MICROBIAL COMMUNITY COMPOSITION OF THE UTERINE HORNS OF BEEF COWS ON DAY 15 OF THE ESTROUS CYCLE

by

MADISON BLAKE WALER

(Under the Direction of Pedro Levy Piza Fontes and Todd Riley Callaway)

ABSTRACT

The objective of this study was to evaluate the uterine microbiota composition of cows on day 15 of the estrous cycle. Non-pregnant *Bos taurus* beef cows (n = 23) were exposed to an estrus synchronization protocol to exogenously induce synchronized ovulation. Transrectal ultrasonography was performed to evaluate ovarian structures, ensure synchrony, and determine the side of ovulation. Cows were harvested on day 15 of the estrous cycle and individual swabs were collected from each uterine horn using aseptic techniques. DNA was extracted and the entire (V1-V9 hypervariable regions) 16s rRNA gene was sequenced. Sequences were analyzed using the QIIME2 Pipeline. The composition of the microbial community on day 15 of the estrous cycle differed between the ipsilateral and contralateral horn of cows, and between cows that expressed estrus and cows that failed to express estrus.

INDEX WORDS: Bovine, Uterine environment, Uterine microbiome, Reproduction

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DEDICATION

I would like to dedicate this to my family. Thank you for all the love and support throughout my education. Without you none of this would have been possible. I strive to continue learning each day and pass a love of knowledge on to the next generation.

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CHAPTER 1

INTRODUCTION

Profitability of a cow-calf beef operation hinges upon its ability to maintain a calving interval of 365 days or less (Pulina et al., 2021). There are many management decisions that can aid or hinder this goal (Perry et al., 2011), but there are also underlying biological factors at play. Fertility is a word often used to describe these biological variables and can be defined as an individual's ability to reproduce. In other words, given the same management and environmental conditions some animals are more likely to successfully reproduce than others. Georgia producers implement reproductive technologies along with high quality nutrition to maximize the fertility of their herds. Some examples of these technologies are; estrous synchronization, artificial insemination, embryo transfer, and pregnancy diagnosis. The key to realizing greater improvements lies in better understanding the biological factors affecting fertility and finding ways to manipulate them to increase productivity.

In the past decade, access to advanced technologies and high-power computing has greatly increased and the cost of utilizing these technologies is rapidly decreasing. As a result, a new perspective of studying biology, often termed "-omics" research, has become widespread (Misra et al., 2019). For example, transcriptomics allows scientists to not only see what genes are present in an organism, but also to what extent those genes are being expressed within a particular tissue. This has already shown that even animals that appear to be identical from a gross physiological and genetic standpoint can be

different in the way they express their genes resulting in different performance. In one experiment the endometrial transcriptome of beef heifers differed between highly fertile and sub-fertile populations (Geary et al., 2016). The results of this study show that there is still room for improvement when selecting replacement animals, and that our methods currently used in industry fail to account for all the biological differences between individuals.

Another next generation sequencing technology that has recently become popular is 16s rRNA sequencing. Sequencing part or all of this gene, and matching those sequences to an established data base, allows the identity of the organism a piece of DNA originated from to be identified. DNA from a given sample can be analyzed and an estimate of the composition of the prokaryotic community can be made. The DNA that makes up this community is often called the "microbiome" and includes all the biotic and abiotic factors that make up a specific location of interest (Bokulich et al., 2020). The microbiome has been studied in many organ systems and species. In cattle there is a large body of research surrounding the gut microbiome and its effect on animal performance or the effects of nutritional interventions on the gut microbiome (Appiah et al., 2020; Khalil et al., 2022; O'Hara et al., 2020). It has also been shown that the microbiome of the gut can impact various other organs in the body (Cryan et al., 2019; Forsythe et al., 2014; Li et al., 2022; Mayer et al., 2015; Welch et al., 2022). The results of these studies suggest that the microbiome could play a role in other aspects of animal performance besides the commonly studied growth parameters studied by nutritionist, including reproductive performance or "fertility".

The study of the reproductive microbiome itself is a newer development compared to the gut microbiome. In fact, prior to next generation sequencing technologies, it was commonly believed that the upper reproductive tract of a healthy mammal was sterile, which we now know is not the case (Baker et al., 2018). The relationship between the gut microbiome and the reproductive microbiome remains to be explored, however some hypothesize the two are closely linked due to the proximity of the anus to the vagina in all livestock species (Jones et al., 2022). It has been shown that the uterine microbiome may impact fertility (Ault et al., 2019b; Heil et al., 2019), but the biology underlying this remains poorly understood.

Early embryonic mortality is one of the biggest obstacles beef producers must overcome to achieve high pregnancy rates. In fact, about 90% of beef cows serviced via artificial insemination have a successful fertilization event, but only approximately half of those embryos develop into healthy pregnancies (Diskin et al., 2011; Reese et al., 2020). Many believe the critical point in pregnancy establishment in cattle occurs around day 15 of the estrous cycle and is a process known as the maternal recognition of pregnancy (Moraes et al., 2018a). The maternal recognition of pregnancy is a complex process that involves the immune and endocrine systems of both the mother and the conceptus communicating and resulting in the conceptus successfully developing and implanting into the uterus (Spencer and Hansen, 2015a). The role of the uterine microbiome at this critical timepoint is unknown and warrants further investigation.

An experiment was conducted to investigate and evaluate the uterine microbiome of the non-pregnant uterus on day 15 of the estrous cycle in mature beef cows. Additionally, the study evaluated the differences between cows that displayed and failed

to display estrus as well as the differences between sampling location relative to the corpus luteum. It was hypothesized that the uterine microbiome would differ between the ipsilateral and contralateral horns. Moreover, within the ipsilateral horn, it was hypothesized that the microbiome of cows that displayed estrus would differ from that of cows that failed to display estrus.

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CHAPTER 2

REVIEW OF THE LITERATURE

The Bovine Estrous Cycle

The cow is a polyestrous animal. Polyestrous animals ovulate and become sexually receptive on a regular cycle continuously from the onset of puberty until death unless something disrupts this cycle. Periods in which the female is not cycling are referred to as periods of anestrous. Cows may become anestrous for several reasons including, pregnancy, age, improper nutrition, disease, and more (Senger, 2012). The average length of the estrous cycle in cows is 21 days, with some animals having cycles as short as 17 days or as long as 24 days (Armstrong and Hansel, 1959).

During the estrous cycle the female is preparing for pregnancy by modulating both the uterine environment and ovarian structures. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) recruit and promote the development of the female gamete throughout the cycle until there is a preovulatory dominant follicle. This dominant follicle will secrete large amounts of estrogen and cause the animal to exhibit behavioral estrus (Sartori et al., 2001). Around this time there will be an LH surge that causes ovulation of the dominant follicle. Ovulation is the process whereby the follicle basement membrane ruptures, releasing the oocyte to be collected by the infundibulum (Senger, 2012).

After ovulation, the ruptured follicle forms a structure known as the *corpus hemorrhagicum* which under the influence of LH will become the *corpus luteum* (CL).

The CL is a critical structure that secretes progesterone (P4), which is the hormone that is required for pregnancy establishment and maintenance (Bazer et al., 2010). The CL will continue to increase in size and steroidogenic capacity until the process of luteolysis begins (Hafs and Armstrong, 1968).

Luteolysis is initiated by increased secretion of prostaglandin $F_{2\alpha}$ (PGF) by the uterine endometrium (McCracken et al., 1999). Approximately twelve hours later, the production of P4 and the physical size of the CL decrease (Meidan et al., 1999). The removal of the negative feedback of P4 allows for estradiol to rise rapidly. High circulating concentrations of estradiol results in behavioral estrus expression. Luteinizing hormone also rises rapidly without P4 negative feedback, this surge in LH induces ovulation, and the start of a new cycle (Gonçalves et al., 2012; Quintal-Franco et al., 1999).

