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Exploring the vaginal microbiome in relation to pregnancy status and reproductive performance in Brangus heifers

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Most research evaluating the effects of the reproductive tract microbiota on reproductive performance has been done in humans, thus far. In bovids, reproductive microbiota research is not as advanced, with preliminary conclusions, not supported by contamination checks or repeatability. Our studies concluded that endogenous reproductive hormones, days of gestation, and pregnancy status does not change the overall vaginal microbiota composition. Although, the overall composition did not change there were species level differences. These differences could have implications in reproductive performance and fertility in heifers. Heifers that undergo nutrient restriction have similar vaginal microbiota to adequately fed heifers with no species differences. The most impactful finding is that exogenous supplementation of melatonin was associated with changes in the vaginal microbiota in Brangus heifers during late gestation. The implications of this finding are not yet clear, but to date, this is the first hormone, in bovids, determined to change the composition of the vaginal microbiota.

DEDICATION

I would like to dedicate this thesis to my parents, Davis and Dawn Messman, who have given me constant support throughout my graduate studies. I could not have accomplished this goal without them.

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CHAPTER I

REPRODUCTIVE PERFORMANCE AND THE REPRODUCTIVE TRACT MICROBIOTA IN BEEF CATTLE

Introduction

A new and expanding area of research in both human medicine and animal science is evaluating microbiomes within different organ systems. In humans, the gut microbiome has been referred to as a supporting organ because of the numerous associations between the gut microbiome and overall host health (Guinane et al., 2013; Ley et al., 2008). Additionally, negative impacts on host microbiomes throughout the body can have detrimental effects on overall health (Citrin, 2016). Therefore, reproductive physiologists have begun investigating the roles that microorganisms could play in fertility and reproductive performance. Current research into the microbiome of the reproductive tract is promising but incomplete. The objective of this review is to highlight current impactful studies evaluating the microbiomes of the reproductive tract and provide direction for future research.

Microbiome Research Methodology

Microbiology refers to the study of microorganisms including bacteria, archaea, fungi, and viruses. A recent focus in microbiology is evaluating and characterizing microbiomes. Researchers evaluating the microbiome are analyzing the roles, by gene sequencing, of microorganisms in a specific biome. These microorganisms can also be referred to as microbiota. A common misconception within the literature is that microbiome research refers to studies

focused on identifying compositional and characterizing changes within the microbiome. However, research that only characterizes a microbiome is referred to as characterization studies, not microbiome studies, because their findings typically do not elude to a microbe's role or contain gene-expression data. Therefore, the majority of the data presented in this review is not classified as microbiome research. These studies are purely characterization focusing on the confirming the existence of a reproductive tract microbiome, exploring the species present, and recognizing changes within the reproductive tract microbiome. After compositional studies, then microbiome researchers can further evaluate the genes of species present which may elude to their role in the biome. Further iterating the need for a continuation of research in this area. The majority of studies published focuses on bacterial community composition, therefore, this review will focus on bacteria within the reproductive tract microbiota.

Bacteria, both pathogenic and commensal, that directly inhabit and co-exist in any environment is referred to as the bacterial microbiota of that environment's microbiome (Lederberg and McCray, 2001). Researchers heavily agree that commensal bacteria are crucial for the maintenance of homeostasis in both human and livestock species (Mccann et al., 2014; Gilbert and Neufeld, 2014). It has been determined that each body site harbors a unique microbial community that can differ completely from one site to another (HMP Consortium, 2012). Therefore, researchers have migrated from evaluating the entire microbiome of a host to instead evaluating specific microbiomes within the host's physiological systems (Bull et al., 2014). Throughout the development of microbiology research, specific terminology, methodology, and data presentation have been adapted. Therefore, before current literature can be discussed in depth, an overview of methods and data analyses unique to microbial research must be presented.

Firstly, obtaining a pure uncontaminated sample requires stringent methods dependent on the specific microbiota being sampled. The results of a study can be heavily impacted by sample collection methods. For example, the gut microbiota can easily be evaluated by collecting a stool sample but sampling the microbiota of a specific organ would require sterile invasive procedures. Additionally, direct collection methods, such as stool collections, yields an extremely microbial dense sample with minimal contamination risk (HMP Consortium, 2012). Conversely, utilizing swabs or lavages to collect samples prove more challenging to obtain without contamination and are less studied regarding protocol consistency and accuracy (Tong et al., 2014). Typically, swabs are the least invasive but retrieve the smallest microbial biomass (Aagaard et al., 2013), whereas biopsies are more invasive but collect the most microbial biomass (HMP Consortium, 2012). Therefore, when determining how to sample, the researcher must consider sampling site, contamination risk, and how invasive their sampling procedure is.

After sample collection, the most common analysis used for evaluating microbiota composition is gene sequencing utilizing the 16S rRNA gene (Petti et al., 2005). This method is not perfect, but has been proven to be an objective, accurate, and reliable method for bacterial identification (Clarridge, 2004). A highly conserved component of the transcription machinery of microorganism's DNA is the 16S rRNA gene (LC Sciences, 2019). Therefore, 16S rRNA is a great target gene for sequencing DNA in samples that contain thousands of different microbial species (LC Sciences, 2019). Additionally, the 16S rRNA gene is large enough (1500 base pairs) for information purposes, its function has not changed over time, and it is present in almost all bacteria and archaea further attesting its use for studying microorganisms (Patel, 2001). The 16S rRNA gene has both variable and conserved regions, the variable regions differ between specific

microorganisms (Janda and Abbott, 2007); this allows for differentiation between microbial species.

The methodology for the bioinformatic analysis of the microbiota is referred to as a pipeline. A pipeline utilizes bioinformatic software programs and laboratory kits which can change dependent on individual preference and the goal of the study, but the general steps taken within analysis is the same.

The first step within microbial analysis is DNA extraction from collected samples, the extracted DNA then undergoes quality/quantity check, next the universal 16S rRNA primers are used to amplify all bacterial DNA via polymerase chain reaction (PCR), then the 16S rRNA is sequenced from the bacterial DNA, finally similar 16S gene sequences are clustered into operational taxonomic units (OTU) and identified (D'Argenio et al., 2015). Operational taxonomic units are groups of similar 16S rRNA reference sequences (Schloss and Westcott, 2011); typically, they are grouped at a certain percentage of similarity, such as 95% or 98%. After clustering into OTU, a reference database, that has current information about what 16S gene sequences match specific bacterial taxonomy, is utilized to identify the species each OTU represents (NIH, 2019). It is important to mention that not all sequences identified as an OTU will have a taxonomic match within the database. This occurs because that specific sequence has yet to be identified and assigned taxonomy. After the bacterial community has been characterized, statistical analysis for significant differences is the next step (D'Argenio et al., 2015).

Most researchers present data analyzing the alpha and beta diversity of samples, as this is a quantitative analysis of comparable differences within and between samples, respectively. First, alpha diversity is the diversity within a sample that specifies species richness and evenness

(Moreno and Rodriguez, 2010). Alpha diversity is commonly measured via Shannon diversity index, a statistical method which accounts for species abundance/richness and evenness within a sample (Magurran, 1988). Richness, or abundance, refers to the number of observed species within an individual sample. Evenness is the relative abundance of species within a sample (Buford et al., 2018). Further explanation of alpha diversity is illustrated in Figure 1. Beta diversity is the extent of change in species composition in different samples (Whittaker, 1977). The statistical methods to analyze differences in beta diversity are dependent on researchers, commonly researchers utilize a non-permutational index (such as Bray-Curtis) accompanied with a PERMANOVA. Normally beta diversity is visualized using a Principal Component Analysis (PCA) (Braak, 1983) which allows a visualization of the of similarities between each sample. This statistical graph allows readers to visualize the beta diversity between samples.

The aforementioned methods have provided insight about microbial composition throughout physiological systems in many species and the innerworkings of microbial presence on overall health. Although many species and physiological systems have been analyzed, there is still much room for growth and the expanding of knowledge in microbiota research. A recent trend in microbiota research is to evaluate the microbiota of the reproductive tract and its impacts on fertility and reproductive performance.

The Microbiota of the Human Reproductive Tract

To date, the most in depth research characterizing reproductive tract microbiotas, including the vagina, uterus, placenta, and amniotic fluid, has been performed in humans. Specifically, researchers are focusing on first determining if a microbiota exists, then

characterizing the microbiotas located within the reproductive tract, and lastly, determining how the microbiota can affect fertility.

The vaginal microbiota and physiological environment of the vaginal tract was characterized by Ravel et al. (2011). In this study, the vaginal bacterial communities of reproductive age women (n=396) were analyzed using the 16S rRNA gene. Subjects were of different ethnic and racial backgrounds (Caucasian, African American, Hispanic, and Asian), and all were currently living in North America. Results found that the majority of bacteria within the subjects' vaginal tracts had the common physiological ability to produce lactic acid. It was concluded that Caucasian and Asian women had different vaginal microbiotas than African American and Hispanic women. Results also showed that the vaginal tracts of Caucasian and Asian women were primarily dominated by *Lactobacillus* species, but the vaginal tracts of African American and Hispanic women had fewer *Lactobacillus* species. Previously, it was proposed that the production of lactic acid by *Lactobacillus* species in the women's vaginal microbiota was crucial for vaginal health (Redondo-Lopez et al., 1990), but Ravel et al. (2011) disputes this claim and states that many factors including ethnicity and environment could contribute to the composition of the vaginal microbiota. Lastly, authors found that the pH of Caucasian and Asian women was significantly different from African American and Hispanic women (4.2 compared to 5.0). Therefore, the two groups clearly had different amounts of lactic acid production in the vagina.

Lactic acid facilitates an acidic vaginal environment, that can prevent the growth and attachment of potential pathogens (Osset et al., 2001). Ronnqvist et al. (2006) evaluated the role of *Lactobacilli* species in the vaginal tract and their relationship to other genital microbes and vaginal pH. In 191 women evaluated, the overall conclusion was that *Lactobacilli* species

contribute to a low vaginal pH which has a negative impact on the growth of Group B Streptococci. Group B Streptococci is associated with preterm birth, fetal injury, and neonatal mortality in newborns; recently, it was discovered this pathogen also has the ability to infect fetuses in utero (Whidbey et al., 2013). Therefore, it can be inferred that an acidic vaginal environment is beneficial in women for preventing growth of pathogenic bacteria, but as mentioned in Ravel et al. (2011), the physiologically normal pH and bacteria that induce the acidic environment is different amongst ethnic groups. Therefore, further characterization of the normal vaginal microbiota in different demographics of women is necessary.

After the original characterization studies, vaginal microbiota researchers shifted to determining if a microbiota is present within the uterus. Similar to animal species, obtaining sterile samples from the uterus in women is difficult due to anatomical barriers such as the vaginal tract and cervix. Data showing microbial populations in the uterus have been published and presented in the literature, but many researchers are still skeptical of the presence of a uterine microbiota and attribute current findings to contamination (Baker et al., 2018). Additionally, if a microbiota exists, it is debatable if it has positive or negative effects on reproductive performance, fertility, or pregnancy establishment.

Within the uterus, the most abundant phyla reported include Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Chen et al., 2017; Walther-Antonio et al., 2016). The sampling pool included patients with both healthy and unhealthy reproductive tracts, therefore, attributing microbial composition to uterine health appears difficult and unreliable at this time. Additionally, *Lactobacillus*, from the Firmicutes phyla, is the most abundant genus in the uterine in the aforementioned studies and others (Mitchell et al., 2015; Miles et al., 2016; Tao et al., 2017). Recall that the colonization of the vaginal tract with *Lactobacillus* species led to

decreased vaginal pH to prevent growth of pathogens, but within the uterus its role is up for debate.

Fang et al. (2016) reported that women with endometrial polyps and chronic endometriosis had a higher relative abundance of *Lactobacillus* species in the uterus than healthy women, concluding that *Lactobacillus* has a negative effect on reproductive performance. However, Moreno et al. (2016a) reported that high levels of *Lactobacillus* species in the uterus increased success rates in women undergoing in vitro fertility treatments and embryo implantation success. Interestingly, there are regular findings of *Lactobacillus* species within the uterine tract but an obvious function or physiological role within the uterus is unknown. It is plausible that vaginal contamination of sample occurred, and the research being reported is not actually the uterine microbiota but a result of contamination. Therefore, more in depth research with multiple contamination checks needs to be implemented before a uterine microbiota can be confirmed.

Finally, researchers have analyzed tissue from the placenta and amniotic fluid during pregnancy to detect the presence of a microbial community. If a microbiota does exist, there could be major implications surrounding fetal microbial inoculation, fetal development, and fetal health both in utero and after birth (Cao et al., 2019).

Researchers studying the placenta and amnion in humans have found microbial populations present, but there is still much debate to whether it is colonization or contamination. In a study by Stinson et al. (2019), amniotic fluid and fetal meconium was collected from women undergoing non-emergency cesarean sections (n=50). Each sample was characterized via 16S rRNA gene sequencing. They found that all meconium samples contained high number of reads that matched *Pelomonas puraquae,* and amniotic fluid samples had a low number of reads and

microbial diversity with only skin commensals being present. Within their discussion, the authors acknowledged the possibility of contamination of meconium samples with *P. puraquae* within the laboratory, but largely concluded that the overall presence could not solely be due to external contaminants (Stinson et al., 2019). The authors attributed the presence of *P. puraquae* only in the meconium to the bacteria's ability to survive in the harsh environmental composition of meconium; whereas skin commensals were mostly found in the amniotic fluid because amniotic fluid typically contains high amounts of sloughed fetal skin cells (Akiyama and Holbrook, 1994).

Conversely, Lim et al., (2018) found that all microbes present within the amniotic fluid were due to sample contamination either in the birthing suite, laboratory, or transport. This study was in agreement with previous literature regarding the low bacterial biomass in amniotic fluid samples (Lim et al., 2018; Stinson et al., 2019). Due to the low bacterial biomass, current researchers in this field are still determining the best way to process and analyze these samples. Recent discrepancies with the materials and methods of Lim et al., (2018) have been published in the literature (Payne et al., 2019). Overall, concrete evidence supporting presence or absence of a microbiota in the amniotic fluid has yet to be presented in the literature.

Lastly, similar to the amniotic fluid, there is a debate in the literature of the existence of a placental microbiota. Aagaard et al. (2014) found that the placenta contained a unique microbiota when sampling 320 women. The placenta was primarily inoculated with commensal bacteria. This study included samples from 320 women with both healthy and pre-term births, and it was concluded that all placentas sampled had a low microbial biomass. Although interesting, the Aagaard et al. (2014) study had some limitations that severely compromised the integrity of the paper in terms of validity; due to no contamination checks being reported or analyzed, there is no

possible way to know if the microbial communities reported in this study were due to contamination or colonization.

The most impactful research evaluating the placental microbial population was done by Goffau et al. (2019) which refutes claims from previous researchers that the placenta contains a microbiota. In this study, placentas (n=537) from women that had vaginal deliveries, pre-labor Cesarean section (CS), and CS after labor onset were evaluated. Rigorous contamination evaluations were performed to determine if contamination occurred in either the delivery room or laboratory. Results showed that all microbiota present on the placenta samples were from contamination. However, native pathogens, specifically Group B Streptococcus, were discovered on the placenta samples. Therefore, authors concluded that while the placenta does not have a resident microbiota, it can harbor pathogens that can have fatal consequences to infant's post-partum.

Interestingly, through their evaluation of contamination, authors also found that the placenta was naturally inoculated with vaginal microbiota during natural birth and CS after labor onset only. This suggests the ascension of vaginal microbiota through the cervix during labor to inoculate the placenta and fetus. Ultimately, if valid, the vaginal microbiota would be the first contact the fetus has with microorganisms; suggesting that the composition of the vaginal microbiota could be associated with the cause or prevention of neonatal diseases. While more research is needed to verify this study, it can be speculated that the vaginal microbiota is extremely important in the reproductive and overall health of women and their neonates.

Clearly, the research and evaluation of the role and presence of microbiota in the reproductive tract of humans is incomplete; however, previous research in humans can assist

animal scientists in designing studies to evaluate the microbiota of the reproductive tract in livestock species.

The Microbiota of the Bovine Reproductive Tract

Literature covering the characterization and implication of the microbiota within the reproductive tract of bovids is scarce. Unfortunately, most of the literature in bovids and livestock species available regarding the reproductive tract microbiota has severe limitations, such as no contamination checks or fewer numbers of animals. Similar to humans, the colonization of the reproductive tract with microbiota could reveal linkages between microbial communities and fertility, reproductive tract health, and overall animal health. To date, the most researched microbiota of the reproductive tract is the vaginal microbiota and its role in reproductive performance.

