Characterization of the equine microbiome during late gestation and the early postpartum period, and at various times during the estrous cycle in mares being bred with raw or extended semen

by

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Abstract

Two experiments were conducted to investigate the microbiome of the mare and foal in the late gestational to early postpartum period as well as the effects of breeding on the mare uterine and vaginal microbiota. In the first experiment the objectives were to investigate the microbial composition of the perinatal foal gut and its similarity to the maternal microbiome, and to characterize the temporal dynamics of the microbial composition of mare feces and the vagina during late gestation and the mare vagina and uterus during the early postpartum period. Nine Quarter Horse mare/foal pairs were utilized in this study. Starting 6 weeks prior to parturition, mare feces, vaginal swabs, and milk secretions were collected at 2-week intervals until parturition. At parturition, mare fecal, colostral and placental, and foal meconium samples were collected. Uterine efflux and vaginal swabs were collected from mares on day 7 and 25 postpartum. Samples were analyzed using NGS of the V4 region of the 16S rRNA gene to determine microbial composition. Analysis of sequencing data and statistics were performed using QIIME2 and R. The mare fecal and vaginal microbiomes were generally stable during late gestation. The neonatal foal gut was dominated by the Bacteroidetes and Firmicutes phyla as well as genera commonly found in adult feces. Meconium appeared to be more similar to mare feces and placenta than colostrum. In general, the postpartum mare uterine and vaginal microbiomes were stable postpartum and had many shared taxa with the placental microbiome. The equine neonate is born with a unique gut microbiome compared to dam feces, placenta and colostrum; however, the foal gut may be colonized *in utero* by some of the dam's gut and uterine microbes.

The objectives of the second experiment were to investigate the microbiome of the uterus and vagina in healthy mares and to determine the effect of breeding with either raw or extended

semen on the uterine/vaginal microbiomes. Sixteen Quarter Horse mares and one stallion were utilized in this study. Mares were separated into 2 treatments: artificially inseminated (AI) with 10 mL of raw semen only (RAW, n=8) or 10 mL of semen and 10 mL of extender (EXT, n=8). Uterine efflux and vaginal swabs were collected when a follicle measuring ≥ 35 mm was first observed. Mares were then inseminated within 24 hours of initial sample collection and uterine and vaginal samples were collected again 48 hours post-AI. The uterine and vaginal protocol was repeated in the next estrous cycle. Feces were collected once from mares, immediately following the first detection of a follicle measuring ≥ 35 mm. Semen samples were collected for analysis prior to insemination. Samples were analyzed using the same protocol as the first experiment. Microbes were detected in all sample types. Feces and semen were distinct from all other sample types, however there were few differences between the uterine and vaginal microbiomes. The uterine microbiota was not significantly impacted by breeding and there were no differences between breeding with raw or extended semen. The vaginal microbiome did shift post-AI in the first cycle and between the first and second cycle. Although there were differentially abundant taxa between the uterus, vagina and semen, Actinobacteria, Bacteroidetes, and Firmicutes the were dominant phyla and *Corynebacterium* and *Prophyromonas* were dominant genera in all three sample types. In healthy mares the uterus appeared to return to the pre-AI microbial composition by 48 hours post-AI and there was no shift in composition between estrous cycles. However, the vaginal microbiome is dynamic and displays more shifts following breeding and throughout the estrous cycle than the uterine microbiota. The semen, vaginal and uterine microbiomes shared dominant taxa, indicating that there may be similar control mechanisms in mares and stallions to recognize commensal bacteria within the reproductive tract.

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Dedication

I would like to dedicate this work to my parents, Mary and Colin Jones, who have supported my dreams and aspirations unconditionally and have always pushed me to be my best self. I would also like to dedicate this work to the first horse I foaled out, little Wasabi, whose birth set me on the path to finding my passion for equine reproduction.

Chapter 1 - Review of Literature

Introduction

The microbiome has been defined as the "microbial organisms located in or on a defined anatomic space or surface" [1]. These microbes are thought to have two roles within the host: gatekeeper and watchman. As gatekeeper microbes are the "front line" against chemicals, toxins and nutrients, influencing what is absorbed through mucosal surfaces into the host body. They act as watchman through responding to, and sending signals to, the host immune system [2]. Decreasing cost of analysis, increased speed of microbial DNA sequencing, and advancements in bioinformatic pipelines used to analyze sequencing data have resulted in a rapid rise in the number of studies investigating the microbiota within the different body sites of various species and the role that these microbes play in host health [3].

The human microbiome has been the most extensively studied of all species but there has been considerable interest in the equine microbiome in recent years. The equine gut microbiome garners the most attention as horses are hindgut fermenters and rely on microbes within the large intestine and cecum to break down complex carbohydrates to provide energy in the form of volatile fatty acids, inhibit overgrowth of pathogens, and modulate of the immune system [4]. Alterations to the adult equine gut microbiota have been associated with colitis, laminitis and colic, however determination of the "normal" equine gut microbiome is elusive as there are typically interindividual based on location, age and management [4].

The importance of gut microbes to the host have sparked an interest into how and which microbes initially colonize the neonate. While only two previous studies have utilized meconium as an indicator for early gut colonization in foals [5,6], human studies have demonstrated that *in utero* transfer of commensal gut microbes may be essential for development of the adult gut

microbiome [7]. Only a few studies have utilized next generation sequencing (NGS) for analysis of the equine uterine microbiome [8–10], and to date, none have investigated the vaginal or seminal microbiome. However it is becoming an area of interest in humans as the relationship between the microbiota of the reproductive tract and fertility/reproductive disorders is becoming more clear [11]. Overall the microbiota of both the gut and reproductive tract in the equine warrants further research to fully understand what is "normal", consequences to alterations of the normal microbiome and which microbes are of importance for maintenance of the microbial composition in these body sites.

Next Generation Sequencing Technology

Microbial organisms were first detected as early as the 1600's by Antony van Leeuwenhoek [12]. Since that time various methods have arisen to determine the phylogenetic makeup, diversity, and physiological characteristics of microbiomes or microbial communities. Microbial communities that inhabit different areas of the body, such as the gastrointestinal and reproductive tracts, are of particular interest because they can have both synergistic and pathogenic relationships with the host and alterations to the microbial community can have negative health consequences [12]. Culture based methods, while still of value, are limited in the microbes that can be studied because the majority of bacteria cannot be cultivated and it is costly and time consuming to determine the ideal conditions for growth of new bacterial species [13]. In contrast, sequencing based technologies account for the total genetic material of the microbes within a community or environment; allowing researchers to more accurately define and compare microbial populations [13].

The gene of interest utilized in next generation sequencing (NGS) is the 16S ribosomal RNA (rRNA) gene. It is highly conserved among prokaryotes which allows for the creation of

polymerase chain reaction (PCR) primers that can accurately replicate the gene [14]. The gene also contains nine highly variable regions that can be utilized to differentiate between bacterial taxa; the V4 region is most commonly used due to its high level of reliability when determining taxonomic level [15]. There are several technologies or platforms that can be utilized for isolation of the 16S rRNA gene, however the Illumina technology is the most popular based on the largest market share [16].

The Illumina workflow begins with sample preparation. First DNA is extracted, and then undergoes PCR to amplify the 16S rRNA gene. Adapter sequences, sequencing binding sites, indexes, and regions complementary to the flow cell are also added through PCR. The next step is to load samples into the flow cell where clustering occurs. Clustering begins when templates hybridize at their adapter sequences to oligos on the surface of the flow cell, they are then copied by a polymerase and washed away [17]. The remaining strands are clonally amplified via bridge amplification [17]. This process is followed by "sequencing by synthesis" in which fluorescently tagged nucleotides are added one at a time during each cycle; the clusters of nucleotide bands are then excited by a light source and the emission wavelength is used to determine the base call [17]. Finally, raw data from sequencing is analyzed using a bioinformatics pipeline such as QIIME.

While NGS technology has greatly expanded our knowledge of microbial communities, there are significant limitations and areas for bias in NGS. The sampling process can introduce bias if the person collecting the samples is not careful to avoid contamination, especially in samples that are low in microbial biomass such as blood and other bodily fluids [13]. Extraction of DNA can also introduce contaminants and bias as there are several methods and commercial kits for DNA extraction [13]. Bias is also present in PCR as there are numerous 16S rRNA

primers available and certain primers may skew the population structure by more favorably binding to the 16S rRNA gene of certain microbes over others [12]. The final area for bias is in selection of the variable region to be targeted during sequencing; several studies using the same ecosystem of interest have found different taxonomic results when different regions of the 16S rRNA gene were targeted [11,18,19]. While these areas of bias can influence the results of a study they can be overcome through careful, sterile sample collection, removal of contaminants from laboratory reagents during data analysis, and choosing a variable region that has been previously utilized in a researchers area of interest to ensure the ability to compare results effectively.

Data analysis also presents challenges: the large volume of sequencing data is a computational issue, data needs to be filtered for quality and chimeras, and there will be many sequences that have no match within the chosen taxonomic database [12,13]. These unidentified microbes may play an important role in the environment of interest but as they are unable to be classified, their role also remains a mystery. Results from NGS are a snapshot in time of the microbial community and there is typically a high amount of variation between individuals or between samples taken from the same individual, making it difficult to produce replicable results [12]. Lastly, NGS technology only measures the genetic material of microbes within a sample so it cannot differentiate between live/dead, active/inactive microbes, or provide any information about the physiological characteristics of the microbes. Despite these limitations, NGS technologies are a valuable tool in furthering our understanding of the microbial communities that are present within different body sites through the ability to recognize species that are unable to be cultured and the capacity to sequence hundreds of samples in a short amount of time.

Neonatal Gut Microbiome

It was previously assumed that the mammalian fetus develops in a sterile environment *in utero* and that upon exposure to the external environment, it obtains the microbes that ultimately colonize the gastrointestinal tract. This mentality has both been challenged and supported by both classic and modern work using culture-based, NGS and PCR-based methods. The debate between bacterial colonization *in utero* vs. bacterial colonization postpartum is ongoing and remains to be definitively proven one way or the other. There may be significant differences between host species in terms of the timing of colonization due to differences in placental structure when comparing hemochorial in humans, or epitheliochorial in monogastric animals (pigs and horses) and ruminants (cows and sheep) [20]. Regardless of when initial colonization takes place, it is well established that the neonatal gut microbiome undergoes a shift in microbial composition so that it ultimately refelcts that of the mature animal, however the time to reach the mature microbiome is variable among species [21–24]. Several factors influence the early recruitment of these gut microbes that ultimately play a significant role in the future health of the animal.

In Utero Colonization - Humans

Meconium is the first stool of a neonate; it is produced in utero and passed following parturition. The first reported microbial analysis of meconium was by Theodor Escherich in 1886 [25]. Using culture-based methods, he was unable to find any microbial flora in the meconium of newborn infants and was only able to detect microbes as early as day 8 postpartum. His findings established the concept of the “sterile womb”, indicating that colonization of the gut only occurs postpartum due to exposure to the external environment. His findings were supported by other early work, until the 1980’s when researchers reported bacteria in the meconium of humans,

piglets, mice, and ruminants using culture-based methods [21,26,27]. Despite their findings of microbes within meconium, which may suggest microbial colonization *in utero,* all of these researchers explained their findings as rapid colonization postpartum due to exposure to the extra-uterine environment. It is worth noting that the exact timing of sampling was not always clearly stated, or it was indicated that samples were taken up to 24 hours after birth; which would allow for possible colonization or contamination from the environment. Although lacking in detail and consistency, these early findings laid the foundation for current research that uses more advanced methods to determine the composition of the gut microbial community of neonates.

Work in the late 1990's and early 2000's found contradictory evidence of the sterile womb. Bacterial DNA typically found in the gut, Bifidobacteria and Lactobacilli, were found in the placenta of normal human pregnancies (vaginal and cesarean section birth) using species-specific PCR [28]. Hitti et al. [29] found bacteria in the amniotic fluid of women with negative culture results in the fluid using PCR to amplify the 16s rRNA region. *Enterococcus faecium, Propionibacterium acnes, Staphylococcus epidermidis, and Streptococcus sanguinis* were detected in the umbilical cord blood of neonates delivered through sterile cesarean section resulting from healthy pregnancies [30]. These bacteria were detected through culture-based techniques and were also found in neonatal meconium. They are generally considered to be commensal as they provide nutrients to the host and help to inhibit growth of pathogenic bacteria. This same research team investigated the microbial composition of meconium in newborns, only utilizing meconium passed within 2 h of birth, with strict inclusion parameters. They found bacterial growth for every subject on certain culture plates, while other types of plates had no detectable growth [31]. The same group also used a genetically labeled *E. faecium*

to test the transmission from the maternal gut to the neonate *in utero* in two separate but similar experiments. Four pregnant mice were orally inoculated with *E. faecium*, then amniotic fluid [30] or meconium from the fetus [31] were taken aseptically during cesarean section and plated on MSR agar, followed by PCR of isolates from plates to detect the labeled strain. In both experiments the labeled strain was only found in the inoculated mice and absent from the controls. These results indicate that transmission of microbes from the dam to the developing fetus is possible in the murine model. One possible explanation of this transmission from the gut to the fetus is dendritic cells [32]. These cells can take up live bacteria from the lumen of the digestive tract; the cells are then transported to other parts of the body through circulation of the lymphatic system. Another possible explanation for this transfer of microbes is that the fetus swallows a significant amount of amniotic fluid, especially in late gestation [33]. If amniotic fluid truly does contain a microbial community (possibly delivered by dendritic cells) then this would be a direct pathway of transmission of microbes *in utero*.

Despite the growing body of evidence that there is potential for microbial colonization of the human gut *in utero*, there is also research contradictory to these findings. Hansen et al. [34] used rigorous inclusion/exclusion standards in an infant meconium study to ensure only healthy subjects were included. Only 66% of meconium samples were FISH (flouresece in situ hybridization) positive and of those only one provided enough bacterial DNA upon extraction to be amplified via PCR. These results would indicate that in truly normal, healthy pregnancies meconium has very little to no bacteria, which suggests colonization may occur rapidly after delivery rather than *in utero*. However, using strict contamination controls, Lauder et al. [35] found that there was no significant difference between DNA extraction and PCR reagent negative controls and placental samples, while vaginal and oral samples were clearly different

from the controls when using qPCR. The authors did note that their results do not rule out the presence of placental microbes, only that it was not possible to distinguish between the placental samples and controls. While these results appear to give evidence of a sterile environment *in utero*, it is important to note that the placenta of ruminants and horses, which is epitheliochorial and has 6 tissue layers seperating the fetal and maternal blood supply, is vastly different from the human placenta, which is classifeid as hemochorial and fetal tissues are in direct contact with maternal blood supply [20]. Given this difference in placental tissue layers, direct comparisons between studies of the human placenta and ruminant/equine placenta cannot be made as there may be differences in the level of microbial translocation that can be achieved.

In Utero Colonization - Livestock

Research into the colonization of the gut in livestock animals is still in the early stages. Studies focusing on the gut microbial community of newborn livestock using PCR and next generation sequencing have only just started in the past 10 years or so, with a heavy focus on the transition from the neonatal gut microbiome to the adult microbiome and how different nutritional strategies affect this transition. Evidence in the calf shows the potential for *in utero* transfer of microbes, potentially through placentomes. The first study to utilize PCR-based techniques to determine the microbial community of the calf gut was Mayer et al. in 2012. They used sequence-specific PCR to determine the microbial profile of calf meconium directly after delivery. Samples were low in diversity and dominated by *Citrobacter spp.* and lactic acid producing bacteria [36]. Diversity increased rapidly however, with a completely different composition present by 48 hours [36]. A study sampling Simmental calves from 12 hours after birth to post-weaning using 454 pyrosequencing found that at the 12 hour time point, the calf gut microbiome had the highest relative abundance of Proteobacteria, with Bacteroidetes second,

followed by Firmicutes [37]. Similar to the results seen by Mayer et al., diversity and richness were low at the 12 hour time point and increased with age. In the most recent bovine study, using a stringent protocol with several negative and contamination controls, authors investigated the microbial community composition of newborn calf meconium [25]. They found that calf meconium consists of a low abundance, high diversity bacterial community containing some known adult intestinal microbiota and being most similar to the dam's oral microbiome. The bacterial community diversity drops within 24 hours and increases again by day 7. These results are contradictory to a previous calf study and many human studies that found meconium to have very low diversity [37–39]. A potential route of the transmission of the microbes found in meconium is through the uterus and placenta of the cow. Similar to human studies that have isolated bacteria from the human placenta, bacteria have also been isolated from the endometrium and placentomes (sites of attachment between the placenta and the endometrium of the uterus) of healthy pregnant cows using FISH [40].

While fecal samples in calves are reflective of the microbial profile of the distal digestive tract, the rumen is of greatest interest as this is the site of the majority of digestion in ruminants. Rumen fluid samples from calves have been utilized to determine the colonization of the rumen early in life with pyrosequencing [41]. The authors found that essential rumen microbes are present at birth including cellulolytic bacteria, bacteria of the *Prevotella* genus which are able to utilize starch to produce succinate, as well as soluble sugar and lactate utilizers. Authors also noted a decrease in aerobic and facultative anaerobic microbes and an increase in strictly anaerobic microbes between day 1 and day 3 [21]. This change is important because the mature rumen relies on anaerobic microbes to convert plant fiber into usable energy sources. Overall

previous work in the neonatal calf has shown that the neonate is born with commensal gut bacteria that are essential for digestion and energy outside the womb.

In Utero Colonization - Equine

Studies using an equine model have also questioned the idea that the fetus develops in a sterile environment. Early culture-based studies found foal meconium to be sterile, and researchers using PCR-DGGE found that foal feces collected some time between 0-24 hours after birth had no or very few bands upon gel examination [42,43]. Researchers using automated ribosomal intergenic spacer analysis to examine the microbial composition of foal meconium found bacteria in 3 out of 5 foals with a high degree of variability between foals in terms of the number of bands [44]. These studies, although contradictory, prompted further investigations into the sterility of the uterus and fetal foal gut during pregnancy.

More recently, the microbiome of the placenta and other fetal membranes have been an area of interest in equine studies. Using culture-based techniques, researchers isolated bacteria from amniotic fluid and umbilical cord blood collected at foaling [45]. Bacteria were present regardless of the health status of the foal, indicating the possibility of a normal bacteria flora in fetal tissues, even in normal pregnancies. Xia et al. (2017) conducted a profiling study in which mare fecal, oral, vaginal, and placental (gravid and non-gravid horn) samples were analyzed using NGS to determine the bacterial composition. The authors found that the fecal and placental microbial communities were the least similar. The vaginal, oral, and placental microbiomes shared the most phyla, although there were minor differences between the gravid and non-gravid portions of the placenta. Most recently, NGS was used to determine the microbial composition of mare feces, milk, amniotic fluid, and foal meconium [5]. Mare feces had the most diverse community and amniotic fluid was the least diverse. Each sample type was unique in its

community composition, however 75 OTUs were shared between mare feces and foal meconium and 32 OTUs were shared between amniotic fluid and foal meconium. In our lab, the fecal and milk microbiome of the mare at parturition was compared to the foal fecal microbiome [6]. We found that, similar to other studies, the mare fecal and milk microbiome were significantly different in both diversity and relative abundance of bacteria, while meconium collected at birth mirrored that of the milk (E. Jacquay, 2017). Similar to studies in calves, there appears to be the possibility of *in utero* colonization in foals due to presence of bacteria within meconium, the placenta, and amniotic fluid; however, studies utilizing healthy vs. non-healthy pregnancies and a greater numbers of horses need to be conducted in order to achieve a definitive answer.

External Influences on the Microbiome during Pregnancy

The influence of extrinsic factors, such as pre or probiotic and antibiotic use during pregnancy and the perinatal period, on the development of the neonatal gut microbiome is still beng investigated. There is currently no research into the effects of external factors on the microbiome during pregnancy in livestock animals but it has been investigated in the human and mouse models. Researchers have found that prenatal use of probiotics by the mother is associated with reduced allergies and disease in the offspring, however the direct impact on the infant microbiome is still being investigated [7]. Administration of *Lactobacillus rhamnosus* probitoic in late pregnancy has been shown to increase colonization of the infant gut with *Bifidobacterium spp.*, a bacteria found in healthy infants but lacking in those that are prone to allergies [47]. The mechanism by which this increase occurred remains to be determined. In a murine model, offspring of mice that were given an oligosaccharide prebiotic during pregnancy and lactation had a signifcantly different gut microbial community and lower incidence of dermititis than those that did not receive the prebiotic [48]. However, in a human study,

administration of prebiotics during pregnancy only resulted in an increase in Bifidobacteria in the maternal gut but had no effect on the infant gut microbial community [49]. Results of these studies indicate that pre/probiotic use during pregnancy and lactation may confer health benefits to the offspring, but the exact mechanism through which this occurs and which pre/pro biotics are most useful is unclear. Studies utilizing species other than humans and development of techniques to determine how these pre/probiotics actually influence or effect the host microbial community are necessary to fully understand the relationshp between microbes and these extrinisc factors.

The impact of maternal antibiotic usage during pregnancy has been more extensively studied than pre/probiotic usage. There is concern that antibiotic use during pregnancy reduces comensal bacteria and increases antibiotic-resistant bacteria in the infant gut [7]. Maternal antibiotic usage also can reduce *Lactobacillus spp.* and increase *Citrobacter spp., Enterobacter spp., and E. coli*, which represent a more antibiotic-resistant population [50,51]. This shift in the vaginal microbiome can then directly effect the neonatal gut microbiota by reducing the abundance of Lactobacilli and Bifidobacteria as well as decreasing the overall diversity of the infant gut bacterial community [50,52,53]. Use of antibiotics during pregnancy has also been associated with several illnesses in the resulting offspring, including asthma [54–56], obesity [57] and necrotizing enterocolitis [58]. While there are variations in these effects depending on the antibiotic used, dosage, and length of time of administration, it is imperative that antibiotic usage during pregnancy be reduced as much as possible to avoid causing potentially life-threatening diseases in offspring. Further research is also needed in other species, including livestock species where antibiotics are given routinely during pregnancy to treat infections that are not related to pregnancy.

There are still many questions to be answered in terms of the neonatal gut microbiome. The debate between *in utero* colonization and colonization postpartum is still ongoing and a clear answer has not yet been determined. In contrast, the transition to the adult gut microbiome is well-documented in most species and an understanding of this transition will allow medical professionals, veterinarians and animal producers to make choices that ensure the ideal gut microbiome is established in order to promote the health and well-being of the individual. These choices should include avoidance of unneccessary antibiotic usage and potentially the use of pre/probiotics, although the mechanisms behind the shifts caused by these products are not yet fully understood.

Reproductive Tract Microbiota

Vaginal Microbes

It is well-established and accepted that the vagina of mammals is host to a community of microbes that is variable between and within species. The vaginal microbiome in women in particular has been well-documented and extensively studied. Culture-based studies in the 1970s and 80s first identified the *Lactobacillus* species within the vagina and its significance to maintaining a healthy vaginal microbiome [59,60]. More recent studies have utilized sequencing technologies to not only confirm the dominance of *Lactobacillus* in the vaginal microbiome of women, but to also differentiate 6 different "grades" or community state types (CST) based on the species of *Lactobacillus* that is dominant [61,62]. *L. crispatus*, *L. gasseri*, *L. iners*, or *L. jenseii* respectively dominate CST I-III and V. There is a low abundance of *Lactobacillus* in CST IV-A as well as anaerobic bacteria such as *Anaerococcus*, *Corynebacterium, Finegoldia* and *Streptococcus in.* Higher proportions of the genus *Atopobium* are found in *CST IV-B* as well as *Prevotella, Parvimonas, Sneathia, Gardnerella, Mobiluncus* or *Peptoniphilus* and other taxa

[62–64]. There is a high degree of variability between women in terms of which CST represents their individual microbiome, and this can be effected by age, race, phase of the menstrual cycle, pregnancy, sexual activity, and exogenous hormones [63,64]. It is well-established that dominance by *Lactobacillus* species is associated with a "normal" or "healthy" vaginal microbiome because these microbes produce bacteriostatic or bacteriocidal compounds as well as lactic acid, which in turn maintains a low pH of 3.5-4.5, all of which helps to limit the growth of pathogenic bacteria and prevent conditions such as bacterial vaginosis [11,61,63,64]. While our understanding of the human vaginal microbiome continues to delve deeper into implications for human health and fertility, studies in livestock species are only beginning to investigate the "core" microbiome of the vagina.

Of all of the livestock species, the vaginal microbiome of cattle has been the most extensively studied, focusing frequently on its relation to postpartum issues such as metritis [65,66]. The first study to utilize sequencing of the 16S rRNA gene to determine the vaginal microbiota of ewes and cattle found several similarities between the two species, however, there were vast differences between both species and women [67]. Authors found that *Aggregatibacter* spp., *Streptobacillus* spp., *Cronobacter* spp., *Phocoenobacter* spp., and *Psychrilyobacter* spp. were the most abundant bacterial genera of the ewe vagina. *Aggregatibacter* spp., *Streptobacillus* spp., *Phocoenobacter* spp., *Sediminicola* spp., and *Sporobacter* spp. were the most common genera in cow samples with the cow vaginal microbiome being more diverse as well. When authors compared their results to culture-based studies they noted that species commonly cultured from ewe and cattle vaginas were only present at very low abundances, calling into question the validity of conclusions from culture-based techniques alone. Although present, a low abundance of *Lactobacillus* could be explained by the greater vaginal pH observed in cattle

(6.5-8.7) and ewes (5.6-7.1) compared to humans [67]. It is unclear if the higher pH inhibits the growth of Lactobacillus or if a low abundance of Lactobacillus results in a higher pH due to less lactic acid production. A more recent study also investigated the vaginal microbiota of cattle;, utilizing a different variable region with different results [18]. Laguardia-Nascimento et al. [18] found that the most abundant bacteria at the phylum level were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*; the dominant genera were *Aeribacillus, Bacteroides, Clostridium* and *Ruminoccocus*. When authors compared heifers to cows, and pregnant to non-pregnant animals, they found no difference between heifers and cows however pregnant animals had a higher abundance of *Archaea* compared to non-pregnant animals. They also found several OTUs that were shared between the vaginal microbiome and the gastrointestinal/fecal microbiome; this could be explained by the close proximity of the anus and vagina in cattle and could further explain why the occurrence of postpartum endometritis is so prevalent in cows [18].