Pregnancy Establishment

For a pregnancy to occur many things must happen in the proper order, the oocyte must be fertilized by the sperm and the uterus must provide an adequate environment for the conceptus to grow and develop properly (Senger, 2012). In the bovine, fertilization rates tend to be around 90% of females inseminated via artificial insemination (AI), however just over half of those embryos will develop into healthy pregnancies (Diskin et al., 2011; Reese et al., 2020).

Fertilization occurs in the oviduct very close to the time of ovulation (day 0). The conceptus then travels down the oviduct and arrives at the most cranial tip of the uterine horn around day 5. On day 8, the embryo hatches from the zona pellucida (Guillomot, 1995) and between days 8-14 the embryo continues to grow and elongate prior to

attaching to the endometrium. Between days 14 and 17 the conceptus shows exponential increase in length, growing from 2-3 mm to over 20 cm (Betteridge and Fléchon, 1988b; Moraes et al., 2018b). During this time of exponential growth, the conceptus begins to secrete interferon-τ (IFNτ). Interferon-τ is a protein that is required for pregnancy establishment as it suppresses the production of PGF, preventing luteolysis (Moraes et al., 2018b). The conceptus continues to elongate and starts to attach itself into the uterine epithelium around day 20 of gestation (Spencer and Hansen, 2015b).

Changes in endometrial transcriptome and histotroph composition that occur during early pregnancy are regulated by circulating P4 produced by the CL during diestrus (Forde & Lonergan, 2012). Endometrial concentration of P4 is greater in the uterine horn ipsilateral to the CL compared with the contralateral horn (Takahashi et al., 2016; Weems et al., 1988). There are also differences in endometrial transcriptome between ipsilateral and contralateral uterine horns during diestrus (José María Sánchez et al., 2019), indicating not only a local effect of the corpus luteum on endometrial progesterone concentrations, but also an effect on endometrial function. Interestingly, pregnancy establishment is decreased when embryos are transferred in the contralateral horn compared with transfers performed in the ipsilateral horn (Del Campo et al., 1983), further highlighting the unequivocal role of progesterone modulating local uterine function and pregnancy establishment.

Recent research has shown that females have inherently different capacities to successfully establish pregnancy, and that these differences are reflected in the transcriptome of the uterine endometrium (Bazer et al., 2018a; Geary et al., 2016).

Female Bovine Reproductive Microbiome

The microbiome is often colloquially referred to as the “last organ” and is defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties (Berg et al., 2020). Though traditionally studied in the gastrointestinal system, recently the term has been applied to other organ systems including the reproductive tract. The reproductive tract microbiomes of a variety of mammals have been studied and there are distinct differences between species as well as within individuals of a given species (Heil et al., 2019).

The reproductive tract can be further broken down into the upper and lower portions, separated by the cervix, due to their vastly different environmental conditions and functions. The exterior genitalia, the vagina, and the caudal face of the cervix comprise the lower reproductive tract. The cranial portion of the cervix, uterus, and oviducts make up the upper reproductive tract (Senger, 2012). The lower reproductive tract is responsible for defending the upper reproductive tract from the outside world, providing a route for urine to exit the body, and accommodating the penis during copulation, serving as the site of semen deposition during natural service breeding events. All these functions make the lower reproductive tract the “dirty” part of the system. The upper reproductive tract is responsible for creating and maintaining an environment suitable for procreation and modulating that environment from conception until parturition. As such the upper reproductive tract is generally thought of as cleaner, and up until recently was believed to be sterile (Baker et al., 2018; Moreno and Franasiak, 2017).

In the bovine the taxonomic composition of the vagina is predominated by the phylum *Firmicutes*, followed by the phyla *Bacteroidetes, Proteobacteria, Tenericutes, and Actino bacteria.* Bacteria in the uterus are less diverse and less dense when compared

to the bacterial community found in the vagina. Uterine communities are most often composed mostly of *Firmicutes, Proteobacteria,* and *Bacteriodetes* (Heil et al., 2019). When comparing the ruminant reproductive microbiome to other mammals a major difference is the method used to defend the vagina from pathogenic organisms. Most mammals harbor high levels of *Lactobacillus* in the vagina to lower the vaginal pH and maintain an acidic environment that is unfavorable to bacterial growth. The bovine and the ovine, however, rely on high levels of bacterial diversity to fully occupy every niche present in the environment and make it challenging for invading pathogens to displace a native organism and cause dysbiosis (Heil et al., 2019). This is a particularly successful strategy that ruminants also use in regulating their gastrointestinal tract. Decreases in bacterial diversity in both the rumen and hindgut are associated with a variety of metabolic diseases. Most notably, acidosis in cattle and irritable bowel syndrome and ulcerative colitis in humans (Major and Spiller, 2014; Monteiro and Faciola, 2020). Similar phenomenon have been studied in relation to uterine disease in cattle, where cows with clinical metritis show decreased bacterial diversity compared with healthy cows (Galvão et al., 2019).

Origins of the Reproductive Microbiome in the Female

The origin of the reproductive microbiome is a topic of great debate. Traditionally the placenta was thought of as a sterile organ; however, recent studies have revealed that the placenta has a unique microbiome (Hummel et al., 2022; Hummel et al., 2021). Therefore, it is reasonable to assume that the colonization of the reproductive tract begins in utero. The vaginal and buccal microbiome of the dam have been shown to have significant impact on the fecal microbiome of the offspring, suggesting that parturition is

a critical timepoint for the colonization of a variety of organ systems in the calf (Dominguez-Bello et al., 2011; Koenig et al., 2011; Owens et al., 2021). Though the colonization of the reproductive tract most likely begins during the peripartum period, puberty presents an opportunity for a change in the community composition. One of the predominate hypotheses suggests that microbiota present in the feces migrate up the reproductive tract from the vulva through the vagina and into the uterus. This hypothesis was tested using advanced sequencing techniques to track clonal isolates of *E. coli* in dairy cows to attempt to understand how the upper reproductive tract was influenced by the gut microbiome. The results were inconclusive and indicated a combination of fecal-vaginal as well as hematogenous routes of transmission (Jones et al., 2022).

It has been well established that the hepatic portal vein can transfer pathogenic bacteria from the rumen to the liver, resulting in the development of liver abscesses and a vast economic loss to the cattle industry (Reinhardt and Hubbert, 2015). Current dogma states that ruminal acidosis leads to "leaky" epithelial cells in the rumen allowing *Fusobacterium* access to the liver via the hepatic portal vein (Tadepalli et al., 2009). A similar pathway may allow other pathogens access to the uterine environment and in specific conditions lead to uterine disease. This is especially likely during parturition as there is a lot of damage to the uterine lining and bleeding. Parturition also provides the opportunity for environmental pathogens to enter the uterus since the cervix must open to allow passage of the fetus.

Work in the rumen has shown that the gut microbiome is not only unique to the individual animal but in some regards is also very stable. This stability has been tested using complete ruminal content exchange experiments. Animals return to their own

"native" microbiome state by around 19 days post exchange (Cox et al., 2021; Zhou et al., 2018). Similar studies have also shown that the individual microbiome stays consistent year after year even in the face of environmental changes (Clemmons et al., 2019; Wallace et al., 2019). Collectively, these studies have led to the idea that the microbiome of an individual is unique, relatively stable barring dramatic events, and can impact a variety of production traits. It is reasonable to assume that the reproductive microbiome would be similar in many ways, especially if colonization is indeed occurring via the GI tract. The reproductive microbiome may provide insight into some of the inherent fertility differences between animals.

