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To the Graduate Council:

I am submitting herewith a thesis written by Taylor Brianne Ault entitled "Effect of uterine and vaginal bacteriomes on fertility in postpartum beef cows." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Ky G. Pohler, Phillip Myer, Major Professor

We have read this thesis and recommend its acceptance:

J. Lannett Edwards

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Effect of uterine and vaginal bacteriomes on fertility in postpartum beef cows

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Taylor Brianne Ault

December 2018

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ABSTRACT

Infertility in beef and dairy cattle costs millions of dollars each year to the animal agriculture industry. The microbiome of the reproductive tract has been indicated to have an effect on pregnancy establishment and maintenance in humans, however the bovine reproductive microbiome and its effect on fertility is not well understood. The objective of the current study is to evaluate bacterial communities of the uterus and vagina of postpartum beef cows undergoing estrous synchronization to determine differences in cows who will become pregnant and those who fail to conceive to fixed time artificial insemination (FTAI). Thirty Angus cows at an average of 82 days postpartum were subjected to a 7 Day Co-Synch protocol with a pre-synchronization step 21 days prior to FTAI (d -21). Uterine and vaginal flushes were collected at each day of the protocol for pH detection and bacterial DNA extraction and sequencing targeting the V1-V3 hypervariable region of the 16S rRNA gene. Pregnancy diagnosis occurred on d 30 by transrectal ultrasound where ten non-pregnant and ten pregnant cows were selected for sequencing. Results indicated a significant decrease between d -9 and d -2 in the number of bacterial species in the uterus of pregnant and non-pregnant cows ($P < 0.0001$). Many significant differences in relative abundance of bacteria phyla and genera were detected between pregnant and non-pregnant cows and over the duration of the protocol. Many bacteria, such as the common pathogenic bacteria *Cornyebacterium*, had relative abundances greater than 1% at d -2 in the uterus of non-pregnant cows, but present in less than 1% in the uterus of pregnant cows ($P < 0.05$). When evaluating pH, uterine pH was lower than vaginal pH on average. Although not significant, uterine pH decreased in

pregnant cows and increased in non-pregnant cows through the duration of the estrous synchronization protocol. In conclusion, our data suggests the bovine reproductive tract microbiome fluctuates over time and differences in bacterial species abundances may affect reproductive outcomes.

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CHAPTER ONE – LITERATURE REVIEW

According to the Food and Agriculture Organization, the world population is expected to increase to almost 10 billion people by 2050. The growing population creates a challenge for the agriculture industry to provide enough food to meet the increased demand. Due to the high energy and nutrient density of livestock products, the animal agriculture industry must increase animal production and improve efficiency. In addition to the growing demand, another challenge to the livestock industry is the prevalence of reproductive losses which has been estimated to cost the beef and dairy industry over 1 billion dollars every year (Bellows et al., 2002). Methods such as advanced reproductive techniques are being used to improve reproductive efficiency in livestock. Current research is evaluating factors such as nutrition, genetics, and health management practices that may influence the fertility of livestock animals to determine approaches for manipulating these factors and improving reproductive outcomes. The following review will focus on the challenge of fertility in postpartum cows and evaluating the potential effect of the reproductive tract microbiome on fertility.

Postpartum Uterine Involution

Normal Uterine Involution

Uterine involution is the process that must occur following parturition to prepare the cow's uterus to be able to successfully develop and maintain another pregnancy. The decline in progesterone and increase in estrogen during parturition results in increased production of prostaglandin (PGF_{2a}) and oxytocin receptors to stimulate uterine

contractions to expel the placenta, with full expulsion occurring ideally before 12 hours postpartum (Beagley et al., 2010). The process of uterine involution can then begin, first with the sloughing of the maternal caruncles of the uterine endometrium. The necrosis of the caruncles results in lochia, a fluid discharge, containing the sloughed tissues and blood from ruptured blood vessels (Sheldon et al., 2008). This discharge will be expelled from the cow within the first two weeks following parturition (Leslie, 1983). Once the caruncles have been sloughed off, the endometrium must be regenerated to develop new caruncles for proper placental attachment and nutrient exchange for the next pregnancy. Regeneration can occur within one month for the epithelial layer, but full regeneration of the deeper uterine tissues to complete uterine involution may take up to two months (Sheldon et al., 2008).

During uterine involution, the cow will be infertile. The process of sloughing and regeneration of the endometrium will not allow for implantation of a potential embryo to occur (Kiracofe, 1980; Short et al., 1990). To prevent monetary losses for the producer due to increased days open after parturition, it is crucial uterine involution occur successfully within a short time period to develop another pregnancy. Normally the cow will be able to develop another pregnancy immediately following the period of uterine involution. However, complications can occur leading to postpartum uterine diseases causing infertility.

Postpartum Uterine Disease

Through parturition and uterine involution, there is high risk for bacterial contamination and infection resulting in uterine diseases, most commonly endometritis

and metritis. Metritis affects multiple layers of uterine tissue resulting in an enlarged uterus, while endometritis is a localized infection of the endometrial layer (Sheldon et al., 2008). In dairy cows, metritis occurred in 40% of cows within 10 days of parturition and endometritis in 15-20% of cows up to 50 days after parturition (Sheldon et al., 2009b). Pathogenic bacteria such as *Escherichia coli* and *Arcanobacterium pyrogenes* are often identified with uterine diseases and delayed uterine involution (Hussain et al., 1990; Huszenicza et al., 1999; Williams et al., 2007). Other bacteria working synergistically with *E. coli* and *A. pyrogenes* such as *Trueperella*, *Fusobacteria*, and *Prevotella* have been identified in higher abundance with uterine infections, although they are also suggested to be present at a lower abundance in normal uterine microbiota (Sheldon and Dobson, 2004; Machado et al., 2012; Karstrup et al., 2017; Sheldon and Owens, 2017). A major consequence of the presence of pathogenic bacteria causing uterine disease and delayed involution have been proven to reduce fertility resulting in increased days open, delayed return to cyclicity, and increased services to conception (Bonnett et al., 1993; LeBlanc et al., 2002; Ribeiro et al., 2013). The reduced fertility in postpartum cows with uterine disease is associated with an exaggerated inflammatory response. Herath et al. (2009) evaluated multiple endometrial biopsies during the postpartum period of fertile and infertile cows. Biopsies from the first week following parturition indicated a higher expression of pro-inflammatory genes in cows that developed endometritis leading to infertility, compared to fertile postpartum cows (Herath et al., 2009). The inflammatory environment in the uterus caused by the presence of pathogenic bacteria following parturition has been proven to be detrimental to fertility, however, further research is

needed to determine the influence of a healthy postpartum reproductive microbiome on fertility.

Microbiomes in the Body

The number of microorganisms in the body have been estimated to be ten times greater than the number human cells (Turnbaugh et al., 2007). As new scientific technologies are continually invented and previous methods improved, the capacity to conduct in depth research into the microbiota is expanded (Chaucheyras-Durand and Ossa, 2014). The development of 16s rRNA sequencing technologies has led to many discoveries of the bacterial communities in the body by a genetic approach to identify bacteria, opposed to phenotypic methods, allowing the ability to discover novel or unculturable bacteria (Clarridge, 2004). In 2008, the National Institutes of Health (NIH) began the Human Microbiome Project using 16s rRNA sequencing to characterize the bacteriome from multiple locations of the body such as the skin, nasal cavity, and digestive and reproductive tracts. Characterizing these communities sets groundwork for further research to determine the importance of the microbiota and its relationship to health issues.

Gastrointestinal Microbiome

The gastrointestinal tract has been a main focus in studying the interaction between the microbiota and its host due to its high abundance of microbes, making up the majority (29%) of the human bacteriome with the greatest species diversity (NIH et al., 2009; The Human Microbiome Project, 2012). In healthy adults, the gut microbiota

contributes to functions such as digestion, metabolism, immune system activation, and protection against pathogens (Bull and Plummer, 2014; D'Argenio and Salvatore, 2015). However, dysbiosis, an imbalance in the core gut microbiome, has been associated with health issues such as obesity and inflammatory bowel disease (IBD) (Bull and Plummer, 2014). The two dominant bacterial phyla of the gut microbiome have been found to differ between lean and obese humans and mice with significantly decreased abundance of Bacteroidetes and increased Firmicutes with obesity (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006). IBD is associated with an overall decrease in the diversity of the gut microbiome, including a decrease in both Firmicutes and Bacteroidetes (Frank et al., 2007; Sheehan et al., 2015; Nishino et al., 2018). Firmicutes and Bacteroidetes are important for their production of short chain fatty acids involved in regulating local immune system activation for anti-inflammatory effects as well as providing energy to epithelial cells for maintenance of proper intestinal lining functions (Tedelind et al., 2007; Ghouri et al., 2014; Nishino et al., 2018). Although the exact mechanism is not understood, the dysbiosis of the microbiome with reduction of beneficial bacteria, Firmicutes and Bacteroidetes, is thought to lead to the inflammatory state of IBD (Nishino et al., 2018). The influence of microbial community shifts on gastrointestinal health prompted current research to focus on manipulation of the microbiome with probiotics. Multiple studies have indicated beneficial effects to prevent obesity by reducing weight gain and treating IBD (Yoo et al., 2013; Zhang et al., 2016; Ganji-Arjenaki and Rafieian-Kopaei, 2018).