In contrast to the ongoing interest in human and cattle vaginal microbiome, only culture-based studies have investigated the equine vaginal microbiota. In 1988, one of the earliest studies to investigate the bacteria within the vagina of clinically healthy mares found that 42% of vaginal swabs were positive on aerobic culture, with only 8% yielding growth of more than 10 colonies [68]. Interestingly, 44% of vestibular swabs and 94% of clitoral fossa swabs had moderate to heavy growth. These swabs also showed presence of pathogenic microbes, such as *Streptococcus zooepidemicus* and *Escherichia coli*. Given these results, the authors concluded that the vulvovaginal fold and the cervix are barriers to ascension of bacteria into the uterus [68]. More recently, the presence of *Lactobacillus* and *Enterococcus* within the equine vagina was investigated using culture-based techniques [69]. Authors detected lactic acid producing bacteria in all of the vaginal samples and further sequencing revealed that the specific strains of the

cultured bacteria were *Lactobacillus pantheris, Lactobacillus mucosae, Lactobacillus equi, Enterococcus faecalis* and *Enterococcus faecium* [69]. All of the strains also exhibited antimicrobial activity against Gram positive and Gram negative bacteria. The lactobacilli growth counts were lower than humans but similar to cattle, this could be explained by the fact that the mare vaginal pH (6-8) is similar to cattle as discussed previously [69]. Authors also concluded that because *L. equi, E. faecalis* and *E. faecium* are also found in the intestine of healthy horses, there may be transference of intestinal microbes to the vagina, as hypothesized in cattle. Further research using NGS technologies is necessary to determine the actual abundance and prevalence of *Lactobacillus* as well as other microbes in the equine vagina other microbes.

Uterine Microbes

The uterus had been thought to be a sterile environment with the presence of intrauterine microbes typically associated with uterine disease or infection [68,70–73]. This idea was challenged by studies that utilized culture-based and NGS technology to show that non-pathogenic microbes were present in the uterus of various mammalian species, resulting in a unique uterine microbiome. Studies in humans have questioned the sterility of the uterus using PCR and sequencing methods [74]. Building off this work, other studies have utilized sequencing technologies to further investigate the microbial population of the human uterus. A core uterine microbiome containing *Bacteroides xylanisolvens, Bacteroides thetaiotaomicron, Bacteroides fragilis, Bacteroides vulgatus, Bacteroides ovatus, Pelomonas,* Betaproteobacteria, *Escherichia/Shigella, Chitinophagaceae, Pseudomonas, Caulobacter,* and *Acidovorax,* was determined using samples collected via transcervical endometrial biopsy and sequencing of the V1-V2 region [19]. In contrast, Moreno et al., (2016) utilized 454 pyrosequencing of the V4-V5 region to analyze endometrial fluid samples and found a slightly different microbiome. Authors

found that in most fertile women *Lactobacillus* (71.7%) was dominant; followed by *Gardnerella* (12.6%), *Bifidobacterium* (3.7%), *Streptococcus* and *Prevotella* (0.866%), which is similar to the typical microbial community of the human vagina. However, in infertile subjects undergoing IVF, approximately half had a *Lactobacillus* dominated (LD) vagina [75]. Those in the non-*Lactobacillus* dominated group had decreased implantation, pregnancy, and live birth rates, indicating a possible link between the uterine microbiome and fertility. Another research team also utilized sequencing of the V4-V5 region but looked at 6 different sites within the reproductive tract, including surgically obtained endometrial samples [11]. In agreement with previous studies, authors found a diverse microbial community within the uterus that included *Lactobacillus* (although it was not dominant), *Pseudomonas*, *Acinetobacter*, *Vagococcus* and *Sphingobium.* This community, similar to the vaginal microbiome, is dependent on the hormones and nutrients that are locally available. Authors also noted correlations between the vagino-utero microbiota and phase of the menstrual cycle as well as diseases such as endometriosis, hysteromyoma, and adenomyosis [11]. As a whole these studies indicate a potential link between the uterine microbiome and fertility/disease, however further research is necessary to explore these correlations.

The microbes within the mare uterus are of interest because they are typically associated with post-breeding induced endometritis (PBIE). In normal, reproductively sound mares there is an inflammatory response to semen, bacteria, and debris that are deposited during either natural service or artificial insemination (AI); this inflammatory response is resolved within 48 hours and most mares are able to expel or eliminate excess fluid and bacteria [76]. A subset of mares are susceptible to PBIE and are unable to effectively clear contaminants from the uterus, significantly decreasing their ability to become pregnant or maintain pregnancy [77]. The

reasons why certain mares are susceptible and others are resistant remain unclear, however it is evident that PBIE has a significant impact on the equine industry as it effects 25-60% of broodmares and is a major reproductive health concern among theriogenologists [70,77]. One of the main diagnostic tools for PBIE is uterine culture, using swabs of the endometrium or fluid collected through low-volume uterine flushing [78]. Both methods present the challenge of false-positives due to contamination during the sampling process or false-negatives due to insufficient sampling. When attempting to diagnose PBIE the number of colonies and the organisms present on culture are taken into account. Microbes that are typically cultured from PBIE mares include *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, ß-hemolytic streptococci, and *Taylorella equigenitalis;* all are thought to be pathogenic with *E.coli* and ß-hemolytic streptococci being the most commonly associated with fertility problems [70,72]. The mode in which these microbes enter the uterus is also not yet fully understood, however poor perineal conformation, trauma during foaling, reduced cervical closure during diestrus/opening during estrus, and impaired immune function could all be contributing factors [70]. In a study looking at 2,123 Thoroughbred mares over the course of three breeding seasons, 72% had negative cultures and of those that were positive, 12.2% were barren, 11.1% were calculated at foaling, and 3.2% were maiden; also, mares with positive culture results had reduced pregnancy rates, and even those with non-pathogenic microbes [79]. These non-pathogenic microbes, as well as pathogenic microbes, have been cultured from normal mares with no reproductive issues, however they typically grow much more slowly and the amount of growth is less than PBIE mares (<10 colonies) [68,78]. The presence of microbes in reproductively normal mares indicates that the equine uterus is not sterile and that it may host a diverse microbial community and could impact fertility.

More recently, two studies sought to investigate the microbiota of the mare uterus through utilization of NGS technologies. Schnobrich et al. [9] compared aerobic culture of endometrial swabs, small volume lavage, and endometrial biopsy to NGS of the same samples from clinically normal mares. The authors found that for swabs 90% showed no growth on culture and 77.8% were negative with NGS (no microbial DNA detected), for small volume lavage 70% had no growth on culture and 70% were negative with NGS, for endometrial biopsy none of the samples recovered growth on culture and only one sample had low levels of microbial DNA with NGS [9]. In contrast, Sathe et al. [8] also used clinically normal mares and found bacteria in all uterine samples using NGS, even in mares with negative cultures. They determined that *Proteobacteria* and *Bacteroidetes* were associated with positive cultures; *Sphingobacteriales (Bacteroidetes)* and *Sphingobium (Proteobacteria)* were associated with mares that produced a viable embryo during embryo transfer, while *Rhodocyclaceae* and *Enterobacteriaceae (Proteobacteria)* were associated with mares that did not produce a viable embryo [8]. Unfortunately, the results from both studies that utilized NGS were only presented within abstracts, which limits interpretation of the results. Further studies utilizing stringent contamination protocols are necessary to further investigate the presence of a microbial population in the equine uterus and the implications of those microbes on reproductive efficiency.

The postpartum uterus of the mare must undergo the process of uterine involution before a new pregnancy can be established, however this process is relatively quick in the mare1, as they are able to become pregnant at their first postpartum estrus, which typically occurs between 7-10 days after foaling [80]. Pregnancy rates for mares bred at the first postpartum estrus are lower than subsequent estrous cycles most likely due to the process of uterine involution postpartum

[81,82]. Previous studies have found that most mares have a positive culture result for potential pathogens such as *Escherichia coli* and *Streptococcus* at the fist postpartum estrus, but the number of mares with positive culture decreases with increased time postpartum and a positive culture result is not statistically correlated with fertility at the first postpartum estrus [81–83]. To date, the microbiome of the mare uterus in the early postpartum period has not been investigated using NGS, however work in cattle has found Firmicutes to be the dominant phylum in the postpartum uterus and vagina, followed by Bacteroidetes and Fusobacteria [84]. This same research group found that there was a distinct difference in the uterine and vaginal microbial population at day 7 postpartum of healthy cows and those that developed endometritis by day 21 postpartum, indicating that the early postpartum microbial community may play an important role in the future reproductive health of animals in the postpartum period.

Male Reproductive Tract and Semen Microbes

It is well-documented that the external genitalia of stallions are not sterile. Several studies have investigated the presence of microbes on external genitalia of stallions including the urethral fossa, penis, prepuce, and urethra pre- and post-ejaculation [85–89]. Rota et al. [87] observed bacterial growth on all of the external genitalia sites; authors also noted that the total bacterial count (TBC) was similar between the urethral fossa and penis/prepuce however the TBC was greater in the urethral fossa than the urethra both pre- and post-ejaculation. These results were supported by a study that investigated the genital microflora of stallions used for artificial insemination over the course of a breeding season [88]. Researchers noted that the number of microbial species isolated changed between February and August, however not in a linear fashion, and the most common bacteria isolated were *Staphylococcus* spp., *Corynebacterium* spp., *Streptococcus* spp., *Acinetobacter* spp., and *Escherichia* spp. Building off

this work, Guimarães et al. [89] compared the genital microbes of breeding stallions (used for both artificial insemination and live cover) to non-breeding stallions. Both groups were positive for bacterial growth on culture, however 16 species were exclusive to breeding stallions and 4 were exclusive to non-breeding stallions, with potential pathogens only being found on breeding stallions. Similar to previous studies, *Corynebacterium* spp. and *Staphylococcus* coagulase-negative were the most common bacterial isolates. The resident microflora of the external genitalia is thought to be the main source of the bacteria found in stallion semen.

Bacteria have been isolated from stallion semen as early as the 1970's using culture-based techniques and sterile collection equipment [90]. The most common non-pathogenic bacteria isolated from semen are *Staphylococcus* coagulase-negative and *Corynebacterium* spp., both of which are also dominant on the external genitalia [87–89,91]. If semen is sterile within the testes it may acquire microbes when it encounters the urethra and urethral fossa. However, Guimarães et al. [89] found non-pathogenic and potentially pathogenic microorganisms in stallion semen that were not present on the external genitalia including, *Staphylococcus* coagulase-negative, *Enterococcus* spp., *Bacillus* spp., *Strep. zooepidemicus* and *Salmonella* spp. Authors postulated that these microbes were present due to contamination during collection and processing rather than inherently present in the semen.

Following the first detection of bacteria in stallion semen, extenders were developed to enhance spermatozoa quality, increase fertility, and reduce the bacterial content. Semen extenders are most commonly milk or egg yolk-based and typically contain antibiotics [92]. The efficacy of various antibiotics on the reduction of the bacterial content of semen has been thoroughly investigated and the most common antibiotics added to stallion semen extender are beta-lactams (potassium penicillin G) and aminoglycosides (amikacin or gentamicin) [90,92–95].

These antibiotics have been shown to effectively reduce the bacterial load of semen by up to 99%, and have other benefits including increased viability of spermatozoa by removing bacteria that may utilize the same substrates and reduction of the microbes introduced to the mare uterus [90]. Bollwein et al. [96] investigated the impact of antibiotic-containing extender and raw semen on the mare through uterine culture and found that insemination with extender resulted in less severe bacterial contamination than raw semen. In contrast, a study that investigated the transmission of bacteria from the stallion to the mare during live cover found that breeding mares to stallions with positive culture vs negative culture did not increase the incidence of positive uterine culture post-breeding, however authors did not report the significance of this conclusion [86]. The results would indicate that semen extender is useful in reducing the bacterial contamination of the mare uterus post breeding however the impact of bacteria transmitted via semen to the mare uterus warrants further investigation.

To date, NGS has not been used to investigate the microbiome of the stallion external genitalia or semen, but it has been used to determine the microbial population of human semen and its potential impact on the female reproductive tract. Human studies have found the most abundant taxa in semen to be *Streptococcus*, *Corynebacterium*, *Finegoldia*, *Veillonella*, *Staphylococcus*, *Anaerococcus*, *Peptoniphilus*, *Lactobacillus*, *Pseudomonas*, *Prevotella*, and *Gardnerella* with semen from normal, fertile men being dominated by *Lactobacillus* [97–99]. Surprisingly, there is limited research into the direct effect of semen on the uterine or vaginal microbiota of women. Borovkova et al. [100] found that staphylococci and streptococci were the most frequent new species isolated from the vagina 8-12 hours post-intercourse in infertile couples using culture-based techniques. However, Mandar et al. [99] utilized NGS and found that the seminal and vaginal microbial communities of couples were highly similar with 85% of

all detected phylotypes being shared, although there was a significant shift in the vaginal microbiome post-intercourse for a decrease in *Lactobacillus crispatus*. This would indicate that although the seminal and vaginal microbiomes are similar between couples, intercourse, allows for introduction of semen into the vagina resulting in alkalization of the typically acidic vagina, which may allow for non-lactobacilli species to become dominant for an unknown period of time. While we have established an understanding that breeding induces certain immunological and physiological responses in the mare, we do yet fully understand the potential effects of stallion semen, whether it is deposited through AI or natural service on the resident microflora of the mare reproductive tract and the possible implications of alterations to the microflora.

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Chapter 2 - Characterization of the mare and foal microbiome at parturition and during the early post-partum period

Summary

Background: To date, only a few studies have focused on the neonatal foal gut microbiota using next generation sequencing (NGS) and none have investigated the uterine and vaginal microbiome of the mare during the early postpartum period.

Objectives: To investigate the microbial composition of the perinatal foal gut and its similarity to the maternal microbiome, and to characterize the temporal dynamics of the microbial composition of mare feces and the vagina during late gestation and the mare vagina and uterus during the early postpartum period.

Study design: Longitudinal study

Methods: Nine Quarter Horse mare/foal pairs were utilized in this study. Starting 6 weeks prior to parturition, mare feces, vaginal swabs, and milk secretions were collected at 2-week intervals until parturition. At parturition, mare fecal, colostral and placental, and foal meconium samples were collected. Uterine efflux and vaginal swabs were collected from mares on day 7 and 25 postpartum. Samples were analyzed using NGS of the V4 region of the 16S rRNA gene to determine microbial composition. Analysis of sequencing data and statistics were performed using QIIME2 and R.

Results: The mare fecal and vaginal microbiomes were generally stable during late gestation. The neonatal foal gut was dominated by the Bacteroidetes and Firmicutes phyla and appeared to be most similar to mare feces and placenta. In general, the postpartum mare uterine and vaginal microbiomes were stable postpartum and had many shared taxa with the placental microbiome.

Main limitations: Possibility for contamination during sampling, and DNA extraction, and PCR, bias of the variable region targeted during sequencing, inability to distinguish live and dead microbes presence of unidentified microbes and the low number of mares/foals utilized in this study.

Conclusions: The equine neonate is born with a unique gut microbiome compared to dam feces, placenta and colostrum; however, the foal gut may be colonized *in utero* by some of the dam's gut and uterine microbes. The mare microbiome is fairly stable prior to foaling and during the early postpartum period.

Introduction

The development of the gut microbial community is important for equines as they rely on microbes for digestion and utilization of complex carbohydrates/starches [1]. These microbes within the cecum and intestines of horses produce volatile fatty acids (VFAs), proteins and vitamins that are utilized by the animal for energy and maintenance [2]. The microbes that colonize the human neonatal gut help also to "program" the immune system to differentiate between commensal and pathogenic bacteria [1]. Disruptions of the equine gut microbiome have been associated with colitis, laminitis, and colic in mature horses, as well as diarrhea in foals; however it is unclear how initial colonization of the foal gut impacts the health of the animal later in life [3,4].

The long-held belief that the fetus develops in a sterile environment *in utero,* and that initial colonization of the gut microbes occurs postpartum, has been questioned in several species, including the equine. Only a few studies have utilized next generation sequencing (NGS) to investigate the microbial colonization of the neonatal foal gut or the mare microbiome before, at, or after foaling [5–9]. Those results support the possibility of *in utero* colonization in

foals due to the presence of microbes within the meconium, placenta, vagina and amniotic fluid; however, none have directly compared meconium, mare feces, and the placenta.

Also of interest is the early postpartum microbiome of the mare uterus and vagina. It is well established that breeding mares during the first postpartum estrus yields lower pregnancy rates compared with subsequent estrous cycles [10]. There is also a higher incidence of positive bacterial culture in the uterus at first postpartum estrus compared to non-lactating mares, however these culture plates are limited in the microbial species that can be detected and practitioners typically select plates capable of detecting known pathogens, such as *Streptococcus* [10]. Katila et al. [11] found that mares with a positive culture postpartum did not have a significantly lower pregnancy rate compared to those with negative culture, and Huhtinen et al. [10] found no significant effect of positive bacterial culture on embryo recovery rate in postpartum mares; indicating that potential pathogens present in the postpartum uterus may not be the cause of reduced pregnancy rates during foal heat. To date, NGS has not been reported to investigate the microbial community of the postpartum uterus and/or vagina of the mare, however studies in cattle have found associations between the uterine and vaginal microbiomes and future fertility and incidence of endometritis [12–14].

Therefore, the first objective of this study was to investigate the microbial composition of the perinatal foal gut and its similarity to the maternal microbiome. The second objective was to characterize the temporal dynamics of the microbial composition of mare feces and the vagina during late gestation and the mare vagina and uterus during the early postpartum period.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Usage Committee at Kansas State University (Protocol No. 4052).

Animals

Nine pregnant Quarter Horse mares 5-14 years of age and their subsequent foals, born between April and May of 2018, were used in this study. Mares were previously dewormed and vaccinated during the prepartum period prior to inclusion in the study. Mares were group-housed at the KSU Horse Unit on a 25-acre brome pasture until approximately 2 weeks prior to their expected foaling date, at which time they were moved closer to the foaling barn to a 5-acre brome pasture where they remained until foaling. Mares were provided ad libitum access to brome hay, salt/mineral blocks, and water. Mares were supplemented with a concentrate pellet to meet NRC requirements for late pregnancy and early lactation. At the time of foaling, mares were moved to a stall for approximately 24 hours and received the same diet. After foaling, mares and foals were group housed in a 3-acre brome pasture with ad libitum water and a concentrate and brome hay diet formulated to meet NRC requirements for early lactation.

Sample Collection

Prepartum Samples

Vaginal swabs, blood, prepartum mammary fluid and fecal samples were collected for each mare at 14 day intervals beginning 42 days prior to the expected foaling date. For vaginal samples tails were first wrapped and held out of the way with a tail tie. The perineum was cleaned with water and dilute Ivory soap and then dried. For vaginal samples, a sterile obstetrical sleeve with sterile lubricant was donned and a double-guarded sterile swab was inserted into the vagina. Once location within the vagina was confirmed through identification of the cervix, a sample was taken from of the epithelium of the floor of the vagina roughly 5 cm from the cervix. Swabs were returned to the protective casing and removed. They were then placed into sterile 50 mL conical tubes and stored at -20° C until further analysis. Ten mL blood samples were

collected via jugular venipuncture into Vacutainer® tubes (Becton, Dickson and Company, Franklin Lakes, NJ) for plasma. Blood was spun at 1,400 x g for 15 min at 25° C and white blood cells/platelets were removed and frozen into 1 mL aliquots. Fecal samples were collected via a rectal grab using a lubricated sterile shoulder-length sleeve. Approximately 5-50 g of feces were placed into a sterile 50 mL conical tube. Prepartum mammary secretions and colostrum samples were collected using aseptic procedures utilized for microbial analysis of milk from dairy cattle [15]. First, 3 streams of milk were stripped from both teats using sterile latex gloves. Next, teats were dipped into a dilute betadine solution and dried with a clean paper towel. Then, teats were scrubbed with gauze pads saturated in 70% isopropyl alcohol until no dirt or debris remained. Finally, an equal volume of 2-5 mL of milk were collected from both teats into a sterile 50 mL conical tube held at a 45° angle below the teat. All fecal and milk/colostrum samples were stored individually at -20° C until DNA extraction.

Parturition Samples

At the time of foaling, blood, fecal, colostrum, and placental samples were collected. Blood, fecal, and colostrum samples were obtained from the mare using the same procedures described above. Approximately 5 g of meconium was collected via rectal grab from foals using a lubricated sterile latex glove and placed in a sterile 50 mL conical tube. Using nitrile gloves, placenta samples were collected by manually scraping a sterile 50 mL conical tube against the surface of the interior (maternal side) of the horns and body of placenta immediately after it was naturally expelled from the mare to gain a representative sample of the entire placenta. Colostrum, blood and meconium samples were collected within 2 h of birth and prior to nursing. Mare fecal samples were collected within 6 h of parturition. All samples were stored individually at -20° C until further analysis.

Postpartum Samples

On days 7 and 25 postpartum, uterine and vaginal samples were collected from each mare. Prior to collection, the tail was wrapped and held out of the way with a tail tie. The perineum was cleaned with water and dilute Ivory soap. Vaginal samples were collected in the same manner as described above. For uterine samples, the investigator donned a sterile obstetrical sleeve with sterile lubricant and a sterile uterine lavage tube was manually passed through the vulva, vagina and cervix into the uterine body. The uterine lavage tube was fixed in place just past the cervix with a balloon filled with 60-100 mL of air. Next, 120 mL of sterile phosphate buffered saline was infused into the uterus via a sterile 60 mL syringe. The fluid was collected via gravity flow into two sterile 50 mL conical tubes. Recovery of fluid ranged from 15-100 mL. Tubes were then centrifuged at 400 x g for 10 min. Supernatant was removed and transferred to a separate sterile conical tube, leaving the pellet and approximately 2 mL of fluid in the original tube. Conical tubes containing supernatant and pellets were stored at -20° C for later DNA analysis.

DNA Extraction and PCR

Total DNA was extracted from the uterine efflux, vaginal swabs, milk and whole blood using the QIAamp® DNA Mini Kit (Qiagen; Hilden, Germany) according to manufacturer protocols for bacteria. Total DNA was extracted from feces and meconium using the E.Z.N.A® Stool DNA Kit (Omega Bio-tek; Norcross, GA). The concentration of DNA was quantified using Quant-iT^{TM} PicoGreeen^{TM} dsDNA Assay Kit (Thermo Fischer Scientific; Waltham, MA).

Amplification of the V4 region of the bacterial 16S rRNA gene was done using PCR primers (515F/926R) according to Earth Microbiome Project protocols [16]. Fecal samples were amplified through triplicate PCR reactions on a Mastercycler® nexus (Eppendorf; Hamburg,

Germany) in a 25 uL solution of 5 uL 5x PCR master mix (Promega; Madison, WI), 0.5 uL of the forward and reverse primers, 0.2 uL of dntps, 1 uL of 1% BSA, 0.2 uL of GoTaq® DNA Polymerase (Promega; Madison, WI), 16.6 uL PCR grade H2O and 1 uL template DNA for 25 cycles. For the low template samples (≤ 10 ng/μL DNA), which included vaginal, uterine, meconium and blood, a modified solution of 12.6 uL PCR grade H2O and 5 uL template DNA was run for 30 or 35 cycles based on previous protocols used in our lab [7]. Each PCR reaction was run with a positive and negative control to ensure amplification and check for bacterial contamination. Agarose gel electrophoresis was utilized to check the PCR products for amplicon length and then PCR product triplicates were pooled for library preparation. Excess primers and unincorporated nucleotides were enzymatically removed using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fischer Scientific; Waltham, MA). Total DNA in each sample was then quantified using the Quant-iT^{TM} PicoGreeen^{TM} dsDNA Assay Kit (Thermo Fischer Scientific, Waltham, MA) and approximately 100 ng of DNA from each sample was combined into a 1.5 mL microcentrifuge tube. The combined library was loaded into a 2% agarose gel and then extracted using the QIAquick® Gel Extraction Kit (Qiagen; Hilden, Germany) to ensure a uniform library size of 500 bp.

Bioinformatics and Statistical Analysis

The final 500 bp library was submitted to the K-State Integrated Genomics Facility for analysis and sequencing. The library was analyzed for amplicon length and bacterial DNA concentration using the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and qPCR. To improve base call quality, 10% PhiX was added to the library. Amplicons were then sequenced using the 500 cycles MiSeq Reagent Kit v2 for a single paired-end run on the Illumina Miseq.

The sequencing data were analyzed using the QIIME2 (version 2019.7) bioinformatics pipeline [17]. Raw sequencing data in the form of FASTQ files were demultiplexed and filtered for quality. Taxonomy and OTUs were assigned according to 97% sequence similarity using the GreenGenes database (version 13.8). Known reagent and laboratory contaminants and contaminants found in negative controls were removed from the data prior to further analysis [18]. All alpha and beta diversity analyses were performed in QIIME2. Alpha diversity was calculated using Shannon index and observed OTUs. Statistical significance of alpha diversity metrics was determined by Kruskal-Wallis one-way ANOVA tests. Beta diversity was calculated using Bray-Curtis dissimilarity which was then ordinated into Principal Coordinates Analysis (PCoA) plots. Pairwise PERMANOVA tests were used to determine compositional differences between body sites and time points based on beta-diversity. Relative abundance of taxa was summarized in plots created in QIIME2. Differential abundance comparisons were calculated in R (version 3.6.1) using ANCOM (Analysis of composition of microbiomes; version 2) [24]. Longitudinal and pairwise comparisons of sample types and were assessed at the phylum and genus level using taxa-wise multiple correlation and $\alpha = 0.05$. The W statistic was used to determine significance of differentially abundant genera; this statistic represents the number of times that the null-hypothesis (the average abundance of a given genus in a group is equal to that in another group) was rejected for a given genus [25–28]. Venn diagrams of shared taxa were constructed using Venny (version 2.0) based on presence/absence tables constructed in QIIME2 [29].

Results

Initial data analysis and quality filtering yielded 1,481,270 sequences in 118 samples. Samples were grouped by sample type and time of collection. Based on rarefication curves, data

analysis was performed at 20,000, 826, 203 and 354 sequences for fecal, vaginal, uterine and milk samples respectively, and at 810 sequences for samples collected at the time of parturition. This was done for normalization of alpha and beta diversity metrics and resulted in the removal of samples with low sequence counts, including 4 colostrum, 3 placenta, and 2 meconium for analysis of samples collected at parturition. For comparison of the vagina and uterus to the placenta, normalization resulted in the removal of 1 placenta sample, 2 vaginal samples from day -14, 6 from day -28, and 4 from day -42, as well as 4 and 2 uterine samples from day 7 and 25 respectively. All samples were utilized for determination of relative abundance of taxa and ANCOM. Foaling was missed in 3 mares, so feces, colostrum, placenta and meconium samples were unable to be collected from those mare/foal pairs at the parturition time point. One mare experienced a retained placenta which resulted in uterine lavage and oral antibiotic treatment post-foaling, so all placenta and postpartum samples were removed from the analysis for that mare. Another mare was bred on her first postpartum estrus so uterine and vaginal samples were not collected from that mare on day 25 postpartum. Blood samples were removed from the analysis and will not be reported on due to insufficient microbial DNA detected in those samples following PCR.

Prepartum

Within fecal samples, Shannon diversity was higher in the day -42 pre foaling samples compared to parturition samples and observed OTUs were higher at the day -42 samples than the day -14 and parturition (Fig. 3.1, $P<0.05$). Vaginal samples did not differ between any pre-foaling time points for Shannon diversity or observed OTUs (Figure 3.3, $P>0.05$). Day -14 prepartum milk did not differ from colostrum collected at foaling for Shannon diversity or observed OTUs ($P>0.05$).

