of DCM foals contained a Rikenellaceae spp., which was not shared with the SFM foals, and the SFM foal core microbiome included four unique taxa groups: *Bacteroides fragilis*, Enterobacteriaceae spp., Erysipelotrichaceae spp., and *Fusobacterium* spp. (Figure 6A). The core microbiome of SFM dams featured 154 OTUs in 16 taxa groups, while that of DCM dams had 54 OTUs in 11 taxa groups (Figure 6B). Unique taxa groups found in the SFM core microbiome for dams included: *Paulibacter* spp., *YRC22* spp., *RFN20* spp., *Oscillospira* spp., *Alphaproteobacteria* spp., and *RFP12* spp. Only one taxon, *Fusobacterium* spp. was unique to the DCM core microbiome for dams. This taxon was found in the core microbiome of SFM foals, but not in that of DCM foals, and was the only taxa group that overlapped between the dams and foals regardless of management.

Alpha and Beta Diversity

Foal samples were grouped into six different age groups determined by the foals' ages in weeks at 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 (PD whole tree, Chao richness, Shannon, and Simpson) was calculated and compared for all foal and dam groups. Mean diversity (PD whole tree, nonparametric t-test, $p>0.05$) 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 all other weeks (PD whole tree, nonparametric t-test, $p<0.01$) (Figure 7).

PCoA of Bray-Curtis dissimilarity by management strategy was plotted for 1-week-old foals, 5 or 6-week- old foals, and dams using multidimensional scaling (Figure 8). As foals age, their microbiomes become more similar to that of their dams, however the domestic dams and their 5/6 weeks old foals (Figure 8A) clustered more tightly than the semi-feral dams and their 6-week-old foals (Figure 8B) with higher amount of overlap in the ellipsoids of the domestic dams and their 5/6-week-old foals indicating differences between the two groups in the progress of microbiome development.

Community similarities between and within DCM and SFM foal groups, compared using multivariate ANOSIM and PERMANOVA indicated significant differences between and within DCM and SFM foals based on age, grazing access and housing as well as within each domestication group between age groups (Table S1, $p<0.05$, ANOSIM, PERMANOVA). These findings show that between study groups, both age and management type affected the foals' hindgut microbiomes. 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 indicating that management affects adult horse microbiomes as well as foals.

Pairwise comparisons by age for SFM and DCM foals found significant differences for DCM foals 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 (Table S1, $p<0.05$, ANOSIM, PERMANOVA). 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. There was more variance between ages in DCM foals, which 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 foals' gut microbiomes had not yet stabilized to that of an adult at 6 weeks of age.

Differences in Community Composition

Significantly different OTUs between SFM and DCM foals at different ages as well as SFM and DCM dams. The most highly significant (Kruskal-Wallis, $p<0.01$) taxa belonging to the Firmicutes and Bacteroidetes phyla were plotted (Figures 1, 2 and 3). Lactobacillaceae gen. was found to be significantly more abundant in DCM foals than in SFM foals and semi-feral and domestic dams (Table 2). This is interesting because it is a family that contains many lactic acid producing bacteria which have been associated with the onset of starch-induced laminitis [27].

Enriched taxa were also analyzed using LEfSe (Linear Discriminant Analysis Effect Size) [35]. DCM and SFM foals were analyzed separately for each of their 6 age groups (Tables 3 and 4). 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 archaea 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.

Predicted Functional Analysis of Foal and Dam Hindgut Microbiome

Functional potential of communities was inferred using PICRUSt [28] to generate predictions based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways at Level 3 [29]. These predictions were then sorted into 6 different digestion related categories (Table S2).

Significant differences were found between the 6 different age groups and DCM and SFM for all types of digestion: general carbohydrate, lipid, protein, complex carbohydrate, starch and simple carbohydrate ($p<0.05$, Kruskal-Wallis). Week 1 DCM and SFM 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 9). 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.

