The Vaginal Microbiome Related to Reproductive Traits in Beef Heifers

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The Vaginal Microbiome Related to Reproductive Traits in Beef Heifers

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

by

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University of Tennessee at Martin
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This thesis is approved for recommendation to the Graduate Council

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ABSTRACT

The greatest impact on profitability of a commercial beef operation is reproduction. In the human vaginal microbiome, dominance by Lactobacillus is a sign of reproductive health and fitness. In other species (non-human primates and ewes), Lactobacillus is found in low amounts and dominators of these microbial communities are considered to be pathogenic in humans. In beef heifers, little is known about the vaginal and fecal microbiota with respect to their relationship with fertility. To this end, we followed heifers through gestation to examine the dynamics of vaginal and fecal microbial composition throughout pregnancy.

Heifers were exposed to an estrus synchronization period, including 12 days of artificial insemination eligibility, and subsequently exposed to bulls for a 50 day breeding season. Vaginal samples were taken at pre-breeding (n=72), during the first (n=72), and second trimester (n=72) for all individuals, and third trimester for individuals with confirmed pregnancies (n=56). Fecal samples were taken at pre-breeding (n=32) and during the first trimester (n=32) and included bred and open individuals. Next generation sequencing of the V4 region of the 16S rRNA gene via the Illumina MiSeq platform was applied to all samples. Shannon indices and the number of observed OTUs were used as alpha-diversity measures resulting in no significant differences in fecal samples (P = 0.95, P = 0.66) and significant differences for vaginal samples due to pregnancy status and/or time (P = 0.0056, P = 0.0015). No differences in beta-diversity were seen in vaginal or fecal samples regarding pregnancy status and/or time. Random Forest was used to identify predictors of pregnancy status and/or time in fecal and vaginal samples and included but are not limited to: Histophilus, Paludibacter, unclassified Ruminococcaceae and Bacteroides. In conclusion, pregnancy status and/or time period altered alpha-diversity measures in vaginal sam-
ples. No changes due to pregnancy status were seen in alpha-diversity measures of fecal samples or beta-diversity measures in fecal and vaginal samples. Random Forest can be used to identify OTUs predictive of pregnancy status and gestational stage in vaginal and fecal samples.
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DEDICATION

I would like to dedicate this thesis to my parents. Mom and Dad, you are my biggest supporters and best friends. You always go above and beyond to make sure I am taken care of, happy, and being the best version of myself. I cannot put into words how thankful I am God chose me to be yours. My experiences in graduate school have given me an opportunity to grow and mature into a person I hope makes you proud. I never realized, and probably never will, how much you do for me. There’s no way to say thank you enough. This is for you. I love you times a million.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter 1:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Factors affecting reproduction in beef cattle</td>
<td>6</td>
</tr>
<tr>
<td>Seasonality</td>
<td>6</td>
</tr>
<tr>
<td>Genetics</td>
<td>8</td>
</tr>
<tr>
<td>Nutrition</td>
<td>8</td>
</tr>
<tr>
<td>Female Factors</td>
<td>9</td>
</tr>
<tr>
<td>Vaginal Microbiome</td>
<td>10</td>
</tr>
<tr>
<td>Human Vaginal Microbiome</td>
<td>12</td>
</tr>
<tr>
<td>Unexplained Infertility</td>
<td>13</td>
</tr>
<tr>
<td>Sperm (motility, longevity and fertilization)</td>
<td>15</td>
</tr>
<tr>
<td>Pre-term Birth</td>
<td>15</td>
</tr>
<tr>
<td>During Gestation</td>
<td>16</td>
</tr>
<tr>
<td>Effects on Neonatal Health</td>
<td>17</td>
</tr>
<tr>
<td>Vaginal Microbiome of Other Species</td>
<td>19</td>
</tr>
<tr>
<td>Bovine Vaginal Microbiome</td>
<td>20</td>
</tr>
<tr>
<td>Reproductive Disorder</td>
<td>21</td>
</tr>
<tr>
<td>Potential Use of Probiotics</td>
<td>23</td>
</tr>
<tr>
<td>Conclusion</td>
<td>26</td>
</tr>
</tbody>
</table>

| Chapter 2: Materials and Methods                                         |      |
| Introduction                                                             | 27   |
| Materials and Methods                                                    | 30   |
|   Ethics Statement                                                       | 30   |
|   Breeding Strategy                                                      | 30   |
|   Sample Collection                                                      | 31   |
|   DNA Extraction and Pyrosequencing                                     | 32   |
|   Library Preparation                                                    | 32   |
|   Sequencing                                                             | 33   |
|   Sequence Analysis                                                      | 33   |
|   Ecological and Statistical Analysis                                    | 34   |
Chapter 3: Results and Discussion

Results .................................................................................................................. 35
  Sequencing Depth and Alpha-diversities ......................................................... 35
  Community Membership and Structure ......................................................... 36
  OTU Distribution ............................................................................................. 37
  Identifying Predictive Bacterial Signatures ....................................................... 37

Discussion ............................................................................................................. 40

Conclusion ........................................................................................................... 50

Tables
  Table 1. P-values related to alpha-diversity measures in vaginal samples ........ 52
  Table 2. P-values related to alpha-diversity measures in fecal samples ............ 53

Figures
  Figure 1. Vaginal community alpha-diversity comparisons .............................. 54
  Figure 2. Fecal community alpha-diversity comparisons ................................. 55
  Figure 3. PCoA of community membership and structure ............................... 56
  Figure 4. Relative abundance of OTUs in the vaginal microbiota of beef heifers 57
  Figure 5. Relative abundance of OTUs in the fecal microbiota of beef heifers .... 58
  Figure 6. OTUs predictive of pregnancy status from vaginal microbiota ........... 59
  Figure 7. OTUs predictive of gestation from vaginal microbiota ....................... 60
  Figure 8. OTUs predictive of pregnancy status and gestation from fecal microbiota 61

References ............................................................................................................. 62

Appendix
  IACUC Letter .................................................................................................... 78
Chapter 1

Review of Literature

Introduction

Financial stability and long term production of a livestock enterprise are related to the producers ability to meet reproductive challenges. The two most important factors of a cow-calf operation effecting economic sustainability are reproduction and nutrition (Hess et al., 2005). While nutrition accounts for a large percentage of the costs associated with commercial beef cow operations, reproduction is the factor with the greatest influence on profitability (Hess et al., 2005). Net calf crop is defined as the number of calves weaned expressed as a percentage of cows exposed in the breeding herd, and can be used as a measure of production (Dziuk and Bellows, 1983). Losses occurring at any given state of the production cycle are represented by net calf crop percentages less than 100% (Dziuk and Bellows, 1983).

De Vries (2006) defined the value of an individual cow’s pregnancy by comparing the differences in discounted future cash flows when she is pregnant and when she is not. The value of the new pregnancy averages $200 (Eicker and Fetroew, 2003) while the value of the loss of a new pregnancy averages $555 (De Vries, 2006). Reproductive loss in the cattle industry can be due to one or multiple of the following factors: female infertility, dystocia, abortions/stillbirths, retained placentas and metritis/pyometra (Bellows et al., 2002). In the United States, reproductive loss due to these conditions is estimated to range from $441 to $502 million and from $473 to $484 million annually in the beef and dairy industries, respectively (Bellows et al., 2002). Combining these losses results in a $1 billion loss in yearly income for the cattle industry making
the failure to reproduce six times more costly than loss associated with respiratory disease and the single largest economic cost to the cattle industry as a whole (Bellows et al., 2002).

Improvements in reproductive efficiencies are validated through the economic impact this factor imparts on the industry. Investigation of management strategies to combat the first and second most costly reproductive disorders in beef cattle, female infertility and dystocia, respectively, are warranted to aid in reducing four-fifths of the financial loss associated with reproductive issues (Bellows et al., 2002). Dystocia resulting in the death of calves, cows and decreased production rates cost the U.S. beef industry $185 million per year (Bellows et al., 2002). While infertility is not easily quantified, it’s impact has the greatest effect on reproductive efficiency and cost (Bellows et al., 2002). Because infertility most often results in culling infertile females, the financial impact of a female lacking the ability to establish a pregnancy in a set breeding period, averages a national loss of $249 million per year (Bellows et al., 2002).

Incorporating reproductive technologies that allow producers to maximize the potential of existing resources into modern production systems can act as a mechanism to improve reproductive efficiency. Combining current assisted reproductive technologies, such as artificial insemination, with estrus synchronization or sex-sorted semen gives producers the ability to tighten the calving season, and plan matings with genetically superior individuals, resulting in an increase in economic return of a calf born from AI breeding (Lamb et al., 2016). Breeding soundness exams (BSE), pregnancy diagnosis, and managing cattle to implement a defined breeding season also provide managers with the ability to increase reproductive performance of their beef herds (Lamb et al., 2016).
Management strategies involving selection, culling and planned matings have given beef managers the ability to alter the genetic makeup of a given population and improve reproductive performance (Dziuk and Bellows, 1983). Genetics give the producer the opportunity to introduce heterosis to produce cows with calving rates and net calf crop percentages greater than that of straightbred females (Dziuk and Bellows, 1983). Hawken et al. (2015) used the bovine SNP50 chip in a genome wide association study (GWAS) to explore the genetics related to female reproduction traits. Numerous single nucleotide polymorphisms (SNP’s) affect multiple reproductive traits such as: age at puberty, postpartum anestrous interval, and preweaning postpartum ovulation in the first rebreeding period, suggesting genetics plays a role in reproductive performance that is yet to be understood (Hawken et al., 2015). While heritability values of most reproductive/fertility traits are low and epigenetic challenges have an effect on management and gestation, age at puberty shows higher heritability, and can be used to measure genetic progress (Dziuk and Bellows, 1983).

Nutrition and seasonality have been well studied as factors that affect the onset of puberty in beef heifers (Schillo et al., 1992). Age at puberty and nutritional availability are inversely proportional, making plane of nutrition the limiting factor for reproduction (Short and Adams, 1988; Schillo et al., 1992). Seasonality has also shown to impact age at puberty. Angus X Holstein heifer calves born during the fall season with subsequent exposure to spring weather conditions by 6 months of age, reached puberty faster than heifer calves born during the spring (Schillo et al., 1983).

The vaginal microbiota, though less thoroughly investigated in cattle, has been shown to impact fertility, preterm birth, and neonatal health in humans. *Lactobacillus* spp. dominates the
vaginal microflora of women in a normal state of health and reproductive fitness, yet the dominant species is likely to change over time, with menstruation and sexual activity to a community dominated by bacterial species other than *Lactobacillus* (Romero et al., 2014; Johnson et al., 1985; Eschenbach et al., 2000). Unlike humans, the vaginal microbiota in ewes and cows are dominated by *Aggregatibacter* spp. and *Streptobacillus* spp. (Swartz et al., 2014), while in pigs, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteriodetes*, and *Tenericutes* dominate the vaginal microbiota (Lorenzen et al., 2015). These species are associated with common pathogens of the human vaginal microbiota (Swartz et al., 2014). In humans, *Lactobacillus* spp. act as antagonists to maintain a mutualistic microbiota in the lower genital tract of females by preventing the colonization of pathogenic bacteria via adherence to the vaginal epithelium and/or producing antimicrobial agents, such as hydrogen peroxide and lactic acid, to control the growth of other inhabitant bacterial species (Verstraelen et al., 2009; Boris and Barbés 2000).

