Colonization and maturation of the foal fecal microbiota from birth through weaning and the effect of weaning method

by

Erica Jacquay

B.S., Virginia Polytechnic Institute and State University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

Major Professor
Dr. Joann Kouba

Abstract

The objectives of these studies were to (1) characterize mare milk and fecal bacteria, and foal fecal microbiota from birth to 4 mo and (2) determine the effect of weaning and weaning method on foal fecal bacterial composition. Next generation sequencing of the V4 region of the bacterial 16S rRNA gene was performed using the Illumina Miseq according to Earth Microbiome Project protocols and sequencing data was analyzed using QIIME. In experiment 1, mare milk, mare fecal, and foal fecal samples were collected from 9 mare and foal pairs at birth (d 0), d 2, 7, and 1, 2, 3 and 4 mo. In experiment 2, 9 foals were separated into 2 treatments: abrupt (n = 5) and gradual (n = 4) weaning methods. Fecal samples were collected the day before weaning (d-1), the day of weaning (d 0) and post-weaning on d 1, 2, 3, 4, and 7. Blood was collected for analysis of cortisol concentration at 0800 h on d -1, 1, 2, and at 0800 h and 1100 h on d 0 and 4. Heart rate was recorded in 10 min intervals on the day of weaning starting 1 h before weaning to 2 h post-weaning, and again for 1 h starting 24 h after weaning. Results from experiment 1 showed newborn foal meconium and mare milk were similar in species diversity and composition; however, large shifts in composition and increases in foal fecal bacterial diversity occurred within the first week. By 1 mo, foal fecal bacterial composition did not differ in composition from mare feces at the phylum level ($P = 1.0$). Firmicutes, Bacteriodetes, Verrucomicrobia, and Spirochaetes were the dominant phyla found in feces of foals 1 mo and older and adult mare feces. For experiment 2, there were no differences in species diversity ($P > 0.05$) or separations in bacterial community structure between weaning methods or before and after weaning. There were minor shifts in relative abundance of specific phyla and genera in response to weaning. Foals in the abrupt treatment group had increased cortisol concentrations on d 1 ($P < 0.05$) and increased heart rate for 50 min after weaning on d 0 ($P < 0.05$). The foal is

born with fecal microbial communities similar to milk that rapidly change during the first week of life, reaching the same composition of its dam by the first month. The foal fecal microbiota matures prior to weaning, therefore weaning did not cause drastic changes in bacterial composition. Although acute stress was increased in abruptly weaned foals, stress associated with different weaning methods did not influence the fecal microbiota within the first week post-weaning.

Table of Contents

List of Figures vii
List of Tables ix
Acknowledgements x
Dedication xi
Chapter 1 - Review of Literature 1
Introduction 1
Human and Livestock Gut Microbiota 2
Technological Advances 4
Non-Culture Based Techniques 5
Community Fingerprinting 5
In Situ Hybridization 7
Next Generation Sequencing 7
Limiting Factors 9
Factors Influencing the Equine Intestinal Microbiota 13
Diet 13
Probiotics 15
Diseases 16
Laminitis 16
Colic 17
Other 18
Development of the Fecal Microbiota 19
Initial Colonization 19
Foal Fecal Microbiota 22
Weaning 24
Weaning and Stress 25
Nutrition 25
Physiological Changes 25
Behavior 26
Weaning Methods 27

Housing 27
Abrupt versus Gradual 28
Weaning and the Foal Fecal Microbiota 29
Literature Cited 31
Chapter 2 - Characterization of foal fecal microbiota from birth to weaning and the relationship to mare milk and mare feces 41
Abstract 41
Introduction 42
Materials and Methods 44
Animals 44
Fecal and Milk Sampling 44
DNA Extraction and 16S rRNA Gene Amplification 45
Sequencing and Analysis 46
Statistical Analysis 47
Results 48
Alpha Diversity 48
Beta Diversity 49
Relative Abundance 50
Discussion 56
Conclusion 59
Literature Cited 60
Chapter 3 - The effect of weaning and acute stress associated with abrupt weaning on foal fecal microbiota 64
Abstract 64
Introduction 65
Materials and Methods 66
Animals 66
Treatment and Weaning Management 67
Fecal Sampling 68
Cortisol Concentration 68
Heart Rate 69

DNA Extraction and 16S rRNA Gene Amplification 69
Sequencing and Analysis 70
Statistical Analysis 71
Results 72
Alpha Diversity 72
Beta Diversity 73
Community Composition 74
Physiological Parameters 77
Discussion 80
Conclusion 84
Literature Cited 85
Appendix A - Mare and foal supplemental data 89
Appendix B - Weaning supplemental data 90

List of Figures

Figure 2-1. Observed operational taxonomic units (OTU), representing number of different bacterial species, between mare milk, foal fecal and mare fecal samples within time points from birth (d 0) to 4 mo. Least square means with different letters within time points differ, ^{a,b} $P < 0.001$ and ^{c,b} < 0.05. Error bars represent SEM. 48

Figure 2-2. Nonmetric multidimensional scaling (NMDS) with 95% confidence interval ellipses based on Bray-Curtis distances of mare milk, mare fecal and foal fecal samples collected at different time points following birth (d 0). Each point represents an individual milk or fecal sample. Point and ellipse colors represent mare milk and mare feces combined across time points, and foal fecal samples from birth (d 0) to 4 mo of age. Non-overlapping ellipses are significantly different ($P < 0.05$). 49

Figure 2-3. Relative abundance of the dominant bacteria at the phylum level found in mare milk, mare feces, and foal feces from birth (d 0) to weaning at 4 mo of age. 51

Figure 2-4. Relative abundance of the dominant bacteria at the genus level, or next lowest taxonomic resolution, found in mare milk, mare feces, and foal feces from birth (d 0) to 4 mo of age. Taxa with o. represent order as the lowest taxonomic level classified whereas f. represents family. 52

Figure 3-1. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance of foal feces from different time points relative to weaning (d 0), from the day before weaning (d -1) to d 7 post-weaning, and different treatments: abrupt (n=5) and gradual (n = 4) weaning. Points with different colors represent different time points before and after weaning. Points with different shapes represent different weaning methods: gradual (circle) and abrupt (square). 73

Figure 3-2. Relative abundance of bacteria from foal feces at the phylum level before and after weaning and between treatments: abrupt and gradual weaning. Foal fecal samples from the day before weaning and day of weaning were combined and into the group Pre and samples from d 1 to 7 following weaning were grouped as Post. Treatment groups consist of abrupt (A) and gradual (G) weaning methods. 74

Figure 3-3. Relative abundance of bacteria in foal feces at the genus level, or lowest taxonomic resolution, before and after weaning and between treatments: abrupt and gradual weaning.

Foal fecal samples from the day before weaning and day of weaning were combined and into the group Pre and samples from d 1 to 7 following weaning were grouped as Post. Treatment groups consist of abrupt (A) and gradual (G) weaning methods. Taxa with o. represent order as the lowest taxonomic level classified whereas f. represents family. 75

Figure 3-4. Mean concentrations of cortisol relative to weaning for foals assigned to 2 treatments: abrupt and gradual weaning. Weaning occurred directly after time point d 0 (a). Blood pull occurred at 0800 h on days without letters and d 0 (a) and 4 (a). Days with (b) indicate a second blood pull 3 h after initial, taken at 1100 h. Points lacking a common superscript differ, $^{a,b}P < 0.05$..... 78

Figure 3-5. Mean heart rate (bpm) relative to weaning for foals assigned to 2 treatments: abrupt and gradual weaning, on the day of weaning (d 0) and 24 h post-weaning (d 1). * Indicates blood pull and weaning on d 0 and + indicates blood pull on d 1 for both treatment groups. Different superscripts at specific time points differ, $^{a,b}P < 0.05$ and $^{c,d}P < 0.01$..... 79

List of Tables

Table 1-1. Classification of bacterial genera in the equine hindgut using next-generation sequencing techniques[1] (Julliand and Grimm 2016) 12

Table 2-1. Differences in the relative abundance of specific bacteria phyla found in foal feces between two specific time points from birth (d 0) to 4 mo 53

Table 2-2. Relative abundance (%) of the main bacteria phyla of mare fecal and milk samples as they compare to foal feces from d 0 to 7 after birth 54

Table 2-3. List of dominant bacterial genera found in mare milk, mare fecal, and foal fecal samples from birth (d 0) to 4 mo 55

Table 3-1. Alpha diversity metrics[1] (LSM ± SEM) of fecal bacteria from pre-weaning[2] and post-weaning[3] in foals weaned abruptly (n = 5) or gradually (n = 4) 72

Table 3-2. Relative abundance (%) of bacterial phyla (LSM ± SEM) in foal feces with differences between treatments, abrupt and gradual weaning, and/or time relative to weaning 76

Table 3-3. Relative abundance (%) of bacterial genera (LSM ± SEM) in foal feces with differences between treatments, abrupt and gradual weaning, and/or time relative to weaning 76

Acknowledgements

This research would not have been possible without the gracious support and help that I received. I would first like to thank Dr. Joann Kouba for taking me on as a graduate student and for her continuous support throughout this project. I would also like to thank my other committee members, Dr. Lydia Zeglin and Dr. Jim Lillich, as well as Dr. Jeffrey Stevenson, for allowing me to work in their laboratories. Thank you to my fellow equine graduate students, Chelsea Sinclair, Courtney Douthit, Laura Fehlberg, Taylor Horne, Katie Jordan, Molly Lass, and Ciana Scaletti, and the horse unit manager, Kallie Emig, who helped me with the mommy and baby horses. I could not have collected all of my samples without your assistance. I also appreciated all of the help and input I received from the students and faculty in the microbial ecology lab group of the K-State Division of Biology. Finally, I would like to thank my friends and family for their unwavering support and encouragement throughout this process.

Dedication

I would like to dedicate this work to my parents, Kathy and Ken Jacquay. Thank you for allowing my horse crazy obsession to continue and for your unconditional love and support. You both mean the world to me and I will never be able to thank you enough.

Chapter 1 - Review of Literature

Introduction

The gastrointestinal tract (GIT) contains one of the most complex and diverse microbial communities. The microbes in the GIT are responsible for aiding in digestion and extraction of nutrients from the diet and they also protect against pathogens and support immune function (Lozupone et al., 2012). Determining the intestinal microbial composition of a healthy adult horse is important for the measurement of the impact of diseases and intestinal problems. Currently, the variability in gut microbiota composition among host species, within different intestinal compartments, and among individuals makes it difficult to make general assumptions (Lozupone et al., 2012).

The intestinal microbiota of the horse plays a critical role in health and disease. Disturbances of the equine intestinal microbiota have been associated with a variety of diseases including colitis (Costa et al., 2012), laminitis (Moreau et al., 2014), equine grass sickness (Garrett et al., 2002) and post-partum colic (Weese et al., 2015). Equine fecal samples are used as a non-invasive way to measure intestinal microbiota (Costa et al., 2015a). Researchers initially relied on culture-based techniques to evaluate fecal bacteria despite inaccurate estimations of microbial communities and the inability to culture most bacteria. Initial studies aimed to categorize the normal fecal microbiome, with the Firmicutes phylum making up the highest percentage of bacteria (Shepherd et al., 2012). Recent advances in next-generation technology and bioinformatics make it possible to further classify the complex microbiota of the equine intestine (Costa and Weese, 2012).

In addition to diseases, an area of particular interest is when and which bacterial taxa are present in the initial colonization of the equine gastrointestinal tract (GIT) beginning at birth.

Although more extensively studied in humans, mice, and other livestock species, there is limited research on the intestinal microbial development in foals (Costa et al., 2015b). Furthermore, the influences of bacteria present in colostrum and mare feces on the initial colonization and early development of the foal intestinal microbiota warrants further research (Earing et al., 2012).

Human and Livestock Gut Microbiota

There are similarities and differences in the intestinal microbiota between humans and livestock species. The variation in microbial composition in the gut of different animals can be largely attributed to differences in diets and digestive system function. Furet et al. (2009) looked at the differences in human and farm animal fecal bacteria and found human fecal microbiota to be distinguishable from farm animals.

The most dominant bacterial phyla in the human gut are Bacteriodetes and Firmicutes followed by Actinobacteria, Proteobacteria, and Verrucomicrobia (Lozupone et al., 2012). Members of the Firmicutes phylum found in the GIT are highly variable in function with species ranging from fibrolytic to pathogenic (Jandhyala et al., 2015). The Bacteriodetes phylum contains bacteria capable of degrading polysaccharides, such as cellulose and pectin, found in plant cell walls (Thomas et al., 2011). Numerous studies have looked at factors such as age, diet, and disease and how they influence the human gut microbiota. The human gut microbiota is rapidly colonized after birth and continues to increase in species diversity, number of different species, and undergoes compositional changes until it reaches stability within the first few years (Palmer et al., 2007). In healthy adults there is generally increased abundance of Prevotella spp., associated with high fiber and plant based diets, whereas Bacteroides are more abundant in high protein and fat diets (Wu et al., 2011). Although not necessarily caused by changes in gut microbiota, there is increased abundance of Firmicutes in people who are obese versus lean (Ley

et al., 2006). In addition, differences in gut microbiota can also be correlated to gastrointestinal problems, such as Crohns's disease, ulcerative colitis, and irritable bowel syndrome (Frank et al., 2007; Dicksved et al., 2008; Carroll et al., 2011).

In the pig there is a shift in bacterial composition throughout the GIT, particularly between the small and large intestine. The ileum consists of Firmicutes and Proteobacteria whereas the cecum and colon are made up of Firmicutes, Proteobacteria, Bacteriodetes and Spirochaetes (Looft et al., 2014). Through metagenomic analysis Looft et al. (2014) also found that the microbiota in the small intestine encode for genes associated with monosaccharide and amino acid uptake. In contrast, the large intestinal microbiota contains more genes for polysaccharide degradation, associated with Bacteriodetes. These observations show variation in composition in the different compartments in the GIT and further illustrate differences in how bacteria aid in digestion. Similar to the large intestine, pig fecal samples are also made up of mostly Firmicutes and Bacteriodetes (Lamendella et al., 2011).

Most studies in cattle related to GIT microbiota focus on the rumen, as it is the major site of microbial fermentation. The rumen contains trillions of bacteria that are responsible for breaking down plant fiber and producing volatile fatty acids (VFAs) and microbial protein (Deusch et al., 2015). At the phylum level, the rumen is made up of mostly Firmicutes and Bacteriodetes, followed by Proteobacteria, Actinobacteria and Tenericutes; however, there is variation in abundance of these phyla between individuals (Jami and Mizrahi, 2012). There are also production implications associated with rumen microbial composition in cattle. For example, Jami et al. (2014) observed increased milk fat yield to be associated with an increased Bacteriodetes to Firmicutes ratio, suggesting that rumen bacteria are linked to host physiological parameters. Meale et al (2016) also observed correlations between physiology and fecal bacterial

composition in cattle. The Bacteroides genus was negatively correlated with body weight, feed intake and VFA production, whereas the genera Pretovella, Succinivbrio, Sharpea and Ruminococcus were all positively correlated.

Although mammalian gut microbiota share the same three dominant phyla (Firmicutes, Bacteriodetes, and Proteobacteria), there are numerous differences in relative abundance and bacterial community composition at lower taxonomic levels (Jami et al., 2014). Furet et al. (2009) found the horse to be intermediate between the cow and pig in terms of fecal microbiota composition. These differences between livestock species may be related to GIT anatomy and function. Although the horse is classified as a monogastric like pigs, it relies on bacterial fermentation in the cecum and colon to produce VFAs, more similarly to the bacterial fermentation in the rumen of cattle.

Horses are hindgut fermenters that rely on bacteria to break down plant structural carbohydrates and produce VFAs that account for 65% of energy production (Al Jassim and Andrews, 2009; Steelman et al., 2012). The microbes in the equine GIT are essential to health due to their role protecting against pathogens, maintaining immune tolerance of beneficial microbiota, neutralizing toxins, and regulating host gene expression in epithelial cells (Wu et al., 2012).

Technological Advances

Initially, culture-dependent assessments were used to evaluate the intestinal microbiota. These culture-based studies provided information only on a limited range of bacteria that could be cultured, such as Lactobacilli and Streptococcus (Muhonen et al., 2009). In addition, since not all bacteria were accounted for in culture-based evaluations, it is possible that influential changes in GIT bacteria went undetected. Although beneficial to detecting specific culturable species, the

use of culture plating to evaluate the entire composition of the equine intestinal microbiota is not feasible (Costa and Weese, 2012). For example, White and Prior (1982) administered a broad spectrum antibiotic to horses, but saw no decrease in the culturable bacteria, suggesting that the antibiotics administered were targeting bacteria that could not be cultured.

With the advancement of technology came the increased ability to accurately and more completely evaluate the intestinal microbiota. Numerous molecular technologies, including the use of the 16S rRNA gene PCR based methods, have made it possible to identify bacterial communities and specific taxa (Costa and Weese, 2012). Unique to prokaryotes, the 16S rRNA is a component of the small 30S ribosomal subunit. The 16S rRNA gene contains conserved regions that are the same across all bacterial species as well as hypervariable regions that are species specific (Patel, 2001). Specific PCR primers can be designed to bind to the conserved regions on the 16S rRNA gene and once bacterial 16S rRNA genes are sequenced, bacterial communities can be subsequently identified based on the nucleotide base differences in the hypervariable regions. The 16S rRNA gene can be used to measure bacterial communities in a number of ways including community fingerprinting techniques and high-throughput sequencing.

Non-Culture Based Techniques

Community Fingerprinting

Community fingerprinting consists of molecular techniques that are used to analyze microbial community structure (Ramette, 2009). Following PCR amplification of the 16S rRNA gene, community fingerprinting separates diverse sequences based on guanine-cytosine (G-C) content or a small number of base pair differences and a profile of microbial communities is

generated to measure variation in bacterial diversity and changes over time (Rastogi and Sani, 2011).

There are a number of fingerprinting techniques that have been used to profile equine intestinal bacterial diversity. Terminal restriction fragment length polymorphism (T-RFLP) has been used to profile the community composition of the equine intestine using restriction enzymes and electrophoresis to separate DNA fragments by length (Rastogi and Sani, 2011). Willing et al. (2009) compared fecal bacteria of horses fed concentrate versus forage-only diets using T-RFLP and found that horses fed the forage-only diet had a more temporally stable microbial composition. Recent studies have also used T-RFLP to profile the bacterial diversity between different regions of the equine GIT (Dougal et al., 2012; Schoster et al., 2013).

Another community fingerprinting method is denaturing gradient gel electrophoresis (DGGE). Through DGGE, DNA sequences are separated based off variation in G-C content (Rastogi and Sani, 2011). Although this method is useful in comparing differences between samples, it does not measure relative abundance of specific species present or identify specific taxa (Costa and Weese, 2012). This fingerprinting method has been used to identify shifts in cecal bacterial populations associated with laminitis in the horse (Milinovich et al., 2008). Earing et al. (2012) used DGGE in comparing the number of different species in fecal samples of mare and foal pairs.

An additional fingerprinting technique used in determining equine intestinal bacteria communities is automated ribosomal intergenic spacer analysis (ARISA), which determines bacterial community structure based off of differences in length between the 16S and 23S rRNA genes. Sadet-Bourgeteau et al. (2014) found that bacterial community structure from equine fecal samples were significantly different from the cecum and colon when using ARISA. Changes in

bacterial community structure before and after weaning in foals have also been evaluated with the use of ARISA (Faubladier et al., 2013). Although community fingerprinting methods can determine differences between samples and estimate the number of species, these techniques lack the ability to characterize taxa and measure abundance (Proudman et al., 2015).

In Situ Hybridization

In situ hybridization has been used to target nucleotide sequences of specific bacterial species. Millinovich et al. (2006) monitored changes in the equine hindgut bacterial populations during oligofructose-induced laminitis using fluorescent in situ hybridization (FISH), which specifically targets organisms of low abundance, in this case Streptococcus spp. Oligonucleotide-RNA hybridization can also be used to detect the relative sizes of microbial populations. Daly et al. (2012) used this method to compare the abundance of predominant bacterial groups in response to diet and intestinal disease in the horse. They found that differences in diet correlated with alterations in the intestinal microbiota (Daly et al., 2012).

Next Generation Sequencing

The development of next generation sequencing (NGS) has allowed for a greater understanding of microbiota found in the GIT. Through the use of next-generation high-throughput sequencing, the taxonomic classification of microbial communities in the equine GIT can now be identified (Proudman et al., 2015).

The first step to sequencing bacterial DNA is to extract DNA from the sample material, which in the horse is usually fecal. The second step involves amplifying the 16S rRNA gene though PCR. Specific barcoded primers are used to computationally identify samples after sequencing. Then PCR products are cleaned and the amplicons are combined into a library based

on equal amounts of DNA. The library is then purified and quantified, to make sure the library is a uniform size, and finally analyzed through NGS technology.

The two main platforms of NGS used in the evaluation of the equine intestinal microbiota are Roche 454 and Illumina. Roche 454 uses pyrosequencing technology where pyrophosphate is released and converted to light that corresponds to different nucleotides (Balzer et al., 2010). Although Roche 454 was the first commercially available NGS platform in 2004, it is not currently as popular because of higher reagent costs (Liu et al., 2012). Illumina is currently the most widely used platform in NGS (Metzker, 2010). Illumina uses a sequencing-by-synthesis technology by which amplified template sequence is measured using a four-colored cyclic reversible termination method (Metzker, 2010). Each color corresponds to a nucleotide base that is detected through fluorescence (Illumina, 2012).

High-throughput sequencing was first used in the evaluation of equine intestinal microbiota through the use of 16s rRNA cloned libraries. Daly et al. (2001) sequenced cloned 16S rRNA genes to determine the bacterial diversity of the equine large intestine. They found a high genetic diversity from bacterial populations found in the cecum and colon; however, the majority of sequences recovered could not be assigned to a recorded sequence in the database, therefore unique species could not be identified (Daly et al., 2001).Shepherd et al. (2012) were the first to evaluate the equine fecal bacterial community through pyrosequencing of 16S rRNA gene amplicons from fecal samples of two adult horses fed a forage diet. Firmicutes (43.7%) was found to be the dominant phylum followed by Verrucomicrobia (4.1%), Proteobacteria (3.8%), and Bacteriodetes (3.7%). Although this study contained novel information about the equine intestinal bacteria, there were many limitations including a high percentage of unclassified sequences, and sequencing was only performed on fecal samples from two horses (Shepherd et

al., 2012). Costa et al. (2012) also used 454 pyrosequencing to compare the fecal microbiota of healthy horses and those with colitis. In agreement with the previous study, Costa et al. (2012) found that the Firmicutes phylum predominated in the fecal samples of six healthy horses; however, Bacteriodetes and Proteobacteria were found in greater abundance than previously reported. In more recent years the Illumina platform has been used to determine the equine fecal microbiota after administration of antibiotics, to compare different compartments of the GIT, and to understand changes during transportation, fasting and when under anesthesia (Costa et al., 2015a; Costa et al., 2015c; Schoster et al., 2015). The use of NGS technologies to identify specific bacterial taxa allows for a better understanding of the healthy equine intestinal microbiota structure as well as factors that can alter the normal state.

Limiting Factors

Advances in technology have allowed for a greater understanding of the composition of the equine intestinal microbiota; however, there are still limitations in its evaluation. Although some studies have used samples from the GIT of euthanized horses or those with cecal cannulas to evaluate the microbial composition of the intestine, most research relies on fecal samples due to the ease of collection. The use of fecal samples is convenient, but may not be representative of the whole intestinal microbiota, as the structure and function throughout the GIT is different for individual compartments (Costa and Weese, 2012). Schoster et al. (2013) were the first to compare the microbial composition of different segments of the GIT to fecal samples using community fingerprinting techniques. There were few similarities in the species richness (estimated number of bacterial species) found throughout the equine GIT with species richness in feces being most similar to the cecum. Dougal et al (2013) observed differences in the core microbiome (key microbes that are present in all or the majority of individuals within a

population) in different compartments of the equine large intestine at the phylum level. The core microbiome in regions of the proximal large intestine differed from distal intestinal regions and feces. These changes might be attributed to the pelvic flexure in between the left ventral colon and the left dorsal colon and further suggests that feces are not representative of the intestinal contents from more proximal regions of the GIT (Dougal et al., 2013). Similarly to the previous study, Costa et al. (2015a) found the Firmicutes phylum to be predominant in all compartments of the equine GIT. In addition, they did not observe statistical differences in the bacterial communities in the compartments of the hindgut proximal to the rectum and feces at higher taxonomic levels, suggesting that feces are partially representative of the dorsal and small colon. The taxonomic classifications of bacteria found in the hindgut are summarized in Table 1-1. Firmicutes and Bacteriodetes were the most abundant phyla found in the colon and cecum (Dougal et al., 2013; Moreau et al., 2014; Hansen et al., 2015); however, Costa et al (2015b) found Verrucomicrobia in greater abundance than Bacteriodetes.