Discussion

We report significant effects of management type and age on the hindgut microbiome in foals and dams. Differences were found in abundances of specific OTUs between SFM and DCM foals as well as in their hindgut microbial communities as a whole. PCoA plots indicated that DCM foals possess a microbiome more similar to that of an adult at an earlier age than SFM foals. This could be due to the DCM dams and foals having more limited and uniform diets than SFM foals and dams. The accessibility of a variety of plant materials as well as numerous other horses in social groups likely provided a more varied exposure to the SFM foals from the beginning of life, and suggests that the wider environment plays an important role in shaping the gut microbiome in foals. The expanded membership and distribution of taxa found in the core microbiomes of SFM dams and foals points to differences in community structure based on management that could confer greater redundancy, and thus enhanced resilience to dietary change and/or stress.

To 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, the gut microbiomes of both SFM and DCM foals remained significantly different than their dams at five or six weeks of life (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 with respect to composition and function during this study period but may have established a stable one in the subsequent weeks after sampling had ended.

PICRUSt analysis [28] to infer the digestion functionality of the foals' microbiomes found that week 1 foals had the greatest amount of general carbohydrate-, lipid-, protein-, complex carbohydrate-, starch- and simple carbohydrate-digesting capability. 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. Mare milk in the first week of lactation was estimated to contain 2.64% protein, 2.07% fat, 6.15% lactose, 23.16% milk urea nitrogen and a somatic cell count of 40,640 cells/mL [30]. 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.

Despite the relatively small number of foals and dams in this study (n_{foals}=20, n_{dams}=20), there were clear differences between SFM and DCM groups. Management factors such as diet were likely a major driver of microbiome structure. This is because DCM foals had access to their dam's concentrate feed as well as hay and limited forage while SFM foals only had access to natural forage. Differences between DCM and SFM foals' microbiomes over time could be due to the changing diet of the DCM group throughout the study period; from no grazing in week 1 to limited access for the remaining weeks as well as increasing access to the dams' grain. These changes in diet may also contribute to the differences found between ages in DCM foals.

The differences found in this study between SFM and DCM horses were shown to be related to management and not breed, in agreement with previous reports [24]. No taxa was shown to be significantly different based on breed (Kruskal-Wallis, corrected $p>0.05$). PCoA plots of Bray-Curtis distances show almost complete overlap between the pony and the

Standardbred samples (Figure 5A), while there were two distinct groups of samples based on management (Figure 5B). There was also clustering due to study (Figure 5C), which points to differences due to sample handling between the EMP (Equine Microbiome Project) [protocols and the current study.

This study provides insight into how management affects the structure, function, and development of the equine microbiome starting at birth. Since SFM and DCM dams also had distinct microbiomes from one another, it is apparent that management factors such as diet, socialization and housing impact horses in their adult life as well. Further study is needed to determine the relative importance of management differences in shaping the microbiomes of horses. In this study SFM foals and dams had a higher amount of social interaction and grazing access than DCM foals and dams as well as greater variability in climate, environmental exposure to pathogens and stress levels. Horses are adapted to be continuous grazers, which can be difficult to achieve in the domestic setting. Domestic horse diets are higher in starch and other easily fermented sugars, leading to higher prevalence of diseases like starch-induced laminitis and gastric ulcers [23]. Management strategies more closely resembling SFM may modulate the microbiome toward a healthier balance and reduce the incidence of diet related illnesses.

Conclusions

This study demonstrates that management impacts the structure and inferred function of the foal hindgut microbiome during development in the first 6 weeks of life as well as for adult horses. The enhanced diversity of key groups (Verrucomicrobia (RFP12), Ruminococcaceae, *Fusobacterium* spp., and *Bacteroides* spp.), higher number of taxa and OTUs found in SFM dams and foals, and expanded inferred functional repertoire suggest greater functional redundancy, stability, and digestive capacity for the gut microbiomes of SFM horses. Greater

abundance of lactic acid bacteria in DCM dams and foals indicates early community adaptation to concentrate feeds. Further research is needed to identify specific management factors that are most significant for gut microbiome health and function in horses, and how the management of domestic horses may be informed by knowledge of semi-feral horses in a more natural state.