Failure of *Lactobacillus* spp. to dominate the vaginal microbiota causes overgrowth of pathogenic bacteria that can result in the presence of bacterial vaginosis (BV), a condition impacting 10-50% of women worldwide (Verstraelen et al., 2009; Sobel, 2000; Koumans et al., 2007). Bacterial vaginosis has been shown to occur more in women experiencing infertility (45.5%, 398/874 subjects) than control women (15.4%, 59/382 subjects, *p < 0.001*) suggesting an association between vaginal dysbiosis and infertility (Salah et al., 2012). Intrauterine infection is a cause of preterm birth (Romero, 2001). The leading cause of intrauterine infection is the ascension of pathogenic bacteria from the vagina, making BV an associate in the manifestation of intrauterine infection and subsequent preterm labor and delivery (Romero, 2001). In dairy cattle,
the presence of vaginal dysbiosis can result in purulent vaginal discharge (PVD) causing endometrial inflammation which negatively effects reproductive efficiency (Gilbert et al., 2005).

Romero et al. (2014) characterized the microbial community structure in the vagina of pregnant women. The ability of *Lactobacillus* spp. to dominate the vaginal ecosystem of these women throughout gestation may show an adaptation for increased reproductive fitness by maintaining the stability of the microflora during pregnancy and preventing ascending infections which are linked to preterm delivery (Romero et al., 2014; Romero et al., 2001; Gonclaves et al., 2002).

Dominguez-Bello et al. (2010) suggest neonates delivered vaginally are less susceptible to certain pathogens when compared to infants delivered C-section. Vaginally delivered infants are naturally exposed to the microbes present in the mother’s vagina during delivery providing the first exposure to microbial communities that establish the neonatal skin microbiota (Dominguez-Bello et al., 2010). Of newborns experiencing methicillin-resistant *Staphylococcus aureus* (MRSA) infections, 64-82% are born via C-section (Watson et al., 2006). This suggests the colonization of the bacteria received via vaginal transmission from the mother impacts the health of the neonate by reducing the ability of pathogens to colonize after delivery (Dominguez-Bello et al., 2010).

Little research has been conducted to explore the vaginal microbiota of beef heifers. Therefore, the purpose of this study was to evaluate vaginal microbial populations before breeding and determine whether or not these microbial signatures are associated with the female’s ability to establish a pregnancy via estrus synchronization with artificial insemination, natural service, or failure to become pregnant. Additionally, the vaginal microbiomes of heifers with
confirmed pregnancies were followed longitudinally to describe the composition of the vaginal microflora as gestation progresses over time. Using second generation 16s rRNA gene-based sequencing of the vaginal microbiota allows the investigation of microbial populations using culture-independent techniques and a deeper understanding of the representation and role of specific communities of bacteria and their abilities to contribute to reproductive efficiencies in beef cattle.

**Factors affecting reproduction in beef cattle**

In the cow-calf segment of the beef cattle industry, lifetime production (total weight of calves a cow weans in her lifetime) is the most important factor when considering the reproductive output of the operation (Cundiff et al., 1992). Reproductive efficiency is greatly affected by the age of the animal at puberty and first conception, the duration of postpartum anestrus and lifetime productivity (Burns et al., 2010). Optimizing reproductive efficiency in the herd is essential to economic profitability and sustainability of a beef cow-calf herd. Reducing the female’s age at first calving, the postpartum anestrous period, fertilization failure, embryo mortality, and postnatal calf mortalities decreases the amount of time a female spends in an unproductive period and decreases loss associated with calf production (Burns et al., 2010).

**Seasonality**

Seasonal and environmental effects impact the male, female and calf’s ability to produce. With males and females, extended periods of drought can lead to hypovitaminosis (Holroyd et al., 2005; Hill et al., 2009). The lack of Vitamin A in males impacts testicular weight, sperm morphology and production, and epididymal sperm reserves which leads to fertilization failure and increased rates of embryo mortality (Rode et al., 1995; Ross et al., 2000). In a study report-
ing a 41% mortality rate from partition to 15 weeks postpartum, 87% of these mortalities were associated with a Vitamin A deficiency in the cow during gestation as a consequence to a drought period (Hill et al., 2009). Prolonged periods of low-rainfall lead to pregnant heifer/cow mortalities resulting in calf mortality from confirmed pregnancy to weaning (Fordyce et al., 1990; McCosker et al., 1991). In a study reporting 33% calf mortality from confirmed pregnancy to weaning, 22% of that mortality resulted from drought associated cow mortality (Fordyce et al., 1990).

The seasonal condition in which a female calf is reared during her first year of life influences the age at which she will reach puberty. During the first 6 months, exposure to environmental conditions that stimulate growth increase the heifer’s growth rate, in turn allowing them to reach puberty at a young age since body weight and age at puberty are related (Schillo et al., 1983). Results from a study by Schillo et al., (1983) show that September born heifers grew at a faster rate than heifers born in the spring. Regardless of the season of birth, heifers exposed to spring and fall-like conditions between 6 and 9 months of age and reached the onset of puberty at an increased age (Schillo et al., 1983). This stunted puberty is likely do to light exposure or environmental temperature effects, or a combination of both (Schillo et al., 1983). While cattle do not experience anestrous periods due to season (like sheep), seasonal factors that influence reproductive traits, such as age at puberty, may act as a mechanism to target calving dates to particular times of the year (Schillo et al., 1983).

Heat stress associated with high environmental temperature and humidity impact both males and females. Though some bovine breeds are inherently more tolerant of heat stress, this factor still has the ability to compromise steriodogenesis, decrease fertilization rates and reduce the morphology and viability of oocytes (Zeron et al., 2001; Post, 1980; Hansen, 2002; Al-
Katanani et al., 2002). In the female, heat stress can act in one of two ways on reproductive performance: hyperthermia on the reproductive axis and/or an indirect effect related to decreased feed consumption and dry matter intake (Rensis and Scaramuzzi, 2003). Decreased levels of circulating LH and FSH which lead to decreased levels of estrogen are seen in heat stressed cows (Rensis and Scaramuzzi, 2003). Low levels of reproductive hormones decrease oocyte quality and inhibit fertilization and implantation resulting in impaired fertility (Rensis and Scaramuzzi, 2003).

**Genetics**

Maternal genetics have the ability to influence fertilization rates and impact embryo and fetal mortalities. Interferon tau is associated with maternal recognition of pregnancy around day 15 gestation in the cow (Mathialagan and Roberts, 1994). This interferon, expressed in the trophectoderm layer of the blastocyst from hatching (days 8-9) until implantation is the signal for the dam’s recognition of the embryo (Mathialagan and Roberts, 1994). Genetic difference due to sire effects (of the calf as well as of the dam in relation to her sire) can impact the female’s ability to maintain a pregnancy (Bar-Anan et al., 1980). Unrelated to the sire or dam, genetics still have the ability to impact the calf through spontaneous chromosomal changes during gametogenesis, fertilization, or development (King, 1990).

**Nutrition**

Different aspects of a plane of nutrition impact beef cattle’s ability to reproduce. Following periods of starvation (from food restriction or drought), the plants that grazing bovines consume may contain high nitrate levels which act as a toxic agent resulting in embryo mortality (Burns et al., 2010; McKenzie 2002). Hay, fed during time periods when pasture grazing is not
available, can contain mycotoxins such as zearalenone, an aflatoxin associated with abortions (Osweiler, 1990; Kallela and Ettala, 1984; Zavy, 1994). Intensively managed herds report acute negative energy balance as a course of embryo mortality (Vanroose et al., 2000; Bilodeau-Goeseeels and Kastelic, 2003). Nutritional availability throughout an animal’s life influences its mature size and liveweight (Moran et al., 1989). Age at puberty is a function of liveweight, drawing a direct connection between nutritional planes and the onset of reproductive function.

Nutrition and body condition also have the ability to impact the length of postpartum anestrous (Rutter and Randel, 1984; Ruiz-Cortes et al., 1997; Randel, 1990).

**Female Factors**

Puberty attainment by replacement heifers often hints at the reproductive potential for the beef herd (Burns et al., 1992; Cundiff et al., 1992). Fordyce and colleagues (1994) report that heifers that calve within the first 2 years of life have increased lifetime production values when compared to heifers that calve for the first time at 3 years of age or older. Age at the first ovulation and corpus luteum formation should be the basis for confirming a female has reached puberty (Johnston et al., 2009). Puberty is likely due to physiological age rather than a chronological age (Moran et al., 1989). Reaching puberty, in the sense of female maturation, is attributed to an interaction of endocrine, genetic and environmental factors (Moran et al., 1989). Endocrine factors work to develop the female reproductive tract where as the genetic and environmental factors (such as nutritional plane) effect the rate at which the endocrine factors can work (Moran et al., 1989). This characteristic emphasizes the importance of nutrition in relation to developmental and reproductive factors.
During a given estrus cycle, the number of follicular waves a female experiences impacts conception rate (Ahmad et al., 1997). In animals with 2 follicular waves, the dominance of the ovulatory follicle can last 2-3 days longer than that of a female with 3 follicular waves (Ginther et al., 1989). With this extended period of dominance, the health and competency of the dominant follicle decreases, in turn, decreasing pregnancy rates in the female (Mihm et al., 1994; Ahmad et al., 1995; Oussaid et al., 2000). Using targeted supplementation to increase nutrition levels, resulting in increased body condition, have been shown to increase the prevalence of 3 follicular waves per cycle in females (Burns et al., 2010).

Postpartum anestrous interval, the length of time between parturition and the first ovulation, is an issue beef cattle producers face when evaluating cow fertility. Prolonged periods of postpartum infertility can be attributed to unexplained infertility, failure of complete uterine involution, short estrus cycles and anestrus (Short et al., 1990). Increased postpartum intervals (PPI) cause the female to breed later than the rest of the herd, increasing the length of the calving season and decreasing the uniformity of the calf crop (Short et al., 1990).

**Vaginal Microbiome**

Though less explored, the bovine vaginal microbiome could impact the female’s ability to establish a pregnancy and influence the health of the calf during gestation. A mutualistic relationship between the host and the microbes inhabiting the vagina exists, establishing the first line of defense against pathogenic colonization on the vaginal mucosa (Smith and Ravel, 2016). Gametic and early embryonic growth, maturation, transport, and long-term survival depend on the mucus present in the vaginal ecosystem (Rutllant et al., 2005). Some species of bacteria that reside in the vagina have capabilities to adhere to the vaginal mucus. These species form biofilms to
stabilize the microenvironment which plays an important role in working as immunological barriers and growth frame works for microorganisms involved in gamete distribution (Salminen et al., 1998; Rullant et al., 2002, 2005).