The Bray-Curtis dissimilarity index was used to create Principle Coordinate Analysis (PCoA) plots of fecal, milk, vaginal, and placental samples. There were no differences in community composition between the prepartum vaginal samples, however there was a significant difference between each of the prepartum vaginal samples and the placenta (Fig. 3.5, $P<0.05$). There was also a significant clustering of day -42 fecal samples compared to parturition ($P<0.01$).

Analysis using ANCOM at the phylum and genus level found no differentially abundant taxa between the different time points for fecal samples. There were also no differentially abundant genera between prepartum vaginal samples, however *Arcanobacterium, Agrobacterium, Helcococcus, Leptospira, Prophyromonas and Sediminibacterium* were differentially abundant when vaginal and placenta samples were compared (Table 3.2). The families Oxalobacteraceae and Enterobacteriaceae were the only differentially abundant taxa in milk and both were more abundant in colostrum than the -14 day prepartum milk samples. Relative abundance of the main phyla and genera of the vagina and placenta are displayed in Figures 3.8 and 3.9 respectively. Relative abundance of the main phyla and genera of milk/colostrum samples is displayed in Figures 3.8 and 3.12 respectively**.**

Parturition

There were no differences between meconium and mare feces for Shannon diversity; however, mare fecal samples were higher in diversity than colostrum and placenta samples (Fig. 3.2, $P<0.05$). Mare feces had higher observed OTUs than placenta and colostrum, however there was no difference between mare feces and meconium (Fig. 3.2, $P<0.05$). According to Bray-Curtis dissimilarity, which is represented as a PCoA plot in Figure 3.6, mare feces had a distinct

community composition compared to all other sample types ($P<0.05$), however there was no difference between meconium and placenta or colostrum ($P>0.05$).

Figures 3.8 and 3.10 display the relative abundance of the main phyla and genera, respectively, for samples collected at parturition. Meconium was compared to mare feces, colostrum and placenta samples using phylum and genus as the lowest taxonomic level for ANCOM. At the phylum level, Acidobacteria and Proteobacteria were more abundant in meconium than feces and Tenericutes were more abundant in feces than meconium. Verrucomicrobia, Bacteroidetes, and Spirochaetes were all more abundant in meconium than colostrum. Actinobacteria were more abundant in placenta than meconium. Using genus as the lowest taxonomic level, there were 14, 7, and 15 differentially abundant taxa between foal meconium and mare feces, placenta, and colostrum, respectively (Table 3.1). The differentially abundant taxa between mare feces and meconium were order RF39; families Christensenellaceae, Comamonadaceae, Enterobacteriaceae Oxalobacteraceae and Veillonellaceae; and genera *02d06, Caulobacter, Curvibacter, Comamonas, Cupriavidus, Clostridium, Staphylococcus* and *Sphingobacterium.* Differentially abundant taxa between meconium and placenta included families p.2534-18B5 and RF16; and genera *Agrobacterium, Corynebacterium, Facklamia, Jeotgalicoccus* and *Ruminococcus*. Between colostrum and meconium orders Bacteroidales, Clostridiales; families Lachnospiraceae, Mogibacteriaceae, Paraprevotellaceae, RF16, RFP12, Ruminococcaceae; and genera *Oscillospira, Phascolarctobacterium, Prevotella, Ruminococcus* and *Treponema* were differentially abundant.

In order to determine possible links between the sample types we determined common genera which is represented as a Venn diagram (Fig. 3.7). There were 88 genera common to meconium, milk, mare feces, and the placenta, however 6, 5, and 9 genera were exclusively

shared between meconium and mare feces, placenta, and colostrum, respectively. There were 11, 32, 27, and 19 genera that were exclusive to meconium, mare feces, colostrum, and placenta, respectively.

Postpartum

There were no significant differences in Shannon diversity or observed OTUs when vaginal and uterine samples were each compared between day 7 and 25 postpartum (Fig. 3.3 and 3.4, $P>0.05$). There were also no differences in alpha diversity when the vaginal and uterine samples were compared to the placenta or to each other (Fig. 3.3 and 3.4, $P>0.05$).

PCoA of Bray-Curtis dissimilarity showed that day 7 and 25 vaginal community composition was significantly different (Fig. 3.5, $P<0.05$). Day 7 and 25 vaginal swabs were collectively different from placenta samples (Fig. 3.5, $P<0.05$). In contrast, there was not a significant separation between the placenta and the postpartum uterus, nor was there a difference in community composition of the uterus between day 7 and 25 postpartum ($P>0.05$). There was also no significant separation between uterine and vaginal postpartum samples ($P>0.05$).

Relative abundance of the main phyla and genera of the vagina, uterus, and placenta are presented in Fig. 3.8 and Fig. 3.9 respectively. The placenta was compared to the vagina and uterus individually; with the ANCOM results at the genus level presented in Table 3.2. *Arcanobacterium, Helcococcus, Leptospira, Porphyromonas,* and *Sediminibacterium* were all more abundant in the vagina than the placenta, however *Agrobacterium* was more abundant in the placenta than the vagina. Only *Agrobacterium* was more abundant in the placenta compared to the uterus. There were no differentially abundant taxa between the uterus and the vagina postpartum, nor between the day 7 and 25 postpartum uterine samples. Four genera,

Sphingobacterium, Mycobacterium, Brevundimonas and Delftia, were all more abundant in the vagina on day 25 compared with day 7 postpartum (Fig. 3.13).

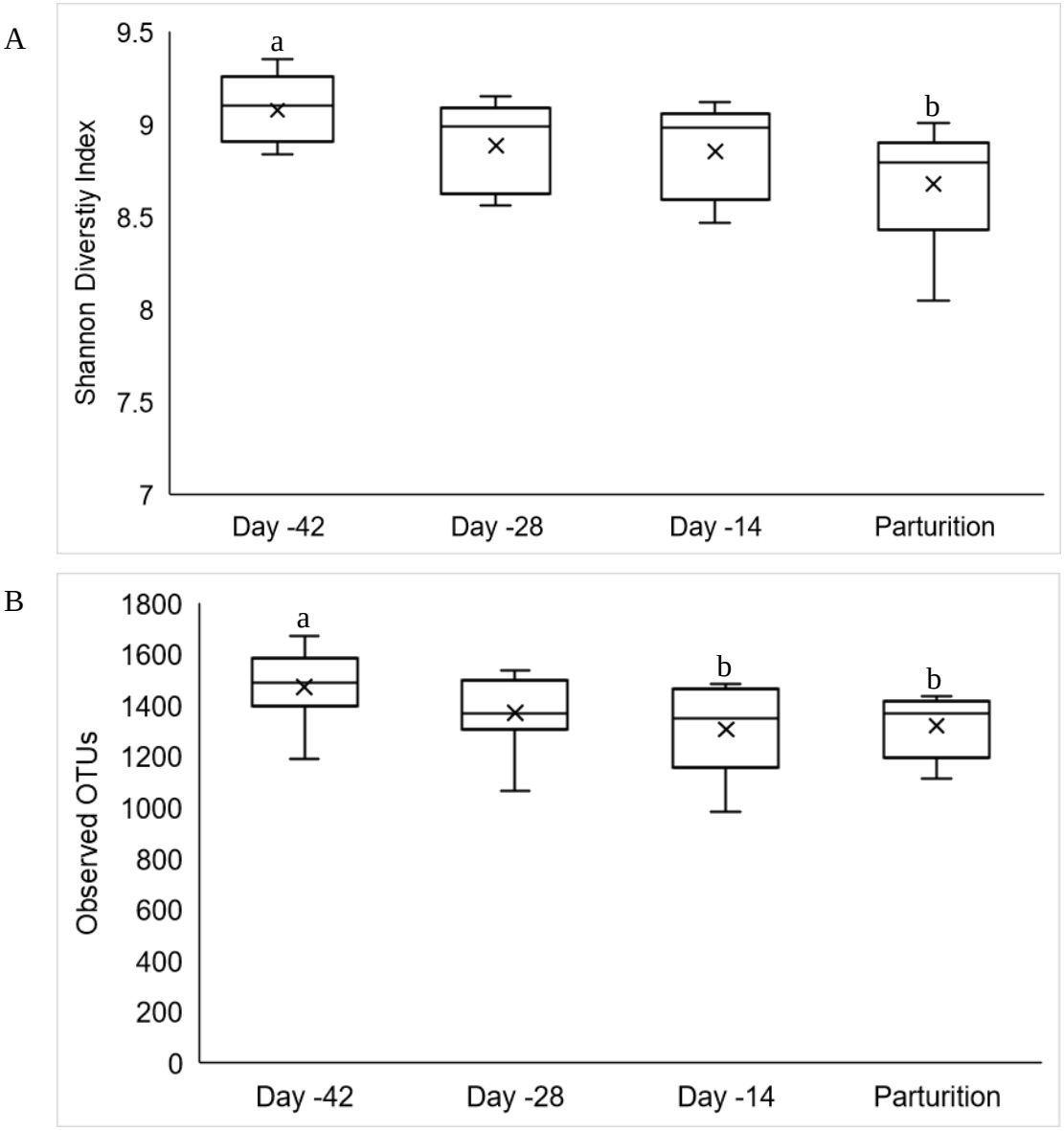
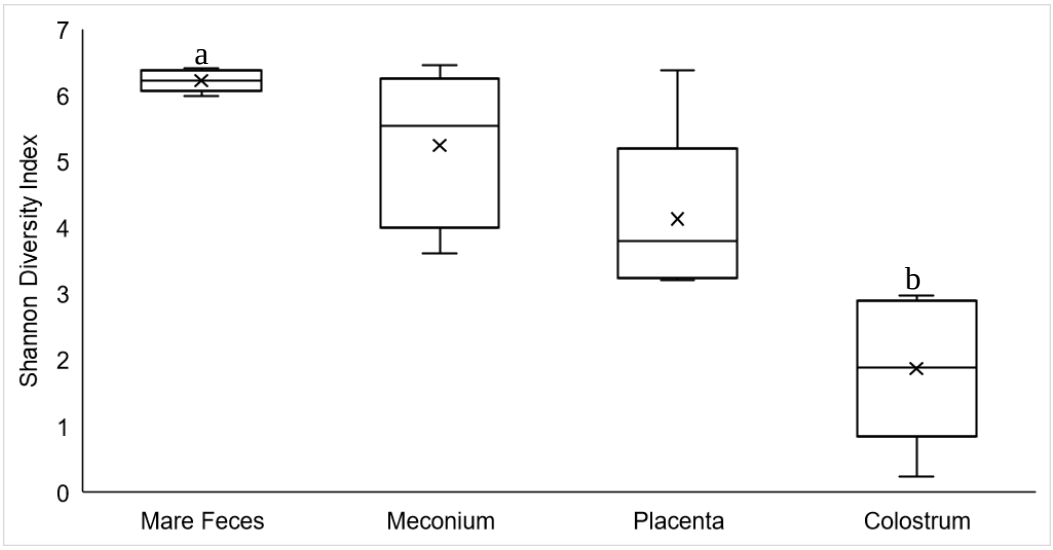


Figure 2.1 Diversity of the fecal microbiome of pregnant mares. Alpha diversity, as measured by Shannon diversity (A) and observed OTUs (B), of samples collected at 14 day intervals from 42 days prepartum to parturition (day-42,-28,-14, n=9; parturition, n=6). Boxes represent the interquartile range (the horizontal line within the box is the median, the x represents the mean). Time points within each individual graph lacking a common superscript differ, $^{a,b}P < 0.05$.

A



B

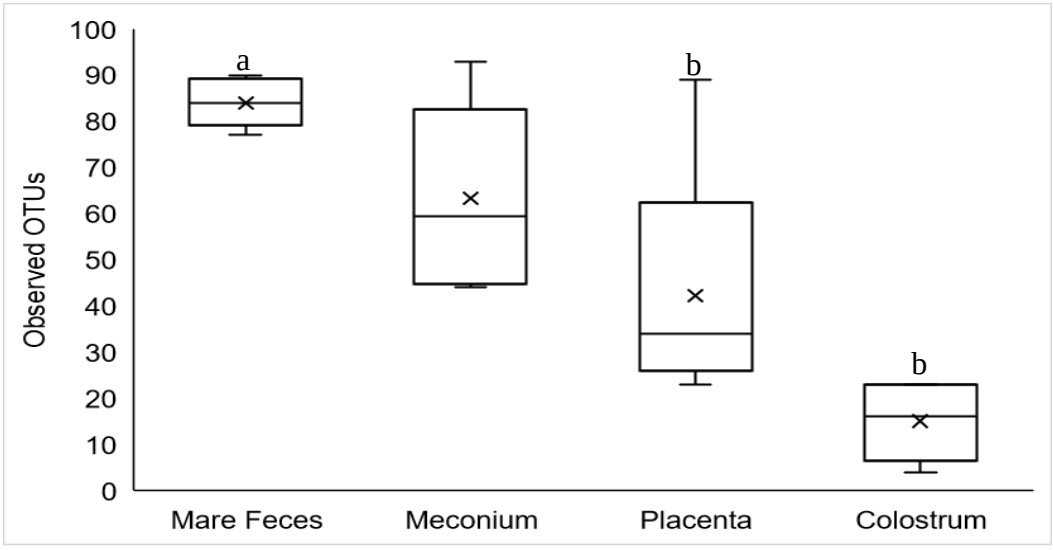


Figure 2.2 Diversity of microbiome of pregnant mare fecal, placental and colostral samples as well as foal meconium samples. Alpha diversity, as measured by Shannon diversity (A) and observed OTUs (B), of samples collected at parturition (mare feces, n=6; meconium, n=4; placenta, n=3; colostrum, n=2). Boxes represent the interquartile range (the horizontal line within the box is the median, the x represents the mean). Time points within each individual graph lacking a common superscript differ, $^{a,b}P < 0.05$.

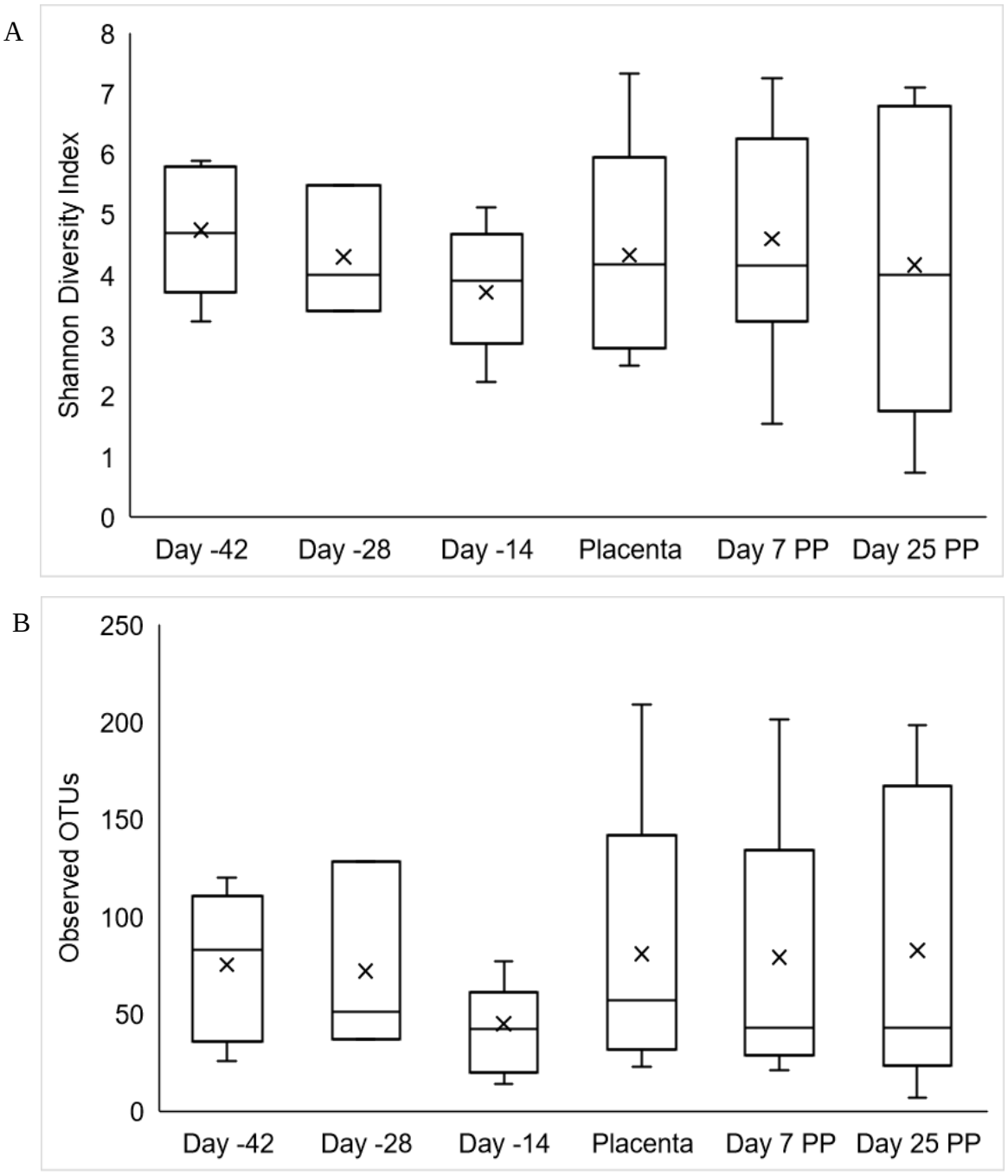


Figure 2.3 Microbial alpha diversity, as measured by Shannon diversity (A) and observed OTUs (B) of pregnant mare placental samples in addition to vaginal samples collected at 14 day intervals, starting 42 days prepartum, and vaginal samples collected on day 7 and 25 postpartum (day -42, n=4; day -28, n=3; day -14, n=6; placenta, n=4; day 7 PF, n=6; day 25 PF, n=6). Boxes represent the interquartile range (the horizontal line within the box is the median, the x represents the mean). No significant differences were detected between any time points. PP: postpartum

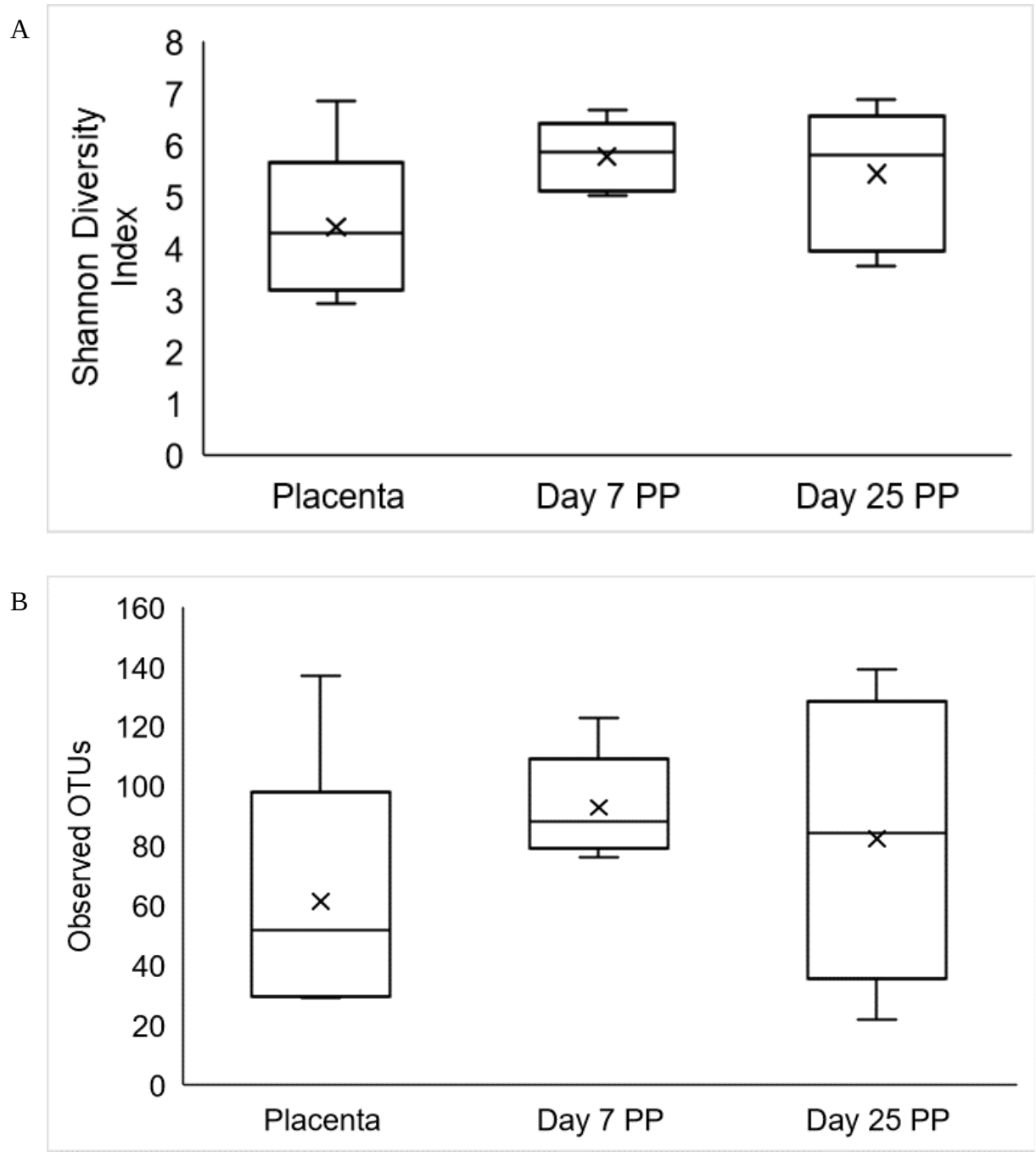


Figure 2.4 Microbial alpha diversity, as measured by Shannon diversity (A) and observed OTUs (B) of pregnant mare placental samples in addition to uterine samples collected on day 7 and 25 postpartum (placenta, n=2; day 7 PF, n=3; day 25 PF, n=4). Boxes represent the interquartile range (the horizontal line within the box is the median, the x represents the mean). No significant differences were detected between any time points. PP: postpartum.

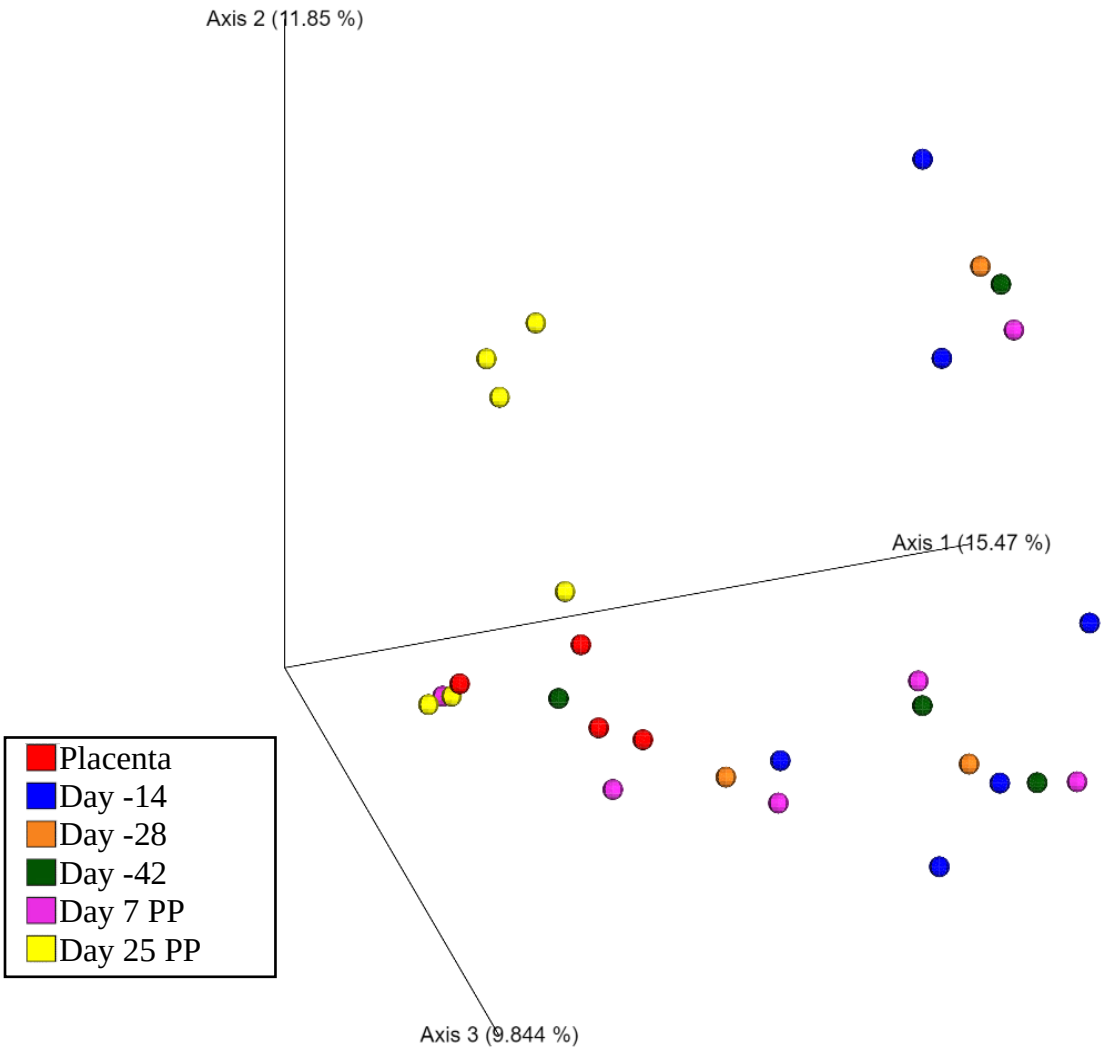


Figure 2.5 Principle Coordinates Analysis (PCoA) of Bray-Curtis Dissimilarity representing comparisons of the microbial composition of vaginal samples collected at different time points (Placenta, n=4; Day -14, n=6; Day -28, n=3; Day -42, n=4; Day 7 PF, n=6; Day 25 PF, n=6). Points in three-dimensional space represent individual samples, which are colored according to time point/type. The percent variance explained by PCoA is indicated on the axes. Placenta and Day 25 postpartum (PP) were significantly separated from all other time points ($P<0.05$).