In addition to differences based on sample location in the GIT, one of the initially limiting factors in the utility of NGS data was due to low sample size. For example, the first study to use NGS in characterizing the equine fecal microbiota only used two horses (Shepherd et al. 2012), while the highest number reported used 42 (Bordin et al., 2013). Since it has been shown that there is a wide variation of bacterial composition among individuals under the same environmental conditions it is not always possible to extrapolate certain research results to all horses. It also limits the ability to see less dramatic changes occurring between treatment groups.

Another limitation in the analysis of the equine intestinal microbiota includes low taxonomic resolution. Although some studies show differences in microbiota at the phylum to genus level (Table 1-1), there are some changes that could be occurring among species that do

not have known sequences in the database and therefore cannot be identified. In addition, there could be species that are at such low abundance they cannot be detected through current sequencing technology, even though they may be necessary or detrimental to equine intestinal health.

Although some potential functional pathways of bacteria communities can be determined through metagenomics, it is unknown if there is a causal relationship between changes in the taxonomic identity of the equine microbiota and physiological changes in the horse. Therefore, cause and effect cannot be determined through current sequencing methods alone.

Table 1-1. Classification of bacterial genera in the equine hindgut using next-generation sequencing techniques[1] (Julliand and Grimm 2016)

Phylum	Class	Order	Family	Genus
Firmicutes (20–59%)	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
			Lactobacillaceae	Lactobacillus
		Bacillales	Bacillaceae	Bacillus
				Solibacillus
	Clostridia	Clostridiales	Catabacteraceae	
			Clostridiacaee	Clostriduim Sarcina
			Eubacteriaceae	Eubacterium
			Lachnospiraceace	Butyrivibrio and Pseudobutyrivibrio
			Ruminococcaceae	Ruminococcus and Sporbacter
	Erysipelotricia	Erysipelotrichales	Erysipelotrichaceae	
	Negativicutes	Selenomondales	Acidomonoccaceae	
			Veillonellaceae	Megasphaera, Mitsuokella, and Veillonella
Bacteroidetes (2–65%)	Bacteroida	Bacteroidales	Porphyromonadaceae	
			Prevotellaceae	Prevotella
			Rikenellaceae	
	Sphingobacteria	Sphingobacteria		
Proteobacteria (0–14%)	Beta-proteobacteria	Burkholderiales	Alcaligenaceae and Oxalobacteraceae	
	Deltaproteobacteria			
	Gamma-proteobateria	Aeromondales	Succinivibrionaceae	
		Enterobacteriales	Enterobacteriaceae	Serratia
		Pasteurellales	Pasteurellaceae	Actinobacillus
		Pseudomonales	Pseudomanadaceae	Pseudomonas
Verrucomicrobia (0–24%)	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
			Akkermansiaceae	Akkermansia
Spirochaetes (1–9%)	Spirochaetes	Spirochaetales	Spirochaeteceae	Treponema
Fibrobecteres (1-7%)	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	Fibrobacter
Actinobacteria (0–2%)	Actinobacteria	Coriobacteriales	Coriobacteriaceae	
Tenericutes	Mollicutes	Anaeroplamatales	Anaeroplasmataceae	
		Mycoplamatales	Incertaesedis	

[1]Results from Dougal et al. (2013), Moreau et al. (2014), Costa et al. (2015c), and Hansen et al. (2015).

Factors Influencing the Equine Intestinal Microbiota

The equine intestinal microbiota can be altered by numerous factors including diet, disease, and age (Willing et al., 2009; Costa et al., 2012; Steelman et al., 2012; Costa et al., 2015b). Although we know it is sensitive to change and differs based on location within the GIT, it is still unclear what the specific composition of a "normal" equine bacterial population is because of the high variation in intestinal microbiota among individuals (Steelman et al., 2012). There can be both breed differences as well as individual variation in composition in horses under the same environmental and management conditions (Proudman et al., 2015). Understanding the variability of the equine intestinal microbiota is crucial because diseases affecting the GIT are the leading cause of death in horses (Al Jassim and Andrews, 2009). The high individual variation in intestinal microbial composition could make it more difficult to use changes in microbiota as a diagnostic tool. A more detailed understating of the factors that influence the intestinal microbiota in horses could be beneficial in improving management practices, such as formulating diets to help decrease occurrences of disease.

Diet

Dietary changes can influence the composition of the equine intestinal microbiota. Horses rely on microbes in the hindgut to break down fiber to produce VFAs, and different diets favor the establishment of specific bacterial communities. Using T-RFLP, Willing et al. (2009) evaluated the relationship between diet and fecal microbiota by comparing the effect of a high energy forage-only diet with a forage-concentrate diet. There were no changes in diversity between diets; however, horses on the forage-only diet had fewer changes in fecal microbial composition and lower number of lactic acid producing bacteria compared to the forage-concentrate diet. Similar to studies with oligofructose-induced laminitis, the concentrate diet had

an increased relative abundance of Streptococcus spp., suggesting forage-only diets may be more beneficial in avoiding intestinal disorders since horses evolved to be continuously grazing on pasture and their intestinal tract is not designed to handle high-starch diets (Willing et al., 2009).

Abrupt changes in the diet have also been shown to change fecal microbiota in horses. Fernandes et al. (2014) observed changes in fecal bacterial abundance due to an abrupt dietary transition from a fermented forage-grain diet to a pasture diet. The diversity of bacterial communities in fecal samples increased within 4 d of converting from a forage-grain diet to pasture and the relative abundance of individual taxa changed in response to the change in diet. Hansen et al (2015) also observed an increase in diversity in cecal bacteria of horses fed only hay compared to those supplemented with oats. Ecological theories suggest that higher diversity increases community stability; therefore, lower nutrient availability should stabilize the intestinal microbiota (Hansen et al., 2015). These results further support the notion that forage and pasture diets are more beneficial to maintaining a healthy equine intestinal microbial balance.

The equine intestinal microbiota can also be altered depending on carbohydrate concentrations in the diet. Increased starch in concentrate diets can potentially lead to problems in the equine GIT. Day et al. (2011) found increased relative abundances of Lachnospiraceae, Bacteriodetes, and lactic-acid producing bacteria in horses fed concentrates. These bacterial taxa readily ferment carbohydrates, subsequently producing lactic acid and decreasing cecal and colonic pH. In addition, this study also reported decreased abundance of fibrolytic, acid-intolerant bacteria such as Fibrobacter and Rumminococeae due to the decrease in pH and reduction in fiber digestion associated with high-concentrate diets. Changes in the microbial composition can alter fermentation products which could lead to intestinal disease (Daly et al., 2011). In addition, Destrez et al. (2015) evaluated changes in the equine hindgut microbiota

through culture techniques and demonstrated that high-starch low-fiber diets can not only modulate the microbiota, but also affect horse behavior through increased stress. Proudman et al. (2015) detected changes in the fecal microbiota following dietary supplementation of amylase-rich malt extract. There were changes in certain taxa of lower abundance, including an increase in Veillonellaceae. Veillonellaceae are lactate-utilizing bacteria that could be beneficial to buffering decreased pH in the cecum caused by increase in lactic acid production from microbial fermentation of carbohydrates (Proudman et al., 2015). Although there are definite differences in fecal microbiota of horses fed high-forage versus concentrate diets, or diets with increased carbohydrates, there needs to be further research evaluating the influence of different diets on the equine intestinal bacteria.

Probiotics

Probiotics are live microorganisms that are fed to an animal in order to elicit health benefits (FAO/WHO, 2002, p. 8). The use of probiotics can benefit livestock during stressful events such as weaning, lactation, and shifts in diets through increasing stability of the microbiota (Chaucheyras-Duran and Durand, 2010). Although there are numerous equine probiotic supplements available on the market there is currently little to no research on the efficacy and standard quality control practices are lacking (Schoster et al., 2014). Most studies involving probiotic supplementation in the horse use the yeast Saccharomyces cerevisiae with mixed results on whether the supplement improves fiber fermentation and digestibility (Coverdale, 2016). Increased understanding of the equine intestinal microbiota will improve the potential development of more beneficial probiotics. Further research needs to be done to determine the beneficial bacterial strains and dosages in the horse. Currently, types and amounts

of probiotics are currently based on those developed for ruminants, which may not be in high enough concentrations to colonize the equine hindgut (Coverdale, 2016).

Diseases

Laminitis

One of the best characterized conditions in which changes in the equine intestinal microbiota can be observed is oligofructose-induced laminitis. Laminitis is caused by various factors and characterized by inflammation of the laminae in the hoof, which in severe cases can lead to failure of attachment of the laminae and subsequent rotation of the coffin bone. One condition associated with laminitis is lactic acidosis. High concentrations of starch in the diet can escape digestion in the small intestine and be rapidly fermented in the hindgut, thus greatly increasing microbial lactic acid production (Al Jassim and Andrews, 2009). Another potential cause of laminitis is through the production of endotoxins in the hindgut. In the carbohydrate-induced laminitis model, vasoactive amines and endotoxins are formed by bacteria in the intestine and can potentially enter the circulation, contributing to inflammation in the hoof (Eades, 2010).

Changes in cecal microbiota associated with acute laminitis were first documented by Garner et al. (1978) using culture based methods. In cecal samples from horses fed a carbohydrate-overloaded diet there was an overgrowth of Streptococcus and Lactobacillus as well as decreases in cecal pH (Garner et al., 1978). Millinovich et al. (2006) used FISH to monitor changes in Streptococcal, oligofructose-utilizing bacteria, in horses after administration of oligofructose to induce laminitis. Considerable changes occurred within 6 to 8 h of oligofructose administration, with Streptococcus infantarius subsp. coli increasing dramatically before the onset of lameness. Millinovich et al. (2008) further evaluated equine hindgut

Streptococcal species using DGGE. They found Streptococcus lutentiensis to be the predominant species before the onset of laminitis, whereas Lactobacilli and Escherichia coli were not determined to be major contributors (Milinovich et al., 2008).

The first study to utilize NGS technologies used pyrosequencing to measure bacterial communities in fecal samples of horses with chronic laminitis. Steelman et al. (2012) observed an increase in bacterial diversity in cecal samples from horses with chronic laminitis. In addition, two Clostridiales genera were in greater abundance in laminitc horses compared with controls (Steelman et al., 2012). In agreement with previous studies, Moreau et al. (2014) found that the relative abundance of Lactobacillus spp. and Streptococcus spp. increased following cornstarch and oligofructose administration. There was also an increase in potentially pathogenic gram-negative bacteria, which could be contributing to entercolitis, pyrexia, and lameness associated with carbohydrate-overload induced laminitis (Moreau et al., 2014).

Colic

There are numerous potential causes of colic including parasites, change in diet, and transportation (Al Jassim and Andrews, 2009). Shifts in microbial composition have been associated with colic in horses. Costa et al. (2012) found Bacteriodetes to be the dominant phylum along with an increase in abundance of Fusobacteria in horses with colitis, whereas the Firmicutes phylum and Clostridiales class were the most abundant taxa in healthy horses. The differences between horses with colitis and healthy horses may be due to dysbiosis, an imbalance of microbiota present, rather than an increase in specific pathogenic bacteria. It is still unclear whether changes in the equine intestine microbiota are causative of colitis (Costa et al., 2012).

One of the greatest incidences of colic occurs in mares within 60 d prior to foaling. It is possible that metabolic and hormonal changes associated with pregnancy disrupt the normal

intestinal microbiota composition. In women, there is an increase in abundance of Proteobacteria leading up to parturition, and increases in Proteobacteria have been associated with intestinal diseases (Koren et al., 2012). Weese et al. (2015) observed changes in fecal microbiota in post-partum mares prior to development of colic. Changes in fecal microbiota in mares that developed colic after foaling were detected within 10 days of onset. This included a decreased abundance of Firmicutes and Bacteriodetes with increased abundance of Proteobacteria (Weese et al., 2015). There have been documented changes in the equine intestinal microbiota associated with colic; however, it remains to be determined if there is a causal relationship and if changes in the microbiota are similar for different forms of colic (Costa et al., 2012). In addition, because of the high individual variation, it is unknown whether these changes in fecal microbiota would be seen in horses of a different production class or only in postpartum mares. The changes in fecal microbiota observed before a colic episode could potentially be used to predict specific types of colic to determine surgical options or in choosing probiotics to stabilize intestinal microbiota. Further research needs to be done to determine how useful these changes are in predicting colic and preventing or treating incidences of colic in horses.

Other

Numerous other factors could potentially disrupt the normal composition of the equine intestinal microbiota. One of the most apparent changes in the equine fecal microbiota is seen with the use of antimicrobial drugs. Significant changes occurred in the fecal microbiota of horses following administration of antimicrobials (Harlow et al., 2013). Costa et al. (2015c) reported that oral administration of Trimethoprim sulfadiazine, a broad spectrum antibiotic, had the greatest reduction in total number of bacterial species in fecal microbiota, with a prominent decrease in the relative abundance of Verrucomicrobia. After antibiotic treatment ended,

bacterial profiles did not completely return to pre-treatment composition by 30 d post-administration, suggesting a recovery period is needed before the intestinal microbiota returns to baseline. There is also potential for variability in response of horses treated with antimicrobials due to differences in individual intestinal microbial composition prior to treatment (Costa et al., 2015c).

There is little research on the effects of stress on equine intestinal microbiota even though gastrointestinal diseases can develop in association with stressful events. Schoster et al. (2015) recently observed that transport, fasting, and anesthesia significantly changes the relative abundances of bacterial communities in horses. There was a decrease in abundance of Clostridiales after transport and this might be negatively impacted by stress. Stressful events could negatively contribute to horse health through changes in the intestinal microbiota and warrants further investigation.

Development of the Fecal Microbiota

Age is an important factor in the microbial composition of the GIT of all mammals. There are dramatic changes in the intestinal microbiome that occur starting at birth as the microbial composition reaches that of a healthy adult. The composition and time at which the intestinal microbiota reach stability varies by species and is largely influenced by changes in diet (Lozupone et al., 2012; Jami et al., 2013; Pajarillo et al., 2014; Costa et al., 2015b).

Initial Colonization

The initial colonization of the GIT is heavily influenced by the environment. It was first thought that the intrauterine environment is sterile; however, bacteria have been isolated from human placentas and umbilical cords that could influence the newborn microbiota (Meropol and Edwards, 2015). Although only present in low levels, the bacteria from amniotic fluid and fetal

membranes could be important in preparing the immune system for bacterial exposure and in contributing to gut maturation (Thum et al. 2012; Romano-Keller, 2014).

After birth there is a rapid succession of bacterial species that colonize the GIT through environmental and maternal exposure. The high richness in intestinal bacteria after birth could be attributed to environmental exposure to thousands of microbial species (Slifertz et al., 2014). The greatest instability in the intestinal microbiota occurs early in life which could lead to a higher susceptibility of diseases in young animals and potentially create problems with the gut microbiota that affects the animal later in life (Lozupone et al., 2012). The initial bacteria to colonize the developing GIT could contribute to immune function by blocking access to gut epithelial cells, through enhancing tight junctions between cells, and stimulating antibody, Immunoglobulin A, production (Kelly et al., 2007). They may also serve to allow the immune system to distinguish between helpful symbiotic and harmful pathogenic bacteria (Meropol and Edwards, 2015).

In humans, the mode of delivery greatly influences the initial colonization of bacteria. Vaginally-delivered infant intestines are colonized through fecal-oral and vaginal transfer of bacteria, whereas those delivered through caesarian section are colonized by bacterial species found on their mothers skin (Meropol and Edwards, 2015). Vaginal microbiota is dominated by Lactobacillus, whereas Staphylococcus is the main bacteria found on skin (Aagard et al., 2012). Initially, infants born via C-section have decreased bacterial richness and diversity as compared with vaginal delivery (Romanno-Keller, 2014). There is also a delayed colonization from members of the Bacteriodetes phylum in infants born through C-section (Jakobsson et al., 2014).

In addition to exposure through the birthing process, the infant will also be exposed to bacteria through nursing. Commensal bacteria found in milk could be explained by an entero-

mammary pathway, where bacteria are transferred from the GIT to the mammary gland through the lymphatic system (Addis et al., 2016). Breast milk is dominated by the Proteobacteria and Firmicutes phyla (Urbaniak et al., 2016). Bacteria found in breast milk are similar to the early colonizing bacteria found in infant feces, suggesting that this is another route in which microbes are introduced to the intestines (Thum et al., 2012). Furthermore, bacteria in milk could also be priming the infantile immune system to recognize commensal microbial species (Addis et al., 2016). The bacterial composition of mare milk and its influence on the developing intestinal microbiota in the horse has yet to be determined.

Prior to nursing, a complex microbial community can be isolated from meconium (Rodriguez et al., 2015). Bacteria found in meconium are not influenced by mode of delivery and are low in species diversity (Hu et al., 2013). However, there was increased relative abundance of the genus Parabacteroides in offspring of mothers with diabetes. In addition, meconium dominated by lactic acid bacteria was associated with respiratory problems in the infant (Gosalbes et al., 2013). Initial colonizers of the GIT in humans are known to be Bacteroides, Clostridium and Bifidobacterium (Rodriguez et al., 2015).

Development of the intestinal microbiota can influence occurrence of metabolic and immune mediated diseases with lifetime impacts on health (Rodriguez et al., 2015). Exposure to diverse bacteria is necessary for normal maturation of the immune system and decreased microbial exposure can lead to abnormal immune function (El Aidy et al., 2013). In addition, there are differences in the gut microbiota of infants with allergies versus those that are allergy-free; however, no specific taxa have are associated with allergies (West et al., 2014). Children with asthma have decreased species diversity compared to non-asthmatic children at 1 mo of age (Abrahamsson et al., 2014). Increased relative abundance of Staphylococcus spp. and lower

abundance of Bifidobacterium spp. have been found in obese infants during the first year of life (Kalliomaki et al., 2008).

Following initial colonization, there is variation between species on the timing of when a stable, mature microbial intestinal environment is reached. In pigs, this occurs about 2 to 3 wk post-weaning (Slifierz et al., 2015), whereas in horses stability is reached by 60 d (Costa et. al., 2015b). The rumen microbiota of cattle is not considered to reach that of an adult animal until 2 year of age (Jami et al, 2013). Humans are more similar to cattle in that their microbiota doesn't appear to reach stability until the age of 3 (Lozupone et al., 2012).

Foal Fecal Microbiota

Within the first few weeks of life there is an intense and rapid change in the fecal bacteria, which has been reported in studies that use culture, fingerprinting, and next-generation sequencing techniques. This temporal change in fecal microbiota continues during the first months of life and is correlated to the change in diet that begins to occur as the foal gradually transitions from a milk-based diet to consuming grain and forages (Costa et al., 2015b).

When using culture techniques to evaluate mare and foal fecal samples, bacteria could be isolated in culture on the day of birth, with similarities in the cultivable bacterial community structure to that of a mature horse by four weeks (Kuhl et al., 2011). Earing et al. (2012) were the first to use non-culture based techniques to estimate changes in bacterial community structure between mares and foal pairs. Through PCR-DGGE, bacteria were detected in foal feces as early as 24 h postpartum. It was also found that there was high similarity between mare-foal pairs by 84 d. In addition, the species richness of foal feces was similar to that of mature horses by six wk (Earing et al., 2012). Another study that used DNA fingerprinting to evaluate foal fecal bacteria also found commensal bacteria in foal meconium (Faubladier et al., 2013). The highest

variability within individual foals occurred in the first 2 d of life, potentially explained by environmental and maternal exposure to numerous and diverse bacteria. It is thought that changes that occurred between 2 to 30 d are attributed to the introduction of solid feed. Faubladier et al. (2013) also found no differences in species richness between foal fecal samples after 30 d, suggesting maturation towards an adult bacterial community. Although these studies provide insight into the initial development of the equine intestinal microbiota, they have several limitations including the inability to determine species abundance and identify specific bacterial species.

The first study to utilize NGS technologies to classify foal fecal bacteria evaluated the changes between 2 and 30 d after birth (Bordin et al., 2013). The number of operational taxonomic units (OTU), representative of bacterial species, increased with age with an increase in relatice abundance of the phylum Bacteriodetes between 2 to 30 d. There was also a significant decrease in Proteobacteria with age. Increase in the Bacteriodetes phylum, which contain fibrolytic and cellulolytic bacteria, in the first month could be attributed to the introduction of forages to the diet. Although specific bacteria present within the first month were determined and differences with age were seen, this study was limited by only having two time points to compare. Costa et al. (2015b) were the first to characterize the foal fecal microbiota through next-generation sequencing over an extended period of time. A high bacterial richness was observed 24 h after birth which is in contrast with studies that used DNA fingerprinting. Fingerprinting techniques have a limited detection of low abundance bacteria which could underestimate bacteria present. In agreement with previous studies, it was found that foals reached a stable intestinal microbiota around 60 days, with the Firmicutes and Verrucomicrobia phyla accounting for the majority of the bacteria at this time. Although stability was reached

around 60 d, there were still differences in the abundance of certain genera found between 9 month old horses and adults. The most prevalent species seen from samples taken between 2 to 30 days was Akkermansia municiphilla. This mucin-degrading bacterium, from the Verrucomicrobia phylum, is associated with a healthier metabolic status in humans and inversely related to body fat in mice (Dao et al., 2016). Akkermansia spp. was also found to be in high proportion in the rumen of calves on an exclusively milk diet (Jami et al., 2013). The function of this species in the early colonization of the GIT warrants further research. A study by Whitfield-Cargile et al. (2016) classified the foal fecal microbiota and predicted metagenome, used to predict functional pathways, during the first month of life. The largest changes seen from 3 to 5 wk were a decrease in abundance of Firmicutes and an increase in Bacteriodetes. These findings further support the theory that changes in the microbiota composition are correlated to the changes in diet that are occurring over the first month of life. There is a shift in the gene functional potential of the microbiota from metabolizing simple sugars in milk to more complex fermentation of insoluble fibers in forages (Whitefield-Cargile et al., 2016). Although the limited research generally agrees that the foal fecal microbiota stabilizes between 30 to 60 d as the animal beings to incorporate grains and forages into its predominantly milk diet, the effect of weaning on the intestinal microbiota of the horse is still unclear.

Weaning

Weaning occurs when the foal ceases to consume milk from the dam. The most common age to wean domestic horses is between 4 to 6 m (Waran et al., 2008). It is a physiologically and nutritionally stressful transition and has been shown to impact the intestinal microbiota of monogastric animals such as rabbits and pigs. In these species the intestinal microbiota converges towards that of an adult post-weaning (Slifierz et al., 2015). In pigs, Firmicutes is the

dominant phyla pre-weaning with a shift towards Bacteriodetes post-weaning. At the genus level there is also a shift from Bacteroides to Prevotella at weaning (Pajarollo et al., 2014). Species in the Bacteroides genus use simple sugars like those found in milk whereas species in the genus Protovella degrade insoluble fiber found in plant-based feed sources (Lamendella et al., 2011). This shift in GIT bacteria in pigs corresponds to the change in diet from milk to solid feed.

Weaning and Stress

Nutrition

The most profound biological change that occurs at weaning is the shift in diet. Although foals begin consuming forages and concentrates early in life, they still heavily rely on nutrients from milk until weaning (Ladewig et al., 2005). It has also been noted that weaning can be associated with a significant, although temporary, weight loss (Waran et al., 2008). The introduction of creep feed, the practice of offering concentrated grain prior to weaning, may reduce stress associated with weaning (Hoffman et al., 1995). In addition, foals fed creep feed prior to weaning gain more weight compared to those not offered a concentrate (McCall et al., 1987). The composition of the diet at weaning may also play an important role. High starch, low fiber diets increased the risk of foals developing abnormal and aggressive behaviors (Zeyner et al., 2004, Bachmann et al., 2003). When fed a high fat and fiber diet, foals had calmer temperaments than foals fed a high sugar and starch diet (Nichol et al., 2005). The introduction of a low sugar and starch creep feed could be beneficial to both maintaining body condition and alleviating stress in foals during weaning.