Methods

Subjects

Ten DCM Standardbreds and ten SFM Shetland-type pony foals and dams were included in this study. 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.

DCM dams were Standardbred broodmares maintained by Winbak Farm, Chesapeake City, Maryland. Each DCM foal was born and 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 was enclosed in a stall with free access to hay. After their first 45 days of life, the DCM foals and dams were permanently located in a large pasture with other foal-dam pairs. DCM foals had access to their dam's feed (Table 1) throughout the study period and had access to grass at the beginning of their second week of life.

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). At the time of this study, the herd consisted of 11 harem groups and one bachelor band with a total of 105 animals. The ponies had no history of laminitis or major gastrointestinal diseases. Handling by humans in the semi-feral herd was 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 at week 5 or 6 post-partum during the study period. Swab samples were collected in triplicate using sterile cotton-tipped swabs, stored on ice for no more than an hour, then placed in a bead tube

containing 750 microliters of bead solution (MO BIO Laboratories Inc., Carlsbad, CA). The tubes were then stored in a freezer at -20^{o}C until extraction.

DNA Extraction and Sequencing

Genomic DNA was extracted from each swab sample using MO BIO Laboratories PowerFecal DNA Isolation Kit® (MO BIO Laboratories Inc., Carlsbad, CA) as directed 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 to maximize yield. Total DNA concentration in each sample was determined using a Qubit® (ThermoFisher Scientific, Waltham, MA) fluorometer and sample quality was determined using a Nanodrop® (ThermoFisher Scientific, Waltham, MA) 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 low DNA quantity and quality were concentrated and cleaned by ethanol precipitation. The V4-V5 variable region of the 16S rRNA gene was amplified using universal primers (515yF 3'-GTGYCAGCMGCCGCGGTAA-5'/926pfR 3'-CCGYCAATTYMTTTRAGTTT-5') and sequenced using Illumina MiSeq (RTL Genomics, Lubbock, TX).

Bioinformatics Analysis

QIIME1 (Quantitative Insights Into Microbial Ecology) was used for microbial data processing and statistical analysis [31]. FLASh (Fast Length Adjustment of SHort reads) was used with default parameters to merge paired-end reads [32] and FastQC was used to determine the quality of reads [33]. In QIIME1, sequence reads were filtered and trimmed for quality and to remove primers.

Open reference OTUs were picked with UCLUST [34] using the Greengenes version 13_8 database [35]. OTUs observed only once or twice were filtered out of the OTU table and the OTU table was normalized using cumulative sum scaling (CSS). Alpha diversity (PD whole tree, Chao richness index, Shannon, and Simpson) was calculated and compared between groups and time points using Kruskal-Wallis. Beta diversity was calculated (Bray-Curtis distances) and compared using ANOSIM and PERMANOVA to determine differences over time. Differences in OTU abundance (group significance) were tested using Kruskal-Wallis in QIIME1 [31]. The core microbiomes of SFM and DCM foals and dams (taxa present in 95% of samples in each group) were identified in QIIME1 [31].

Enriched taxa by management group and time were identified using LEfSe [36]. Statistical analysis and visualization were completed using R [37]. PICRUSt [28] was used on the Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/) to infer functional potential of each sample's gut bacterial community using closed reference OTUs generated against the Greengenes version 13_5 database [35]. Briefly, OTUs were normalized by copy number, metagenome predictions were made and categorized to identify enriched KEGG functions [29].

Effect of Breed on Horse's Hindgut Microbiome

Comparison of breed and management effects on the gut microbiome was conducted in order to justify the use of Standardbred foals and dams to Shetland-type pony foals and dams in this study. Shetland-type ponies and Standardbred comparators were selected from the EMP database [26] to include only healthy individuals that had not received deworming medication or antibiotics within 30 days of sampling. Samples from eight adult ponies and nineteen Standardbred adult horses were compared with the horses in this study. Per EMP protocols,

freshly voided fecal samples were collected in 20% DNA Shield (Zymo, Irvine, CA) and stored at 4$_{o}$C. DNA was extracted, sequenced and analyzed as described above.