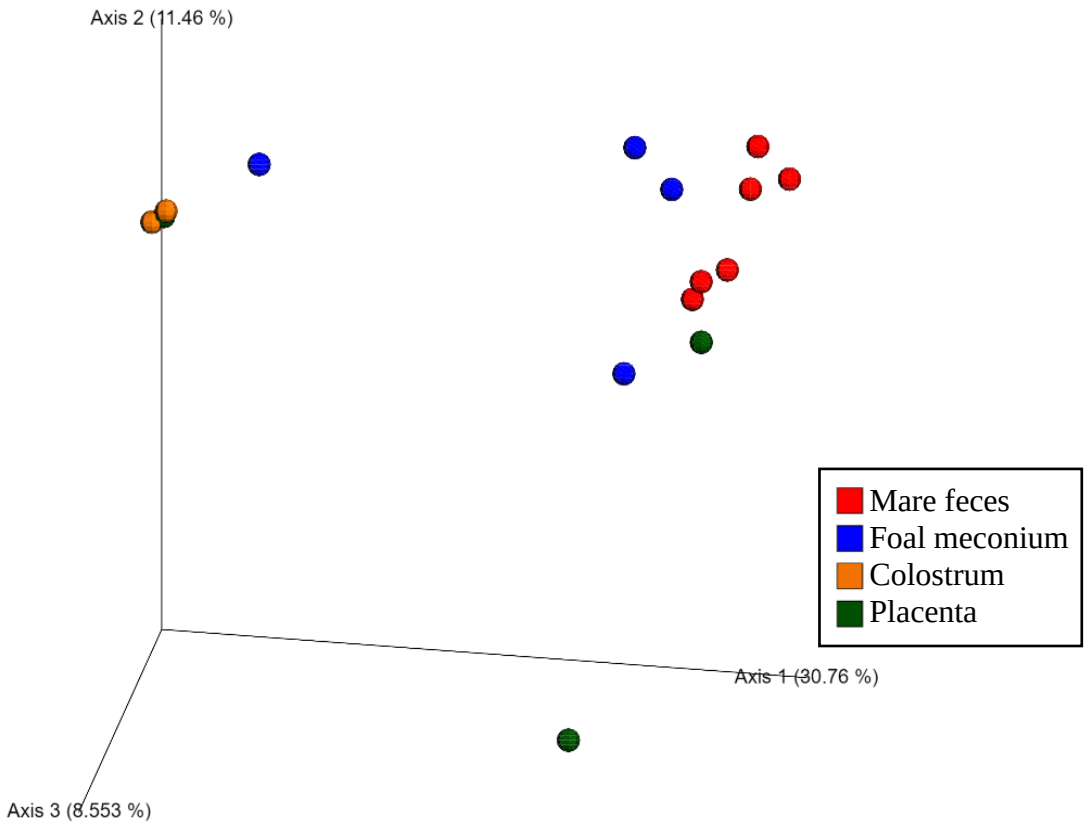


Figure 2.6 Principle Coordinates Analysis (PCoA) of Bray-Curtis Dissimilarity representing comparisons of the microbial composition of different samples collected at parturition in mares and foals (mare feces, n=6; meconium, n=4; placenta, n=3; colostrum, n=2). Points in three-dimensional space represent individual samples, which are colored according to sample type. The percent variance explained by PCoA is indicated on the axes. Mare feces clustered separately from all other sample types ($P<0.05$). There was not a clear separation between meconium, placenta, or colostrum ($P>0.05$).

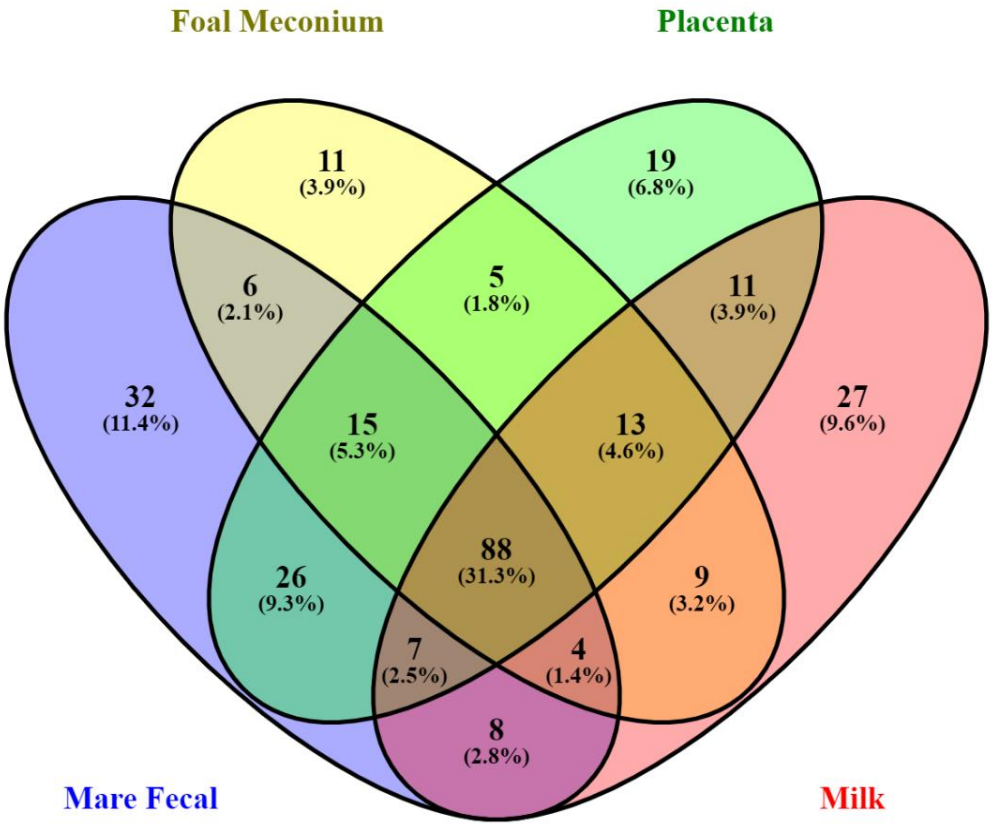


Figure 2.7 Venn diagram of shared genera between different sample types collected at parturition in mares and foals (mare fecal, n=33; milk, n=14; foal meconium, n=6; placenta, n=5). Overlapping circles indicate the number of shared genera in bold and the percentage of the total observed genera shared between each sample type.

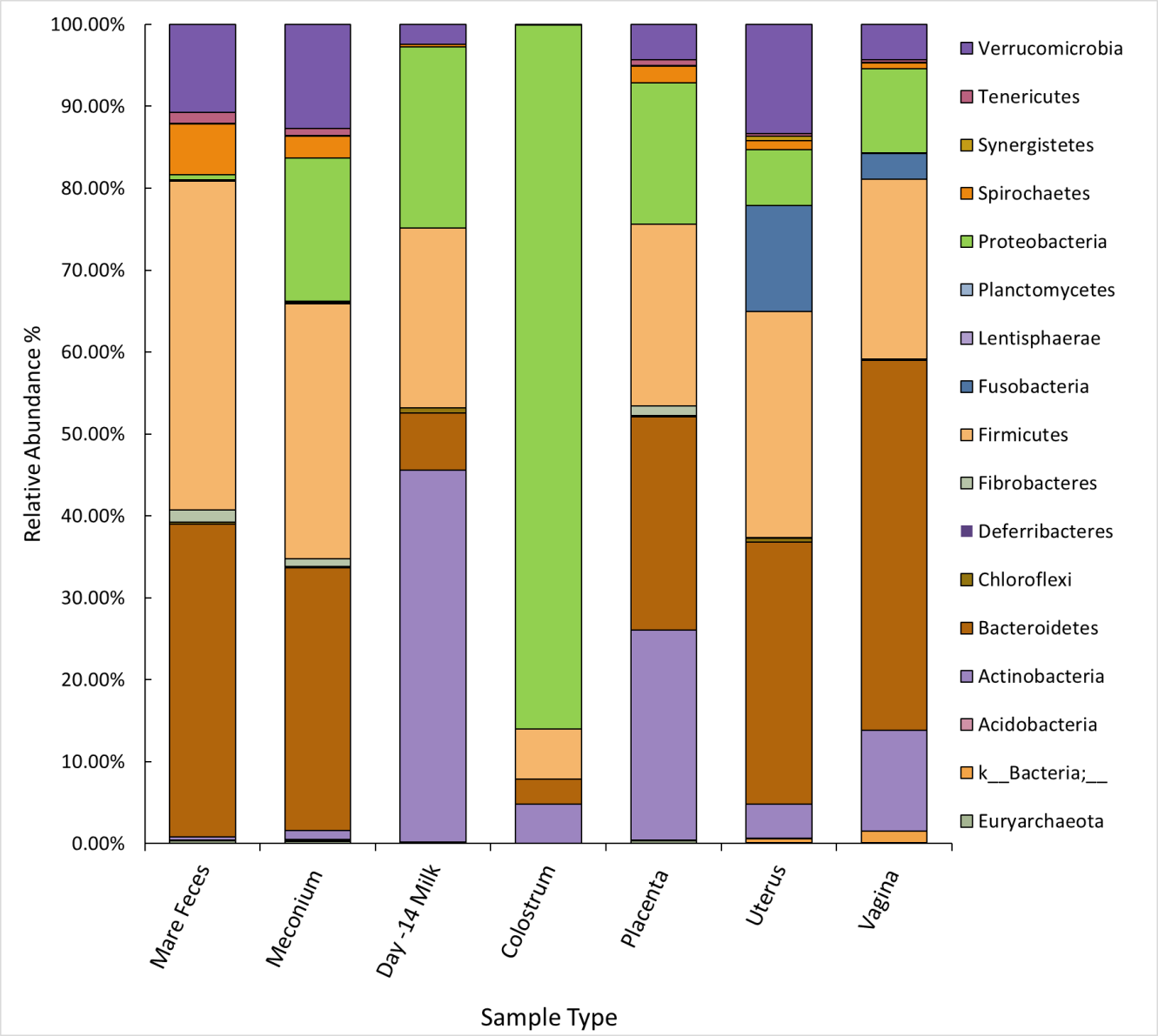


Figure 2.8 Relative abundance of dominant microbes at the phylum level identified in pregnant mare fecal samples collected prepartum and at parturition (n=33); milk samples collected day-14 prepartum (n=8); meconium (n=6), colostral (n=6) and placental (n=5) samples collected at parturition; postpartum uterine samples (n=15) and prepartum/postpartum vaginal samples (n=48). Samples were pooled by sample type if there were no differences at the phylum level in taxa present at >5% mean relative abundance. Taxa with k__ represent kingdom as the lowest taxonomic level.

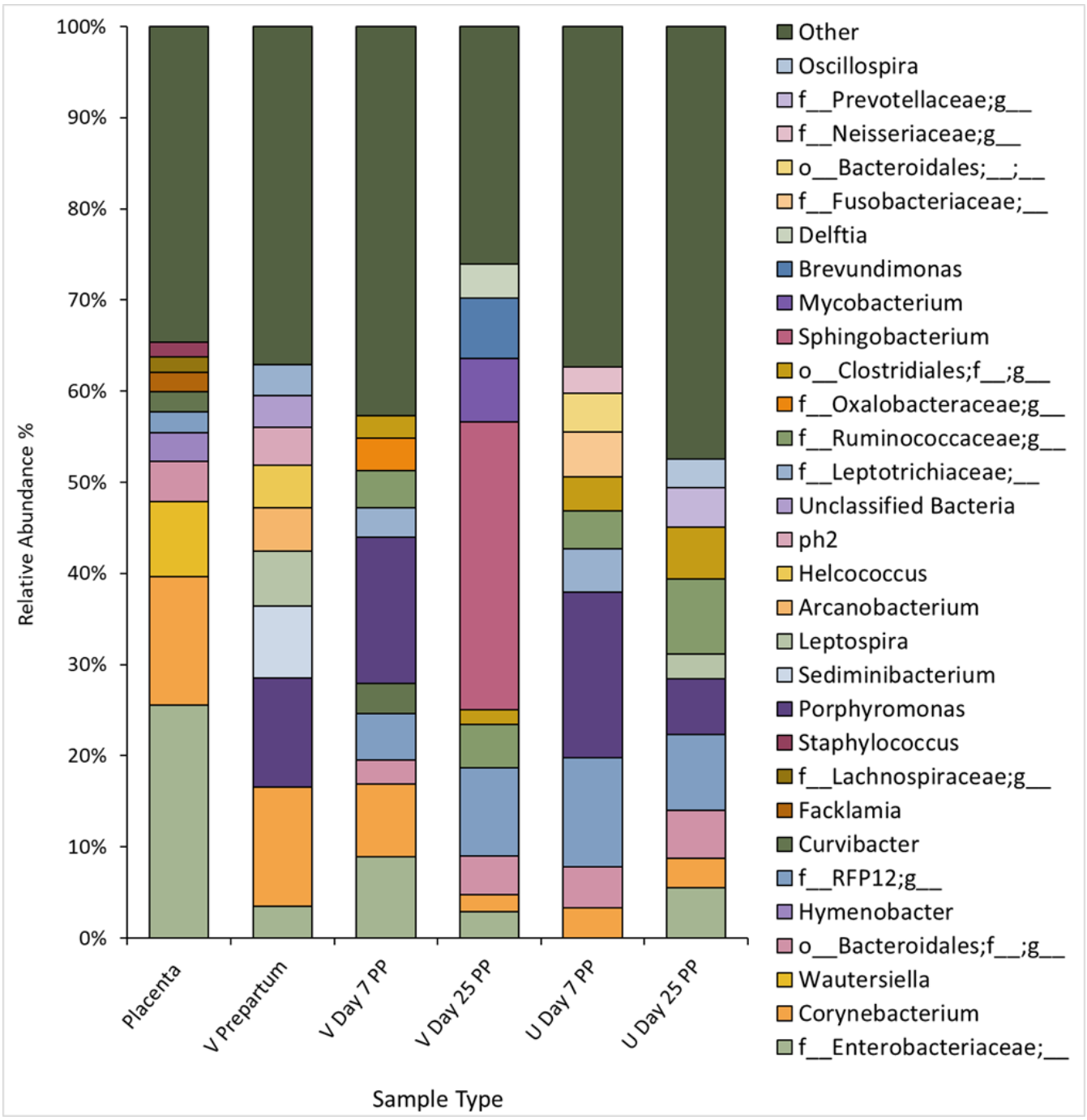


Figure 2.9 The 10 most relatively abundant microbial genera or lowest taxonomic level identified in vaginal samples collected during the prepartum period (V prepartum, n=33) and on day 7 (V day 7 PP, n=7) and 25 postpartum (V day 25 PP, n=7) as well as the placental samples collected at parturition (n=5); and uterine samples collected on day 7 (U day 7 PP, n=7) and 25 postpartum (U day 25 PP, n=7). All other genera are grouped into "Other". Taxa with o__ represent order as the lowest taxonomic level, f__ represents family. PP: Postpartum

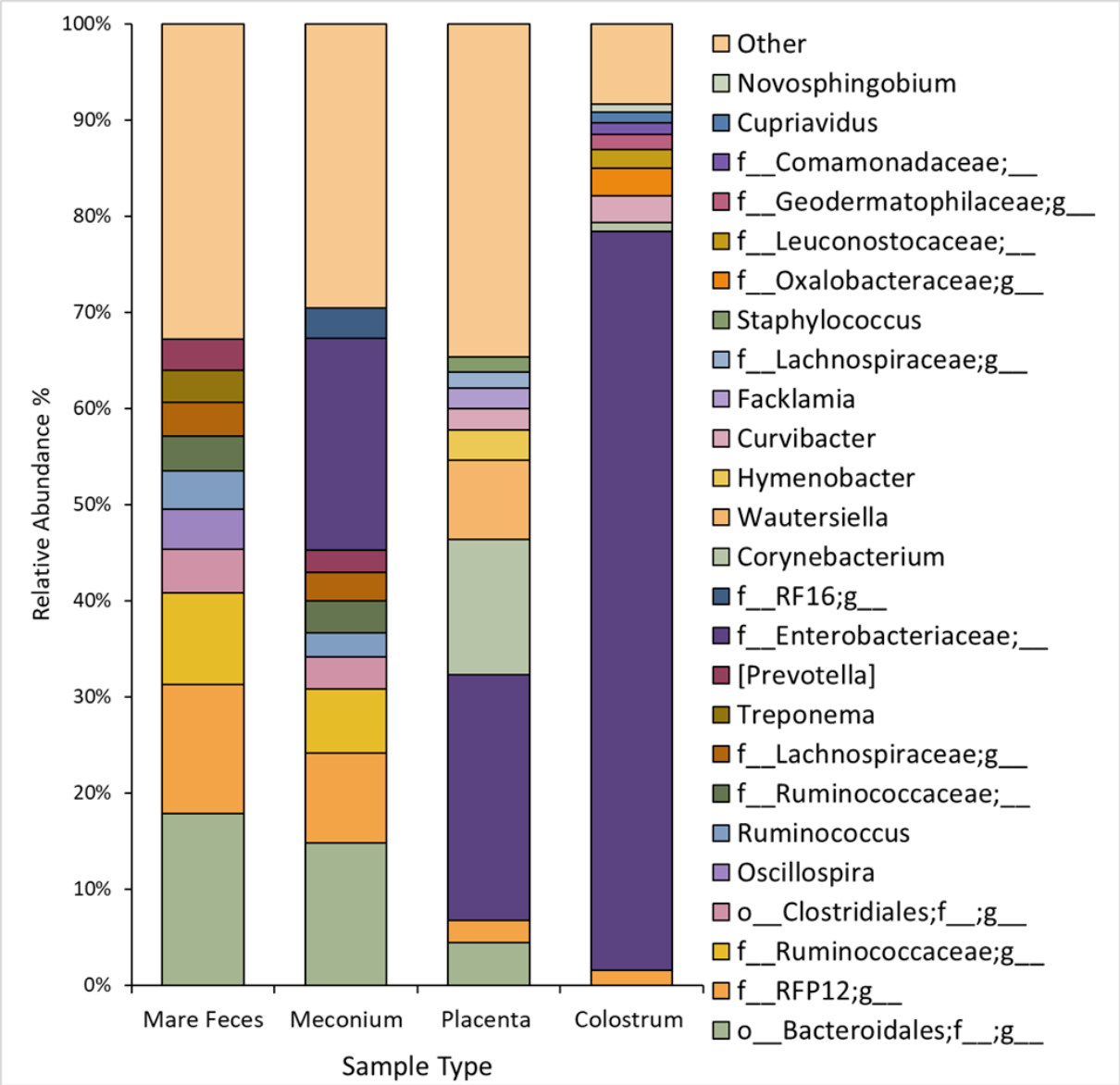


Figure 2.10 The 10 most relatively abundant genera or lowest taxonomic level identified in samples collected from mares and foals at parturition, which included mare feces (n=6) and foal meconium (n=6), as well as placental (n=5), colostral (n=6) samples. All other genera are grouped into "Other". Taxa with o__ represent order as the lowest taxonomic level, f__ represents family.

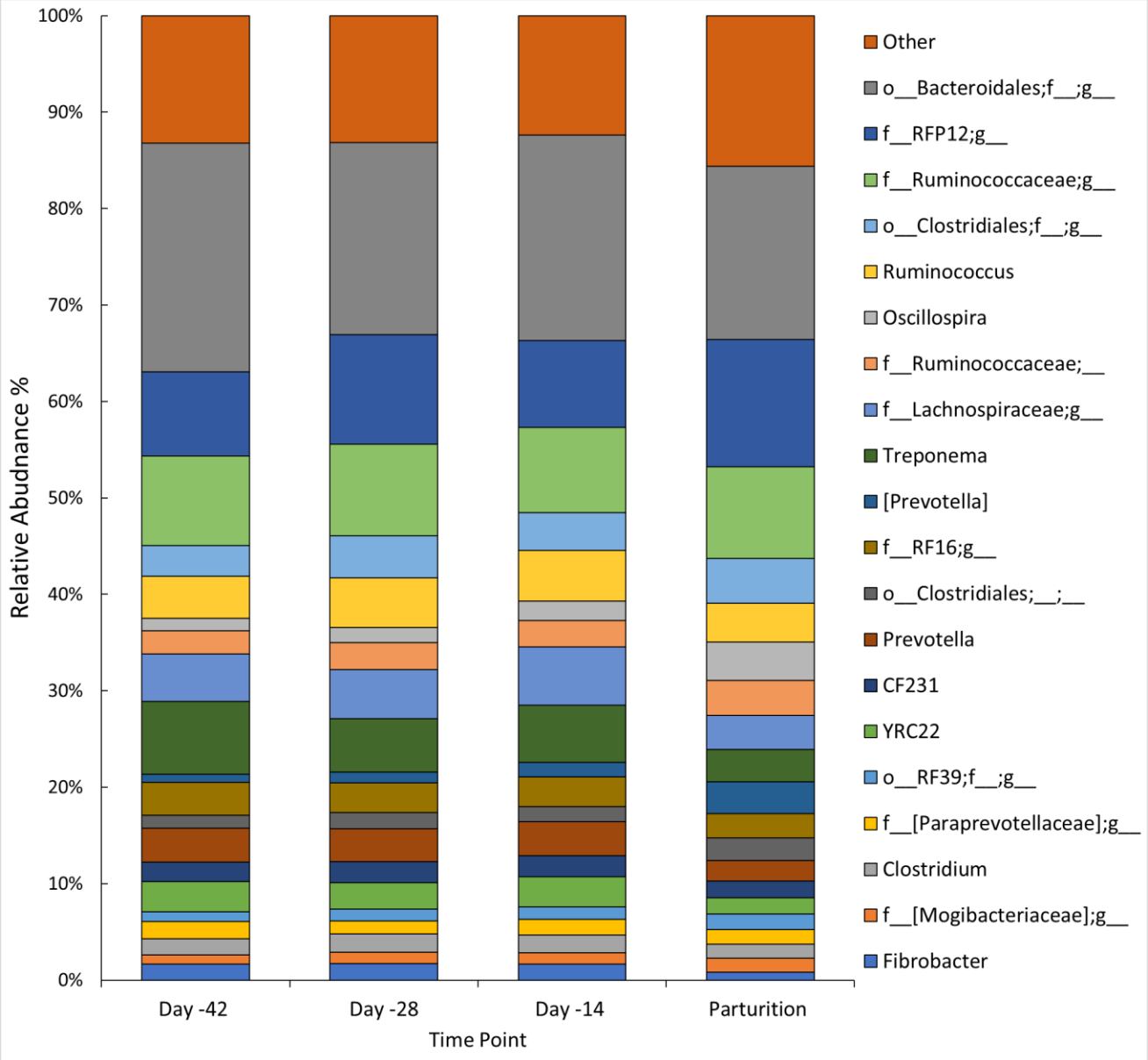


Figure 2.11 The 10 most relatively abundant genera or lowest taxonomic level identified in mare fecal samples collected at 14 day intervals through parturition, starting 42 days prepartum (day-42,-28,-14, n=9; parturition, n=6). All other genera are grouped into "Other". Taxa with o__ represent order as the lowest taxonomic level, f__ represents family.

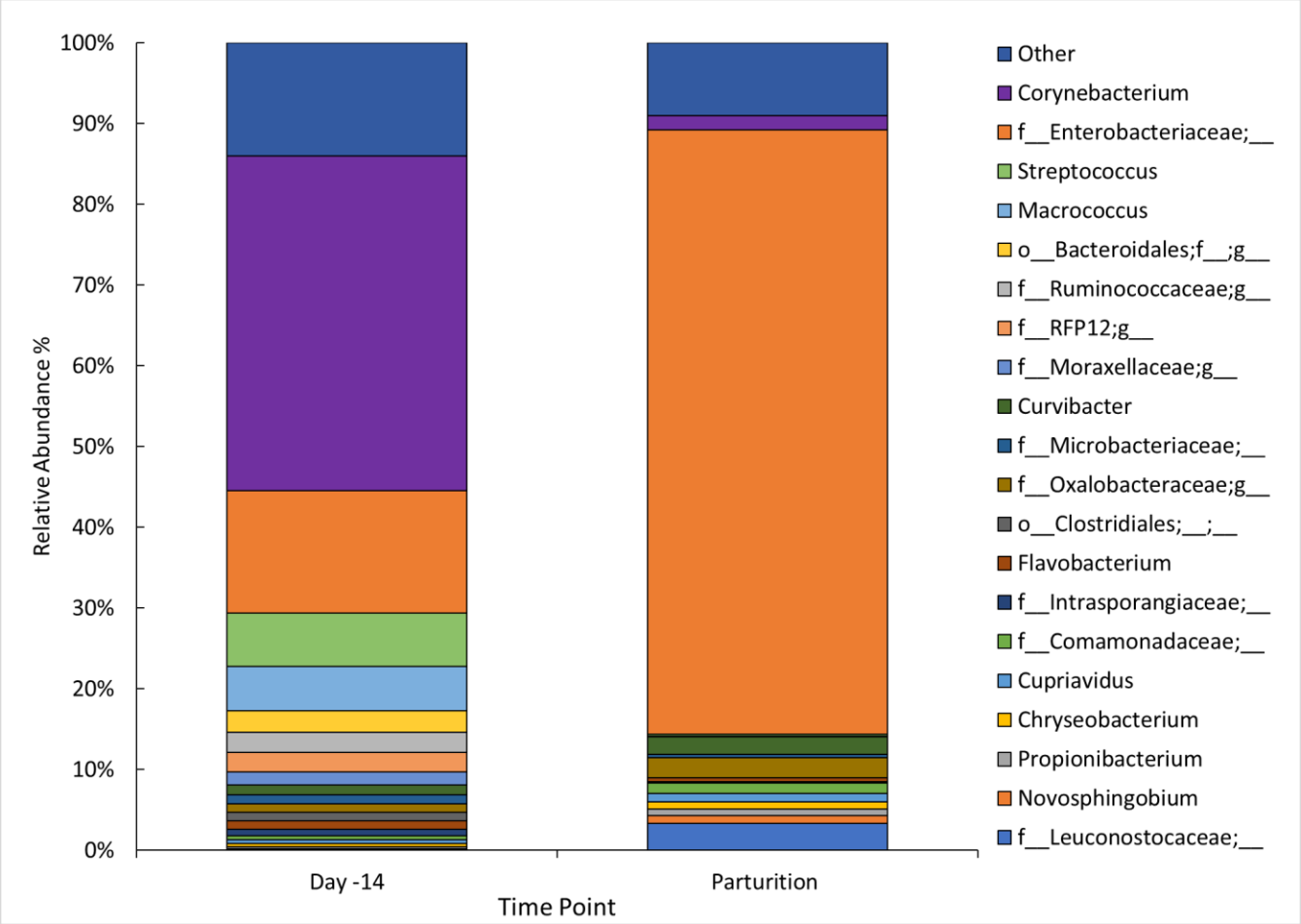


Figure 2.12 The 10 most relatively abundant genera or lowest taxonomic level identified in pregnant mare secretions collected 14 days prior to parturition (n=8) and colostrum samples collected at parturition (n=6). All other genera are grouped into "Other". Taxa with o__ represent order as the lowest taxonomic level, f__ represents family.