Firstly, Swartz et al. (2014) found that the vaginal environment and microbial community composition of humans and bovids differ. This study utilized crossbred cows (n=20) and compared the microbial data results to previous literature characterizing women's vaginal microbiota. These researchers found that bovid's vaginal tracts have low levels of *Lactobacilli* and near neutral pH compared to humans. Due to the change in pH, it can be expected that the commensal vaginal bacteria within bovids produce less lactic acid and require a near neutral pH for optimal growth. This was further demonstrated in the characterization of the bovine vaginal microbiota, with the three most abundant phyla being Bacteroidetes, Fusobacterium, and Proteobacteria (Swartz et al., 2014). Therefore, previous literature associating the human vaginal microbiota to fertility (Sirota et al., 2014) may not be directly applicable to bovids.

A study by Nascimento et al. (2015) evaluated the vaginal microbiota in four subsets of bovids, each unique in their reproductive status. The Nellore cattle breed was utilized with

pregnant cows (n=5), nonpregnant cows (n=5), pregnant heifers (n=5), and nonpregnant heifers (n=5) included in the study. In agreeance with Swartz et al. (2014), the most abundant phyla in all animals were Firmicutes, Bacteroidetes, and Proteobacteria. Within this study, they found that great microbial variability exists among animals, and no statistical differences were found as a result. Additionally, this study was the first to mention the potential role of reproductive hormones influencing the composition of the vaginal microbiota, but no evidence was provided. Perhaps the most interesting findings from the study was the lack of obligate anaerobes in nonpregnant heifers' vaginal microbiotas. Typically, obligate anaerobes are the last bacterial community to colonize a specific environment (Noor and Khetarpal, 2019). Authors attributed this finding to the potential of an underdeveloped vaginal microbiota in heifers that could lead to decreased fertility in these animals (Nascimento et al., 2015).

Estrus synchronization protocols in cattle could potentially impact the vaginal microbiota, more specifically protocols that utilize vaginal inserts to deliver hormones. Therefore, Moreno et al. (2016b) investigated the difference in vaginal microbial communities post-progesterone releasing intravaginal devices (PRID) in cows and heifers. Post- PRID removal the animals were classified as having either vaginitis or no vaginitis (healthy); this was determined by counting polymorphonuclear neutrophils in vaginal cytobrush samples. They found that cows and heifers with vaginitis had higher Proteobacteria, a more uniform microbiota, and an increase prevalence of opportunistic pathogens within their vaginal microbiota. Overall, vaginitis caused by the use of PRIDs could increase the prevalence of bacteria in the vaginal microbiota that could act as uterine pathogens and negatively impact fertility.

In addition to maternal health and fertility, the vaginal microbiota could have impacts on the dam's offspring as well. Lima et al. (2018) sampled the vaginal and fecal microbiota of

Holstein cows (n=81) and compared it to the fecal and upper respiratory tract (URT) microbiota of their calf (n=81), at 3, 14, and 35 days old, to evaluate if the maternal microbiota could influence calf health. They found that the URT microbiota of calves and the dams' vaginal microbiota were highly similar (63% overlap with 253 shared OTU) regardless of days of life. *Mannheimia* and *Moraxella,* bacteria involved in bovine respiratory disease in calves, were some of the most prevalent shared between dam and calf, suggesting that the health of a calf could be affected by mother-to-offspring transmission during parturition.

Messman et al. (2020) evaluated the role of estradiol and pregnancy status on bacterial community composition of the vaginal microbiota. Researchers sampled Brangus heifers (n=78) at timed artificial insemination to determine if there were differences in bacterial composition between pregnant and non-pregnant heifers or heifers with divergent estradiol concentrations. It was determined that at the bacterial composition of the vaginal microbiota was not changed by estradiol concentrations at time of artificial insemination. Additionally, heifer that became pregnant vs. nonpregnant did not have a different vaginal microbiota at time of artificial insemination.

Moreover, a study by Ault et al. (2019), investigated the role of progesterone concentrations altering the vaginal and uterine microbiota, and compared the vaginal and uterine microbiotas of pregnant and nonpregnant post-partum cows prior to timed artificial insemination (TAI). Samples were collected from pregnant (n=10) and nonpregnant (n=10) cows at 3 time points (d-21, d-9, and d-2 prior to TAI) from the vagina and uterus via lavage; blood serum was also collected at the aforementioned time points for progesterone concentration analysis. The estrus synchronization protocol utilized in this study did not incorporate CIDRs.

There were no differences in the alpha diversity of vaginal microbiota species abundance at any sample date prior to TAI, but authors found that OTU significantly decreased in the uterus over time (Ault et al., 2019). Ault et al. (2019) utilized a principal coordinate analysis to visualize beta diversity within the samples. Results showed significant clustering at d-2 in the uterus (P=0.005) and in the vagina at d-21 (P= 0.002) between pregnant and nonpregnant cows. Authors proposed the differences in the uterus at d-2 could be affecting pregnancy establishment in these animals. They also found that the composition of the uterine and vaginal microbiotas varied in beta diversity dependent on day of collection. Lastly, authors found no direct relationship to progesterone concentrations and the vaginal and uterine microbiotas.

Overall, Ault et al. (2019) agrees with previous literature stating that there is high variation and diversity within the microbiotas of the bovine reproductive tract (Nascimento et al., 2015; Swartz et al., 2014). Authors suggested that close clustering of nonpregnant animals at d-2 prior to TAI is due to a group of closely related bacteria within the uterus that can prevent or decrease the likelihood of pregnancy establishment. Limitations from this study are the small sample size and lack of contamination checks.

Ault et al. (2019) is one of the few studies within bovids evaluating the uterine microbiota in live animals before parturition. Moore et al. (2017) sampled the entire reproductive tract of virgin heifers and pregnant cows. This study aimed to establish that the uteri of pregnant cows and virgin heifers possessed a resident microbiota. Researchers collected the endometrial samples of 10 virgin dairy heifers via biopsies, and collected amniotic fluid, placentomes, intercotelydonary placenta, cervical lumen, and vaginal tissue from 5 pregnant dairy cows after slaughter. All samples generated a microbiota, leading researchers to conclude that both pregnant and virgin uteri contained a resident microbiota. They found the three most abundant phyla in

both the pregnant and virgin uteri were Firmicutes, Bacteroidetes, and Proteobacteria. These results led them to conclude that the uterine microbiota is established before females reach reproductive maturity. Therefore, the uterus is not a sterile environment at the establishment of pregnancy and could potentially inoculate the calf in utero (Moore et al., 2017). However, no contamination checks were mentioned in the publication, and tracts of pregnant animals were collected on a processing floor where the possibility of contamination is extremely high.

Lastly, the most literature evaluating bovine uterine bacterial communities has been studied in post-partum cows to evaluate the microbial abundance/composition and the risk of developing uterine disease, such as metritis. A study by Wang et al. (2018) focused on characterizing the uterine microbiota of post-partum dairy cows with clinical (CE) and subclinical (SE) endometritis. Researchers collected uterine flush samples from 13 healthy, 5 SE, and 9 CE cows at 30 days post-partum. All cows had a similar microbiota with 293 of 445 shared OTUs. Clinical endometritis was characterized by an increased abundance of *Fusobacterium* and presence of *Trueperella* and *Peptoniphilus.* Subclinical cows had no uterine pathogens, but an increased abundance of *Lactococcus* and *Acinetobacter.* This study concluded that healthy, subclinical, and clinically infected cows share a similar microbiota; however, the competitive and cooperative interactions within the microbiota still need to be established to determine how to combat uterine pathogens and promote a healthy uterine microbiota.

Santos et al. (2011) also found that the phylum *Fusobacteria* was affiliated with metritic post-partum cows, but in this study, no differences were found between healthy and metritic cows. Interestingly, Jeon et al. (2017) evaluated the possibility of blood introducing pathogens into the uterine post-partum. They compared bacterial communities from blood, feces, and uterine samples at day 0 and day 2 post-partum and vaginal samples at 7 days prior to calving.

Results of the principal coordinate analysis showed distinct clustering of blood and fecal bacterial communities, but more scattered clustering of uterine and vaginal communities indicating greater variability (Jeon et al., 2017). Additionally, major uterine pathogens were detected in blood samples and there was a strong and significant interaction of uterine pathogens and the blood microbiota. Authors concluded that in addition to fecal and vaginal microbial contaminates, uterine bleeding post-partum can also lead to the introduction of pathogens to the uterus.

Researchers who evaluate post-partum uterine microbiota generally assume that any microbial populations present are contaminates that contribute to decreased reproductive performance. This is in stark contrast compared to the Ault et al. (2019) and Moore et al. (2017) studies that proposed that the presence of a commensal uterine microbiota could facilitate pregnancy. Ultimately, further research will be needed to determine if a uterine microbiota exists in bovids; these studies need to implement contamination checks similar to human studies and be cognizant of contamination throughout the entire analytical process.

Implications of the Vaginal Microbiota in Compromised Pregnancy

Literature evaluating the role of the reproductive tract microbiota in bovine pregnancies that are compromised is sparse. The negative consequence of compromised pregnancy is intra-uterine growth restriction (IUGR) of the conceptus that has negative effects both in-utero and after birth. There are many mechanisms that cause IUGR, but one of the most notable and prevalent in the beef cattle industry is nutrient restriction. Therefore, researchers have spent decades studying nutrient restriction, IUGR pregnancies, and potential supplements to mediate the effects of both. Despite this breadth of knowledge, the implications of the vaginal microbiota in compromised pregnancies is unknown.

Nutrient partitioning, or energy partitioning, describes the physiological function wherein the body decides which organs, tissues, or cellular processes have the highest demand for energy to support homeostasis; typically, the vitality of the physiological or metabolic process to the homeostatic state of the animal is the determining factor of nutrient partitioning (Hammond, 1947; Bauman and Currie, 1980). This concept is relevant throughout the life of an animal. Typically, animals must first meet their maintenance energy requirements to sustain life before energy can be partitioned to growth (Lifshitz, 2010), gestation (Wallace, 2000) or lactation (Hart, 1983).

During pregnancy, the vascularity of the small intestine of the dam is increased which allows for greater nutrient absorption (Scheaffer et al., 2003). The increased uptake of nutrients during pregnancy is conducive to supporting the maintenance energy of the dam, as well as, the growth and develop of the conceptus (Vonnahme et al., 2015). Additionally, during pregnancy nutrient partitioning is dependent on the metabolic rate of tissues (Redmer et al., 2004). Tissues that are highly metabolically active have increased blood flow (Redmer et al., 2004). During pregnancy, the maternal cardiovascular system undergoes dramatic changes (Vonnahme et al., 2015). Specifically, it has been shown that maternal arterial blood pressure and vascular resistance is decreased, while cardiac output, heart rate, stroke volume, and blood volume is increased (Magness, 1988). These physiological changes are congruent with systemic vasodilation within the maternal cardiovascular system during gestation, specifically late gestation (Vonahhme et al., 2015). The increased cardiac output is largely to provide increased blood flow to the uterus; this phenomenon has been observed in multiple species (Dowell and Kauer, 1997; Rosenfeld et al., 1974; Lees et al., 1971). The increase in uterine blood flow during pregnancy indicates a highly metabolically active tissue; therefore, due to its high priority status,

increased nutrients are partitioned to the uterine placental unit during pregnancy (Redmer et al., 2004). In many cattle pregnancies, these physiological adaptations are sufficient in providing enough energy for maintenance of the dam along with the development of the conceptus. However, Godfrey and Barker (2000) found that nutrient partitioning favors the growth and development of the conceptus at the expense of the dam, which more often than not results in a compromised pregnancy in humans. Therefore, in cattle that experience decreased nutrient intake during pregnancy could have similar consequences. Inadvertent nutrient restriction is common within cattle; researchers have shown that the nutrient content of grazed forages, especially during winter, is insufficient to support reproductive performance (Patterson et al., 2003), and gestating heifers in feedlots do not have adequate nutrient intake to support optimal fetal growth (Kreikermeier and Unruh, 1993). Therefore, compromised pregnancies due to nutrient restriction are likely to be common in cattle production systems that utilize free-range grazing or feedlots that house gestating animals.

Intra-uterine growth restriction is defined as the retardation of growth and development of a mammalian conceptus during pregnancy (Wu et al., 2006). The offspring of dams who experience nutrient restriction, especially during the third trimester when the majority of fetal growth occurs, are extremely susceptible to IUGR (Robinson et al., 1999). Primarily, IUGR is caused by the limitation of uterine capacity, translating to a decrease in nutrient flux to the feto-placental-maternal unit (Bazer et al., 1969). This results in diminished nutrient availability to the fetus. The limited nutrients available to the fetus are shunted to primary organs, the brain, and necessary physiological systems that are critical for life (Puchner et al., 2006). As a result, IUGR calves characteristically have a smaller birth weight, larger head to body ratio, and are metabolically altered throughout their life (Long et al., 2009). Additionally, these calves tend to

be weak, struggle earlier on in life, have a higher mortality rate, and can be an economic burden to producers (Ogata et al., 1999). Therefore, researchers have begun to focus on mitigating the effects of nutrient restriction during pregnancy to counteract the negative effects of IUGR on calf performance.

Nutrient flux to the placental unit is dependent on nutrient availability from maternal intake, adequate uterine blood flow, adequate maternal blood perfusion to the placentome, and the surface area of nutrient exchange within the placentome (Sibley et al., 2010). During nutrient restriction, maternal nutrient intake is decreased, but normally producers are not financially capable to provide excess supplementation. Therefore, researchers began to focus on inexpensive therapeutics that increase blood flow to the uterus and promotes placental angiogenesis to increase surface area within the placentome. One potential therapeutic is the hormone melatonin (Lemley et al., 2012). Melatonin is produced in the pineal gland within the central nervous system at nighttime in diurnal mammals (Emet et al., 2016). Melatonin plays a role in regulating sleep-wake cycles, seasonality, puberty, and the circadian rhythm (Pandi-Perumal et al., 2008). Melatonin can also alter peripheral blood circulation by mechanisms of either vasodilatation or vasoconstriction dependent on the target cell (Dubocovich et al., 2003). The most interesting application of melatonin in preventing IUGR is its role as a modulator of oxidative stress within endothelial cells (McCarty et al., 2018). Melatonin is an endogenous antioxidant; in endothelial cells, melatonin will bind to the MT2 receptor causing an increase in nitric oxide production resulting in vasodilation (Zhao et al., 2017). Previous literature holds promise that melatonin could be utilized to prevent IUGR by increasing uterine artery blood flow and therefore increasing nutrient delivery to the fetus. Lemley et al. (2012) supplemented melatonin in ovine IUGR dams; data showed increased umbilical artery blood flow compared to control dams. In

dairy cows, melatonin supplementation increased uterine artery blood flow, did not alter calf birth weight, but calves from melatonin supplemented dams weighed more at 8-9 weeks of age (Brockus et al., 2016). Similarly, in beef cows, melatonin supplementation increased uterine artery blood flow during mid to late gestation, but there was no increase in fetal weight compared to control dams (McCarty et al., 2018). Therefore, melatonin supplementation could be a viable option for the prevention of IUGR in cattle, but more research to evaluate the overall mechanism of melatonin within the pregnant bovid is needed.

Although nutrient restriction and melatonin supplementation has been studied, there has been limited research evaluating how the reproductive tract microbiota is affected in IUGR pregnancies. In mice, pups experiencing IUGR had different intestinal microbiota (Berthon et al., 2010). In piglets experiencing IUGR, researchers found an unbalanced gut microbiota, impaired small intestine structure and abnormal inflammatory and metabolic profiles 12 hours after birth (Huang et al., 2019). A model for how microbial communities could influence IUGR pregnancies in women was described by Bardos et al. (2020). They postulated that dysbiosis throughout the maternal microbiota caused by pathogens can result in systemic inflammation, including inflammation of uterine and placental tissues (Bardos et al., 2020). The inflammatory response in these tissues could lead to decreased surface area for nutrient exchange, resulting in an IUGR fetus. Overall, these studies indicate that the maternal microbiota is in a state of dysbiosis during pregnancy, resulting in systemic inflammation. Furthermore, during parturition the fetus is inoculated with the dam's microbiota resulting in the newborn being exposed to a potentially pathogenic microbiota that ensues alterations in metabolic and inflammatory responses.

Houdijk et al. (2001) found that during gestation metabolizable protein becomes scarce, and nutrient partitioning will prioritize protein transport to the reproductive tract instead of the immune system. Thereby, an animal that is nutrient restricted will also be immunocompromised to a certain extent. This immunocompromised state, accompanied by the already immunosuppressive state of the reproductive tract during gestation (Oliveira et al., 2012), provides the perfect situation for the inoculation of the vaginal microbiota with potential pathogens. If the vaginal microbiota does become inoculated with pathogenic bacteria during gestation then localized inflammation in the reproductive tract will be present, the calf will be exposed to the pathogens during parturition, and the potential for a post-partum infection is severely increased. Therefore, the implications of the vaginal microbiota in IUGR pregnancies is overwhelming, yet there is a paucity in the literature.