Physiological Changes

There are numerous physiological changes that occur at the time of weaning in foals due to stress. Some biological responses include behavioral changes, increased heart rate, increased

respiration, and increased secretion of adrenal glucocorticoids such as cortisol. Physiological responses to stress are generally associated with the hypothalamic-hypophyseal-adrenal axis. The release of ACTH, and subsequently cortisol, increase following a stressful event and remain elevated until the stress is removed or the animal adapts (McCall et al., 1987). In the horse, a number of stressors have been shown to cause an increase in circulating cortisol concentrations, such as exercise, trailering, and disease (Gold et al., 2012; Kedzierski et al., 2014; Tadich et al., 2015). Chronic stress, and subsequent release of glucocorticoids, can suppress the cell-mediated immune response (Malinowski et al., 1990). Stress at weaning has been measured in foals through increased concentrations of cortisol in plasma, saliva, and feces when compared with pre-weaning concentrations (McCall et al., 1987; Moons et al., 2005; Merkies et al., 2016). Fazio et al. (2009) found that fillies had higher levels of stress as seen through increased cortisol concentrations 24 h and 48 h post weaning compared to colts. In contrast, Hoffman et al. (1995) found no differences in cortisol concentrations post-weaning between genders, but blood samples were taken monthly. In addition to changes in hormone concentrations, increased heart rates at weaning are observed in foals in response to increased stress and activity at weaning (Erber et al., 2011).

Behavior

Some of the most obvious signs of stress at weaning are seen through behavioral changes, most notably increased locomotion, vocalization, and defecation (Moons et al., 2005). These behavioral changes can have a negative transient effect on appetite, immune function, and growth during weaning (Fazio et al., 2009). Certain foals have a tendency to develop stereotypical and aggressive behavior during weaning. Equine stereotypies are repetitive, abnormal behaviors that can be classified as oral or locomotor. Oral behaviors that have been

shown to increase during weaning include cribbing and biting (Nichols et al., 2005), while weaving is an example of a locomotor stereotypic behavior (Walters et al. 2002). Although some changes in behavior at weaning could be viewed as adaptive, the development of stereotypical behavior further supports the notion that weaning is a stressful event (Waran et al., 2008).

Weaning Methods

There are numerous methods for weaning horses. They all take into consideration multiple factors such as housing environment, foal development, available resources, and foal-mare attachment (Waran et al., 2008). The goal is to reduce the potential for injury and stress to both mare and foal while still being able to wean in a timely, efficient, and cost effective manner.

Housing

There are two categories of housing options for weaning which include weaning in a stall versus paddock or pasture and weaning individually or in groups. Foals weaned singly have increased vocal and locomotor responses compared with those weaned with another foal (Houpt et al., 1984). In addition to increased stress, foals weaned in stalls have decreased exercise, which if housed in stalls permanently could be correlated to a decrease in bone mineral content and susceptibility to orthopedic diseases (Reichman et al., 2004). There is also a greater occurrence of stereotypic and abnormal behaviors in stall-weaned foals (McGeevy et al 2005, Heleski et al., 2002). One might assume that weaning in pairs would reduce stress through companionship; however, foals stall weaned in pairs engaged in more aggressive behavior and had increased serum cortisol compared to foals stall weaned individually (Hoffman et al., 1995). Group weaning is generally associated with lower stress, if foals have enough space and if consideration is given to the social hierarchy to minimize aggression between foals (Waran et al., 2008).

Abrupt versus Gradual

The most common weaning practice is to abruptly remove the mare from the foal. This involves complete visual, auditory, and tactile removal of the mare. This method is favored because it is simple and cost-effective (Dubcova et al., 2015). On the other hand, gradual separation involves physical separation of the mare for an increasing amount of time each day until there is no longer contact between mare and foal. This method reduces the amount of time nursing, but still allows some daily physical contact. Gradual weaning methods reduce behavioral and physiological stress compared to abrupt weaning (McCall et al., 1987). However, short-term separation of mare and foal pairs prior to weaning did not have an effect on stress response in foals (Moons et al., 2005). Dubcova et al. (2015) found that a stepwise approach of moving a foal to a novel weaning environment reduced acute stress post weaning, but had negative impacts on growth rate. Merkies et al. (2016) used a two stage weaning method that first prevented nursing and then physically separated the mare and foal; however, this approach did not reduce stress at weaning. This suggests that physical separation is more stressful than the cessation of nursing that occurs at weaning (Merkies et al., 2016). These results support earlier findings by Malinowski et al. (1990) that indicated maternal separation at weaning is the major stressor.

Other variations in weaning methods can be utilized to reduce stress caused by maternal separation in horses. Interval weaning is a popular method which involves removing one dam from a unit of mares and foals overtime. This allows foals to remain with other horses and in a familiar environment during weaning (Holland et al., 1996). Fenceline weaning is another method that reduces stress by allowing foals to have visual and auditory contact with mares while being physically separated by a fence (McCall et al., 1985).

Another factor that helps eliminate stress at weaning is the addition of an unrelated adult horse, referred to as a babysitter. Henry et al. (2012) recorded behavioral and physiological responses in foals weaned in groups with other foals or with other foals and an adult horse. In all groups there was an increase in vocalization, locomotion and salivary cortisol concentrations at weaning; however, stress responses were less noticeable and for a shorter interval when foals were weaned with an adult (Henry et. al, 2012).

Weaning and the Foal Fecal Microbiota

There is very limited research on the response of the foal intestinal microbiota to weaning. Faubladier et al. (2014) used community fingerprinting to evaluate the differences in number of bacteria species pre and post-weaning and did not see any significant changes. It is possible that changes in the intestinal microbiota associated with the adaptation from consuming milk to a forage-based diet takes place gradually and therefore is not influenced by weaning. In addition, community fingerprinting is limited in that it only identifies of species richness and cannot detect specific bacteria taxa. Costa et al. (2015) found an increase in abundance of Proteobacteria and Fibrobacteres in post-weaning fecal samples through NGS; however, it is unknown how close to weaning this change in abundance occurred since samples taken within a 30 d period were grouped together and not from a specific time before and after weaning.

Stress is associated with significant behavioral and physiological changes. Schoster et al. (2015) observed changes in fecal microbiota in adult horses 12 h after transport and fasting, and 24, 48 and 72 h after anesthesia recovery. These are considered stressful events and transportation has also been correlated with increased cortisol (Tadisch et al., 2016).

It is unclear whether changes in fecal microbiota occur in response to weaning. It is possible that such a stressful event could have a greater impact on the intestinal bacteria in foals

with a compromised immune function versus healthy foals. There is also a possibility that the equine intestinal microbiota transitions to that similar of an adult prior to weaning with the gradual introduction to forages and grain and the method of weaning will not impact the intestinal microbiota. More frequent sampling is needed to determine whether the fecal microbiota can be altered by the dietary and environmental changes that occur at weaning. Different weaning methods could have different effects on the foal intestinal microbiota. In addition, the impact of weaning on the microbial composition of unhealthy foals could aid in management decisions to minimize problems that could occur at weaning. Knowing whether there is an effect of weaning itself or the method of weaning on fecal bacteria can aid in making management decisions at weaning.

Literature Cited

Aagaard, K., J. Ma, K. M. Antony, R. Ganu, J. Petrosino, and J. Versalovic. 2014. The placenta harbors a unique microbiome. Sci. Transl. Med. 6(237):237ra65. doi:10.1126/scitranslmed.3008599

Abrahamsson, T. R., M. Sandberg Abelius, A. Forsberg, B. Bjorksten, and M. C. Jenmalm. 2011. A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization. Clin. Exp. Allergy. 41(12):1729-1739. doi:10.1111/j.1365-2222.2011.03827.x

Addis, M. F., A. Tanca, S. Uzzau, G. Oikonomou, R. C. Bicalho, and P. Moroni. 2016. The bovine milk microbiota: Insights and perspectives from -omics studies. Mol. Biosyst. 12(8):2359-2372. doi:10.1039/c6mb00217j

Al Jassim, R. A., and F. M. Andrews. 2009. The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic, and stomach ulcers. Vet. Clin. North Am. Equine Pract. 25(2):199-215. doi:10.1016/j.cveq.2009.04.005

Bachmann, I. L. Audige, and M. Stauffacher. 2003. Risk factors associated with behavioural disorders of crib-biting, weaving and box-walking in Swiss horses. Equine Vet. J. 35(2) 158-163. doi:10.2746/042516403776114216

Balzer, S., K. Malde, A. Lanzen, A. Sharma, and I. Jonassen. 2010. Characteristics of 454 pyrosequencing data--enabling realistic simulation with flowsim. Bioinformatics. 26(18):i420-5. doi:10.1093/bioinformatics/btq365

Bordin, A. I., J. S. Suchodolski, M. E. Markel, K. B. Weaver, J. M. Steiner, S. E. Dowd, S. Pillai, and N. D. Cohen. 2013. Effects of administration of live or inactivated virulent rhodococccus equi and age on the fecal microbiome of neonatal foals. PLoS One. 8(6):e66640. doi:10.1371/journal.pone.0066640

Carroll, I. M., T. Ringel-Kulka, T. O. Keku, Y. H. Chang, C. D. Packey, R. B. Sartor, and Y. Ringel. 2011. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. Am. J. Physiol. Gastrointest. Liver Physiol. 301(5):799-807. doi:10.1152/ajpgi.00154.2011

Chaucheyras-Durand, F., and H. Durand. 2010. Probiotics in animal nutrition and health. Benef Microbes. 1(1):3-9. doi:10.3920/BM2008.1002

Costa, M. C., L. G. Arroyo, E. Allen-Vercoe, H. R. Stampfli, P. T. Kim, A. Sturgeon, and J. S. Weese. 2012. 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. 7(7):e41484. doi:10.1371/journal.pone.0041484

Costa, M. C., G. Silva, R. V. Ramos, H. R. Staempfli, L. G. Arroyo, P. Kim, and J. S. Weese. 2015a. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet. J. 205(1):74-80. doi:10.1016/j.tvjl.2015.03.018

Costa, M. C., H. R. Stampfli, E. Allen-Vercoe, and J. S. Weese. 2015b. Development of the faecal microbiota in foals. Equine Vet. J. 48(6):681-688. doi:10.1111/evj.12532

Costa, M. C., H. R. Stampfli, L. G. Arroyo, E. Allen-Vercoe, R. G. Gomes, and J. S. Weese. 2015c. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC Vet. Res. 11:19. doi:10.1186/s12917-015-0335-7

Costa, M. C., and J. S. Weese. 2012. The equine intestinal microbiome. Anim. Health. Res. Rev. 13(1):121-128. doi:10.1017/S1466252312000035

Coverdale, J. A. 2016. HORSE SPECIES SYMPOSIUM: Can the microbiome of the horse be altered to improve digestion? J. Anim. Sci. 94(6):2275-2281. doi:10.2527/jas.2015-0056

Daly, K., C.S. Stewart, H.J. Flint, and S.P. Shirazi-Beechey. 2001. Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes, FEMS Microbiol. Ecol. 38(2-3):141-151. doi:10.1016/S0168-6496(01)00178-7

Daly, K.,C.J. Proudman, S.H. Duncan, H.J. Flint, J. Dyer, and S.P. Shirazi-Beechey. 2012. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. Br. J. Nutr. 107(7):989–995. doi:10.1017/S0007114511003825.

Dao, M. C., A. Everard, J. Aron-Wisnewsky, N. Sokolovska, E. Prifti, E. O. Verger, B. D. Kayser, F. Levenez, J. Chilloux, L. Hoyles, MICRO-Obes Consortium, M. E. Dumas, S. W. Rizkalla, J. Dore, P. D. Cani, and K. Clement. 2016. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: Relationship with gut microbiome richness and ecology. Gut. 65(3):426-436. doi:10.1136/gutjnl-2014-308778

Destrez, A., P. Grimm, F. Cezilly, and V. Julliand. 2015. Changes of the hindgut microbiota due to high-starch diet can be associated with behavioral stress response in horses. Physiol. Behav. 149:159-164. doi:10.1016/j.physbeh.2015.05.039

Deusch, S., B. Tilocca, A. Camarinha-Silva, and J. Seifert. 2014. News in livestock research - use of omics-technologies to study the microbiota in the gastrointestinal tract of farm animals. Comput. Struct. Biotechnol. J. 13:55-63. doi:10.1016/j.csbj.2014.12.005

Dicksved, J., O. Schreiber, B. Willing, J. Petersson, S. Rang, M. Phillipson, L. Holm, and S. Roos. 2012. Lactobacillus reuteri maintains a functional mucosal barrier during DSS treatment despite mucus layer dysfunction. PLoS One. 7(9):e46399. doi:10.1371/journal.pone.0046399

Dougal, K., P. A. Harris, A. Edwards, J. A. Pachebat, T. M. Blackmore, H. J. Worgan, and C. J. Newbold. 2012. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol. Ecol. 82(3):642-652. doi:10.1111/j.1574-6941.2012.01441.x

Dougal, K., G. de la Fuente, P. A. Harris, S. E. Girdwood, E. Pinloche, and C. J. Newbold. 2013. Identification of a core bacterial community within the large intestine of the horse. PLoS One. 8(10):e77660. doi:10.1371/journal.pone.0077660

Dougal, K., G. de la Fuente, P. A. Harris, S. E. Girdwood, E. Pinloche, R. J. Geor, B. D. Nielsen, H. C. Schott 2nd, S. Elzinga, and C. J. Newbold. 2014. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 9(2):e87424. doi:10.1371/journal.pone.0087424

Dubcová,J., J. Bartošová, and M. Komárková. 2015. Effects of prompt versus stepwise relocation to a novel environment on foals' responses to weaning in domestic horses (Equus caballus). J Vet. Behav. 10(4):346-352. doi:10.1016/j.jveb.2015.03.003.

Eades, S. C. 2010. Overview of current laminitis research. Vet. Clin. North Am. Equine Pract. 26(1):51-63. doi:10.1016/j.cveq.2010.01.001

Earing, J.E., A.C. Durig, G.L. Gellin, L.M. Lawrence, and M.D. Flythe. 2012. Bacterial colonisation of the equine gut; Comparison of mare and foal pairs by PCR-DGGE. Adv Microbiol 2:79-86. doi:10.4236/aim.2012.22010.

El Aidy, S., P. Van den Abbeele, T. Van de Wiele, P. Louis, and M. Kleerebezem. 2013. Intestinal colonization: How key microbial players become established in this dynamic process: Microbial metabolic activities and the interplay between the host and microbes. Bioessays. 35(10):913-923. doi:10.1002/bies.201300073

Erber, R., M. Wulf, S. Rose-Meierhofer, M. Becker-Birck, E. Mostl, J. Aurich, G. Hoffmann, and C. Aurich. 2012. Behavioral and physiological responses of young horses to different weaning protocols: A pilot study. Stress. 15(2):184-194. doi:10.3109/10253890.2011.606855

Faubladier, C., S. Sadet-Bourgeteau, C. Philippeau, E. Jacotot, and V. Julliand. 2014. Molecular monitoring of the bacterial community structure in foal feces pre- and post-weaning. Anaerobe. 25:61-66. doi:10.1016/j.anaerobe.2013.11.010

Fazio, E., P. Medica, L. Grasso, C. Messineo,and A. Ferlazzo. 2009. Changes of circulating β-endorphin, adrenocorticotrophin and cortisol concentrations during growth and rearing in Thoroughbred foals. Livest. Sci. 125(1):31-36. doi:10.1016/j.livsci.2009.02.021.

Fernandes, K. A., S. Kittelmann, C. W. Rogers, E. K. Gee, C. F. Bolwell, E. N. Bermingham, and D. G. Thomas. 2014. Faecal microbiota of forage-fed horses in new zealand and the

population dynamics of microbial communities following dietary change. PLoS One. 9(11):e112846. doi:10.1371/journal.pone.0112846

Food and Agricultural Organization/World Health Organization (FAO/WHO). 2002. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Frank, D. N., A. L. St Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc. Natl. Acad. Sci. U. S. A. 104(34):13780-13785. doi:0706625104

Furet, J. P., O. Firmesse, M. Gourmelon, C. Bridonneau, J. Tap, S. Mondot, J. Dore, and G. Corthier. 2009. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol. Ecol. 68(3):351-362. doi:10.1111/j.1574-6941.2009.00671.x

Garner, H. E., J. N. Moore, J. H. Johnson, L. Clark, J. F. Amend, L. G. Tritschler, J. R. Coffmann, R. F. Sprouse, D. P. Hutcheson, and C. A. Salem. 1978. Changes in the caecal flora associated with the onset of laminitis. Equine Vet. J. 10(4):249-252. doi:10.1111/j.2042-3306.1978.tb02273.x

Garrett, L. A., R. Brown, and I. R. Poxton. 2002. A comparative study of the intestinal microbiota of healthy horses and those suffering from equine grass sickness. Vet. Microbiol. 87(1):81-88. doi:S0378113502000184

Gold, J. R., T. J. Divers, M. H. Barton, S. V. Lamb, N. J. Place, H. O. Mohammed, and F. T. Bain. 2007. Plasma adrenocorticotropin, cortisol, and adrenocorticotropin/cortisol ratios in septic and normal-term foals. J. Vet. Intern. Med. 21(4):791-796. doi:10.1111/j.1939-1676.2007.tb03023.x

Gosalbes, M. J., S. Llop, Y. Valles, A. Moya, F. Ballester, and M. P. Francino. 2013. Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. Clin. Exp. Allergy. 43(2):198-211. doi:10.1111/cea.12063

Hansen, N. C., E. Avershina, L. T. Mydland, J. A. Naesset, D. Austbo, B. Moen, I. Mage, and K. Rudi. 2015. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb. Ecol. Health Dis. 26:27216. doi:10.3402/mehd.v26.27216

Harlow, B. E., L. M. Lawrence, and M. D. Flythe. 2013. Diarrhea-associated pathogens, lactobacilli and cellulolytic bacteria in equine feces: Responses to antibiotic challenge. Vet. Microbiol. 166(1-2):225-232. doi:10.1016/j.vetmic.2013.05.003

Heleski, C.R,, A.C. Shelle, B.D. Nielsen, and A.J. Zanella. 2002. Influence of housing on weanling horse behavior and subsequent welfare. App. Anim. Behav. Sci. 78(2-4):291-302. doi:10.1016/S0168-1591(02)00108-9

Henry, S., A. J. Zanella, C. Sankey, M. A. Richard-Yris, A. Marko, and M. Hausberger. 2012. Adults may be used to alleviate weaning stress in domestic foals (equus caballus). Physiol. Behav. 106(4):428-438. doi:10.1016/j.physbeh.2012.02.025

Hoffman, R. M., D. S. Kronfeld, J. L. Holland, and K. M. Greiwe-Crandell. 1995. Preweaning diet and stall weaning method influences on stress response in foals. J. Anim. Sci. 73(10):2922-2930. doi:10.2527/1995.73102922x

Holland, J.L., D.S. Kronfeld, R.M. Hoffman, K.M. Greiwe-Crandell, T.L Boyd, W.L Cooper, and P.A. Harris. 1996. Weaning stress is affected by nutrition and weaning methods. Pferdeheilkunde. 3:257-260.

Houpt, K.A., H.F. Hintz, and W.R. Butler. 1984. A preliminary study of two methods of weaning foals. App. Anim. Behav. 12(1-2):177-181. doi:10.1016/0168-1591(84)90107-2

Hu, J., Y. Nomura, A. Bashir, H. Fernandez-Hernandez, S. Itzkowitz, Z. Pei, J. Stone, H. Loudon, and I. Peter. 2013. Diversified microbiota of meconium is affected by maternal diabetes status. PLoS One. 8(11):e78257. doi:10.1371/journal.pone.0078257

Jakobsson, H. E., T. R. Abrahamsson, M. C. Jenmalm, K. Harris, C. Quince, C. Jernberg, B. Bjorksten, L. Engstrand, and A. F. Andersson. 2014. Decreased gut microbiota diversity, delayed bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 63(4):559-566. doi:10.1136/gutjnl-2012-303249

Jami E., and I. Mizrahi. 2012. Composition and similarity of bovine rumen microbiota across individual animals. PLoS One. 7(3):e33306. doi:10.1371/journal.pone.0033306

Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J.. 7(6):1069-1079. doi:10.1038/ismej.2013.2

Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS One. 9(1):e85423. doi:10.1371/journal.pone.0085423

Jandhyala, S. M., R. Talukdar, C. Subramanyam, H. Vuyyuru, M. Sasikala, and D.N. Reddy. 2015. Role of the normal gut microbiota. World J. Gastroenterol. 21(29):8787–8803. doi:10.3748/wjg.v21.i29.8787

Julliand, V., and P. Grimm. 2016. HORSE SPECIES SYMPOSIUM: The microbiome of the horse hindgut: History and current knowledge. J. Anim. Sci. 94(6):2262-2274. doi:10.2527/jas.2015-0198

Kalliomäki, M., P. Kirjavainen, E. Eerola, P. Kero, S. Salminen, and E Isolauri. 2001. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J. Allergy Clin. Immunol. 107:129–34. doi:10.1067/mai.2001.111237

Kedzierski, W., A. Cywinska, K. Strzelec, and S. Kowalik. 2014. Changes in salivary and plasma cortisol levels in purebred arabian horses during race training session. Anim. Sci. J. 85(3):313-317. doi:10.1111/asj.12146

Kelly, D., T. King, and R. Aminov. 2007. Importance of microbial colonization of the gut in early life to the development of immunity. Mutat. Res. 622(1-2):58-69. doi:S0027-5107(07)00170-4

Koren, O., J. K. Goodrich, T. C. Cullender, A. Spor, K. Laitinen, H. K. Backhed, A. Gonzalez, J. J. Werner, L. T. Angenent, R. Knight, F. Backhed, E. Isolauri, S. Salminen, and R. E. Ley. 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 150(3):470-480. doi:10.1016/j.cell.2012.07.008

Kuhl, J., N. Winterhoff, M. Wulf, F. J. Schweigert, I. Schwendenwein, R. M. Bruckmaier, J. E. Aurich, P. Kutzer, and C. Aurich. 2011. Changes in faecal bacteria and metabolic parameters in foals during the first six weeks of life. Vet. Microbiol. 151(3-4):321-328. doi:10.1016/j.vetmic.2011.03.017

Ladewig, J., E. Søndergaard, and J.W. Christensen. 2005. Ontogeny: preparing the young horse for its adult life. In: D.S. Mills and S.M. McDonnell, editors, The Domestic Horse: The Origins, Development and Management of its Behaviour. Cambridge University Press. p 139–149.

Lamendella, R., K. C. Li, D. Oerther, and J. W. Santo Domingo. 2013. Molecular diversity of bacteroidales in fecal and environmental samples and swine-associated subpopulations. Appl. Environ. Microbiol. 79(3):816-824. doi:10.1128/AEM.02535-12

Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon. 2006. Microbial ecology: Human gut microbes associated with obesity. Nature. 444(7122):1022-1023. doi:4441022a

Liu, L., Y. Li, S. Li, N. Hu, Y. He, R. Pong, D. Lin, L. Lu, and M. Law. 2012. Comparison of next-generation sequencing systems. J. Biomed. Biotechnol. 2012:251364. doi:10.1155/2012/251364

Looft, T., H. K. Allen, B. L. Cantarel, U. Y. Levine, D. O. Bayles, D. P. Alt, B. Henrissat, and T. B. Stanton. 2014. Bacteria, phages and pigs: The effects of in-feed antibiotics on the microbiome at different gut locations. ISME J.. 8(8):1566-1576. doi:10.1038/ismej.2014.12

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature. 489(7415):220-230. doi:10.1038/nature11550

Malinowski, K., N.A. Hallquist, L. Helyar, A.R. Sherman, C.G. Scanes. 1990. Effect of different separation protocols between mares and foals on plasma cortisol and cell-mediated immune response. J. Equine Vet. Sci. 10(5):363-368. doi:10.1016/S0737-0806(06)80098-5

McCall, C.A., G.D. Potter and J.L. Kreide., 1985. Locomotor, vocal and other behavioral responses to varying methods of weaning foals. App. Anim. Behav. Sci. 14(1):27-35. doi:10.1016/0168-1591(85)90035-8

McCall, C.A., G.D. Potter, J.L. Kreider, W.L. Jenkins. 1987. Physiological responses in foals weaned by abrupt or gradual methods. J. Equine Vet. Sci. 7(6):368-374 doi:10.1016/S0737-0806(87)80007-2

McGreevy, P.D., P.J. Cripps, N.P. French, L.E. Green, and C.J. Nicol. 1995. Management factors associated with stereotypic and redirected behaviour in the thoroughbred horse. Equine Vet. J. 27:86–91. doi:10.1111/j.2042-3306.1995.tb03041

Meale, S.J., S. Li, P. Azevedo, H. Derakhshani, J.C. Plaizier, E. Khafipour, and M.A. Steele. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. Front. Microbiol. 7:582. doi:10.3389/fmicb.2016.00582

Meropol, S. B., and A. Edwards. 2015. Development of the infant intestinal microbiome: A bird's eye view of a complex process. Birth Defects Res. C. Embryo. Today. 105(4):228-239. doi:10.1002/bdrc.21114

Merkies,K.,C. DuBois, K. Marshall, S. Parois, L. Graham, and D. Haley. 2016. A two-stage method to approach weaning stress in horses using a physical barrier to prevent nursing. App. Anim. Behav. Sci. 183:68-76. doi:10.1016/j.applanim.2016.07.004.