List of Abbreviations

DCM: domestic conventionally managed

SFM: semi-feral managed

OTU: operational taxonomic unit

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PCoA: Principle Coordinate Analysis

LEfSe: Linear Discriminant Analysis Effect Size

EMP: Equine Microbiome Project

QIIME1: Quantitative Insights Into Microbial Ecology

FLASh: Fast Length Adjustment of SHort reads

CSS: Cumulative Sum Scaling

KEGG: Kyoto Encyclopedia of Genes and Genomes

Declarations

Ethics Approval and Consent to Participate

All animal procedures were conducted following Institutional Animal Ethics Committee guidelines.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets generated during this study have been deposited in the NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra, Bioproject: PRJNA647744, Accession numbers: SAMN15597322- SAMN15597482

Competing Interests

None of the authors have any conflict of interest to declare.

Funding

We thank the University of Delaware Equine Science Program for supporting this research.

Authors' Contributions

MT designed and conducted the study as well as wrote the majority of the manuscript. ASB designed and provided the workflow for bioinformatics analysis of data and assisted with the design of the study and writing of the manuscript. SM managed SFM subjects, assisted with subject sampling, and helped with the design of the experiment. All authors read and approved the final manuscript.

Acknowledgements

We thank the University of Delaware Equine Microbiome Project for supplying comparative sequence data.

References

[1] Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nature Reviews Immunology 2016;16:341–52. doi:10.1038/nri.2016.42.

[2] Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nature Reviews Immunology 2017;17:219–32. doi:10.1038/nri.2017.7.

[3] Ocallaghan T, Ross R, Stanton C, Clarke G. The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology. Domestic Animal Endocrinology 2016;56. doi:10.1016/j.domaniend.2016.05.003.

[4] Vuong HE, Yano JM, Fung TC, Hsiao EY. The microbiome and host behaviorThe microbiome and host behavior. Annual Review of Neuroscience 2017. doi:10.1146/annurev-neuro-072116-031347.

[5] Galland L. The gut microbiome and the brain. Journal of Medicinal Food 2014;17:1261–72. doi:10.1089/jmf.2014.7000.

[6] Lee W-J, Hase K. Gut microbiota–generated metabolites in animal health and disease. Nature Chemical Biology 2014;10:416–24. doi:10.1038/nchembio.1535.

[7] Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-Gut Microbiota Metabolic Interactions. Science 2012;336:1262–7. doi:10.1126/science.1223813.

[8] Toh MC, Allen-Vercoe E. The human gut microbiota with reference to autism spectrum disorder: considering the whole as more than a sum of its parts. Microbial Ecology in Health & Disease 2015;26. doi:10.3402/mehd.v26.26309.

[9] Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. Microbial Ecology in Health & Disease 2015;26. doi:10.3402/mehd.v26.26191.

[10] Gagnière J. Gut microbiota imbalance and colorectal cancer. World Journal of Gastroenterology 2016;22:501. doi:10.3748/wjg.v22.i2.501.

[11] Steelman SM, Chowdhary BP, Dowd S, Suchodolski J, Janečka JE. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Veterinary Research 2012;8:231. doi:10.1186/1746-6148-8-231.

[12] Oliver OE, Stämpfli H. Acute Diarrhea in the Adult Horse: Case Example and Review. Veterinary Clinics of North America: Equine Practice 2006;22:73–84. doi:10.1016/j.cveq.2005.12.008.

[13] Costa MC, Arroyo LG, Allen-Vercoe E, Stämpfli HR, Kim PT, Sturgeon A, et al. Comparison of the Fecal Microbiota of Healthy Horses and Horses with Colitis by High Throughput Sequencing of the V3-V5 Region of the 16S rRNA Gene. PLoS ONE 2012;7. doi:10.1371/journal.pone.0041484.