Lastly, no literature on the effects of melatonin on the vaginal microbiota in cattle has been documented. The antioxidant properties of melatonin could have major implications in decreasing maternal stress during nutrient restriction (Hardeland and Fuhberg, 1996). In fact, Kumar et al. (2014), found that melatonin supplementation to summer stressed anestrus water buffalo increased their total antioxidant capacity, decreased oxidative stress biomarkers, and induced estrus in these animals. The ability of melatonin to mitigate stress could strengthen the immune system which prevents the inoculation of pathogens into the vaginal microbiota leading to a healthier pregnancy. Therefore, future studies evaluating the effects of melatonin on the vaginal microbiota during IUGR pregnancies are vital to expanding the knowledge in this area of research.

Conclusion

In conclusion, the study of the microbial communities of the reproductive tract is a new and emerging area of research. Most of the published literature, especially in bovids, has limitations that negatively impacts the validity of the research. However, these studies are important for characterization of the microbiota. More studies will be needed to characterize the reproductive tract microbiota throughout the estrous cycle, pregnancy, post-partum, and any other significant reproductive events. Once characterization is completed, comparison studies can occur and perhaps mechanisms of pathogenesis will be revealed. Ultimately, the long-term goal is to determine if a certain commensal bacteria community within the bovine vaginal tract facilitates the establishment of pregnancy or if the presence of pathogenic bacteria negatively impacts fertility, fetal development, or pregnancy establishment. More research will be needed to reveal the answers to such questions, but this field has promise for furthering knowledge in reproductive physiology and improving current production methods to increase pregnancy rates and economic profitability.

Statement of the Problem

In 2017, the total economic contribution of the beef industry to the U.S. economy was approximately $165 billion (Census of Agriculture, 2017). It is predicted this economic impact will continue to increase as beef consumption is expected to increase by 8.9% by 2020 (Census of Agriculture, 2017). In order to meet the future demand for beef products, producers must be operating at maximum efficiency. Achieving maximum efficiency entails improving the efficiency of management, nutrition, genetics, and reproduction within animal production. Currently, losses in reproduction, such as low conception rates, early embryonic death (EED),

low pregnancy rates, reproductive tract infections, and infertile cattle are causing significant economic burden to producers (Diskin, 2008).

Efficiency is defined as minimal inputs yielding maximum outputs. Therefore, reproductive efficiency would be defined as the costs associated with the minimum number of services to yield a successful pregnancy and birth of a calf. To date, there are numerous publications in the literature striving to solve the mystery of the cause of EED, low conception rates, and to implement management practices to improve reproductive efficiency (Perry et al., 2007) Although these studies provide valuable insights and recommendations, they all fall short of solving the problem of decreased reproductive performance in beef cattle.

An overlooked but crucial component of the reproductive tract is the presence of microorganisms. Nutritionists have found that feed efficiency can be influenced by the rumen microbiota (Jami et al., 2014) and more recently that bacterial communities within the rumen can be inherited (Li et al., 2019). However, researchers investigating the reproductive tract microbiotas in cattle are limited; many of the current studies have small animal numbers and do not implement contamination checks which limits the validity of the research.

Therefore, this review and the research we conducted contributes valuable knowledge to the literature by highlighting the discrepancies between studies and providing direction for a new and growing area of research. If the microbiota of the bovine reproductive tract has as much impact on efficiency and health as the rumen microbiota does, it could be a key factor in improving reproductive and overall efficiency in beef cattle. Ultimately, the end-goal of microbiota research in the reproductive tract would be to identify bacterial communities that facilitate and inhibit pregnancy. Next, a supplement or treatment, delivered vaginally, that promotes the growth of healthy bacteria could be developed for producer use. Ideally, this

supplement would increase conception rates, decrease early embryonic death, and increase the number of calves born by facilitating the correct physiological environment in the vaginal tract and uterine body. Such a development would translate to significant economic gains for the producer and have major economic implications for the beef industry as a whole.

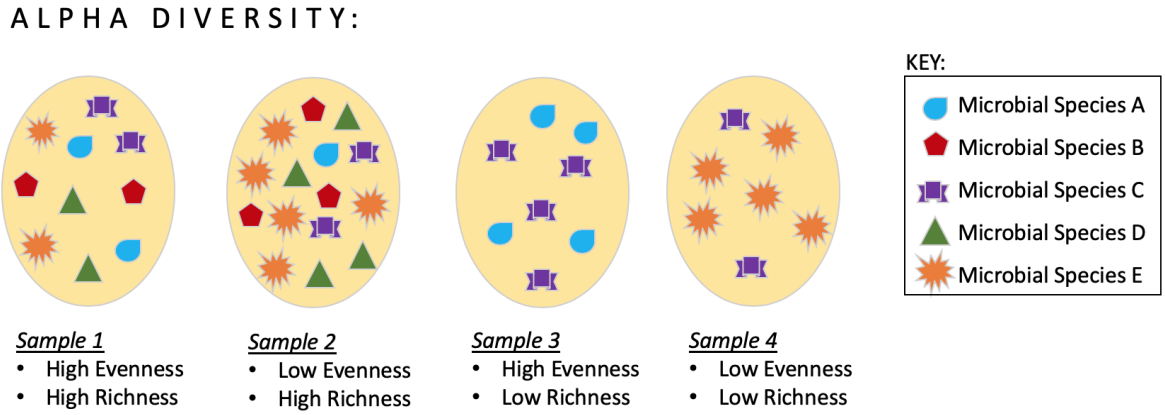

Figure 1.1 Alpha Diversity

Alpha diversity is the microbial diversity within a sample. Alpha diversity is measured in evenness and richness. High evenness is when the population proportions are similar within the samples. For example, in samples 1 and 3 there are two of each microbial species and three of each microbial species, respectively. Therefore, the microbial proportions are even within these samples. High richness is when there are a high number of microbial species within a sample. For example, in samples 1 and 2 all microbial species are represented, so these samples have high richness compared to samples 3 and 4 that only have two microbial species represented. See text for citations.

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This chapter of the thesis has been submitted and will be published as Chapter 68- the reproductive tract microbiome: implications for fetal and neonatal health in the second edition of Bovine Reproduction by Dr. George Hopper.

CHAPTER II

VAGINAL BACTERIAL COMMUNITY COMPOSITION AND CONCENTRATIONS OF ESTRADIOL AT TIME OF ARTIFICIAL INSEMINATION IN BRANGUS HEIFERS

Abstract

The knowledge surrounding the bovine vaginal microbiota and its implications on fertility and reproductive traits remains incomplete. The objective of the current study was to characterize the bovine vaginal bacterial community and estradiol concentrations at time of artificial insemination (AI). Brangus heifers (n = 78) underwent a 7-day Co-Synch + controlled internal drug release (CIDR) estrous synchronization protocol. At AI, a double guarded uterine culture swab was used to sample the anterior vaginal tract. Immediately after swabbing the vaginal tract, blood samples were collected by coccygeal venipuncture to determine concentrations of estradiol. Heifers were retrospectively classified as pregnant (n = 29) versus nonpregnant (n = 49) between 41-57 days post-AI. Additionally, heifers were classified into low (1.1 to 2.5 pg/mL; n = 21), medium (2.6 to 6.7 pg/mL; n = 30), and high (7.2 to 17.6 pg/mL; n = 27) concentration of estradiol. The vaginal bacterial community composition was determined through sequencing of the V4 region from the 16S rRNA gene using the Illumina Miseq platform. Alpha diversity was compared via ANOVA and beta diversity was compared via PERMANOVA. There were no differences in the Shannon diversity index (alpha diversity; P = 0.336) or Bray-Curtis dissimilarity (beta diversity; P = 0.744) of pregnant versus nonpregnant heifers. Overall bacterial community composition in heifers with high, medium, or low

concentrations of estradiol did not differ ($P = 0.512$). While no overall compositional differences were observed, species level differences were present within pregnancy status and estradiol concentration groups. The implications of these species level differences are unknown, but these differences could alter the vaginal environment thereby influencing fertility and vaginal health. Therefore, species level changes could provide better insight rather than overall microbial composition in relation to an animal's reproductive health.

Introduction

The role and importance of the vaginal microbiota in fertility and reproduction has been recently explored in sheep (Martinez-Roz et al., 2018), cattle (Clemmons et al., 2017), horses (Fraga et al., 2011) and humans (Chen et al., 2017). In general, the vaginal microbiota is dynamic and constantly changing in the aforementioned species. Furthermore, the vaginal microbiota composition differs between human and livestock species. Swartz et al. (2014) compared the vaginal microbiomes of bovine and ovine species to that of humans. They concluded that the microbiomes of ovine and bovine species are characteristically and compositionally different from humans and that these differences were attributed to varying physiological environments within the vaginal tract. Specifically, human vaginal microbiota has more *Lactobacillus* species which drive the acidic vaginal environment in humans. In comparison, the bovine and ovine species have a vaginal environment closer to neutral pH due to a lower abundance of *Lactobacillus* (Swartz et al., 2014). Therefore, previous research associating the human vaginal microbiome to fertility (Sirota et al., 2014) may not be directly applicable to livestock species.

Research in bovids analyzing the vaginal microbiome is limited. Previous studies have focused on the characterization of bovids with reproductive disorders (Moreno et al., 2016),

pregnant and nonpregnant status (Ault et al., 2019; Nascimento et al., 2015), and the post-partum stage (Lima et al., 2019). However, inference from these studies is limited due to the small sample size with many including less than 25 animals.

The implication of hormones, specifically estrogen, on the vaginal microbiome has only been speculated. It is well known that reproductive hormones significantly affect the reproductive physiology overall. Specifically, estradiol has been shown to change the uterine pH in cattle (Perry and Perry, 2008). Additionally, in humans, it has been determined that estrogen increases the acidity of the vaginal environment (Gorodeski et al., 2004). Shifts in pH will greatly impact the ability of bacteria to survive within a certain environment. In humans, the acidity of the vaginal environment is considered a defense mechanism against many pathogens associated with sexually transmitted diseases (Miller et al., 2016). Therefore, estradiol could have significant impacts on the vaginal microbiome and changes in the microbial composition could be dependent on circulating levels of estradiol.

There is a significant lack of research evaluating the vaginal microbiome's role in bovine fertility and how the microbiome could be influenced by reproductive hormones. Therefore, the objectives of this study were to characterize the vaginal microbiota and to determine if vaginal microbiota composition differed across divergent concentrations of estradiol at time of artificial insemination (AI) in 78 Brangus heifers. Additionally, we also aimed to compare the vaginal microbiota composition at time of AI between heifers that were retrospectively classified as pregnant or nonpregnant. We hypothesized that different vaginal microbiota communities will either facilitate or inhibit pregnancy. Additionally, we hypothesized heifers with high, medium, and low concentrations of estradiol at the time of AI will differ in vaginal microbiota composition.

Materials and Methods

Animal Management and Treatment

Animal care and use were approved by the Mississippi State University Institutional Animal Care and Use Committee (#18-386). Brangus heifers (n = 78) were housed at the H.H. Leveck Animal Research Center (Mississippi State, MS) in a 25-acre pasture grazing seasonal grasses. All heifers were fed the same concentrate diet twice daily with ad libitum water and minerals. Heifers were approximately 18 months old at time of breeding with an average weight of 405 ± 38 kgs. All heifers underwent a 7-day Co-Synch + CIDR estrous synchronization protocol. Heifers were synchronized and artificially inseminated in six groups over a three-week period. All heifers were housed in the same pasture throughout the study. All heifers were artificially inseminated with commercially available semen from a single Angus sire. Trans-rectal ultrasonography was used to determine pregnancy status between days 41 and 57 post-artificial insemination dependent on breeding group (Fig 2.1).

Vaginal Swab Collection

A double guarded equine uterine culture swab (Minitube Ref. 17214/2950) was utilized to sample the anterior vaginal tract of each heifer immediately before AI. Heifers were restrained in a hydraulic chute and the vulva was cleaned, by wiping with a paper towel, to prevent swab contamination. The double guarded unit containing the swab was removed from sterile packaging and immediately inserted through the vulva into the vaginal tract. The swab was angled upward, over the pelvic shelf, and towards the anterior vagina. Once the swab would not move forward with pressure, the cotton swab was exposed from the sterile guarding to make direct contact with the anterior vagina. The swab was rotated for approximately 30 seconds then retracted back into the sterile guarding. The entire double guarded swab unit was removed from

the heifer's vaginal tract. After removal, the unit was broken down, by removing the external layer to expose the swab in the sterile tubing. The sterile tube containing the swab was snapped at a pre-determined length, then capped with sterile caps to prevent airborne contamination. The swab was closely examined in the sterile guarding for any urine (yellow staining) or feces. If contamination looked possible, the heifer was re-swabbed. Two uncontaminated swabs were collected per animal. The swabs were stored in -80°C with the swab end upright in a sterile tube until further analysis.

Blood Collection and Estradiol Assay

At time of AI, blood was collected via coccygeal venipuncture. Blood was allowed to clot at room temperature and placed on ice until transported to the laboratory for processing. Approximately two hours after collection, blood tubes were centrifuged at 2000xg at 4°C for 10 minutes. Serum was immediately collected and transferred into sterile 1.5 mL tubes and then stored at -80°C until further analysis. Circulating serum concentrations of estradiol were analyzed via radioimmunoassay with methodology previously published by Perry et al. (2004). The intra- and inter-assay CV for estradiol assays were 4.7% and 6.1% respectively. Heifers were classified into low (1.1 to 2.5 pg/mL; n = 21), medium (2.6 to 6.7 pg/mL; n = 30), and high (7.2 to 17.6 pg/mL; n = 27) concentration of estradiol based off previous literature (Jinks et al., 2013) (Fig 2.1).

Bacterial Community Analysis

The bacterial community analysis was performed by Microbiome Insights Co. located in Vancouver, Canada. Genomic DNA was extracted using the MoBio PowerMag Soil DNA Isolation Bead Plate (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) on a KingFisher robot

following the manufacturer's protocol. Blank control swabs (n=2) were included in the analysis for contamination checks. Bacterial amplicons from the V4 region of the 16S rRNA gene were amplified using the 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and the 806R 5'-GGACTACHVGGGTWTCTAAT-3' primers as described by Kozich et al. (2013). Sequencing was done with an Illumina MiSeq (Illumina, San Diego, CA, USA) using the 300-bp paired-end kit (v.3). Quality filtering, taxonomic classification using the Greengenes (v. 13_8) database, and clustering into 97%-similarity operational taxonomic units (OTU) were done using the mothur software package (v. 1.39.5) (Schloss et al., 2009).

Statistical Analysis

The vaginal bacterial community comparisons of interest were between pregnant and nonpregnant heifers and across the low, medium, and high estradiol concentrations. The R software program (R Core Team, 2013) was used to conduct the statistical analyses. Alpha diversity was calculated using the Shannon index and significance was tested using ANOVA. Beta diversity was computed using the Bray-Curtis dissimilarity and visualized using the principle coordinate analysis (PCoA) plot. Differences in community structure were assessed using the permutational multivariate analysis of variance (PERMANOVA) with pregnant vs nonpregnant or low, medium, or high estradiol group as the main fixed factor and using 9999 permutations for significance testing in R (adonis function from the Vegan package). After estradiol classification, the PROC Mixed procedure in SAS 9.4 Software was utilized to determine significance between classification groups.

Results

Sequencing Information

A total of 80 swabs were analyzed including two blank control swabs. Vaginal swabs from heifers grouped retrospectively as pregnant (n=29) or nonpregnant (n=49). A total of 1,156,788 quality-filtered reads were obtained with an average of 14,459 quality-filtered reads per sample that were assigned to 8,358 OTU. Control samples had minimum quality-filtered reads (80 and 218), thus contamination was considered absent. Samples with less than 1000 quality-filtered reads were removed from the analyses (3 nonpregnant and 2 pregnant samples).

Taxonomic Composition

The four most abundant phyla were Tenericutes (36%), Proteobacteria (30%), Fusobacteria (7.6%), and Firmicutes (1.8%), respectively. There were no compositional differences (Fig. 2.2) or proportional differences (Fig. 2.6) between nonpregnant and pregnant heifers Additionally, no compositional differences (Fig. 2.3) or proportional differences (Fig. 2.7) were seen across heifers with high, medium, or low estradiol concentrations

Comparing Vaginal Microbiota of Pregnant vs. Nonpregnant Heifers

There were no differences in alpha diversity between pregnant and nonpregnant heifers (Fig. 2.4A, $P = 0.366$). The Shannon diversity index showed pregnant heifers with a mean of 2.18 ± 1.68 and nonpregnant heifers with a mean of 2.22 ± 1.48. The PCoA displayed no clustering of samples by pregnancy status (Fig. 2.4B). Furthermore, the PERMANOVA showed similar ($P = 0.744$) overall bacterial profiles between pregnant and nonpregnant heifers. There were three OTUs that were different between pregnant and nonpregnant heifers which included *Pasteurella multocida* ($P < 0.001$), *Pasteurellaceae unclassified* ($P < 0.001$), and *Fusobacterium unclassified*

($P = 0.001$). *Pasteurellaceae unclassified* and *Fusobacterium unclassified* were more abundant in nonpregnant heifers whereas *Pasteurella multocida* was more abundant in pregnant heifers. Noteworthy, only three animals lacked the presence of at least one of these species within their reproductive tract.