Metzker, M. L. 2010. Sequencing technologies - the next generation. Nat. Rev. Genet. 11(1):31-46. doi:10.1038/nrg2626

Milinovich, G. J., D. J. Trott, P. C. Burrell, A. W. van Eps, M. B. Thoefner, L. L. Blackall, R. A. Al Jassim, J. M. Morton, and C. C. Pollitt. 2006. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. Environ. Microbiol. 8(5):885-898. doi:EMI975

Milinovich, G. J., P. C. Burrell, C. C. Pollitt, A. V. Klieve, L. L. Blackall, D. Ouwerkerk, E. Woodland, and D. J. Trott. 2008. Microbial ecology of the equine hindgut during oligofructose-induced laminitis. ISME J. 2(11):1089-1100. doi:10.1038/ismej.2008.67

Moons,C.P.H., K. Laughlin, A.J. Zanella. 2005. Effects of short-term maternal separations on weaning stress in foals. Appl. Anim. Behav. Sci. 91(3):321-335. doi:10.1016/j.applanim.2004.10.007.

Moreau, M. M., S. C. Eades, C. R. Reinemeyer, M. N. Fugaro, and J. C. Onishi. 2014. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in

bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet. Microbiol. 168(2-4):436-441. doi:10.1016/j.vetmic.2013.11.017

Muhonen, S., V. Julliand, J. E. Lindberg, J. Bertilsson, and A. Jansson. 2009. Effects on the equine colon ecosystem of grass silage and haylage diets after an abrupt change from hay. J. Anim. Sci. 87(7):2291-2298. doi:10.2527/jas.2008-1461

Nicol, C.J., A.J. Badnell-Waters, R. Bice, A. Kelland, A.D. Wilson, and P.A. Harris. 2005. The effects of diet and weaning method on the behaviour of young horses. Appl. Anim. Behav. Sci. 95(3):205-221. doi:10.1016/j.applanim.2005.05.004.

Pajarillo, E.A.., J. P. Chae, M. P. Balolong, H. Bum Kim, and D. K. Kang. 2014a. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J. Gen. Appl. Microbiol. 60(4):140-146. doi:DN/JST.JSTAGE/jgam/60.140

Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown. 2007. Development of the human infant intestinal microbiota. PLoS Biol. 5(7):e177. doi:07-PLBI-RA-0129

Patel, J.B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. J. Mol. Diagn. 6(4): 313-321. doi:10.1007/BF03262067

Proudman, C. J., J. O. Hunter, A. C. Darby, E. E. Escalona, C. Batty, and C. Turner. 2015. Characterisation of the faecal metabolome and microbiome of thoroughbred racehorses. Equine Vet. J. 47(5):580-586. doi:10.1111/evj.12324

Ramette, A. 2009. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. Appl. Environ. Microbiol. 75(8):2495-2505. doi:10.1128/AEM.02409-08

Rastogi, G., and R.K. Sani. 2011. Molecular techniques to assess microbial community structure, function, and dynamics in the environment. In: Microbes and microbial technology. Springer New York. p 29-57.

Reichmann, P., A. Moure, and H.R. Gamba. 2004. Bone mineral content of the third metacarpal bone in Quarter Horse foals from birth to one year of age. J. Equine Vet. Sci. 24(9):391-396. doi:10.1016/j.jevs.2004.08.002

Rodriguez, J. M., K. Murphy, C. Stanton, R. P. Ross, O. I. Kober, N. Juge, E. Avershina, K. Rudi, A. Narbad, M. C. Jenmalm, J. R. Marchesi, and M. C. Collado. 2015. The composition of the gut microbiota throughout life, with an emphasis on early life. Microb. Ecol. Health Dis. 26:26050. doi:10.3402/mehd.v26.26050

Romano-Keeler, J., and J. H. Weitkamp. 2015. Maternal influences on fetal microbial colonization and immune development. Pediatr. Res. 77(1-2):189-195. doi:10.1038/pr.2014.163

Sadet-Bourgeteau, S., C. Philippeau, S. Dequiedt, and V. Julliand. 2014. Comparison of the bacterial community structure within the equine hindgut and faeces using automated ribosomal intergenic spacer analysis (ARISA). Animal. 8(12):1928-1934. doi:10.1017/S1751731114001943

Schoster, A., L. G. Arroyo, H. R. Staempfli, and J. S. Weese. 2013. Comparison of microbial populations in the small intestine, large intestine and feces of healthy horses using terminal restriction fragment length polymorphism. BMC Res. Notes. 6: 91. doi:10.1186/1756-0500-6-91

Schoster, A., J.S. Weese and L. Guardabassi. 2014. Probiotic use in horses – what is the evidence for their clinical efficacy? J. Vet. Inter. Med. 28:1640-1652. doi:10.1111/jvim.12451/epdf

Schoster, A., M. Mosing, M. Jalali, H. R. Staempfli, and J. S. Weese. 2015. Effects of transport, fasting and anaesthesia on the faecal microbiota of healthy adult horses. Equine Vet. J. 48(5):595-602. doi:10.1111/evj.12479

Shepherd, M. L., W. S. Swecker Jr, R. V. Jensen, and M. A. Ponder. 2012. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol. Lett. 326(1):62-68. doi:10.1111/j.1574-6968.2011.02434.x

Slifierz, M. J., R. M. Friendship, and J. S. Weese. 2015. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol. 15(1):184-015-0512-7. doi:10.1186/s12866-015-0512-7

Steelman, S. M., B. P. Chowdhary, S. Dowd, J. Suchodolski, and J. E. Janecka. 2012. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet. Res. 8:231. doi:10.1186/1746-6148-8-231

Tadich,T., F. Leal, and C. Gallo. 2015. Preliminary Study on the Effects of Long Distance Road Transport on Some Blood Constituents in Recently Weaned Thoroughbred Foals. J. Equine Vet. Sci. 35(8):697-699. doi:10.1016/j.jevs.2015.06.011

Thomas, F., J. H. Hehemann, E. Rebuffet, M. Czjzek, and G. Michel. 2011. Environmental and gut bacteroidetes: The food connection. Front. Microbiol. 2:93. doi:10.3389/fmicb.2011.00093

Thum, C., A. L. Cookson, D. E. Otter, W. C. McNabb, A. J. Hodgkinson, J. Dyer, and N. C. Roy. 2012. Can nutritional modulation of maternal intestinal microbiota influence the development of the infant gastrointestinal tract? J. Nutr. 142(11):1921-1928. doi:10.3945/jn.112.166231

Urbaniak, C., M. Angelini, G. B. Gloor, and G. Reid. 2016. Human milk microbiota profiles in relation to birthing method, gestation and infant gender. Microbiome. 4: 1. doi:10.1186/s40168-015-0145-y

Waran,N.K., N.Clarke, and M. Farnworth. 2008. The effects of weaning on the domestic horse (Equus caballus). Appl. Anim. Behav. Sci. 110(1):42-57. doi:10.1016/j.applanim.2007.03.024

Weese, J. S., S. J. Holcombe, R. M. Embertson, K. A. Kurtz, H. A. Roessner, M. Jalali, and S. E. Wismer. 2015. Changes in the faecal microbiota of mares precede the development of post partum colic. Equine Vet. J. 47(6):641-649. doi:10.1111/evj.12361

West, C.E., M.C. Jenmalm, and S.L. Prescott. 2014. The gut microbiota and its role in the development of allergic disease: a wider perspective. Clin. Exp. Allergy. 25(1):45-53. doi:10.1111/cea.12332.

White, G., and S. D. Prior. 1982. Comparative effects of oral administration of trimethoprim/sulphadiazine or oxytetracycline on the faecal flora of horses. Vet. Rec. 111(14):316-318.

Whitfield-Cargile, C. M., N. D. Cohen, J. Suchodolski, M. K. Chaffin, C. M. McQueen, C. E. Arnold, S. E. Dowd, and G. P. Blodgett. 2015. Composition and diversity of the fecal microbiome and inferred fecal metagenome does not predict subsequent pneumonia caused by rhodococcus equi in foals. PLoS One. 10(8):e0136586. doi:10.1371/journal.pone.0136586

Willing, B., A. Voros, S. Roos, C. Jones, A. Jansson, and J. E. Lindberg. 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. Equine Vet. J. 41(9):908-914. doi:10.2746/042516409X447806

Wu, G. D., J. Chen, C. Hoffmann, K. Bittinger, Y. Y. Chen, S. A. Keilbaugh, M. Bewtra, D. Knights, W. A. Walters, R. Knight, R. Sinha, E. Gilroy, K. Gupta, R. Baldassano, L. Nessel, H. Li, F. D. Bushman, and J. D. Lewis. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science. 334(6052):105-108. doi:10.1126/science.1208344

Wu, H. J., and E. Wu. 2012. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 3(1):4-14. doi:10.4161/gmic.19320

Zeyner, A., C. Geißler, and A. Dittrich. 2004. Effects of hay intake and feeding sequence on variables in faeces and faecal water (dry matter, pH value, organic acids, ammonia, buffering capacity) of horses. J. Anim. Physiol. and N. 88:7–19. doi:10.1111/j.1439-0396.2004.00447

Chapter 2 - Characterization of foal fecal microbiota from birth to weaning and the relationship to mare milk and mare feces

Abstract

The objectives of this experiment were to characterize the development of the foal fecal microbiota from birth to 4 mo and determine its relationship to mare milk and fecal bacteria. Mare milk, mare fecal, and foal fecal samples were collected from 9 mare and foal pairs at birth, d 0, 2, and 7 postpartum, and then monthly until 4 mo. Next generation sequencing of the V4 region of the bacterial 16S rRNA gene was performed using the Illumina Miseq according to Earth Microbiome Project protocols. Sequencing data was analyzed using QIIME. Bacteria found in mare milk and mare feces were different in diversity ($P < 0.001$) and composition ($P < 0.05$) across all time points. Newborn foal meconium contained low species diversity and a bacterial composition similar to mare milk consisting of the genera Enterococcus, Bacillus, Pseudomonas and Lactococcus. The Firmicutes and Proteobacteria phyla comprised the majority of bacteria in mare milk and foal feces on d 0 and 2. At the genus level, the bacterial communities shifted within the first week to be dominated by Bacteroides and Escherichia. By 1 mo the foal fecal composition did not differ in composition at the phylum level from mare feces; however, species diversity was still lower in foals and the relative abundance of several genera were different ($P < 0.05$). Firmicutes, Bacteriodetes, and Verrucomicrobia were the dominant phyla found in both foal feces older than 1 mo and mare feces. These results demonstrate the foal is born with fecal microbial communities similar to milk that rapidly change within the first week of life. Within the first month of life there is a gradual transition of the foal fecal bacterial structure until it resembles that of its dam, remaining constant over the next 3 months.

Introduction

Horses are dependent on microbes in the hindgut to produce volatile fatty acids (VFAs) that are then used for energy production (Al Jassim and Andrews, 2009). Bacteria found in the gastrointestinal tract (GIT) are also responsible for the synthesis of B and K vitamins, aiding in protection against pathogens, and contributing to immune function (NRC, 2007; Louzupone et al., 2012). Feces are used as a proxy for intestinal bacteria due to the ease of collection; however, the bacterial composition of feces is only partially representative of those found in luminal contents of distal portions of the intestine (Costa et al., 2015a). With the advancement of next generation sequencing (NGS) technology, the taxonomic classification of equine fecal microbiota has become possible (Costa and Weese, 2012).

The microbial composition of the equine hindgut can be influenced by numerous factors. Diseases, such as colic and laminitis, have been associated with disruptions in normal fecal microbial structure in the horse (Costa et al., 2012; Steelman et al., 2012). Alterations in diets have also been associated with changes in equine fecal microbial composition (Dougal et al., 2014, Fernandes et al., 2014). Gastrointestinal diseases are very common in the horse and understanding the initial development of the intestinal microbial composition could lead to improved management through disease prevention and treatment.

The most notable natural change in intestinal microbiota occurs with age. It was originally thought that neonates were born sterile; however, it is now believed that microbiota can be passed through the placenta in utero and through the birthing process (Dominguez-Bello et al., 2010). One mode of transfer of bacteria from mother to infant is proposed to occur through exposure to maternal vaginal and fecal sources during delivery (Metropol and Edwards, 2015). The initial colonization of the infantile intestinal microbiota is important to animal health and

problems with its development could lead to increased susceptibility to disease or metabolic disorders (Louzupone et al., 2012). Milk also has an important influence on the developing intestinal microbiota (Metropol and Edwards, 2015). Bacteria found in milk can be directly transferred to the neonatal GIT through nursing. Milk microbiota can prepare the neonatal GIT to become familiar with commensal bacteria and program the immune system against pathogenic bacteria (Addis et al., 2016).

The equine intestinal microbiota undergoes rapid changes starting at birth until it reaches a composition similar to an adult (Costa et al., 2015b). Differences in fecal microbial composition can be observed between 2 and 30 d after birth (Bordin et al., 2013; Costa et al., 2015b). In previous studies the Bacteriodetes phylum increased in foals from 2 d to 5 wk, which could be attributed to the addition of solid feed to an exclusively milk diet (Bordin et al., 2013; Whitfield-Cargile et al., 2015). The foal fecal microbiota seems to be similar to that of an adult by 60 d (Costa et al., 2015b); however, the exact changes in composition need to be confirmed through further research to better understand the temporal phases of GIT bacterial communities in healthy foals. Recognizing the normal age-related changes in microbial composition of healthy foal feces could allow for better management and prevention of intestinal diseases by knowing the ages that foals might be most susceptible to intestinal problems.

The objectives of this study were to understand the initial colonization and development of foal fecal microbiota from birth until 4 mo of age, and to compare foal fecal microbiota to bacteria found in mare milk and mare feces to determine the influence that the dam has in inoculating the foal with intestinal microbes.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol No. 3571).

Animals

Nine Quarter Horse mares and their corresponding foals, born between April and May of 2015, were used in this study. Mares and foals were stalled for the first 2 d after parturition and group housed in pasture with ad libitum access to brome hay and water. Mares were supplemented with a pelleted concentrate, Purina Strategy (Purina Mills, Inc., St. Louis, MO), to meet dietary requirements for late pregnancy and early lactation (NRC, 2007). Although foals were not specifically fed concentrate, they did have access to their dams feed bins.

Fecal and Milk Sampling

Sampling from mares and foals occurred on d 0 (at parturition), 2, and 7, and then 1, 2, 3, and 4 mo post-foaling. At each time point, mare milk, mare feces and foal feces were collected. Foal meconium and mare colostrum samples collected on d 0 were taken within 2 h of birth and prior to nursing. Mare fecal samples were collected by rectal palpation using a sterile shoulder-length Ag-Tek PolySleeve (Neogen, Lexington, KY) and sterile lubricating jelly (First Priority, Inc., Elgin, IL). Foal fecal samples were collected manually using sterile latex gloves. Between 5 and 50 g of feces were collected and placed directly into 50 mL sterile conical tubes. In order to reduce potential for contamination of milk samples, mare udders were cleaned and samples were obtained according to aseptic procedures used to collect milk samples from dairy cattle for microbial analysis (Bewley et al., 2014). Using sterile latex gloves, 3 streams of milk were stripped from both teats. Next, teats were dipped in betadine solution and dried with a clean paper towel 20 sec after application. Then both teats were scrubbed with a gauze pad moistened

in 70% isopropyl alcohol until no debris remained on the gauze pad, cleaning the far side first to further prevent contamination. Finally, an equal volume of 5 to 10 mL of milk was collected into a sterile 50mL conical tube held at a 45° angle below the teat. After collection, fecal and milk samples were immediately stored at -20° C until further analysis.

DNA Extraction and 16S rRNA Gene Amplification

Total DNA was extracted from 200 mg of frozen fecal and milk samples using the EZNA Stool DNA Kit (Omega Bio Tek, Norcross, GA) and the stool DNA protocol for pathogen detection, previously validated for use in the horse (Costa et al. 2012). After extraction, DNA was quantified using the Nanodrop 800 spectrophotometer (Thermo Scientific, Wilmington, DE).

The V4 region of bacterial 16S rRNA gene was amplified using modified universal PCR primers (515F/806F) according to Earth Microbiome Project protocols (Caporaso et al., 2012). Fecal and milk DNA samples were then amplified in triplicate through PCR reactions run on a Mastercycler nexus (Eppendorf, Hamburg, Germany) in a 25 µL solution containing 5 µL 5x PCR master mix (Promega; Madison, WI), 0.5 µL of each primer (10 uM), 0.5 µL dntps, 1 uL 1% BSA, 0.25 µL GoTaq Polymerase (Promega; Madison, WI), 16.25 µL PCR grade H2O and 1 µL template DNA for 25 cycles. Milk samples and some early foal fecal samples with low template DNA were carried out with 5 µL DNA for 30 cycles. All PCR reactions were run with a negative control to ensure there was no bacterial contamination and appropriate amplification of PCR products was checked using agarose gel electrophoresis. Amplicon triplicates were combined and non-PCR products, such as residual reagents and primers, were removed from the amplicon using the EXO-SAP-IT kit (Thermo Fischer Scientific, Waltham, MA). Each sample was individually quantified using Quant-iT PicoGreeen dsDNA Assay Kit (Thermo Fischer

Scientific, Waltham, MA). Approximately 100 ng of DNA from each sample was combined in a 1.5 mL microcentrifuge tube. The combined library was purified and extracted from 2% agarose gel using the QIAquick gel purification kit (Quiagen, Valencia, CA) to ensure a uniform library size of 400 bp.

Sequencing and Analysis

The combined library was submitted to the K-State Integrated Genomics Facility for sequencing. The library was first analyzed for quality and fragment length using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and qPCR. Amplicon sequencing was performed using the 300 cycles MiSeq reagent v3 kit for a single 2 x150 paired-end cycle run on the Illumina Miseq, with a 10% PhiX spike to increase library diversity and overall read quality.

Sequencing analysis was performed using QIIME version 2.06 (Quantitative Insights into Microbial Ecology). MiSeq data was received in the form of three FASTQ files R1 (forward), R2 (reverse) and I1 and the paired end reads were joined. A mapping file was created to link nucleotide barcodes with corresponding sample identification. The library was then demultiplexed as samples were assigned to their barcode sequence. Next, reads were assigned to operational taxonomic units (OTUs) using an open-reference OTU picking protocol based on 97% sequence similarity and aligned to the GreenGenes sequence database (version 13.5). Taxonomic assignments were given using the Ribosomal Database Project (RDP) classifier (Wang et al. 2007). Then OTU assignment was completed on RDP taxonomy data by aligning OTU sequences with the GreenGenes database and any unassigned OTUs were removed. Chimeras were identified using ChimeraSlayer and removed from the data matrix.

Data analysis was performed on all samples at 20,300 reads based on rarefaction curves constructed in QIIME. The rarefied data set included 284,660 total OTUs. Alpha rarefaction

indices were calculated to determine within-sample differences and beta diversity plots were also constructed to compare between-sample differences. Using QIIME, observed OTUs, Chao1 richness, and Shannon diversity were calculated to determine species richness, number of different species, and evenness, abundance of those species. Nonmetric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities was used to measure differences between samples based on OTU relative abundance. From QIIME, summary plots of taxonomy were created to report relative abundances of dominant taxa.

Statistical Analysis

All statistical analysis was performed using R (version 3.2.2). Alpha diversity metrics and differences in relative abundances between individual phyla and genera were measured with using linear mixed-effects model ANOVA with the lme function in nlme R package. Post hoc comparisons using Bonferroni adjustments were used to compare sample types and time points using the glht function in the multcomp R package. Least-square means and standard error were calculated using lsmeans function in the lsmeans R package. Changes in community composition between sample type and foal fecal samples from birth to 4 mo were determined through PERMANOVA using the adonis function in the Vegan package in R. Using the ellipse and ggplot2 packages in R, 95% confidence intervals were added to NMDS plots for different mare milk and fecal, and foal fecal samples taken at different time points.

Results

Alpha Diversity

Alpha diversity was estimated by calculating the observed OTUs per sample, number of different species, Shannon, species diversity, and Chao1, total species richness, across sample type and foal age. Milk and younger foals (d 0 to 7) had lower diversity in their fecal bacteria when compared with foals older than 1 mo and mare fecal samples. Mare feces were significantly different from mare milk across all measured alpha diversity metrics at each time point ($P < 0.001$). For observed OTUs, there were significant differences between time points for foal feces from d 0 to 7 and 1 to 4 mo ($P < 0.001$). Observed OTUs for foal feces from d 0, 2, and 7 were not different from mare milk ($P = 1.0$). Conversely, mare feces did not differ from foal feces from 2 mo to 4 mo ($P = 1.0$). Shannon and Chao1 showed similar patterns; however, there were no significant differences between 1 mo foal fecal and mare fecal for Shannon diversity ($P = 0.11$).

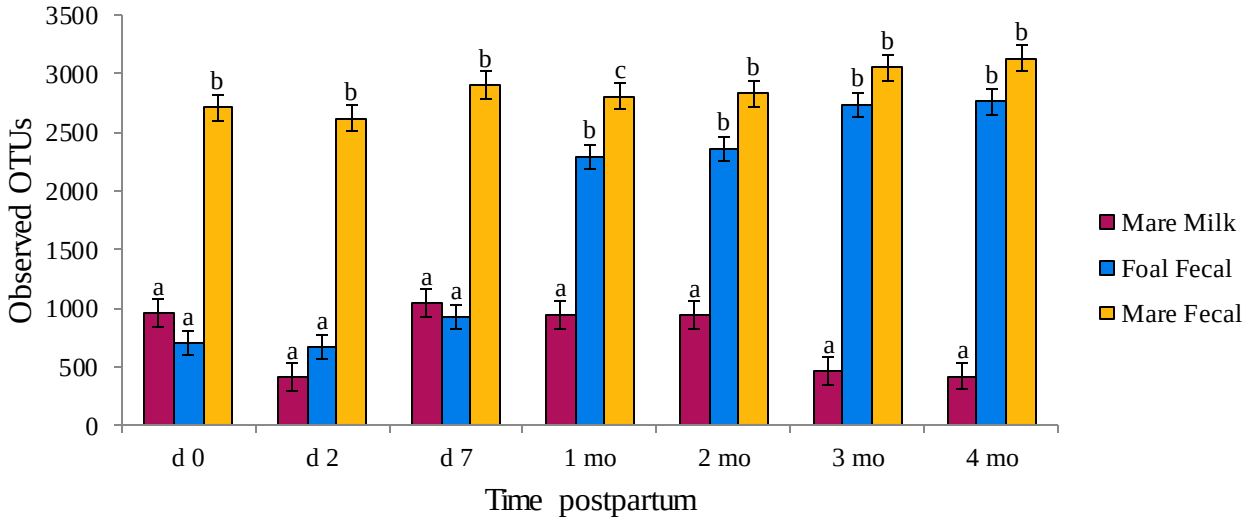

Figure 2-1. Observed operational taxonomic units (OTU), representing number of different bacterial species, between mare milk, foal fecal and mare fecal samples within time points from birth (d 0) to 4 mo. Least square means with different letters within time points differ, $^{a,b}P < 0.001$ and $^{c,b} < 0.05$. Error bars represent SEM.