[14] Milinovich GJ, Trott DJ, Burrell PC, Eps AWV, Thoefner MB, Blackall LL, et al. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. Environmental Microbiology 2006;8:885–98. doi:10.1111/j.1462-2920.2005.00975.x.

[15] Jassim RAA, Andrews FM. The Bacterial Community of the Horse Gastrointestinal Tract and Its Relation to Fermentative Acidosis, Laminitis, Colic, and Stomach Ulcers. Veterinary Clinics of North America: Equine Practice 2009;25:199–215. doi:10.1016/j.cveq.2009.04.005.

[16] Gritz EC, Bhandari V. The Human Neonatal Gut Microbiome: A Brief Review. Frontiers in Pediatrics 2015;3. doi:10.3389/fped.2015.00017.

[17] Malmuthuge N, Griebel PJ, Guan LL. The Gut Microbiome and Its Potential Role in the Development and Function of Newborn Calf Gastrointestinal Tract. Frontiers in Veterinary Science 2015;2. doi:10.3389/fvets.2015.00036.

[18] Jacquay E. Colonization and maturation of the foal fecal microbiota from birth through weaning and the effect of weaning method. thesis. 2017.

[19] Costa MC, Stämpfli HR, Allen-Vercoe E, Weese JS. Development of the faecal microbiota in foals. Equine Veterinary Journal 2015;48:681–8. doi:10.1111/evj.12532.

[20] Quercia S, Freccero F, Castagnetti C, Soverini M, Turroni S, Biagi E, et al. Early colonisation and temporal dynamics of the gut microbial ecosystem in Standardbred foals. Equine Veterinary Journal 2018;51:231–7. doi:10.1111/evj.12983.

[21] Schoster A, Guardabassi L, Staempfli HR, Abrahams M, Jalali M, Weese JS. The longitudinal effect of a multi-strain probiotic on the intestinal bacterial microbiota of neonatal foals. Equine Veterinary Journal 2015;48:689–96. doi:10.1111/evj.12524.

[22] Bordin AI, Suchodolski JS, Markel ME, Weaver KB, Steiner JM, Dowd SE, et al. Effects of Administration of Live or Inactivated Virulent Rhodococccus equi and Age on the Fecal Microbiome of Neonatal Foals. PLoS ONE 2013;8. doi:10.1371/journal.pone.0066640.

[23] Ward S, Sykes BW, Brown H, Bishop A, Penaluna LA. A comparison of the prevalence of gastric ulceration in feral and domesticated horses in the UK. Equine Veterinary Education 2015;27:655–7. doi:10.1111/eve.12491.

[24] Metcalf JL, Song SJ, Morton JT, Weiss S, Seguin-Orlando A, Joly F, et al. Evaluating the impact of domestication and captivity on the horse gut microbiome. Scientific Reports 2017;7. doi:10.1038/s41598-017-15375-9.

[25] Yamano H, Koike S, Kobayashi Y, Hata H. Phylogenetic analysis of hindgut microbiota in Hokkaido native horses compared to light horses. Animal Science Journal 2008;79:234–42. doi:10.1111/j.1740-0929.2008.00522.x.

[26] Berg J, Chambers B, Siegel H, Biddle A. Equine microbiome project: Understanding differences in the horse gut microbiome related to diet. Journal of Equine Veterinary Science 2017;52:94. doi:10.1016/j.jevs.2017.03.138.

[27] Garner HE, Moore JN, Johnson JH, Clark L, Amend JF, Tritschler LG, et al. Changes in the Caecal Flora associated with the Onset of Laminitis. Equine Veterinary Journal 1978;10:249–52. doi:10.1111/j.2042-3306.1978.tb02273.x.

[28] Langille MGI, Zaneveld J, Caporaso JG, Mcdonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnology 2013;31:814–21. doi:10.1038/nbt.2676.

[29] Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research 2000;28:27-30. doi:10.1093/nar/28.1.27.