Impact of the Uterine Microbiome on Fertility

There is human data which suggests an impact of both the vaginal and uterine microbiome are correlated with and may impact a variety of reproductive outcomes, specifically preterm delivery, miscarriage, and implantation rates in an IVF setting (Giudice, 2016; Green et al., 2015). In the dairy industry, one the greatest challenges producers face in maintaining high fertility in a herd is uterine disease (metritis and endometritis), which has a 20% prevalence in the US dairy herd and costs the industry more than $500 per case (Pérez-Báez et al., 2021). For this reason, there is a large body of research that investigates the microbiome postpartum of healthy cows and cows that will develop metritis in an attempt to understand the process by which this disease develops (Bicalho et al., 2017; Bicalho et al., 2012; Jeon et al., 2015; Santos and Bicalho, 2012b).

Traditionally the incidence of metritis and endometritis in beef cattle was assumed to be very low. This was due to the low number of clinical cases; however, a

recent study indicated that the prevalence of subclinical metritis in beef herds could be as high as 31%. In this study, cows diagnosed with subclinical metritis had decreased pregnancy rates at 130 days postpartum compared to cows without subclinical endometritis (13% and 57% respectively). All of the cows without subclinical metritis were pregnant by 150 days postpartum compared to the 250 days it took for every cow with subclinical endometritis to become pregnant again (Ricci et al., 2015). Clearly, clinical and subclinical uterine infections impact herd fertility. Additionally, data to support the idea that the uterine microbiome is associated with the development of these diseases (Baker et al., 2018). It is possible that the uterine microbiome influences the likelihood of a cow developing subclinical endometritis during the peripartum period leading to decreased fertility.

A recent study attempted to investigate the impact of both the uterine and vaginal microbiomes on pregnancy rate in a timed AI setting in beef cows. Uterine and vaginal microbiomes differed significantly from each other and changed significantly over time during the synchronization protocol. In addition, significant clustering of the uterine microbiome was observed two days prior to AI in cows that failed to become pregnant compared with cows that became pregnant (Ault et al., 2019b). This same group further analyzed the samples from this study to try to understand what differences in microbial communities between the cows that become pregnant cows that failed to become pregnant. The phyla *Actinobacteria* was significantly more abundant (14.4% vs. 1.33%) in nonpregnant compared with pregnant cows two days prior to AI (Ault et al., 2019a).

It is well established that cows that display estrus have increased plasma concentrations of estradiol prior to ovulation and increased pregnancy rates in both

artificial insemination (Madureira et al., 2019; Richardson et al., 2016) and embryo transfer settings (Pereira et al., 2016). Cows that express estrus have decreased pregnancy loss after a pregnancy was initially confirmed via ultrasonography during early gestation (Madureira et al., 2019; Pereira et al., 2016). Hence, the greater fertility observed in cows that express estrus is not explained only by improved follicular and oocyte development (Perry et al., 2005; Pohler et al., 2012), but also by changes in subsequent luteal development and the resulting uterine environment (Davoodi et al., 2016).

The composition of the uterine microbial community remains a novel environment and investigations into this community is constrained by similar limitations. Transvaginal uterine flush or uterine swab provides an opportunity for sample contamination, which can drastically alter results when examining a low biomass environment like the uterus. In addition, DNA extraction techniques as well as sequencing technologies applied may have been the best available at the time, but the cost of microbiome sequencing has continued to decrease, and more precise technologies are now available.

There is still not enough data to establish a "core" uterine microbiome of healthy fertile cows (Heil et al., 2019), and more research is needed in this field to truly understand the impact of the bovine uterine microbiome composition and activity on fertility.

Endocrine Impact on the Microbiome

The uterine microbiome has been shown to change temporally throughout the estrous cycle (Ault et al., 2019a). The temporal nature of the vaginal microbiome has been more extensively studied. We know that the vaginal community also varies through

the estrous cycle as well as through the life cycle of the female (Adnane and Chapwanya, 2022; Laguardia-Nascimento et al., 2015). In Nelore cattle, there was a tendency for bacterial populations in the vagina to decrease during pregnancy and populations of archaea to rise (Laguardia-Nascimento et al., 2015). Though this study used a culture dependent methodology, the results still indicate that the endocrine environment of the female impacts the microbiome composition.

Circulating concentrations of P4 varies during the estrous cycle and differs significantly between pregnant and nonpregnant animals starting on day 18 of the estrous cycle (Pugliesi et al., 2014). Progesterone has an inhibitory effect on the host immune system (Hall and Klein, 2017), which is beneficial to the host since when under a high progesterone environment, the animal expects a conceptus to be present in the uterus. However, prior to attachment the mother will not recognize the conceptus as "self" and may initiate an immune response which will likely prevent the conceptus from implanting. High levels of progesterone have been linked to higher fertility in cattle and this could in part be due to higher levels of immune system suppression in a very location dependent fashion in the uterus (Spencer et al., 2016; Spencer et al., 2007; Wiltbank et al., 2016). This hypothesis is supported by transcriptome studies that show genes related to immunoglobulin production are significantly upregulated in sub-fertile and infertile heifers when compared to highly fertile heifers (Geary et al., 2016). In addition, the development of P4 resistance leads to decreased success in an embryo transfer (ET) setting, and may be linked to the uterine microbiome (Sirota et al., 2014). A recent study indicates that exogenous P4 and estradiol are able to alter the uterine microbiome of heifers around the time of ovulation (Poole et al., 2023).

Estradiol (E2) is another hormone of interest when studying fertility in cattle. Greater circulating concentrations of E2 secreted by the dominant follicle are observed shortly during the proestrus and estrus phase of the estrous cycle, and greater concentrations of E2 are observed in cows with greater estrus intensity (Nogueira et al., 2019). Females that display standing estrus have increased conception rate and decreased pregnancy loss in both AI and ET settings (Bó and Cedeño, 2018; Núñez-Olivera et al., 2022). In a study evaluating the vaginal microbiome of Brangus heifers on the day of AI, there were no differences in bacterial community composition between high, medium, and low (7.2 - 17.6 pg/ml; 2.6 - 6.7 pg/ml; 1.1 - 2.5 pg/ml respectively) concentrations of E2 (Messman et al., 2020). However, this study did not investigate the uterine microbiome, nor did they collect concentrations of E2 during estrus expression so the true impact of E2 on the reproductive microbiome is not clear. In ovariectomized rats, a significant decrease in bacterial load, especially *Lactobacillus,* was demonstrated when compared to normal non-ovariectomized cycling controls. Under E2 replacement therapy *Lactobacillus* levels were restored (Bezirtzoglou et al., 2008). Therefore, E2 plays a role in modulating the reproductive microbiome and this may partially explain the differences in fertility between cows that display estrus and those that do not display estrus upon exogenously induced ovulation (Núñez-Olivera et al., 2022; Richardson et al., 2016).

Differences in the pH of the reproductive tract have been shown to influence semen motility and fertilization rates. The greatest sperm motility was seen at pH 6.5 and 7 indicating that excessively acidic and basic environments have negative impacts on fertility (Contri et al., 2013; Rizvi et al., 2009). In humans, colonizing the ET catheter tip with *Lactobacillus* was shown to increase blastocyst implementation rate (Sirota et al.,

2014). This indicates that the relative activity of lactic acid producing bacteria, which have a direct impact on the pH of both the uterus and vagina, could have impacts on fertility by modulating sperm function and the implantation process. Thus, introducing the idea that utilizing probiotics in assistive reproductive technologies might have the potential to increase pregnancy success. However, little is understood about the native microbiome and there are no established recommended protocols to alter the reproductive microbiome to optimize fertility.