The primary example of a microbial-host interaction in bovine is the rumen microbiome. Cattle rely on the diverse and abundant microbial communities in the rumen to convert their diet to nutrients and energy. The diet consists of forages and grains high in complex carbohydrates, such as cellulose, that are not able to be broken down by the animal's own enzymes. Microbes are able to use fermentation to break down these molecules producing volatile fatty acids (VFA) that can then be absorbed and used by the animal for energy production. The rumen microbiome is not stable, however, shifting depending on the composition of the diet (de Menezes et al., 2011; Thoetkiattikul et al., 2013; Henderson et al., 2015). For example, the ratio between concentrates and forages alters the microbial communities present due to the difference in substrates they provide for fermentation, therefore altering the end products of fermentation and affecting the rumen environment (Carro et al., 2000; Petri et al., 2012; Petri et al., 2013). When the diet proportion of concentrates is increased, higher abundances of starch-fermenting bacteria will be present resulting in rapid and increased production of lactic acid and VFAs, lowering ruminal pH (Nagaraja and Titgemeyer, 2007; Fernando et al., 2010). *S. bovis*, a starch fermenting bacteria, often increases in abundance with a continuously higher concentrate proportion in the diet. The low pH of the rumen from lactic acid accumulation by *S. bovis* can lead to lactic acidosis, negatively affecting the animal's overall health and performance (Nagaraja and Lechtenberg, 2007). Probiotics have indicated beneficial effects to improve growth performance in calves and increase fermentation measures in mature cattle, although results are inconsistent due to the

variation of diet and pre-existing ruminal microbial communities and environment (Desnoyers et al., 2009; Frizzo et al., 2011; Uyeno et al., 2015; Retta, 2016).

The actions of microbial communities in the human digestive tract and rumen of cattle has indicated the symbiotic relationship between microbes and their host is vital for optimal health and performance. Studies using probiotics has suggested positive results by manipulating the microbiome to return to a healthy state. Current research is exploring the functions of additional microbiomes found throughout the body and how they interact with other processes, such as reproduction.

Human Reproductive Microbiome

The Human Microbiome Project found that 9% of the total bacteriome of the body is present in the reproductive tract (NIH et al., 2009). This newly discovered microbiome is a recent focus in human health research to discover its influence on the microbial colonization of young and relationship to fertility.

The vaginal microbiome has been well defined in women. In contrast to the highly diverse microbiome of the digestive system, the vagina has a low species diversity consisting of predominately *Lactobacillus* in healthy non-pregnant and pregnant women (The Human Microbiome Project, 2012; Walther-António et al., 2014). The low pH of the reproductive tract of women is attributed to *Lactobacillus* species fermentation products, hydrogen peroxide and lactic acid (Rnnqvist et al., 2006). Many studies indicate these products may provide a defense mechanism to prevent pathogen entry and colonization, suggesting *Lactobacillus* is a predictor of reproductive tract health (Klebanoff et al., 1991; Osset et al., 2001; Rnnqvist et al., 2006). In a study by Ravel et

al. (2011), five major core vaginal microbiomes were identified in reproductive age asymptomatic women. Four of the five groups were dominated by *Lactobacillus* species with the remaining group less dominated by *Lactobacillus*, containing higher species diversity and greater proportion of strictly anaerobic bacteria. Women in the non-*Lactobacillus* dominated core microbiome group were correlated to a higher Nugent score, a diagnosis for bacterial vaginosis (Ravel et al., 2011). *Lactobacillus* abundance has also been suggested as a potential predictor of fertility. Moreno et al. (2016) evaluated the reproductive tract microbiome of women undergoing in vitro fertilization. *Lactobacillus* species dominated in both vaginal and uterine endometrial samples. They found women with greater than 10% non-*Lactobacillus* species had significantly lower pregnancy rates, implantation rates, on-going pregnancies, and live births with a tendency of higher miscarriage rates (Moreno et al., 2016). The established dominance of *Lactobacillus* species in the vagina has indicated its importance in health and fertility of women, but further studies are needed to determine the cause of lower abundance in women that develop bacterial vaginosis or infertility.

The uterine microbiome, in contrast to studies of the vaginal microbiome, has been highly debated. Previously, the "sterile womb hypothesis" suggested the uterus is a sterile environment in healthy and pregnant women and animals with bacterial colonization leading to complications (Perez-Muñoz et al., 2017). Bacterial transmission to young was thought to first occur at birth as differential communities have been shown to develop between vaginal and cesarean deliveries (Dominguez-Bello et al., 2010; Dogra et al., 2015). However, recent studies with advanced technologies have begun to disprove

the sterile hypothesis by identifying a placental microbiome, leading to the "in utero colonization hypothesis" suggesting colonization of bacterial communities in the young begins in the uterus (Stout et al., 2013; Aagaard et al., 2014; Wassenaar and Panigrahi, 2014). Due to the role of commensal bacteria in immune system stimulation, defense against pathogens, and providing fermentation products for use by the host, the uterine microbiota is also of interest for its potential effect on the establishment of pregnancy. However, results from uterine microbiome studies are conflicting due to difficulty and variation in collection methods. In addition, the majority of research into the uterine microbiome has been conducted on women undergoing hysterectomies or in vitro fertilization for various reasons that may influence results. Further research is needed to determine the composition of the healthy uterine microbiome of women and how shifts in its composition can affect fertility.

Bovine Reproductive Microbiome

As previously discussed, bacteria have been identified that frequently cause infections during uterine involution affecting the future fertility of postpartum cows. However, few studies have evaluated the healthy bovine reproductive microbiome to determine the presence of bacterial species that may be beneficial to reproductive tract health and pregnancy establishment, or detrimental to fertility without presence of a clinical infection. One of the first studies evaluating the reproductive tract microbiome of bovine using 16s rRNA sequencing was Swartz et al. (2014) evaluating the vaginal microbiome of cows and ewes. Overall, they found high bacterial species diversity, contrasting results from human studies. Interestingly, *Lactobacillus* was common among

samples but had an average relative abundance of less than 1%, resulting in a more neutral pH of the vagina (Swartz et al., 2014). In 2015, Laguardia-Nascimento et al. compared the vaginal microbiome between pregnant and non-pregnant cows and heifers. They found pregnant cows and heifers had significantly lower bacterial species diversity than in non-pregnant animals. However, non-pregnant animals were not controlled for their stage of the estrous cycle, possibly affecting results due to physiological differences in hormone concentrations. Laguardia-Nascimento et al. (2015) and multiple other studies agree the reproductive tract is dominated by the phyla Firmicutes, Bacteroidetes, and Proteobacteria, but with low levels of *Lactobacillus* species (Clemmons et al., 2017; Moore et al., 2017). Clemmons et al. (2017) evaluated the bacterial community differences between the uterus and vagina of non-pregnant cows two days prior to fixed time artificial insemination (FTAI). Firmicutes had the highest average relative abundance and the only significantly different phyla between the uterus (31.3%) and vagina (65.9%). The greater abundance in the vagina has been attributed to its anatomical location on the cow, allowing entry of bacteria from the digestive system and the environment. The cervix acts as a physical barrier to prevent entry of these potential pathogenic bacteria into the uterus. The abundance of unassigned bacteria was also significantly higher in the uterus (16.1%) than the vagina (3.4%), indicating there could be a novel bacteriome not detected prior to 16s rRNA sequencing and contributing evidence to disprove the sterile womb hypothesis (Clemmons et al., 2017). Although these studies have begun to characterize the reproductive microbiome, the uterine and vaginal bacterial composition's effect on fertility has not been determined.

The following study evaluates the reproductive tract microbiome of postpartum beef cows through the duration of an estrous synchronization protocol. Our goal is to determine the microbiome's potential effect on pregnancy establishment by identifying differences in the microbiome between cows who become pregnant and those who fail to conceive to artificial insemination.

CHAPTER TWO – REPRODUCTIVE BACTERIAL COMMUNITIES BETWEEN PREGNANT AND NON-PREGNANT POSTPARTUM COWS THROUGH ESTROUS SYNCHRONIZATION

Abstract

Reproductive losses are caused by various factors costing the beef and dairy industry an estimated total of one billion dollars each year. Few studies have evaluated the domestic animal reproductive microbiome and its relationship to fertility. The present study evaluates the vaginal and uterine microbiome throughout an estrous synchronization protocol to compare cows who will become pregnant and those who fail to conceive to fixed time artificial insemination (FTAI). Thirty postpartum Angus cows were synchronized beginning with a pre-synchronization step on day -21 (d -21) followed by the 7-Day Co-Synch protocol, ending with FTAI on d 0. Uterine and vaginal flushes were collected on each day of the protocol for pH measurement and bacterial DNA extraction. Pregnancy diagnosis occurred on d 30 by transrectal ultrasound. A total of 10 non-pregnant and 10 pregnant cows were selected for sequencing. Extracted bacterial DNA was sequenced targeting the V1-V3 hypervariable region of the 16s rRNA gene. Results indicated a significant decrease in the number of bacterial species from d -9 to d -2 in the uterus of pregnant and non-pregnant cows ($P < 0.0001$). Principal coordinate analyses using unweighted UniFrac metrics indicated significant clustering of samples by day in the uterus and vagina ($P < 0.0001$). Many significant differences in the relative abundance of bacterial species at the phylum and genus levels of taxonomic composition

occurred between pregnant and non-pregnant cows and among days of the protocol. At d -2, the most abundant classified genera of bacteria in the uterus of non-pregnant cows was *Corynebacterium* at 9.62 ± 4.97% relative abundance, however it was only present with 0.50 ± 0.36% relative abundance in pregnant cows ($P = 0.003$). Many other genera were also present in >1% abundance of non-pregnant cows but <1% abundance in pregnant cows. When evaluating pH, the uterus had an overall more acidic pH than the vagina. Although not significant, pH increased in the uterus of non-pregnant cows but decreased in the uterus of pregnant cows. Overall, the current study suggests the shift in the bacterial microbiome and differences in relative abundances of bacterial species of the reproductive tract may be important for successful pregnancy establishment and maintenance. Further research is needed to evaluate the factors causing these changes and how to manipulate them to improve reproductive efficiency.