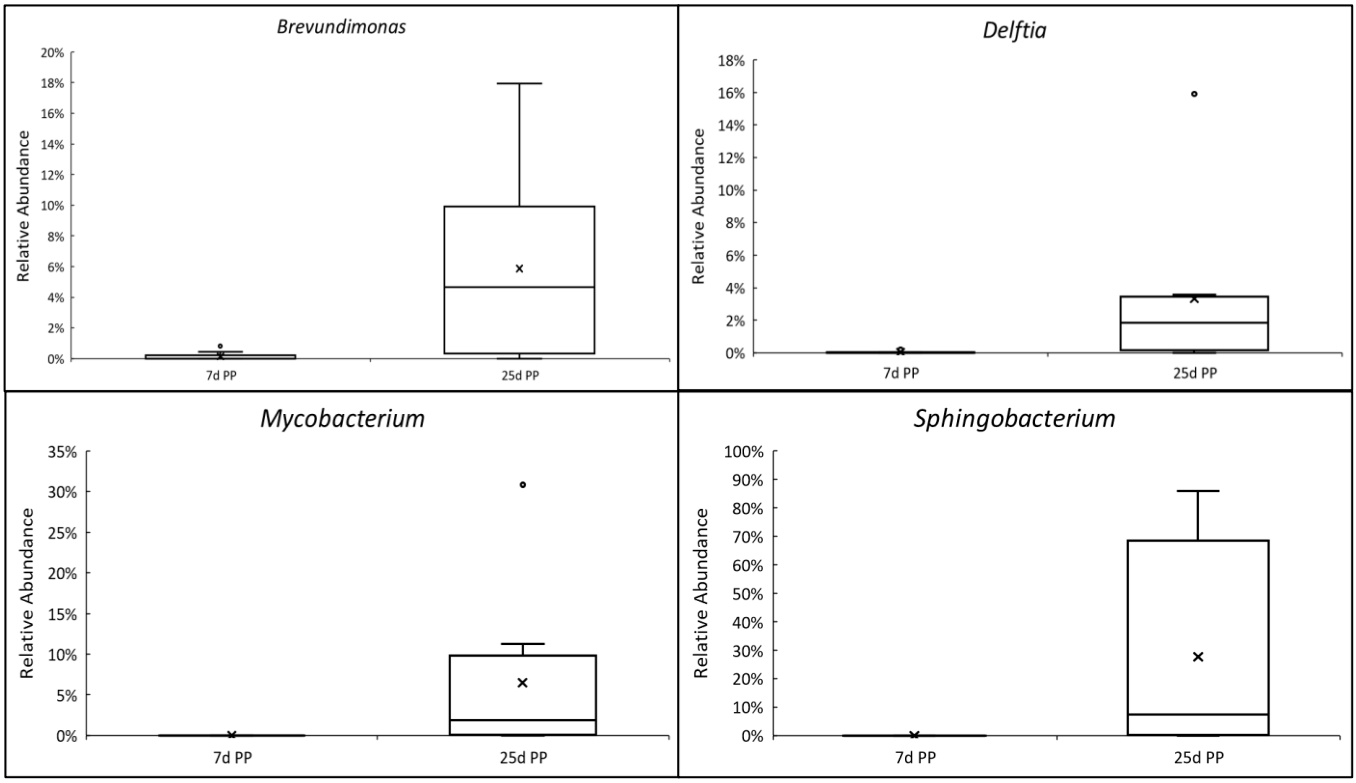


Figure 2.13 Percent relative abundance of differentially abundant taxa according to Analysis of Composition of Microbes (ANCOM) between mare vaginal samples collected on day 7 (n=9) and day 25 (n=8) postpartum (PP). Boxes represent the interquartile range (the horizontal line within the box is the median, the x represents the mean), dots outside boxes are outliers. Each genus was more abundant in the vagina on day 25 compared to day 7 postpartum.

Table 2.1 Summary of Analysis of Composition of Microbes (ANCOM) results of differential abundance using genus as the lowest taxonomic level in samples collected from mare/foal pairs at parturition. Meconium samples (n=6) were compared to mare feces (n=6), placenta (n=5) and colostrum (n=6) samples.

Taxa[a]	Meconium[b]	Mare Feces[b]	Placenta[b]	Colostrum[b]
o__Bacteroidales.f__.g__	14.82 ± 4.81	17.87 ± 2.21	4.44 ± 6.97	**0 ± 0***
o__Clostridiales.__.__	1.66 ± 0.96	2.40 ± 1.23	0.34 ± 0.73	**0 ± 0***
o__Clostridiales.f__.g__	3.35 ± 2.28	4.62 ± 1.13	0.44 ± 0.74	**0 ± 0***
o__RF39.f__.g__	0.34 ±0.38	**1.61 ± 0.70***	0.26 ± 0.50	0 ± 0
f__Enterobacteriaceae.__	22.07 ± 20.56	**0.03 ± 0.05***	25.52 ± 24.14	76.79 ± 14.74
f__Lachnospiraceae.g__	3.03 ± 1.71	3.51 ± 0.74	1.66 ± 2.68	**0 ± 0***
f__RF16.g__	3.17 ± 1.81	2.54 ± 2.17	**0.12 ± 0.27***	**0 ± 0***
f__RFP12.g__	9.34 ± 3.54	13.39 ± 3.96	2.33 ± 2.90	**1.59 ± 3.89***
f__Ruminococcaceae.__	3.31 ± 2.15	3.61± 0.58	0.34 ± 0.71	**0 ± 0***
f__Ruminococcaceae.g__	3.31 ± 2.15	9.53 ± 1.53	1.35 ± 2.60	**0 ± 0***
Clostridium	0.75 ± 0.52	**1.47 ± 0.58***	0.20 ± 0.39	0 ± 0
Corynebacterium	0.26 ± 0.60	0 ± 0	**14.13 ± 21.92***	0.96 ± 1.06
Facklamia	0 ±0	0 ± 0	**2.13 ± 2.68***	0.09 ± 0.21
Oscillospira	1.76 ± 0.82	4.09 ± 3.23	0.40 ± 0.66	**0 ± 0***
Prevotella	1.27 ± 0.80	2.08 ± 0.97	0.55 ± 0.98	**0.12 ± 0.30***
Ruminococcus	2.48 ± 0.56	3.98 ± 2.22	**0.33 ± 0.44***	**0.15 ± 0.29***
Staphylococcus	0.07 ± 0.10	**0 ± 0***	1.62 ± 2.04	0.24 ± 0.37
Treponema	1.62 ± 1.26	3.33 ± 1.77	0.66 ± 1.22	**0 ± 0***

[a]Only taxa with mean relative abundance >1% in at least one sample type were included
[b]Percent mean relative abundance ± standard deviation
*Denote taxa that were found to be significantly different from meconium

Table 2.2 Summary of Analysis of Composition of Microbes (ANCOM) results of differential abundance using genus as the lowest taxonomic level in samples collected from mares in late gestation, at parturition and during the early postpartum period. Placenta samples collected from mares at parturition (n=6) were compared to prepartum/postpartum vaginal (n=33) and postpartum uterine (n=14) samples.

Taxa[a]	Placenta[b]	Vagina[b]	Uterus[b]
Arcanobacterium	0 ± 0	**3.41 ± 5.36***	0.13 ± 0.27
Helcococcus	0 ± 0	**3.26 ± 6.27***	0.32 ± 0.46
Leptospira	0 ± 0	**4.08 ± 7.87***	2.00 ± 3.37
Porphyromonas	0.89 ± 1.89	**10.93 ± 14.60***	12.13 ± 14.09
Sediminibacterium	0 ± 0	**5.27 ± 10.25***	1.20 ± 2.69

[a]Only taxa with mean relative abundance >1% in at least one sample type were included
[b]Percent mean relative abundance ± standard deviation
*Denote taxa that were found to be significantly different from the placenta

Discussion

First, vaginal, milk and fecal samples collected biweekly during the last 6 weeks of gestation were analyzed for microbial composition and diversity. To our knowledge this is the first study to analyze the microbiome of the pregnant mare vagina using NGS. We found the vaginal microbiome of the late gestational mare to be stable in terms of diversity and microbial community composition, which is similar to results in human studies [19,20]. The dominant phyla of the prepartum vagina were Firmicutes, Actinobacteria, and Bacteroidetes, while the dominant genera were *Corynebacterium* and *Porphyromonas. Corynebacterium* is a diverse genus containing over 110 species, some of which are pathogenic in animals and humans; this genus has been found in human skin, oral, and seminal microbiomes as well as the equine uterus, vagina, and semen in our lab [21–23]. *Porphyromonas* is typical of the oral microbiome in humans however it has also been isolated from the uterus, cervix, and vagina of humans, as well as the vagina of cattle [24,25]. In late gestational mares, placentitis is an area of concern as

ascending infections can cause abortions, premature birth, and compromised foals [26]. Bacteria typically cultured from mares with placentitis include *Streptococcus equi* subspecies *zooepidemicus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella* spp., *Leptospira* spp., *Enterobacter* spp., α-haemolytic *Streptococci*, and *Staphylococci* spp. [26]. While some of these bacteria were found in the prepartum vagina of a few mares, they were present at a low abundance and all 6 mares that were included in the parturition analysis were healthy and had a normal foaling. Taxa that were isolated from feces in this study were also found to be abundant in the prepartum vagina, including phyla Bacteroidetes and Firmicutes and family RFP12. Jeon et al. [27] found similar results in dairy cattle in that there were shared taxa between the fecal and vaginal microbiomes of dairy cattle prepartum, indicating that fecal microbes may be translocated to the vagina during defecation, however the impact that this has on pregnancy is unknown.

In contrast to the vagina, there was a shift in diversity in the prepartum fecal samples as -42 day feces were higher in Shannon diversity and observed OTUs than feces collected at parturition; -42 day feces were also higher in observed OTUs than -14 day feces. Similar results were also seen in beta diversity as there was a distinct clustering based on community composition between -42 day feces and feces collected at parturition. While their diet did not change in terms of type of forage or concentrate, these differences might be explained by the change in environment and grass composition as mares were moved to a pasture closer to the foaling barn and given more supplemental hay than fresh forage, approximately 2 weeks prior to their expected foaling date. Our results are in contrast to work done by Salem et al. [28], where researchers found mare feces to be stable in terms of alpha diversity prepartum, however they only utilized fecal samples up to 3 weeks prior to the expected foaling date. We found no

changes in relative abundance between prepartum and parturition feces, and the microbial composition of mare feces is consistent with what has been reported in previous studies [3,9,28,29].

Prepartum mammary secretions were only able to be collected from mares at the -14 day time point; but they were compared to colostrum collected at parturition. The prepartum milk secretions were dominated mostly by *Corynebacterium*, followed by *Streptococcus* and the family Enterobacteriaceae; however, there was a major shift to almost complete dominance by the family Enterobacteriaceae in colostrum. *Streptococcus* and *Corynebacterium* are typically dominant in human milk and have been isolated from mare colostrum as well [6,30]. Enterobacteriaceae are facultative anaerobes that ferment glucose and are typically isolated from the lower gastrointestinal tract [31]. A possible mechanism for the translocation of Enterobacteriaceae and other gut microbes to the mammary glands is thought to occur through dendritic cells. These cells can take up live bacteria from the lumen of the digestive tract; the cells are then transported to other parts of the body, including the mammary glands, through circulation of lymph [30,32]. While the dominance of Enterobacteriaceae in colostrum is contradictory to previous work done in our lab, it is consistent with the findings of Quercia et al. [6] who also determined Enterobacteriaceae to be dominant in mare colostrum [7]. This could be explained by the use of different DNA extraction kits between the two projects conducted in our lab; the previous work done by Jacquay et al. [7] utilized the EZNA Stool DNA Kit (Omega Bio Tek, Norcross, GA) to extract DNA from colostrum samples, however in this study we utilized the QIAamp® DNA Mini Kit (Qiagen; Hilden, Germany), which is similar to the QIAamp DNA Stool Mini Kit (Qiagen; Hilden, Germany) utilized by Quercia et al. [6]. Previous studies have highlighted the differences in reported taxa from different DNA extraction methods, which

points to both the potential difficulty in comparing results and the importance of developing standardized procedures for sample analysis [33,34].

Secondly, the microbial composition of mare feces, colostrum and placenta were compared to that of meconium from neonatal foals. Microbes were detected in all sample types, which is in agreement with equine, bovine and human studies that have also isolated bacteria from meconium, milk and the placenta [5–7,35,36]. There were no differences between mare feces and meconium in terms of alpha diversity, however feces were higher in diversity than placenta and colostrum. This is in contrast to previous work comparing mare feces and meconium, where mare feces were found to be higher in diversity than meconium [6,7]. Previous studies have found meconium to be distinct from mare feces, colostrum and amniotic fluid according to beta diversity [6,7]. Interestingly, we found the microbial composition of meconium was not different from the placenta or colostrum according to Bray-Curtis dissimilarity, however it was distinct from feces. This could be explained by low power when comparing meconium to milk and placenta samples since only 2 colostrum and 3 placenta samples were included after normalization for alpha and beta diversity analysis due to low sequence count.

In terms of relative abundance at the different sample types, meconium was dominated by Bacteroidetes and Firmicutes, followed by Proteobacteria and Verrucomicrobia. Feces was also dominated by Bacteroidetes and Firmicutes but lacked substantial Proteobacteria. Colostrum was discussed earlier, and the placenta was fairly evenly split between Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla. Other dominant taxa in meconium included typical gut microbes such as orders Bacteroidales and Clostridiales, and families Enterobacteriaceae, RFP12 and Ruminococcaceae. At the genus level, the placenta was mostly dominated by *Corynebacterium*, which could be attributed to the high abundance of *Corynebacterium* within

the vagina. The placenta also had the least differentially abundant taxa compared to meconium; with the placenta being more abundant in *Corynebacterium* and *Facklamia* while meconium was more abundant in typical gut microbes such as RF16 and Ruminococcus. The majority of the differentially abundant taxa between meconium and colostrum were also typical gut microbes, including orders Bacteroidales and Clostridiales, families Lachnospiraceae, RF16, RFP12, Ruminococcaceae, and genera *Oscillospira*, *Prevotella*, *Ruminococcus* and *Treponema*. Most of the taxa that were differentially abundant between meconium and mare feces were abundant at very low levels (< 1%) except for Enterobacteriaceae, which was present at a moderate abundance in meconium and very low abundance in mare feces. Surprisingly, 88 (31.3%) genera were shared between the 4 sample types, in contrast to previous studies that found only 6 shared OTUs between mare feces, meconium and amniotic fluid in horses and 14% shared genera between meconium, adult feces, adult mouth, and vaginal samples in dairy cattle [6,35]. Meconium shared the most genera with the placenta (121), followed by colostrum (114) and mare feces (113), which does agree with a previous study in dairy cattle that found meconium shared the least number of genera with dam feces [35].

Interestingly, in the one mare that had a retained placenta, 2 pathogens known to cause placenttis and abortions were the second and third most abundant taxa in her placenta [26,37]. *Actinobacillus* was present at 20.5% and *Streptococcus equi* was present at 15.5% relative abundance. These pathogens were absent from all other mares' placentas. Although his mare was not included in the postpartum analysis due to oral antibiotic treatment to prevent complications associated with the retained placenta, there was no *Streptococcus equi* detected in her postpartum uterus. However, it was present at a low abundance in her day 7 postpartum vaginal sample, but absent by day 25. *Actinobacillus* was absent from her postpartum vagina and uterus. Although

this data is only from a single mare it is possible that these pathogens, which were solely isolated from this mare, could have played a role in the incidence of retained placenta, which may warrant further investigation.

The presence of bacteria in meconium, and the shared taxa between meconium and the other sample types, would indicate an *in utero* transfer of microbes to the developing fetus. These microbes could potentially reach the fetus in the same manner that microbes from the gut theoretically travel to the mammary gland via dendritic cells as discussed previously [32]. Another possible explanation for this transfer of microbes is that the fetus swallows a significant amount of amniotic fluid, especially in late gestation [1]. If amniotic fluid were to contain a microbial community, as previously reported and possibly delivered by dendritic cells, then this would be a direct pathway of transmission of microbes *in utero* between the dam and the fetus [6]. While it is unlikely that the dam's immune system allows functional microbes to reach the fetus, *in utero* inoculation with typical gut microbes may play an important role in programming the fetal immune system to recognize these commensal microbes when the foal takes in live bacteria through suckling, coprophagy, and general exposure to the external environment [7,8]. This programming could potentially prevent the fetal immune system from seeking out and destroying microbes necessary for digestion and metabolism, ensuring the foal gut is adequately prepared for life outside the womb [1].

As a third objective we investigated the temporal dynamics of the mare uterine and vaginal microbiome in the early postpartum period. There were no differences in alpha or beta diversity or community composition in the postpartum uterus. The most dominant family in the uterus was Fusobacteriaceae, which has been isolated from the vaginal, gut and oral mucosa of humans and other animals [38]. *Porphyromonas*, which was also dominant in the vagina, was the

dominant genus in the uterus postpartum. This is similar to non-lactating mares where *Porphyromonas* was the 2nd most dominant genus and several of the 10 most dominant genera were shared between the early postpartum and non-lactating mares according to results found in another study in our lab. Several typical gut microbes were also included in the 10 most dominant taxa including RFP12, Bacteroidales, Ruminococcaceae, *Prevotella*, and *Clostridiales*. The presence of these microbes could be explained through an entero-utero pathway via dendritic cells as discussed previously. Another route for these microbes could be through translocation from the vagina to the uterus during the early postpartum period when the cervix is relaxed and open due to dilation that occurs during parturition, possibly allowing for increased migration of typical fecal microbes through the vagina and into the uterus.

The stability of the uterine microbiome is surprising considering previous studies have found that most mares have a positive culture result for potential pathogens such as *Escherichia coli* and *Streptococcus* at the fist postpartum estrus, but the number of mares with positive culture decreases with increased time postpartum [11,39]. While there is a reduced pregnancy rate when mares are bred during the first postpartum estrus compared to subsequent cycles, this phenomenon is not statistically correlated to the incidence of positive cultures [11,40]. Our findings would support the idea that uterine involution and recovery of uterine tissues, which is generally complete by day 9 or 10 postpartum, may be the limiting factor in pregnancy establishment rather than the presence of potentially pathogenic bacteria, as we found the uterus to have a diverse microbial community postpartum that included some known pathogens such as *Leptospira* [41].

There were no differences in alpha diversity in the vagina postpartum; however, there was a distinct shift in community composition between day 7 and 25 postpartum. The day 7

postpartum vagina was similar in terms of microbial composition and dominant phyla/genera as the prepartum vagina described previously. There were increases in *Sphingobacterium multivorum, Mycobacterium, Brevundimonas diminuta* and *Delftia* from day 7 to 25 in the vagina. *Brevundimonas diminuta* has been isolated from human blood and urine but is not believed to be a significant pathogen as its virulence is low; it has not been reported as a pathogen in the equine [42]. Similarly, *Delftia* has been found in the human oral microbiome, and it has not been recognized as pathogenic in horses [43]. *Sphingobacterium multivorum* and *Mycobacterium* have been associated with suppurative meningitis, meningoencephalomyelitis, intracranial abscessation, abortion and granulomatous colitis in adult horses [44,45], however all mares were clinically healthy and all became pregnant following study completion with the exception of one mare that experienced fluid retention issues post-foaling. These microbes were not present in mare feces, so it is unlikely that these microbes originated from the gut. Since NGS is not capable of differentiating live and dead bacteria, the high abundance of these pathogens in the absence of clinical symptoms could be explained by the possibility of an influx of these microbes from an unknown source, followed by immune-mediated removal, but residual DNA remained in the vagina. The physiological reason why these particular genera/species increased in abundance between day 7 and 25 in the vagina remains unclear, however the lack of clinical symptoms and the lack of a corresponding shift in the uterine microbiome would indicate that their presence had little, if any, impact on the subsequent reproductive efficiency of these mares.

Lastly, we compared the postpartum vagina and uterus to each other and to the placenta. We found there were few differences between these sites. This is similar to a recent study in dairy cattle, where researchers found no differences between the uterine and vaginal microbiome

or between samples taken 7, 21, or 50 days postpartum in healthy cows, although there was significant variation between individual animals [14]. Another study that utilized dairy cattle found that the postpartum vagina and uterus had significant differences in alpha and beta diversity which is contradictory to our results, however, consistent with our study, the postpartum vagina and uterus of dairy cattle were similar in terms of community composition [46]. We found no differentially abundant taxa between the uterus and vagina, however there were a few that were different between the placenta and vagina/uterus. Only *Agrobacterium* was differentially abundant between the uterus and placenta, however it was abundant at less than 1% in each site and is most likely a contaminant introduced through sampling/laboratory analysis, as this genus is most typically found in plants and has not been isolated from placenta or uterine samples in prior studies [47]. Between the vagina and placenta *Sediminibacterium*, which was abundant at 5% in the vagina but absent from the placenta, is also most likely a contaminant as it has been found to be highly abundant in DNA extraction kit reagents [33]. *Arcanobacterium* and *Leptospira* have both been associated with abortion in the mare and *Helcococcus* is pathogenic in humans, however they were more abundant in the vagina than the placenta, so they were most likely introduced postpartum or during the foaling process [48–50]. *Porphyromonas* which was more abundant in the vagina, has been associated with bacterial vaginosis in women and bacterial infections of the uterus in cattle, however mares did not show any clinical symptoms of bacterial infection [27,51]. We found the uterus, vagina and placenta to be highly similar to each other, indicating that the microbial composition of these sample types may be interdependent on each other and the detection of potentially pathogenic microbes using sequencing does not necessarily indicate clinical disease.

Conclusion

This study confirmed the presence of bacteria within meconium, placental, and colostral samples, in addition to the early postpartum uterus and vagina of the mare. Results indicate that there are temporal changes in mare feces prior to foaling that may be related to a change of physical location. There is also a large shift between the -14 day prepartum milk secretions and colostrum. Meconium had a unique microbiome, however, there were some similarities between meconium and mare feces, placental, and colostral samples in terms of alpha and beta diversity as well as community composition. The vagina is relatively stable in terms of microbial composition prepartum, however there is a slight shift between day 7 and 25 postpartum towards an increase in potentially pathogenic bacteria. The uterus appeared to be stable between day 7 and 25 postpartum and was similar to the postpartum vagina, with both being similar to the placenta. Although several differences in composition were discovered, the specific functions of the bacteria detected and the physiological significance of shifts in microbial composition remain unclear and warrant further investigation. Comparing these results to mares under different management practices or to those experiencing clinical disease during the late gestational and early postpartum period could better define the relevance of the mare's microbiome in establishing normal reproductive function.

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Chapter 3 - Effect of breeding on the mare uterine and vaginal microbial composition

Summary

Background: Microbial contamination of the mare uterus and vagina associated with breeding can potentially be detrimental to fertility. To date, the effect of breeding on the mare uterine or vaginal microbiome has not been investigated using next generation sequencing (NGS).

Objectives: To investigate the microbiome of the uterus and vagina in healthy mares and to determine the effect of breeding with either raw or extended semen on the uterine and vaginal microbiota.

Study design: Longitudinal study

Methods: Sixteen Quarter Horse mares and one stallion were utilized in this study. Mares were separated into 2 treatments: artificially inseminated (AI) with 10 mL of raw semen only (RAW, n=8) or 10 mL of semen and 10 mL of extender (EXT, n=8). Uterine efflux and vaginal swabs were collected when a follicle measuring ≥ 35 mm was first observed. Mares were then inseminated within 24 hours of initial sample collection and uterine and vaginal samples were collected again 48 hours post-AI. The uterine and vaginal protocol was repeated in the next estrous cycle. Feces were collected once from mares, immediately following the first detection of a follicle measuring ≥ 35 mm. Semen samples were collected for analysis prior to insemination. Samples were analyzed using NGS of the V4 region of the 16S rRNA gene to determine microbial composition. Analysis of sequencing data and statistics were performed using QIIME2 and R.

Results: Microbes were detected in all sample types. Feces and semen were distinct from all other sample types, however there were few differences between the uterine and vaginal

microbiomes. The uterine microbiome was not significantly impacted by breeding and there were no differences between breeding with raw or extended semen. The vaginal microbiome did shift post-AI in the first cycle and between the first and second cycle. Although there were differentially abundant taxa between the uterus/vagina and semen, *Corynebacterium* and *Prophyromonas* were dominant in all three sample types.

Main limitations: Possibility for contamination during sampling, and DNA extraction, and PCR, bias of the variable region targeted during sequencing, inability to distinguish live and dead microbes, and presence of unidentified microbes.

Conclusions: In healthy mares the uterus appeared to return to the pre-AI microbial composition by 48 hours post-AI and there was no shift in composition between estrous cycles. However, the vaginal microbiome is dynamic and displays more shifts following breeding and throughout the estrous cycle than the uterine microbiome. The semen, vaginal and uterine microbiomes shared similar dominant taxa, indicating that there may be similar control mechanisms in mares and stallions to recognize commensal bacteria within the reproductive tract.

Introduction

The microbes within the mare uterus and vagina are of interest because certain species, including *Streptococcus equi subsp. zooepidemicus, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacteroides fragilis and Bacteroides ureolyticus,* are associated with post breeding-induced endometritis (PBIE) with *Streptococcus equi subsp. Zooepidemicus* being most commonly cultured microorganism in effected mares [1]. In normal, reproductively sound, mares there is an inflammatory response in the uterus to semen, bacteria, and debris that are deposited during breeding via either natural service or AI; this inflammatory response is typically resolved within 48 hours and most mares are able to expel or

eliminate fluid and bacteria introduced during breeding [2]. In mares that are susceptible to PBIE, this inflammation is not resolved within 48 hours post-breeding, resulting in subfertility and economic losses, making PBIE the leading reproductive health concern among equine veterinary practitioners [3]. However, non-pathogenic and pathogenic microbes have been cultured from clinically normal mares with no reproductive issues, indicating that the equine uterus may host a diverse microbial community that could include commensal bacteria [4,5].

In semen, the most common non-pathogenic bacteria are *Staphylococcus* coagulase-negative and *Corynebacterium* spp, both of which are also dominant on the external genitalia [6–9]. Following the detection of bacteria in stallion semen, extenders were developed with the goal of decreasing potential bacterial contamination of the mare, as well as enhancing spermatozoa longevity and improving conception rates. The most common antibiotics added to stallion semen extenders are beta-lactams (potassium penicillin G) and aminoglycosides (amikacin or gentamicin) [10–14]. The addition of these antibiotics has been shown to reduce the severity of uterine bacterial contamination post-AI compared to raw semen alone [15].

Utilizing NGS technologies, only two studies have investigated the microbial community of the mare uterus and none have characterized the vagina or semen microbiome [16,17]. Both uterine studies profiled clinically healthy mares, however neither investigated the effect of breeding on the microbiome. We sought to establish the “normal” mare uterine and vaginal microbiome for comparison to future studies. We also wanted to investigate the effects of breeding on the microbiome to gain a better understanding of the immune response that is initiated post-breeding and the potential effects of that immune response on pathogenic and commensal microbes. The objective of this study was to investigate the microbiome of the uterus

and vagina in healthy mares and to determine the effect of breeding with either raw or extended semen on the uterine and vaginal microbiomes.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Usage Committee at Kansas State University (Protocol No. 4052).