Estradiol Concentrations

There were significant differences between heifer group classifications for their serum estradiol concentrations; heifers classified in the low, medium, or high serum estradiol group had significantly different ($P < 0.05$) serum estradiol concentrations. Alpha diversity was measured using Shannon Index to evaluate differences between the vaginal microbiotas of heifers with high, medium, and low serum estrogen concentrations (Fig. 2.5A). No differences were found across the high (2.16 ± 1.46), medium (2.13 ± 1.53), and low (2.37 ± 1.74) samples (Fig. 2.4A, $P= 0.512$). There were no differences in beta diversity across these samples either ($P = 0.48$). A principal component analysis was utilized to visualize the relationship across samples and no clusters were observed (Fig. 2.5B). There were eight statistically different OTUs observed in animals with varying estradiol concentrations. *Leptotrichiaceae unclassified* ($P < 0.0001$), *Pasturella multocida* ($P < 0.001$), and *Pasteurellaceae unclassified* ($P < 0.001$) were more abundant in heifers with high concentrations of estradiol compared to heifers with low and medium concentrations of estradiol. *Histophilus somni* ($P < 0.001$), *Actinobacillus seminis* ($P < 0.001$) and *Fusobacterium unclassified* ($P = 0.001$) were more abundant in the vaginal microbiota of heifers with medium and low estradiol concentrations compared to heifers with high concentrations of estradiol. *Bacteroidetes unclassified* ($P < 0.001$) were more abundant in the vaginal microbiota of heifers with low estradiol concentrations compared to heifers with medium and high estradiol concentrations.

Discussion

There were no differences in the overall bacterial community composition between heifers that became pregnant versus nonpregnant. However, the taxonomic profiles within our study are different compared to current literature. Other studies commonly report Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria as the most abundant phyla within the vaginal tract of the bovids (Lima et al., 2019; Nascimento et al., 2015). In contrast, the phyla Tenericutes and Fusobacterium were more abundant, than Bacteroidetes and Actinobacteria, in our heifers. The implications of this difference are not yet known. Differences could be attributed to environmental factors, age, sexual maturity, or fertility issues within the sample groups, but more research is needed to confirm these speculations.

Nevertheless, species level differences in this study between pregnant and nonpregnant heifers could have implications surrounding fertility and reproductive performance. Specifically, the presence of *Fusobacterium spp.* and *Pasteurella multocida* in nonpregnant heifers. *Fusobacterium* species have been found to be causative agents in morbidity and mortality in both humans and animals (Afra et al., 2013; Citron, 2002). *Fusobacterium spp.* are anaerobic, gram-negative rods that are considered pathogenic in humans (Huggan and Murdoch, 2008). Specifically, *Fusobacterium necrophorum* is a known causative agent of puerperal metritis in post-partum cows (Machado et al., 2014). Therefore, nonpregnant heifers in this study with colonization of *Fusobacterium spp.* could have had vaginal infections inhibiting pregnancy success. Next, *Pasteurella multocida*, is an opportunistic pathogenic, gram-negative rod that has previously been associated with bovine respiratory disease in cattle (Dabo et al., 2007). It is debatable whether the colonization of *P. multocida* has negative or positive impacts on fertility. It is still undetermined if *P. multocida* is pathogenic in the reproductive tract. Lima et al (2019), found that calves

inoculated with upper respiratory tract potential pathogens, such as *P. multocida,* at birth were less likely to develop disease later in life. It could be speculated, that *P. multocida* is considered pathogenic in nonpregnant animals, but during gestation it increases for inoculation of the calf at parturition. Further studies following the vaginal microbiota throughout gestation are necessary to provide evidence to support this hypothesis.

There were no differences in the microbial composition of heifers with high, medium, and low concentrations of estradiol. These results are in agreement with a study published by Ault et al, 2019 where they compared progesterone levels to the microbiota, they found no differences in the microbiota with varying progesterone concentrations. These conclusions are quite shocking considering the significant impacts hormones have on the vaginal tract throughout the estrous cycle (Jinks et al., 2013). Therefore, while we may not have seen any direct differences at time of AI, we cannot rule out with complete certainty that hormones have no impacts on the vaginal microbiota throughout gestation.

In the current study, all heifers had undergone an estrus synchronization protocol with a CIDR. All heifers had a new CIDR inserted for 7-days according to the protocol. The CIDR applicator was cleaned with Nolvasan solution between heifers to prevent contamination. To date, there is a lack of knowledge surrounding how an estrus synchronization protocol, especially using CIDRs, could influence the vaginal microbiota. In microbiology, it is widely accepted that specific bacteria require a certain pH range to grow regardless of their environment. In cattle, the pH of the vaginal tract fluctuates during the estrous cycle; the pH of the vaginal tract is the most acidic during ovulation when estradiol is peaking (Lewis and Newman, 1984). Estradiol causes a change in vaginal secretions and causes a more acidic vaginal environment compared to a vaginal environment under the influence of progesterone (Gorodeski et al., 2008). A limitation to this study

is that vaginal pH was not measured in these heifers. Moreover, there has also been speculation that the immunosuppressive role of progesterone in the reproductive tract environment could allow for the easy colonization of pathogenic bacteria (Padula and Macmillan, 2006). Studies in humans, have eluded to this concept where they have found women in the luteal phase of their menstrual cycle are more susceptible to sexually transmitted diseases versus women in the follicular phase (Wessels et al., 2018). It is not clear the influence that exogenous progestins have in bovine species, but Martinez-Ros et al. (2018) showed that CIDRs altered the vaginal pH in ewes.

In the majority of bovine estrus synchronization protocols there is a very short window between the removal of the CIDR and artificial insemination (Beef Reproductive Task Force, 2019). Therefore, a CIDR insert could alter the vaginal pH by decreasing the acidity which inhibits growth of commensal bacteria. Commensal bacteria often inhibit the growth of pathogens in environments by competing for nutrients and attachment sites (Abt and Pamer, 2014). Due to insufficient time before artificial insemination, recolonization of commensal bacteria that may be conducive to pregnancy by preventing the growth of pathogens is not possible and therefore, decreased fertility results. Additionally, studies have shown that CIDRs can cause vaginosis and improper handling could introduce bacteria into the vaginal tract (AgriHealth, 2018). Dias et al. (2019) found that vaginosis caused by CIDR usage does not affect fertility in beef cattle; it is possible that the symptoms of vaginosis commonly found after CIDR insertion could be an immune response to the foreign object itself. Nevertheless, an immune response in the vaginal tract during CIDR insertion could result in a decrease in commensal bacteria translating to an environment that is more susceptible to colonization of pathogens. Therefore, more research focused on CIDR usage and its relationship with bacterial colonization of the reproductive tract is needed.

Similarly, to the pregnant and nonpregnant heifers, there were differences in specific OTUs between heifers with different estradiol concentrations. *P. multocida, Leptotrichiaceae unclassified,* and *Histophilus somni* have all been associated with BRD by being found in the lungs of infected animals post-mortem (Johnston et al 2017; Hellenbrand et al., 2013). The significance of these bacteria present in the vaginal microbiota at time of AI is not clear. These bacteria were more commonly present in heifers with medium to high concentrations of estradiol compared to heifers with low concentrations of estradiol. In theory, these heifers should have been more receptive to breeding and pregnancy, but the pregnancy rate in the current study was very poor (36%); it is possible these bacteria were acting as opportunistic pathogens at timed artificial insemination (TAI) in these Brangus heifers. Furthermore, these bacteria commonly grow in a pH that is near neutral, heifers with high or medium estradiol should have had a slightly acidic vaginal pH to inhibit the growth of these opportunistic pathogens (Bandara et al., 2018; Cho et al., 2017; Shah et al., 2008). The presence of these bacteria makes it more plausible that vaginal pH was not physiologically decreased enough at time of artificial insemination to inhibit the growth of these opportunistic pathogens which eludes to the heifers with high estradiol not having more pregnancies (Figure 2.1). A potential mechanism for these heifers having a pH closer to neutral is CIDR usage with inadequate time for estradiol to alter the vaginal environment before AI. More directly in humans, estradiol acidifies the vaginal tract by active proton secretion through the apical vaginal membrane by utilizing tight junctions to maintain an acidic environment (Gorodeski et al., 2008). Progesterone, the hormone on CIDRs, inhibits the insertion of tight junction proteins in the reproductive tract (Garfeild et al., 1978). Therefore, it is plausible that CIDR insertion prevents normal physiological processes from occurring before ovulation leading to an increased vaginal pH at AI accompanied by increased opportunistic pathogens in the vaginal microbiota.

Additionally, *Actinobacillus seminis* was significantly increased in heifers with low to medium estradiol concentrations at TAI compared to heifers with high concentrations of estradiol. In sheep and goats, *A. seminis* causes infections in both the male and female reproductive tract that leads to decreased fertility and abortions (Cebra and Cebra, 2012). Therefore, this bacterium could be negatively affecting the reproductive tract by decreasing the cyclicity and fertility of heifers.

The differences in OTUs could indicate that vaginal dysbiosis was occurring in some groups of heifers. However, the microbiota of infected or normal bovine vaginal tracts throughout the estrous cycle has yet to be fully characterized. Therefore, dysbiosis cannot be confirmed because there is no standard healthy vaginal composition for comparison to our heifer's vaginal microbiota. Normally, dysbiosis occurs when the commensal bacteria die due to unfavorable environmental conditions. These conditions include changes in pH, decreased nutrient availability, introduction of chemicals, stress, antibiotic usage, dietary changes, and many other factors (Levy et al., 2017). The introduction of CIDRs, stress due to animal handling, and other environmental factors could have led to vaginal dysbiosis in the heifers used in the current study. Africa et al. (2014) evaluated the effects of bacterial vaginosis in women, finding that this infection altered the equilibrium of the normal vaginal microbiota and caused dysbiosis; furthermore, bacterial vaginosis has been reported to cause pelvic inflammatory disease, adverse pregnancy outcomes, increased susceptibility to reproductive tract infections, and infertility. Therefore, the decreased fertility observed in this study could be a result of vaginal dysbiosis, but conformation of this requires more characterization research specifically evaluating healthy heifers at TAI.

Lastly, it is important to note that in all heifers, the phyla Tenericutes were the most abundant within the vaginal microbiota, specifically Mycoplasma and Ureaplasma species. These bacteria are small pleomorphic obligate intracellular cocci that lack a cell wall (Combaz-Sohnchun

and Kuhn, 2017). Therefore, the identification of these bacteria using a microscope is extremely difficult. Due to the difficulty to culture and identify, these species could be easily overlooked in the analysis of fertility issues. Both species, Mycoplasma and Ureaplasma are commensals of the urogenital tract. *U. urealyticum* has been found to cause spontaneous abortions in women between 10-20 weeks of gestation (Ahmadi et al., 2014). Mycoplasma species have been associated with spontaneous abortions, polyarthritis, and mastitis in cattle (Hum et al., 2008). Normally, these species are more abundant in certain environments and inoculate the vaginal tracts of animals in those environments; therefore, certain farms are more susceptible to negative reproductive effects from these bacteria than others based on location (Murai and Higuchi, 2018). As previously mentioned, the pregnancy rates of the heifers utilized in this research were surprisingly low. The decreased fertility of these heifers may be attributed to the accidental introduction of Mycoplasma and Ureaplasma species to the uterine body by the artificial insemination gun; Hughes and Cranes (2018), found a potential for Ureaplasma species to infect embryos resulting in abortion, gestational losses, and pathologies in calves after birth. Therefore, many heifers on this project could have had successful conception, but once the embryo migrated to the uterine body these opportunistic pathogens caused early embryonic death leading to low pregnancy rates at 41-57 days post-AI.

In conclusion, no differences were found in the vaginal bacterial community profiles of heifers with different pregnancy status or estradiol concentrations. Overall, it can be concluded that the vaginal microbiota is diverse and finding significant differences in the composition as a whole has proven difficult. However, species level differences can provide insights to the innerworkings of the vaginal microbiota to determine if an animal is more susceptible to infection, spontaneous abortion, or successful conception. To fully understand the vaginal

microbiota, more research is needed; especially research focused on the species level changes in the vaginal microbiota throughout the estrus cycle, gestation, immediately following parturition, and any other significant reproductive events.

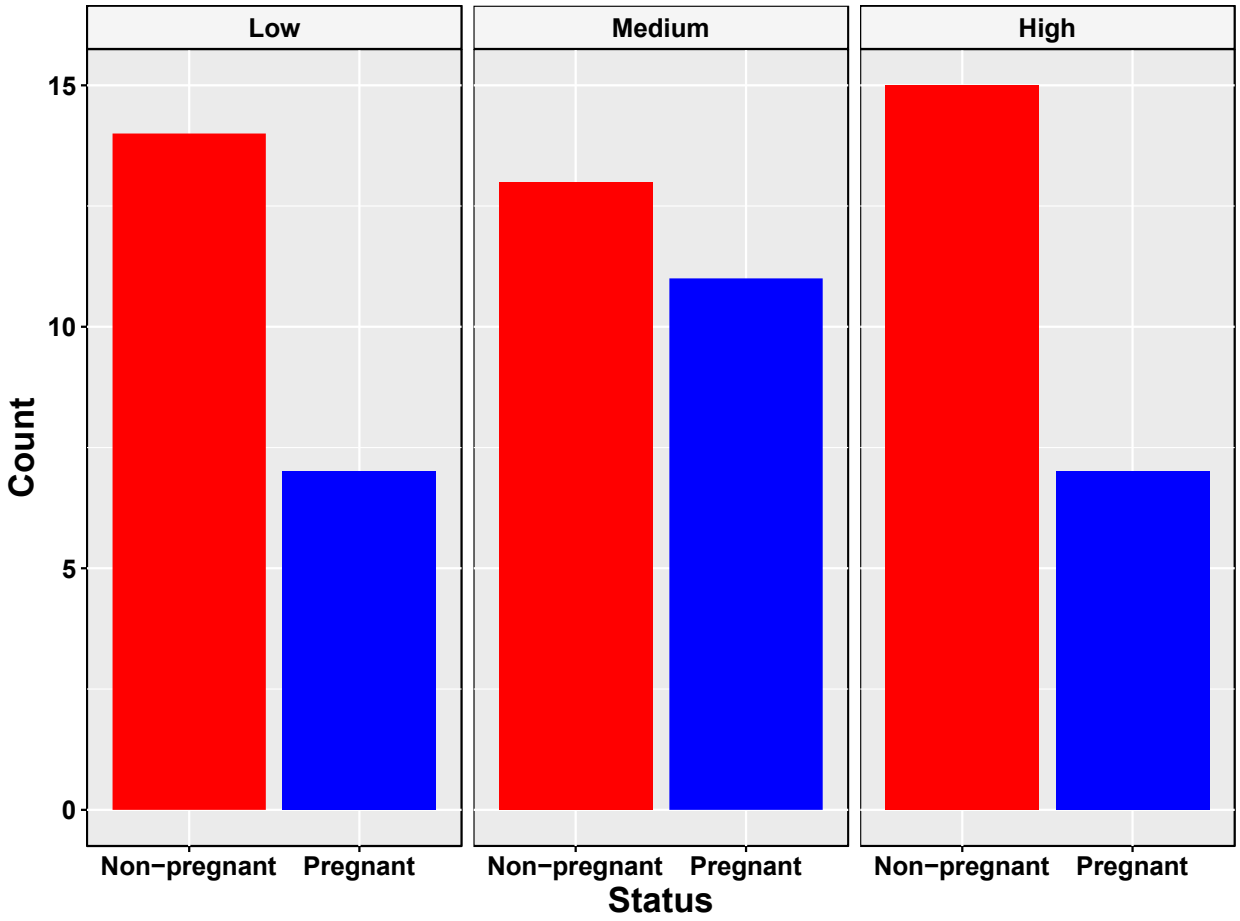

Figure 2.1 Heifer Pregnancy Status within Estradiol Concentration

A bar graph representing the number of nonpregnant (red bar) and pregnant (blue bar) heifers in each group (high, medium, or low) of estradiol concentration.