Introduction

Reproductive losses cost the beef and dairy industry over one billion dollars every year (Bellows et al., 2002). Fertility can be affected by many factors such as nutrition, heat stress, parity, and potentially microbes (Richards et al., 1986; Wolfenson et al., 2000; Meikle et al., 2004; Rodney et al., 2018). In postpartum cows, the effects of these factors are particularly challenging due to the rapid changes during uterine involution that must occur after parturition for the cow to develop another successful pregnancy (Sheldon et al., 2008). During this time, there is a high probability of bacterial contamination that is most often cleared by uterine immune cells (Sheldon et al., 2006). Colonization of the pathogenic bacteria that are not cleared can lead to uterine diseases

which may result in decreased fertility of beef and dairy cows (Griffin et al., 1974; Bonnett et al., 1993; Földi et al., 2006; Sheldon et al., 2009a; Potter et al., 2010).

Bacteria reside in multiple systems throughout the body contributing vital roles to health such as modulating the pH of their environment, providing nutrients, and providing protection against harmful pathogens (NIH et al., 2009; Beecher et al., 2014). An imbalance, termed dysbiosis, in the normal bacteriome (the collection of bacterial genetic material present in a specific environment) can result in increased risk for health issues (Ojetti et al., 2009; Zhang et al., 2015). The reproductive tract contains its own distinct bacteriome, with dysbiosis of these communities potentially affecting fertility (The Human Microbiome Project, 2012; Moreno et al., 2016; Clemmons et al., 2017). Human studies have shown a high diversity of bacteria in the reproductive tract can affect establishment and maintenance of a pregnancy. *Lactobacillus* accounts for over 90% of the vaginal reproductive microbiome in healthy women, with reduced *Lactobacillus* dominance leading to disease and infertility (Rnnqvist et al., 2006; Sirota et al., 2014; Moreno et al., 2016).

Although many human studies have characterized and demonstrated the relationship between the bacteriome of the reproductive tract and fertility, it is relatively unknown in bovine. Postpartum cows have been shown to be at risk for bacterial infections that can cause various uterine diseases and result in infertility. Studies characterizing various livestock reproductive tracts in a non-diseased state have identified a lower percentage of *Lactobacilli* and closer to neutral pH compared to humans (Otero et al., 1999; Otero et al., 2000; Swartz et al., 2014). Laguardia-Nascimento et al. (2015)

evaluated the vaginal microbiome between pregnant and non-pregnant *Bos indicus* heifers and cows and found reduced bacterial species diversity in pregnant animals. Our group recently characterized the microbiome two days prior to fixed-time artificial insemination (FTAI) of non-pregnant postpartum beef cows to determine the difference in bacteriomes between the uterus and vagina. Results indicated the bacterial phylogenetic diversity in the vagina was significantly greater than in the uterus with many significant genus-level differences (Clemmons et al., 2017). As this characterized the healthy postpartum cow uterine and vaginal bacteriome, the effect of the differences in bacteriomes on fertility has not been determined. The objective of the current study was to characterize the bacteriome of the uterus and vagina of postpartum cows throughout an estrus-synchronization protocol and evaluate differences between those that successfully established a pregnancy and those that failed to conceive. We hypothesized the bacteriome differs in the uterus and the vagina of cows that become pregnant versus those that do not and that the bacteriome will change over time during the synchronization protocol.

Materials and Methods

This study was performed under an approved protocol by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville. The following methods are as described in Clemmons et al. (2017) with slight modifications.

Experimental Design

Thirty Angus beef cows located at the East Tennessee Research and Education Center, with an average of 80 ± 2.6 days postpartum (DPP) and 4.6 ± 0.57 years old at FTAI, were used for the study. Figure 1 presents the estrus-synchronization protocol and sampling periods as described. An industry standard estrus-synchronization protocol was used with a pre-synchronization step on d -21 (21 days before AI, average of 59 DPP) with an intramuscular administration of prostaglandin F2α (Lutalyse, 5 mL; 5mg/mL). A standard 7 Day Co-Synch Protocol began on d -9 (9 days before AI, average of 71 DPP) with gonadotropin releasing hormone (GnRH; Factrel, 200 mg) followed on d -2 (2 days before AI, average of 78 DPP) with an intramuscular injection of prostaglandin F2α (Lutalyse, 5 mL 25 mg/mL). Controlled internal release devices (CIDR) were not used in this study due to collection methods of uterine and vaginal flush samples and to prevent bacterial growth on the device or within the vagina influencing results. During the synchronization protocol, uterine and vaginal flush samples were collected at d -21, d -9, and d -2. For vaginal flush collection, 60 mL of 0.9% saline (Brand name; pH = 5.6) was passed into the vagina using a syringe and recovered via vaginal lavage. For uterine flush collection, a Foley catheter was placed in the body of uterus to prevent vaginal contamination of the sample. Saline (180 mL) was flushed through the catheter into the uterus and fluid was collected by rectal massage. Uterine and vaginal flush pH was measured by UltraBasic pH meter (Denver Instruments, Arvada, CO, United States) and recorded immediately following collection. Samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. Blood samples were also collected in BD Vacutainer

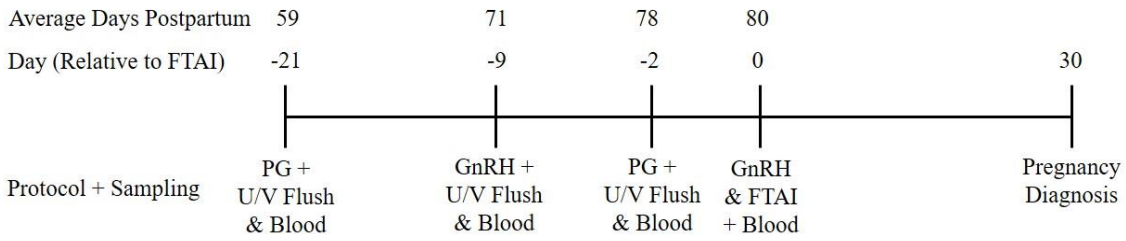

Figure 1. Timeline of estrus-synchronization protocol and sampling occurring on each day of the experiment with the average days postpartum at each time point.

tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) at each time point of the synchronization protocol. Additionally, transrectal ultrasound was conducted at each time point to map ovarian structures. On d 0 of the protocol, 2mL 100 mg/mL of GnRH was administered by intramuscular injection followed by FTAI using a single sire and technician. Pregnancy diagnosis by transrectal ultrasound occurred on d 30 following FTAI by observation of embryonic heartbeat. Following collections twenty (10 pregnant and 10 non-pregnant) of the thirty cows met all the criteria for the study and were used for sample sequencing and analysis. The criteria for inclusion in the study included: 1) CL present on day -21 or d-9 as well as P4 greater than 1ng/mL, 2) response to GnRH on day -9 measured by the absence of an ovulatory follicle on day -2, 3) CL present on day -2, 4) ovulatory follicle present on d 0 (FTAI).

DNA Extraction and Sequencing

A total of 120 samples (10 pregnant and 10 non-pregnant, 3 time points, 2 sampling locations) were used from the uterus and vagina of pregnant and non-pregnant cows from flush collections. DNA extraction and sequencing were performed similar to Clemmons et al. (2017). Once thawed at room temperature (22°C), samples were vortexed and aliquots of 5 mL were removed, placed in 15 mL tubes and centrifuged at 4,696 x g and 4℃. The resulting pellet was resuspended in 180 µL of sterile saline solution to concentrate the cells. The Qiagen DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany) was used according to manufacturer protocol for DNA extraction. Samples were stored at -20℃ until the amplification process. Polymerase Chain Reaction (PCR) was used for both DNA amplification and library construction. The hypervariable

regions V1-V3 of the 16S rRNA bacterial gene were targeted for bacterial identification. Sequencing libraries were produced using modified universal primers 27F (5′-Adapter/Index/AGAGTTTGATCCTGGCTCAG) and 519R (5′-Adapter/Index/GTATTACCGCGGCTGCTG) which included TruSeq indices and adapters, and was performed using Accuprime Taq high fidelity DNA Polymerase (LifeTechnologies, Carlsbad, CA, United States). The PCR annealing temperature was 58°C for 30 cycles. Libraries were quality checked by gel electrophoresis. Products were purified and quantified with AmPure beads and Nanodrop 1000 spectrophotometer (Agencourt, Beverly, MA, United States and ThermoScientific, Wilmington,DE, United States) as well as real time PCR on LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Sequencing of the PCR libraries was performed at the United States Meat Animal Research Center (USDA-ARS-USMARC; Clay Center, NE, United States) using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, United States) with the 2 x 300, v3 600 cycle kit.

Sequence Reading and Analysis

Resulting sequence reads were processed using the Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010) and Mothur version 1.36.1 (Schloss et al., 2009) programs. The Galaxy server was used for quality filtering to retain all sequences with quality scores ≥ Q 25. Adapter and index sequences were trimmed and sequences <300 bp were removed. Chimeric sequences were identified and removed by the usearch61 command (Edgar, 2010). The sequences which classified as chloroplasts and mitochondria were removed from analyses. Each sample was then

subsampled to 30,000 sequences to remove sequence depth bias. Operational taxonomic unit (OTU) picking occurred with a pairwise identity threshold of 97% using UCLUST in QIIME with taxonomy assignment by UCLUST and the Greengenes v13_8 16S rRNA database (Caporaso et al., 2010). Phylogenetic trees were built with FastTree (Price et al., 2010) to determine α- and β-diversity. Alpha-diversity, the measurement of bacterial species diversity within a sample, was analyzed using observed OTU, Faith's phylogenetic diversity, Shannon's diversity index, and Chao1 richness indices. Observed OTU indicates the number of species that are present from the subsampled sequences while Chao1 estimates the total number of species that are present. Shannon's diversity index uses the number of species present accounting for their abundance and evenness of distribution. Faith's phylogenetic diversity measures the diversity within a sample by using the total branch lengths of all bacteria detected. Beta-diversity analyses, comparing the bacterial species diversity between samples using phylogenetics, were conducted using unweighted UniFrac distance matrices to generate principal coordinates analyses (PCoA) utilizing QIIME (Lozupone and Knight, 2005).