[30] Wells S, Ferwerda N, Timms LL. Evaluation of Mare Milk Composition / Quality during Lactation 2012. doi:10.31274/ans_air-180814-7.

[31] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. QIIME Allows Analysis of High-Throughput Community Sequencing Data 2010;7:335–6. doi:10.1038/nmeth.f.303.

[32] Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 2011;27:2957–63. doi:10.1093/bioinformatics/btr507.

[33] FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (2015), "FastQC," https://qubeshub.org/resources/fastqc

[34] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1. doi:10.1093/bioinformatics/btq461.

[35] Desantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Applied and Environmental Microbiology 2006;72:5069–72. doi:10.1128/aem.03006-05.

[36] Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biology 2011;12. doi:10.1186/gb-2011-12-6-r60.

[37] R Core Team. R: A Language and Environment for Statistical Computing 2012; Vienna, Austria: R Foundation for Statistical Computing. Available: http://www.R-project.org/

Figures, tables and additional files

Figure legends

Figure 1. Comparison of the microbiomes semi-feral and domestic dams and foals at different age groups at the phylum level. Low abundance phyla (represented in fewer than 60% of samples) are not shown: Armatimonadetes, Chlamydiae, Cyanobacteria, Elusimicrobia, Lentisphaerae, Planctomycetes, Synergistetes, WPS-2.

Figure 2. A. Average relative abundances of Firmicutes by family for DCM and SFM dams and foals across the time course. Families with less than 0.01% relative abundance for all samples are not shown. **B.** Firmicutes families that were significantly different between DCM and SFM dams and foals ($p<0.05$, Two factor t-test).

Figure 3. A. Average relative abundances of Bacteroidetes by family for DCM and SFM dams and foals across the time course. Families with less than 0.01% relative abundance for all samples are not shown. **B.** Bacteroidetes families that were significantly different between DCM and SFM dams and foals ($p<0.05$, Two factor t-test).

Figure 4. A. Average relative abundances of non-Firmicutes/ Bacteroidetes by family for DCM and SFM dams and foals across the time course. Families with less than 0.01% relative abundance for all samples are not shown. **B.** Families that were significantly different between DCM and SFM dams and foals ($p<0.05$, Two factor t-test).

Figure 5. DCA/PCoA plot of the relationships between the beta diversity of the DCM and SFM dam microbiota and comparative samples from the EMP database using Bray-Curtis Dissimilarity. Ellipsoids representing a 95% confidence interval. Color by: **A.** Breed: Pony (red), Standardbred (blue), **B.** Management: Domestic (red), Semiferal (blue), **C**. Farm: EMP (red), Winback Farm (blue), New Bolton Center (green)

Figure 6. Numbers of OTUs by taxa group in the core microbiomes (present in 95% or more of samples in each group) of SFM (SF) and DCM (D) managed horses. **A.** Foals, **B.** Dams

Figure 7. Alpha diversity (PD_Whole_tree) of gut microbiome communities for DCM and SFM dams and foals across the time course. Standard error indicated.

Figure 8. MDS/PCoA plot of the relationships between 1-week-old and 5/6-week-old foals as and dams using Bray-Curtis Dissimilarity. Ellipsoids representing a 95% confidence interval were used to surround each dam or foal group. **A.** DCM **B.** SFM

Figure 9. 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 indicated.

Tables

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

Table 2. Highly significantly different groups 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.