Animals and Treatment

Sixteen non-pregnant stock-type mares 3-18 years of age and one 5-year-old Quarter Horse stallion were used in this study. Mares were group-housed in two pens at the KSU Horse Unit; they were not separated by treatment and were evenly distributed between the two pens. They were provided ad libitum access to brome hay, salt/mineral blocks, and water. The stallion was housed individually and fed a diet consisting of brome grass hay, concentrate formulated for maintenance, a salt/mineral block and water. Prior to inclusion in the study all horses were evaluated through a physical examination to ensure they were healthy; mares did not have any obvious reproductive issues upon ultrasound examination, and semen collected from the stallion yielded 424 million sperm/ml and 61.36% progressive motility using computer-assisted sperm analysis. Mares were blocked by age and weight and assigned to one of two treatment groups: bred via artificial insemination (AI) with fresh semen only (RAW, n=8) or bred with fresh semen and extender (EXT, n=8).

Mare Management

Beginning in early February of 2018, mare follicular status was monitored via transrectal ultrasonography 3 days/week (M, W, F) until ovulation was observed. Following confirmation of ovulation, mares were administered Lutalyse® (1 mg/100 lb BW, IM) on day 6 post-ovulation to short cycle each mare and bring them back into estrus. Following Lutalyse®, mares received an

were ultrasounded every day until a follicle measuring ≥ 35 mm was observed. Mares were inseminated once, depending on their treatment, within 24 hours of initial detection of a follicle ≥ 35 mm. After insemination, ultrasound examinations were performed daily until ovulation was detected. On day 6 post-ovulation, mares received another dose of Lutalyse® (1 mg/100 lb BW, IM) to induce lysis of the corpus luteum and prevent pregnancy establishment. Eleven mares (RAW: n=7; EXT: n=4) were then tracked using ultrasound examination through a second estrous cycle in the manner described above and the breeding process was repeated. The number of mares was reduced from 16 to 11 in the second cycle due to various reasons including change of ownership or to avoid delay in establishing a desired pregnancy. Also, one mare developed a hoof abscess that required oral antibiotic treatment between the first and second estrous cycle so she was removed from the study for the second cycle; all other mares remained healthy throughout the duration of the study. Following the final sample collection and Lutalyse® administration on day 6 post-ovulation, after either the first cycle or second cycle for those that continued on, mares were removed from the study.

Semen Collection and Mare Insemination

All semen samples were collected from one stallion. The stallion was first exposed to a teaser mare through face to face interaction only to stimulate an erection. Thereafter, the penis was washed with warm water only and dried with paper towels. The stallion was re-exposed to the teaser mare and then directed to mount a phantom to facilitate semen collection into an artificial vagina (AV) that was only used with this stallion during the breeding season. A Missouri model AV was used, equipped with a collection bottle. The collection bottle contained a sterile bottle liner and a non-sterile gel filter. The AV, wash bucket, and collection bottles were cleaned using 70% isopropyl alcohol after each use. The collected semen was processed and

analyzed immediately for volume, concentration and motility. For EXT mares the commercially available semen extender, INRA 96 (IMV Technologies; L'Aigle, France), was used at a 1:1 semen:extender ratio with a total dose of 20 mL (10 mL semen and 10 mL extender). For RAW mares, a total dose of 10 mL of semen only was used. All insemination doses were prepared using sterile syringes. In some instances, the stallion did not produce a total volume of 10 mL of semen. In these cases the semen included in the insemination dose ranged from 7-9 mL for 3 mares, in the first cycle (RAW=2, EXT=1) and 2 mares in the second cycle (RAW=2). Despite the lower volume in some instances, sperm concentration and motility were evaluated prior to every insemination to ensure mares were bred with a minimum of 500 million progressively motile sperm.

Prior to insemination, the perineal area was cleaned with water and dilute ivory soap and dried, then a sterile pipette was inserted through the cervix into the uterus using a lubricated sterile shoulder-length sleeve. Raw or extended semen was deposited, and the pipette was removed. Care was taken to ensure that the glove and pipette only made contact with the mare prior to semen deposition.

Sample Collection

Vaginal and Uterine Samples

Vaginal samples were collected immediately following initial detection of a follicle ≥ 35 mm and then again 48 hours post-insemination for cycles 1 and 2. Uterine efflux was collected immediately after collection of both vaginal samples. Prior to sample collection the tail was wrapped and held out of the way with a tail tie. The perineum was cleaned with water and dilute Ivory soap and dried. For vaginal samples, a sterile obstetrical sleeve with sterile lubricant was donned and a double-guarded sterile swab was inserted into the vagina. Once location within the

vagina was confirmed through identification of the cervix, a sample was taken from of the epithelium of the floor of the vagina roughly 5 cm from the cervix. Swabs were returned to the protective casing and removed. They were then placed into sterile 50 mL conical tubes and stored at -20° C until further analysis. For uterine samples the investigator donned a sterile obstetrical sleeve with sterile lubricant and a sterile uterine lavage tube was manually passed through the vulva, vagina and cervix into the uterine body. The uterine lavage tube was fixed in place just past the cervix with a balloon filled with 60-100 mL of air. Next, 120 mL of sterile phosphate buffered saline was infused into the uterus via a sterile 60 mL syringe. The fluid was collected via gravity flow into two sterile 50 mL conical tubes. Recovery of fluid ranged from 48-100 mL. Tubes were then centrifuged at 400 x g for 10 min. Supernatant was removed and transferred to a separate sterile conical tube, leaving the pellet and approximately 2 mL of fluid in the original tube. Conical tubes containing supernatant and pellets were stored at -20° C for later DNA analysis.

Fluid and Fecal Samples

Blood and fecal samples were collected only once from each mare following detection of a follicle ≥ 35 mm during the first estrous cycle. A 10 mL blood sample was collected via jugular venipuncture into Vacutainer® tubes for plasma (Becton, Dickson and Company, Franklin Lakes, NJ). Blood was spun at 1,400 x g for 15 min at 25° C and white blood cells/platelets were removed and frozen into 1 mL aliquots. Fecal samples were collected via a rectal grab using a lubricated sterile shoulder-length sleeve. Approximately 5-50 g of feces were placed into a sterile 50 mL conical tube. Five semen collections resulted in sufficient volume post-AI for a 2-5 mL sample to be taken from raw semen using a sterile pipette and deposited into a 10 mL sterile

conical tube. All fecal, blood, and semen samples were stored individually at -20° C for later DNA analysis.

DNA Extraction and PCR

Total DNA was extracted from the uterine efflux, vaginal swabs, and blood using the QIAamp® DNA Mini Kit (Qiagen; Hilden, Germany) according to manufacturer protocols for bacteria. Total DNA was extracted from feces using the E.Z.N.A® Stool DNA Kit (Omega Bio-tek; Norcross, GA). The concentration of DNA was quantified using Quant-iT^{TM} PicoGreeen^{TM} dsDNA Assay Kit (Thermo Fischer Scientific; Waltham, MA).

Amplification of the V4 region of the bacterial 16S rRNA gene was done using PCR primers (515F/926R) according to Earth Microbiome Project protocols [18]. Fecal samples were amplified through triplicate PCR reactions on a Mastercycler® nexus (Eppendorf; Hamburg, Germany) in a 25 uL solution of 5 uL 5x PCR master mix (Promega; Madison, WI), 0.5 uL of the forward and reverse primers, 0.2 uL of dntps, 1 uL of 1% BSA, 0.2 uL of GoTaq® DNA Polymerase (Promega; Madison, WI), 16.6 uL PCR grade H2O and 1 uL template DNA for 25 cycles. For the low template samples (≤ 10 ng/μL DNA), which included blood, vaginal, uterine, and semen samples, a modified solution of 12.6 uL PCR grade H2O and 5 uL template DNA was run for 30 or 35 cycles based on previous protocols used in our lab [19]. Each PCR reaction was run with a positive and negative control to ensure amplification and to check for contamination. Agarose gel electrophoresis was utilized to check the PCR products for amplicon length and then PCR product triplicates were pooled for library preparation. Excess primers and unincorporated nucleotides were enzymatically removed using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fischer Scientific; Waltham, MA). Total DNA in each sample was then quantified using the Quant-iT^{TM} PicoGreeen^{TM} dsDNA Assay Kit (Thermo Fischer Scientific, Waltham,

MA) and approximately 100 ng of DNA from each sample was combined into a 1.5 mL microcentrifuge tube. The combined library was loaded into a 2% agarose gel and then extracted using the QIAquick® Gel Extraction Kit (Qiagen; Hilden, Germany) to ensure a uniform library size of 500 bp.

Bioinformatics and Statistical Analysis

The final 500 bp library was submitted to the K-State Integrated Genomics Facility for analysis and sequencing. The library was analyzed for amplicon length and bacterial DNA concentration using the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and qPCR. To improve base call quality, 10% PhiX was added to the library. Amplicons were then sequenced using the 500 cycles MiSeq Reagent Kit v2 for a single paired-end run on the Illumina Miseq.

The sequencing data were analyzed using the QIIME2 (version 2019.7) bioinformatics pipeline [20]. Raw sequencing data in the form of FASTQ files were demultiplexed and filtered for quality. Operational Taxonomic Units (OTUs) and taxonomy were assigned according to 97% sequence similarity using the GreenGenes database (version 13.8). Known reagent and laboratory contaminants and contaminants found in the negative controls were removed from the data prior to further analysis [21]. All alpha and beta diversity analyses were performed in QIIME2. Alpha diversity was calculated using Shannon index and observed OTUs. Statistical significance of alpha diversity metrics was determined by Kruskal-Wallis one-way ANOVA tests and linear mixed effects models for body site, time point, and treatment [22]. Beta diversity was calculated using Bray-Curtis dissimilarity which was then ordinated into Principal Coordinates Analysis (PCoA) plots. Pairwise PERMANOVA tests were used to determine compositional differences between sample types, time points, and treatments based on beta-

diversity. Relative abundance of taxa was summarized in plots created in QIIME2. Differential abundance comparisons were calculated in R (version 3.6.1) using ANCOM (Analysis of composition of microbiomes; version 2) [23]. Longitudinal and pairwise comparisons of body sites and treatments and were done at the phylum and genus level using taxa-wise multiple correlation and $\alpha = 0.05$. The W statistic was used to determine significance of differentially abundant genera; this statistic represents the number of times that the null-hypothesis (the average abundance of a given genus in a group is equal to that in another group) was rejected for a given genus [24–27]. Venn diagrams of shared taxa were constructed using Venny (version 2.0) based on presence/absence tables constructed in QIIME2 [28].

Results

Initial data analysis and quality filtering yielded 1,356,938 sequences in 128 samples, consisting of 16 fecal, 4 semen, 54 vaginal and 54 uterine samples. Blood samples were removed from the analysis and will not be reported on due to insufficient microbial DNA detected in those samples following PCR. Samples were grouped by type, treatment and time of collection. Data analysis was performed at 407 sequences in each sample for normalization of alpha and beta diversity metrics based on rarefication curves. Therefore 21 samples were excluded, of which 19 were uterine and 2 were vaginal samples. On average, there were 19 days between sample collections for the mares that went through 2 estrous cycles.

Alpha Diversity

There were no differences in Shannon diversity or observed OTUs between time points or treatments for both the uterus and vagina, so all uterine and vaginal samples were pooled for comparison to other sample types. Fecal samples were higher in diversity according to the Shannon index than all other sample types (Fig. 2.1, $P<0.01$). The uterus and vagina were also

greater in diversity than semen (Fig. 2.1, $P<0.01$), however there was no difference between the uterus and vagina ($P>0.05$). Similar results were seen when alpha diversity was evaluated using observed OTUs (Fig. 2.2). Feces had greater observed OTUs than all other sample types (Fig.2.2, $P<0.05$). Semen had fewer observed OTUs than the uterus and vagina (Fig. 2.2, $P<0.05$). There was no difference between the uterus and vagina ($P>0.05$).

Beta Diversity

The Bray-Curtis dissimilarity index was used to create Principle Coordinate Analysis (PCoA) plots to compare microbial composition. There was a significant clustering according to sample type when mare fecal, uterine, vaginal and stallion semen samples were compared (Fig. 2.3, $P<0.01$). When vaginal samples were compared across four time points over two consecutive estrous cycles there was significant separation in microbial composition between the two cycles pre-AI and post-AI (Fig. 2.4, $P<0.01$). There was no difference between the pre-AI or post-AI vaginal samples between cycle 1 and 2 ($P>0.05$). There were no differences between treatments for vaginal samples ($P>0.05$). There were also no differences in beta diversity for uterine samples between treatments or time points ($P>0.05$).

Differential Abundance

Relative abundance of the main phyla of each location/time point/treatment are summarized in Figure 2.5 with the main genera summarized in Figure 2.6.

To investigate possible links between body sites, shared genera were determined and summarized in a Venn diagram in Figure 2.7. Between fecal, uterine, vaginal, and semen samples 28 genera were shared. The uterus and vagina shared 169; and 25 and 4 were shared between feces and the vagina and uterus, respectively. Between the vaginal, uterine, and semen

samples, 26 genera were shared. There were 35, 38, 14, and 0 genera exclusive to the uterine, vaginal, fecal, and semen samples, respectively.

Analysis using ANCOM found all detected genera and phyla to be differentially abundant between feces and the uterus and vagina (Table 2.1). Uterine and vaginal samples from each treatment were compared across the four time points using ANCOM. In both EXT and RAW uterine samples *Mobiluncus* was more abundant pre-AI than post-AI for cycle 1 (Fig. 2.8). There were no differentially abundant taxa between cycles or in cycle 2 for uterine samples. Unclassified kingdom bacteria, order Bacteroidales, and genera *Cupriavidus, Staphylococcus, Comamonas, Caulobacter, Curvibacter, Mobiluncus, Porphyromonas* and *Phascolarctobacterium* were differentially abundant across time points in EXT vaginal samples (Fig. 2.8). The families Propionibacteriaceae and Coriobacteriaceae, and genera *Streptococcus, Porphyromonas, Mobiluncus, Arcanobacterium* and *Comamonas* were differentially abundant across time points in RAW vaginal samples (Fig. 2.9). When the post-AI uterus and vagina were compared between the EXT and RAW groups the only differentially abundant genus was *Helcococcus*, which was more abundant in the uterus of EXT mares post-AI in cycle 2 compared to RAW mares. When semen was compared to the post-AI uterus and vagina, there were several differentially abundant taxa between semen and EXT and RAW at both the phylum (Table 2.2) and genus level (Uterus: Table 2.3, Vagina: Table 2.4).

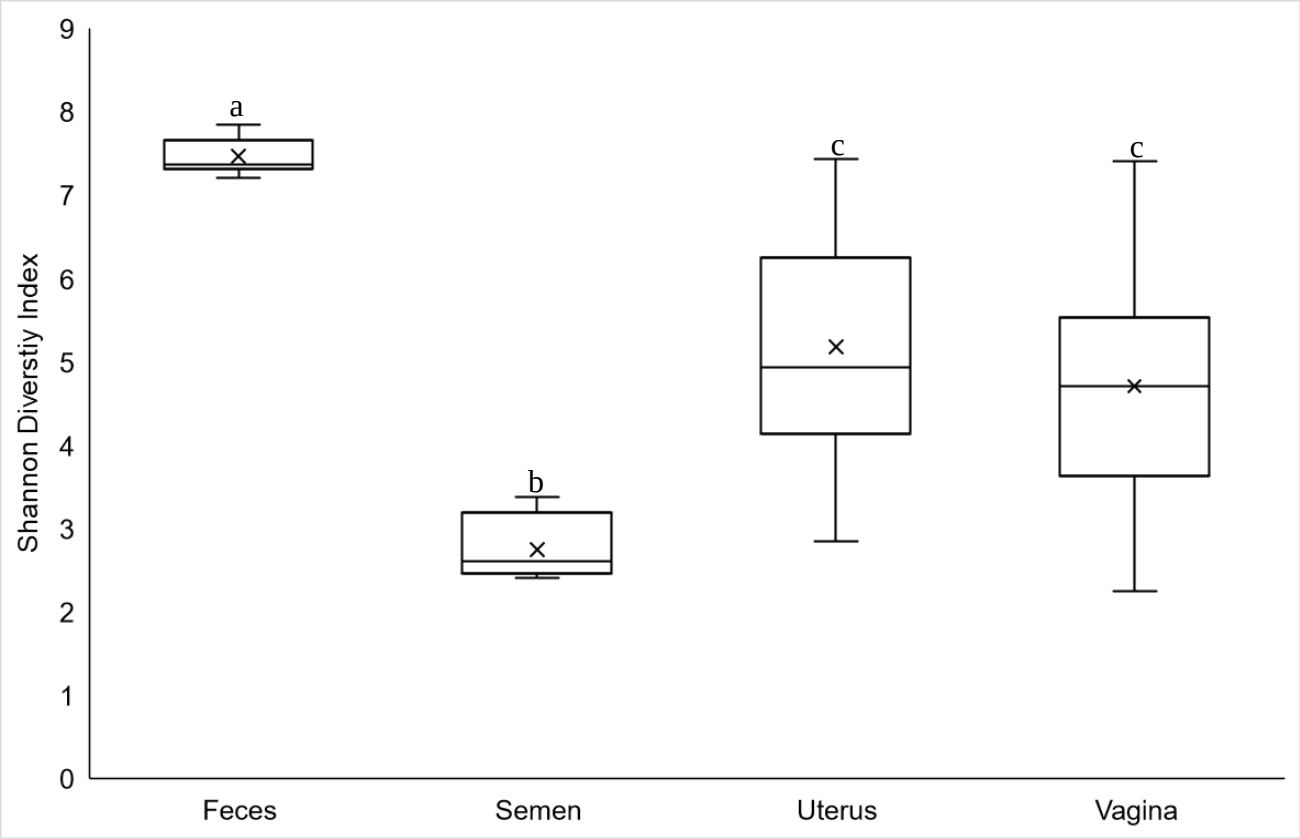

Figure 3.1 Alpha diversity, as measured by the Shannon diversity index, of mare fecal (n=16), uterine (n=35) and vaginal (n=52) samples, and stallion semen (n=4) samples. Fecal and semen samples were collected prior to artificial insemination (AI); uterine and vaginal samples were collected pre and post-AI. No differences were detected between time points or treatments in vaginal or uterine samples, so they were pooled by body site. Boxes represent the interquartile range, the horizontal line within the box is the median, and the x represents the mean. Sampe types lacking a common superscript differ ($^{a,b,c}P<0.01$).

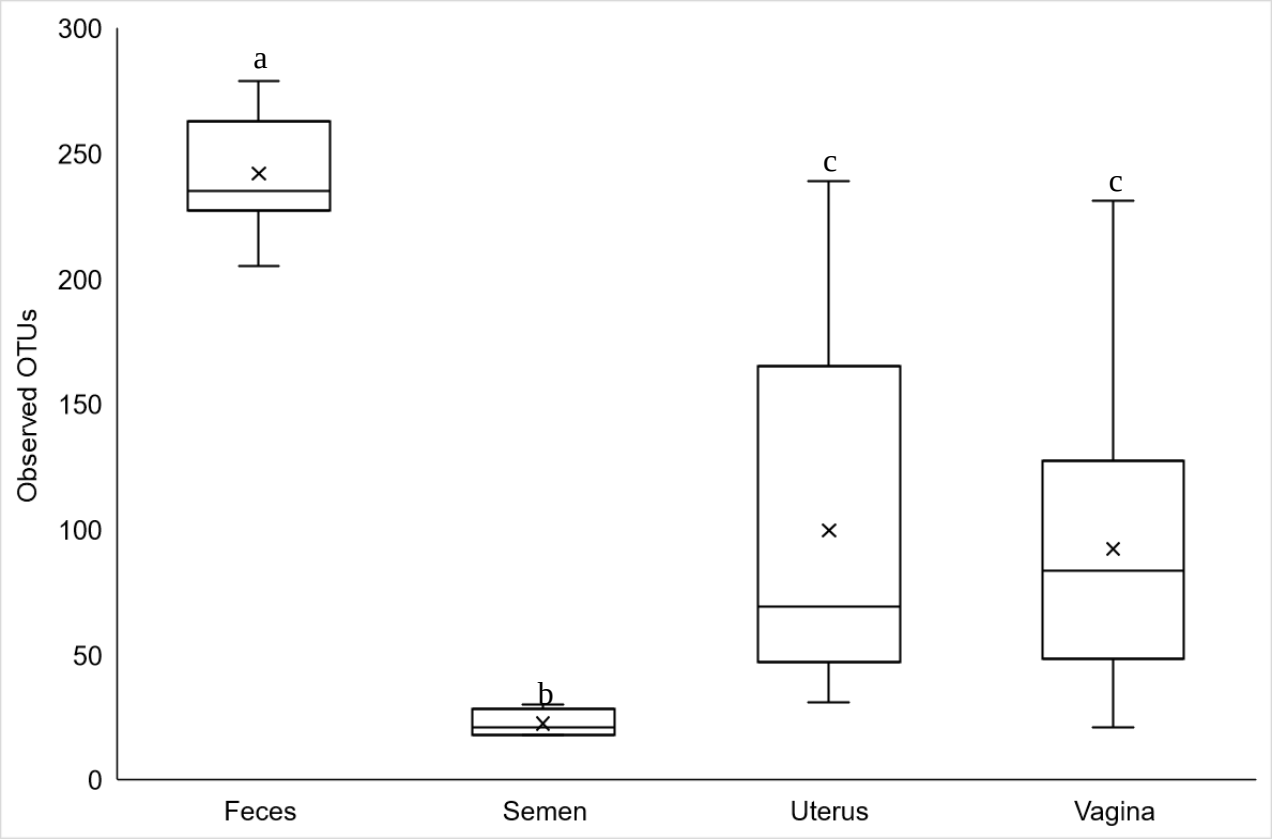

Figure 3.2 Alpha diversity, as measured by observed OTUs, of mare fecal (n=16), uterine (n=35) and vaginal (n=52) samples, and stallion semen samples (n=4). Fecal and semen samples were collected prior to artificial insemination (AI); uterine and vaginal samples were collected before and after AI. No differences were detected between time points or treatments in vaginal or uterine samples, so they were pooled by body site. Boxes represent the interquartile range, the horizontal line within the box is the median, and the x represents the mean. Sample types lacking a common superscript differ ($^{a,b,c}P<0.01$).

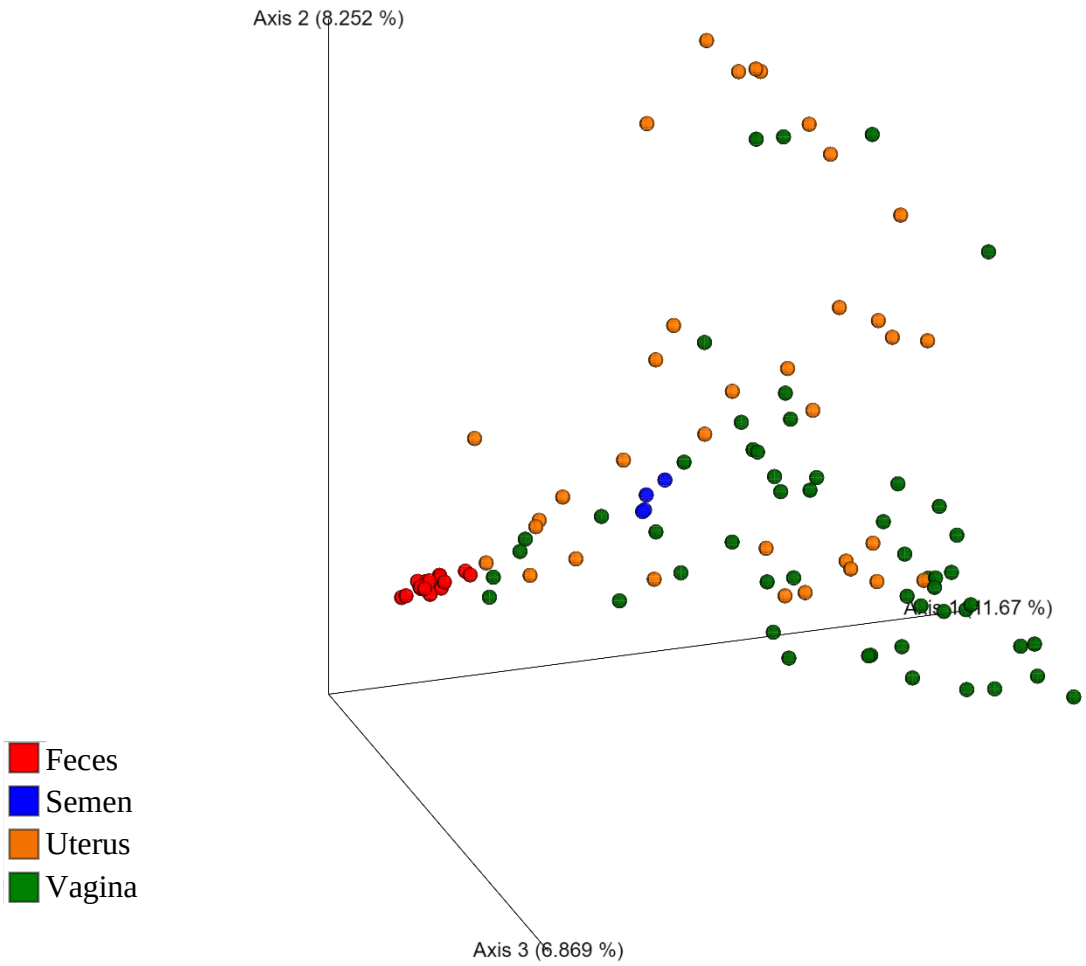

Figure 3.3 Principle coordinates analysis (PCoA) of Bray Curtis dissimilarity representing comparisons of the microbial composition of mare fecal (n=16), uterine (n=35) and vaginal (n=52) samples, and stallion semen samples (n=4). Fecal and semen samples were collected prior to artificial insemination (AI); uterine and vaginal samples were collected before and after AI. Uterine and vaginal samples were pooled according to body site. Points in three-dimensional space represent individual samples, which are colored according to sample type. Percent variance explained by the PCoA is indicated on the axes. There was a significant clustering according to sample type ($P<0.01$).