Progesterone Assay

Progesterone was measured to determine the physiologic response to the protocol. Progesterone RIA was performed according to the previously described protocol (Pohler et al., 2016) using a double antibody RIA kit from MP Biomedicals. A standard curve (ranging from 0ng/mL to 50ng/mL) were used to calculate sample concentrations and in house controls we used for quality control. Inter and intra assay CV were less than 10%.

Statistical Analysis

Alpha diversity metrics including observed OTU, Chao1, and Faith's Phylogenetic Diversity were transformed to a normal distribution using lambda values obtained from Box-Cox transformations using the PROC TRANSREG procedure in SAS 9.4 and confirmed as normal using the PROC UNIVARIATE procedure. Transformed data, including Observed OTU, Chao1, and Faith's Phylogenetic Diversity metrics, were analyzed by one-way ANOVA using Tukey LSD in SAS Enterprise Guide 7.1. Data that non-normally distributed and could not be transformed were analyzed by non-parametric one-way ANOVA in SAS Enterprise Guide. Non-parametric ANOVAs were used for determining differences in Shannon's Diversity Index, bacterial community abundances at phylum and genus levels, pH measurements, and progesterone concentrations. Significance was determined by Wilcoxon exact test for comparisons between pregnancy statuses and Kruskal Wallis test for comparisons among days of the protocol. Independent variables for all analyses were day or protocol or pregnancy status. DPP and age were determined to have no significant effect on pregnancy status and were removed from analysis. Significance level was set at $P \leq 0.05$. Correlations were performed in SAS Enterprise Guide 7.1 using Pearson correlation. Beta diversity differences between day of sample and pregnancy status by environment were analyzed using QIIME analysis of similarity (ANOSIM) with 9999 permutations.

Results

Sequence Information

After quality control and chimera removal, 10,448,316 total clean sequences remained among all samples, with an average of 92,463 ± 2,976 per sample. The total number of sequences ranged from 42 to 232,160 among individual samples. Table 1 indicates the average number of clean sequences and standard error of the mean (SEM) for each similar sample day, sample type, and pregnancy status group.

Alpha Diversity

A total of 112,953 OTU were detected among all samples. Seven samples were removed from alpha diversity analyses due to very low observed OTU detected, likely due to sample contamination.

Between Pregnant and Non-pregnant Cows

In the uterus, the number of observed OTU, indicating the number of observed species, did not differ between pregnant and non-pregnant cows at any day of the protocol ($P > 0.05$; Table 1, Supplementary File 1). Similarly, Chao1, Faith's Phylogenetic Diversity, and Shannon's Diversity Index supports the number of observed OTU with no difference in diversity between pregnancy statuses. In the vagina, non-pregnant cows had significantly lower number of observed OTU than pregnant animals at d -21 ($P = 0.05$) with no difference at d -9 or d -2 ($P > 0.05$; Table 1, Supplementary File 1). Faith's Phylogenetic Diversity also indicated greater diversity in pregnant cows at d -21 in the vagina ($P = 0.04$; Table 1, Supplementary File 1). However, Chao1 indicates

Table 1. Sequence and alpha diversity statistics between pregnant and non-pregnant cows.[1]

Day	Type	Status	Cleaned Sequences	Observed OTUs	Chao1	Shannon's Diversity Index	Faith's Phylogenetic Diversity
-21	Uterine	Non-pregnant	88,494 ± 12,926	1,150 ± 138.97 [a]	1,550.3 ± 114.8 [a]	7.8 ± 0.38 [a]	106.5 ± 9.05 [a]
		Pregnant	101,430 ± 6,947	1,210 ± 126.35 [a]	1,552.4 ± 103.4 [a]	8.4 ± 0.13 [a]	109.5 ± 7.8 [a]
	Vaginal	Non-pregnant	98,085 ± 10,238	1,058 ± 72.64 [a]	1,548 ± 64.4 [a]	8.7 ± 0.06 [a]	97.5 ± 5.28 [a]
		Pregnant	94,675 ± 7,847	1,260 ± 61.47 [b]	1,638 ± 36.9 [a]	8.2 ± 0.5 [a]	111.3 ± 3.4 [b]
-9	Uterine	Non-pregnant	88,024 ± 7,185	1,215 ± 67.19 [a]	1,564.1 ± 51.4 [a]	8.4 ± 0.1 [a]	107.6 ± 3.44 [a]
		Pregnant	89,521 ± 4,474	1,135 ± 52.08 [a]	1,564.2 ± 38.4 [a]	8.2 ± 0.31 [a]	105.2 ± 3.1 [a]
	Vaginal	Non-pregnant	93,705 ± 6,077	1,106 ± 41.92 [a]	1,460.1 ± 33.8 [a]	7.8 ± 0.13 [a]	100.3 ± 2.66 [a]
		Pregnant	94,353 ± 4,624	1,015 ± 60.03 [a]	1,372.9 ± 57.7 [a]	7.8 ± 0.08 [a]	94.6 ± 4.29 [a]
-2	Uterine	Non-Pregnant	114,937 ± 5,304	401 ± 92.73 [a]	659.4 ± 107.8 [a]	5.1 ± 0.75 [a]	51.5 ± 9.41 [a]
		Pregnant	82,814 ± 22,483	354 ± 130.24 [a]	574.6 ± 148.4 [a]	4.1 ± 1.02 [a]	44 ± 11.74 [a]
	Vaginal	Non-pregnant	93,118 ± 6,730	1,165 ± 115.13 [a]	1,454.9 ± 115 [a]	8.7 ± 0.06 [a]	108.3 ± 7.75 [a]
		Pregnant	71,270 ± 10,776	987 ± 142.84 [a]	1,273.5 ± 143.6 [a]	8.2 ± 0.5 [a]	93.2 ± 11.55 [a]

[1]Non-transformed means. Significance determined by transformed data and analysis presented in Supplementary File 1.

[ab]Between each day of each column indicates $P \leq 0.05$.

there was no difference in the predicted number of species in the vagina between pregnant and non-pregnant cows at all days ($P > 0.05$; Table 1, Supplementary File 1).

Days of the estrus synchronization protocol

Observed OTU significantly decreased in the uterus of both pregnant and non-pregnant cows ($P < 0.0001$) between d -21 to d -2 and d -9 to d -2 with no significant difference between d -21 and d -9 (Table 2, Supplementary File 2). Chao1, Faith's Phylogenetic Diversity, and Shannon's Diversity Index also significantly decreased in the uterus of pregnant and non-pregnant cows from d -21 and d-9 to d -2 ($P < 0.05$; Table 2, Supplementary File 2). In the vagina, observed OTU and Faith's Phylogenetic Diversity did not differ over time in pregnant and non-pregnant cows. Chao1 indicated a significantly higher predicted species richness in the vagina of non-pregnant cows at d -21 compared to d -9 and d -2 ($P < 0.05$) with no difference over time in pregnant cows ($P > 0.05$; Table 2, Supplementary File 2). Shannon's Diversity Index significantly changed over time in the vagina of pregnant and non-pregnant cows with a decrease in diversity at d -9 ($P < 0.05$; Table 2, Supplementary File 2).

Beta Diversity

Principal Coordinate Analysis (PCoA) using UniFrac unweighted metrics were generated to analyze and visualize beta diversity to determine the phylogenetic relationship between samples in the uterus and vagina.

Table 2. Alpha diversity statistics among days of the protocol.[1]

Type	Status	Day	Observed OTU	Chao1	Shannon's Diversity Index	Faith's Phylogenetic Diversity
Uterine	Pregnant	-21	1,210 ± 126.35 [a]	1,552.4 ± 103.4 [a]	8.4 ± 0.13	109.5 ± 7.8 [a]
		-9	1,135 ± 52.08 [a]	1,564.2 ± 38.4 [a]	8.2 ± 0.31	105.2 ± 3.1[a]
		-2	354 ± 130.24 [b]	574.6 ± 148.4 [b]	4.1 ± 1.02	44 ± 11.74 [b]
	Non-pregnant	-21	1,150 ± 138.97 [a]	1,550.3 ± 114.8 [a]	7.8 ± 0.38	106.5 ± 9.05 [a]
		-9	1,215 ± 67.19 [a]	1,564.1 ± 51.4 [a]	8.4 ± 0.1	107.6 ± 3.44 [a]
		-2	401 ± 92.73 [b]	659.4 ± 107.8 [b]	5.1 ± 0.75	51.5 ± 9.41 [b]
Vaginal	Pregnant	-21	1,260 ± 61.47 [a]	1,638 ± 36.9 [a]	8.2 ± 0.5	111.3 ± 3.4 [a]
		-9	1,015 ± 60.03 [a]	1,372.9 v 57.7 [a]	7.8 ± 0.08	94.6 ± 4.29 [a]
		-2	987 ± 142.84 [a]	1,273.5 ± 143.6 [a]	8.2 ± 0.5	93.2 ± 11.55 [a]
	Non-pregnant	-21	1,058 ± 72.64 [a]	1,548 ± 64.4 [a]	8.7 ± 0.06	97.5 ± 5.28 [a]
		-9	1,106 ± 41.92 [a]	1,460.1 ± 33.8 [b]	7.8 ± 0.13	100.3 ± 2.66 [a]
		-2	1,165 ± 115.13 [a]	1,454.9 ± 115 [b]	8.7 ± 0.06	108.3 ± 7.75 [a]

[1]Non-transformed means. Significance determined by transformed data and analysis presented in Supplementary File 2.

[ab]Between each day of each column indicates $P \leq 0.05$.