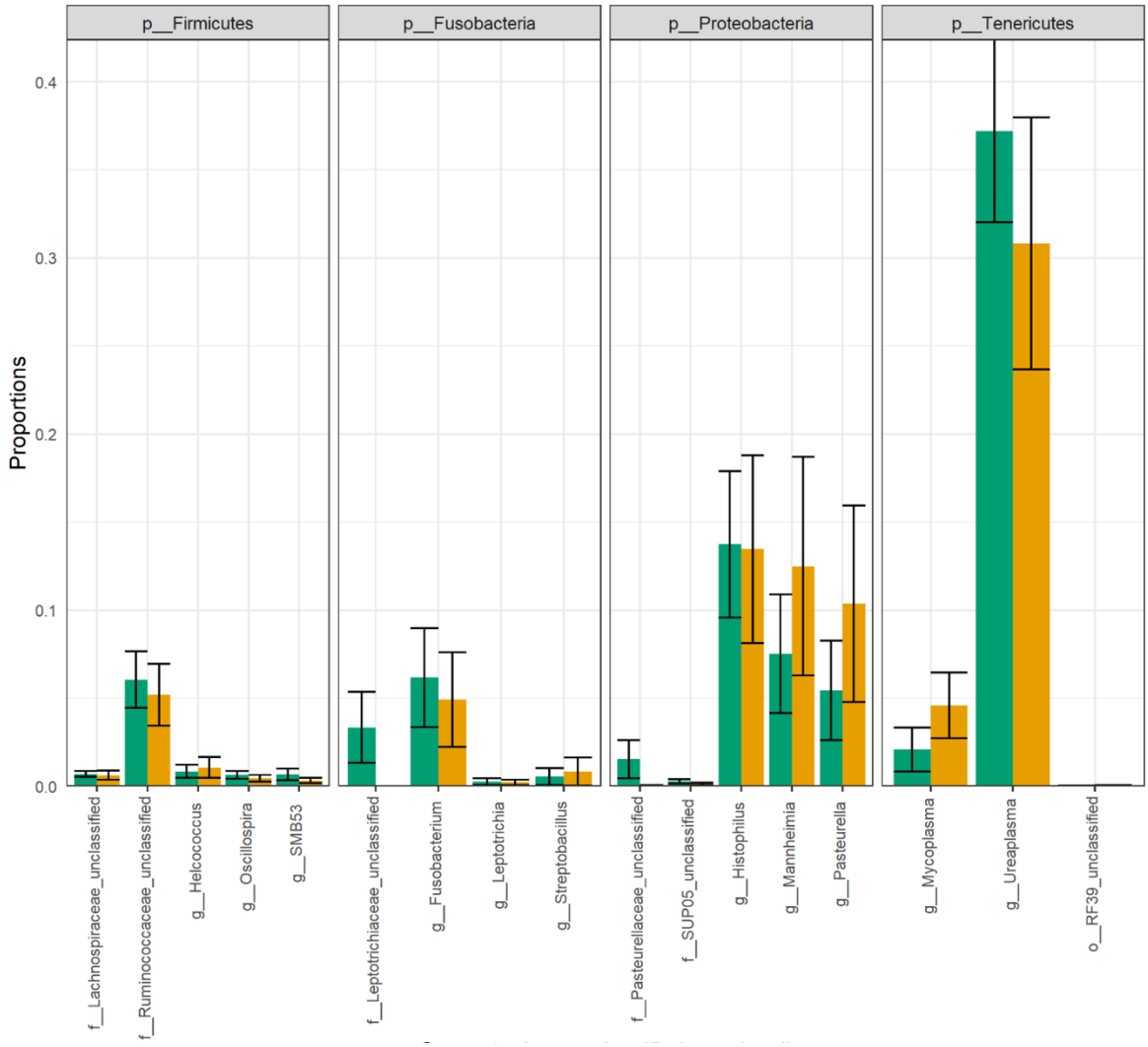

Figure 2.2 Relative Microbial Abundance in Pregnant and Nonpregnant Heifers

Relative abundance of the 5 most abundant genus-level taxa within the 4 most abundant phyla. The orange bars represent nonpregnant heifers and the green bars represent pregnant heifers.

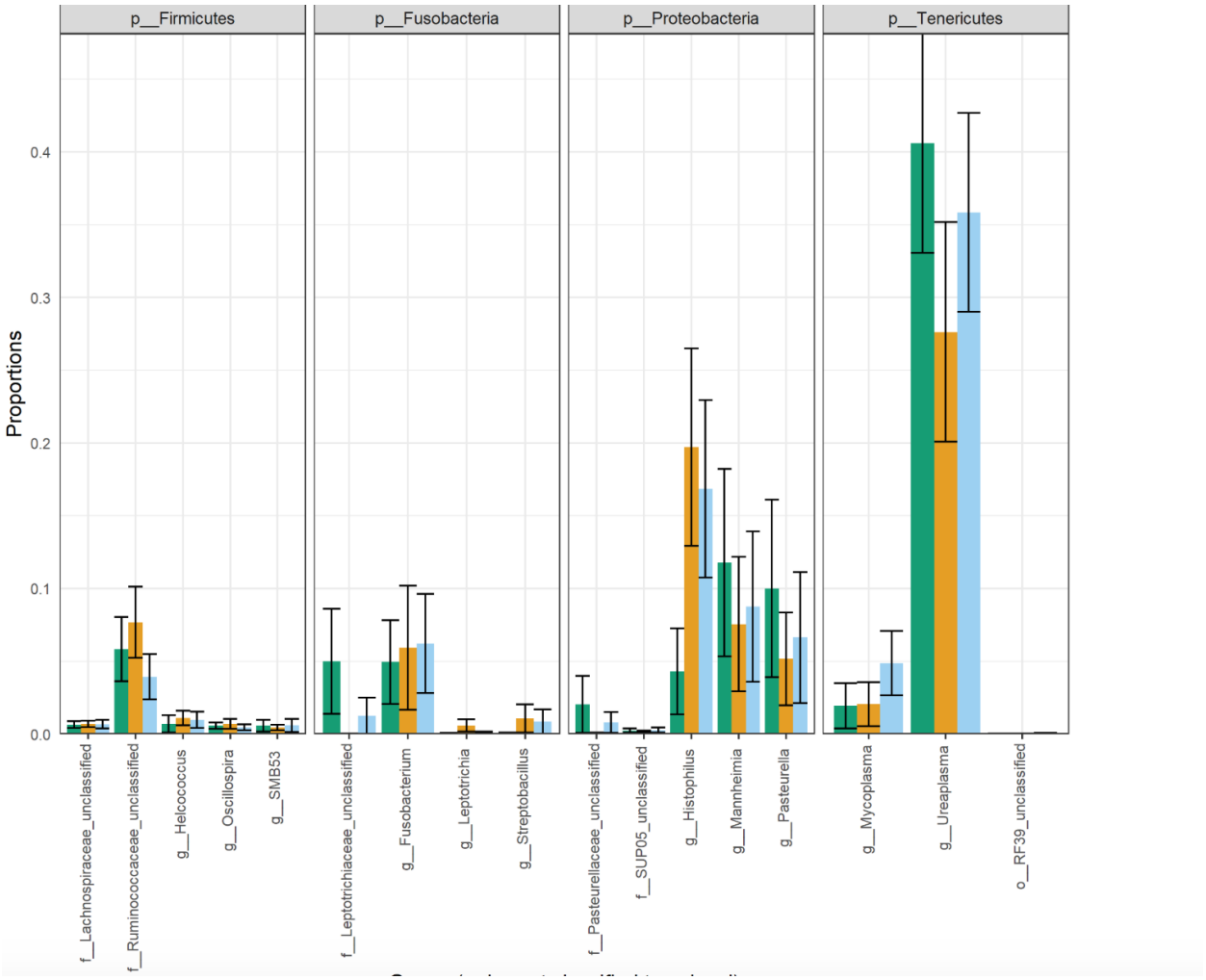

Figure 2.3 Relative Microbial Abundance in Heifers with Divergent Estradiol Concentrations

Relative abundance of the 5 most abundant genus-level taxa within the 4 most abundant phyla. The bars represent heifers with high (green), medium (blue) and low (orange) estradiol concentrations.

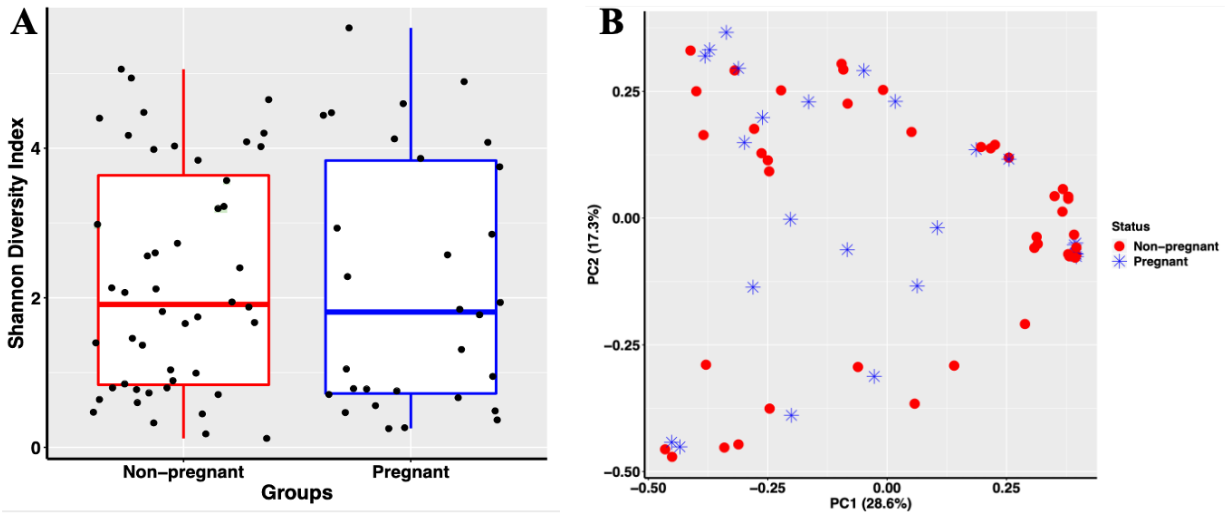

Figure 2.4 Alpha and Beta Diversity in Pregnant vs. Nonpregnant Heifers

Alpha diversity boxplot of the vaginal bacterial microbiota in Brangus heifers measured by the Shannon Diversity Index (Panel A). The red box represents nonpregnant heifers and blue box represents pregnant heifers. Black dots represent values for individual samples. Alpha diversity did not differ (P= 0.366) between pregnant vs. nonpregnant heifers. Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples (Panel B). Nonpregnant heifers are represented by red circles and pregnant heifers are represented by blue stars. There were no differences in beta diversity between pregnancy status (P= 0.74).

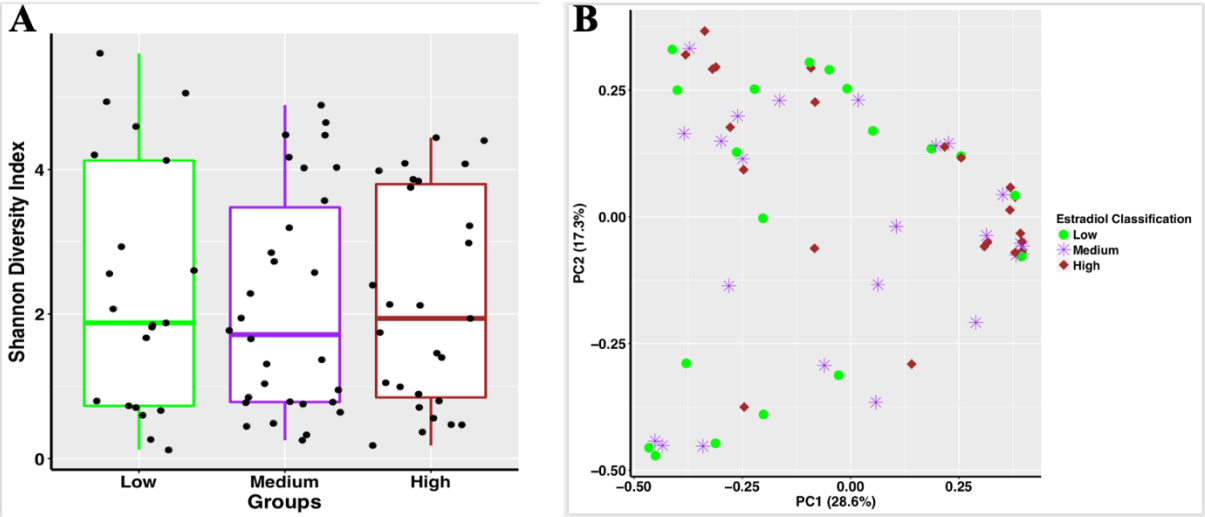

Figure 2.5 Alpha and Beta Diversity in Heifers with Divergent Estradiol Concentrations

Alpha diversity boxplot of vaginal bacterial microbiota in Brangus heifers measured by the Shannon Diversity Index (Panel A). The green box represents heifers with low estrogen concentration (1.1 - 2.5 pg/mL), the purple box represents heifers with medium estrogen concentration (2.6 - 6.7 pg/mL), and the brown represents heifers with high estrogen concentration (7.2 - 17.6 pg/mL). Black dots represent values for individual samples. Alpha diversity did not differ across groups (P= 0.51). Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples (Panel B). Heifers with high (maroon diamond; 7.2 - 17.6 pg/mL), medium (purple star; 2.6 - 6.7 pg/mL), and low (green circle; 1.1 - 2.5 pg/mL) estradiol concentrations are represented. There were no differences in beta diversity between estradiol groups (P= 0.48)

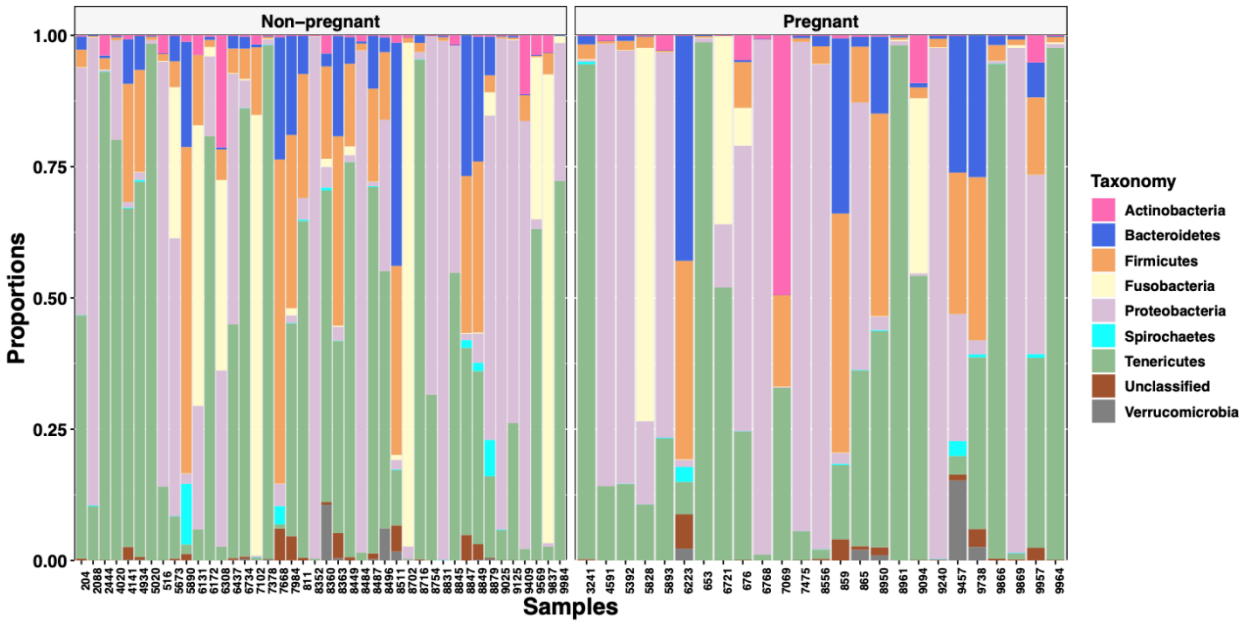

Figure 2.6 Taxonomy of Nonpregnant and Pregnant Heifers

A taxonomic bar plot of the phyla within the vaginal bacterial community of each sample.