Lactobacilli are widely recognized in the vaginal niche of mammals because of their ability to produce compounds that aid in preventing the colonization of pathogenic microorganisms (Stykov et al., 2014). The reduction in relative abundance or absence of Lactobacillus spp. can hint at signs of infection or shifts in microbial populations that could be detrimental to vaginal health and reproduction (Stykov et al., 2014). *Lactobacillus*-dominated vaginal microbial communities, are associated with healthy women of active reproductive age (Smith and Ravel, 2016). While there is great variation across the vaginal microbiome of women, an accepted signature for a healthy vaginal environment is a community dominated by a Lactobacillus spp. The same cannot be said for other species, including cattle. In female bovine, the vaginal niche is reported to be dominated by *Bacteroides*, Enterobacteriaceae, *Victivallis*, Ureaplasma, Peptostreptococcaceae, Rikenellaceae, *Firmicutes*, and *Proteobacteria* (Rodrigues et al., 2015; Clemmons et al., 2017; Laguardia-Nascimento et al., 2015).

In cattle, microbiome studies more commonly surround the bacterial contents of the rumen, digestive and respiratory tracts, whereas there is a paucity of information in regards to microbes inhabiting the vaginal niche. The relationship microbes have with the host in terms of fertility and gestation remain unknown. In humans, the vaginal microbiome is used, in part, to explain the prevalence of disease, unexplained infertility, pre-term births, and neonatal health during gestation and following birth.
Human Vaginal Microbiome

A core vaginal microbiome that can be used to describe the “normal” flora in females is yet to be established (Ravel et al., 2011). The vaginal microbiome of women can be grouped into two categories: (1) a community that is dominated by *Lactobacillus* spp. and (2) a community that is dominated by a species other than *Lactobacillus* (Ravel et al., 2011). These differences are likely due to ethnic group. Ravel et al. (2011) explored the vaginal microbiomes of white, black, Asian and Hispanic women living in North America. A higher proportion of *Lactobacillus* spp. dominated communities were found in white and Asian women where 89.7% and 80.2% of sampled subjects had vaginal microflora dominated by *Lactobacillus* spp., respectively (Ravel et al., 2011). These findings agree with the studies by Zhou et al. (2007, 2010) where the vaginal microbial communities were evaluated in black, white and Japanese women. Ravel et al., (2011) speculated these differences in the vaginal bacterial communities of different ethnic groups is likely due to genetics.

The “normal” vaginal flora of different groups of women are not the same, however 73% of vaginal microbial communities in humans are dominated by a *Lactobacillus* spp. (Ravel et al., 2011). These *Lactobacillus* spp. dominated groups can be subdivided based on the species of *Lactobacillus* that is present in the largest proportion. The amount of *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* in a community serve as the parameter for subdivision into categories known as community state types (CST’s) (Ravel et al., 2011). In women with *Lactobacillus* spp. dominated communities, 34.1% are dominated by *L. iners* (Ravel et al., 2011). Dominance by the other *Lactobacillus* species are less common with *L. crispatus*, *L. gasseri* and *L. jensenii* representing 26.2%, 6.3% and 5.3% of women respectively (Ravel et al., 2011). The 27% of women
not represented by communities dominated by *Lactobacillus* spp. show higher proportions of anaerobic bacteria such as *Prevotella*, *Dialister*, and *Atopobium* (Ravel et al., 2011). Interestingly, all community types house members of bacterial genera that are known to produce lactic acid, suggesting that lactic acid plays a role in maintaining a “healthy” vaginal microflora (Ravel et al., 2011).

**Unexplained Infertility**

Although defining a “normal” or “healthy” microbiome is challenging, vaginal dysbiosis or unbalance of the microbial communities has shown to cause infertility, infections and other debilitating disorders (Mor et al., 2015). Infertility in individuals can be caused by a number of different conditions.

While the exact mechanism linking BV to infertility is unknown, its prevalence in infertile women raises concern and warrants further investigation. BV is a change in the vaginal flora characterized by decreased concentrations of *Lactobacillus* spp. and increased concentrations of *G. vaginalis*, *Mycoplasma hominid*, *Prevotella*, *Porphyromonas*, *Bacteroides* and *Peptostreptococcus* (Eschenbach et al., 1989; Hawes et al., 1996; Thorsen et al., 1998; Hill, 1993). Bacterial vaginosis has the potential to affect implantation and embryo development by disrupting the immune-endocrinological environment and shifting reproductive tract cytokine signatures (Mandar et al., 2015). Salah et al., (2012) explored the impact of BV on infertile women and evaluated the effects of single dose BV treatment. The presence of BV in infertile women is 30.1% higher than that of fertile women (Salah et al., 2012). Of the women with infertility caused by different conditions such as polycystic ovarian disease (PCOD), as well as women with unexplained infertility, there is a significantly higher presence of BV with 60.1% ($p = 0.0001$) and 37.4% ($p = 0.001$)
of infertile women with reported BV, respectively (Salah et al., 2010). With a single dose of BV treatment, women with PCOD ($p = 0.001$) and unexplained infertility ($p = 0.04$) had pregnancy rates that were lower than BV negative women, but higher than women with BV that had not been treated (Salah et al., 2012). These findings suggest that BV, caused by shifts in vaginal microflora, significantly impairs pregnancy rate (Salah et al., 2012).

Pathogenic bacteria, introduced through medical procedures such as catheterization or reproductive technologies such as embryo transfer, impact the microflora of the vagina. Implantation rates in females undergoing in vitro fertilization (IVF) were higher in women testing negative for bacterial contamination than women contaminated by one or more species of pathogenic bacteria (Franasiak et al., 2015). Sampling prior to embryo transfer yielded implantation rates of 14% for women without bacterial contamination and 12.4% for women with bacterial contamination ($p < 0.001$). The lowest pregnancy rates were found in women that tested positive for Enterobacteriaceae and Staphylococcus contamination (Franasiak et al., 2015). These findings suggest certain bacterial species can negatively impact female fertility. A challenge associated with this study lies in the determination of bacterial contamination. The culture-based techniques used in this study are unable to represent the diversity of the microbiome (Franasiak et al., 2015). The negative association between bacterial contamination and pregnancy rate warrant further investigation by culture-independent techniques.

The ascension of the vaginal microbiota can extend into the remainder of the female reproductive tract (Franasiak et al., 2015). As these bacteria move through the reproductive tract, they have the ability to impact the reproductive axis and gametogenesis (Franasiak et al., 2015).
These pathogenic bacteria have been shown to inhibit gonadotropin response and negatively impact follicular development (Franasiak et al., 2015).

**Sperm (motility, longevity and fertilization)**

The vaginal ecosystem is an open environment which is influenced by the male genital tract microbial populations via unprotected sex (Mandar et al., 2015). Although diversity in the seminal microbiota of humans is greater than that of the vagina, total bacterial concentrations are lower (Mandar et al., 2015). Vaginal bacterial communities share 85% of all detected phylotypes with seminal bacterial communities (Mandar et al., 2015). Unlike the vagina, the seminal microbial ecosystem is composed of more Firmicutes, Bacteroidetes and Actinobacteria (Mandar et al., 2015). These differences in composition allows the seminal microbiome to cause significant shifts in vaginal microbial make-up (Mandar et al., 2015). The introduction of the male genital microflora to the vagina causes decreases in the relative abundance of *L. crispatus*, in turn allowing opportunistic pathogens to overgrow and possibly dominate the vaginal ecosystem (Mandar et al., 2015).

**Pre-term Birth**

Pre-term birth (PTB; delivery prior to 37 gestational weeks) affects 12% of births in the United States and can be associated with intrauterine infection resulting from the ascension of pathogenic bacteria from the vagina to the uterus in some pregnant females (Martin et al., 2012; Romero et al., 2001; Goncalves et al., 2002). Hyman et al. (2014) found decreased alpha diversity in the vaginal microbiome of Caucasian women with pregnancies resulting in PTB. As pregnancy progresses, the vaginal microbiome changes to a population more closely related to a non pregnant vaginal microbiome with increased beta diversity (Prince et al., 2014; Romero et al., 2001; Goncalves et al., 2002).
2014). This suggests that a changing vaginal flora may be associated with parturition (Prince et al., 2014).

During Gestation

Gajer et al. (2012) found the vaginal communities of healthy, reproductive-age women are characterized by species turnover, little consistency in community composition over time and large differences in microbial composition, even in those that cluster into the same community type. Among many other unexplored factors, these fluctuations in microbial communities are likely due to menses, community cluster and sexual involvement (Gajer et al., 2012). While the community composition changes, the functionality of the community remains, likely because a fraction of bacterial species serve the same purpose in their given communities (Gajer et al., 2012). While certain events in the reproductive cycle may initiate shifts in vaginal microbial populations, pregnancy alters the vaginal microbiome with a characteristic of decreased species diversity (Mendez et al., 2016).

MacIntyre et al. (2015) used the 5 vaginal CST’s previously described to characterize populations among women with a healthy, single pregnancy. All five CST’s were found in pregnant women with L. crispatus being the most commonly observed (43%) followed by L. iners (30%), L. jensenii (14%), L. gasseri (9%) and communities dominated by a species other than Lactobacillus spp. (in this case Prevotella spp., Clostridium spp., Atopobium spp., and Megasphaera spp.; 2%) (MacIntyre et al., 2015). These findings closely resemble findings from the work of other groups evaluating the microbial composition of the vaginal microbiome during pregnancy (Romero et al., 2014; Verstraelen et al., 2009; Huang et al., 2014; Walther-Antonio et al., 2014; Aagaard et al., 2012). The lowest alpha diversity and richness were seen in vaginal
communities dominated by *L. crispatus*, correlating to the most common CST in pregnant women, characterized by increased vaginal flora stability (MacIntyre et al., 2015). When *L. crispatus* dominated the vaginal flora, the community was 5 times less likely to shift to an abnormal community type (*p* = 0.04) (Verstraelen et al., 2009). During pregnancy, the vaginal epithelium matures due to increased levels of circulating estrogen, produced by the placenta, establishing an accumulation of glycogen (Boskey et al., 2001). The glycogen is broken down by host α-amylase in the vaginal epithelium to produce products that support the colonization of *Lactobacillus* spp. and hence, a more stable vaginal microbiome during pregnancy (Spear et al., 2014). Conversely, the greatest vaginal community diversity and richness was reported in females with vaginal floras dominated by species other that *Lactobacillus* spp. (MacIntyre et al., 2015). Vaginal microbial populations dominated by *L. gasseri* or *L. iners* were 10 times more likely to shift to an abnormal community type (Verstraelen et al., 2009).

**Effects on Neonatal Health**

Mode of delivery is the primary factor in determining the initial bacterial community composition of a newborn (Dominguez-Bello et al., 2010). In vaginally delivered infants, their initial skin microbiota most closely resembles that of their mother’s vaginal microflora, while infants delivered via cesarean section have a skin microbiota more similar to the mother’s skin microflora (Dominguez-Bello et al., 2010). This suggests the vertical transmission of bacteria from mother to infant during delivery provides a natural first colonization of bacteria across all body habitats of the newborn (Dominguez-Bello et al., 2010).