Between Pregnant and Non-pregnant Cows

Significant clustering between pregnant and non-pregnant cows occurred in the uterus at d-2 (R = 0.28, *P* = 0.005; Fig. 2). Samples from non-pregnant cows clustered tightly while pregnant cows were less clustered, but distinctly separate from the non-pregnant cow samples. In the vagina, significant clustering was observed at d -21 between pregnant and non-pregnant cow samples with slight overlap (R = 0.24, *P* = 0.002; Supplementary Figures Fig. 1a). No significant clustering by pregnancy status was observed in the uterus at d -21 (R = -0.007, *P* = 0.46; Supplementary Figures Fig. 1b) or d -9 (R = -0.024, *P* = 0.64 Supplementary Figures Fig. 1c), or in the vagina at d -9 (R = -0.004, *P* = 0.43; Supplementary Figures Fig. 1d) or d -2 (R = -0.004, *P* = 0.43; Fig. 3).

Days of the estrus synchronization protocol

Significant clustering by day was observed in both uterine and vaginal samples. In the vaginal samples, there was tight clustering of d -21 and d -9 samples with a separation of d -2 samples (R = 0.27, *P* = 0.0001, Fig. 4). Uterine samples clustered by day, but in contrast to the vaginal samples, d -21 and d -9 samples were clustered together and separate from d -2 (R = 0.23, *P* = 0.0001; Fig. 5).

Phylum Level Taxonomic Composition

Among all samples, 34 total phyla were detected. Figure 6 presents the phyla relative abundance in the uterus and vagina of pregnant or non-pregnant cows over time.

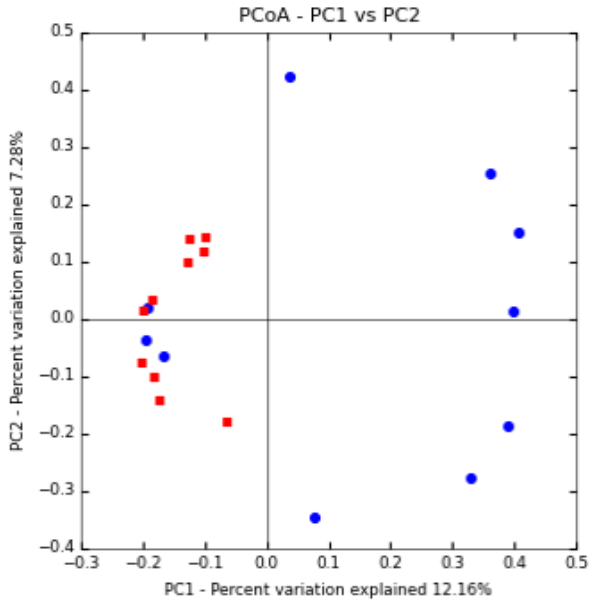

Figure 2. PCoA of d -2 uterine samples between pregnant and non-pregnant cows using Unifrac unweighted metrics.

Non-pregnant cows are indicated by red squares and pregnant cows by blue circles.

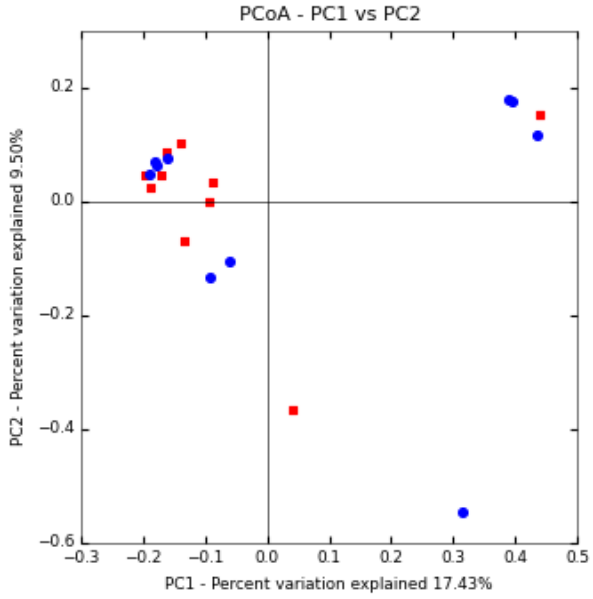

Figure 3. PCoA of d -2 vaginal samples between pregnant and non-pregnant cows using Unifrac unweighted metrics.

Non-pregnant cows are indicated by red squares and pregnant cows by blue circles.

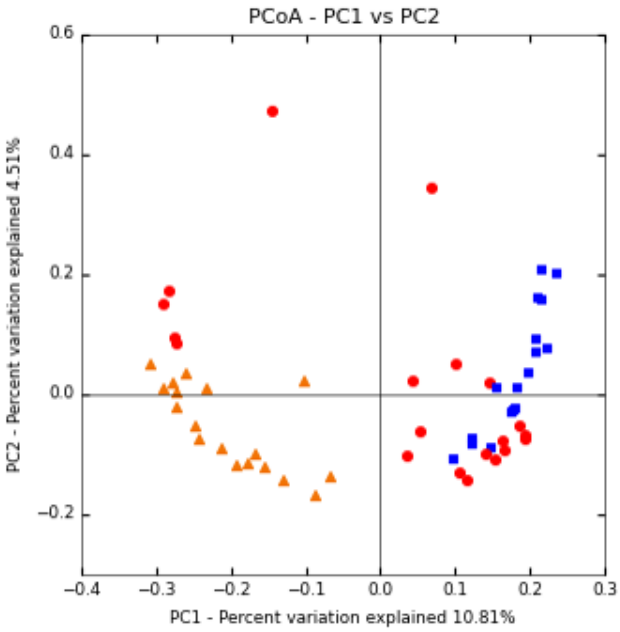

Figure 4. PCoA of vaginal samples by day of the protocol using Unifrac unweighted metrics.

Legend: d -21, Orange triangles; d -9, blue squares; d -2, red circles.

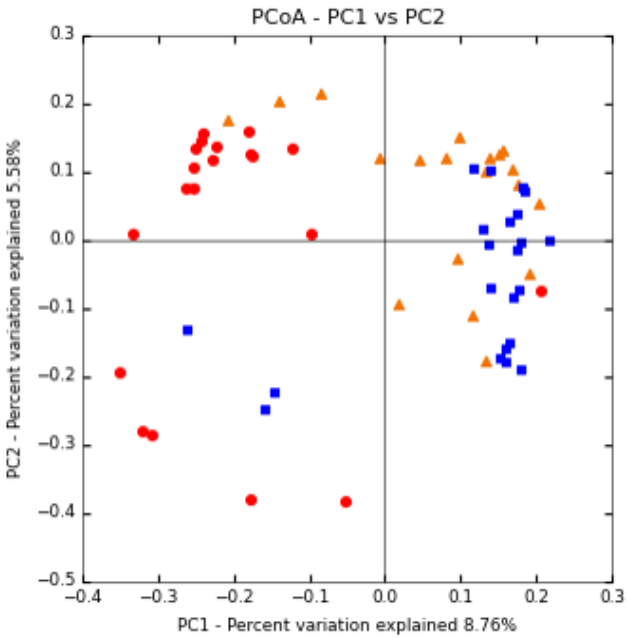

Figure 5. PCoA of vaginal samples by day of the protocol using Unifrac unweighted metrics.

Legend: d -21, Orange triangles; d -9, blue squares; d -2, red circles.

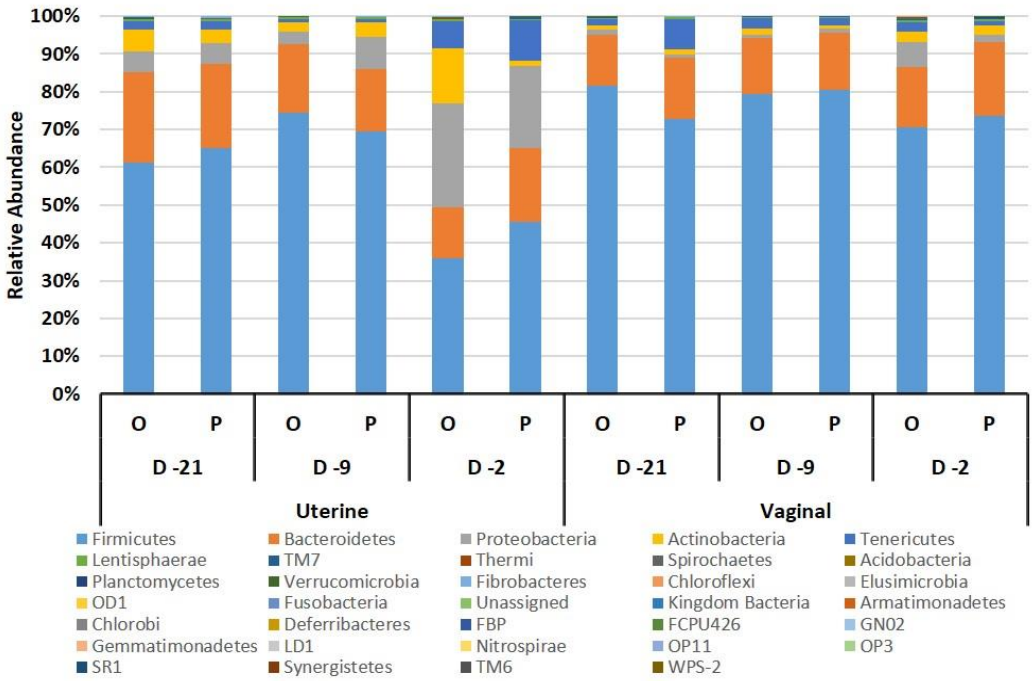

Figure 6. Relative abundance of phyla in the uterus and vagina at each day of the protocol in pregnant (P) and non-pregnant (O) cows.