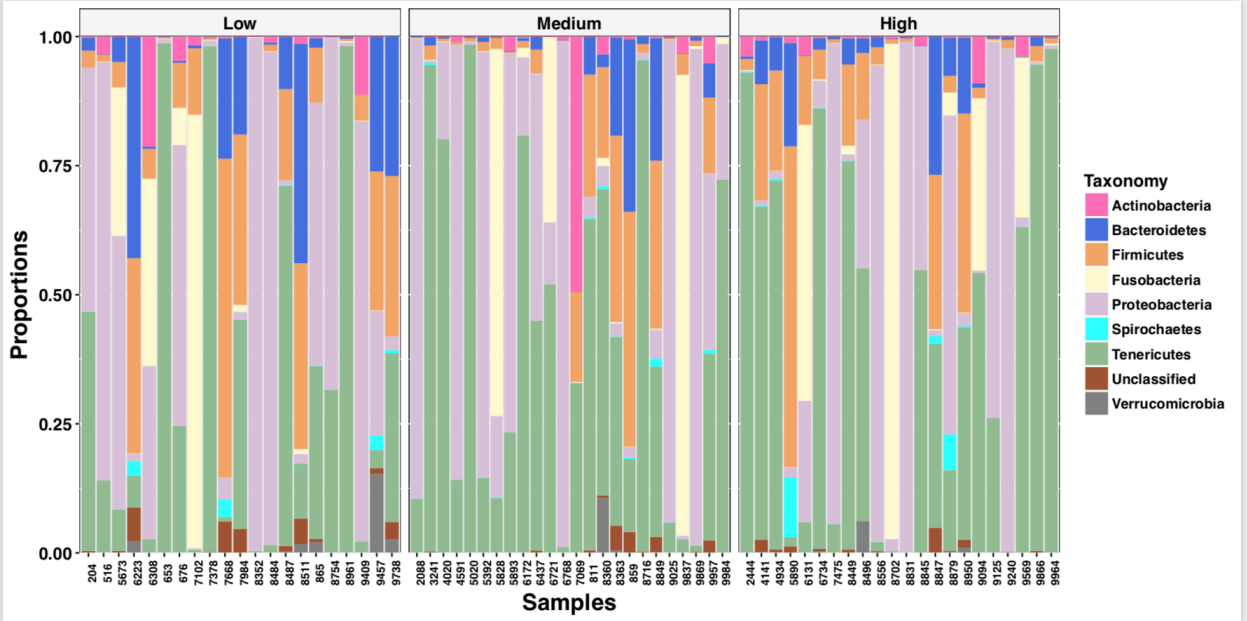

Figure 2.7 Taxonomy of Heifers with Divergent Estradiol Concentrations

A taxonomic bar plot of the phyla within the vaginal bacterial community of each sample. Starting at the left are samples from heifers with low estrogen concentration (1.1 - 2.5 pg/mL), medium estrogen concentrations (2.6 - 6.7 pg/mL) are represented in the middle bar, and high estrogen concentrations (7.2 - 17.6 pg/mL) within the right bar.

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CHAPTER III

MELATIONIN-ASSOCIATED CHANGES IN THE BOVINE VAGINAL MICROBIOTA DURING MATERNAL NUTRIENT RESTRICTION

Abstract

The objective of this study was to determine if gestational age, serum concentrations of progesterone, maternal nutrient restriction, or dietary melatonin supplementation alters the composition of the vaginal microbiota in Brangus heifers. First, we hypothesized that gestational age and concentrations of progesterone would influence the composition of the vaginal microbiota. Secondly, we hypothesized that maternal nutrient restriction or dietary melatonin supplementation during the late gestation would influence the composition of the vaginal microbiota. Brangus heifers (n=29) were sampled for vaginal microbiota analysis on day 0 (just prior to AI), day 140 (prior to dietary treatments), and day 220 of gestation (60 days post-treatment initiation) using a double guarded culture swab. At day 160 of gestation, heifers were assigned to dietary treatments in a 2 x 2 factorial design, in which animals were assigned to either an adequate (ADQ; n = 14) or restricted (RES; n = 15) nutritional plane and were either supplemented dietary melatonin (MEL; n =15) or control (CON; n = 14). The vaginal bacterial community composition was determined through sequencing of the V4 region from the 16S rRNA gene using the Illumina Miseq platform. Alpha diversity was compared via two-way ANOVA and beta diversity was compared via PERMANOVA. Gestational age did not alter the alpha (P= 0.87) or beta diversity (P= 0.11) of the vaginal microbiota. There were no differences

in alpha (P=0.27) or beta diversity (P= 0.60) in the vaginal microbiota of heifers with high vs. low serum concentrations of progesterone during mid-gestation. There were no differences in alpha diversity for maternal nutrient restriction (P=0.73) or melatonin treatment (P=0.49) or their interaction (P=0.22). There was a difference for melatonin treatment within beta diversity (P=0.02), but no difference in diet (P=0.40) or their interaction (P=0.52). This is the first study reporting alterations in the bovine vaginal microbiota following 60 days of dietary melatonin supplementation, while composition was not influenced by maternal nutrient restriction.

Introduction

The vaginal microbiota within the reproductive tract of cattle remains elusive despite an increase in research within this area. In recent years, researchers have sampled the vaginal microbiota at various points within the production and reproductive cycle, including immediately prior artificial insemination (Ault et al., 2019), during late gestation (Nascimento et al., 2015), and post-partum (Bicalho et al., 2017). Additional studies have analyzed if common reproductive hormones, such as progesterone or estradiol, can impact the composition of the vaginal microbiota at artificial insemination (Ault et al., 2019; Messman et al., 2020). The overwhelming conclusion of these studies is that the vaginal microbiota of the reproductive tract is dynamic. To date, the literature provides no evidence that any reproductive hormones significantly influence the bovine vaginal microbiota.

Moreover, little information exists evaluating how concentrations of progesterone or pregnancy itself can alter the vaginal microbiota. In humans, the vaginal microbiota was evaluated throughout gestation; researchers concluded that species level differences could occur between trimesters (Walther-Antonio et al., 2014). Additionally, the supplementation of exogenous progesterone during pregnancy in humans had no impact on the vaginal microbiota

(Kindinger et al., 2017). However, the impacts of naturally occurring concentrations of progesterone during gestation on the vaginal microbiota has yet to be investigated in any species. Therefore, the objectives, for experiment 1, were to compare the vaginal microbiota prior to conception and at mid-gestation and compare the vaginal microbiota of heifers with low (1.85 to 3.39 ng/mL) versus high (3.55 to 6.19 ng/mL) concentrations of serum progesterone at mid-gestation. We hypothesized, for experiment 1, that the vaginal microbiota would be in heifers between day 0 and day 140 of gestation and with low vs. high concentrations of serum progesterone.

Nutrient restriction during pregnancy negatively affects both the dam and fetus (Meyer et al., 2010; Camacho et al., 2014). Nutrient restriction can lead to a weakened immune system due to prolonged stress (Brown and Vosloo, 2017). Immunosuppression renders an animal more vulnerable to the inoculation of bacterial pathogens (George et. al., 2014). However, in sheep, the hormone melatonin has many impacts in rescuing fetal development, despite nutrient restriction throughout the last third of pregnancy (Lemley et al., 2012). Melatonin, a hormone produced in the pineal gland, has antioxidant capacity (Hardeland and Fuhberg, 1996), meaning it has implications for strengthening the immune system of the dam. In fact, Kumar et al. (2014), found that melatonin supplementation to summer-stressed, anestrus water buffalo increased their total antioxidant capacity, decreased oxidative stress biomarkers, and induced estrus in these animals. The ability of melatonin to mitigate stress could strengthened the immune system which prevents the inoculation of pathogens into the vaginal microbiota leading to a healthier pregnancy. Lastly, studies have found the nasopharyngeal tract of the calf to become inoculated with the vaginal microbiota at birth (Lima et al., 2018). Therefore, decreasing the risk of the vagina being inoculated with pathogenic bacteria could lead to a healthier dam and calf. Thus,

the objective, for experiment 2, was to determine if maternal nutrient restriction or dietary melatonin supplementation altered the composition of the vaginal microbiota. We hypothesized that both maternal nutrient restriction and dietary melatonin supplementation would change the composition of the vaginal microbiota.

Materials and Methods

Animal Management Timeline

Animal care and use were approved by the Mississippi State University Institutional Animal Care and Use Committee (#18-386). Brangus heifers (n = 78) were housed at the H.H. Leveck Animal Research Center (Mississippi State, MS) in a 25-acre pasture grazing seasonal grasses. All heifers were fed the same concentrate diet twice daily with ad libitum water and minerals. All heifers underwent a 7-day Co-Synch + CIDR estrous synchronization protocol. Heifers were synchronized and artificially inseminated in six groups over a three-week period. Trans-rectal ultrasonography was used to confirm pregnancy between days 41 and 57 post-artificial insemination dependent on breeding group. The nonpregnant animals were excluded from project (n = 49).

Pregnant Brangus heifers (n = 29) were housed at the H.H. Leveck Animal Research Center (Mississippi State, MS) in a 25-acre pasture grazing seasonal grasses until day 120 of gestation. At this time, heifers were moved into (15x45) foot pen with 4 heifers per pen. From day 120-140 of gestation, heifers underwent a 2-week Calan gate training period. At this time heifers were acclimated to being fed the concentrate diet, hay, and water sources within the pen. No treatments were imposed during this training period. When heifers started treatment, they were all able to open their respective Calan gate with a collar to obtain their feed. On day 160 of gestation, heifers began their treatment diets fed from the their respective Calan gates. Heifers

were fed once per day in the morning; pens were cleaned daily in the evening. Therefore, heifers were closely observed twice daily throughout the treatment period.

Dietary Treatments

On day 160 of gestation, heifers (n=29) were assigned to one of four treatment groups, stratified by body weight, in a 2x2 factorial arrangement. The treatment groups were as follows: nutrient restricted with melatonin supplementation (RES/MEL; n=8), adequately fed with melatonin supplementation (ADQ/MEL; n=7), nutrient restricted with no melatonin supplementation (RES/CON; n=7), or adequately fed with no melatonin supplementation (ADQ/MEL; n=7).

Concentrates containing treatments were fed at 09:00 hours daily in Calan gates, followed by a total mixed ration (TMR). Melatonin supplemented heifers received 0.9 kg of concentrate top-dressed with 2 mL of melatonin concentrated in ethanol (10 mg/mL). Control heifers also received 2 pounds of the same dietary concentrate top-dressed with 2 mL of ethanol containing no melatonin. Heifers were closely monitored to ensure they were consuming all of the correct treatment before being fed their TMR, and only after the treatment concentrate was consumed entirely was the TMR provided.

Each heifer was given one of two treatments consisting of 100% (adequate fed; ADQ; n = 14) or 60% (nutrient restricted; RES; n = 15) of net energy requirements for gestating cattle (NRC, 2000). Based on NRC (2000), in order for heifers to gain 1kg/day, the net requirements were calculated to be 0.012 Mcal/kg of body weight (BW). For maintenance energy, net energy requirements were calculated to be 0.018 Mcal/kg of BW. Heifers were fed a TMR (89.9 % DM and on a DM basis 20.7% grass hay, 7.65% vitamin mix, and 71.65% base diet). ADQ heifers were fed the TMR at a rate of 2.4% BW/day and chopped hay at a rate of 0.5% BW/day. For

RES heifers, the TMR was fed at a rate of 1.2% BW/day and hay at 0.3% BW/day. Both TMR (1.0% BW and 0.6% BW for ADQ and RES, respectively) and hay (0.25% BW and 0.15% BW for ADQ and RES, respectively) were offered twice daily and heifers had *ad libitum* access to water. Hay and TMR amounts were determined based on body weight; body weights were updated every week and diets were adjusted accordingly. The TMR was composed of corn, cotton seed hulls, soy hulls, molasses, limestone, and salt; the diets in this study are consistent with previous dietary treatments in nutrient restriction studies (Long et al., 2010).

Vaginal Swab Collection

A double guarded equine uterine culture swab (Minitube Ref. 17214/2950) was utilized to sample the anterior vaginal tract of each heifer on day 0, 140, and 220 of gestation. Samples were collected according to the protocol in Messman et al. (2020).

Blood Collection and Progesterone Assay

At day 140, blood was collected via coccygeal venipuncture immediately following vaginal swab collection. Blood was allowed to clot at ambient temperature then placed on ice until transported to the laboratory for processing. Approximately two hours after collection, blood tubes were centrifuged at 2,000 xg at 4°C for 10 minutes. Serum was immediately collected and transferred into sterile 1.5 mL tubes and then stored at -80°C until further analysis. Circulating serum concentrations of progesterone were analyzed according to Owen et al. (2018) using a radioimmunoassay kit from MP Biomedicals (0727010-CF). The intra-assay CV was 11.6%. Heifers with a concentration under 3.5 ng/mL progesterone were considered low progesterone animals (n = 14) whereas heifers with progesterone concentrations over 3.5 ng/mL were considered to be high progesterone animals (n = 15).

Bacterial Community Analysis

The bacterial community analysis was performed by Microbiome Insights Co. located in Vancouver, Canada. Genomic DNA was extracted using the MoBio PowerMag Soil DNA Isolation Bead Plate (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) on a KingFisher robot following the manufacturer's protocol. Blank control swabs (n=2) were included in the analysis for contamination checks. Bacterial amplicons from the V4 region of the 16S rRNA gene were amplified using the 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and the 806R 5'-GGACTACHVGGGTWTCTAAT-3' primers as described by Kozich et al. (2013). Sequencing was done with an Illumina MiSeq (Illumina, San Diego, CA, USA) using the 300-bp paired-end kit (v.3). Quality filtering, taxonomic classification using the Greengenes (v. 13_8) database, and clustering into 97%-similarity operational taxonomic units (OTUs) were done using the mothur software package (v. 1.39.5) (Schloss et al., 2009).

Statistical Analysis

For experiment 1, the vaginal bacterial community comparisons of interest were among heifers with low and high progesterone and from d 0 to d 140 of gestation. The R software program (R Core Team, 2013) was used to conduct the statistical analyses. Alpha diversity was evaluated using the Shannon index and significance was tested using ANOVA, whereas beta diversity was evaluated using the Bray-Curtis dissimilarity and visualized using the principle coordinate analysis (PCoA) plot. Differences in community structure were assessed using the permutational multivariate analysis of variance (PERMANOVA) with treatment group as the main fixed factor and using 9999 permutations for significance testing in R (adonis function from the Vegan package). After progesterone classification, the PROC Mixed procedure in SAS 9.4 Software was utilized to determine significance between classification groups.

For experiment 2, a 2 x 2 factorial statistical design was utilized to compare the vaginal bacterial community among treatment groups. The R software program (R Core Team, 2013) was used to conduct the statistical analyses. Alpha and beta diversity were evaluated with similar methods to concentrations of progesterone analysis. Significance was set at $P \leq 0.05$.

Results

Experiment 1 Results

For the gestational age analysis, a total of 58 vaginal swabs (n = 2 per heifer) were included. Bacterial community composition of the sample obtained at day 0 of gestation (n = 29) was compared to the swab collected on day 140 of gestation (n = 29). A total of 475,687 quality-filtered reads were obtained with an average of 8,201 quality-filtered reads per sample that were assigned to 10,668 OTUs. There were no differences in the composition of the vaginal microbiota from day 0 compared to day 140 of gestation. The most abundant phylum in all samples was Proteobacteria; the most abundant species was *Cupriavidicus spp*. There were no differences in alpha diversity ($P = 0.87$; Fig 3.1A) or beta diversity ($P = 0.11$; Fig 3.1B) between the vaginal bacterial composition of heifers at day 0 or 140 of gestation. Lastly, the relative abundance of any OTUs identified were not different between day 0 and 140 of gestation in these heifers.

As anticipated, the groups classification of heifers with low (1.85 to 3.39 ng/mL) or high (3.55 to 6.19 ng/mL) concentrations of serum progesterone were significantly different ($P < 0.05$). A total of 29 vaginal swabs from d 140 were analyzed from heifers grouped by high (n = 15) versus low (n = 14) concentrations of serum progesterone. A total of 259,361 quality-filtered reads were obtained with an average of 8,943 quality-filtered reads per sample that were assigned to 10,668 OTUs. The four most abundant phyla were Proteobacteria, Firmicutes,

Bacteroidetes, and Actinobacteria, respectively. There were no overall compositional or proportional differences between heifers with high versus low concentrations of serum progesterone. There were no differences in alpha diversity ($P = 0.27$; Fig 3.2A) or beta diversity ($P = 0.61$; Fig 3.2B) between heifers with high vs. low concentrations of serum progesterone at day 140 of gestation. Lastly, none of the OTUs identified were different between heifers with high vs. low concentrations of serum progesterone (Fig 3.3).

Experiment 2 Results

The maternal body weights of nutrient restricted heifers were different from adequately fed heifers at time of swab collection ($P < 0.005$). Additionally, the fetal body weights were different with nutrient restricted heifers having a decreased fetal body weight ($P < 0.005$) compared to adequately fed. Therefore, the nutrition treatments were sufficient in achieving an intra uterine growth restricted (IUGR) model.