The species residing in the mother’s habitats (vaginal and skin) that effect the newborn microbiota can influence the health of the neonate. The composition of the initial colonizing mi-
crobiota can illicit immune function and alter nutrient uptake, as well as effect the long-term development of the microbiota of multiple body habitats (Dominguez-Bello et al., 2010). The operational taxonomic units (OTU’s) present in the microbiome of an infant can be described as kept, lost, gained or regained when compared to the initial acquisition of microbial communities (Stewart et al., 2017). In vaginally delivered infants, the number of kept OTU’s were significantly higher than infants delivered via C-section when sampled at two months of age (Stewart et al., 2017). This increased stability is reflective of an increased stability in the gut microbiome of vaginally delivered infants compared to C-section delivered infants (Backhed et al., 2015). *Lactobacillus* abundance in the vaginally delivered neonate’s distal gut is positively correlated with the abundance of *Lactobacillus* in the dam’s vagina (*p* < .0001) (Banks, 2015). Maternal stress during pregnancy alters the vaginal microbiome by decreasing the amount of *Lactobacillus* spp. (*p* = .0398) (Banks, 2015). With an altered vaginal bacterial populations, infants are unable to colonize specific bacterial communities imperative for infant GI tract development which indirectly affects neonatal neurochemistry and amino acid availability (Banks, 2015). Babies born via C-section are colonized by an abundance of *Staphylococcus* spp. which can explain their increased susceptibility to certain species of pathogenic bacteria when compared to vaginally delivered infants (Dominguez-Bello et al., 2010). Regardless of the mode of delivery, the initial colonization of the neonate’s microbiota is homogenous across all body habitats increasing the importance for colonization of bacterial communities that positively impact neonatal development (Dominguez-Bello et al., 2010).
Vaginal Microbiome of Other Species

With little exploration to date of the bacterial species that inhabit the vagina of species other than humans, their relationships with reproductive performance, maternal health and neonatal health remain unknown. The vaginal microbiota of several other species harbor bacterial strains that have been associated with disease and perinatal morbidity in humans, raising questions surrounding the role of bacteria in the reproduction.

Little is known about the microbes that inhabit the reproductive tracts of non-human primates. Yildirim and colleagues (2014) set out to view the microbial composition of the primate vagina in attempts to understand microbial evolution. Their results show lower richness and diversity levels in the microbes that inhabit the human vagina when compared to that of the primate (Yildirim et al., 2014). Firmicutes were detected across all primates showing high relative abundance in all species (20-30% of the total microbiota; Yildirim et al., 2014). Genera dominating the primate species included: Sneathia, Aerococcus, Anaerococcus, Porphyromonas, Fusobacterium, Atopobium and Prevotella (Yildirim et al., 2014). Despite the physiological similarities between primates and humans, the vaginal microbial populations differ (Rivera et al., 2010). The normal microbiota found in some species of primates, particularly baboons, is indicative of a disease state in humans (Rivera et al., 2010). Relative abundances of lactobacilli in the primate vaginal microbial community was significantly lower than that of humans (Yildirim et al., 2014). Chimpanzees (the closest non-human relative to humans) had vaginal microbiomes with less than 3.5% lactobacilli (Yildirim et al., 2014). These results have implication in understanding the host-microbe interaction and in particular, the role of species like Lactobacillus in vaginal health.
The ewe vaginal microbiome is dominated by *Aggregatibacter* spp., *Streptobacillus* spp., *Cronobacter* spp., *Phocoenobacter* spp., and *Psychrilyobacter* spp. (Swartz et al., 2014). *Aggregatibacter* spp. has been linked to periodontal disease, infective endocarditis and brain abscesses and has not been reported as a common member of vaginal bacterial communities in humans (Gonzales-Marin et al., 2011; Norskov-Lauritsen, 2014). Like humans, *Lactobacillus* spp. were found in the ewe vagina, but in a low relative abundance (0.53 ± 0.65%) (Swartz et al., 2014). The populations of bacteria, including the low yet detectable presence of lactobacilli, found in ewes most closely resembles CST IV of the human vagina dominated by species other than *Lactobacillus* spp. and characteristic of women diagnosed with bacterial vaginosis (Swartz et al., 2014; Ravel et al., 2011). Another comparison of human and ewe vaginal microbiota can be made in terms of pH. The human vaginal pH corresponding to CST IV is maintained at 5.3 (Manes et al., 2010). Surprisingly, the ewe vaginal pH is maintained at a near-neutral level between 7.0 and 7.6 despite the lack of *Lactobacillus* spp. in the vaginal communities (Swartz et al., 2014).

**Bovine Vaginal Microbiome**

The microbial populations that reside in a ‘normal’ bovine vagina are a mixture of aerobic, anaerobic, and facultative anaerobic bacterium (Otero et al., 2000). Although the microbial flora of this niche is dynamic, the ecosystem remains stable under natural conditions, preventing the proliferation of pathogenic microorganisms (Otero et al., 2000). Dominant colonizing species vary among studies. Swartz et al. (2014) reports the cow vaginal microbiota is dominated by *Aggregatibacter* spp., *Streptobacillus* spp., *Phocoenobacter* spp., *Sediminicola* spp., and *Sporobacter* spp. These particular species of bacteria have been shown to adhere to the collagen...
present in the vaginal tissue, which could explain their prevalence in this particular niche, especially during gestation (Tang et al., 2008; Swartz et al., 2014). Hafez (1993) found the dominant species to include *Staphylococcus*, *Streptococcus* and coliforms. Firmicutes, Bacteroidetes were present in a study conducted by Moreno et al. (2016) and has been associated with bacterial vaginosis in humans (Eschenbach et al., 1989; Hawes et al., 1996; Thorsen et al., 1998; Hill, 1993; Franasiak et al., 2015). Identified species from this study include *Ruminococcus* spp., *Di-alister* spp., *Aeribacillus* spp., and *Porphyromonas* spp. which have also been associated with BV and infertility in humans (Moreno et al., 2016; Franasiak et al., 2015). Regardless of the bacterial species present with the most relative abundance in the vagina, *Lactobacillus* spp., were seen in low relative abundance (0.36 ± 0.66%), similar to lactobacilli content in the vaginal niche of ewes (Swartz et al., 2014; Otero et al., 2000). These contrast the characteristic ‘normal’ vaginal microbiome in that is well established in humans and primates (Reid et al., 1985; Herthelius et al., 1989). Furthermore, vaginal levels of *Lactobacillus* spp. were shown to increase in cattle during the estrus cycle and in the uterus, have been shown to stimulate the immune function (Otero et al., 1999; Kummer et al., 1997).

**Reproductive Disorder**

Bacteria genra such as, *Bacteroides*, *Mycoplasma*, *Histophilus*, *Fusobacterium*, and *Prevotella* as well as *Escherichia coli* and *Streptococcus* species, among others, have been shown to cause reproductive tract disease in cattle (Pfutzner and Sachse, 1996; Corbeil, 2007; LeBlanc, 2008). These diseases can be linked to interactions of the vaginal ecosystem and human interference (Garbeva et al., 2004; Lynch et al., 2004). Often, reproductive tract diseases can be at-
tributed to changes in the composition of bacterial populations in niches where disease is present (Seksik et al., 2003, Ott et al., 2004; Turnbaugh and Gordon, 2009).

Reproductive disorder and genital disease have been shown to increase the relative abundance of core microbiota and increase the number of bacterial taxa present on vaginal epithelial tissue. Rodrigues et al. (2015) found the *Bacteroides* and *Enterobacteriaceae* to be the most abundant species in healthy cows, with relative abundances of 28.3% and 17.8%, respectively. In females with reproductive disorder, abundance of these core microbiota were increased to levels of 35.83% and 18.62% for *Bacteroides* and *Enterobacteriaceae*, respectively (Rodrigues et al., 2015). In addition to the enrichment of the core species, 53 additional taxa were present in the females with reproductive disorder (Rodrigues et al., 2015). *Histophilus* was only found in the group with this disorder (Rodrigues et al., 2015). This genus of bacterium has been described as an opportunistic pathogen in the reproductive tracts of cattle and has been associated with the pathogenesis and progression of reproductive disease that negatively impacts fertility (van der Burgt et al., 2007).

The microflora of female bovine without reproductive disorder is characterized by a community totally dominated by anaerobic species with only a small presence of other microbes, while the communities of females in a state of disease present species that thrive in aerobic or facultative anaerobic environments (Rodrigues et al., 2015). *Enterobacteriaceae, Victivalles* and *Bacteroides*, present in healthy female bovine, direct fermentation to produce acidic compounds resulting in a change of environmental pH that favors the inhabitance for bacterial species of *Lactobacillus* and *Fibrobacter* that contribute to a healthy vaginal microflora (Rodrigues et al., 2015; Chow and Russell, 1992; Zoetendal et al., 2003). The ability of certain species to produce
nutrients for pathogenic bacteria could indirectly alter the microbial ecosystem. *Histophilus* uses compounds produced by bacteria inhabiting the vaginal niche possessing methane, nitrogen and sulfur metabolism that contribute to infection (Rodrigues et al., 2015).

Overall, the interrelationship of microbes and their hosts is an important concept for disease pathogenesis and progression and, when considering the reproductive tract microflora, explaining reproductive failure. The ecological balance of microbial communities can shift from commensal interactions to a pathogenic infection with a slight alteration in community composition (Rodrigues et al., 2015). Disease pathogenicity arises from both suppression and over colonization of certain bacterial species which further explains the importance of understanding the association between the way microorganisms interact with each other and the host environment (Ott et al., 2004; Manichanh et al., 2006; Fredricks et al., 2005; Rodrigues et al., 2015). The belief that *Lactobacillus* dominance is crucial to a healthy vaginal ecosystem is accepted in humans, but not among other species giving added insight into species diversity and raising more questions surrounding the role microbes play in health and reproduction (Yildirim et al., 2014).

**Potential Use of Probiotics**

The World Health Organization/Food and Agricultural Organization define probiotic as live microorganisms that confer health benefits to the host when administered in adequate amounts (2001). In the past two decades, the use of probiotic bacteria in yogurts and fermented milks has increased due to improved intestinal microbial balance and overall health of the consumer (Fuller, 1989; Saarela et al., 2000). These health promoting, live microbial food supplements, stimulate the growth of micro-organisms that are preferred in the intestinal nice, act to reduce the amount of unwanted or potentially harmful bacteria and reinforce the body’s immune
mechanisms (Saarela et al., 2000). Most of the successful strains of cultured probiotic used with human application are derived from human origin (Saarela et al., 2000). This eliminates some of the safety concern associated with the introduction of bacteria to the digestive system and aids in improving their functionality (Saarela et al., 2000).

During pregnancy, the maternal microbiota shifts to one showing signs of inflammation and characteristic of obesity (Gomez Arango et al., 2015). Manipulating the gut microbiome of expectant mothers by manipulating diet and introducing prebiotics, pharmaceutical agents, antibiotics and probiotics could prevent pregnancy associated complications by altering the microbial composition of the digestive tract (Musso et al., 2010; Thum et al., 2012; Thomas et al., 2010). Alone, probiotics can alter the vaginal and gut microbiome to produce metabolites and promote metabolic activity that is favorable during advanced stages of pregnancy (Gerritsen et al., 2011).