Between Pregnant and Non-pregnant Cows

Firmicutes was the most abundant phyla across all samples, but no significant differences in the relative abundance was observed between pregnant and non-pregnant cows in the uterus or vagina. The most significant differences in relative abundance of phyla between pregnant and non-pregnant cows occurred at d -9 in the uterus with Bacteroidetes ($P = 0.05$), Actinobacteria ($P = 0.05$), Spirochaetes ($P = 0.05$), Fusobacteria ($P = 0.009$), and unassigned taxa ($P = 0.03$; Table 3). Only three phyla were significantly different at d -21 and d -2 in the uterus between pregnant and non-pregnant cows with Thermi ($P = 0.005$), Acidobacteria ($P = 0.03$), and FBP ($P = 0.05$) at d -21, and Actinobacteria ($P = 0.005$), Fibrobacteres ($P = 0.02$), and Unassigned ($P = 0.009$) at d -2 (Table 3). In the vagina, only two phyla were significantly different with Verrucomicrobia ($P = 0.01$) and Fusobacteria ($P = 0.03$) at d -9 and Tenericutes ($P = 0.03$) and Acidobacteria ($P = 0.05$) at d -2 between pregnant and non-pregnant cows (Table 3); no significant differences between pregnant and non-pregnant cows in phyla relative abundance were detected at d -21.

Days of the estrus synchronization protocol

The most abundant phyla, Firmicutes, significantly shifted over time in the uterus of pregnant ($P = 0.04$) and non-pregnant ($P = 0.0002$) cows as well as in the vagina of non-pregnant cows over time ($P = 0.03$, Table 4). When observing the change in phyla relative abundance over time of the synchronization protocol, numerous differences occurred in the uterus and vagina of pregnant and non-pregnant animals (Table 4).

Table 3. Percent relative abundance of significant phyla (Mean ± SEM) between non-pregnant and pregnant cows in the uterus and vagina at each day.

Type	Day	Phylum	Non-pregnant	Pregnant	*P* Value
Uterine	-21	Thermi	0.003 ± 0.001	0.03 ± 0.01	0.005
		Acidobacteria	0.002 ± 0.001	0.01 ± 0.006	0.029
Uterine	-9	Bacteroidetes	18.3 ± 0.64	16.4 ± 0.83	0.047
		Actinobacteria	2.4 ± 0.72	3.8 ± 0.7	0.047
		Spirochaetes	0.03 ± 0.004	0.03 ± 0.009	0.049
		Unassigned	0.003 ± 0.001	0.01 ± 0.003	0.027
		Fusobacteria	0.002 ± 0.001	0.02 ± 0.009	0.009
Uterine	-2	Actinobacteria	14.4 ± 7.36	1.34 ± 0.76	0.005
		Fibrobacteres	0.27 ± 0.21	0.041 ± 0.03	0.018
		Unassigned	0.009 ± 0.004	0.0005 ± 0.0005	0.009
Vaginal	-9	Fusobacteria	0.02 ± 0.01	ND	0.029
		Verrucomicrobia	0.005 ± 0.003	0.02 ± 0.005	0.01
Vaginal	-2	Tenericutes	2.6 ± 1.19	1.01 ± 0.17	0.032
		Acidobacteria	0.009 ± 0.005	0.002 ± 0.001	0.053

Significance determined by $P \leq 0.05$. No significant differences were detected at Day -21 in the vagina. ND: Not detectable at current depth.

Table 4. Percent relative abundance of significant phyla (Mean ± SEM) among protocol days in the uterus and vagina of non-pregnant and pregnant cows.

Type	**Status**	**Phylum**	**Day -21**	**Day -9**	**Day -2**	***P* Value**
Uterine	Non-pregnant	Firmicutes	61.3 ± 4.69	74.3 ± 2.22	36 ± 5.97	0.0002
		Proteobacteria	5.4 ± 2.1	3.3 ± 1.65	27.7 ± 9.46	0.013
		Tenericutes	2.3 ± 0.54	0.93 ± 0.16	7.4 ± 6.4	0.027
		Verrucomicrobia	0.22 ± 0.06	0.01 ± 0.003	0.12 ± 0.03	0.011
		Fusobacteria	0.02 ± 0.005	0.002 ± 0.001	0.03 ± 0.02	0.033
		Thermi	0.003 ± 0.001	0.07 ± 0.02	0.03 ± 0.01	0.043
		OD1	0.001 ± 0.0009	ND	0.01 ± 0.008	0.008
		FBP	ND	0.003 ± 0.002	ND	0.047
Uterine	Pregnant	Firmicutes	65.2 ± 3.13	69.5 ± 3.27	45.5 ± 9.28	0.043
		Actinobacteria	3.7 ± 0.99	3.8 ± 0.7	1.34 ± 0.76	0.011
		Lentisphaerae	0.51 ± 0.08	0.45 ± 0.06	0.25 ± 0.15	0.014
		Fibrobacteres	0.3 ± 0.13	0.05 ± 0.02	0.04 ± 0.03	0.004
		Spirochaetes	0.13 ± 0.03	0.03 ± 0.009	0.13 ± 0.08	0.037
		Chloroflexi	0.03 ± 0.005	0.06 ± 0.02	0.02 ± 0.01	0.015
		Unassigned	0.01 ± 0.004	0.01 ± 0.003	0.0005 ± 0.0005	0.006
		WPS-2	0.005 ± 0.003	0.002 ± 0.001	ND	0.044
		Armatimonadetes	0.002 ± 0.001	ND	ND	0.015
		OD1	ND	ND	0.005 ± 0.003	0.047
Vaginal	Non-pregnant	Firmicutes	81.7 ± 1.53	79.5 ± 2.29	70.7 ± 3.84	0.032
		Proteobacteria	1.2 ± 0.38	0.9 ± 0.28	6.6 ± 3.38	0.045
		TM7	0.1 ± 0.03	0.03 ± 0.008	0.28 ± 0.08	0.0009
		Thermi	0.001 ± 0.0008	0.02 ± 0.006	0.02 ± 0.007	0.033

Table 4 continued.

Type	Status	Phylum	Day -21	Day -9	Day -2	*P* Value
Vaginal	Non-pregnant	Spirochaetes	0.05 ± 0.02	0.02 ± 0.005	0.13 ± 0.04	0.004
		Acidobacteria	ND	0.0009 ± 0.0009	0.009 ± 0.005	0.0036
		Planctomycetes	0.04 ± 0.02	0.008 ± 0.003	0.08 ± 0.01	0.005
		Verrucomicrobia	0.18 ± 0.14	0.005 ± 0.003	0.17 ± 0.06	0.001
		Fibrobacteres	0.04 ± 0.02	0.02 ± 0.008	0.12 ± 0.05	0.034
		OD1	ND	ND	0.07 ± 0.05	0.027
Vaginal	Pregnant	Proteobacteria	0.96 ± 0.15	1.05 ± 0.35	2.00 ± 0.36	0.014
		TM7	0.08 ± 0.02	0.05 ± 0.02	0.32 ± 0.12	0.02
		Planctomycetes	0.01 ± 0.006	0.006 ± 0.003	0.08 ± 0.02	0.001
		Verrucomicrobia	0.08 ± 0.04	0.02 ± 0.005	0.20 ± 0.09	0.026
		Unassigned	0.01 ± 0.003	0.003 ± 0.001	0.003 ± 0.001	0.018
		SR1	0.012 ± 0.011	ND	ND	0.047
		Synergistetes	0.002 ± 0.0009	ND	ND	0.015

Significance determined by $P \leq 0.05$. ND: Not detectable at current depth.

Genus Level Taxonomic Composition

At the genus level, 792 total genera were detected among all samples. Figure 7 presents the genera relative abundance in the uterus or vagina and pregnant or non-pregnant cows over time.

Between Pregnant and Non-pregnant Cows

Relative abundances for each significantly different genera between pregnant and non-pregnant cows in the uterus and vagina at each day of the protocol are listed in Supplementary File 3. The undetermined genus of the family Ruminococcaceae had the greatest abundance of all samples, differing between pregnant and non-pregnant cows only at d -21 in the vagina. However, overall, the most differences in genera relative abundance between pregnant and non-pregnant cows occurred at d -2. The genera in the uterus at d -2 in pregnant cows with relative abundance greater than 1%, other than the undetermined genus of the family Ruminococcaceae, included the classified genera of *Ureaplasma*, *Helcococcus*, *Bacteroides*, *5-7N15*, and *Oscillospira*, as well as unassigned genera of order Burkholderiales, order Clostridiales, family Bacteroidaceae, family Lachnospiraceae, order Bacteroidales, and family Rikenellaceae. However in non-pregnant cows, as similarly reported in Clemmons et al. (2017), *Corynebacterium* had the greatest abundance in the uterus at d -2 (9.62 ± 4.97%). The current study indicates a significantly lower relative abundance of *Corynebacterium* at 0.50 ± 0.36% in the uterus at d -2 of pregnant cows ($P = 0.003$). Genera with relative abundance greater than 1% in the uterus at d -2 of non-pregnant cows that significantly differed from pregnant cows, other than *Corynebacterium*, included classified genera *Staphylococcus* ($P = 0.0002$),