A total of 29 vaginal swabs of heifers at day 220 of gestation were included in the analysis. These heifers were within the treatment groups of RES/MEL, ADQ/MEL, RES/CON, or ADQ/CON. A total of 360,615 quality-filtered reads were obtained with an average of 12,435 quality-filtered reads per sample that were assigned to 10,668 OTUs. The interaction of melatonin supplementation and nutrition did not cause any species level differences in the vaginal microbiota (Fig 3.4). The most abundant phyla in all treatment groups was Proteobacteria with the most abundant species being *Cupriavidicus spp*. There were no differences in alpha or beta diversity for the interaction of melatonin supplementation and nutrition status ($P= 0.22$). There were no differences in alpha diversity ($P = 0.13$; Fig 5) or beta diversity ($P = 0.40$; Fig 6) between heifers that were RES vs. ADQ fed. Additionally, there was no difference in alpha diversity (0.49; Fig 5) for heifers that were supplemented with MEL vs.

CON. The beta diversity of the vaginal microbiota was significantly different ($P = 0.02$; Fig 6) between MEL vs. CON heifers. Lastly, none of the OTUs identified were differently abundant among treatment groups.

Discussion

Interestingly, the overall composition of vaginal microbiota did not change from day 0 to day 140 of gestation in Brangus heifers. Due to the drastic physiological changes within the reproductive tract during this time, we expected to observe differences in bacterial composition. Reproductive hormones, such as estrogen and progesterone, are the drivers of physiological changes within the reproductive tract during pregnancy (Jinks et al., 2013). However, in the study endogenous concentrations of progesterone during gestation did not influence the vaginal microbiota. This finding agrees with previous literature done by Ault et al. (2019) and Messman et al. (2020) who found that circulating concentrations of progesterone and estradiol do not change the vaginal microbiota at TAI, respectively. These studies suggest that endogenous physiological concentrations of reproductive hormones do not alter the vaginal microbiota. Therefore, since physiological levels of the reproductive hormones, that drive changes during gestation, do not affect the vaginal microbiota, it is logical to conclude that this is could explain why no differences in the composition of the vaginal microbiota throughout gestation from day 0 to 140. A potential limitation to this study is that heifers underwent treatments during the third trimester of pregnancy; therefore, although swabs were obtained, they could not be used for comparison to day 0 and day 140 swabs.

A notable difference between the vaginal microbiota at day 0 of gestation and day 140 was the appearance of the *Cupravidicus spp.* This species was present in heifers on day 140 and 220 of gestation; additionally, this species was present in the majority of heifers. The treatment

groups had similarly elevated levels of *Cupravidicus spp.* within their vaginal microbiota compared to other bacterial species (Fig. 6). *Cupravidicus spp.* are gram negative bacteria that were recently isolated from the soil, ground water, and human clinical specimens (Vandamme and Coenyne, 2004). This species is known for its affinity of metal, ability to survive in harsh environments, and use for biodegradation of persistent aromatic compounds (Moriuchi et al., 2019). There is a lack of literature detailing *Cupravidicus spp*'s. role as a pathogen within mammals, but some studies postulate that the species is capable of causing disease. Yahya and Mushannen (2019) found that *Cupravidicus pauculus* is capable of infecting both immunocompromised and healthy people presenting as pneumonia, sepsis, meningitis, bacteremia, and cardiogenic shock. However, it is difficult to determine if the *Cupravidicus spp.* presence in these heifers is pathogenic or merely a side-effect of the heifer's housing. The heifers were kept in concrete pens with Calan gates surrounded by metal fencing and gates; the pens were thoroughly cleaned every evening and power washed every two weeks. Therefore, *Cupravidicus spp.,* which have an affinity for metal, could have been on the metal bars surrounding each pen. This allows for easy colonization the vaginal tract either through direct contact with the metal bars, heifers laying in feces or soil near metal, or through power-washing causing *Cupravidicus spp.* to spread all over the pen. Therefore, it is more likely that heifers on this project were colonized with *Cupravidicus spp.* due to their environment. However, more research characterizing the vaginal microbiota in heifers during mid to late gestation is needed to confirm if the presence of *Cupravidicus spp.* is relevant or purely circumstantial in this study.

The objective of nutrient restriction in these heifers was to cause IUGR. Intra uterine growth restriction is defined as the failure of the conceptus to grow and develop to its genetic potential during pregnancy (Wu et al., 2006). Typically, IUGR results when physiological

stresses on the dam, such as nutrient restriction, cause decreased nutrient flux to the placenta resulting in less nutrient availability to the fetus (Redmer et al., 2004). There is limited literature evaluating how IUGR can be related to the vaginal microbiota. Studies in piglets (Huang et al., 2019) and mouse pups (Berthon et al., 2010), found that IUGR fetuses had an altered gut microbiota and abnormal inflammatory and metabolic profiles. These studies led Bardos et al. (2020) to suggest that dysbiosis throughout the maternal microbiota could result in systemic inflammation; if systemic inflammation occurred in uterine and placental tissues the surface area of nutrient transfer would be decreased further increasing IUGR.

In this study, there were no differences in vaginal microbiota composition between nutrient restricted versus adequately fed heifers. These results indicate that nutrient restriction does not influence the vaginal microbiota to cause compositional changes. However, the vaginal microbiota could influence IUGR; if there is dysbiosis in the vaginal microbiota then inflammation of the reproductive tract could occur translating to inflammation of placental tissues. Therefore, while no compositional changes were observed there could be underlying physiological effects of vaginal microbiota on IUGR pregnancies. More research evaluating specific pathogens, inflammation, and microbial composition of the vaginal microbiota in IUGR pregnancies is necessary.

Moreover, in this study, the beta diversity of melatonin supplemented heifers' vaginal microbiota was significantly different than control heifers. Melatonin is a steroid hormone that is produced in the pineal gland of the brain; it functions to regulate sleep-wake cycles (Pandi-Perumal et al., 2008), functions as an antioxidant (Zhao et al., 2017), and can alter peripheral blood circulation (Dubocovich et al., 2003). Melatonin's capability to function as an antioxidant could decrease the immunosuppressive effects experienced by dam's during gestation (Oliveira

et al., 2012). If the dam's immune system can decrease the ability of pathogens to inoculate the vaginal microbiota during gestation, this greatly reduces the risk of inflammation in the reproductive tract. It is unclear how melatonin altered the vaginal microbiota in this study, it appeared to decrease the presence of *Ureaplasma spp.*, but further analysis is needed to confirm. While this study was conducted in late gestation heifers, it would be interesting to evaluate the effects of melatonin on heifers prior to artificial insemination. If the vaginal microbiota could be altered to decrease the abundance of potential pathogens, especially *Ureaplasma spp.*, there are major implications for producer usage. More research evaluating the usage of melatonin as a therapeutic to alter the vaginal microbiota is needed to fully understand the mechanism by which the hormone is causing compositional changes.

In conclusion, this study confirmed that physiological circulating concentrations of hormones do not alter the vaginal microbiota. Moreover, there were no differences between the vaginal microbiota on day 0 and day 140 of gestation. More research evaluating the relationship between the vaginal microbiota and IUGR pregnancies is needed to fully understand the physiological effects dysbiosis could have on a growing fetus. Lastly, to date, melatonin is the only known exogenous hormone to alter the vaginal microbiota. As always, further research is needed to fully understand why melatonin significantly changes the vaginal microbiota, but the implications of melatonin in bovine reproductive microbiology could be important.

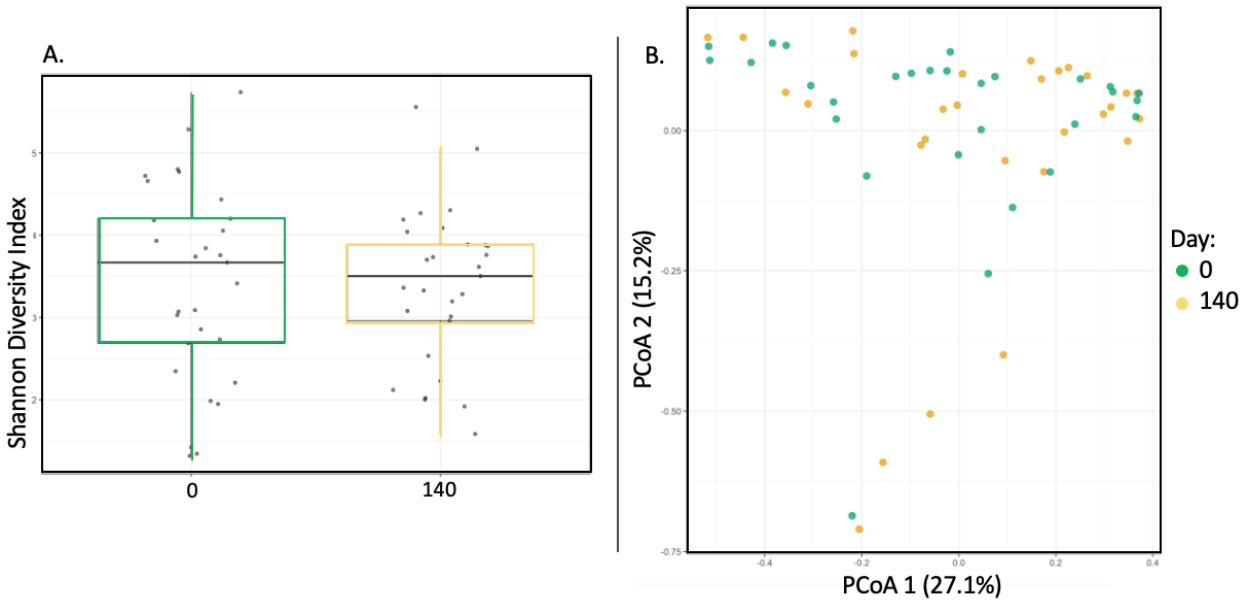

Figure 3.1 Alpha and Beta Diversity in Heifers at Day 0 and Day 140 of Gestation

Alpha diversity boxplot of the vaginal bacterial community in Brangus heifers measured by the Shannon Diversity Index (Panel A). The left box represents the vaginal bacterial community on day 0 of gestation and the right box represents the vaginal bacterial community on day 140 of gestation. Black dots represent values for individual samples. Alpha diversity of the vaginal bacterial community did not differ (P= 0.870) between day 0 and day 140 of gestation. Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples (Panel B). The vaginal bacterial community on day 0 of gestation (green circle) and the vaginal bacterial community on day 140 (yellow circle) are represented. There were no differences in beta diversity between day 0 and 140 of gestation (P= 0.11).

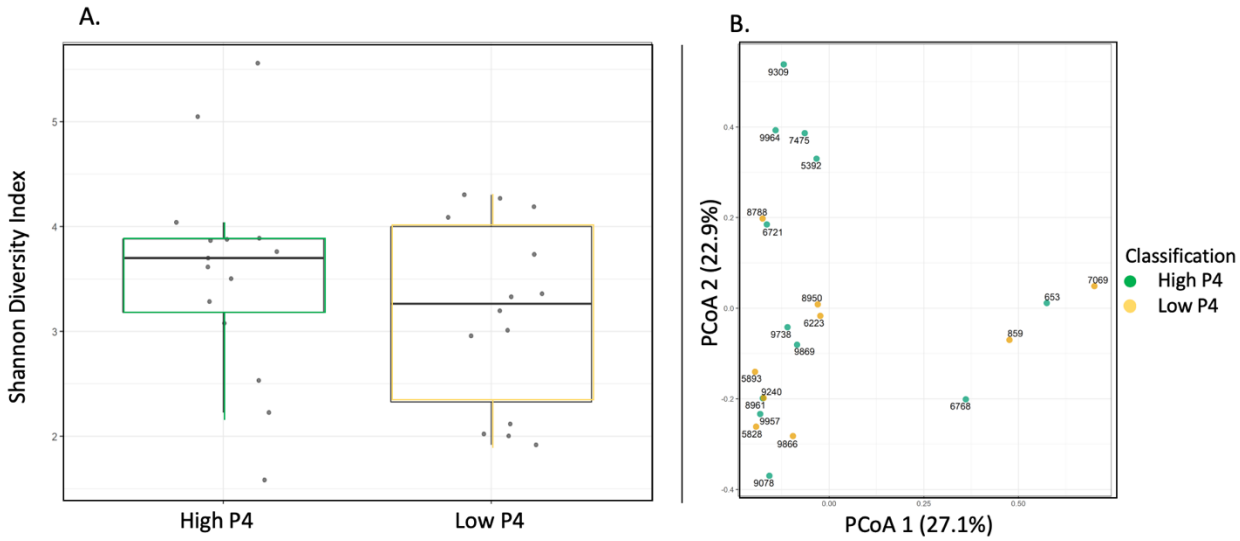

Figure 3.2 Alpha and Beta Diversity of Heifers with High vs. Low Serum Concentrations of Progesterone

Alpha diversity boxplot of the vaginal bacterial microbiota in Brangus heifers measured by the Shannon Diversity Index (Panel A). The left box represents vaginal bacterial community of heifers with high (3.55 to 6.19 ng/mL) concentrations of serum progesterone and the right box represents the vaginal bacterial community of heifers with low (1.85 to 3.39 ng/mL) concentrations of serum progesterone on day 140 of gestation. Black dots represent values for individual samples. Alpha diversity of the vaginal bacterial community did not differ (P= 0.273) between heifers with high vs. low concentrations of serum progesterone. Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples (Panel B). The vaginal bacterial community of heifers with high (green circle; 3.55 to 6.19 ng/mL) and low (yellow circle; 1.85 to 3.39 ng/mL) concentrations of progesterone are represented. There were no differences in beta diversity between progesterone groups (P= 0.606).

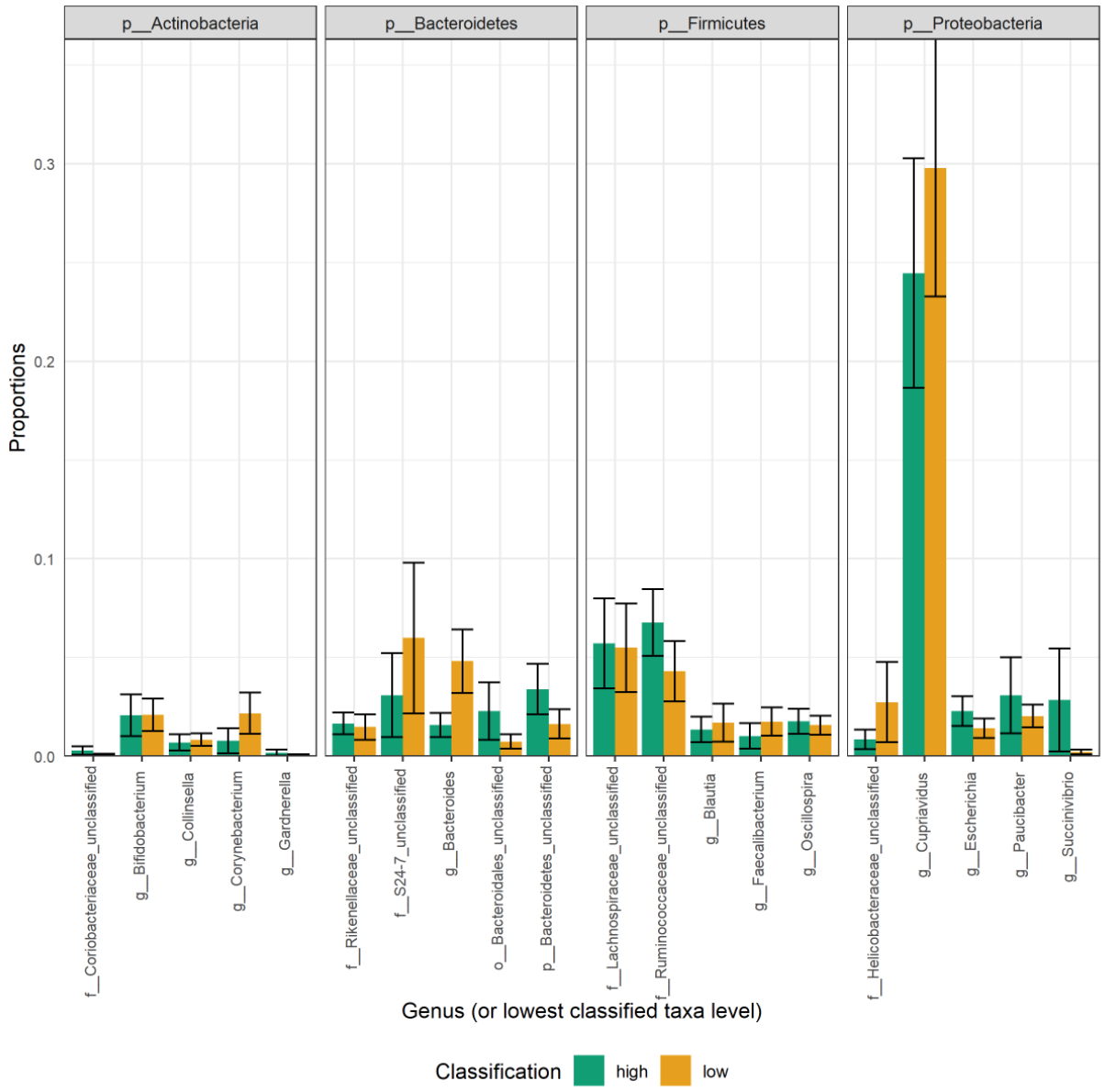

Figure 3.3 Relative Microbial Abundance in Heifers with High vs. Low Serum Concentrations of Progesterone

Relative abundance of the 5 most abundant genus-level taxa within the 4 most abundant phyla. The orange bars represent heifers with low concentrations of serum progesterone (1.85-3.39 ng/mL) and the green bars represent heifers with high concentrations of serum progesterone (3.55-6.19 ng/mL).