Probiotic intervention during pregnancy can impact outcomes of both the mother and infant. Identifying women with increased risk for PTB related to intrauterine infection and utilizing microbial treatment of abnormal genito-urinary flora may aid in preventing pre-term labor and delivery (Gonclaves et al., 2002). Probiotics can shift microbial communities to inhibit the growth of pathogenic microbes, modifying the inflammatory response associated with pre-term birth (Othman et al., 2007). In infants, the risk of contracting atopic eczema at 6 months of age or rhinoconjunctivitis between 18 and 36 months of age was reduced (when mothers consumed probiotic milk (L. acidophilus LA-5, B. lactis BB12 and L. rhamnosus) during pregnancy (Bertelsen et al., 2014). Additionally, children 2-7 years of age showed a reduced prevalence of
atopic eczema (-5.7%, $P = 0.02$) when mothers were administred *Lactobacilli* during gestation (Doege et al., 2012).

In cattle, an animal’s health surrounding the time of calving is directly related to subsequent fertility (Genis et al., 2017). In the dairy industry, the use of probiotics to treat postpartum uterine infection and endometritis have been studied to reduce the reliance on antibiotics that decrease the quality of the milk produced by treated females. The traditional use of intra-uterine antibiotics to treat puerperal metritis and non-pathological endometritis can decrease milk quality, inhibit uterine immune function, and act as irritants (Otero et al., 2000; Campero et al., 1992). Using a probiotic as a preventative treatment would negate the antibiotic influence on milk quality and improve reproductive rates in the cows (Otero et al., 2000).

Post calving, the uterus undergoes tissue repair and involution during a time when the female is in a period of negative energy balance (Genis et al., 2017). Beta-defensins are antimicrobial peptides that reside on the endometrium that work in the event of bacterial invasion (Sheldon et al., 2014). *MUC1* is part of the innate immune system that works as a component to the first line of defense against microbial challenges on the epithelial surface (Kasimanickam et al., 2014). Both *B-defensins* and *MUC1* in the endometrium of postpartum dairy cows have shown reduction when the females were treated with an intravaginal probiotic composed of *Lactobacillus rhamnosus*, *Pediococcus asidilactici*, and *Lactobacillus reuteri* (Genis et al., 2017). This reduction in indicators of uterine infection could be explained by the modulation of the vaginal microbial ecosystem (Genis et al., 2017). Infection and prolonged inflammation of the uterus post calving could challenge the reproductive efficiency of the female. The use of a probi-
otic creates a vaginal niche that aids in preventing the growth of pathogenic bacteria that could ascend into the uterus and delay the recovery process (Genís et al., 2017).

Conclusion

In summary, many factors and their interactions impact reproductive success of humans and similarly bovine. Genetics, seasonally, nutrition and physiological parameter’s impact on female reproduction have been well documented in cattle. In humans, the colonization of bacteria in the vagina have been associated with attempts to answer questions surrounding unexplained fertility, pre-term birth and neonatal health. Since successful reproduction plays such an important role in the financial stability of a cow-calf operation, investigation of the bovine vaginal microbiome and it’s potential to impact reproductive to success or failure is warranted.
CHAPTER 2

Introduction

In commercial beef operations, the factor with the greatest impact on profitability is reproduction (Hess et al., 2005). Combing the losses in dairy and beef cattle due to reproductive failure, results in a $1 billion annual loss in income for the cattle industry and makes the failure to reproduce six times more costly than loss associated with respiratory disease (Bellows et al., 2002). Although a myriad of other factors contribute to this financial loss, infertility, due to culling infertile females averages a national annual loss of $249 million alone (Bellows et al., 2002). Incorporating reproductive technologies, management strategies involving genetic selection and taking into account nutrition and seasonality can impact reproductive efficiency in a beef herd (Lamb et al., 2016; Duzik and Bellows, 1983; Schillo et al., 1992). However, the less explored vaginal microbiota of female bovine may also provide insights to explain reproductive failure and success.

The extensively characterized human vaginal microbiome is divided into 5 community state types (CSTs) based on the dominating species of bacteria (Zhou et al., 2007; Ravel et al., 2001; Gajer et al., 2012). Four of these CSTs are dominated by the hydrogen peroxide and lactic acid producing family of Lactobacillus species (Verstraelen et al., 2009; Boris and Barbés, 2000). In the fifth CST, the failure of Lactobacillus dominance can lead to the overgrowth of pathogenic bacteria resulting in bacterial vaginosis (BV) which negatively impacts the ability of females to establish a pregnancy (Verstraelen et al., 2009; Sobel, 2000; Koumans et al., 2007; Salah et al., 2012).
Unlike humans, the vaginal microbiota in other species have not been reported to possess *Lactobacillus* dominance. In non-human primates, the vaginal microflora presents a decrease in both richness and diversity (Yildirim et al., 2014). These populations show dominance by bacterial genera that are considered pathogenic in the human vaginal ecosystem, such as *Firmicutes*, *Porphyromonas*, *Fusobacterium*, *Atopobium* and *Prevotella* (Yildirim et al., 2014). The ewe vaginal microbiome is dominated by *Aggregatibacter* species, *Streptobacillus* species, *Cronobacter* species, *Phocoenobacter* species and *Psychrilyobacter* species (Swartz et al., 2014). Unlike humans, the relative abundance of *Lactobacillus* species in the vaginal ecosystem is low at less than 3.5% and 0.53% for chimpanzees and ewes, respectively (Yildirim et al., 2014; Swartz et al., 2014).

In cattle, various studies report a variety of microbial compositions related to the vagina in female bovine. Dominance by *Aggregatibacter*, *Streptobacillus*, *Phocoenobacter*, *Sediminicola* and *Sporobacter* species are reported in a study by Swartz et al. (2014). Hafez (1993) presents dominance by *Staphylococcus*, *Streptococcus* and coliforms. *Firmicutes*, *Bacteroidetes*, *Ruminococcus*, *Dialister*, *Aeribacillus*, and *Porphyromonas* were dominant colonizers in a study by Moreno et al. (2016) and have been associated with BV in humans (Eschenbach et al., 1989; Hawes et al., 1996; Thorsen et al., 1998; Hill, 1993; Franasiak et al., 2015).

Differences in relative abundance of certain genera in the vaginal microbiota in female bovine have been linked to distinction between healthy cows and those suffering from reproductive disorder. Increased relative abundance signatures in *Bacteroides* and *Enterobacteriaceae* (35.83% and 18.62%, respectively) have been shown in females with reproductive disease compared to healthy individuals with relative abundance values of 28.3% and 17.8%, respectively.
(Rodrigues et al., 2015). *Histophilus* has also been isolated from vaginal communities in bovine with reproductive disorder, and not from those with healthy reproductive function (Rodrigues et al., 2015).

Overall, the interrelationship between hosts and their microbes is important to consider when exploring disease presence and pathogenesis, especially related to female fertility. In humans and non-human species alike, the suppression and over colonization of certain bacterial species in a niche results in disease pathogenicity and emphasizes the importance of understanding the way the host environment and inhabiting microbes interact (Ott et al., 2004; Manichanh et al., 2006; Fredricks et al., 2005; Rodrigues et al., 2015). The belief that *Lactobacillus* dominance is crucial to vaginal health in human, but not other species raises questions of the role microbes play in health and reproduction (Yildirim et al., 2014). Studies have shown positive effects of using probiotics to shift microbial communities in gestating humans to inhibit the growth of microbes that modify the host inflammatory response and signal for pre-term birth (Othman et al., 2007). When ingested, these live organisms can alter the vaginal and gut microbiomes to produce metabolites and products that promote favorable metabolic activity during late stages of gestation (Gerritsen et al., 2011). Understanding the role and function certain species of bacteria play in terms of fertility and overall reproductive performance in female cattle could help increase the reproductive fitness of cow herds worldwide.

Therefore, the purpose of this study is to characterize the vaginal and fecal microbiome of commercial beef heifers and relate their vaginal microbial signatures to the ability to establish a pregnancy or not. Furthermore, this study seeks to understand the dynamic communities of both fecal and vaginal environments in the gestating heifer by following individuals throughout
pregnancy. Using second generation 16S rRNA sequencing allows investigation of microbial communities and a deeper understanding of the roles community members play in contributing to reproductive function in beef cattle.

**Materials and Methods**

**Ethics statement:**

All animal work was approved by the Institutional Animal Care and Use Committee of the University of Arkansas under permit number 16024.

The University of Arkansas Division of Agriculture’s Beef Research Unit near Fayetteville, AR, housed 72 crossbred beef heifers averaging $420.88 \pm 17.42$ days of age and $328.036 \pm 25.45$ kg for this study.

**Breeding Strategy:**

At the onset of breeding season, a 25 mg PGF2α injection (Lutalyse®, Zoetis, Parsippany, NJ) was administered intramuscularly in the neck. A heat detection patch (Estrotect Heat Patches®, Melrose, MN) was placed on the tailhead of each female. Heifers were then allocated to 1 of 6, 1 ha grass pastures. Each day for the subsequent 7 days, all heifers were monitored for estrus activity at 8:30 am and 4:30 pm. Within 12 to 18 hours of estrus detection, heifers were artificially inseminated.

Following day 7 of estrus detection, those individuals not showing signs of estrus like behavior were administered a second PGF2α injection. This group of heifers were monitored and artificially inseminated as described above for 5 additional days. The heifers were then moved to 6, 2.4 ha fescue-bermuda grass mixed pastures and were rotated every 28 days.
Seven days after transfer to the pastures, 6 fertile bulls were introduced to initiate a 50 day breeding season. The bulls were rotated among the pasture every 7 days. A breeding soundness examination was preformed on each bull no greater than 30 days before introduction to the heifer herd and following the 50 day breeding season. After 50 days of exposure all bulls passes breeding soundness examinations.

Sixty-three days after the onset of breeding season, ultrasound was used to determine the heifer’s pregnancy status and if the pregnancy was due to artificial insemination or natural breeding.

Sample Collection:

At the onset of breeding season, fecal samples were taken and immediately placed in 50 mL conical tubes and placed on ice. The vulva was wiped clean with a paper towel and vaginal swabs were collected by inserting a double guarded culture swab (Jorgensen Labs, Loveland, Colorado, USA) at a $45^\circ$ angle into the vagina and moving to the posterior cervix. At the posterior cervix, the swab and inner guard were maneuvered through the outer guard. The swab was then pushed out of the inner guard and rolled on the surface of the vaginal epithelium for approximately 15 seconds. The swab was retracted back into the inner guard. The inner guard (containing the swab with sample) was retracted into the outer guard and the mechanism was removed from the animal. The swab was cut from the handle, placed in a 2 mL snap-cap tube with 1 mL of AMIES transport buffer and placed on ice. All samples were stored at -80°C. Fecal and vaginal samples were taken from all individuals, as described previously at a second time point during the first trimester of gestation. Vaginal swabs were also taken on all heifers during the second trimester of gestation and again for those with confirmed pregnancies during the third gestational trimester.
Detailed health records were maintained for each heifer throughout the entirety of the trial to ensure the health status of each individual was maintained. Each female was vaccinated and treated for external and internal parasites according to the University of Arkansas Division of Agriculture’s Beef Research Unit cattle management protocol.