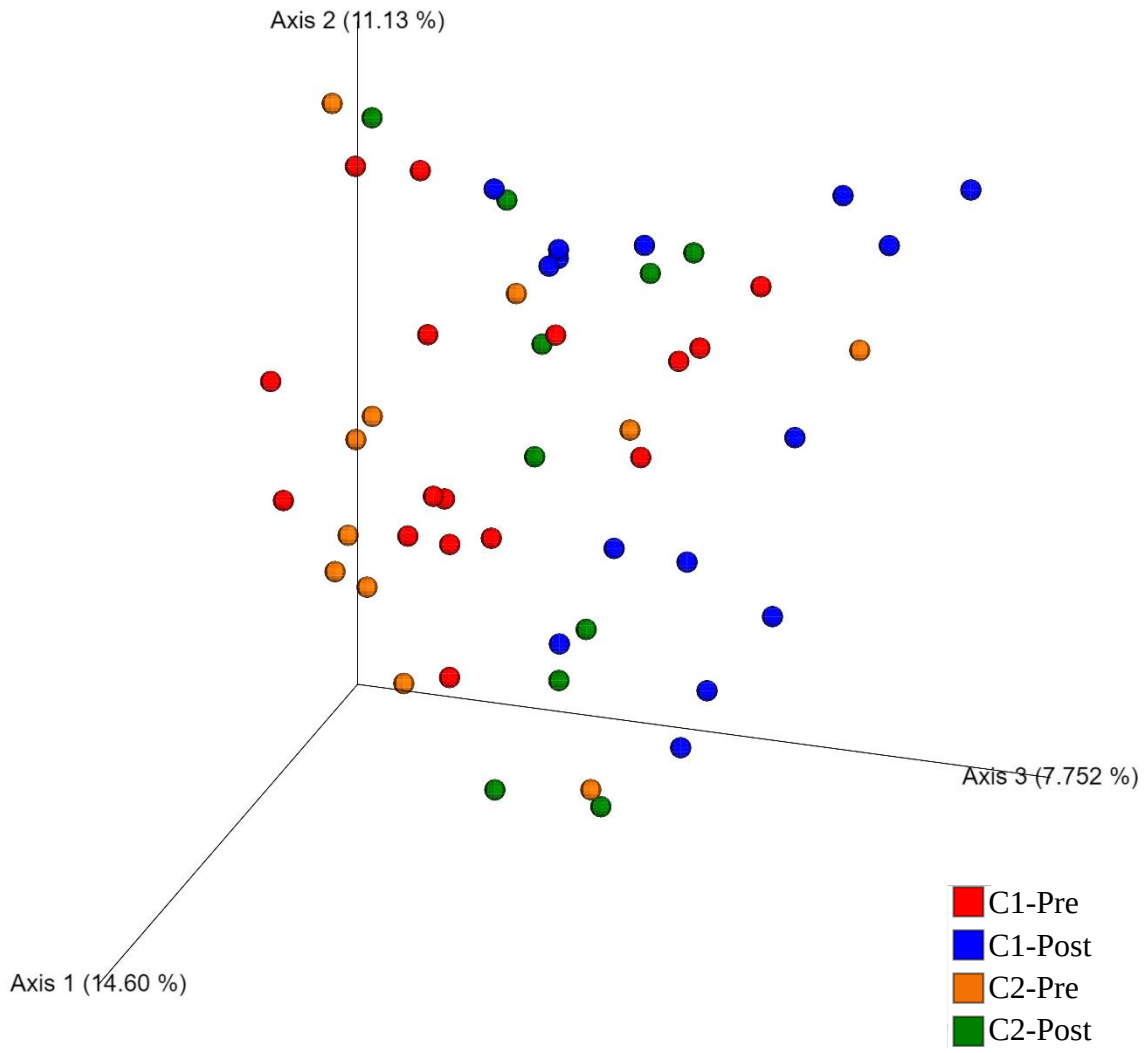

Figure 3.4 Principle coordinates analysis (PCoA) of Bray Curtis dissimilarity representing comparisons of the microbial composition of mare vaginal samples over two consecutive estrous cycles. Points in three-dimensional space represent individual samples, which are colored according to time point: C1-Pre (n=16), C1-Post (n=15), C2-Pre (n=7), C2-Post (n=10) where "C" denotes the first or second estrous cycle, "Pre" indicates samples collected ≤ 24 hours prior to artificial insemination (AI) and "Post" indicates samples collected 48 hours post AI. The percent variance explained by the PCoA is indicated on the axes. There is significant separation between C1-Pre and both C1-Post and C2-Post ($P<0.05$). There is also a significant separation between C2-Pre and both C1-Post and C2-Post ($P<0.05$).

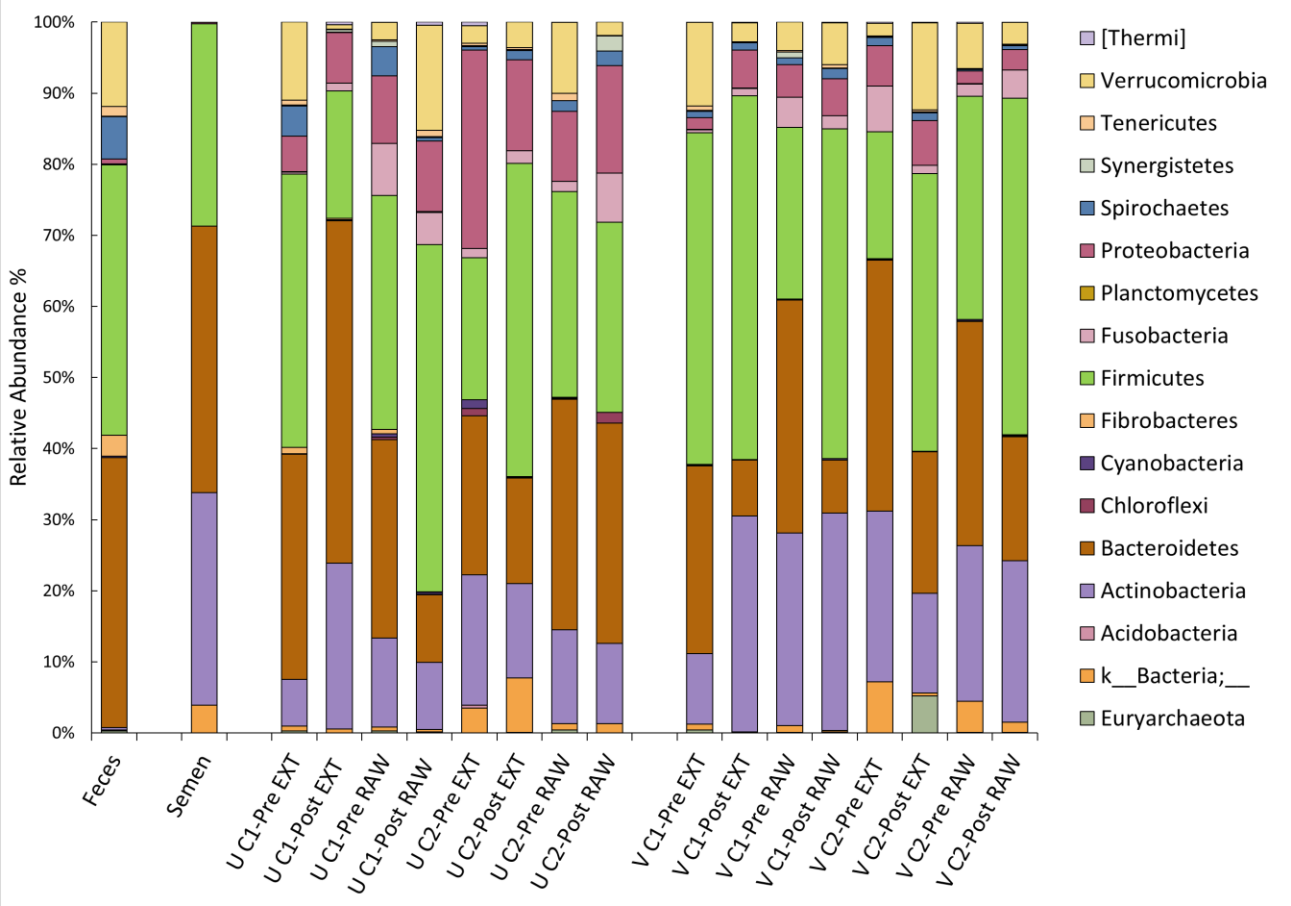

Figure 3.5 Relative abundance at the phylum level of mare fecal (n=16) and stallion semen samples (n=4) collected prior to AI; uterine samples collected from mares bred with extended semen: U C1-Pre/Post EXT (n=8) and U C2-Pre/Post EXT (n=4); uterine samples collected from mares bred with raw semen only: U C1-Pre/Post RAW (n=8) and U C2-Pre/Post RAW (n=7); vaginal samples collected from mares bred with extended semen: V C1-Pre/Post EXT (n=8) and V C2-Pre/Post EXT (n=4); vaginal samples collected from mares bred with raw semen only: V C1-Pre/Post RAW (n=8) and V C2-Pre/Post RAW (n=7). U: Uterus; V: Vagina; C: the first or second estrous cycle; Pre: samples collected ≤ 24 hours prior to artificial insemination (AI); Post: samples collected 48 hours post AI.

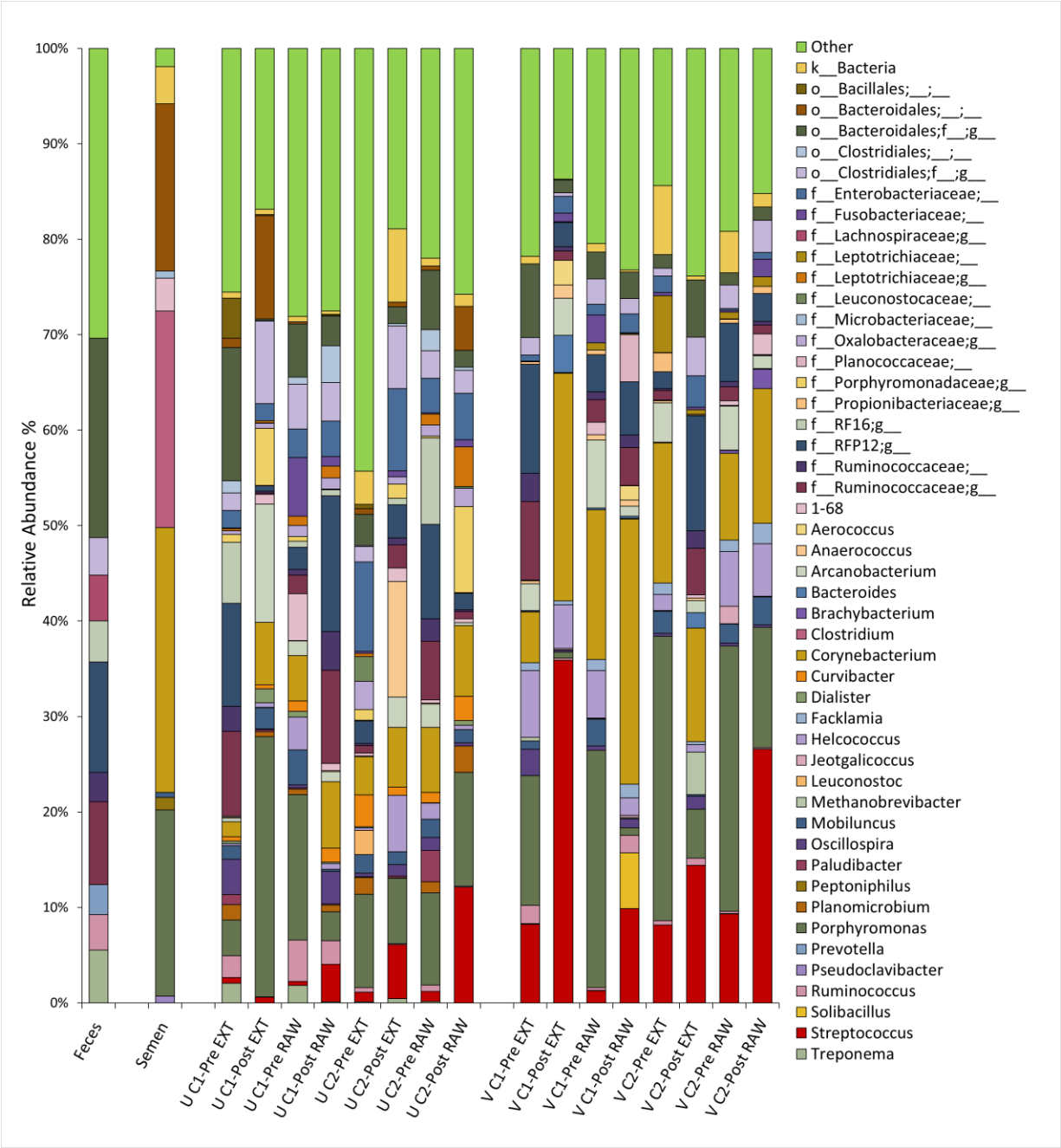

Figure 3.6 The 10 most relatively abundant genera or lowest taxonomic level of mare fecal (n=16) and stallion semen samples (n=4) collected prior to AI; uterine samples collected from mares bred with extended semen: U C1-Pre/Post EXT (n=8) and U C2-Pre/Post EXT (n=4); uterine samples collected from mares bred with raw semen only: U C1-Pre/Post RAW (n=8) and U C2-Pre/Post RAW (n=7); vaginal samples collected from mares bred with extended semen: V C1-Pre/Post EXT (n=8) and V C2-Pre/Post EXT (n=4); vaginal samples collected from mares bred with raw semen only: V C1-Pre/Post RAW (n=8) and V C2-Pre/Post RAW (n=7). U: Uterus; V: Vagina; C: the first or second estrous cycle; Pre: samples collected ≤ 24 hours prior to artificial insemination (AI); Post: samples collected 48 hours post AI. All other genera are

grouped into “Other”. Taxa with k__ represent kingdom as the lowest taxonomic level, o__ represents order, and f__ represents family.

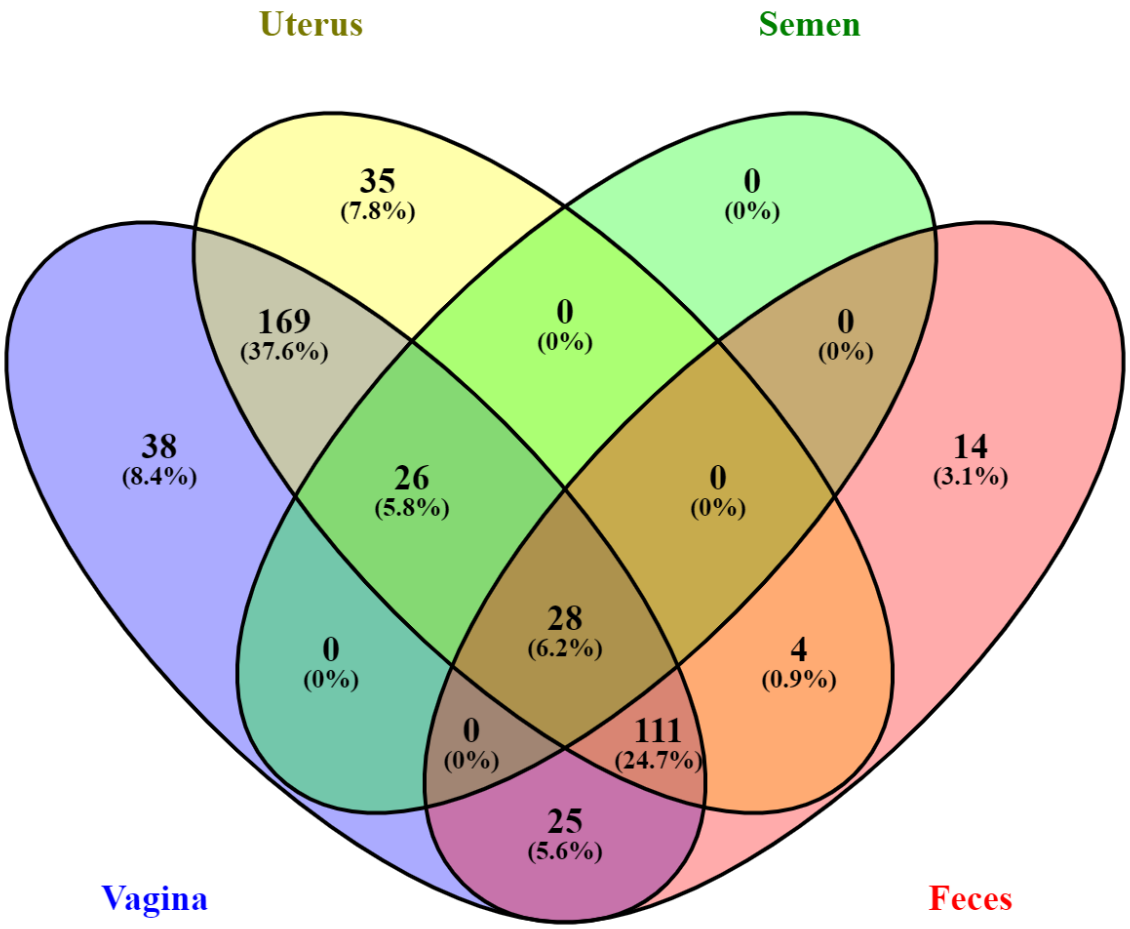

Figure 3.7 Venn diagram of shared genera between mare fecal (n=16), uterine (n=35) and vaginal (n=52) samples, and stallion semen samples (n=4). Fecal and semen samples were collected prior to artificial insemination (AI); uterine and vaginal samples were collected before and after AI. Vaginal and uterine samples were pooled according to body site. Overlapping circles indicate the number of shared genera and percentage of total genera shared between sample types.

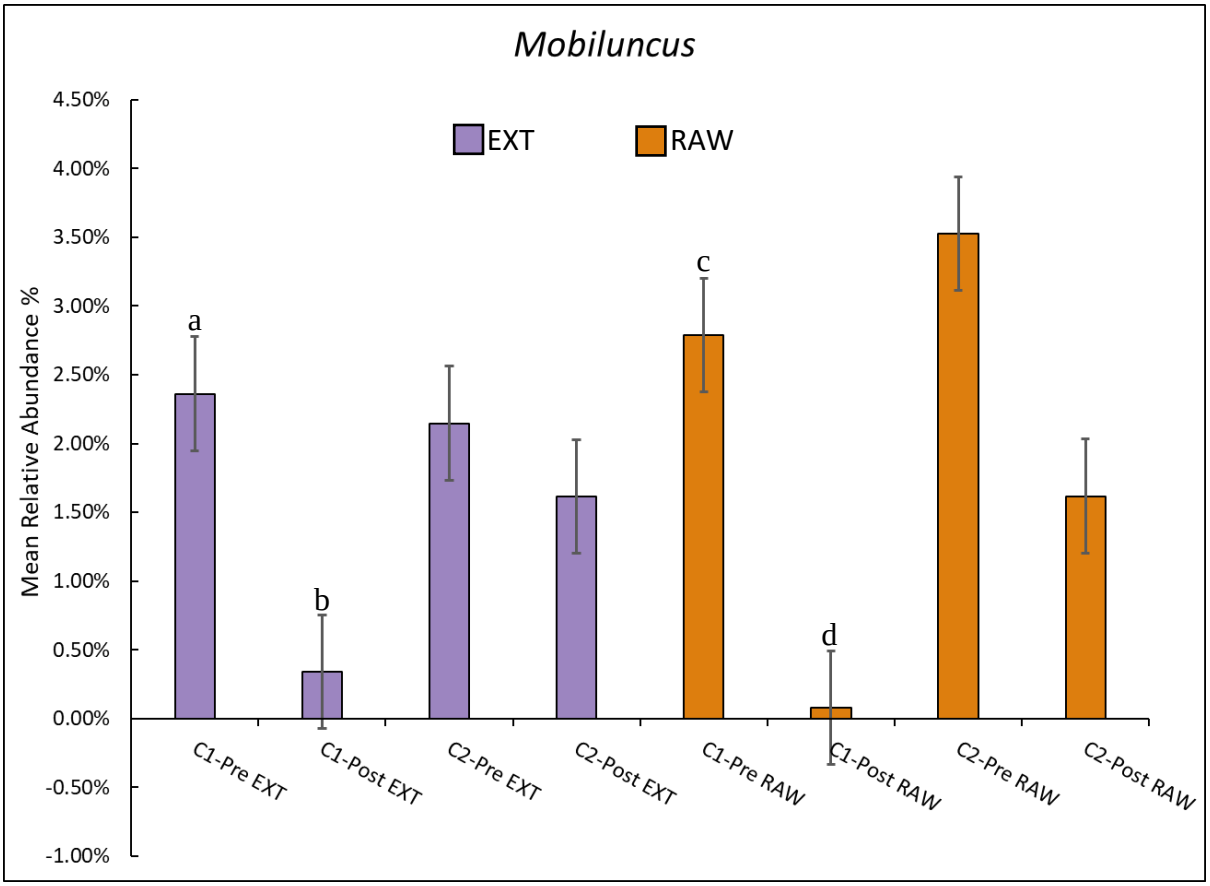

Figure 3.8 Genus that differed between pre-artificial insemination (AI) and post-AI in the uteri of mares bred with extended semen (EXT, n=8) or raw semen only (RAW, n=8) according to longitudinal Analysis of Composition of Microbiomes (ANCOM). C: Cycle; Pre: samples taken ≤ 24 hours prior to artificial insemination (AI); Post: samples taken 48 hours post AI. The genus was differentially abundant at time points within EXT or RAW with different superscripts (EXT:^{a,b}; RAW:^{c,d})

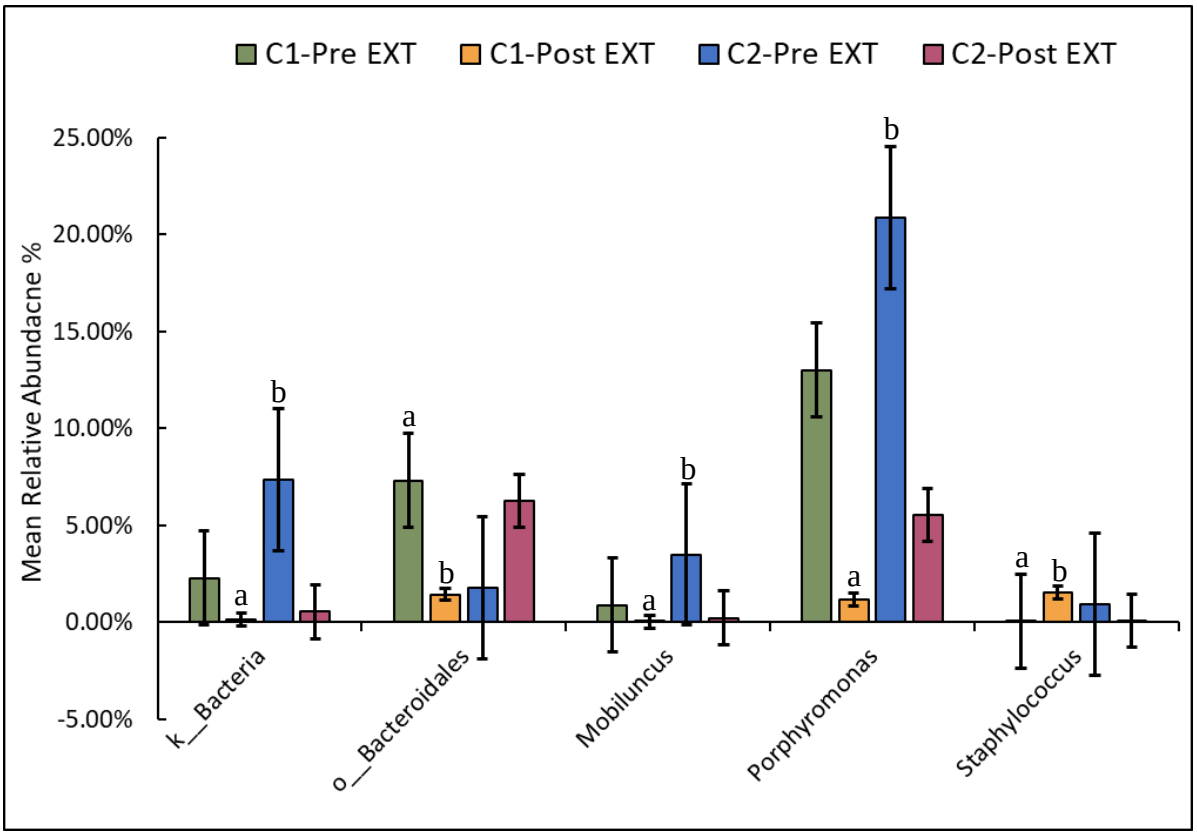

Figure 3.9 Genera or lowest taxonomic level that differ in the vagina of mares bred with extended semen (EXT) across different time points over the course of two estrous cycles according to longitudinal Analysis of Composition of Microbiomes (ANCOM). C1: cycle 1 (n=8); C2: cycle 2 (n=4); Pre: samples taken ≤ 24 hours prior to artificial insemination (AI); Post: samples taken 48 hours post AI. Titles of taxa with k__ represents kingdom. Only taxa with mean relative abundance >1% in at least one time point were included in this figure. Error bars represent SEM. Time points within individual taxa with different superscripts are differentially abundant (^{a,b}).

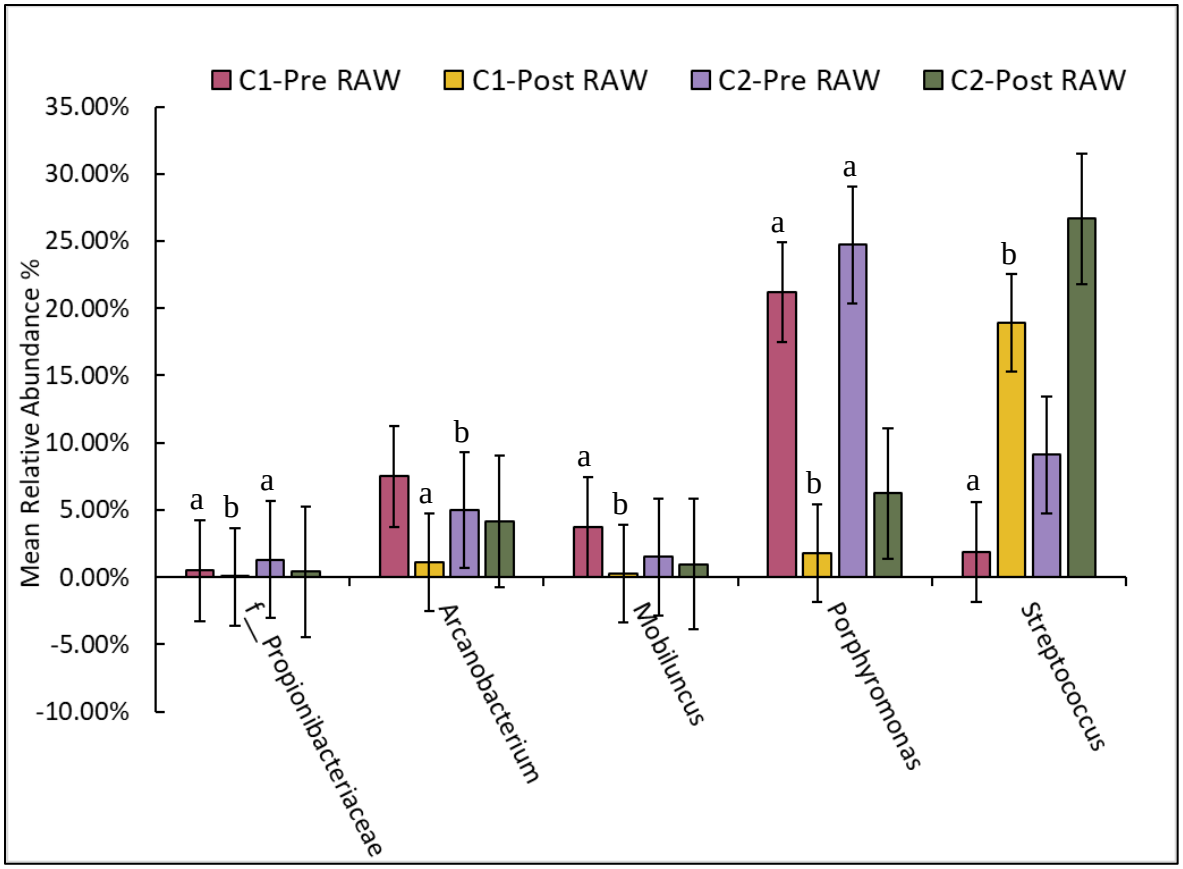

Figure 3.10 Genera or lowest taxonomic level that differ in the vagina of mares bred with raw semen only (RAW) across different time points over the course of two estrous cycles according to longitudinal Analysis of Composition of Microbiomes (ANCOM). C1: cycle 1 (n=8); C2: cycle 2 (n=7); Pre: samples taken ≤ 24 hours prior to artificial insemination (AI); Post: samples taken 48 hours post AI. Titles of taxa with f__ represents family. Time points within individual taxa with different superscripts are differentially abundant (^{a,b}). Only taxa with mean relative abundance >1% in at least one time point were included in this figure. Error bars represent SEM.