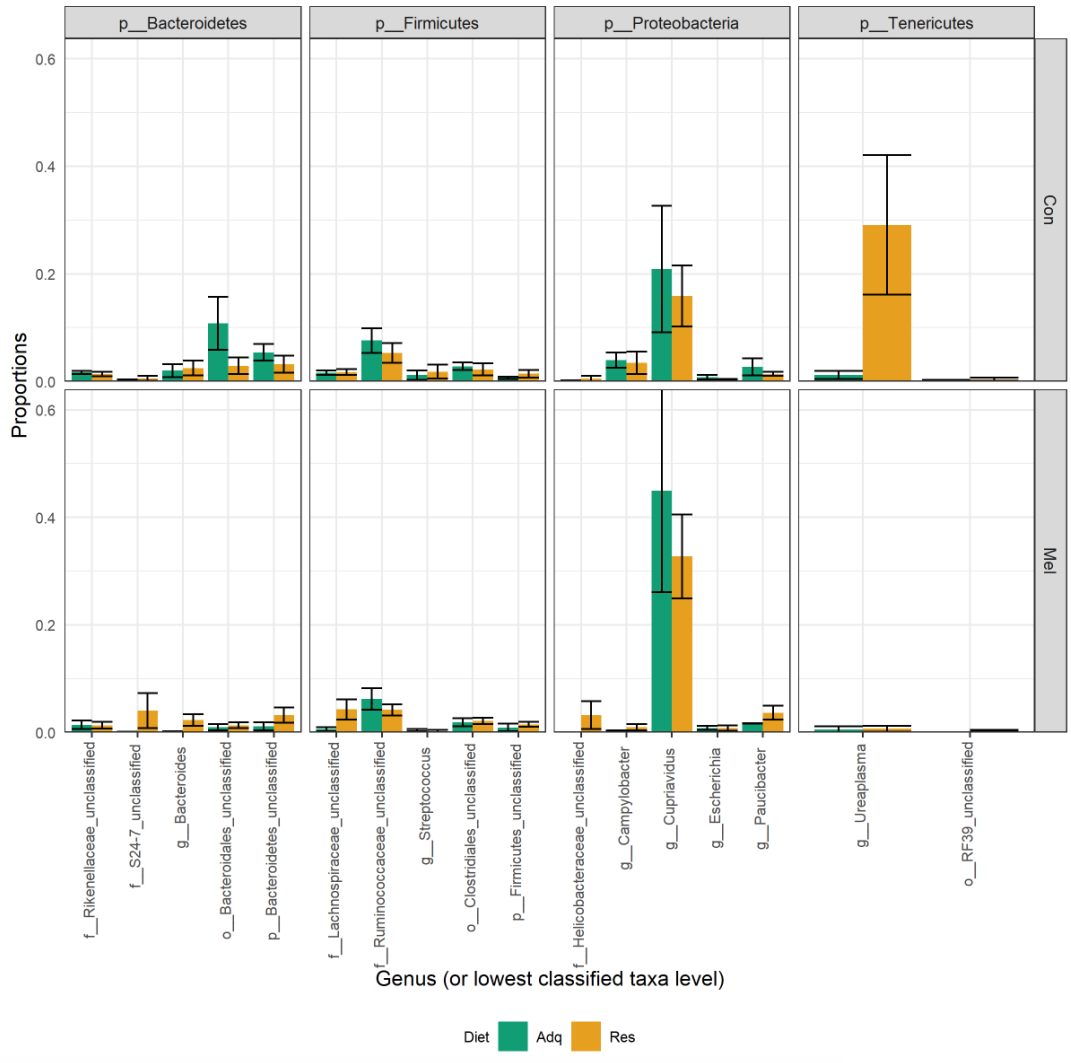

Figure 3.4 Relative Microbial Abundance Between Melatonin Supplemented vs. Control and Nutrient Restricted vs. Fed Heifer Treatment Groups

Relative abundance of the 5 most abundant genus-level taxa within the 4 most abundant phyla for control heifers (CON; top box) or heifers supplemented with melatonin (MEL; bottom box). The orange bars represent heifers that were nutrient restricted from day 160-220 of gestation, and the green bars represent heifers that were adequately fed from day 160-220 of gestation. Each bar represents a treatment group: CON/ADQ (top box, green bar), CON/RES (top box, yellow bar), MEL/ADQ (bottom box, green bar), and MEL/RES (bottom box, yellow bar).

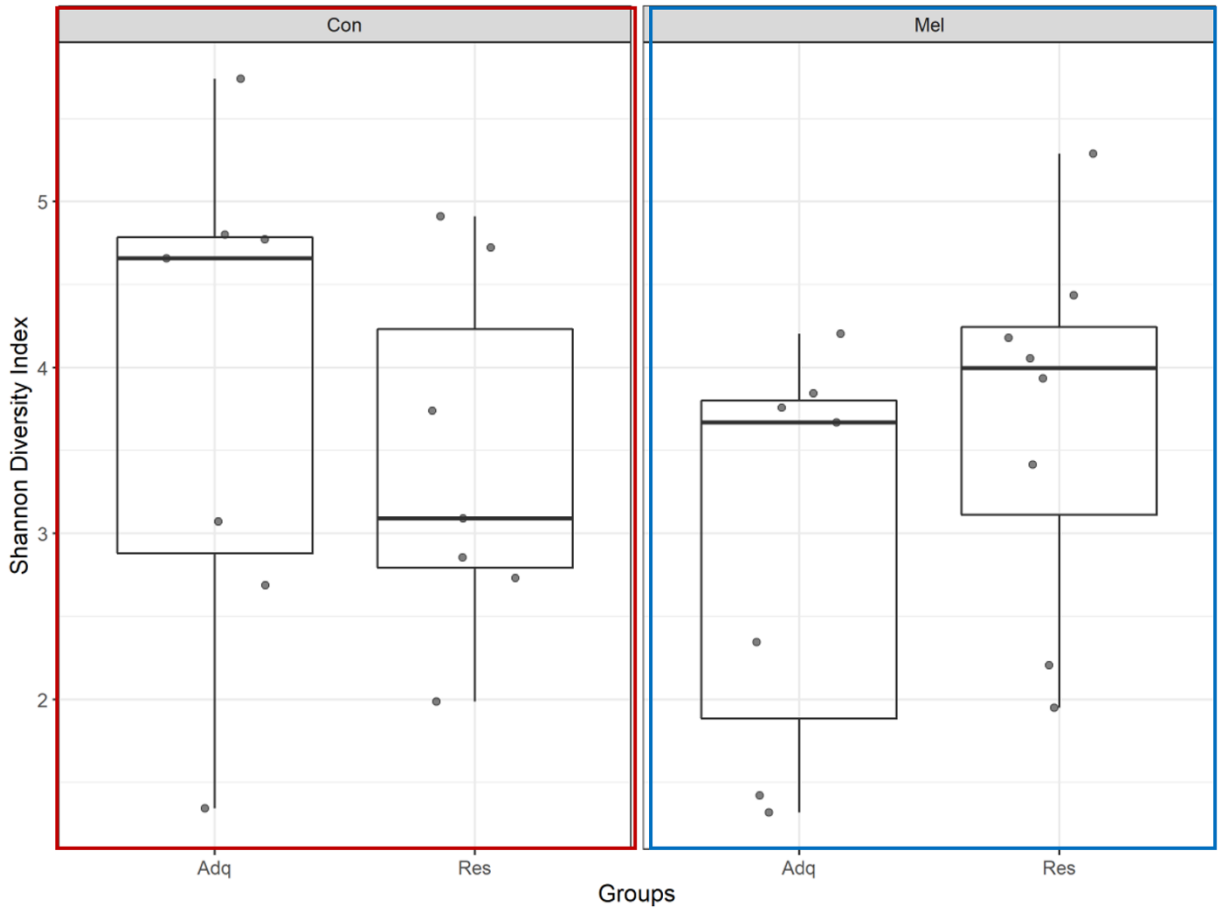

Figure 3.5 Alpha Diversity of Melatonin Supplemented vs. Control and Nutrient Restricted vs. Fed Treatment Groups

Alpha diversity boxplot of the vaginal bacterial microbiota in Brangus heifers measured by the Shannon Diversity Index. The red box represents the vaginal bacterial community of heifers that were not supplemented with melatonin and the blue box represents the vaginal bacterial community of melatonin supplemented heifers. Within the red and blue boxes, the box to the left represents the vaginal bacterial community of adequately fed heifers and the box to the left represented the vaginal bacterial community nutrient restricted heifers. Black dots represent values for individual samples. Each box and whisker plot represents a treatment group: CON/ADQ (red box, left bar), CON/RES (red box, right bar), MEL/ADQ (blue box, left bar), and MEL/RES (blue box, right bar). There were no differences in alpha diversity between adequately fed vs. nutrient restricted (P = 0.126), heifers supplemented with melatonin vs. control (P = 0.494), or the interaction between the diet and melatonin treatment (P = 0.216).

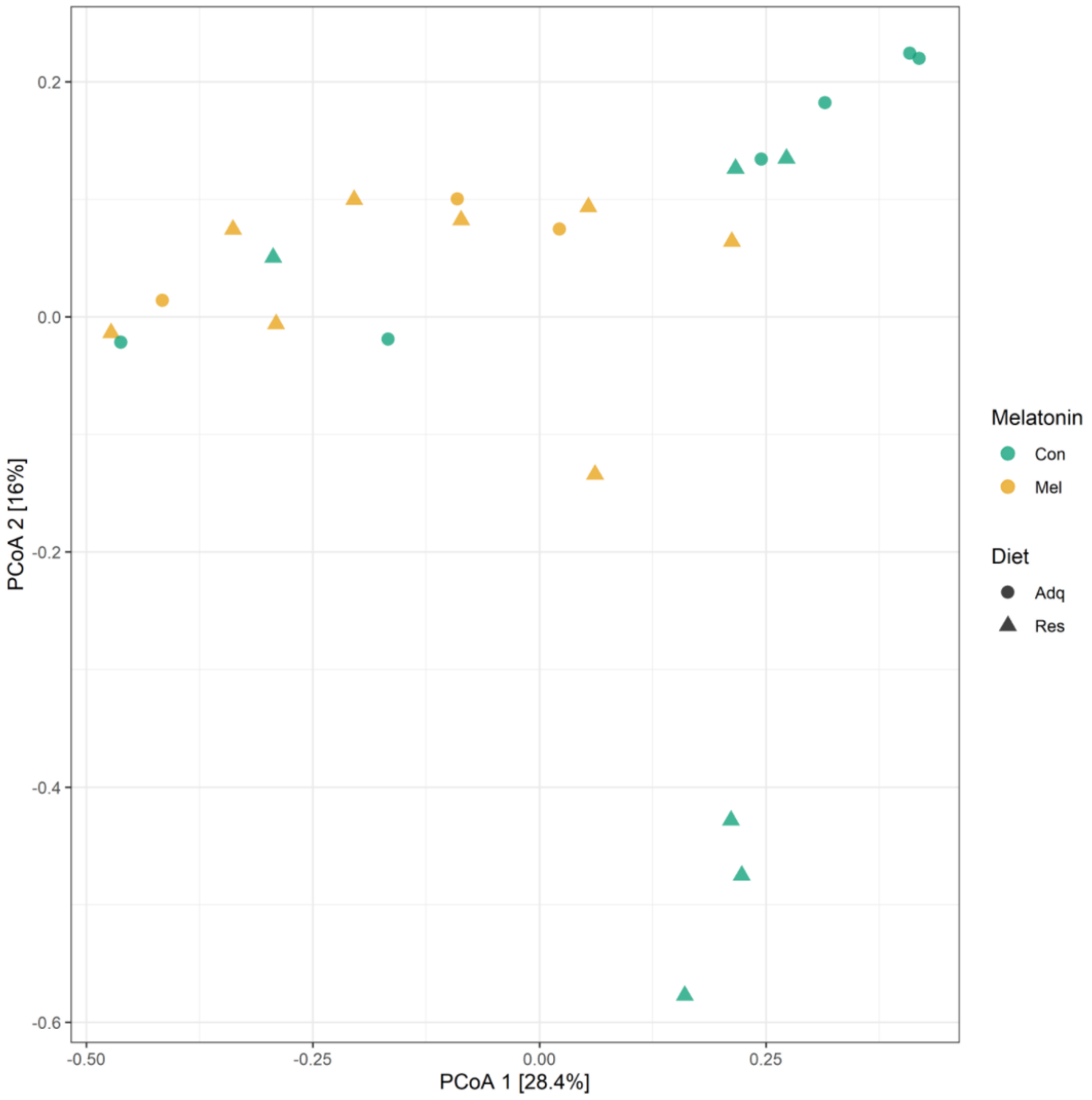

Figure 3.6 Beta Diversity of Melatonin Supplemented vs. Control and Nutrient Restricted vs. Fed Treatment Groups

Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples. Each point represents the vaginal bacterial community of each treatment group CON/ADQ (green circle), CON/RES (green triangle), MEL/ADQ (yellow circle), and MEL/RES (yellow triangle). There were no significant differences in beta diversity between nutrient restricted heifers and adequately fed (P= 0.402). There was a significant difference in beta diversity between heifers supplemented with melatonin vs. control (P= 0.019).

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CHAPTER IV

GENERAL DISCUSSION

The data and literature presented in this thesis provides valuable novel information and draws conclusions that can provide direction for future studies in bovine reproductive tract microbiota research. It is important to recognize that previous literature in reproductive microbiota research has been predominately studied in humans. Literature in bovine reproductive tract microbiota research is sparse but growing. Additionally, the methodology in bovine reproductive microbiota studies has not been fully refined to meet the rigorous contamination requirements typical of human studies. Current literature in bovine reproductive tract microbiota research acknowledges the presence of a vaginal, uterine, placental, and amniotic microbiota. However, in humans, there is evidence that refutes the existence of all reproductive tract microbiotas except the vaginal microbiota. Hence, until more bovine studies with rigorous contamination checks are published, it is difficult to conclude the value of studying other reproductive tract microbiotas besides the vaginal microbiota. Little is known about the role of the vaginal microbiota in bovine reproductive performance, specifically regarding microbial composition changes due to treatments, pregnancy status, day of gestation, or endogenous hormones. Therefore, the research presented in this thesis had contamination checks in place and was purposefully designed to address gaps within the current literature

Firstly, data indicates that concentrations of endogenous reproductive hormones, specifically estradiol and progesterone, do not change the composition of the bovine vaginal

microbiota. Therefore, future studies should direct their focus to evaluating if exogenous reproductive hormones at supraphysiological concentrations have an effect on the bovine vaginal microbiota.

Moreover, the composition of the bovine vaginal microbiota does not change between pregnant or nonpregnant heifers or from day 0 to day 140 of gestation. While the vaginal environment does radically change during early gestation and throughout the estrous cycle; these changes are driven by concentrations of endogenous estradiol and progesterone which had been previously determined to not alter the vaginal microbiota. Therefore, concluding that the vaginal microbiota does not change throughout gestation or between pregnant and nonpregnant heifers is due to the lack of hormonal influence is both logical and supported within the literature.

Before the studies within this thesis, there was no literature, to our knowledge, evaluating how nutritional or hormonal treatments imposed on bovids could change the vaginal microbiota. Specifically, within this study we wanted to evaluate how compromised pregnancy established through maternal nutrient restriction altered the bovine vaginal microbiota, if melatonin supplementation had any implications on the vaginal microbiota, or their interaction. Nutrient restriction did not alter the vaginal microbiota, but melatonin supplemented heifers did have a change in the beta diversity of the vaginal microbiota. The underlying mechanism to explain this change is unknown but suggests an implication of melatonin to decrease microbial abundance of pathogens through its antioxidant capacity.

The overwhelming conclusions thus far in bovine vaginal microbiota research is that the vaginal microbiota is dynamic. Therefore, finding overall compositional differences is difficult and may not hold as much merit as species level differences. Due to a decreased microbial abundance within the reproductive tract, species level differences are more sixgnificant in

impacting overall reproductive performance in bovids. Future research should focus on characterization to the species level changes within the vaginal tract microbiota during any significant reproductive event. In conclusion, this thesis has provided clarity for future research and has major implications in reproductive physiology. Perhaps this emerging area of research could be critical in solving the current enigmas in reproductive physiology.