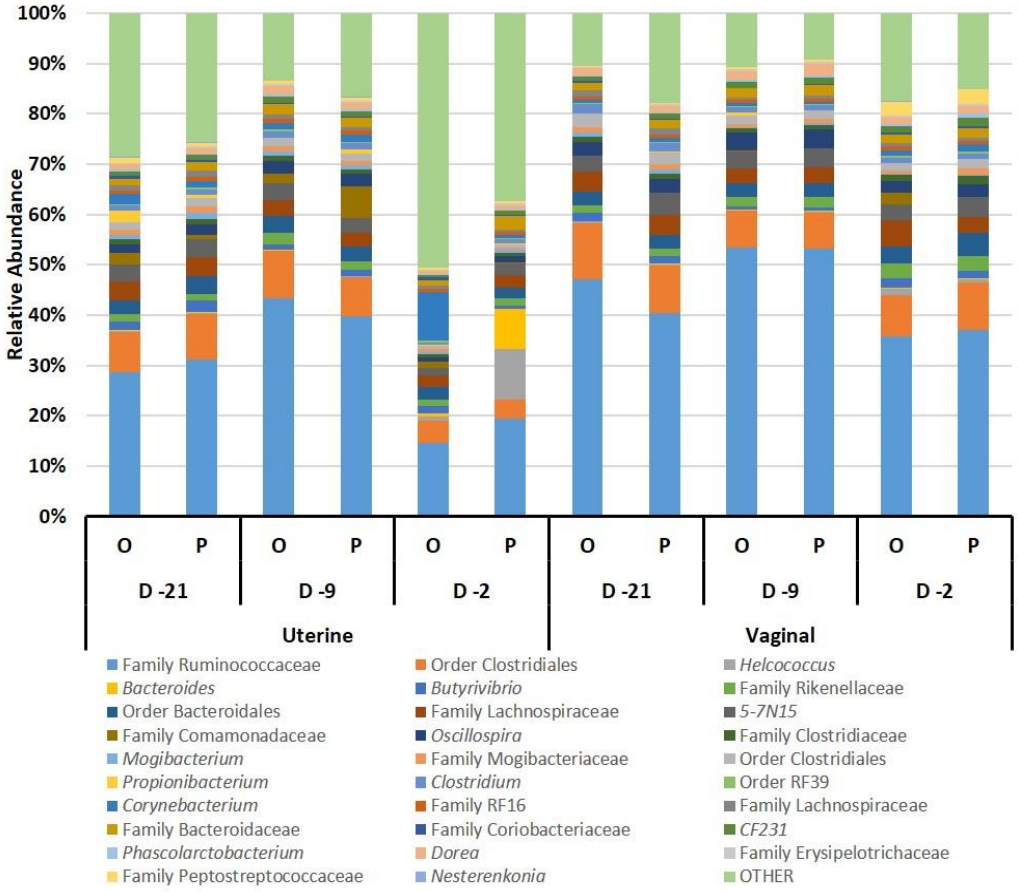

Figure 7. Relative abundance of genera in the uterus and vagina at each day of the protocol in pregnant (P) and non-pregnant (O) cows.

Prevotella ($P = 0.03$), *Microbacterium* ($P = 0.01$), *Butyrivibrio* ($P = 0.05$), *Ralstonia* ($P = 0.01$), and unassigned genera from family Alcaligenaceae ($P = 0.0001$), and family Comamonadaceae ($P = 0.02$; Table 5). Interestingly, these genera in non-pregnant cows with greater than 1% relative abundance had a relative abundance less than 1% in pregnant cows (Table 5). Fewer differences in the relative abundances of genera occurred between pregnant and non-pregnant cows at d -9 and d -21 in the uterus and vagina compared to d -2 in the uterus (Supplementary File 3).

Days of the estrus synchronization protocol

The most abundant genera detected, unassigned from the family Ruminococcaceae, significantly changed over time in the uterus and vagina of both pregnant and non-pregnant animals ($P < 0.05$). Numerous other genera relative abundances significantly changed over the duration of the protocol (Supplementary File 4).

Circulating Progesterone Concentrations and Correlation to Bacteria Phyla

Circulating progesterone concentrations indicated no significant difference between pregnant and non-pregnant cows at d -21 and d -2. Progesterone was significantly higher in non-pregnant cows than pregnant cows at d -9 ($P = 0.01$; Table 6).

In addition, correlation of circulating progesterone concentration to the relative abundance of phyla indicated significance with Firmicutes. In the vagina of open cows, as circulating progesterone concentration decreased, the relative abundance of Firmicutes increased at both d -9 ($r = -0.79$, $P = 0.02$) and d -2 ($r = -0.81$, $P = 0.004$). However, at d -

Table 5. Significantly different genera between non-pregnant and pregnant cows at d -2 with a relative abundance greater than 1%.[1]

Genus	Non-pregnant	Pregnant	P Value
Corynebacterium	9.62 ± 4.97	0.50 ± 0.36	0.003
Family Alcaligenaceae	9.49 ± 9.41	0.01 ± 0.01	0.0001
Staphylococcus	3.28 ± 2.89	0.02 ± 0.02	0.0002
Prevotella	2.82 ± 2.61	0.03 ± 0.02	0.031
Microbacterium	2.22 ± 2.18	0.009 ± 0.007	0.009
Butyrivibrio	1.36 ± 0.43	0.79 ± 0.39	0.049
Ralstonia	1.35 ± 0.78	0.04 ± 0.02	0.007
Family Comamonadaceae	1.13 ± 0.48	0.34 ± 0.21	0.023

[1]All genera relative abundances that significantly differ between non-pregnant and pregnant cows at d -2 are presented in Supplementary File 3.

Table 6. Progesterone concentrations (ng/ml) between pregnant and non-pregnant cows.

Day	Status	Mean ± SEM	*P* Value
-21	Non-pregnant	2.34 ± 0.62	0.361
	Pregnant	2.21 ± 0.74	
-9	Non-pregnant	7.58 ± 1.30	0.014
	Pregnant	3.83 ± 0.68	
-2	Non-pregnant	6.11 ± 1.81	0.342
	Pregnant	4.76 ± 1.77	
0	Non-pregnant	0.83 ± 0.31	0.051
	Pregnant	0.31 ± 0.09	

2 an increase in progesterone was significantly correlated to an increase in relative abundance of Proteobacteria in the vagina of open cows (r = 0.70, *P* = 0.03).

pH and Correlation to Bacterial Genera

Regardless of day, the uterus had a lower pH, with means ranging from 5.86 ± 0.11 to 6.06 ± 0.09, compared to the vagina with means ranging from 6.31 ± 0.09 to 7.04 ± 0.17.

In the uterus, the only significant difference in pH between non-pregnant and pregnant cows occurred at d -9 (*P* = 0.05; Supplementary File 5). Although pH values were not significantly different among days of the protocol (*P* > 0.05), uterine pH increased in non-pregnant cows from 5.86 ± 0.11 at d -21 to 6.06 ± 0.09 at d -2 and decreased in pregnant cows from 6.04 ± 0.20 at d -21 to 5.92 ± 0.13 at d -2 (Table 7).

No difference in vaginal pH was detected between pregnancy statuses at any day of the protocol (*P* > 0.05; Supplementary File 5). Vaginal pH changed significantly in non-pregnant cows (*P* = 0.01; Table 7). However, although no significant change occurred in pregnant cows (*P* = 0.16), the pattern of pH change over time was similar to the non-pregnant cows with the highest pH at d -21 and the lowest pH at d -9 (Table 7).

Additionally, pH values were tested for correlation with genera abundances in the uterus and vagina. In the uterus, genera *Mogibacterium* (r = -0.25, *P* = 0.057) and unassigned genera from the family Lachnospiraceae (r = -.0.35, *P* = 0.008) and family Clostridiaceae (r = -0.27, *P* = 0.04) were correlated with pH. As the abundances of these genera increased, uterine pH decreased. However in the vagina, genera *Clostridium* (r =

Table 7. Change of uterine and vaginal pH of non-pregnant and pregnant cows over time of the synchronization protocol.

Type	Status	Day	Mean ± SEM	P Value
Uterine	Non-pregnant	-21	5.86 ± 0.11	0.275
		-9	5.88 ± 0.10	
		-2	6.06 ± 0.09	
	Pregnant	-21	6.04 ± 0.20	0.916
		-9	5.95 ± 0.07	
		-2	5.92 ± 0.13	
Vaginal	Non-pregnant	-21	7.04 ± 0.17	0.011
		-9	6.31 ± 0.09	
		-2	6.69 ± 0.14	
	Pregnant	-21	6.76 ± 0.24	0.164
		-9	6.29 ± 0.11	
		-2	6.58 ± 0.14	

Differences between means were determined significant by Non-parametric ANOVA Wilcoxon exact test ($P \leq 0.05$).

0.28, $P = 0.04$), unassigned genera from the family Lachnospiraceae (r = 0.31, $P = 0.02$), and two different genera from the unassigned from the order Clostridiales (r = 0.30, $P = 0.02$ and r = 0.36, $P = 0.007$) were correlated to pH with an increase in abundance associated with an increase in pH.

Discussion

The known importance of the symbiotic relationship between microbes and their host has prompted deeper investigation into the novel bacterial communities throughout the body, such as the reproductive tract microbiome. However, few studies have explored the bacterial communities in the uterus and vagina of domestic livestock species and their relationship to fertility.