Factors Influencing the Composition of the Reproductive Microbiome

There are a number of other practices that are common on the beef production industry that have the potential to impact the reproductive tract microbiome. Assistive reproductive technologies usually require a technician to bypass the cervix and deposit semen or an embryo in the uterus itself. This introduces contamination from the vagina, to combat this transfer medias and semen extenders are dosed with antibiotics (Morrell and Kumaresan, 2022; Schulze et al., 2020). There is potential for these antibiotics to cause drastic dysbiosis in the native population of the uterus and present an opportunity for pathogenic organisms to colonize.

Even less is understood about the male reproductive microbiome than the female. There are a number of studies that show decreased fertility of semen when the seminal plasma is removed, indicating that the seminal plasma is crucial to fertilization and placental formation (Bromfield, 2016). Seminal plasma also housed the majority of the male's reproductive microbiome and may interact with the vaginal microbiome in natural service settings.

The impact of sexually transmitted diseases on the reproductive tract microbiome has yet to be studied but could help to explain some of the fertility consequences associated with those diseases. In addition, other immunological stressors like respiratory disease have been shown to cause dysbiosis in unrelated organisms like the gut and should presumably have similar impacts on the reproductive microbiome, which may explain some of the decreased fertility seen in BRD cows. In general, whenever there is dysbiosis in the gut the entire body will enter a proinflammatory state that can lead to a cascade of negative consequences for the host, to whom reproduction is the last priority (van der Meulen et al., 2016).

Overview of 16s rRNA Sequencing Technology

High-throughput sequencing platforms have allowed for revolutionary study of microbial communities. To apply these technologies, marker genes like the 16s rRNA gene in bacteria are amplified and then sequenced. These sequences are then matched to databases of known sequences to identify from which organism the sequence was originated. Every step of the process, from sample collection to final analysis, introduces the opportunity to bias the results and not accurately reflect the microbial community present in the environment of interest. Therefore, it is important to utilize best practices to minimize the chance of inaccurate results (Pollock et al., 2018).

Though there is debate on sample collection methodology it is generally accepted that studies can be compared when similar sampling methods are utilized. Studies utilizing different sampling techniques rarely yield similar results. There is consensus, however, that the best method for sample storage is rapid freezing and then storage at -80 C° (a cryoprotectant is not necessary) until DNA extraction (Fouhy et al., 2015). During

DNA extraction a mechanical lysis method (bead beating) should be used to maximize DNA yield and bacterial diversity (Pollock et al., 2018).

Once the DNA is extracted the 16s rRNA gene needs to be amplified. When utilizing short-read sequencing platforms, investigators must choose which of the nine variable regions to target with their polymerase chain reaction (PCR) primers. However, when utilizing PacBio and Oxford Nanopore sequencing it is possible to amplify the entire 16s rRNA gene and gain greater specificity of sequence composition for downstream analysis reducing the risk of unassigned or misassigned sequences (Overholt et al., 2020; Weirather et al., 2017).

The PCR process itself can also affect the results of the study. Certain PCR inhibitors can prevent sequence amplification, and increasing numbers of PCR cycles leads to increased presence of chimeras (Kanagawa, 2003). There is not an agreed upon value for the number of cycles a PCR should be run for in microbiome studies; however, it is important to keep in mind when comparing studies that bacterial richness increases with PCR cycles as well as the formation of chimeras.

For downstream analysis QIIME 2 is generally accepted at the current time as the fastest and easiest method to analyze sequence reads (Nilakanta et al., 2014; Plummer et al., 2015). Generally speaking, amplicon sequence variants (ASVs) are the preferred method to operational taxonomical units (OTUs) in that they provide more accurate assignment to the species level and have a higher threshold of similarity to group sequences together (Pollock et al., 2018). Of course, as with all techniques it is important to include a negative control in all analysis to ensure that reagents, supplies, and the environment are free from contamination

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CHAPTER 3

DIFFERENCES IN MICROBIAL COMMUNITY COMPOSITION BETWEEN UTERINE HORNS IPSILATERAL AND CONTRALATERAL TO THE CORPUS LUTUEUM IN BEEF COWS ON DAY 15 OF THE ESTROUS CYCLE

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Abstract

The objective of this study was to evaluate the uterine microbiota composition of cows on day 15 of the estrous cycle. Non-pregnant *Bos taurus* beef cows (n = 23) were exposed to an estrus synchronization protocol to exogenously induce synchronized ovulation. Transrectal ultrasonography was performed to evaluate ovarian structures, ensure synchrony, and determine the side of ovulation. Cows were harvested on day 15 of the estrous cycle and individual swabs were collected from each uterine horn using aseptic techniques. DNA was extracted and the entire (V1-V9 hypervariable regions) 16s rRNA gene was sequenced. Sequences were analyzed using the QIIME2 Pipeline. Across all samples, 2 bacterial domains, 24 phyla, and 265 genera were identified. *Butyribirio, Cultibacterium, BD7-11, Bacteroidales BS11 gut group, Ruminococcus, Bacteroidales RF16 group* and *Clostridia UCG-014* differed in relative abundances between uterine horns. *Rikenellaceae RC9 gut group, Bacteroidales UCG-001, Lachnospiraceae AC2044 group, Burkholderia-Caballeronia-Paraburkholderia,, Psudobutyribibrio,* and an unidentified genus of the family *Chitinophagaceae* and *dgA-11 gut group* differed between cows that displayed estrus and those that did not. The composition of the microbial community on day 15 of the estrous cycle differed between the ipsilateral and contralateral horn of cows, and between cows that expressed estrus and cows that failed to express estrus.

Lay Summary

The microbiome is the collection of microscopic organisms and environmental conditions that make up a specific location. Often these organisms can have positive or negative impacts on the host. Early embryonic mortality is one of the main drivers of infertility in mammals, including humans and livestock species. Most embryonic mortality in the bovine occurs around day 15 of gestation while the embryo relies on the uterine environment to develop and successfully establish pregnancy. Although the uterine environment during key periods of embryo development has been extensively investigated, the uterine microbiome during this critical time point remains unknown. The present study characterized the uterine microbiome of beef cows on day 15 of the estrous cycle using advanced DNA sequencing technologies. Our results indicate that there are differences in the microbial communities between each uterine horn depending on their location relative to the corpus luteum. Moreover, microbial communities were different in cows that expressed estrus behavior compared with cows that did not express estrus.

Introduction

Embryonic mortality is a major contributor to infertility and subfertility in all mammal species, including humans (Pohler et al., 2015). In cattle, reproductive failure costs beef and dairy producers more than $1 billion USD annually (Bellows et al., 2002). In beef production systems, fertilization rates typically exceed 80% of females exposed to artificial insemination; however, only approximately 50% of females are able to successfully establish pregnancy (Reese et al., 2020), indicating that pregnancy loss during early embryonic development is a major contributor to reproductive failure. The

majority of these losses occur between days 6 and 20 of gestation (Diskin et al., 2011; Wiltbank et al., 2016), a pivotal period of pregnancy during which the bovine conceptus undergoes dramatic morphological and functional changes prior to implantation (Bazer et al., 2018b; Betteridge and Fléchon, 1988a; Ribeiro et al., 2016). Collectively, these changes result in an orchestrated paracrine conversation between the elongating conceptus and the endometrium that is required for successful pregnancy establishment (Bazer et al., 2018b; Spencer and Hansen, 2015b). Disruptions to the uterine environment that impedes adequate growth and development of the conceptus, leads to early embryonic mortality (Spencer and Hansen, 2015b).