Beta Diversity

Differences in community composition between mare milk, mare fecal and foal fecal over time were determined through NMDS using Bray-Curtis dissimilarity (Figure 2-2). The NMDS ordination shows clear separations between type of sample and foal fecal samples from d 0 to 7. There are compositional differences between mare milk and mare fecal samples ($P < 0.05$; Figure 2-2). In addition, foal fecal samples from d 2 and d 7 were different from all other samples ($P < 0.05$; Figure 2-2). Mare fecal and foal feces from 1 to 4 mo were different from milk and foal fecal from d 0 to 7 ($P < 0.05$; Figure 2-2).

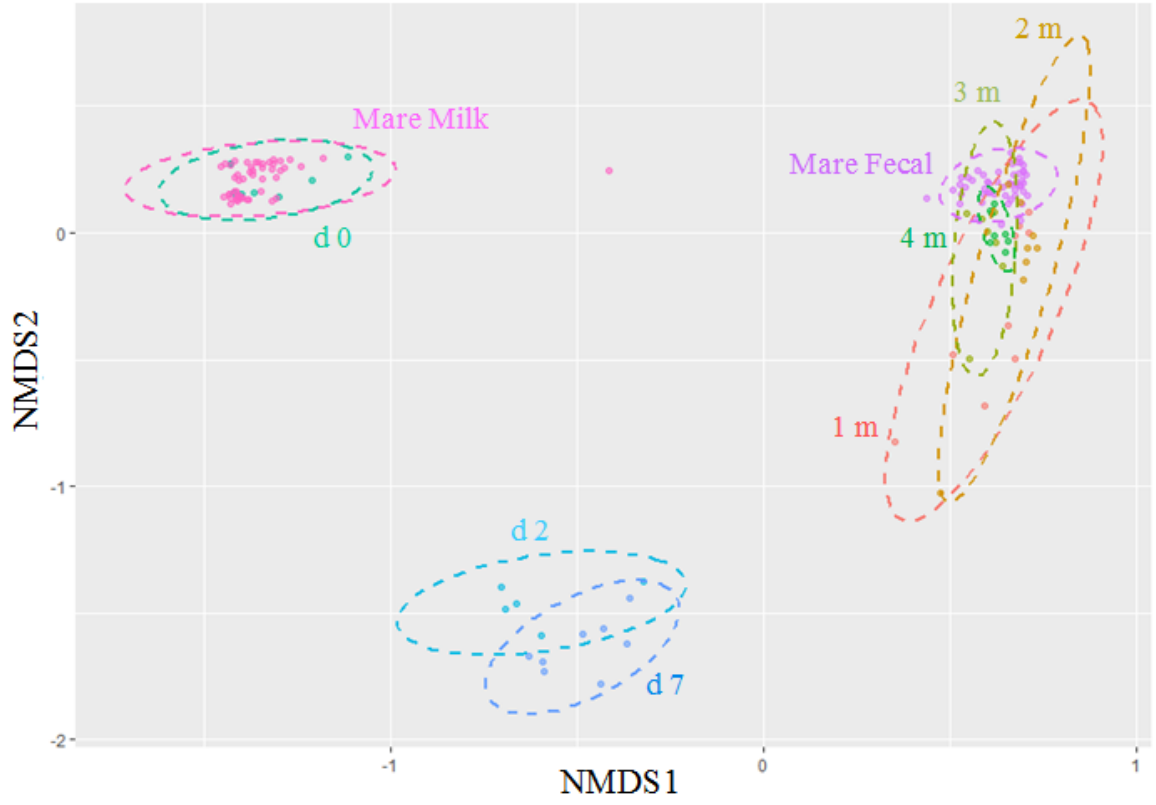

Figure 2-2. Nonmetric multidimensional scaling (NMDS) with 95% confidence interval ellipses based on Bray-Curtis distances of mare milk, mare fecal and foal fecal samples collected at different time points following birth (d 0). Each point represents an individual milk or fecal sample. Point and ellipse colors represent mare milk and mare feces combined across time points, and foal fecal samples from birth (d 0) to 4 mo of age. Non-overlapping ellipses are significantly different ($P < 0.05$).

Relative Abundance

The main bacteria phyla present in mare fecal samples included Firmicutes, Bacteriodetes, Verrucomicrobia, and Spirochaetes (Figure 2-3). Although the phyla Firmicutes made up the majority of all mare milk samples there were differences in composition with samples from d 0, 7, 1 and 4 mo having increased relative abundances of Proteobacteria. Mare milk and mare feces relative abundances at the phylum level were significantly different across all major bacteria phyla ($P < 0.05$; Figure 2-3). There were no differences in relative abundance of mare milk or mare feces at different time points when compared with foal fecal samples, therefore mare samples from different time points were pooled according to source. Foal feces differed across time points between various dominant phyla (Table 2-1). Differences in relative abundance of phyla comparing foal feces, from d 0 to 7, to mare fecal and mare milk are presented in Table 2-2. There were no significant differences in relative abundance between mare milk and foal fecal samples on d 0 ($P = 1.0$; Table 2-2). Relative abundance of bacteria phyla between mare feces and foal feces from 1 to 4 mo did not differ.

There were major shifts in the relative abundances of genera found in mare milk and feces and foal feces from birth (d 0) to 4 mo (Figure 2-4; Table 2-3). Differences in community composition were found across all genera between mare milk and mare feces ($P < 0.05$). Mare milk did not differ from foal fecal samples on d 0 ($P = 1.0$). Genera of mare feces were different from foal feces from d 0 to 7 ($P < 0.05$); however, mare feces did not differ from foal fecal samples from 1 to 4 mo ($P = 1.0$). Foal feces from d 0, d 2 and d 7 had differences in relative abundances of bacteria genera compared with each other and foal fecal samples from 1 to 4 mo ($P < 0.05$). The dominant bacteria genera of mare milk, mare feces, and foal feces from d 0 to 4 mo can be found in Table 2-3.

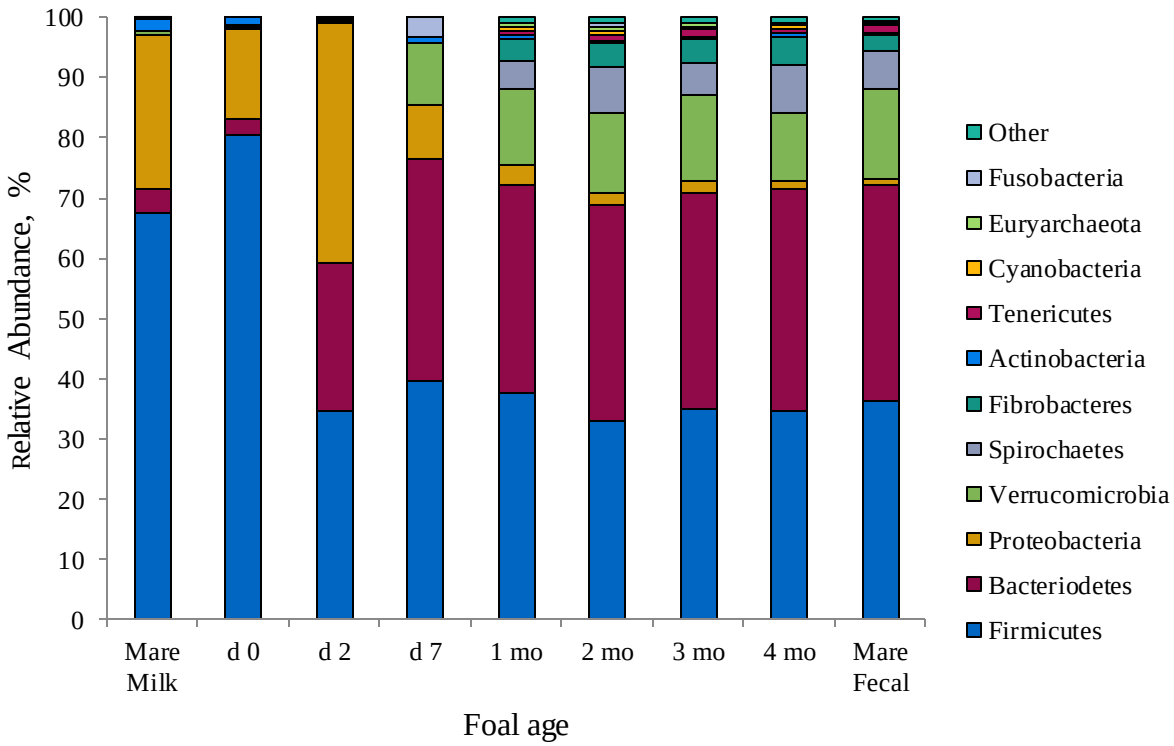

Figure 2-3. Relative abundance of the dominant bacteria at the phylum level found in mare milk, mare feces, and foal feces from birth (d 0) to weaning at 4 mo of age.

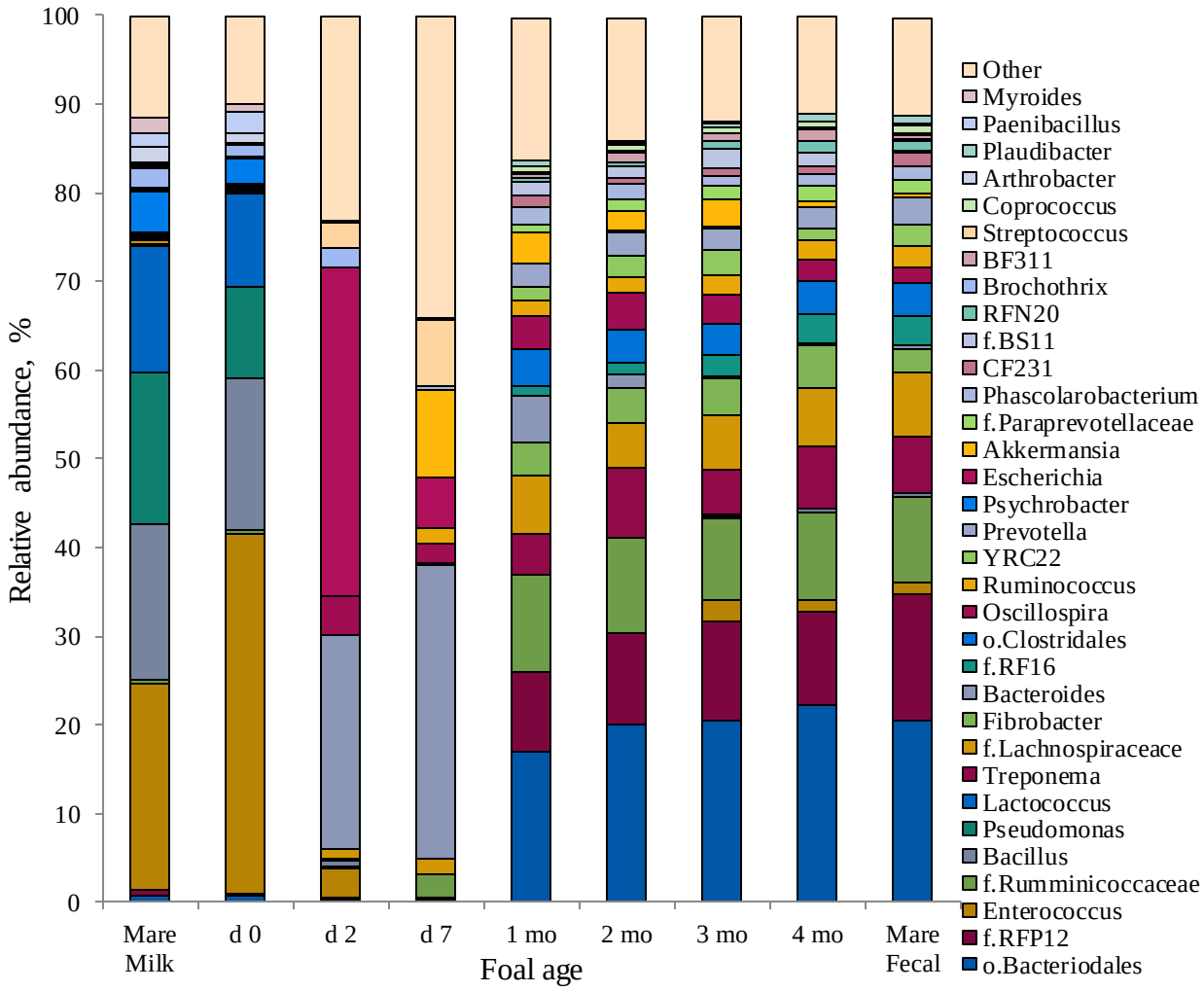

Figure 2-4. Relative abundance of the dominant bacteria at the genus level, or next lowest taxonomic resolution, found in mare milk, mare feces, and foal feces from birth (d 0) to 4 mo of age. Taxa with o. represent order as the lowest taxonomic level classified whereas f. represents family.

Table 2-1. Differences in the relative abundance of specific bacteria phyla found in foal feces between two specific time points from birth (d 0) to 4 mo

Time points	Phyla[1]
d 0 vs. 2	Firm.[2], Bact.[2], Pro.[2]
d 0 vs. 7	Firm[2], Bact.[2], Oth.[2]
d 0 vs. 1 mo	Firm.[2], Bact.[2], Pro.[4], Verr.[2], Spir.[2], Ten.[2], Cyan.[2]
d 0 vs. 2 mo	Firm.[2], Bact.[2], Pro.[3], Verr.[2], Spir.[2], Ten.[2], Eury.[3], Cyan.[2]
d 0 vs. 3 mo	Firm.[2], Bact.[2], Pro.[3], Verr.[2], Spir.[2], Ten.[2], Eury.[2]
d 0 vs. 4 mo	Firm.[2], Bact.[2], Pro.[2], Verr.[2], Spir.[2], Fib.[2], Ten.[2]
d 2 vs. 7	Pro.[2], Verr.[2]
d 2 vs. 1 mo	Pro.[2], Verr.[3], Spir.[2], Ten.[2], Cyan.[3]
d 2 vs. 2 mo	Pro.[2], Verr.[3], Spir.[2], Ten.[2], Eury.[4], Cyan.[3]
d 2 vs. 4 mo	Pro.[2], Verr.[2], Spir.[2], Fib.[2], Ten.[3]
d 2 vs. 4 mo	Pro.[2], Verr.[3], Spir.[2], Fib.[4], Ten.[2]
d7 vs. 1 mo	Spir.[2], Ten.[2], Cyan.[2]
d 7 vs. 2 mo	Spir.[2], Ten.[2], Eury.[3], Cyan.[2]
d 7 vs. 3 mo	Spir.[2], Ten.[2], Eury.[2]
d 7 vs. 4 mo	Spir.[2], Fib.[3], Ten.[2]

[1]Bacteria phyla: Firm. = Firmicutes, Bact. = Bacteriodetes, Pro. = Proteobacteria, Verr. = Verrucomicrobia, Spir. = Spirochaetes, Fib. =Fibrobacteres, Ten. = Tenericutes, Eury. = Euryarchaeota, Cyan. = Cyanobacteria, Oth. = Other.
[2]Difference between time points ($P \leq 0.001$).
[3]Difference between time points ($P \leq 0.01$).
[4]Difference between time points ($P \leq 0.05$).

Table 2-2. Relative abundance (%) of the main bacteria phyla of mare fecal and milk samples as they compare to foal feces from d 0 to 7 after birth

	Phyla (mare/foal)	
Foal fecal	Mare Fecal[1]	Mare Milk[2]
d 0	Firmicutes (36/80.4)	Firmicutes (67.6/80.4)
	Bacteriodetes (35.7/2.8)	Bacteriodetes (3.8/2.8)
	Verrucomicrobia (15.1/0.35)	Verrucomicrobia (0.41/0.35)
	Spirochaetes (6.4/0.18)	Proteobacteria (25.7/14.9)
	Fibrobacteres (2.7/0.04)	Spirochaetes (0.13/0.18)
	Tenericutes (1/0.04)	Fibrobacteres (0.06/0.04)
	Cyanobacteria (0.4/0.02)	Actinobacteria (2.0/1.2)
		Tenericutes (0.03/0.04)
		Cyanobacteria (0.02/0.02)
		Other (0.1/0.02)
d 2	Verrucomicrobia (15.1/0.3)	Verrucomicrobia (0.41/0.3)
	Proteobacteria (1/39.8)	Proteobacteria (25.7/39.8)
	Spirochaetes (6.4/0.14)	Spirochaetes (0.13/0.14)
	Tenericutes (1/0.002)	Fibrobacteres (0.06/0.04)
		Tenericutes (0.03/0.002)
		Cyanobacteria (0.02/0.006)
		Other (0.1/0.01)
d 7	Spirochaetes (6.4/0)	Spirochaetes (0.13/0)
	Fibrobacteres (2.7/0.04)	Fibrobacteres (0.06/0.04)
	Tenericutes (1/0.01)	Tenericutes (0.06/0.01)
	Cyanobacteria (0.4/0.2)	Cyanobacteria (0.02/0.02)
	Other (0.4/3.4)	

[1]Relative abundances between mare and foal feces differ, $P \leq 0.05$.

[2] Relative abundances between mare milk and foal feces did not differ, $P \geq 0.05$.

Table 2-3. List of dominant bacterial genera found in mare milk, mare fecal, and foal fecal samples from birth (d 0) to 4 mo

	Dominant genera
Type/Time point	
Mare Milk[1]	Enterococcus, Bacillus, Pseudomonas, Lactococcus
Foal fecal, d 0[1]	
Foal fecal, d 2	Escherichia, Bacteroides, Oscillospiria, Enterococcus
Foal fecal, d 7	Bacteroides, Akkermansia, Streptococcus, Escherichia
Foal fecal, 1 mo	Bacteriodales[3], RFP12[4], Ruminococcaceae[4], Lachnospiraceace[4], Treponema, Fibrobacter, Bacteroides, Oscillospiria, Akkermansia
Foal fecal, 2 to 4 mo[2]	Bacteriodales[3], RFP12[4], Ruminococcaceae[4], Treponema,
Mare Fecal[2]	Lachnospiraceace[4], RF16[4], Fibrobacter, Clostridiales[3], Oscillospiria, YRC22

[1]No differences in genera between mare milk and foal feces d 0, $P \geq 0.05$.
[2]No differences in genera between foal feces 2 to 4 mo and mare fecal, $P \geq 0.05$.
[3]Lowest taxonomic resolution is at the order level.
[4]Lowest taxonomic resolution is at the family level.

Discussion

Bacteria are present in foal meconium at birth; however, dramatic shifts in fecal microbial composition and diversity occur within the first month of life. The foal fecal bacterial community transitions from being similar to mare milk to becoming indistinguishable from that of an adult horse. To our knowledge, this is the first study to report the bacteria taxa found in equine milk using next generation sequencing. The distinct differences between bacteria found in mare milk and feces can be attributed to differences in function between the udder and intestinal tract. Low species diversity is found in milk with the commensal bacteria present thought to contribute to the initial colonization of the neonatal gut (Martin et al., 2003), whereas adult equine feces contain diverse and predominately fibrolytic bacteria responsible for the fermentation of fiber (Julliand and Grimm, 2016).

Although initially low in species diversity during the first week of life, results from this study indicate diversity increased and did not differ from an adult horse by 2 mo. This is similar to studies using community fingerprinting techniques (Earing et al., 2012; Faubladier et al., 2014), but in contrast with a previous study that used next generation sequencing and did not find any differences in diversity with age (Costa et al., 2015b). There were no differences between mare milk and foal fecal bacterial diversity in meconium (d 0) or feces from d 2 and 7, suggesting less diverse bacteria species are needed to aid in digestion during the first week of life. This could be because the foal is consuming an exclusively milk diet and is not yet dependent on a complex bacterial community capable of degrading forages.

Mare milk and foal meconium were dominated by the Firmicutes phylum, consisting primarily of the genera Enterococcus, Bacillus, and Lactococcus. Lactococcus and Enterococcus are part of a group collectively referred to as lactic acid bacteria (LAB), members of which are

generally found in nutrient rich, high sugar environments and use glucose as energy to produce lactic acid (König and Fröhlich, 2009). These genera have also been identified in the milk of dairy cattle (Kuehn et al., 2015; Zhang et al. 2016). In addition LAB, which are classified as facultative anaerobes, have been found in human feces at birth and are thought to promote the later growth of obligate anaerobes, including fibrolytic bacteria (Flint et al., 2012). The similarities between mare milk and foal meconium, prior to nursing, could be explained through endogenous transfer of microbiota to the mammary gland and to the foal in utero. It has been postulated that an entero-mammary pathway could bring microbes from the rumen to the mammary gland through the lymphatic system (Addis et al., 2016). Foal feces on d 0 and 2 contain a greater relative abundance of Proteobacteria than older foals and adults. In humans, the placenta has been found to have a large relative abundance of Proteobacteria, further suggesting possibilities for endogenous transfer of bacteria prior to birth (Aagard et al., 2014). Initial intestinal colonization in humans is thought to be transferred through exposure to vaginal bacteria from the mother during delivery (Dominguez-Bello et al., 2010). A similar transfer of bacteria during birth has not been identified to date in the horse.

The most dramatic changes in fecal microbial composition occurred during the first month of life. Within the first 2 d there was a shift in the dominant phyla of foal feces with an increase in Proteobacteria and Bacteriodetes and a decrease in Firmicutes. Increased relative abundance of Proteobacteria during the first days of life has also been observed in pigs and cattle (Jami et al., 2013; Slifierz et al., 2015). Similarly to foals, the Bacteriodetes phylum increases within the first 2 mo of age in cattle rumen fluid and feces (Jami et al., 2013; Klein-Jöbst et al., 2014). The introduction of solid plant-based food has also been associated with an increase in the Bacteriodetes phylum in human infants (Koenig et al., 2011). Members of the Bacteriodetes

phylum found in the GIT can be associated with degradation of polysaccharides, which make up plant cell walls (Thomas et al., 2011). Bacteroides, a member of the Bacteriodetes phylum, and Escherichia, a member of the Proteobacteria phylum, make up the dominant genera of foal feces from d 2 and 7 respectively. These genera are also dominant in calf and pig feces during the first few weeks of life (Klein-Jöbstl et al., 2014; Mach et al., 2015). Bacteroides and Escherichia can also be found in the intestinal tract of humans after 10 d and are thought to be transferred to newborns through delivery (Nowrouzian et al., 2003; Wexler, 2007). In the first week of life foals are exclusively consuming milk and require microbes, such as Bacteroides, to metabolize oligosaccharides that otherwise could not be digested (Jandhyala et al., 2015). From d 7 to 1 mo, Proteobacteria decreases and Bacteriodetes, Firmicutes and Verrucomicrobia remain the dominant phyla through adulthood. Members of the Bacteriodetes and Firmicutes phylum are vital to the horse as they aid in the metabolism of insoluble fiber to produce VFAs which are then used for energy (Thomas et al., 2011; Biddle et al., 2013).

Foal fecal bacterial composition is similar to an adult mare within the first 60 d of life. By 1 mo, the relative abundance at the phylum level and the diversity in foal feces does not differ from adult mare feces; however, differences in abundances of several genera were found until 2 mo. This agrees with previous studies that observed stability and similarity to mares by 60 d (Kuhl et al. 2011, Costa et al., 2015b). Although the majority of nutritional requirements are met by milk until weaning, the foal will begin to consume forages and grain within the first weeks of life (Ladewig et al., 2005). Additionally, foals practice coprophagy and consume the feces of their dam (Crowel-Davis et al., 1985). The rapid changes in foal fecal microbiota that occur before reaching stability in the first months of life could be explained by inoculation through the consumption of their dam feces, introduction of solid feeds, and environmental exposure. Since

the equine fecal microbiota is influenced by diet and environment (Dougal et al., 2014; Hansen et al., 2015), by keeping the diet and environment of foals on this study constant we were able to isolate the effect of age.

Although there have been advances in the research of equine fecal microbiota there are still many limitations. Feces are currently used to predict the bacterial communities in the equine intestinal tract; however, they may only representative bacteria found in more distal intestinal compartments, such as the small colon (Costa et al., 2015a). Although significance was found, this study used a small sample size which could inaccurately estimate changes in fecal bacteria. Although the advancement of NGS allows for a more comprehensive classification of fecal bacteria as compared to culture techniques, there is a low taxonomic resolution as many intestinal bacteria genera and species in the horse have not yet been classified.