Upon completion of the trial, pregnant heifers were maintained as one group and open heifers were culled. The retained females grazed fescue-bermuda grass pastures and were supplemented with adequate free choice mineral supplements during gestation. Within 24 hours of birth, calf sex and birthweight were recorded.

**DNA Extraction and Pyrosequencing:**

Approximately 0.1 g of thawed feces was used for DNA extraction using the QIAamp PowerFecal DNA Kit (QIAGEN Inc., Germantown, MD, USA) according to the manufacturer’s protocol. DNA was extracted from the vaginal swabs using the QIAAmp BiOStic Bacteremia DNA Kit (QIAGEN Inc., Germantown, MD, USA) according to the manufacturer’s protocol. Nanodrop One C (Fisher Scientific, Hanover Park, IL, USA) was used to measure the DNA concentration.

**Library preparation:**

Ten ng aliquots of DNA were used from each sample to construct a library targeting the V4 region of the 16S rRNA gene. PCR was used to amplify each sample using dual index primers. Amplicons were normalized using a SequaPrep™ Normalization Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s protocol. At this point, each end of the sample’s sequence contains a unique barcode that allows for identification of each individual PCR amplicon when pooled together in a library. To generate the pooled library, 5 µl aliquots
from each normalized sample (vaginal, n=272; fecal, n=64) were combined. The exact size of the library product and the concentration were measured with a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) through quantitative PCR (Eppendorf, Westbury, NY, USA) assay and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA). The library was diluted based on the results from the qPCR and the bioanalyzer.

**Sequencing:**

The 20 nM pooled library, containing 336 individual samples, and a PhiX control v3 (20 nM) (Illumina) were mixed with 0.2 N NaOH and HT1 buffer (Illumina). PhiX control v3 (5%, v/v) (Illumina) was added to the mix and 600 µl were loaded into a MiSeq® v2 (500 cycle) reagent cartridge for sequencing. The sequencing procedure was monitored periodically throughout the assay using the Illumina BaseSpace® website.

**Sequence analysis:**

The demultiplexed R1 and R2 sequencing read files (approximately 250 base pairs in length) were downloaded to a local computer from the Illumina BaseSpace® website and the data was processed following the MiSeq SOP on the mothur wiki (version 1.39.1) (https://www.mothur.org/wiki/MiSeq_SOP) and Kozich et al. (2013). The Uchime algorithm was used to remove chimeric sequences (Edgar et al., 2011). To reduce sequencing noise, sequences were subjected to a preclustering methodology (Huse et al., 2010). Sequences were considered to be high quality if they were at least 200 base pairs in length and passed the error reducing, chimera detection and removal steps. The sequences were assigned to OTUs using a 97% cutoff. These OTUs were classified using the Bayesian method at the genus level (Cole et al., 2009). The number of reads
for fecal samples and vaginal swabs were subsampled to 4,900 and 900, respectively, to reduce sequencing bias before downstream analysis.

**Ecological and statistical analyses:**

For all analyses, significance was determined as $P < 0.05$. Shannon Diversity index (Shannon, 1949), and richness (number of observed OTUs) were calculated using mothur to evaluate alpha diversity. The Kruskal-Wallis test was performed to explore differences in alpha diversities (Shannon Diversity index and richness) between heifers who established a pregnancy and those that did not, and over time for fecal and vaginal samples. Beta diversity was evaluated using Bray Curtis (Bray and Curtis, 1957) and Jaccard (Chao et al., 2005) distances, calculated in mothur, to explore the dissimilarity between the communities’ structure and membership, respectively. Random Forest was used to rank microbial signatures that accurately differentiate between groups of females. This machine learning technique accounts for non linear relationships and dependencies among all microbial features. The relative abundance of the top 500 OTUs and alpha-diversity measures were included as inputs for the Random Forest model. Each input (feature) was given an importance score (MDA: mean decrease accuracy) based on the increase in error caused by removing that feature from the predictors. These features were ranked by the assigned importance scores and those with an MDA $> 3$ were considered highly predictive.
CHAPTER 3

Results

Sequencing depth and alpha diversities

A total of 336 samples were collected from commercial beef heifers prior to breeding and during each trimester of gestation. Vaginal (n=272) and fecal (n=64) were utilized for DNA extraction and bar-coded pyrosequencing of the V4 region of the 16S rRNA gene. After removing low quality reads and chimeras using mothur (versions 1.39.1), 4,925,330 and 2,037,467 high quality reads remained for vaginal and fecal samples respectively. Vaginal samples averaged 18,516 reads per sample ranging from 934 to 223,412. Fecal samples averaged 33,958 reads per sample ranging from 4,966 to 250,987. These sequences were assigned to 13,477 and 13,531 OTUs based on 97% similarity for vaginal and fecal samples, respectively. Sequence number was normalized to 900 for vaginal samples and 4,900 for fecal samples to standardize sampling for downstream alpha and beta diversity analyses.

Alpha (bacterial community) diversity was measured using Shannon index and the number of observed OTU’s. For vaginal samples, indices were significantly different based on time (Figure 1A, Kruskal-Wallis test, P = 0.0056). Animals with established pregnancies had increased indices from pre breeding to the second trimester (P = 0.021), then shows decreased indices from the second to the third trimester (P = 0.048, Table 1). Females that did not establish a pregnancy presented increased Shannon indices from pre breeding to the second sampling time (P = 0.0019). For vaginal samples, the number of observed OTUs, indicating community richness had significant differences based on time (Figure 1B, Kruskal-Wallis test, P = 0.0015). Open individuals showed an increase in the number of observed OTUs from pre breeding to the
second trimester ($P = 0.0014$) and from the first trimester to the second ($P = 0.033$, Table 1). The number of obsessed OTUs decreased in bred females from the second trimester to the third ($P = 0.0014$, Table 1). For fecal samples, there were no significant differences for Shannon indices by pregnancy status or over time (Figure 2A, Kruskal-Wallis test, $P = 0.95$) and no significant differences for total number of observed OTUs (Figure 2B, Kruskal-Wallis test, $P = 0.66$). $P$ values for pairwise comparison of fecal samples are presented in Table 2.

**Community membership and structure**

Beta-diversity analyses were used to examine both community membership and structure for both pregnant and non-pregnant females overtime. The Jaccard dissimilarity matrix was used to evaluate bacterial community membership. To visualize the Jaccard distances, principal coordinate analysis (PCoA) was applied to the dissimilarity matrix. Vaginal samples representative of all time points and each pregnancy status cluster together on principle coordinate axes 1 and 2 (PC1, PC2). The amount of variation explained by PC1 and PC2 are 7.72% and 2.06%, respectively (Figure 3A). No differences based on pregnancy status were seen (stage 1: $P = 0.207$; stage 2: $P = 0.657$; stage 3: $P = 0.827$), but differences in community membership changed based on time ($P \leq 0.05$). The Bray-Curtis index was used to estimate dissimilarities in both community membership and structure. PCoA was also applied to this dissimilarity matrix to visualize calculated distances. From the PCoA for both vaginal and fecal samples, there is no distinct clustering based on pregnancy status or time. For the vaginal samples, PC1 explains 12.25% of the variation and PC2 explains only 3.69% (Figure 3B). No differences based on pregnancy status were seen (stage 1: $P = 0.421$; stage 2: $P = 0.720$; stage 3: $P = 0.770$), but differences in community membership changed based on time ($P \leq 0.005$).
The Jaccard PCoA for fecal samples show similar findings to that of the vaginal samples. There is no distinct clustering and there is little variation among samples explained by the PCoA, were PC1 explains only 5.64% and PC2 explains only 4.22% of the variation among samples (Figure 3C). No differences based on pregnancy status were seen (stage 1: \( P = 0.577 \); stage 2: \( P = 0.915 \)), but differences in community membership changed based on time (\( P \leq 0.001 \)). Similarly, in the fecal PCoA, PC1 explains 12.63% of variation and PC2 explains 8.57% of variation (Figure 3D). No differences based on pregnancy status were seen (stage 1: \( P = 0.325 \); stage 2: \( P = 0.919 \)), but differences in community membership changed based on time (\( P \leq 0.001 \)).

**OTU distribution**

The top 50 bacterial OTUs at the genus level are represented similarly among pregnant and non-pregnant heifers as well as over time. The vaginal microbiome is dominated by *Escherichia/Shigella*, unclassified *Ruminococcaceae* and *Ureaplasma* (Figure 4). The fecal microbiome is dominated by OTUs associated with unclassified *Ruminococcaceae* and unclassified *Bacteroidales* (Figure 5). Interestingly, both vaginal and fecal samples show community dominance by OTUs associated with unclassified *Ruminococcaceae*. This could suggest part of the vaginal microflora is influenced by that of the gut microbiota due to anatomical location, or contamination during sampling. The similarity of OTU distribution based on sample type (vaginal or fecal) agree with the findings of the beta diversity analyses when both community membership and relative abundance are taken into account.

**Identifying predictive bacterial signatures**

Random Forest was used to identify bacterial signatures that can best differentiate between pregnancy status and time of sampling. Features that differentiate between pregnancy sta-
tus and also gestational time point include several OTUs for both vaginal and fecal samples. In the vaginal microbiota, *Histophilus, Paludibacter*, and unclassified *Ruminococcaceae* are good predictors of a females ability to establish a pregnancy at pre-breeding. The abundance and variation of *Histophilus* is less in females that become pregnant than those that do not. The opposite is true when using *Paludibacter* and unclassified *Ruminococcaceae* as predictors as their abundance and variation is greater in females that would establish a pregnancy (Figure 6).

During the first trimester, pregnancy status can be predicted by increased abundance of OTUs associated with *Flavonifractor*, unclassified *Clostridiales*, and unclassified *Lachnospiraceae* (Figure 6). Unclassified *Ruminococcaceae*, and unclassified *Firmicutes* can be used to predict pregnancy status during the second trimester of pregnancy. The abundance of these OTUs is greater in animals that are not gestating than those that are carrying a pregnancy (Figure 6). Stage of gestation can also be predicted in pregnant females using specific OTUs. From pre-breeding, *Corynebacterium, Mycoplasma* and unclassified *Lachnospiraceae* can be used to differentiate from the first trimester of gestation (Figure 6).

From pre-breeding to the second trimester, unclassified *Firmicutes, Leptotrichia* and unclassified *Bacteria* can be used as predictors (Figure 7). To differentiate from pre-breeding to the third trimester, the decrease in abundance in unclassified *Firmicutes, Leptotrichia* and *Clostridium sensu stricto* can be used (Figure 7). During the first trimester of pregnancy, increases in abundance of *Olsenella*, and unclassified *Bacteria*, as well as decreased abundance in *Corynebacterium* can predict changes to the second trimester. (Figure 7). Decreased abundance of unclassified *Lachnospiraceae*, unclassified *Firmicutes* and unclassified *Coriobacteriaceae* can differentiate from the first trimester to the third trimester of pregnancy (Figure 7). Predictors dif-
ferentiating from the second trimester to the third trimester, include: decreased abundance and
variation of unclassified *Lachnospiraceae*, and unclassified *Firmicutes* (Figure 7).