Changes in endometrial transcriptome and histotroph composition that occur during early pregnancy are predominantly regulated by circulating progesterone produced by the corpus luteum during diestrus (Forde and Lonergan, 2012). Endometrial concentration of progesterone are greater in the uterine horns ipsilateral to the corpus luteum compared with contralateral horns (Takahashi et al., 2016; Weems et al., 1988). There are also differences in endometrial transcriptome between ipsilateral and contralateral uterine horns during diestrus (Sánchez et al., 2019a), indicating not only a local effect of the corpus luteum on endometrial progesterone concentrations, but also an effect on endometrial function. Interestingly, pregnancy establishment is decreased when embryos are transferred in the contralateral horn compared with transfers performed in the ipsilateral horn (Del Campo et al., 1983), further highlighting the unequivocal role of progesterone modulating local uterine function and pregnancy establishment.

Cows that display estrus have increased plasma concentrations of estradiol prior to ovulation, and increased pregnancy rates in both artificial insemination (Madureira et

al., 2019; Richardson et al., 2016) and embryo transfer settings (Pereira et al., 2016). Cows that express estrus also have decreased pregnancy loss after a pregnancy is initially confirmed via ultrasonography during early gestation (Madureira et al., 2019; Pereira et al., 2016). Hence, the greater fertility observed in cows that express estrus is not explained only by improved follicular and oocyte development (Perry et al., 2005; Pohler et al., 2012), but also changes in subsequent luteal development and the resulting uterine environment (Davoodi et al., 2016). Next generation sequencing technologies have allowed us to establish the presence and composition of a microbiome in the bovine uterus; however, the reason for its existence or role in reproduction remains poorly understood (Baker et al., 2018; Heil et al., 2019; Moore et al., 2017). We hypothesized that there were differences in the microbial community of the ipsilateral and contralateral uterine horns on day 15 of the estrous cycle in *Bos taurus* beef cows. Moreover, we hypothesized that there are differences in the uterine microbial community composition between cows that display estrus and those that do not express estrus. Therefore, the objectives of this study were to: 1) evaluate the differences in uterine microbial community between the ipsilateral and contralateral uterine horns on day 15 of the estrous cycle, and 2) evaluate the impact of estrus expression on subsequent uterine microbial composition.

Materials and Methods

Animals, Experimental Design, and Diet

All procedures were carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Georgia, Athens (Protocol A2020 02-002-Y3-A0).

Non-pregnant *Bos taurus* cows (n=23; BW = 582 ± 68.6 kg; BCS = 5.46 ± 0.67) from the University of Georgia's Northwest Georgia Research and Education Center (Rome, GA 34.34° N, 85.12° W) were utilized in this experiment. All cows were housed as a single group on native improved bermudagrass (*Cynodon dactylon)* pasture and had ad libitum access to bermudagrass hay. Cows were exposed to a modified estrus synchronization program to induce synchronized ovulation. Briefly, cows received a 25-mg injection of prostaglandin $F_{2\alpha}$ (PG; 2 mL Lutalyse HighCon, Zoetis Animal Health, Parsippany-Troy Hills, NJ) on day -13, followed by a 100-µg injection of gonadotrophin releasing hormone (GnRH; 2mL Factrel ; Zoetis Animal Health) on day -10. Another injection of PG was administered on day -3 to induce luteolysis and an estrus detection patch (Estrotect Breeding Indicator, Rockway Inc., Spring Valley, WI) was applied. Cows received a second GnRH injection on day 0 and estrus detection patches were evaluated. Cows considered to have expressed estrus when at least 50% of the rub off coating was removed from the patch. B-mode ultrasonography (Easi-Scan:Go;IMV Imaging, Rochester, MN) was used to confirm synchrony through ovarian mapping on days -13,-10,-3,0, and 14. Cows were considered to have ovulated to the first GnRH injection (d -10) when a corpus luteum was present on day -3 on the same ovary that a dominant follicle (> 8 mm) was present on day -10. Cows were considered to have ovulated spontaneously or in response to the second GnRH (day 0) when CL was present on day 14 on the same ovary that a dominant follicle (>8mm) was present on day 0. Only cows that responded to both GnRH injections were used for sample collection. Cows were transported to a commercial packing plant (FPL Foods, Augusta, GA (33.46 °N, 81.96 °W)) on day 14 and harvested on day 15 of the study.

Sample Collection

Cows were harvested and the reproductive tracts (including vagina, cervix, uterus, and ovaries) were removed. Each uterus was washed with water and then the incision site was sterilized with 70% ethanol. An aseptic cross-sectional incision was made into each horn at the greater curvature region and a sterile cotton tipped swab inserted into the cranial portion of the uterine horn, rubbed against the uterine epithelial lining until saturated, and immediately flash frozen (-80 °C) using liquid N_2 (Lourenco et al., 2019).

DNA Extraction and Sequencing

Samples were thawed to room temperature, and 1 mL of sterile Phosphate Buffered Saline (PBS) was added to each tube. Tubes were vortexed for 10 minutes to maximize recovery. DNA extraction was performed using a commercial kit (QIAamp BiOstic Bacteremia DNA kit; QIAGEN) according to manufacturer instructions using 0.5 mL of each sample. The concentration of DNA was quantified by spectrophotometry (Synergy H4; BioTek, Winooski, VT, USA). The entire 16S rRNA gene libraries were prepared from genomic DNA using LoopSeq kits (Loop Genomics, San Jose, CA), and synthetic long reads were constructed from the short-read sequences generated by Illumina sequencing technology (Callahan et al., 2021). Analysis of sequences was preformed using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline, version 2-2021.11. Sequences were cleaned and assigned to taxa using a pre-trained naïve Bayes classifier (Bokulich et al., 2020; Robeson et al., 2021) which was trained on the full-length small subunit of the SILVA 138 database (Quast et al., 2013). Samples were rarified to a common depth of 154 sequences for computation of alpha and beta diversity metrices, and to calculate the mean relative abundance of

individual taxa. One cow was removed from the study due to receiving antibiotic treatment during the synchronization protocol, therefore sequence analysis was performed on samples from 22 cows (Table 1). After all quality filtering steps a total of 36 samples (17 contralateral and 19 ipsilateral) were analyzed. There were 15 cows who after filtering and rarefaction retained both ipsilateral and contralateral samples. There were 15 samples from cows that did not display estrus and 21 from cows that did display estrus, including matched pairs of 8 cows that displayed estrus and 7 that did not.

Statistical Analysis

Data were analyzed using a python script (vanRossum, 1995) to preform Kruskal-Wallis test and paired t-test on every taxa for all the explanatory variables (horn and estrus expression). The significant results are displayed in the tables and discussed in the results section. Differences in alpha diversity were calculated using the "qiime diversity alpha-group-significance" function of QIIME2 version 2-2021.11 and differences in beta diversity were calculated using the "qiime diversity beta-group-significance" function on the unweighted unifrac distance matrix. Significance was declared when $P < 0.05$ with $0.05 < P \leq 0.10$ considered a tendency.

Results

After quality filtering and taxonomic classification, a total of 2 kingdoms, 24 phyla, and 265 genera were assigned to sequences. Relative abundances of all phyla did not differ between horns or between estrus expression (Figure 3.1). At the phylum level, samples from the ipsilateral horn of cows that did not express estrus had a greater (~23%) relative abundance of *Proteobacteria*, but lower relative abundances of *Bacteroidota* (Figure 3.1). In addition, there were no differences ($P \geq 0.334$) in alpha diversity metrics

between uterine horns (Table 3.1). The data approached a tendency for the increased (P = 0.131) alpha diversity based on number of observed features and tended to have increased (P = 0.098) alpha diversity based on Shannon index of cows that expressed estrus when compared with cows that failed to express estrus (Table 3.2). There was also no clear clustering in the principal coordinate analysis of unweighted unifrac distances based upon uterine horn (Figure 3.3). Similarly, the principal coordinate analysis for unweighted unifrac distances showed no clear clustering of the ipsilateral samples for cows that displayed estrus and those that did not (Figure 3.4).