Conclusion

This study confirmed that foals are born with fecal bacteria low in species diversity that increases with age. Results indicate that the composition of bacteria found in foal meconium at birth is analogous to mare milk and that rapid changes in composition occur during the first week of life. The foal fecal microbial composition stabilizes and becomes similar to that of an adult within the first two months of life; however, further research is needed to determine the influence of mare vaginal and placenta bacteria on initial foal fecal bacterial development. The shift from oligosaccharide degrading, facultative anaerobic bacteria to fibrolytic, obligate anaerobic bacteria occurred in conjunction with the transition in the consumption of milk to the gradual introduction of forages to the diet of the foal. Although the compositional changes of the foal fecal microbiota after birth were documented, the exact function of specific bacteria is still unknown due to limitations in current research technologies.

Literature Cited

Aagaard, K., J. Ma, K. M. Antony, R. Ganu, J. Petrosino, and J. Versalovic. 2014. The placenta harbors a unique microbiome. Sci. Transl. Med. 6(237):237ra65. doi:10.1126/scitranslmed.3008599

Addis, M. F., A. Tanca, S. Uzzau, G. Oikonomou, R. C. Bicalho, and P. Moroni. 2016. The bovine milk microbiota: Insights and perspectives from -omics studies. Mol. Biosyst. 12(8):2359-2372. doi:10.1039/c6mb00217j

Al Jassim, R. A., and F. M. Andrews. 2009. The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic, and stomach ulcers. Vet. Clin. North Am. Equine Pract. 25(2):199-215. doi:10.1016/j.cveq.2009.04.005

Bewley, J.M., R.J. Harmon, S. Locke, and M. Arnold. 2014. Collection and Preparation of Milk Samples for Microbiological Culturing. Agriculture and Natural Resources Publications. Paper 164.

Biddle, A., L. Stewart, J. Blanchard, and S. Leschine. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. 2013. Diversity. 5:627-640. doi:10.3390/d5030627

Bordin, A. I., J. S. Suchodolski, M. E. Markel, K. B. Weaver, J. M. Steiner, S. E. Dowd, S. Pillai, and N. D. Cohen. 2013. Effects of administration of live or inactivated virulent rhodococccus equi and age on the fecal microbiome of neonatal foals. PLoS One. 8(6):e66640. doi:10.1371/journal.pone.0066640

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the illumina HiSeq and MiSeq platforms. Isme j. 6(8):1621-1624. doi:10.1038/ismej.2012.8

Costa, M. C., L. G. Arroyo, E. Allen-Vercoe, H. R. Stampfli, P. T. Kim, A. Sturgeon, and J. S. Weese. 2012. 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. 7(7):e41484. doi:10.1371/journal.pone.0041484

Costa, M. C., G. Silva, R. V. Ramos, H. R. Staempfli, L. G. Arroyo, P. Kim, and J. S. Weese. 2015a. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet. J. 205(1):74-80. doi:10.1016/j.tvjl.2015.03.018

Costa, M. C., H. R. Stampfli, E. Allen-Vercoe, and J. S. Weese. 2015b. Development of the faecal microbiota in foals. Equine Vet. J. 48(6):681-688. doi:10.1111/evj.12532

Costa, M. C., and J. S. Weese. 2012. The equine intestinal microbiome. Anim. Health. Res. Rev. 13(1):121-128. doi:10.1017/S1466252312000035

Crowell-Davis, S.L. 1985. Nursing behaviour and maternal aggression among Welsh ponies (Equus caballus). Appl. Anim. Behav. Sci. 14(1):11–25. doi: 10.1016/0168-1591(85)90034-6

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U.S.A. 107(26):11971-11975. doi:10.1073/pnas.1002601107

Dougal, K., P. A. Harris, A. Edwards, J. A. Pachebat, T. M. Blackmore, H. J. Worgan, and C. J. Newbold. 2012. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol. Ecol. 82(3):642-652. doi:10.1111/j.1574-6941.2012.01441.x

Dougal, K., G. de la Fuente, P. A. Harris, S. E. Girdwood, E. Pinloche, R. J. Geor, B. D. Nielsen, H. C. Schott 2nd, S. Elzinga, and C. J. Newbold. 2014. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 9(2):e87424. doi:10.1371/journal.pone.0087424

Earing, J.E., A.C. Durig, G.L. Gellin, L.M. Lawrence, and M.D. Flythe. 2012. Bacterial colonisation of the equine gut; Comparison of mare and foal pairs by PCR-DGGE. Adv Microbiol 2:79-86. doi:10.4236/aim.2012.22010.

Faubladier, C., S. Sadet-Bourgeteau, C. Philippeau, E. Jacotot, and V. Julliand. 2014. Molecular monitoring of the bacterial community structure in foal feces pre- and post-weaning. Anaerobe. 25:61-66. doi:10.1016/j.anaerobe.2013.11.010

Fernandes, K. A., S. Kittelmann, C. W. Rogers, E. K. Gee, C. F. Bolwell, E. N. Bermingham, and D. G. Thomas. 2014. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PLoS One. 9(11):e112846. doi:10.1371/journal.pone.0112846

Flint, H. J., K. P. Scott, P. Louis, and S. H. Duncan. 2012. The role of the gut microbiota in nutrition and health. Nat. Rev. Gastroenterol. Hepatol. 9(10):577-589. doi:10.1038/nrgastro.2012.156

Hansen, N. C., E. Avershina, L. T. Mydland, J. A. Naesset, D. Austbo, B. Moen, I. Mage, and K. Rudi. 2015. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb. Ecol. Health Dis. 26:27216. doi:10.3402/mehd.v26.27216

Jandhyala, S.M., T. Rupjyoti, S. Chivkula, V. Harish, S. Mitnala, and D.N. Reddy. 2015. Role of the normal gut microbiota. World J. Gastroenterol. 21(29):8787–8803. doi:10.3748/wjg.v21.i29.8787

Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7(6):1069-1079. doi:10.1038/ismej.2013.2

Julliand, V., and P. Grimm. 2016. HORSE SPECIES SYMPOSIUM: The microbiome of the horse hindgut: History and current knowledge. J. Anim. Sci. 94(6):2262-2274. doi:10.2527/jas.2015-0198

Klein-Jobstl, D., E. Schornsteiner, E. Mann, M. Wagner, M. Drillich, and S. Schmitz-Esser. 2014. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front. Microbiol. 5:622. doi:10.3389/fmicb.2014.00622

Koenig, J. E., A. Spor, N. Scalfone, A. D. Fricker, J. Stombaugh, R. Knight, L. T. Angenent, and R. E. Ley. 2011. Succession of microbial consortia in the developing infant gut microbiome. Proc. Natl. Acad. Sci. U.S.A. 1:4578-4585. doi:10.1073/pnas.1000081107

König, H., and J. Fröhlich. 2009. Lactic acid bacteria. In: Biology of Microorganisms on Grapes, in Must and in Wine. Springer Berlin Heidelberg. p. 3-29.

Kuehn, J. S., P. J. Gorden, D. Munro, R. Rong, Q. Dong, P. J. Plummer, C. Wang, and G. J. Phillips. 2013. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One. 8(4):e61959. doi:10.1371/journal.pone.0061959

Kuhl, J., N. Winterhoff, M. Wulf, F. J. Schweigert, I. Schwendenwein, R. M. Bruckmaier, J. E. Aurich, P. Kutzer, and C. Aurich. 2011. Changes in faecal bacteria and metabolic parameters in foals during the first six weeks of life. Vet. Microbiol. 151(3-4):321-328. doi:10.1016/j.vetmic.2011.03.017

Ladewig, J., E. Søndergaard, and J.W. Christensen. 2005. Ontogeny: preparing the young horse for its adult life. In: D.S. Mills and S.M. McDonnell, editors, The Domestic Horse: The Origins, Development and Management of its Behaviour. Cambridge University Press. p 139–149.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature. 489(7415):220-230. doi:10.1038/nature11550

Mach, N., M. Berri, J. Estellé, F. Levenez, G. Lemonnier, C. Denis, J. Leplat, C. Chevaleyre, Y. Billon, J. Doré, C. Rogel-Gaillard, and P Lepage. 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ. Microbiol. Rep. 7:554–569. doi:10.1111/1758-2229.12285

Martín, R., S. Langa, C. Reviriego, E. Jimínez, M.L. Marín, J. Xaus, L. Fernández, and J.M. Rodríguez. 2003. Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr. 143(6):754-758. doi:10.1016/j.jpeds.2003.09.028

Meropol, S. B., and A. Edwards. 2015. Development of the infant intestinal microbiome: A bird's eye view of a complex process. Birth Defects Res. C. Embryo Today. 105(4):228-239. doi:10.1002/bdrc.21114

NRC. 2007. Nutrient Requirements of Horses. 6th rev. ed. Natl. Acad. Press, Washington, DC.

Nowrouzian, F., B. Hesselmar, R. Saalman, I.L. Strannegard, N. Aberg, A.E. Wold, and I. Adlerberth. 2003. Escherichia coli in infants' intestinal microflora: colonization rate, strain turnover, and virulence gene carriage. Pediatr. Res. 54(1):8-14. doi:10.1203/01.PDR.0000069843.20655.EE

Slifierz, M. J., R. M. Friendship, and J. S. Weese. 2015. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol. 15(1):184-015-0512-7. doi:10.1186/s12866-015-0512-7

Steelman, S. M., B. P. Chowdhary, S. Dowd, J. Suchodolski, and J. E. Janecka. 2012. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet. Res. 8:231. doi:10.1186/1746-6148-8-231

Thomas, F., J. H. Hehemann, E. Rebuffet, M. Czjzek, and G. Michel. 2011. Environmental and gut bacteroidetes: The food connection. Front. Microbiol. 2:93. doi:10.3389/fmicb.2011.00093

Wang, Q., G.M. Garrity, J.M. Tiedje, and J.R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73(16):5261-5267. doi:10.1128/AEM.00062-07

Wexler, H.M. 2007. Bacteroides: the Good, the Bad, and the Nitty-Gritty. Clin. Microbiol. Rev. 20(4):593-621. doi:10.1128/CMR.00008-07.

Whitfield-Cargile, C. M., N. D. Cohen, J. Suchodolski, M. K. Chaffin, C. M. McQueen, C. E. Arnold, S. E. Dowd, and G. P. Blodgett. 2015. Composition and diversity of the fecal microbiome and inferred fecal metagenome does not predict subsequent pneumonia caused by rhodococcus equi in foals. PLoS One. 10(8):e0136586. doi:10.1371/journal.pone.0136586

Zhang, R., W. Huo, W. Zhu, and S. Mao. 2015. Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. J. Sci. Food Agric. 95(5):1072-1079. doi:10.1002/jsfa.6800

Chapter 3 - The effect of weaning and acute stress associated with abrupt weaning on foal fecal microbiota

Abstract

The objectives of this study were to determine the effect of weaning and weaning method on the composition of foal fecal microbiota. Nine foals were blocked by gender and weight and randomly assigned to 2 treatment groups: abrupt (n = 5) and gradual (n = 4) weaning. Fecal samples were collected the day before weaning (d -1), the day of weaning (d 0), and post-weaning on d 1, 2, 3, 4, and 7. Blood was collected for analysis of cortisol concentration at 0800 h on d -1, 1, and 2, and at 0800 h and 1100 h on d 0 and 4. Heart rate was recorded in 10 min intervals on the d of weaning starting 1 h before weaning to 2 h post-weaning, and again for 1 h starting 24 h after weaning. Next generation sequencing of the V4 region of the bacterial 16S rRNA gene was performed on foal fecal samples using the Illumina Miseq according to Earth Microbiome Project protocols. Sequencing data was analyzed using QIIME. There were no differences in species diversity between treatments or when comparing fecal bacteria before and after weaning ($P > 0.05$), and there was no clear separation in bacterial community structure as determined through non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities. The Bacteriodetes, Firmicutes and Verrucomicrobia phyla accounted for over 80% of the relative abundance, regardless of treatment or time. There were minor shifts in composition that occurred in response to treatment, with decreased relative abundance of Firmicutes in the gradual group post-weaning ($P = 0.02$). In addition, the phyla Spirochaetes, comprised of the genus Treponema, and Cyanobacteria decreased following weaning ($P < 0.01$), but were not influenced by weaning method. A significant time by treatment interaction was observed in cortisol ($P<0.05$). Foals in the abrupt treatment had increased cortisol ($P < 0.05$) on d 1 as compared to foals in the gradual group. Heart rate also increased ($P < 0.05$) in foals in the

abrupt weaning group for 50 m post-weaning. Increased cortisol and heart rate after weaning represents and increased level of acute stress for foals weaned abruptly. There were minor differences in foal fecal bacterial composition following weaning and although acute stress was increased in abrupt weaned foals, weaning method did not have an obvious impact on fecal microbiota during the first 7 d post-weaning.

Introduction

Weaning in horses is associated with nutritional stress from the cessation of nursing, as well as mental and physical stress from maternal separation. Stress can be detected through physiological changes, such as increased cortisol secretions (McCall et al., 1987). Increased cortisol concentrations following weaning have been previously reported in foals (McCall et al., 1987; Malinowski et al., 1990; Moons et al., 2005; Merkies et al., 2016). In addition to cortisol, increased heart rates have been detected following weaning, with the highest heart rate observed immediately after weaning (Moons et al., 2005).

Management during weaning can influence stress. Foals paddock weaned in groups have less stress in response to weaning than foals individually weaned in stalls (Helsinki et al., 2002; McGeevy et al., 2005). In addition, foals weaned abruptly had increased stress when compared to foals separated from their dam by a fenceline (McCall et al., 1987). Based on existing data, it appears that abrupt stall weaning is the method associated with highest level of stress.

Prior to weaning, the equine GIT is gradually colonized by fibrolytic and cellulolytic bacteria (Faubledier et al., 2013; Jacquay, unpublished). This coincides with the introduction of forages and concentrates to the foals diet (Ladewig et al., 2005). There is a shift in fecal microbial composition following dietary changes that occur during weaning in pigs and cattle (Pajarillo et al., 2014; Meale et al., 2016). Previous research did not detect changes in microbial

composition following weaning in foals; however, samples were collected from various time points within a 60 d period of when weaning occurred and it is possible that changes were not detected with a broad sampling range (Costa et al., 2015b).

The objectives of this study were to determine if there is a shift in fecal microbiota within the week following weaning and if different weaning methods effect foal stress and influence the fecal microbiota of foals. It was hypothesized that the change in diet that occurs with weaning, coupled with the added stress of abrupt stall weaning would have the greatest influence on changes in the fecal microbiota of foals.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Usage Committee at Kansas State University (Protocol No. 3571).

Animals

Nine Quarter Horse foals, born between April and May 2015, were used in this study. Prior to weaning, foals were group housed with their dams in a pasture with ad libitum access to brome hay and water. Mares were supplemented with a pelleted concentrate, Purina Strategy (Purina Mills, Inc., St. Louis, MO), to meet dietary requirements for early lactation and although foals were not specifically fed concentrate, they did have access to their dams feed bins (NRC, 2007). During weaning, foals were individually housed in 12’ x 12’ stalls or 12’ x 24’ runs from the day before weaning (d -1) to d 4, when they were turned out with the other previously weaned foals in the study and group housed on pasture through d 7 with access to ad libidum brome hay and water throughout the study. Foals were fed the same concentrate as their dams in order to meet nutritional requirements of a growing horse at 4 mo of age (NRC, 2007).

Treatment and Weaning Management

Foals were weaned in pairs according to age (120 ± 5 d). At weaning, foals were blocked by weight and gender and assigned one of two treatment groups: abrupt (n=5) or gradual (n=4). Housing for weaning consisted of a 5 stall barn across from 5 covered runs with an aisle in between. Each run was made of a 5 rail pipe fencing with approximately 4' tall panels with 10" of space between each rail. Stalls consisted of 2 solid wood and concrete walls and 2 walls featuring a vertical bar top and wood filled bottom measuring 7' tall, with a 4' wide full-length sliding door on the aisle wall. The bottom of the stall walls consisted of wood panels approximately 4' tall and the top of the stall contained steel bars 33" in height with 2.25" spaces between each bar.

Foals in the abrupt weaning group were individually placed into a 12' x 12' stall at the end of the barn farthest from any other horses the day of weaning (d 0) and their dam was removed and placed into a paddock out of physical, visual, and auditory range. Abruptly weaned foals then remained stalled, without direct visual and physical contact with another horse for 48 h (d 0 to 1). They were then moved across an aisle and placed into a 12' x 24' run, next to the foal in the gradual treatment group, for another 48 h (d 2 to 3).

On d 0 of weaning, foals in the gradual weaning group were placed in 12' x 24' runs adjacent to their dam with the opportunity for the foal to see, hear, smell, and touch their dam for 48 h (d 0 to 1). After 48 h, the dam was placed in a 12' x 12' stall directly across the aisle from their foal where they could see and hear, but no longer had physical contact with their foal for another 48 h (d 2 to 3). Also occurring on d 2, the foal in the abrupt group was moved into the adjacent run allowing the foal in the gradual group physical contact with the abruptly weaned foal. Following fecal and blood collections on d 4, foals from both groups were turned out

together in a large pen, with the previously weaned foals on the study, and dams of foals in the gradual group were removed from visual and auditory range.

Fecal Sampling

Foal fecal samples were collected from foals 1 d prior to weaning (d -1), at weaning (d 0), and on d 1, 2, 3, 4, and 7 post-weaning. Foal fecal samples were collected manually using sterile latex gloves and sterile lubricating jelly (First Priority, Inc., Elgin, IL). Between 5 and 50 g of feces were collected and placed directly into 50 mL sterile conical tubes. After collection, fecal samples were immediately stored at -20° C until further analysis.

Cortisol Concentration

Foals were handled prior to blood collection and blood samples were taken by the same technician to reduce potential for increased stress and cortisol. Blood was collected the d before weaning (d -1), the day of weaning (d 0) and after weaning on d 1, 2, 3, and 4. Collections occurred between 0800 h and 0900 h to minimize the circadian effect as cortisol concentrations are highest in the morning with peak concentrations between 0600 h and 0900 h. (Cordero et al., 2012). On d 0 and 4 a second blood pull occurred approximately 3 h after the initial sample was taken between 1100 h and 1200 h. A 10 mL blood sample was collected via the jugular vein into heparinized blood tubes (Becton, Dickson and Company, Franklin Lakes, NJ). Whole blood was centrifuged at 1,400 x g for 15 min at 25° C and plasma was separated and frozen into 1.5 mL aliquots and stored at -20° C until further analysis. Cortisol concentrations were determined in duplicate using the ImmuChem Cortisol ^{125}I Coated Tube RIA Kit (MP Biomedicals, Santa Ana, CA) which was previously validated for use in the horse (Gold et al. 2012). The intra-assay C.V. was 8%, with a detection sensitivity of 3.0 ng /ml.

Heart Rate

The day prior to weaning, foals were acclimated to wearing heart rate monitors so that handling and attaching the monitors would not influence heart rates. Heart rate monitors (InZone 93039578, Polar Equine, Lake Success, NY) were attached according to manufacturer instructions. First, Lectron II electrode conductivity gel (Pharmaceutical Innovations, Inc., Newark, NJ) was placed on the surface of the electrodes. Next, the positive electrode was placed behind the shoulder near the withers and the negative electrode was placed behind the elbow at the heart girth area. The transmitter and electrodes were secured to the weanlings with Vetrap bandaging (3M, St. Paul, MN) and Elastikon tape (Johnson & Johnson, New Brunswick, NJ). Heart rate monitors were attached 1 h prior to weaning at 0800 h on d 0 and at 0800 h 24 h post-weaning. Pulse (bpm) was recorded every 10 min for 3 h using wristwatch receivers, secured to the transmitter on the weanling, for 1 h prior to weaning and for 2 h post-weaning on d 0, day of weaning, and again for 1 h 24 h post-weaning (d 1).

DNA Extraction and 16S rRNA Gene Amplification

Total DNA was extracted from 200 mg of frozen fecal samples using the EZNA Stool DNA Kit (Omega Bio Tek, Norcross, GA) and the stool DNA protocol for pathogen detection, previously validated for use in the horse (Costa et al. 2012). After extraction, DNA was quantified through the Nanodrop 800 spectrophotometer (Thermo Scientific, Wilmington, DE).

The V4 region of bacterial 16S rRNA gene was amplified using modified universal PCR primers (515F/806F) according to Earth Microbiome Project protocols (Caporaso et al., 2012). Fecal DNA samples were then amplified in triplicate through PCR reactions run on a Mastercycler nexus (Eppendorf, Hamburg, Germany) in a 25 µL solution containing 5 µL 5x PCR master mix (Promega; Madison, WI), 0.5 µL of each primer (10 uM), 0.5 µL dntps, 1 uL

1% BSA, 0.25 µL GoTaq Polymerase (Promega; Madison, WI), 16.25 µL PCR grade H2O and 1 µL template DNA for 25 cycles. All PCR reactions were run with a negative control to ensure there was no bacterial contamination and appropriate amplification of PCR products was checked using agarose gel electrophoresis. Amplicon triplicates were combined and non-PCR products, such as residual reagents and primers, were removed from the amplicon using the EXO-SAP-IT kit (Thermo Fischer Scientific, Waltham, MA). Each sample was individually quantified using Quant-iT PicoGreeen dsDNA Assay Kit (Thermo Fischer Scientific, Waltham, MA). Approximately 100 ng of DNA from each sample was combined in a 1.5 mL microcentrifuge tube. The combined library was purified and extracted from 2% agarose gel using the QIAquick gel purification kit (Quiagen, Valencia, CA) to ensure a uniform library size of 400 bp.

Sequencing and Analysis

The combined library was submitted to the K-State Integrated Genomics Facility for sequencing. The library was first analyzed for quality and fragment length using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and qPCR. Amplicon sequencing was performed using the 300 cycles MiSeq reagent v3 kit for a single 2 x150 paired-end cycle run on the Illumina Miseq, with a 10% PhiX spike to increase library diversity and overall read quality.

Sequencing analysis was performed using QIIME version 2.06 (Quantitative Insights into Microbial Ecology). MiSeq data was received in the form of three FASTQ files R1 (forward), R2 (reverse) and I1 and the paired end reads were joined. A mapping file was created to link nucleotide barcodes with corresponding sample identification. The library was then demultiplexed as samples were assigned to their barcode sequence. Next, reads were assigned to operational taxonomic units (OTUs) using an open-reference OTU picking protocol based on

97% sequence similarity and aligned to the GreenGenes sequence database (version 13.5). Taxonomic assignments were given using the Ribosomal Database Project (RDP) classifier (Wang et al. 2007). Then OTU assignment was completed on RDP taxonomy data by aligning OTU sequences with the GreenGenes database and any unassigned OTUs were removed. Chimeras were identified using ChimeraSlayer and removed from the data matrix.

Data analysis was performed on all samples at 20,300 reads based on rarefaction curves constructed in QIIME. The rarefied data set included 152,465 total OTUs. Alpha rarefaction indices were calculated to determine within-sample differences and beta diversity plots were also constructed to compare between-sample differences. Using QIIME, observed OTUs, Chao1 richness, and Shannon diversity were calculated to determine species richness, number of different species, and evenness, abundance of those species. Nonmetric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities was used to measure differences between samples based on OTU relative abundance. From QIIME, summary plots of taxonomy were created to report relative abundances of dominant taxa.

Statistical Analysis

All statistical analysis was performed using R (version 3.2.2). Alpha diversity metrics, differences in relative abundances between taxa, cortisol concentrations, and heart rates were measured using linear-mixed effects model ANOVA with the lme function in nlme R package. Post hoc comparisons using Bonferroni adjustments were used to compare treatment and time relative to weaning using the glht function in the multcomp R package. Least-square means (LSM) and standard error were calculated using the lsmeans function in the lsmeans R package.

Results

Alpha Diversity

There were no differences in observed OTUs, Chao1, and Shannon alpha diversity metrics between treatments, abrupt versus gradual, or time periods, pre-weaning, d -1 and d 0, and post-weaning, d 1 to d 7 ($P > 0.4$; Table 3-1).