The Random Forest from fecal samples result in OTUs that can be used to predict the
ability of a female to establish a pregnancy before breeding. Unclassified *Actinobacteria*,
*Clostridium XIVa* and *Bacteroides* were all represented in a higher amount in those animals that
did not establish a pregnancy than those that did (Figure 8A). During the first trimester of gesta-
tion, *Alistipes*, *Mogibacterium* and *Blautia* were more abundant in animals that were not preg-
nant than those animals that were gestating (Figure 8A). For pregnant females, *Mogibacterium*,
unclassified *Coriobacteriaceae*, and *Olsenella* were predictive of stage of gestation. These OTUs
were more abundant at pre-breeding and decreased during the first trimester. Overtime, the
abundance of *Mogibacterium*, unclassified *Clostridiales*, unclassified *Coriobacteriaceae* and
*Olsenella* decreased in both bred and open females (Figure 8B). Unclassified *Ruminococcaceae*
is the only OTU predictor showing increased abundance from pre-breeding to the second sam-
pling time point in both pregnant and non-pregnant females (Figure 8B).
Discussion

Many studies evaluating the microbiota in cattle focus on communities that reside in the nasal and gut (rumen and fecal) niches to understand more about bovine respiratory disease (BRD), feed efficiency and health. These studies have added to the current knowledge of species that contribute to and inhibit the pathogenesis of BRD (Callan and Garry, 2002; Manunsell et al., 2011; Johnston et al., 2017) and those that work to turn feedstuffs into usable energy for the animal (Kim et al., 2011; Mao et al., 2015; Biddle et al., 2013). However, there is little known about the microbes that inhabit the reproductive tract and their functions related to a female’s ability to reproduce. The bovine urogenital tract houses a variety of microbes composed of aerobic, facultative-anaerobic and anaerobic microorganisms (Otero et al., 2000). There is much variation in this niche due to intrinsic and extrinsic factors, and little knowledge explaining the role microbes play in reproduction (Nadar-Macis and Otero, 2009). Therefore, the aim of this study is to characterize the vaginal and fecal microbiota of females that established a pregnancy and those that did not, and follow the communities overtime to explore how microbial populations impact fertility and change throughout gestation.

Differences in bacterial community evenness and richness were observed in vaginal samples comparing bred and open females and over time. Females that did not establish a pregnancy showed decreased richness and evenness at pre-breeding when compared to bred females during the first and second trimester. In humans, the opposite has been observed (Aagaard et al., 2012; Oakley et al., 2008). Of the 5 CST’s in humans, 4 are dominated by different species of Lactobacillus leaving the fifth CST to be dominated by a mixture of strict and facultative anaerobes (Ravel et al., 2001; Gajer et al., 2012). Bacterial vaginosis (BV), which negatively impacts
fertility, has a microbial composition similar to that of the fifth CST (Smith and Ravel, 2016). In a human study comparing the vaginal microbiota of subjects with and without clinically defined BV, those with BV presented an increase in taxonomic richness and diversity measured by the number observed OTU’s \( (P < 0.001) \) and the Shannon index being 1.4 to 4.1 times greater than those without BV (Oakley et al., 2008). The variation among the BV positive subjects and their differences compared to those without BV could be attributed to a high degree of variability within each group (Oakley et al., 2008). These opposing findings from cattle and humans could suggest the increased importance of \textit{Lactobacillus} species in humans and its lack of importance in cattle. The role \textit{Lactobacillus} species play in the bovine vaginal ecosystem is yet to be determined, but it is possible that other species dominating the vaginal niche have similar and possibly improved function. Interestingly, no significant differences in community evenness or richness were observed in fecal samples based on pregnancy status or time.

Overtime, the richness and evenness of vaginal bacterial communities in open individuals increased, while the opposite is true for bred individuals transitioning from the second to third gestational trimester. Similarly, human researchers have reported the development of a more stable vaginal microbiota near the end of the gestation period. Aagaard (2012) reports decreased species richness and diversity that progresses with gestational age. A target set of \textit{Lactobacillus} related OTUs are enriched in women with increased gestational age explaining changes in community membership and structure in late gestating humans (Aagaard et al., 2012). Unlike humans, no change in community membership or structure was observed in the vaginal niche or the feces of female bovine throughout gestation. Also, no changes or clusters were observed to differentiate pregnant from non-pregnant females. With little variation in membership and structure
accounted for based on pregnancy status or time, speculations can be made that microbial communities are effected to a greater extent by other factors.

The individual genera that interact to form a community, play specific roles in maintaining the integrity of the microbial ecosystem. In this study, genera dominating the vaginal microbiota are *Escherichia/Shigella*, *Ruminococcaceae* and *Ureaplasma*. *Escherichia* has been documented as a contributing pathogen to metritis (uterine inflammation) due to its ability to establish residency in the reproductive tract from contamination by feces, ascend up the reproductive axis and maintain a presence in a contaminated uterus (Sheldon et al., 2002; Williams et al., 2005). In dairy cows, metritis is considered to be one of the most costly factors reproductive inefficiency due to increased days open, failure to conceive on the first service, increased number of inseminations, and failure to establish a pregnancy, establishing a link between *Escherichia* and reproductive failure (Gilbert et al., 2005).

*Ruminococcaceae* is a common isolate from the mammalian gut environment (Mao et al., 2015; Malmuthuge et al., 2012). The function of this genera of bacteria involves degradation of starch and fiber and has increased abundance in the large intestine compared to preceding gut organs (Kim et al., 2011; Mao et al., 2015). Members of this genus are not commonly isolated from the mucosa of gut organs, rather the digesta itself, emphasizing its role in feedstuff digestion rather than working with the host mucosa and revealing its presence in this study is likely due to contamination during sampling (Mao et al., 2015).

*Ureaplasma* is a common isolate from cervicovaginal mucosal samples from beef females with healthy reproductive tracts (Mulira et al., 1992). However, *Ureaplasma* has been associated with cows suffering from granular vulvitis syndrome and mastitis, ciliostasis in cultured
oviductal tissues and humans experiencing reproductive failure and infertility (Ruhnke et al., 1978; Stipkovits et al., 1983; Kreplin et al., 1987). A previous study claims that *U. diversum* in combination with *Pasteurella* and/or *Manheimia* species causes lung lesions in calves resulting in pneumonia and consequent reoccurring morbidity (Thomas et al., 2002). This study agrees with the commonality of *Ureaplasma* isolation in vaginal samples, and since it’s presence is similar among bred and open females, could explain a requirement for interaction with other pathogens to cause disease.

In a study by Rodrigues et al., (2015), the bacterial communities dominating the vaginal niche of healthy females consisted of *Bacteroides, Enterobacteriaceae, Victicallis, Streptococcus, Selenomonadales, Treponema, Porphyromonadaceae, Alistipes, Coriobacteriaceae, Clostridium, Betaproteobacteria, Corynebacterium, Cytophagaceae, Oscillibacter, and Planctomycetaceae*. Of the 15 mentioned OTUs, none were considered to dominate the vaginal flora in this study. However, 5 were considered to be good predictors of gestational time point in bred individuals from vaginal samples and in fecal samples could be used to predict pregnancy status at pre-breeding and during the first trimester.

*Bacteroides, Clostridium* and *Alistipes* species can be used to predict the ability to establish a pregnancy in fecal samples. *Bacteroides* species are also associated with a negative health status in cattle. In calves with fatal cases of BRD, *Bacteroides* is found to be one of the most abundant bacteria associated with post-mortem lung samples (Johnston et al., 2017). These species have also been associated with chronic, abscessing lung lesions in BRD cattle suggesting its prevalence in contribution to respiratory disease (Callan and Garry, 2002). This genus of bacterium is also a common inhabitant of the colonic mucosa and has been associated with diarrheal
illness in humans a livestock (Myers et al., 1984, 1987). The pathogenesis of enterotoxigenic \textit{B. fragilis} is due to a metalloprotease dependent toxin (Rhee et al., 2009). This toxin binds to colonic epithelial receptors and activates NF-κB pathways that result in increased cell proliferation, DNA damage and the release of pro inflammatory mediators (Sears, 2009; Wu et al., 2004, 2006). Since altered immune function is accepted as a primary mechanism for female infertility, the ability of a species of bacterium to influence the hosts immune function could explain its role in predicting a female’s reproductive potential (Fair, 2015).

The \textit{Clostridium} genus is a major component of the fecal microbiota in mammalian species (Atarashi et al., 2011). \textit{Clostridium} species that characterize in cluster I, other than \textit{Clostridium sensu stricto}, are involved in suppressing immune disorders (Ling et al., 2014). In fecal samples, the increase in \textit{Clostridium XIVa} at pre-breeding in animals that do not establish a pregnancy could be due to other microorganisms acting as inducers of regulatory T cells or anti-inflammatory commensal bacteria causing dysbiosis in the vaginal ecosystem (Ling et al., 2014).

\textit{Alistipes} is a constituent of the fecal microbiota in cattle (Girija et al., 2012). It is a member of the Bacteroidetes phylum and plays a role in the degradation of complex glycans (Girija et al., 2012). This bacterium is a known commensal of the gut microbiota and is often used as a probiotic due to it’s ability to produce butyrate as part of the lysine pathway (Li et al., 2016). In humans, decreased amounts of butyrate producing bacterium have been linked to increased numbers of established enteric pathogens (Rivera-Chávez et al., 2016). Interestingly, in this study, increased numbers of \textit{Alistipes} are seen in open individuals.

\textit{Coriobacteriaceae}, \textit{Clostridium}, and \textit{Corynebacterium} can be used a predictors in vaginal samples of pregnant animals to determine gestational age. \textit{Coriobacteriaceae} has been isolat-
ed from the vagina of cattle with and without reproductive disorder, but it’s function is more accurately described in its symbiotic relationship with the gut of insects (Rodrigues et al., 2015; Haas and Koing, 1988). This gram-positive, obligate anaerobe works to ferment glucose, and other compounds found in the foodstuffs of insects to produce lactic acid, ethanol, CO₂ and H₂ (Haas and Koing, 1988). During the first trimester of gestation, the relative abundance of *Coriobacteriaceae* is greater than that of the third trimester. This decrease in abundance suggests a decrease in overall lactic acid production and disagrees with the current knowledge in humans (Aagaard et al., 2012).

Particularly in human infants, *Clostridium senso stricto* is strongly associated with food allergies (Ling et al., 2014). In a study exploring the fecal microbiota in IgE-mediated food allergies, increased relative abundance of *Clostridium senso stricto* was associated with decreased relative abundance of *Bacteroides*, disturbing the homeostatic environment surrounding the infant immune system (Ling et al., 2014). The relative abundance of these two genera do not appear to have association in vaginal samples represented in this study, but the ability of *Clostridium senso stricto* to alter an ecosystem could explain its ability to predict gestational age.

*Corynebacterium* is a pathogenic bacteria, residing in the vagina of females and the prepuce of males, that ascend from the bladder into the kidneys causing pyelonephritis (Constable, 2018). Most often, the microorganisms gain entry and colonize the mucosal lining of the bladder after parturition (Constable, 2018). The role *Corynebacterium* plays in disease pathogenesis after parturition is interesting to consider based on the results from this study. In pregnant females, decreased abundance of *Corynebacterium* is useful in predicting gestational age from the first to
the second to the third trimester suggesting less *Corynebacterium* would be present at the time of parturition.