Table 3. 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.	*Holdemania spp.*	*Veillonella dispar*		*Coprobacillus spp.*	*Selenomonas noxia*
	Clostridium spp.		*Veillonella spp.*			*Mogibacterium spp.*
	Ruminococcus gnavus		Christensenellaceae gen.			Mogibacteriaceae gen.
	Ruminococcus spp.		Lachnospiraceae gen.			
Bacteroidetes		*Odoribacter spp.*		S24_7 gen.		*YRC22 spp.*
		CF231 spp.		*Prevotella spp.*		Rikenellaceae gen.
		Paraprevotellaceae gen.		Prevotellaceae gen.		
				Prevotella copri		
				Paraprevotellaceae gen.		
Proteobacteria	Aeromonadaceae gen.	*Desulfovibrio spp.*			Methylobacteriaceae gen.	*Campylobacter spp.*
					Helicobacter spp.	Campylobacteraceae gen.
Euryarchaeota			Dehalobacteriaceae gen.		*Methanobrevibacter spp.*	*vadinCA11 spp.*
					Methanobacteriaceae gen.	Methanomassiliicoccaceae gen.
						Methanocorpusculaceae gen.
						Methanocorpusculum spp.
Actinobacteria			Coriobacteriaceae gen.			
Fibrobacteres				*Fibrobacter spp.*		
				Fibrobacteraceae gen.		
				Fibrobacter succinogenes		
Spirochaetes					*Treponema spp.*	
Planctomycetes						Pirellulaceae gen.
Chlamydiae						*Chlamydia spp.*
Verrucomicrobia						RFP12 gen.

Table 4. 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.*	*Blautia producta*	*Lactobacillus reuteri*	*Selenomonas ruminantium*	Mogibacteriaceae gen.	Veillonellaceae gen.	*Sarcina spp.*
	Butyricicoccus pullicaecorum	*Ruminococcus spp.*	Peptococcaeae gen.	Selenomonas gen.	*Ruminococcus flavefaciens*	*Peptococcus spp.*	*Peptoniphilus spp.*
	Clostridium perfringens	*Eubacterium spp.*	*Holdemania spp.*	*Lactobacillus spp.*	*Ruminococcus spp.*	*Coprobacillus spp. 2*	*Rummeliibacillus spp.*
	Clostridium spp.	*Eubacterium dolichum*	*Anaerotruncus spp.*	*Coprococcus spp.*	*RFN20 spp.*	Mogibacteriaceae gen.	*Pseudobutyrivibrio spp.*
	Peptostreptococcaceae gen.	*Oscillospira spp.*	*rc4_4 spp.*	*Clostridium spp.*			*Mogibacterium spp.*
	Turicibacter spp.		Peptococcaceae gen.	*Phascolarctobacterium spp.*			Mogibacteriaceae gen.
	Enterococcaceae gen.		*Blautia spp.*	*Roseburia spp.*			Leuconostocaceae gen.
	Enterococcus spp.			Lachnospiraceae gen.			*Finegoldia spp.*
	Enterococcus casseliflavus			*Lactobacillus spp.*			Tissierellaceae gen.
	Vagococcus spp.			Lactobacillaceae gen.			
	Blautia spp.			*Dorea spp.*			
Bacteroidetes			*Bacteroides ovatus*	*Butyricimonas spp.*	*5_7N15 spp.*	*Bacteroides plebeius*	RF16 gen.
				Odoribacteraceae gen.	Prevotellaceae gen.	*CF231 spp.*	*YRC22 spp.*
				Porphyromonadaceae gen.	*BF311 spp.*	*Prevotella copri*	*Prevotella spp.*
					Paludibacter spp.	*Prevotella spp.*	Paraprevotellaceae gen.
					Paraprevotellaceae gen.	Prevotallaceae gen.	
						BS11 gen.	
Proteobacteria	Enterobacteriaceae gen.	*Escherichia spp.*	*Actinobacillus spp.*				Oxalobacteraceae gen.
	Erwinia dispersa	*Escherichia coli*	Pasteurellaceae gen.				
	Erwinia spp.	*Sphingomonas spp.*					
	Citrobacter spp.	*Morganella spp.*					
	Proteus spp.	*Klebsiella spp.*					
Actinobacteria	*Eggerthella spp.*				*Actinomyces spp. 2*	Actinomycetaceae gen.	*Corynebacterium spp.*
	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.
Cyanobacteria							Synechococcaceae gen.
							Synechococcus spp.

Additional Files

Table S1. 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
	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

607

608 **Table S2.** 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

609

610