Table 3-1. Alpha diversity metrics[1] (LSM ± SEM) of fecal bacteria from pre-weaning[2] and post-weaning[3] in foals weaned abruptly (n = 5) or gradually (n = 4)

	Observed OTUs	Chao1	Shannon
Time periods			
Abrupt			
Pre-weaning	2839.3 ± 108.8	5971.4 ± 263.2	9.0 ± 0.14
Post-weaning	2767.7 ± 75.1	6021.6 ± 181.6	8.8 ± 0.10
Gradual			
Pre-weaning	2827.2 ± 140.1	5997.6 ± 339.8	9.0 ± 0.18
Post-weaning	2717.8 ± 81.1	5783.7 ± 196.2	8.7 ± 0.10

[1]There were no differences between treatments or time relative to weaning for any alpha diversity metric ($P \geq 0.4$)
[2]Pre-weaning: d -1 and 0.
[3]Post-weaning: d 1, 2, 3, 4 and 7.

Beta Diversity

There were no clear separations between treatments, abrupt or gradual weaning, or time relative to weaning (d 0) based off of NMDS (Figure 3-1). NMDS1 ordinations were significantly different for time point d 0 ($P<0.001$); however, there were no distinct separations between any other time points.

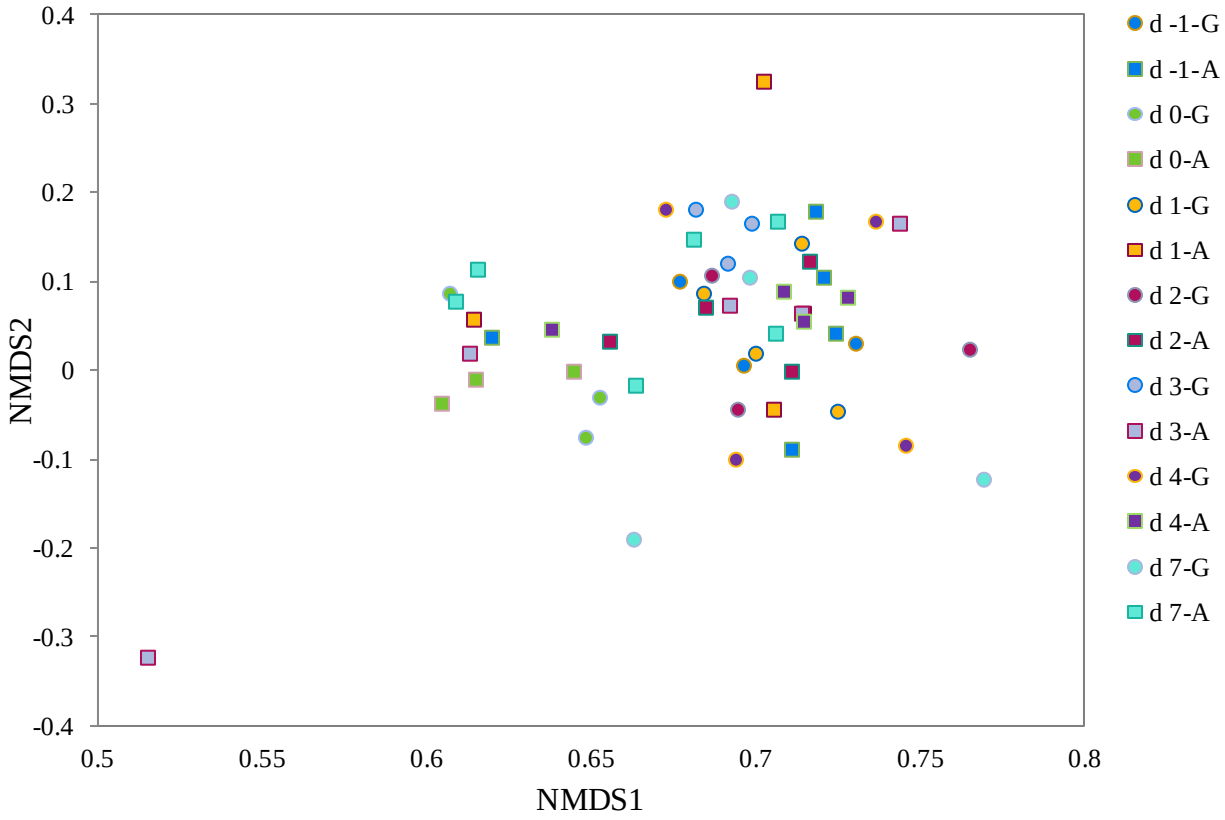

Figure 3-1. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance of foal feces from different time points relative to weaning (d 0), from the day before weaning (d -1) to d 7 post-weaning, and different treatments: abrupt (n=5) and gradual (n = 4) weaning. Points with different colors represent different time points before and after weaning. Points with different shapes represent different weaning methods: gradual (circle) and abrupt (square).

Community Composition

The main bacteria phyla found in foals at weaning were Firmicutes, Bacteriodetes, Verrucomicrobia, Spirochaetes, Fibrobacteres and Proteobacteria (Figure 3-2). Firmicutes was the only phylum that showed a difference between treatments after weaning ($P < 0.05$); however, Cyanobacteria ($P < 0.01$) and Spirochaetes ($P < 0.001$) significantly decreased following weaning (Table 3-2). The relative abundance of bacteria at the genus level are shown in Figure 3-3. There were 3 bacterial genera that changed during weaning with BF311, a genus from the Bacteriodaceae family, being significantly different between treatments after weaning ($P = 0.05$; Table 3-3).

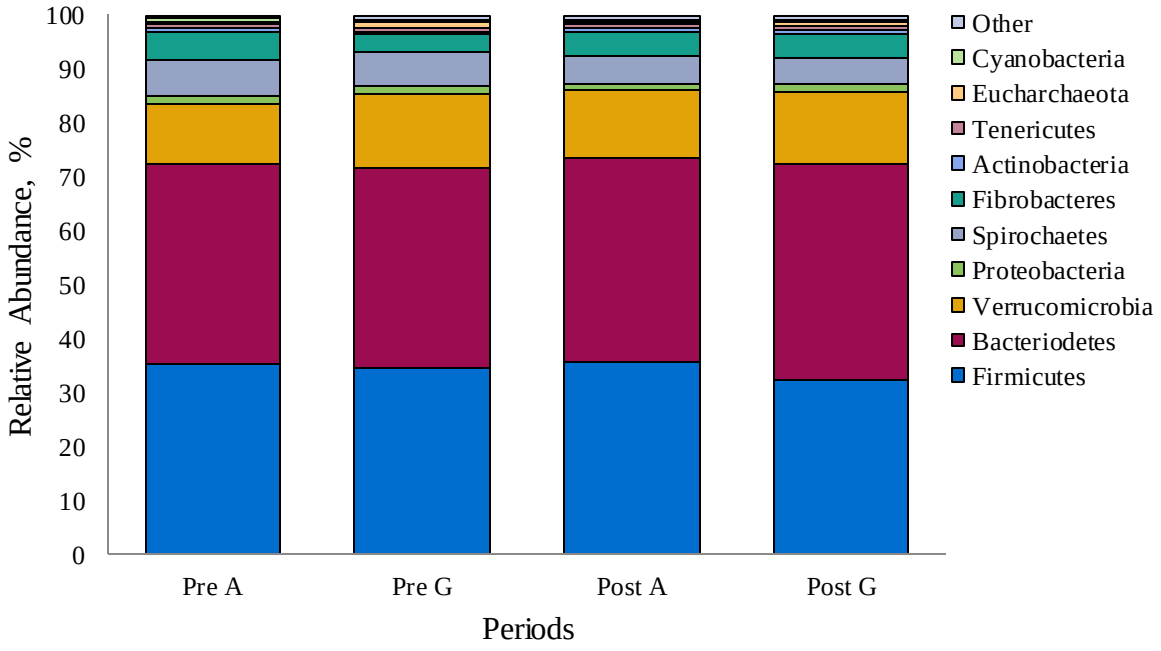

Figure 3-2. Relative abundance of bacteria from foal feces at the phylum level before and after weaning and between treatments: abrupt and gradual weaning. Foal fecal samples from the day before weaning and day of weaning were combined and into the group Pre and samples from d 1 to 7 following weaning were grouped as Post. Treatment groups consist of abrupt (A) and gradual (G) weaning methods.

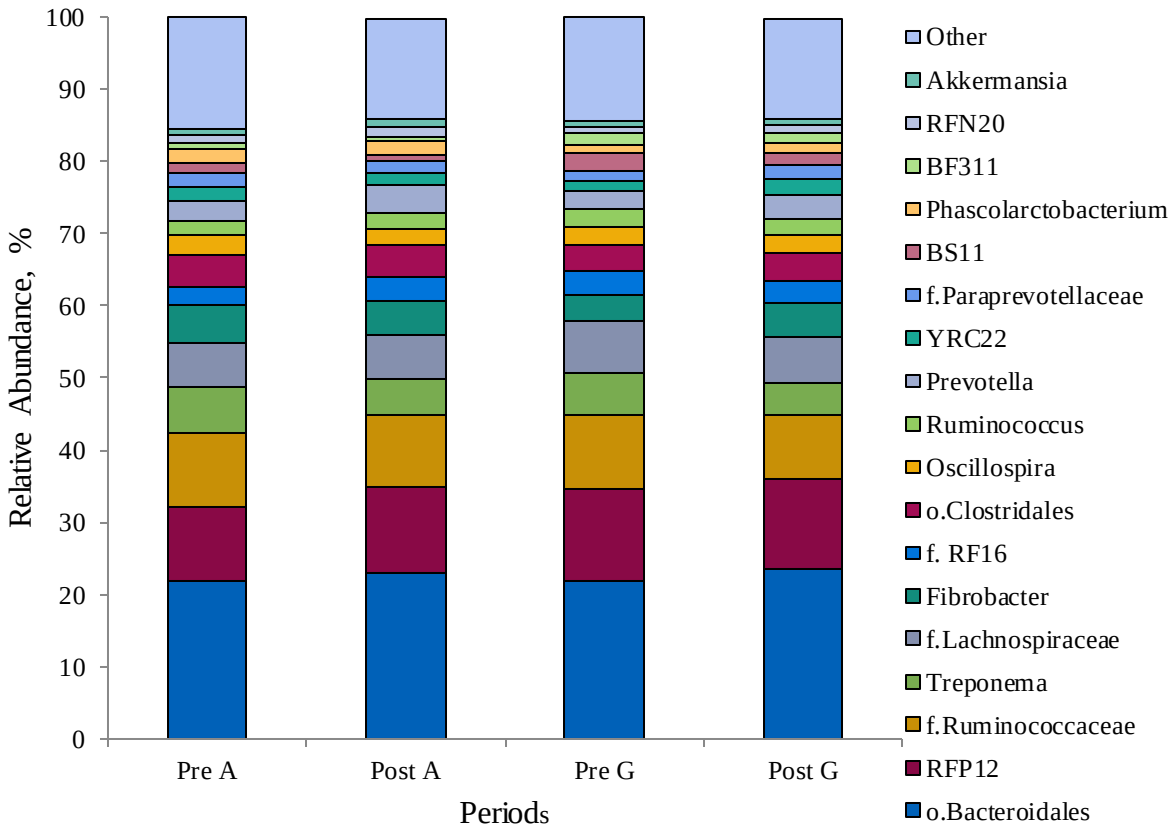

Figure 3-3. Relative abundance of bacteria in foal feces at the genus level, or lowest taxonomic resolution, before and after weaning and between treatments: abrupt and gradual weaning. Foal fecal samples from the day before weaning and day of weaning were combined and into the group Pre and samples from d 1 to 7 following weaning were grouped as Post. Treatment groups consist of abrupt (A) and gradual (G) weaning methods. Taxa with o. represent order as the lowest taxonomic level classified whereas f. represents family.

Table 3-2. Relative abundance (%) of bacterial phyla (LSM ± SEM) in foal feces with differences between treatments, abrupt and gradual weaning, and/or time relative to weaning

	Abrupt		Gradual	
	Pre[1]	Post[2]	Pre[1]	Post[2]
Phyla				
Firmicutes	35.2 ± 1.4	35.4[a] ± 1.1	34.4 ± 1.5	32.3[b] ± 1.2
Spirochaetes	6.8[c] ± 0.57	5.2[d] ± 0.37	6.3[c] ± 0.64	5.2[d] ± 0.40
Cyanobacteria	0.42[c] ± 0.06	0.29[d] ±0.05	0.53[c] ± 0.07	0.28[d] ± 0.05

[a,b] Values within a row with differing superscripts are different ($P \leq 0.05$).
[c,d] Values within a row with differing superscripts are different ($P \leq 0.01$).
[1]Pre-weaning: d -1 and 0.
[2]Post-weaning: d 1, 2, 3, 4 and 7.

Table 3-3. Relative abundance (%) of bacterial genera (LSM ± SEM) in foal feces with differences between treatments, abrupt and gradual weaning, and/or time relative to weaning

	Abrupt		Gradual	
	Pre[1]	Post[2]	Pre[1]	Post[2]
Genus				
Treponema	6.3[a] ± 0.51	5.0 ± 0.35	5.8 ± 0.65	4.4[b] ± 0.38
BF311 (Family Bacteriodaceae)	0.9 ± 0.21	0.6[a,c] ± 0.16	1.8[d] ± 0.28	1.3[b] ± 0.17
YRCC2 (Family Paraprevotellaceae)	1.8 ± 0.19	1.9 ± 0.16	1.3[a] ± 0.23	2.3[b] ± 0.18

[a,b] Values within a row with differing superscripts are different ($P \leq 0.05$).
[c,d] Values within a row with differing superscripts are different ($P \leq 0.01$).
[1]Pre-weaning: d -1 and 0.
[2]Post-weaning: d 1, 2, 3, 4 and 7.

Physiological Parameters

Heart rate and cortisol were measured as a representation of stress levels in response to treatment at weaning. There were no differences in cortisol concentrations between treatments the day prior to weaning (d -1, $P = 0.42$), and directly before weaning at 0800 h (d 0, $P = 0.59$). There was no significant effect of treatment on cortisol concentrations ($P = 0.4$); however, a treatment by time interaction was observed ($P < 0.05$). There was no significant difference in the mean cortisol concentration of foals in the gradual group across time points. Foals in the abrupt weaning group had significantly higher cortisol concentrations at d 1 as compared with foals in the gradual group ($P = 0.02$; Figure 3-4).

There were no differences in heart rate from 0 m to 60 m prior to weaning regardless of treatment group ($P > 0.05$, Figure 3-5). There was no effect of treatment on heart rate ($P = 0.11$), but the treatment by time interaction was significant ($P<0.001$). There was no significant difference in the mean heart rates of foals in the gradual treatment group across time points ($P > 0.99$). Foals in the abrupt treatment group had significantly higher heart rates than foals weaned gradually from 70 m to 110 m ($P \leq 0.04$; Figure 3-5). There were no differences in heart rate 24 h post-weaning ($P > 0.05$).

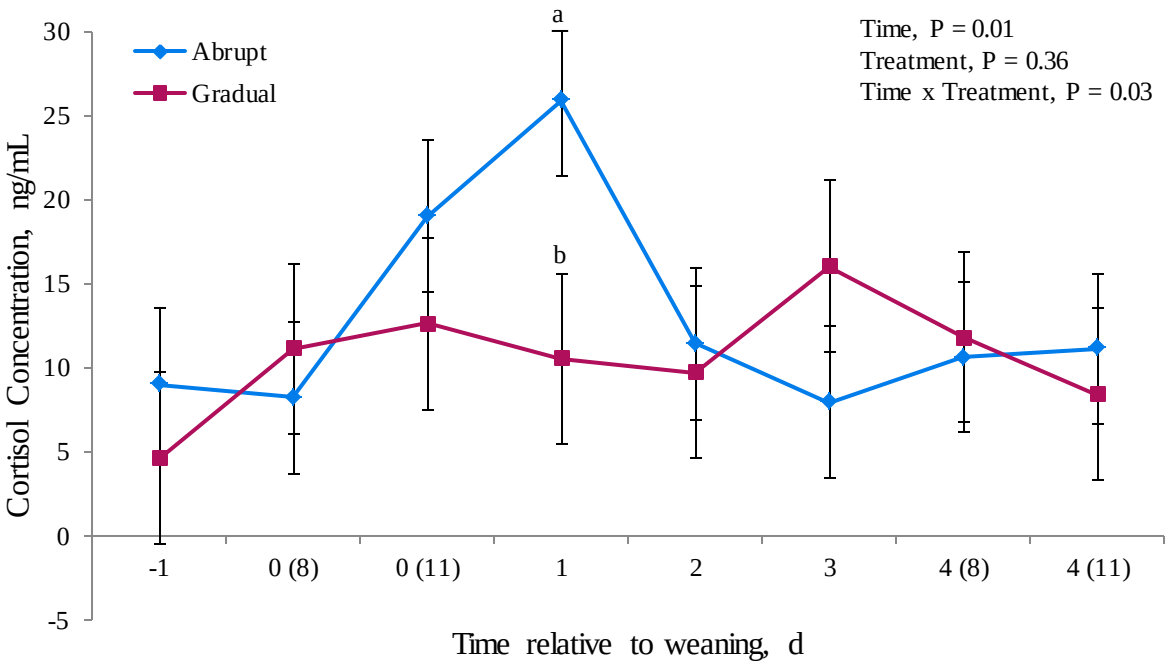

Figure 3-4. Mean concentrations of cortisol relative to weaning for foals assigned to 2 treatments: abrupt and gradual weaning. Weaning occurred directly after time point d 0 (a). Blood pull occurred at 0800 h on days without letters and d 0 (a) and 4 (a). Days with (b) indicate a second blood pull 3 h after initial, taken at 1100 h. Points lacking a common superscript differ, $^{a,b}P < 0.05$.

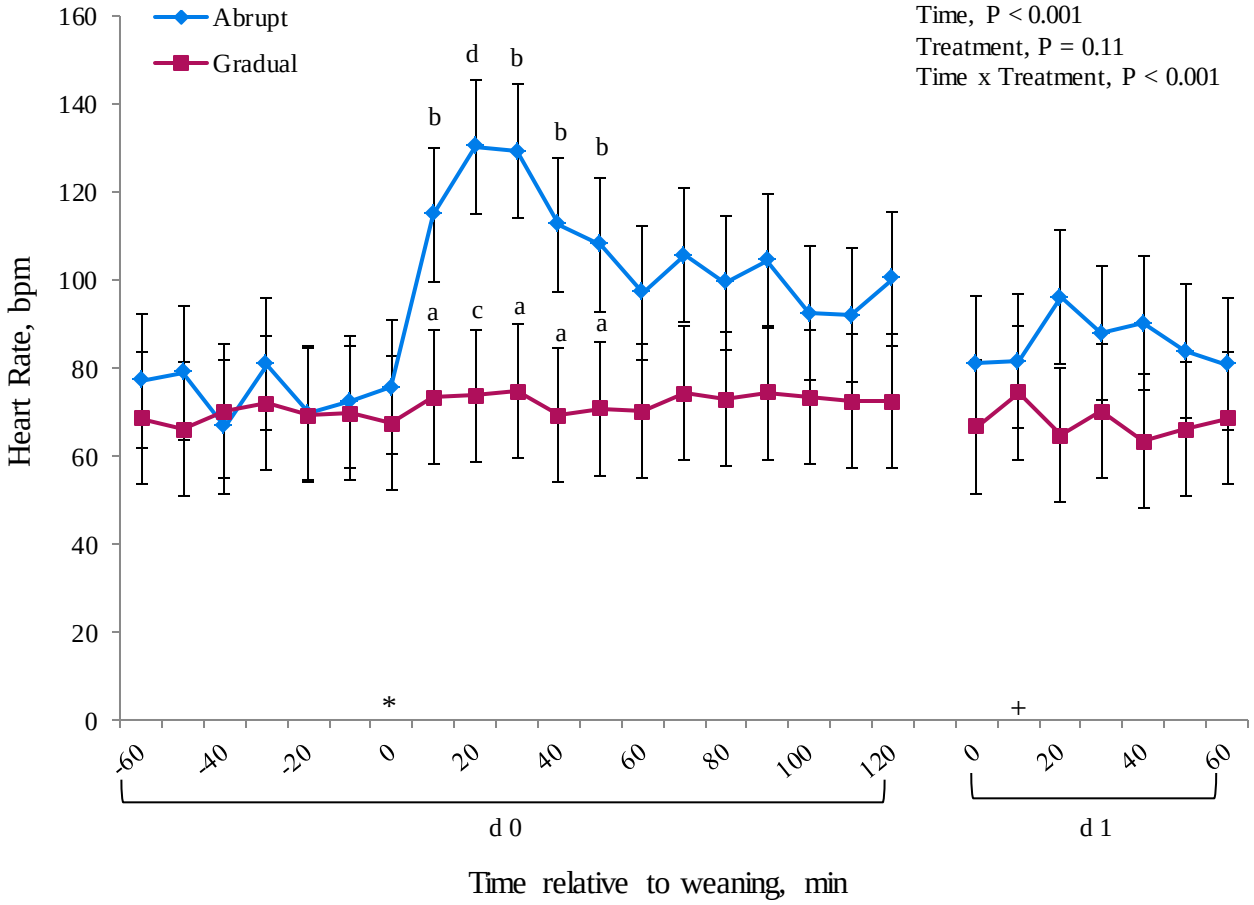

Figure 3-5. Mean heart rate (bpm) relative to weaning for foals assigned to 2 treatments: abrupt and gradual weaning, on the day of weaning (d 0) and 24 h post-weaning (d 1). * Indicates blood pull and weaning on d 0 and + indicates blood pull on d 1 for both treatment groups. Different superscripts at specific time points differ, $^{a,b}P < 0.05$ and $^{c,d}P < 0.01$.

Discussion

Although weaning method had little impact on fecal microbial composition, there were differences in stress between abrupt and gradual weaning methods. Abrupt stall weaning is associated with increased stress in foals compared with gradual paddock weaning (Waran et al., 2008). In this study, foals abruptly weaned in stalls had higher plasma cortisol concentrations 24 h post-weaning. Similar results have been observed with foals abruptly weaned having increased cortisol concentrations 24 h post-weaning compared to pre-weaning cortisol concentrations and cortisol concentrations in gradually weaned foals and (Berger et al., 2013; Dobcova et al., 2015). In addition, the abruptly weaned foals in this study had elevated heart rates for 50 m after weaning when compared to gradually weaned foals. Increased heart rate following weaning has been previously reported in foals abruptly weaned individually in stalls, with a mean heart rate of 86.57 bpm for the 12 h following weaning, whereas mean heart rate was 59.63 bpm pre-weaning (Moons et al., 2005). Similar to our results, using a gradual weaning method also did not influence heart rate the day of weaning (Merkies et al., 2016). The increased heart rate and cortisol concentrations observed in this study support the theory that abruptly weaning in stalls puts excess transient stress on foals as compared to alternative weaning methods. Although there was increased transient stress that occurred within 24 hr in response to abrupt weaning there were minimal changes in the foal fecal microbiota. While stress associated with abrupt weaning did not impact the fecal microbiota within 7 d of weaning in our study, there are still negative implications associated with increased stress at weaning. Although weight was not recorded in this study, increased cortisol concentrations following weaning have been correlated with a decrease in weight gain (Erber et al., 2012). Stress has been shown to influence the equine fecal microbiota 12 h after transportation and fasting, with a decrease in the relative abundance of

Clostridiales (Schoster et al., 2015). Decreased Clostridiales have also been observed in horses with acute colitis, indicating a relationship with intestinal health (Costa et al., 2012). The change in diet from fasting and stress associated with transportation could have a greater impact on fecal bacteria in the adult horse as compared with weaning stress in foals. However, the physiological consequence of a change in relative abundance of one order of bacteria has yet to be determined.

Minor shifts in relative abundance of specific phyla and genera were reported; however, the microbial composition was not altered by weaning of foals in this study. Bacteriodetes was the dominant phyla for all foals across all time points relative to weaning. This is in contrast with a previous study that detected an increase in Proteobacteria in foals post-weaning with Firmicutes as the dominant phyla (Costa et al., 2015b); however, it does agree with a study that found a higher relative abundance of the Bacteriodetes phylum in fecal microbiota in adult horses (Hansen et al., 2015). These differences could be related to differences in diets, management practices, sequencing methods, and intestinal tract location (Daly et al., 2012; Dougal et al., 2014, Fernandes et al., 2014; Hansen et al., 2015). At the phylum level there was a decrease in the relative abundance of Spirochaetes post-weaning, which was accounted for by the decrease in the genus Treponema. The bacteria present in foal feces at weaning collectively aid in the breakdown of insoluble fiber and it is unlikely that minor shifts in abundance have an effect on the overall function of the intestinal microbiota of the weanling.