At the genus level, *Butyribirio, Cultibacterium, BD7-11, Bacteroidales BS11 gut group,* and *Ruminococcus* had greater ($P \leq 0.045$) relative abundances in the contralateral horn compared to the ipsilateral horn (Table 3.3). However, *Bacteroidales RF16 group* and *Clostridia UCG-014* were more abundant ($P \leq 0.025$) in the ipsilateral than the contralateral horn (Table 3.3). Paired t-test comparisons revealed that uterine horns ipsilateral to the CL had greater abundance ($P \leq 0.045$) of *Butyribirio, Cultibacterium, Ruminococcus, Bacillus,* and *Bacteroidales BS11 gut group* compared with the contralateral horns (Table 3.4). In addition, cows that expressed estrus signs had increased ($P \leq 0.045$) abundances of *Rikenellaceae RC9 gut group, Bacteroidales UCG-001, Lachnospiraceae AC2044 group, Burkholderia-Caballeronia-Paraburkholderia,* and *Pseudobutyribibrio* than did cows who did not express estrus (Table 3.5). Estrus expression resulted in decreased ($P \leq 0.035$) relative abundances of an unidentified genus of the family *Chitinophagaceae, Vibrionimonas,* and *dgA-11 gut group* when compared to cows that did not display estrus (Table 3.5).

Discussion

The healthy uterus was long thought to be a sterile environment. However, recent developments in sequencing technology allowed for the characterization of the bovine uterine microbial community (Ault et al., 2019a; Baker et al., 2018; Heil et al., 2019). Most uterine microbiome studies in the bovine utilized short sequence technology and only amplified a portion of the 16s rRNA gene (Ault et al., 2019a; Ault et al., 2019b; Jeon et al., 2015). In the present study, all nine hypervariable regions of the 16s rRNA gene was sequenced in order to increase the specificity and accuracy of taxonomic assignment (Tedersoo et al., 2021). To our knowledge, this is the first bovine uterine microbiome study that utilized a surgical collection procedure to ensure a uterine sample free of fecal and vaginal microbial contamination. The large number of samples with few, or no sequences recovered even after polymerase chain reaction amplification, confirms that this approach produced low-contamination samples. The use of whole 16s rRNA gene sequencing allowed for more taxa to be assigned to the genus and species levels (e.g., *Actinobacillus seminis* and *Brevibacterium casei*). This granularity of data allowed observational differences that were not evident at higher levels analyses (such as phylum or family) which would have not been identified with short sequencing due to a high number of unassigned taxa (Johnson et al., 2019).

Alpha and beta diversity metrics have gained popularity as a convenient way to compare similarities or differences between two or more microbial communities (Kers and Saccenti, 2022). Alpha diversity quantifies the amount of diversity within a particular community, whereas beta diversity quantifies species composition differences between two communities (Whittaker, 1972). Though we lacked the statistical power to detect

differences in alpha diversity in these conditions, it is a common assumption that greater diversity is most often associated with "healthy" microbiomes, and a loss in this biodiversity could be indicative of a disease state (Mosca et al., 2016; Ong et al., 2021; Roslund et al., 2020; Santos and Bicalho, 2012a). The tendency for decreased observed features and Shannon index in the ipsilateral horn of cows that did not express estrus may indicate a "disease" state and be associated with fertility differences seen between cows expressing estrus versus those that do not (Pereira et al., 2016; Richardson et al., 2016). Since we saw no beta diversity differences this implies that the overall structure of the bacterial communities found across both horns and categories of estrus expression were similar, and that these communities only differed in relative abundance of specific taxa to one another.

There was a tendency for an increase in populations of *Proteobacteria* and an accompanying tendency for decreased *Bacteriodota* in the ipsilateral horn of non-estrual cows. This is particularly intriguing since many members of *Proteobacteria* can cause disease (opportunistic), whereas most *Bacteriodota* are considered commensal (Rajilić-Stojanović and de Vos, 2014; Rizzatti et al., 2017). Potentially, differences in the uterine environment between uterine horns are associated with dysbiosis in the ipsilateral horn of non-estrual cows which contributes to decreased fertility compared to their estrual counterparts (Pereira et al., 2016; Richardson et al., 2016). *Actinobacillus seminis* was detected exclusively in the horn ipsilateral to the CL in two cows that did not display estrus. *Actinobacillus seminis* is an opportunistic pathogen that has been shown to cause epididymitis and orchitis in rams, as well as metritis and abortion in ewes (Foster, 2016;

Macaldowie, 2016). This bacterium has not been previously found in cattle, so it is still unclear its effect on fertility in this species.

Chitinophage is a bacterium that was present in nearly every sample and found across uterine horns and estrus expression. Chitin is a carbohydrate that characterizes fungi that some species in the family *Chitinophage* are able to use as a substrate for metabolism (Rosenberg, 2014). This result could indicate that there is some interaction between fungi present in the uterus, and this bacterial population. The presence of fungal species, such as *Aspergillus fumigatus, Penicillium spp., and Candida kefyr* in the uterus of cows have been reported and associated with fungal endometritis (Karstrup et al., 2017; Saini et al., 2019). A decreased abundance of *Chitinophage* in cows that displayed estrus could indicate a lower population of fungi, further supporting the idea that the cows that did not display estrus suffer from a uterine dysbiosis that could be impacting fertility. It must be noted that the present study did not attempt to quantify the presence of fungi in these samples.

The underlying reasons why the ipsilateral and contralateral microbial communities differed remains to be fully understood and is almost certainly a multifaceted (Davoodi et al., 2016; Sánchez et al., 2019a; Takahashi et al., 2016). Even though transcriptome studies show large differences between the endometrial transcriptome of uterine horns during early compared with late diestrus when a conceptus is present (Sánchez et al., 2019a) we were still able to detect differences in the microbial population during late diestrus. In addition, the presence of a conceptus influences the gene expression in the uterine transcriptome (Sánchez et al., 2019a; Sánchez et al., 2019b). Thus, the presence of a conceptus may also impact the composition of the

microbial community in the uterine horn. Local substrate availability and concentrations of various hormones (e.g., progesterone and estradiol), could alter the microbial population differentially within each uterine horn during the late luteal phase. Progesterone concentrations vary throughout different parts of the estrous cycle, reaching its peak between days 8 and 18 (Henricks et al., 1970). Progesterone has an inhibitory effect on innate inflammatory immune response (Cui et al., 2020; Hansen, 1998), which could provide an opportunity for microbes to colonize and proliferate in the uterus during this time.

The results of this study support previous research in concluding that the healthy non-pregnant uterus is not sterile. Taken together, these results indicate that the uterine microbiome is location and individual specific and should be studied with this in mind. In the future, whole uterine flushing should be avoided in studies that wish to draw conclusions about the effect of the microbiome on pregnancy establishment to avoid confounding their results. Differences in the microbiome relative to estrus expression warrant further study. Investigation of the variation in the uterine microbiome at different phases throughout the estrous cycle, especially in correlation with hormonal variation should be carried out. Further investigation is also needed to compare microbiomes of animals with intrinsically greater fertility.

Conflict of Interest Statement

The authors declare no conflict of interest.

Tables and Figures

Table 3. 1. Differences in Alpha Diversity between ipsilateral and contralateral uterine horns on day 15 of the estrous cycle of non-pregnant beef cows.

Diversity Metric	Ipsilateral	Contralateral	P-value (t-test)	p-value (Wilcoxon)
Faith's Phylogenetic Diversity	8.78	9.56	0.176	0.334
Observed Features (ASVs)	36.21	37.65	0.403	0.962
Shannon Index	3.65	4.04	0.188	0.825
Pielou Evenness	0.71	0.79	0.071	0.506

[1]Uterine microbiome samples were collected on day 15 of the estrous cycle from the uterine horns ipsilateral and contralateral to the corpus luteum for entire (V1-V9 hypervariable regions) 16s rRNA gene sequencing. Non-parametric test preformed since data was not normal.