Although bacterial communities have many beneficial contributions to the body, dysregulation of these communities and a change in abundances can have negative consequences. Our study suggests that changes in the bacteriome of the reproductive tract over time leads to differences in genera abundances prior to FTAI that may affect fertility. According to beta diversity analyses, the significant clustering of uterine samples between pregnant and non-pregnant cows at d -2 indicates the differences in bacteria species present in the uterus at this time may be affecting the establishment of pregnancy. The uterine samples from pregnant cows cluster separately from the non-pregnant cows, but with much greater variation as the non-pregnant cow samples cluster very tightly. This large variation in the healthy reproductive tract bacteriome suggests there is no single healthy bacteriome benefiting fertility in the cows that were able to

conceive, supporting previous bovine research of the wider variation and high diversity compared to human studies (Swartz et al., 2014; Laguardia-Nascimento et al., 2015). However, the tight clustering of samples from cows who failed to conceive suggests there is a group of closely related bacteria present potentially preventing the establishment of pregnancy. When evaluating bacteria at the genera level, multiple genera that have previously been determined to be commonly pathogenic, differed between pregnant and non-pregnant cows. These genera were determined to be present in the uterus at d -2 with abundances greater than 1% in cows that did not become pregnant, but these same bacteria were present in less than 1% in cows that became pregnant. Clemmons et al. (2017) found *Corynebacterium* as the most abundant genus in the uterus two days prior to FTAI in cows who did not become pregnant. Previously, *Corynebacterium* was determined to be highly abundant in postpartum cows that had developed uterine infections and led to negative effects on the ability to conceive another pregnancy (Ruder et al., 1981). The present study found significantly lower abundances of *Corynebacterium* in postpartum cows that were able to develop a pregnancy, supporting previous evidence of *Cornyebacterium*'s potential negative effects on fertility. In addition, results indicated *Staphylococcus* as the second most abundant genus detected in the uterus of non-pregnant cows at d -2, which was also significantly less in the uterus of pregnant cows at d -2. *Staphylococcus* is often found to be present in the uterus of postpartum cows that develop acute metritis and known as the most common pathogen causing mastitis (Vasudevan et al., 2003; Otero and Nader-Macías, 2006). Unexpectedly, *Ureaplasma* and *Helcococcus* genera had the greatest abundance detected in the uterus of pregnant cows at d -2, but did

not significantly differ between pregnant and non-pregnant cows. Previously, these bacteria have been shown to have infectious potential and are a factor in the development of uterine metritis with the potential to lead to reproductive issues such as infertility or abortion in cattle (Vasconcellos Cardoso et al., 2000). However, similar results to the current study were found by Jeon et al. (2015) with increased *Ureaplasma* correlated to a healthy uterine environment than those that developed metritis after calving. Further research may be conducted to determine the specific species level differences and how they relate to the development of uterine disease or presence in the healthy uterus.

The current study indicates the presence of pathogenic bacteria in the reproductive tract have negative effects on the development of pregnancy, however other bacteria are also present that may be contributing to a healthy reproductive tract environment. The unassigned genera from the family Ruminococcaceae was determined to have the greatest abundance in the reproductive tract. Although the specific species present has yet to be classified, the Ruminococcaceae family of bacteria is known to be highly abundant in the gastrointestinal tract of healthy humans and cattle (Mao, Zhang, Liu, & Zhu, 2015; Rajilić-Stojanović & de Vos, 2014; Flint, Scott, Duncan, Louis, & Forano, 2012). These bacteria contribute to carbohydrate degradation resulting in the production of short chain fatty acids (SCFA) such as butyrate (Forbes, Van Domselaar, & Bernstein, 2016; Zheng et al., 2017). Butyrate has been previously determined to prevent local inflammation by increasing the population of Treg immune cells (Smith et al., 2013; Zhang et al., 2016). As the regulation of inflammation in the reproductive tract by Tregs has been demonstrated to be important for the establishment of pregnancy and to prevent

rejection of the fetus, the high abundance of bacteria from the family Ruminococcaceae may be contributing fermentation products to regulate immune cell populations in the reproductive tract to maintain the proper inflammatory environment (Aluvihare, Kallikourdis, & Betz, 2004; Shima et al., 2010). Additionally, other bacteria present in lower abundances may also be benefiting the reproductive tract environment. Although *Lactobacillus* is present in much lower abundance in the reproductive tract of cattle than humans, as confirmed by the current study, Otero et al. (2006) found strains of *Lactobacillus* present in the vagina of cattle that were able to inhibit the colonization of pathogenic *Staphylococcus* in culture suggesting that *Lactobacillus* could be beneficial as a probiotic for the reproductive tract due to its inhibition of pathogenic bacteria (Otero and Nader-Macías, 2006).

The environment of the uterus plays a critical role in proper sperm transport for successful fertilization. The present study in cattle indicated a decrease in uterine pH leading up to FTAI in cows that became pregnant, but an increase in pH in those who failed to conceive. These results support evidence that a lower uterine pH at the time of sperm deposition may be beneficial to fertilization. Studies in cattle have shown that sperm motility is increased in higher pH environments and inhibited by a lower pH environment, leading to an increased lifespan (Jones and Bavister, 2000). A decrease in uterine pH at the time of estrus has been shown to lead to a favorable environment for sperm to reside prior to fertilization of the oocyte, allowing for increased pregnancy rates (Perry and Perry, 2008a,b). The relationship between the change in pH and bacterial communities in the reproductive tract has not previously been evaluated in bovine.

Interestingly, our study found significant correlations of bacterial genera to increase the pH in the vagina, with other bacteria contributing to the decrease in pH in the uterus. The decreased species number and phylogenetic diversity with the change in abundances of bacteria occurring in the uterus leading to FTAI, as indicated by the current study, may contribute to the change in pH. Previous studies in humans determined estrogen stimulates the epithelial cells of the reproductive tract to produce glycogen, a fuel source for *Lactobacillus*, which dominates the reproductive microbiota (Boskey et al., 1999; Lamont et al., 2011; Ravel et al., 2011; Brotman et al., 2014). Boskey et al. (1999) indicated the glycogen produced is sufficient for *Lactobacillus* to produce a high concentration of lactic acid to maintain a low pH environment, suggesting the microbiota are the major contributor to the pH of the reproductive tract to potentially protect against pathogen colonization (Eschenbach et al., 1989). Future studies may be able to use bacteria known to lower pH, such as *Lactobacillus*, in probiotic form to manipulate the microbiome of the bovine reproductive tract and evaluate the effect on improving pregnancy rates.

Studies agree that the human uterine and vaginal bacteriome has a low diversity, with an increase in diversity leading to disease and fertility issues (Ravel et al., 2011; The Human Microbiome Project, 2012). However, our study along with previous evidence indicated the bacteriome of the cow's reproductive tract was distinctly different than in humans with a very low abundance of *Lactobacillus*, higher pH, and increased bacterial phylogenetic diversity (Otero et al., 2000; Rodríguez et al., 2011; Swartz et al., 2014; Clemmons et al. 2017). The greatest average of OTU detected in the vagina occurred in

pregnant cows on d -21 (1,260 ± 61.47). However, as stated by Clemmons et al. (2017), many assigned taxa in the vagina were associated with digestive bacterial species possibly due to its proximal location on the cow and opening to the external environment which may contribute to the greater number of OTU observed in the vagina of cows compared to women. These same vaginally-identified OTU were less abundant in the uterus which theoretically is protected by the cervix from the outside environment. Although the bacteriome composition of the bovine reproductive tract has been found to be greatly different than humans, our results indicate reduced alpha diversity and pH of the uterus prior to FTAI on d -2. In the uterus, observed OTU decreased in cows that became pregnant from an average of 1,210 ± 126.35 at d -21 to 354 ± 130.24 at d -2. Unlike the stable bacteriome of humans dominated by *Lactobacillus* species, our study supports previous evidence the reproductive bacteriome of bovine is highly dynamic and fluctuating with shifts in bacterial diversity over time (Santos and Bicalho, 2012; Jeon et al., 2015). Otero et al. (1999) used a culture-based method to evaluate the shift in the bovine vaginal bacteriome, specifically *Lactobacillus* and *Enterococci* genera of the phylum Firmicutes, throughout the estrous cycle. From their results, they suggested hormonal shifts through the phases of the estrous cycle affected the fluctuations of bacteria abundance with *Lactobacillus* and *Enterococci* decreasing during high progesterone phases (Otero et al., 1999). Similarly, our results indicated the shift of Firmicutes in the vagina was correlated to progesterone concentration, with decreased progesterone indicating an increase in Firmicutes. In contrast, an increase in Proteobacteria relative abundance in the vagina was correlated to an increase in

progesterone, suggesting the response to hormonal concentrations may differ by species. No differences in progesterone concentration were detected in the current study between pregnant and non-pregnant cows at d -21, d -2, or d 0 indicating a physiologic response to the protocol prior to breeding and no effect on the bacteriome differences observed at each day. However, the shifts in hormonal concentrations throughout the estrous cycle may influence the change in bacterial abundances over time. Further research is necessary to determine the mechanisms contributing to the fluctuations in the uterine and vaginal bacteriome.

The current study demonstrates bacterial communities of the reproductive tract undergo changes in diversity and abundance during an estrous synchronization protocol leading up to artificial insemination. The uterine environment experiences significant decreases in species richness and phylogenetic diversity with resulting differences in phyla and genera abundance during this time potentially affecting fertility outcomes. Future research should evaluate the cause of variation in the reproductive tract microbiome such as environment, genetics, nutrition, or health management methods. Probiotics may provide potential to maintain a healthy microbiome in the reproductive tract to prevent fertility issues and help producers improve their herd's reproductive efficiency.

CHAPTER THREE – CONCLUSION

As new technologies are developed and improved, such as 16s rRNA sequencing, scientists are better able to understand the microbial communities living in a symbiotic relationship with the host. The composition of the human reproductive microbiome has been thoroughly characterized, as well as determining its effect on fertility. However, there has been few studies evaluating the reproductive microbiome effect on fertility in bovine. The current study suggests the bacterial communities of the uterus and vagina of postpartum cows can affect their ability to develop a pregnancy. The significant decrease in bacterial species of both cows that were able to conceive and those who did not suggests the importance of reducing the microbial presence for pregnancy establishment. Differences were seen between cows that became pregnant and those who failed to conceive at the phylum and genus levels of the taxonomic composition of bacteria. As the number of bacterial species declined in the uterus, the differences detected in phyla and genera abundances suggest the reproductive microbiome shifted to a healthier bacterial environment or to more pathogenic bacterial communities resulting in infertility. Additional research is needed to determine the mechanism leading to the reduction of bacterial species prior to estrus and the cause of the shift to a healthy or pathogenic bacteriome. Further research may lead to the use of probiotics for cows to maintain a healthy bacteriome and prevent pathogenic bacterial community growth prior to breeding.

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VITA

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