Weaning is defined as the cessation of nursing, which constitutes a change of diet for the foal from milk to solid feeds. Horses are hindgut fermenters that rely on fiber-degrading bacteria in the cecum and colon to break down plant sources for the production of VFAs that are used by the horse for energy (Al Jassim and Andrews, 2009). Although most of the nutritional needs are met by milk from the dam prior to weaning, foals begin to consume grain and forages within the

first few weeks of life (Laedwig et al., 2005). The most common time to wean a foal is between 4 to 6 m of age and this coincides with a shift in digestive enzymes from lactase, which breaks down milk sugar, to amylase, needed for digestion of cereal grains (Kapper, 2004). There were no differences in relative abundances of any phyla or genera between foal feces at 4 mo of age or with feces from the foal's dam (Jacquay, unpublished data). In this study, foals had access to pasture, hay, and grain prior to weaning and it is possible that the introduction of solid feed primed the intestinal bacteria and aided in the transition to a microbial composition dominated by fibrolytic bacteria. The lack of changes in microbial diversity and structure as a result of weaning could be attributed to a gradual shift from a milk diet to consuming forages and grain prior to weaning (Faubladier et al., 2013). Cattle experience a similar sequential development of the rumen microbiota with a functionally mature composition present prior to weaning; however, increases in bacterial species diversity were observed post-weaning (Meale et al., 2016). In contrast, there is an abrupt transition from milk to solid feeds that occurs at weaning in pigs, accompanied by a more dramatic shift in microbial composition and function (Frese et al., 2015). Following weaning in pigs, there is a change in dominant phyla from the Firmicutes to Bacteriodetes and increased abundance of the Prevotella genus, which is capable of degrading hemicellulose and xylans (Pajarillo et al., 2014). The nutritional changes that occur at weaning in horses seem to have little impact on the fecal microbiota since the hindgut already contains fibrolytic and cellulolytic bacteria prior to weaning in animals that rely on the fermentation of forages to meet nutritional requirements.

Although not specifically fed creep feed, foals in this study did have access to concentrate grain to replicate standard management practices of foals prior to weaning. Prior research shows that foals fed concentrate prior to weaning had decreased stress responses as

compared to foals that did not receive concentrate feed (Hoffman et al., 1995). In addition, foals fed high sugar and starch diets were more stressed at weaning compared to those fed a diet higher in fat and fiber (Holland et al., 1996; Nicol et al., 2005). Diets with high starch and sugar are associated with increased Bacteriodetes and the lactic-acid producing bacteria Bacillus, Lactobacillus, and Streptococcus (Daly et al., 2012). Increased abundances of Lactobacillus and Streptococcus are also found in the feces of horses with oligofructose induced laminitis, suggesting increases of these intestinal bacteria are linked with disease (Moreau et al., 2014). Although the introduction of creep feed could aid in the development of equine intestinal bacteria, diets high in starch and sugar can negatively influence intestinal microbial health and further increase stress during weaning. Since diet has been observed as one of the main driving factors for changes in the equine microbial composition, changes in diet that occur at weaning could have a greater impact on the foal fecal microbiota than stress associated with weaning method. Therefore, proper development of the foal intestinal microbiota, through exposure to grain prior to weaning, could prevent occurrences of gastrointestinal problems at weaning.

In horses, feces are used as a representative of intestinal contents to determine bacterial composition due to the ease of sampling. When evaluating microbiota results, it should be taken into account that feces are only partially representative of proximal portions of the GIT, specifically the large colon, and differences in bacterial abundances at the genus level are still detected (Costa et al., 2015a). In cattle, differences in rumen microbial composition were identified post-weaning, but no differences were identified in fecal samples from the same animals (Meal et al., 2016). It is possible that there are changes in the equine cecal bacteria during weaning that go undetected in the feces due to differences in microbial composition across different intestinal compartments.

Conclusion

The equine fecal microbiota reaches stability and maturity in composition prior to weaning. There were minor changes in foal fecal microbial composition within the first 7 d following weaning; however, further research is needed to determine the threshold of change in equine intestinal microbiota that leads to functional disruption and a subsequent physiological change in health. Abrupt stall weaning increased acute stress, but did not substantially alter the foal fecal microbiota. The gradual transition in diet from milk to solid feed prior to weaning may have allowed the foal intestinal microbiota to remain unchanged in response to weaning and stress associated with abrupt weaning. The amount and duration of stress required to cause changes in the equine intestinal microbiota has yet to be determined.

Literature Cited

Berger, J.M. S.J. Spier, R. Davies, I.A. Gardner, C.M. Leutenegger, and M. Bain. Behavioral and physiological responses of weaned foals treated with equine appeasing pheromone: A double-blinded, placebo-controlled, randomized trial. J. Vet. Behav. 8(4):265-277. doi:10.1016/j.jveb.2012.09.003.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the illumina HiSeq and MiSeq platforms. ISME J. 6(8):1621-1624. doi:10.1038/ismej.2012.8

Cordero, M., B.W. Brorsen, and D. McFarlane. 2012. Circadian and circannual rhythms of cortisol, ACTH, and α-melanocyte-stimulating hormone in healthy horses. Domest. Anim. Endocrinol. 43(4):317-324. doi:10.1016/j.domaniend.2012.05.005

Costa, M. C., L. G. Arroyo, E. Allen-Vercoe, H. R. Stampfli, P. T. Kim, A. Sturgeon, and J. S. Weese. 2012. 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. 7(7):e41484. doi:10.1371/journal.pone.0041484

Costa, M. C., G. Silva, R. V. Ramos, H. R. Staempfli, L. G. Arroyo, P. Kim, and J. S. Weese. 2015a. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet. J. 205(1):74-80. doi:10.1016/j.tvjl.2015.03.018

Costa, M. C., H. R. Stampfli, E. Allen-Vercoe, and J. S. Weese. 2015b. Development of the faecal microbiota in foals. Equine Vet. J. 48(6):681-688. doi:10.1111/evj.12532

Daly, K.,C.J. Proudman, S.H. Duncan, H.J. Flint, J. Dyer, and S.P. Shirazi-Beechey. 2012. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. Br. J. Nutr. 107(7):989–995. doi:10.1017/S0007114511003825

Dougal, K., G. de la Fuente, P. A. Harris, S. E. Girdwood, E. Pinloche, R. J. Geor, B. D. Nielsen, H. C. Schott 2nd, S. Elzinga, and C. J. Newbold. 2014. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 9(2):e87424. doi:10.1371/journal.pone.0087424

Dubcová, J., J. Bartošová, and M. Komárková. 2015. Effects of prompt versus stepwise relocation to a novel environment on foals' responses to weaning in domestic horses (Equus caballus). J. Vet. Behav. 10(4):346-352. doi: 0.1016/j.jveb.2015.03.003

Erber, R., M. Wulf, S. Rose-Meierhofer, M. Becker-Birck, E. Mostl, J. Aurich, G. Hoffmann, and C. Aurich. 2012. Behavioral and physiological responses of young horses to different

weaning protocols: A pilot study. Stress. 15(2):184-194. doi:10.3109/10253890.2011.606855

Faubladier, C., S. Sadet-Bourgeteau, C. Philippeau, E. Jacotot, and V. Julliand. 2014. Molecular monitoring of the bacterial community structure in foal feces pre- and post-weaning. Anaerobe. 25:61-66. doi:10.1016/j.anaerobe.2013.11.010

Fernandes, K. A., S. Kittelmann, C. W. Rogers, E. K. Gee, C. F. Bolwell, E. N. Bermingham, and D. G. Thomas. 2014. Faecal microbiota of forage-fed horses in new zealand and the population dynamics of microbial communities following dietary change. PLoS One. 9(11):e112846. doi:10.1371/journal.pone.0112846

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome. 3:1-10. doi:10.1186/s40168-015-0091-8

Gold, J. R., T. J. Divers, M. H. Barton, S. V. Lamb, N. J. Place, H. O. Mohammed, and F. T. Bain. 2007. Plasma adrenocorticotropin, cortisol, and adrenocorticotropin/cortisol ratios in septic and normal-term foals. J. Vet. Intern. Med. 21(4):791-796. doi:10.1111/j.1939-1676.2007.tb03023.x

Hansen, N. C., E. Avershina, L. T. Mydland, J. A. Naesset, D. Austbo, B. Moen, I. Mage, and K. Rudi. 2015. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb. Ecol. Health Dis. 26:27216. doi:10.3402/mehd.v26.27216

Heleski, C.R,, A.C. Shelle, B.D. Nielsen, and A.J. Zanella. 2002. Influence of housing on weanling horse behavior and subsequent welfare. App. Anim. Behav. Sci. 78(2-4):291-302. doi:10.1016/S0168-1591(02)00108-9

Hoffman, R. M., D. S. Kronfeld, J. L. Holland, and K. M. Greiwe-Crandell. 1995. Preweaning diet and stall weaning method influences on stress response in foals. J. Anim. Sci. 73(10):2922-2930. doi:10.2527/1995.73102922x

Holland, J.L., D.S. Kronfeld, R.M. Hoffman, K.M. Greiwe-Crandell, T.L Boyd, W.L Cooper, and P.A. Harris. 1996. Weaning stress is affected by nutrition and weaning methods. Pferdeheilkunde. 3:257-260.

Kapper, D.R. 2004. Appendix A - Applied Nutrition. In: S.M. Reed, W.M Baly, and D.C. Sellon editors, Equine Internal Medicine. 2nd ed. W.B. Saunders. Saint Louis, MO. p 1543-1605.

Ladewig, J., E. Søndergaard, and J.W. Christensen. 2005. Ontogeny: preparing the young horse for its adult life. In: D.S. Mills and S.M. McDonnell, editors, The Domestic Horse: The Origins, Development and Management of its Behaviour. Cambridge University Press. p 139–149.

Malinowski, K., N.A. Hallquist, L. Helyar, A.R. Sherman, C.G. Scanes. 1990. Effect of different separation protocols between mares and foals on plasma cortisol and cell-mediated immune response, J. Equine Vet. Sci. 10(5):363-368. doi:10.1016/S0737-0806(06)80098-5

McCall, C.A., G.D. Potter, J.L. Kreider, W.L. Jenkins. 1987. Physiological responses in foals weaned by abrupt or gradual methods, J. Equine Vet. Sci. 7(6):368-374. doi:10.1016/S0737-0806(87)80007-2

McGreevy, P.D., P.J. Cripps, N.P. French, L.E. Green, and C.J. Nicol. 1995. Management factors associated with stereotypic and redirected behaviour in the thoroughbred horse. Equine Vet. J. 27:86–91. doi:10.1111/j.2042-3306.1995.tb03041

Meale, S.J., S. Li, P. Azevedo, H. Derakhshani, J.C. Plaizier, E. Khafipour, and M.A. Steele. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. Front. Microbiol. 7:582. doi:10.3389/fmicb.2016.00582

Merkies, K., C. DuBois, K. Marshall, S. Parois, L. Graham, and D. Haley. 2016. A two-stage method to approach weaning stress in horses using a physical barrier to prevent nursing. App. Anim. Behav. Sci. 183:68-76. doi:10.1016/j.applanim.2016.07.004

Moons, C.P.H., K. Laughlin, and A.J. Zanella. 2005. Effects of short-term maternal separations on weaning stress in foals. Appl. Anim. Behav. Sci. 91(3):321-335. doi:10.1016/j.applanim.2004.10.007

Moreau, M. M., S. C. Eades, C. R. Reinemeyer, M. N. Fugaro, and J. C. Onishi. 2014. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet. Microbiol. 168(2-4):436-441. doi:10.1016/j.vetmic.2013.11.017

Nicol, C.J., A.J. Badnell-Waters, R. Bice, A. Kelland, A.D. Wilson, and P.A. Harris. 2005. The effects of diet and weaning method on the behaviour of young horses. Appl. Anim. Behav. Sci. 95(3):205-221. doi:10.1016/j.applanim.2005.05.004

Pajarillo, E.A.., J. P. Chae, M. P. Balolong, H. Bum Kim, and D. K. Kang. 2014a. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J. Gen. Appl. Microbiol. 60(4):140-146. doi:DN/JST.JSTAGE/jgam/60.140

Schoster, A., M. Mosing, M. Jalali, H. R. Staempfli, and J. S. Weese. 2015. Effects of transport, fasting and anaesthesia on the faecal microbiota of healthy adult horses. Equine Vet. J. 48(5):595-602. doi:10.1111/evj.12479

Wang, Q., G.M. Garrity, J.M. Tiedje, and J.R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73(16):5261-5267. doi:10.1128/AEM.00062-07

Waran, N.K., N.Clarke, and M. Farnworth. 2008. The effects of weaning on the domestic horse (Equus caballus). Appl. Anim. Behav. Sci. 110(1):42-57. doi:10.1016/j.applanim.2007.03.024

Appendix A - Mare and foal supplemental data

Table A-1. Mean relative abundances (%) of mare milk, mare fecal, and foal fecal bacteria, from birth (d 0) to 4 mo, at the phylum and genus level

Phylum	Genus	Mare Milk[3]	d 0	d 2	d 7	1 mo	2 mo	3 mo	4 mo	Mare Fecal[4]
Firmicutes		67.7^{a}	80.4^{a}	34.6^{b}	39.6^{b}	37.6^{b}	34.5^{b}	34.8^{b}	34.5^{b}	36.0^{b}
	Enterococcus	23.2^{a}	40.6^{a}	3.3^{b}	0.21^{b}	0.02^{b}	0.02^{b}	2.3^{b}	1.2^{b}	1.3^{b}
	Ruminococcaceae[2]	0.48^{a}	0.34^{a}	0.31^{a}	2.68^{a}	10.8^{b}	10.7^{b}	9.2^{b}	9.9^{b}	9.7^{b}
	Bacillus	17.5^{a}	17.2^{a}	0.51^{b}	0.02^{b}	0.02^{b}	0.03^{b}	0.39^{b}	0.37^{b}	0.26^{b}
	Lactococcus	14.3^{a}	10.5^{a}	0.15^{b}	0.02^{b}	0.01^{b}	0.02^{b}	0.1^{b}	0.07^{b}	0.07^{b}
	Lachnospiraceae[2]	0.34^{a}	0.22^{a}	1.1^{a}	1.6^{a}	6.6^{b}	5.1^{b}	6.2^{b}	6.6^{b}	7.3^{b}
	Clostridiales[1]	0.25^{a}	0.13^{a}	0.11^{a}	0.24^{a}	4.1^{b}	3.7^{b}	3.6^{b}	3.8^{b}	3.6^{b}
	Oscillospira	0.15^{a}	0.03^{a}	4.3^{b}	2.3^{b}	3.8^{b}	4.1^{b}	3.2^{b}	2.5^{b}	1.8^{b}
	Ruminococcus	0.07^{a}	0.07^{a}	0.1^{a}	1.7^{b}	1.8^{b}	1.8^{b}	2.2^{b}	2.0^{b}	2.3^{b}
	Phascolarcobacterium	0.09^{a}	0.07^{a}	0.09^{a}	0.26^{a}	1.8^{b}	1.8^{b}	1.2^{b}	1.5^{b}	1.5^{b}
	RFN20	0.03^{a}	0.01^{a}	0.02^{a}	0.009^{a}	0.27	0.51	0.93	1.2^{b}	1.1^{b}
	Brochothrix	2.4^{a}	1.3^{a}	2.0^{a}	0.002^{b}	0.003^{b}	0.005^{b}	0.0007^{b}	0.0006^{b}	0.004^{b}
	Streptococcus	0.4^{a}	0.21^{a}	2.9^{a}	7.6^{b}	0.28^{a}	0.23^{a}	0.05^{a}	0.02^{a}	0.1^{a}
	Corprococcus	0.04^{a}	0.02	0.05	0.09	0.6	0.5	0.5	0.7	1.0^{b}
	Paenibacillus	1.4^{a}	2.4^{a}	0.14^{b}	0.002^{b}	0.002^{b}	0.001^{b}	0.08^{b}	0.08^{b}	0.06^{b}
Bacteriodetes		3.8^{a}	3.8^{a}	24.7^{b}	36.9^{b}	34.5^{b}	34.7^{b}	35.9^{b}	36.9^{b}	35.7^{b}
	Bacteriodales[1]	0.69^{a}	0.64^{a}	0.2^{a}	0.06^{a}	17^{b}	20.1^{b}	20.5^{b}	22.2^{b}	20.4^{b}
	Bacteroides	0.12^{a}	0.004^{a}	24^{b}	33.1^{b}	5.3^{a}	1.5^{a}	0.22^{a}	0.1^{a}	0.24^{a}
	RF16[2]	0.1^{a}	0.2^{a}	0.01^{a}	0.0^{a}	1.0^{a}	1.4^{a}	2.4^{b}	3.3^{b}	3.5^{b}
	YRC22	0.07^{a}	0.1^{a}	0.0	0.02^{a}	1.5	2.3^{b}	3.0^{b}	1.4	2.4^{b}
	Prevotella	0.15^{a}	0.21^{a}	0.02^{a}	0.04^{a}	2.59^{b}	2.74^{b}	2.33^{b}	2.4^{b}	3.18^{b}
	Paraprevotellaceae[2]	0.08^{a}	0.08^{a}	0.03^{a}	0.01^{a}	1.0^{b}	1.2^{b}	1.6^{b}	1.7^{b}	1.6^{b}
	CF231	0.07^{a}	0.09^{a}	0.0^{a}	0.0^{a}	1.5^{b}	0.83^{b}	0.91^{b}	0.84^{b}	1.5^{b}
	BS11[2]	0.02^{a}	0.0^{a}	0.0^{a}	0.004^{a}	1.5^{b}	1.2^{b}	2.1^{b}	1.5^{b}	0.4^{b}
	BF311	0.04^{a}	0.004^{a}	0.02^{a}	0.02^{a}	0.56^{a}	1.1^{b}	0.86^{b}	1.5^{b}	0.65^{b}
	Paludibacter	0.09	0.04	0.02	0.02	0.7	0.4	0.6	0.9	0.9
	Myroides	1.8^{a}	0.93^{a}	0.003^{b}	0.004^{b}	0.0^{b}	0.002^{b}	0.0007^{b}	0.0^{b}	0.002^{b}
Verrucomicrobia		0.41^{a}	0.35^{a}	0.3^{a}	10.2^{b}	12.4^{b}	12.5^{b}	14.2^{b}	11.3^{b}	15.1^{b}
	RFP12[2]	0.73^{a}	0.35^{a}	0.26^{a}	0.17^{a}	9.0^{b}	10.3^{b}	11.2^{b}	10.6^{b}	14.4^{b}
	Akkermansia	0.07^{a}	0.003^{a}	0.05^{a}	10^{b}	3.4^{a}	2.2^{a}	3.0^{a}	0.65^{a}	0.35^{a}
Proteobacteria		25.7^{a}	14.9^{b}	39.8^{a}	9.1^{b}	3.4^{c}	2.2^{c}	2.2^{c}	1.5^{c}	1.0^{c}
	Pseudomonas	17.2^{a}	10.2^{a}	0.04^{b}	0.02^{b}	0.01^{b}	0.02^{b}	0.01^{b}	0.01^{b}	0.03^{b}
	Psychrobacter	4.5^{a}	2.8^{a}	0.01^{b}	0.003^{b}	0.003^{b}	0.0006^{b}	0.002^{b}	0.005^{b}	0.006^{b}
	Escherichia	0.03^{a}	0.008^{a}	36.9^{b}	5.6^{a}	0.15^{a}	0.22^{a}	0.2^{a}	0.01^{a}	0.01^{a}
Spirochaetes		0.14^{a}	0.18^{a}	0.13^{a}	0.0^{a}	4.8^{b}	8.0^{b}	5.3^{b}	7.8^{b}	6.4^{b}
	Treponema	0.17^{a}	0.13^{a}	0.12^{a}	0.0^{a}	4.6^{b}	7.8^{b}	5.1^{b}	7.1^{b}	6.3^{b}
Fibrobacteres		0.06^{a}	0.04^{a}	0.04^{a}	0.04^{a}	3.8^{a}	3.9^{a}	4.0^{a}	4.8^{a}	2.7^{a}
	Fibrobacter	0.06^{a}	0.04^{a}	0.04^{a}	0.04^{a}	3.8^{a}	3.9^{a}	4.0^{a}	4.8^{a}	2.7^{a}
Actinobacteria		2.0^{a}	1.2^{a}	0.30^{b}	0.74^{b}	0.41^{b}	0.41^{b}	0.30^{b}	0.40^{b}	0.53^{b}
	Arthrobacter	1.8^{a}	1.1^{a}	0.008^{b}	0.003^{b}	0.003^{b}	0.003^{b}	0.005^{b}	0.002^{b}	0.005^{b}
Tenericutes		0.03^{a}	0.04^{a}	0.002^{a}	0.01^{a}	0.91^{b}	1.1^{b}	1.4^{b}	0.88^{b}	1.0^{b}
Euryarchaeota		0.02^{a}	0.01^{a}	0.01^{a}	0.02^{a}	0.44^{b}	0.60^{b}	0.70^{b}	0.50^{b}	0.32^{b}
Cyanobacteria		0.02^{a}	0.02^{a}	0.006^{a}	0.02^{a}	0.58^{b}	0.59^{b}	0.43^{b}	0.48^{b}	0.38^{b}

^{a,b,c} Values within a row with differing superscripts differ, $P \leq 0.05$.

[1] Order is highest taxonomic level assigned.

[2] Family is highest taxonomic level assigned.

[3] All mare milk samples from different time points combined

[4] All mare fecal samples from different time points combined.

Appendix B - Weaning supplemental data

Table B-1. Mean relative abundances (%) of foal fecal bacteria before and after weaning, in two treatments: abrupt and gradual, at the phylum and genus level

Phylum	Genus	Abrupt		Gradual	
		Pre[3]	Post[4]	Pre[3]	Post[4]
Firmicutes		35.2	35.4[a]	34.4	32.3[b]
	Ruminococcaceae[2]	10.3	10.0	10.2	9.1
	Lachnospiraceae[2]	6.1	6.2	7.4	6.3
	Clostridiales[1]	4.5	4.5	3.6	3.9
	Oscillospira	2.8	2.3	2.4	2.4
	Ruminococcus	1.8	2.1	2.6	2.1
	Phascolarcobacterium	1.9	1.9	1.2	1.4
	RFN20	1.0	1.5	0.9	1.0
Bacteriodetes		36.9	37.9	37.2	40.0
	Bacteriodales[1]	21.9	23.0	21.9	23.4
	RF16[2]	2.6	3.4	3.3	3.0
	YRC22	1.8	1.7	1.3[a]	2.3[b]
	Prevotella	2.9	3.9	2.6	3.4
	Paraprevotellaceae[2]	2.1	1.7	1.2	1.8
	CF231	0.07	0.09	0.0	0.0
	BS11[2]	1.5	0.8	2.5	1.8
	BF311	0.9	0.6[a,c]	1.8[d]	1.3[b]
	Paludibacter	0.09	0.04	0.02	0.02
	Myroides	1.8	0.93	0.003	0.004
Verrucomicrobia		11.3	12.8	13.5	13.4
	RFP12[2]	10.3	11.8	12.6	12.5
	Akkermansia	1.0	1.0	0.8	0.9
Proteobacteria		1.4	1.5	1.1	1.4
Spirochaetes		6.8[a]	5.2[b]	6.3[a]	4.6[b]
	Treponema	6.3[a]	5.0	5.8	4.4[b]
Fibrobacteres		5.3	4.5	3.5	4.8
	Fibrobacter	5.2	4.5	3.5	4.8
Actinobacteria		0.55	0.51	0.43	0.58
Tenericutes		0.37	0.48	0.94	0.77
Cyanobacteria		0.42[a]	0.29[b]	0.53[a]	0.28[b]

[a,b] Values within a row with differing superscripts are different ($P \leq 0.05$).
[c,d] Values within a row with differing superscripts are different ($P \leq 0.01$).
[1]Order is highest taxonomic level assigned.
[2]Family is highest taxonomic level assigned.
[3]Foal fecal samples from d -1 and d 0 combined into Pre-weaning.
[4]Foal fecal samples from d 1 to 7 combined into Post-weaning.