Table 3. 2. Impact of estrus expression on ipsilateral uterine horn alpha diversity metrics on day 15 of the estrous cycle in non-pregnant beef cows.

Diversity Metric	**Estrus Expression**	**No Estrus Expression**	**P-value (t-test)**	**P-value (Kruskal-Wallis)**
Faith's Phylogenetic Diversity	9.45	8.73	0.189	0.344
Observed Features	39.57	33.13	0.151	0.131
Shannon Index	4.17	3.36	0.048	0.098
Pielou Evenness	0.80	0.67	0.034	0.144

[1]Uterine microbiome samples were collected on day 15 of the estrous cycle from the uterine horns ipsilateral and contralateral to the corpus luteum for entire (V1-V9 hypervariable regions) 16s rRNA gene sequencing. Non-parametric test preformed since data was not normal.

Table 3. 3. Differences in mean relative abundance at the genus level between ipsilateral and contralateral uterine horns in non- pregnant cows on day 15 of the estrous cycle.[1]

Genus	**Ipsilateral (%)**	**Contralateral (%)**	**p-value**
Butyrivibrio	0.14	1.53	0.003
Bacteroidales RF16 group	0.37	0.0	0.013
Cultibacterium	3.31	8.11	0.025
Clostridia UCG-014	0.42	0.04	0.025
BD7-11	0.07	0.32	0.033
Bacteroidales BS11 gut group	0.12	0.43	0.037
Ruminococcus	0.40	1.03	0.045

[1]Uterine microbiome samples were collected on day 15 of the estrous cycle from the uterine horns ipsilateral and contralateral to the corpus luteum for entire (V1-V9 hypervariable regions) 16s rRNA gene sequencing. Non-parametric test preformed since data was not normal.

Table 3. 4. Differences in mean relative abundance at genus level between ipsilateral and contralateral uterine horn using paired t-test (paired by cow).[1]

Genus	**Ipsilateral (%)**	**Contralateral (%)**	**p-value**
Butyrivibrio	0.06	1.46	0.000
Bacteroidales RF16 group	0.34	0.00	0.028
Bacillus	0.00	0.64	0.030
Clostridia UCG-014	0.48	0.00	0.031
Cultibacterium	2.60	8.77	0.043
Veillonellaceae UCG-001	0.25	0.00	0.043
Bacteriodales BS11 gut group	0.00	0.40	0.045
Ruminococcus	0.24	0.77	0.016

[1]Uterine microbiome samples were collected on day 15 of the estrous cycle from the uterine horns ipsilateral and contralateral to the corpus luteum for entire (V1-V9 hypervariable regions) 16s rRNA gene sequencing.

Table 3. 5. Differences in mean relative abundance at the genus level between cows that expressed estrus and cows that failed to express estrus.[1]

Genus	Estrus Expressed (%)	No Estrus Expression (%)	p-value
Rikenellaceae RC9 gut group	4.32	1.59	0.002
Bacteroidales UCG-001	1.33	0.06	0.014
Lachnospiraceae AC2044 group	0.98	0.17	0.016
Vibrionimonas	0.94	2.24	0.023
Burkholderia-Caballeronia-Paraburkholderia	0.50	0.04	0.027
Family *Chitinophagaceae*	0.03	0.16	0.028
dgA-11 gut group	0.00	0.06	0.035
Pseudobutyrivibrio	0.34	0.00	0.045

[1]Uterine microbiome samples were collected on day 15 of the estrous cycle from the uterine horns ipsilateral and contralateral to the corpus luteum for entire (V1-V9 hypervariable regions) 16s rRNA gene sequencing. Non-parametric test preformed since data was not normal.

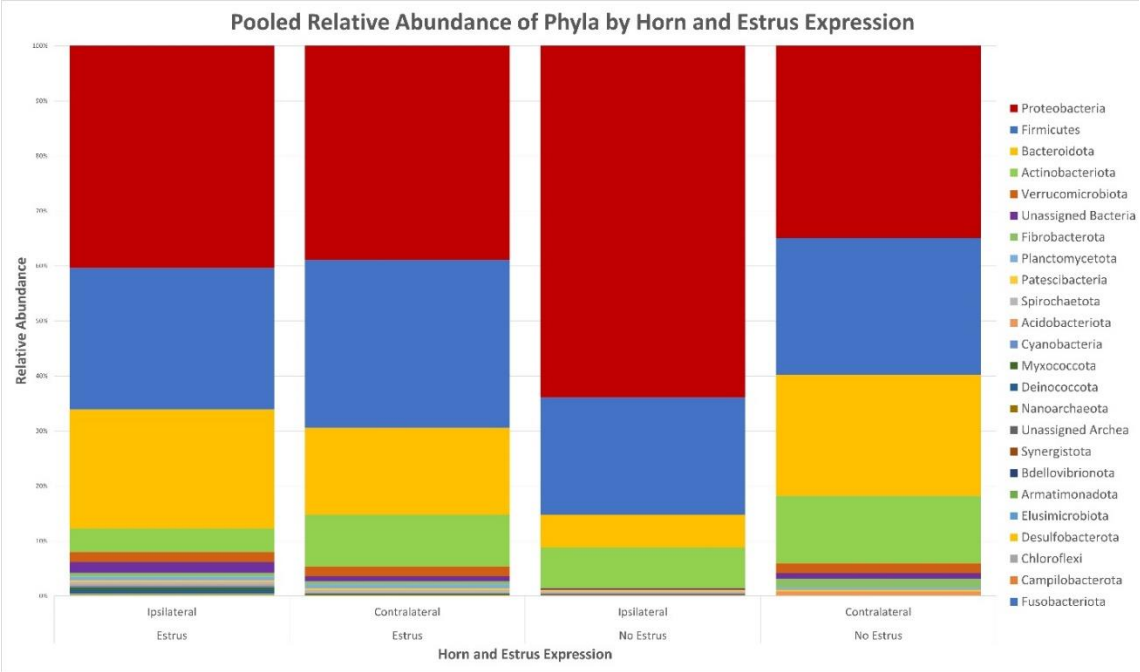

Figure 3. 1. Relative abundance of phyla based on estrus expression and uterine horn position relative to the luteal bearing ovary (ipsilateral or contralateral) in non-pregnant beef cows on day 15 of the estrous cycle. Estrus: expressed estrus prior to ovulation. No estrus: failed to express estrus prior to exogenously induced ovulation.