Table 3.1 Summary of Analysis of Composition of Microbiomes (ANCOM) results of differential abundance, using phylum as the lowest taxonomic level, contrasting mare fecal (n=16), uterine (n=35) and vaginal (n=52) samples. Fecal were collected prior to artificial insemination (AI); uterine and vaginal samples were pooled according to body site and include samples collected pre/post AI in the first and second estrous cycle. All detected phyla were differentially abundant.

Phylum[a]	Feces[b]	Uterus[b]	Vagina[b]
k__Bacteria;__	0.14 ± 0.11	**1.18 ± 2.42***	**1.70 ± 3.77***
Actinobacteria	0.34 ± 0.13	**18.11 ± 17.53***	**23.76 ± 15.96***
Bacteroidetes	38.02 ± 3.81	**20.25 ± 18.27***	**19.92 ± 14.25***
Fibrobacteres	2.98 ± 1.49	**0.19 ± 0.61***	**0.06 ± 0.13***
Firmicutes	37.97 ± 4.27	**26.18 ± 21.16***	**35.44 ± 17.50***
Fusobacteria	0 ± 0	**2.89 ± 4.79***	**2.20 ± 3.13***
Proteobacteria	0.66 ± 0.32	**21.60 ± 23.71***	**8.55 ± 11.33***
Spirochaetes	6.16 ± 3.24	**3.42 ± 6.74***	**1.78 ± 2.92***
Tenericutes	1.38 ± 0.63	**0.46 ± 1.29***	**0.24 ± 0.44***
Verrucomicrobia	11.46 ± 4.45	**4.09 ± 6.18***	**5.35 ± 7.65***

[a]Only taxa with mean relative abundance >1% in at least one sample type were included
[b]Percent mean relative abundance ± standard deviation
*Denotes taxa that were found to be differentially abundant from mare feces

Table 3.2 Summary of Analysis of Composition of Microbiomes (ANCOM) results of differential abundance, using phylum as the lowest taxonomic level, contrasting semen and the post-AI vaginal and uterine samples of mares bred with extended semen in cycle 1 (C1-Post EXT, n=8), bred with raw semen in cycle 1 (C1-Post RAW, n=8), bred with extended semen in cycle 2 (C2-Post EXT, n=4) and bred with raw semen in cycle 2 (C2-Post RAW, n=7).

	Semen[b]	Uterus[b]				Vagina[b]			
Phylum[a]		C1-Post EXT	C2-Post EXT	C1-Post RAW	C2-Post RAW	C1-Post EXT	C2-Post EXT	C1-Post RAW	C2-Post RAW
k__Bacteria;__	3.87 ± 0.86	**0.13 ± 0.24***	3.66 ± 5.59	**9.28 ± 6.77***	**28.47 ± 34.83***	**9.04 ± 8.02***	**22.61 ± 11.01***	**10.06 ± 5.36***	**11.11 ± 6.90***
Actinobacteria	33.94 ± 21.38	**28.80 ± 23.73***	**20.38 ± 21.21***	**20.49 ± 24.66***	**11.45 ± 8.04***	**26.26 ± 11.64***	**13.12 ± 18.42***	**25.24 ± 21.58***	26.90 ± 12.54
Bacteroidetes	35.48 ± 13.23	**12.11 ± 19.22***	**19.37 ± 6.79***	**38.79 ± 21.46***	**23.73 ± 24.41***	**43.98 ± 23.90***	37.95 ± 7.10	**40.30 ± 18.30***	**42.78 ± 10.87***
Firmicutes	26.45 ± 21.14	**29.75 ± 31.32***	37.97 ± 24.58	**0.14 ± 0.36***	**0.72 ± 0.93***	**0.13 ± 0.23***	**0.55 ± 0.71***	**0.16 ± 0.17***	**0.57 ± 0.93***
Spirochaetes	0 ± 0	**0.26 ± 0.59***	**0.84 ± 1.05***	**8.54 ± 8.20***	**1.23 ± 1.21***	**2.79 ± 3.14***	**11.25 ± 12.29***	**5.95 ± 6.96***	**2.91 ± 2.30***
Verrucomicrobia	0 ± 0	**6.38 ± 10.54***	**1.58 ± 2.76***	**1.98 ± 3.25***	**2.25 ± 3.04***	**2.13 ± 2.76***	**1.07 ± 0.24***	**2.33 ± 2.37***	**3.15 ± 6.10***

[a]Only taxa with mean relative abundance >1% in at least one sample type were included

[b]Percent mean relative abundance ± standard deviation

*Denote taxa that were found to be significantly different from semen

Table 3.3 Summary of Analysis of Composition of Microbiomes (ANCOM) results of differential abundance, using genus as the lowest taxonomic level, contrasting semen and the post-AI uterine samples of mares bred with extended semen in cycle 1 (C1-Post EXT, n=8), bred with raw semen in cycle 1 (C1-Post RAW, n=8), bred with extended semen in cycle 2 (C2-Post EXT, n=4) and bred with raw semen in cycle 2 (C2-Post RAW, n=7).

	Semen[b]	Uterus[b]			
Taxa[a]		C1-Post EXT	C1-Post RAW	C2-Post EXT	C2-Post RAW
k__Bacteria; Unclassified	3.87 ± 0.86	**0.13 ± 0.24***	**0.14 ± 0.36***	**3.66 ± 5.59***	**0.72 ± 0.93***
o__Bacteroidales	15.24 ± 15.71	**1.69 ± 4.78***	**0.01 ± 0.02***	**0.53 ± 0.81***	**1.63 ± 2.69***
1-68	3.57 ± 3.11	**0.16 ± 0.44***	**0.36 ± 1.01***	**0.83 ± 0.78***	**0.32 ± 0.64***
Clostridium	20.40 ± 18.49	**0.09 ± 0.23***	**0.35 ± 0.55***	**3.62 ± 7.11***	**0.57 ± 0.79***
Corynebacterium	31.65 ± 22.84	**21.96 ± 24.12***	**16.93 ± 23.48***	**14.29 ± 19.17***	**7.26 ± 4.93***
Helcococcus	0 ± 0	1.99 ± 4.84	**0.85 ± 1.69***	**10.67 ± 12.29***	0.26 ± 0.35
Mobiluncus	0.45 ± 0.43	**0.34 ± 0.96***	**0.08 ± 0.15***	1.85 ± 2.68	**1.62 ± 3.64***
Peptoniphilus	1.40 ± 0.78	**0.10 ± 0.28***	**0.05 ± 0.09***	**0.74 ± 0.94***	**0.51 ± 0.79***
Porphyromonas	19.76 ± 21.93	**5.11 ± 11.89***	**1.9 ± 3.55***	**7.26 ± 7.69***	**7.25 ± 6.91***

[a]Only taxa with mean relative abundance >1% in at least one sample type were included
[b]Percent mean relative abundance ± standard deviation
*Denote taxa that were found to be significantly different from semen

Table 3.4 Summary of Analysis of Composition of Microbiomes (ANCOM) results of differential abundance, using genus as the lowest taxonomic level, contrasting semen and the post-AI vaginal samples of mares bred with extended semen in cycle 1 (C1-Post EXT, n=8), bred with raw semen in cycle 1 (C1-Post RAW, n=8), bred with extended semen in cycle 2 (C2-Post EXT, n=4) and bred with raw semen in cycle 2 (C2-Post RAW, n=7).

	Semen[b]	Vagina[b]			
Taxa[a]		C1-Post EXT	C2-Post EXT	C1-Post RAW	C2-Post RAW
k__Bacteria	3.87 ± 0.86	**0.13 ± 0.23***	**0.55 ± 0.71***	**0.16 ± 0.17***	**0.57 ± 0.93***
o__Bacteroidales	15.24 ± 15.71	**0.004 ± 0.01***	**2.59 ± 5.0***	**0.24 ± 0.42***	**0.32 ± 0.54***
o__Bacteroidales.f__g__	0.01 ± 0.001	1.27 ± 1.47	**6.26 ± 6.83***	2.38 ± 2.70	1.03 ± 1.19
f__Lachnospiraceae	0 ± 0	0.22 ± 0.23	**1.13 ± 1.14***	0.75 ± 0.95	0.29 ± 0.31
f__RFP12	0.004 ± 0.01	**2.58 ± 2.96***	**11.1 ± 12.19***	**5.66 ± 6.54***	**2.69 ± 2.03***
f__Ruminococcaceae	0.001 ± 0.003	0.48 ± 0.42	**1.26 ± 1.57***	1.06 ± 0.98	0.36 ± 0.61
f__Ruminococcaceae	0.001 ± 0.002	0.97 ± 0.95	**3.05 ± 4.63***	3.09 ± 3.78	1.12 ± 1.31
1-68	3.57 ± 3.11	**0 ± 0***	**0.51 ± 0.93***	**0.014 ± 0.04***	**0.71 ± 1.63***
Arcanobacterium	0 ± 0	2.41 ± 3.21	**1.49 ± 1.28***	**1.14 ± 0.79***	4.15 ± 7.34
Bacteroides	0.002 ± 0.003	2.74 ± 7.34	**2.43 ± 4.01***	0.08 ± 0.18	0.07 ± 0.07
Clostridium	20.40 ± 18.49	**0.21 ± 0.26***	**0.19 ± 0.22***	0.46 ± 0.44	0.24 ± 0.22
Corynebacterium	31.65 ± 22.84	**20.68 ± 9.24***	**10.75 ± 18.48***	**22.71 ± 20.61***	**13.03 ± 10.43***
Helcococcus	0 ± 0	2.82 ± 4.22	**1.24 ± 2.14***	1.36 ± 2.19	3.09 ± 4.30
Leptospira	0 ± 0	**2.06 ± 2.78***	**0.76 ± 0.16***	2.20 ± 2.42	**3.13 ± 6.11***
Peptoniphilus	1.4 ± 0.78	**0.10 ± 0.14***	**0.28 ± 0.48***	**0.07 ± 0.15***	**0.10 ± 0.20***
Porphyromonas	19.76 ± 21. 93	**1.07 ± 1.24***	**5.53 ± 6.51***	**1.80 ± 3.63***	6.25 ± 7.23
Streptococcus	0.001 ± 0.002	**30.61 ± 24.80***	**16.00 ± 15.77***	**18.95 ± 20.43***	**26.68 ± 12.22***

[a]Only taxa with mean relative abundance greater than 1% in at least one sample type were included

[b]Percent mean relative abundance ± standard deviation

*Denote taxa that were found to be significantly different from semen

Discussion

As a first objective, the microbial composition and diversity of stallion semen and fecal, uterine and vaginal samples from the healthy mares were analyzed and compared. The dominant phyla of mare feces were Bacteroidetes and Firmicutes, followed by Verrucomicrobia and Spirochaetes. With genus as the lowest taxonomic level, the dominant taxa were Bacteroidales, RFP12, Ruminococcaceae, *Treponema*, Lachnospiraceae and *Prevotella.* Composition of mare feces was similar to previous work done in our lab as well as other studies that have investigated the adult equine gut microbiome [19,29,30].

Although only one animal is represented, to our knowledge this is the first study to characterize the microbial composition of equine semen using NGS. The main phyla in this stallion's semen were Actinobacteria, Bacteroidetes and Firmicutes. The dominant taxa in semen with genus as the lowest level were *Corynebacterium, Clostridium, Porphyromonas,* and Bacteroidales. *Corynebacterium* are aerobic Gram-positive bacteria; they have frequently been isolated from the semen and the genital tract of men using NGS as well as from stallions using culture-based methods and are generally considered to be commensal [8,31,32]. *Clostridium*, which is typically thought to be pathogenic, has been cultured from equine semen however it is more frequently found in feces, indicating that the high abundance found here may be due to a contamination source rather than being inherently present in semen [11,29,33]. The same rationale could be applied to Bacteroidales, which are also more typically isolated from equine feces and have not been cultured from equine semen previously [30]. *Porphyromonas* are obligate anaerobes that have also been detected in semen of men using NGS and stallions using culture-based methods, as well as the vagina and uterus of several species [32,34–36]. A more

in-depth analysis of the semen microbiome utilizing a greater number of stallions and time points throughout the breeding season is necessary to confirm these preliminary findings.

Microbes were detected in all the uterine and vaginal samples. The dominant phyla in the uterus and vagina were Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia. In the uterus *Corynebacterium* was the dominant genus followed by *Porphyromonas,* Enterobacteriaceae, and *Streptococcus. Corynebacterium* was also the dominant genus in the vagina, followed by *Streptococcus, Porphyromonas,* and RFP12. *Corynebacterium* has been previously cultured from the equine uterus and was dominant in the uteri of lactating dairy cows using NGS [37,38]. *Porphyromonas* and *Streptococcus* have also been isolated from the uterus and vagina of dairy cattle, although they were not the most dominant genera [36]. As expected, due to the close proximity and anatomical configuration of the anus and vagina, common fecal microbes, Enterobacteriaceae and RFP12, were also found in the vagina. *Streptococcus* was found in the vagina of all mares and only absent from the uterus in 2 mares. The dominance of *Streptococcus* within both the uterus and vagina was surprising due to the prevalence of *Streptococcus* in mares diagnosed with endometritis; however, none of these mares displayed any symptoms of endometritis and all became pregnant following the first AI after completion of this study [39,40]. One explanation for the presence of these pathogens could be that because NGS is not capable of differentiating between live and dead bacteria, the mares' immune system was able to neutralize them, but their DNA remained in the uterus/vagina.

The microbiome of the mare vagina has not, to our knowledge, been investigated previously using NGS. Culture-based studies have also detected potentially pathogenic microbes, such as *Streptococcus zooepidemicus* and *Escherichia coli,* in vaginal vestibule and clitoral fossa swabs of healthy mares with no reproductive issues [4]. *Lactobacillus* has been detected in the

mare vagina using culture techniques; but growth counts were lower than humans and similar to cattle [41]. Several human studies have demonstrated the dominance and importance of *Lactobacillus spp.* in the vagina and uterus of healthy women [42], however in the present study the genus *Lactobacillus* was present at a very low abundance in the mare uterus and vagina, at 0.02% and 0.01% respectively. As suggested by Fraga et al. [43], the lower abundance of *Lactobacillus* could be related to pH, as the mare has a much higher vaginal pH (6-8) compared to women (3-5).

In terms of the uterine microbiome, our results are consistent with previous work that found bacteria in the uterus of mares with positive and negative culture results [17], however they are in contrast to another recent study that detected bacteria in only 30% of mare uterine lavage samples using NGS [44]. Many of the dominant taxa isolated from the uteri in this study have also been detected in the uterus of healthy mares, and mares with endometritis, using culture-based techniques [5,38,39].

This is the first time, to our knowledge, that the microbiome of the equine uterus, vagina, semen and feces have been compared. We found feces to be the most diverse while semen was the least diverse with no difference in diversity between the uterus and vagina. For beta diversity, each sample type clustered independently from all others. These results are consistent with work in dairy cattle that found feces to be more diverse than the uterus and clustering based on sample type for cow feces, uterus, and vagina in terms of Bray-Curtis dissimilarity [36]. In contrast to our findings, human semen is more diverse, with greater observed species, compared to the vagina; however, authors did not compare human semen to the uterus or feces and did not make any comparisons using beta diversity as we did in this study [45]. Only 28 genera were shared between all 4 sample types including the dominant genera of the uterus/vagina:

Corynebacterium, *Streptococcus*, and *Porphyromonas*. The most genera were shared between the uterus and vagina at 334 total genera shared. Of note, the uterus and vagina shared the same 54 genera with semen. Feces and semen shared the least genera with 28. Differential abundance analysis determined all phyla and genera tested to be differentially abundant between mare feces and the uterus; the same result was found when feces were compared to the vagina. This result is not unexpected due to the clear separation between feces, uterus and vagina in the PCoA plot of Bray-Curtis dissimilarity, which indicated that each sample type has a unique microbiome.

As a second objective we sought to investigate the effects of breeding healthy, PBIE-resistant mares with either raw or extended semen over the course of 2 consecutive estrous cycles on the microbial composition of the reproductive tract. This is an area of importance in the equine industry as bacteria and debris introduced into the mare uterus during breeding have been associated with PBIE; which has a major impact on fertility in mares [3]. Breeding, through artificial insemination or natural service, induces an inflammatory response and uterine contractions which clear bacteria and excess semen from the uterus. Inflammation is typically resolved in 48 hours in most mares and has no adverse effects on pregnancy establishment [3]. The efficacy of various antibiotics on reduction of the bacterial content of semen, and thus a reduction of bacterial contamination of the uterus, has been thoroughly investigated [10–14]. The most common antibiotics added to stallion semen extender are beta-lactams (potassium penicillin G), which target Gram positive organisms including *Streptococcus*, and aminoglycosides (amikacin or gentamicin), which target both Gram positive and negative bacteria and are particularity potent against the family Enterobacteriaceae but are generally ineffective against *Streptococcus* and *Enterococcus* [14,46,47]. The extender utilized in this study, INRA 96,

contains penicillin and gentamicin. To our knowledge, this is the first time NGS has been utilized to compare the effects of raw or extended semen on the mare uterus or vagina.

We found no differences in alpha diversity or beta diversity between treatments or time points in the uterus. This is supported by the results for differentially abundant taxa using ANCOM, where uterine and vaginal samples from EXT and RAW were compared pre-AI and post-AI over the two estrous cycles. Only one genus and one phylum were found to be differentially abundant in the uterus of either treatment between pre and post-AI in the first cycle, none changed between the first and second cycle and none changed between pre and post-AI in the second cycle. *Mobiluncus* decreased in the uterus of EXT and RAW post-AI in cycle 1, and Bacteroidetes decreased in the uterus of RAW post-AI in cycle 1. *Mobiluncus* is interesting as it is typically associated with bacterial vaginosis in women, however to our knowledge, it has not been isolated from the equine uterus using NGS [48,49]. Although there was not a significant increase in *Mobiluncus* between post-AI of cycle 1 and pre-AI of cycle 2, it does appear that *Mobiluncus* returns to pre-AI levels in both EXT and RAW mares in the pre-AI samples of the second cycle. The lack of significant change could be due to the reduced number of mares utilized in the second estrous cycle. Overall there were only 4 phyla and 9 genera that were differentially abundant between the uterus and semen (excluding differentially abundant taxa that were present at <1% abundance in both sample types), and the majority were more abundant in semen. This might suggest that the uterus and semen share a similar core microbiome, which could possibly explain the lack of change seen post-breeding in these mares.

The overall stability of the mare uterine microbiome post-AI is not altogether surprising considering the inflammatory response that is initiated post-breeding is typically resolved by 48 hours post-breeding in clinically normal mares [3]. Previous work using culture-based techniques

and mares resistant to PBIE has demonstrated that breeding with natural service does not result in increased bacterial contamination or inflammatory reaction in samples collected 48 hours post-breeding compared with those bred using extended semen via AI; which is consistent with our findings [2,50]. In contrast, Bollwein et al. [15] found that insemination with semen and antibiotic-containing extender resulted in less severe bacterial contamination of the uterus at 48 hours post-AI compared to insemination with raw semen using culture techniques. It is worth noting however, that only 6 mares were utilized in that study and the significance level of their claim was not stated. Our results indicate the healthy mare uterus contains a core microbiome that is stabilized, perhaps by the immune response to breeding, by 48 hours post-AI and the use of extender with semen does not result in a significant difference in microbial community composition compared to insemination with raw semen. Since our results are limited to samples that were collected 48 hours post-AI, it is unclear if a more significant, but temporary, shift from the pre-AI microbial composition may have occurred at some point before 48 hours. There may also be more significant changes in the uterine microbiome following repeated inseminations throughout a breeding season or with breeding to different stallions. Both areas warrant further investigation in order to determine the effects of breeding on the mare uterine microbiome.

In the vagina, ANCOM revealed several differentially abundant taxa in both treatments between pre-AI and post-AI for cycle 1, and between post-AI cycle 1 and pre-AI cycle 2. There were no differentially abundant taxa between pre-AI and post-AI in cycle 2. In the vagina of EXT mares, *Phascolarctobacterium* and Bacteroidales decreased post AI in cycle 1 while *Staphylococcus*, *Cupriavidus*, *Comamonas*, and *Caulobacter* increased post-AI. In the vagina of RAW mares *Mobiluncus*, *Porphyromonas* and Propionibacteriaceae decreased while *Streptococcus* and *Comamonas* increased post-AI in cycle 1. *Phascolarctobacterium*,

Bacteroidales, and Propionibacteriaceae are all gut microbes and their decrease post-AI is most likely due to the immune response associated with breeding or through natural expulsion from the vagina [3,30,51,52]. Of the bacteria that increased in the vagina, *Cupriavidus*, *Comamonas*, and *Caulobacter* are all Gram-negative bacteria that are typically found in environmental samples [53–55]. Given their low relative abundance in the vagina (<3.5%) these are all possible contaminants introduced during the breeding process. Also, these bacteria did not increase in the uterus post-AI and were present at very low abundance in the uterus (<1.5%). *Staphylococcus*, which is typically considered a pathogen as it has been associated with pneumonia, metritis, and blood infections in horses, and *Streptococcus*, which is a known reproductive tract pathogen, increased post-AI in the vagina of EXT and RAW mares respectively [56]. However, *Staphylococcus* was present at only 1.52% mean relative abundance in the post-AI EXT mares. *Streptococcus* is of more concern as it was present at approximately 19% mean relative abundance in the post-AI RAW mares. *Staphylococcus* species have become increasingly resistant to antimicrobials, including penicillin; however gentamicin is considered to have good activity against *Staphylococcus* [47,56]. The opposite case is true for *Streptococcus*, in that penicillin is generally effective against these microbes while gentamicin is not [47]. The extender utilized in this study, INRA 96, contains both penicillin and gentamicin and would have encountered the vaginal microbes as the semen/extender was removed from the uterus through contractions. Perhaps the antibiotic dosage was not sufficient to inhibit growth of *Streptococcus* or *Staphylococcus* in the vagina post-AI, or both antibiotics reduced the abundance of other inhibitive bacteria, allowing for the growth of these pathogens. Another explanation could be that the antibiotics were successful at eliminating an influx or resident population of these pathogens, but bacterial DNA/debris remained in the vagina. In RAW mares, both *Mobiluncus*

and *Porphyromonas* decreased post-AI. As discussed above, *Mobiluncus* has been associated with bacterial vaginosis in women and *Porphyromonas* is part of the core semen, uterine, and vaginal microbiomes. The decrease in these genera could also be attributed to the inflammatory response to breeding, however it is surprising that *Porphyromonas* decreased post-AI considering its high abundance in semen.

When post-AI vaginal samples of cycle 1 were compared to the pre-AI samples of cycle 2 there were differentially abundant taxa in each treatment. Interestingly, *Porphyromonas* returned to the pre-AI level seen in cycle 1 for RAW mares, which would indicate that these bacteria are able to recover from losses, possibly due to the inflammatory response to breeding, indicating their possible necessity to the core vaginal microbiome. In the vagina of EXT mares, *Mobiluncus* and *Porphyromonas* increased between the two cycles, again indicating that there may be some physiological control mechanism that attempts to maintain a stable microbial population within the vagina.

Lastly, the vaginal and seminal microbiomes were compared. One kingdom, 5 phyla, and 17 genera were differentially abundant between the vagina and semen. Typical gut microbes, Bacteroidales, Lachnospiraceae, RFP12, Ruminococcaceae, *Bacteroides*, and *Clostridium* varied between being more abundant in the semen or vagina, indicating that certain gut microbes, such as RFP12 and Ruminococcaceae may be more easily translocated to the vagina than to the external genitalia/semen of stallions. As expected, certain microbes that are more typically associated with semen, 1-68 (from the urine microbiome), *Corynebacterium*, *Peptoniphilus*, and *Prophyromonas,* were more abundant in semen than the vagina [7,57–59]. Known reproductive tract pathogens, *Arcanobacterium*, *Leptospira*, and *Streptococcus,* were all more abundant in the vagina than semen, but with no clear differentiation between EXT or RAW mares and with no

apparent clinical symptoms, indicating that they had little to no impact on the reproductive efficiency of these mares [60–62]. The relatively low number of differentially abundant taxa would indicate that like the uterus, the core microbiomes of semen and the vagina are similar. This is supported by work done in humans that found couples to have highly similar seminovaginal microbiomes with 85% shared phylotypes [45]. Given our results and previous studies there may be a physiological or immunological system responsible for maintaining the uterine, vaginal and seminal microbiome.

Conclusion

This study confirmed the presence of microbes within the uterus, vagina, feces and semen of equines using NGS. There was a distinct microbial composition when fecal and seminal samples were compared to uterine and vaginal samples. We demonstrated for the first time the impact of breeding with raw or extended semen on the uterine and vaginal microbiomes. The uterine microbiome was fairly stable across timepoints and between treatments, indicating that the uterus is able to effectively stabilize the microbial population by 48 hours post-AI despite introduction of foreign bacteria within semen and antibiotics present in extender. The vaginal microbiome is more dynamic and susceptible to fluctuations compared to the uterus as it was less stable across time points and treatments. Lastly, there was a high level of similarity between the seminal microbiome and that of both the uterus and vagina, indicating that there may be similar control mechanisms within the mare and stallion to recognize commensal bacteria within the reproductive tract or it could simply be due to the similar environment that the mares and stallion were exposed to. Further research is necessary to determine the specific roles of bacteria that make up the core microbiome of the uterus, vagina, and semen of equines and the physiological mechanisms that maintain the stable microbial composition at these sites.

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