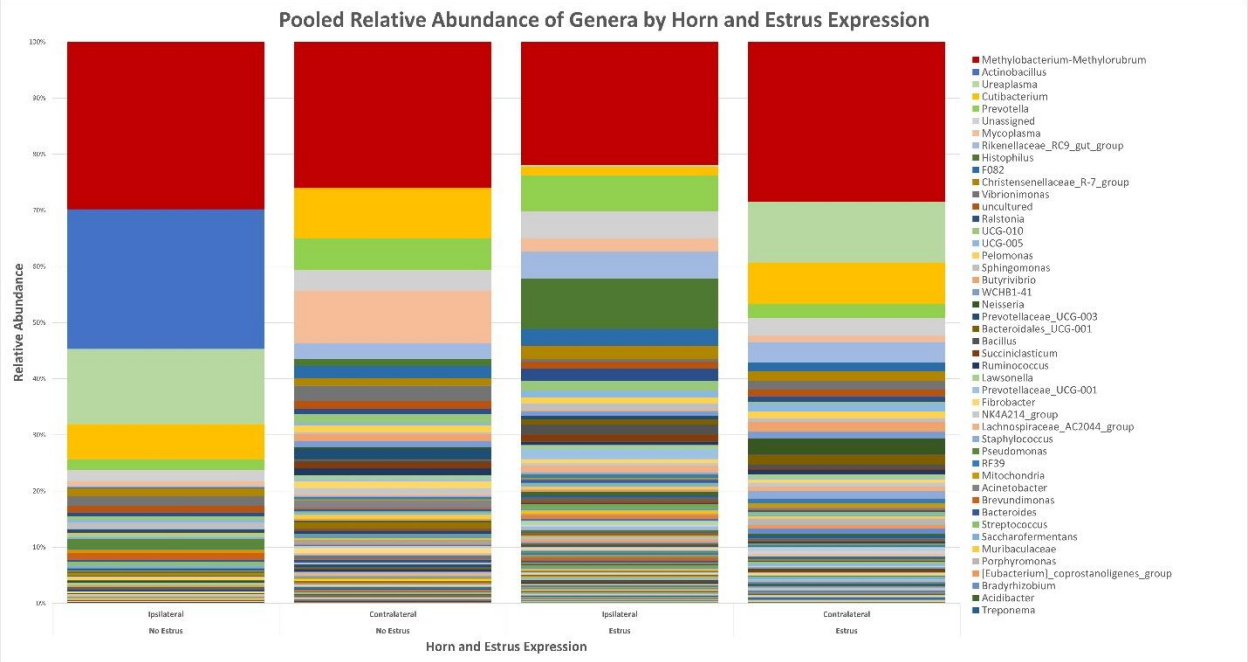

Figure 3. 2. Relative abundance of genera based on estrus expression and uterine horn position relative to the luteal bearing ovary (ipsilateral or contralateral) in non-pregnant beef cows on day 15 of the estrous cycle. Estrus: expressed estrus prior to ovulation. No estrus: failed to expressed estrus prior to exogenously induced ovulation.

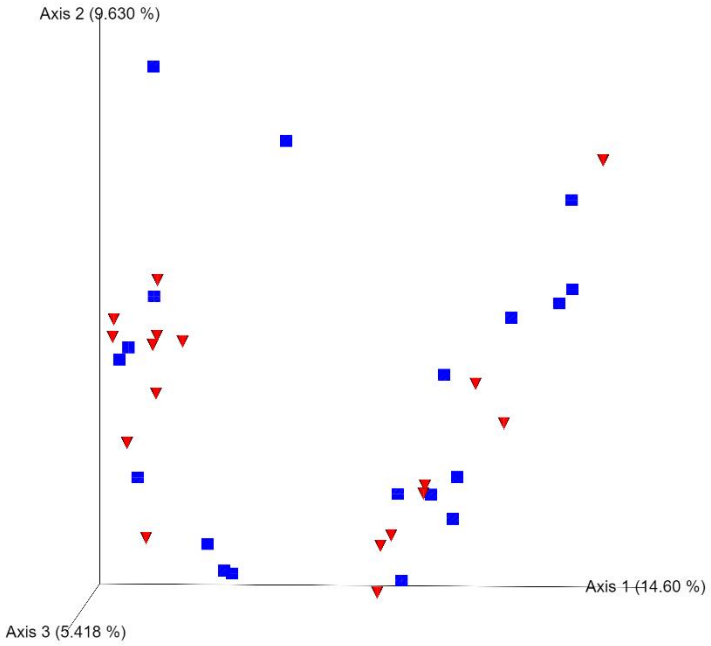

Figure 3. 3. Principal coordinate analysis plot for unweighted unifrac-distances based on horn position relative to the luteal bearing ovary (ipsilateral or contralateral) in non-pregnant beef cows on day 15 of the estrous cycle. Blue squares and red triangles represent ipsilateral and contralateral uterine horns, respectively.

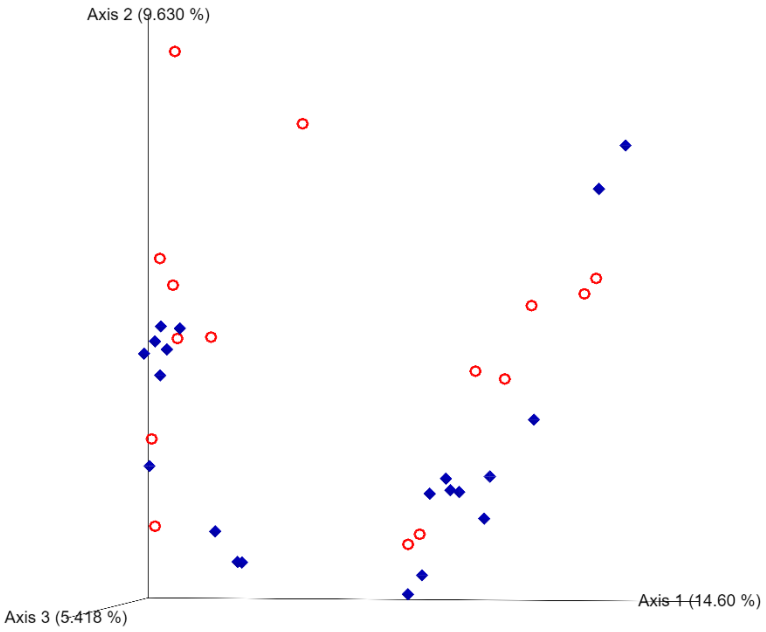

Figure 3. 4. Principal cooordinate analyses plot for unweighted unifrac-distances based on estrus expression. Blue diamonds represent cows that expressed estrus and red circles represent cows that failed to express estrus.

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CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

In conclusion, the microbiome of the uterine horn ipsilateral to the corpus luteum on day 15 of the estrous cycle in non-pregnant beef cows differed from the contralateral horn. There was a higher *Firmicutes* to *Bacteroidetes* ratio in the ipsilateral horn of cows that failed to display estrus compared to cows that displayed estrus. There were no differences in the alpha or beta diversity between uterine horns. Within the ipsilateral uterine horn, cows that displayed estrus had greater Shannon index values compared to those that did not display estrus. Therefore, there is a loss of bacterial diversity, often indicative of a disease state, in cows that fail to display estrus. This could contribute to the lower observed fertility in cows that fail to display estrus; suggesting the loss of diversity may also decrease the likelihood of a female displaying signs of estrus.

These results indicate the need for further research into the composition of the microbiome at different points throughout the estrous cycle. Little is known about the effect of hormones on the composition of the microbiome or the impact these bacteria may have on the function of the endocrine system. In addition, further investigation is merited to examine differences in microbiome composition when a conceptus is present compared to when it is absent. These studies would investigate the effect the microbiome has on successful pregnancy establishment, to try to elucidate what a “healthy” uterine microbiome composition is.

The relationship between estrus expression and the microbiome of the uterine horn ipsilateral to the corpus luteum is the most interesting result found here. This relationship should be further explored especially during proestrus and estrus to more thoroughly understand the impact estrus expression has on fertility. Failure to display estrus may be the physiological expression of a subclinical disease that is negatively impacting fertility and a "normal" state needs to be established before treatments for abnormal states can be developed.

In the future, understanding the differences in the microbiome throughout a successful reproductive event and a failed one will provide a myriad of benefit to the industry. A variety of technologies could be developed to identify animals that are more likely to successfully reproduce than others to help with culling decisions. Other approaches could include attempting to return a dysbiosis to a healthy state prior to breeding using antibiotics, probiotics, or prebiotics. Adding these technologies to the current commercially available assistive reproductive technologies would increase the efficiency of the beef herd in Georgia and minimize the environmental impact of feeding the world's growing population.