The study by Rodrigues and colleagues also described the vaginal microflora of female bovine with reproductive disorder. They found *Bacteroides, Enterobacteriaceae*, and *Histophilus* to be the top three dominant OTUs in unhealthy animals. Based on Random Forest predictors from this study, *Histophilus* can be used to predict pregnancy status in vaginal samples before breeding. *Histophilus* species are gram-negative, nonsporeforming bacterium that can exist in both pathogenic and non-pathogenic forms (Janzen, 2018). Both forms of *H. somni* are isolated from the bovine mucous membranes of nasal passages, the prepuce and sheath of males and in the vagina of females (Janzen, 2018). Reproductive disease manifestation, most likely due to venereal spread, results in abortion, mastitis, and granular vulvovaginitis (Janzen, 2018). The increased abundance of *Histophilus* and *Bacteroides* in females that do not establish a pregnancy in vaginal and fecal samples, respectively, agree with the presence of these two OTUs in animals with reproductive disorder and could be used as markers for reproductive disorder.

Based on finding from a Japanese plant study, *Plaudibacter*, is an anaerobic bacterium that produces propionate, acetate and succinate from the fermentation of various sugars (Ueki et al., 2006). The presence of this bacterium in vaginal swabs could be attributed to its ability to use glycogen to synthesize its fermentation products (Ueki et al., 2006). Increased abundance of glycogen in the vagina is correlated with a healthy vaginal status and increased ovarian activity which can be explained by *Plaudibacter*’s increased abundance in females that establish a pregnancy (Cruickshank and Sharman, 1934).
Pregnancy status at pre-breeding and during the first trimester can be predicted by an abundance in unclassified *Ruminococcaceae*, in both vaginal and fecal samples, and *Lachnospiraceae*, in vaginal samples, respectively. Both of these families have genera that are common isolates of healthy mammalian gut microbial populations and are known to share a common role as plant degraders (Biddle et al., 2013). This explains dominance of unclassified *Ruminococcaceae* in fecal samples. These bacteria work to decompose plant substrates that are indigestible to the host and produce acetate, butyrate and propionate through fermentation (Biddle et al., 2013). These bacteria harbor the same function as *Alistipes*, yet are seen in the vaginal microbiota and are increased in bred individuals, whereas the opposite is true for *Alistipes* in fecal samples. This finding makes an interesting connection between the vaginal and fecal microbiota’s and the ability of their composition to predict pregnancy status.

Similar to *Lachnospiraceae*, increased abundance of *Flavonifractor*, in vaginal samples, and unclassified Clostridales, in vaginal and fecal samples, can be predictive of pregnancy status during the first trimester. *Flavonifractor* is an anaerobic bacilli that uses glucose, fructose, and ribose to produce acetic and butyric acids (Carlier et al., 2010). *Clostridales* are a hallmark bacterium of a healthy gut microbiota in humans and cattle and include the family of *Lachnospiraceae* (Jewell et al., 2015; Biddle et al., 2013). The presence of these bacteria, along with those that share the same function, are likely due to contamination from feces during sampling but are good predictors of pregnancy status in vaginal samples, drawing a link between the vaginal and fecal microbial populations.

*Firmicutes* dominate the healthy gut microbiota in humans and have been used in combination with other bacteria as probiotics for the elderly due to its ability to establish and maintain
a healthy gut flora (Marx, 2015). The Firmicute phylum includes the family Lactobacillaceae, which includes the genus *Lactobacillus* (Marx, 2015). The extensive research surrounding the importance of *Lactobacillus* species in a healthy human vagina can help explain the presence of *Firmicutes* in the bovine vagina, yet the increased abundance of *Firmicutes* in open individuals disagrees with the development of a gestational microbiome dominated by lactic acid producing species (Aagaard et al., 2012).

*Mycoplasma* is a pathogenic bacteria most commonly causing respiratory disease in stressed and feedlot calves (Maunsell et al., 2011). In humans, *Mycoplasmas* are common inhabitants of the respiratory and urogenital tracts (Razin, 1996). *Mycoplasma hominis* has been isolated from the vagina of healthy women, but has been shown to ascend up the reproductive tract where it can colonize as a pathogen and cause symptoms similar to those associated with pelvic inflammatory disease (Razin, 1996). In this study, the relative abundance of *Mycoplasma* increases in open individuals and from pre-breeding to the first trimester of pregnancy. The ability of *Mycoplasma* species to cause pathogenesis into the upper reproductive tract explains its prevalence in open females, yet its increased abundance during gestation remains unexplained.

*Olsenella*, in vaginal and fecal samples, and *Leptotrichia*, in vaginal samples, can be used to predict gestational period from pre-breeding to first trimester or to second trimester, respectively. *Olsenella* is commonly isolated from the rumen of sheep and the jejunum of pigs and has function related to fermenting feedstuffs to produce lactic acid (Kraatz et al., 2011). It’s role in lactic acid production could explain it’s presence in the vaginal mucosa. In this study, *Olsenella* can be used to predict gestational age from pre-breeding to the first gestational trimester. The decrease in relative abundance with the establishment of pregnancy could aid in explaining how the
vaginal ecosystem changes with breeding, the establishment of pregnancy and the progression of pregnancy. *Leptotrichia*, in cattle, is associated with calves suffering from BRD (Johnston et al., 2017). Little is known about the *Leptotrichia* genus but its prevalence as an opportunistic pathogen in the respiratory system is documented in both humans and bovine (Johnston et al., 2017; Eribe and Olsen, 2008; Outurier et al., 2012). In samples from lungs of cattle with BRD, the presence of *Leptotrichia* always occurred with either *Pasteurellaceae, Mycoplasma*, or *Fusobacterium* (Johnston et al., 2017). The relationship between the present of *Mycoplasma* and *Leptotrichia* is interesting based on findings in this study because both genera are predictors of gestational age.

Similar to *Clostridium XIVa* and *Bacteroides, Actinobacteria* is predictive of pregnancy with increased abundance in open animals in fecal samples. The finding supports what is seen in humans with patients suffering from BV presenting 4.5 times higher related abundance values for Actinobacteria than healthy patients (Oakley et al., 2008). These findings could suggest that *Actinobacteria* are characteristic of an unhealthy vaginal ecosystem and are detrimental to reproductive success.

During the first trimester of pregnancy, *Mogibacterium* and *Blautia*, along with *Alistipes*, were predictive of pregnancy with higher relative abundance in open individuals. *Mogibacterium* is an anaerobic, gram-positive bacterium commonly isolated from the oral niche in humans (Nakazawa et al., 2000). Species in this genus are not involved in fermentation and produce phenyl acetate as a product (Nakazawa et al., 2000). Based on the function of the bacteria, contamination from feces is not likely to have occurred in this study. *Mogibacterium* has been isolated from the human vagina with higher relative abundance in patients experiencing BV than
those with healthy vaginal ecosystems (Salas and Chang, 2014). The findings in this study support the prediction that increased abundances of *Mogibacterium* in the feces reflect non-pregnant individuals. *Blautia* and *Bacteriodales* are common isolates of both human and bovine gut microbiota (Koskey et al., 2014). They have been used as predictors to distinguish water contamination in Brazil where agriculture and sewage run-off negatively impacts the water systems (Koskey et al., 2014). *Blautia* inhabits the mucosal surfaces of internal organs (Murphy and Frick, 2012). Previous studies have associated *Blautia* with antibiotic resistance and virulence factors that are associated with clinical infections in humans (Murphy and Frick, 2012). The decrease in abundance of *Blautia* in pregnancy females compared to open females during the first trimester suggest this genus could negatively impact the health of the vaginal ecosystem.

Koskey and colleagues reported the presence of both genera in cattle and human samples, but specifically increased relative abundance of *Blautia* in human feces (Koskey et al., 2014). These findings support the result from our study of *Bacteriodales* being a dominant order in the fecal microflora. *Bacteriodales* work in the bovine gut to degrade plant components such as hemicellulose and pectin (Ormerod et al., 2016).

**Conclusion**

In conclusion, the ability to use the vaginal microbiome in beef heifers to predict reproductive potential and gestational period is confirmed. The differences in alpha-diversities among females that were able to establish a pregnancy and those that were not, as well as gestational period can be used in selection strategies when selecting replacement heifers and used to determine pregnancy status. Using Random Forest to identify specific bacterial strains that can predict pregnancy status or gestation time period, for both vaginal and fecal niches, allows insight into
the roles and functions particular microbes play in the vaginal ecosystem and with reproduction. Findings from this study advance the knowledge of the microbial communities residing in the vagina of beef heifers before breeding and throughout pregnancy and highlight specific genera of bacteria that could be used to determine reproductive potential, unexplained infertility, and estimate time of gestation.
Table 1. *P* values related to alpha diversity measures in vaginal samples.\(^1\)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Change in Diversity</th>
<th><em>P</em> value</th>
<th>Comparison</th>
<th>Change in Diversity</th>
<th><em>P</em> value</th>
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<td>0.11</td>
<td>1 Open</td>
<td>2 Open</td>
<td>0.11</td>
</tr>
<tr>
<td>1 Open</td>
<td>3 Open Increase</td>
<td>0.002*</td>
<td>1 Open</td>
<td>3 Open Increase</td>
<td>0.001*</td>
</tr>
<tr>
<td>2 Bred</td>
<td>3 Bred</td>
<td>0.058</td>
<td>2 Bred</td>
<td>3 Bred Increase</td>
<td>0.015*</td>
</tr>
<tr>
<td>2 Bred</td>
<td>4 Bred</td>
<td>0.79</td>
<td>2 Bred</td>
<td>4 Bred</td>
<td>0.31</td>
</tr>
<tr>
<td>2 Open</td>
<td>3 Open Increase</td>
<td>0.04*</td>
<td>2 Open</td>
<td>3 Open Increase</td>
<td>0.033*</td>
</tr>
<tr>
<td>3 Bred</td>
<td>4 Bred Decrease</td>
<td>0.048*</td>
<td>3 Bred</td>
<td>4 Bred Decrease</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

\(^1\) Vaginal samples were obtained from 72 beef heifers. Individuals that established a pregnancy (n=56) were samples before breeding (stage 1) and at 3 time points during gestation (stages 2, 3, and 4). Individuals that failed to establish a pregnancy (n=16) were sampled before breeding (stage 1) and during the first and second trimesters of gestation (stages 2 and 3).

* Pair-wise comparisons between stage and pregnancy status were determined to be statistically significant at *P* < 0.05.
Table 2. $P$ values related to alpha diversity measures in fecal samples.\textsuperscript{1}

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$P$ value</th>
<th>Comparison</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shannon Index</strong></td>
<td></td>
<td><strong>Observed OTUs</strong></td>
<td></td>
</tr>
<tr>
<td>1 Bred 1 Open</td>
<td>0.97</td>
<td>1 Bred 1 Open</td>
<td>0.71</td>
</tr>
<tr>
<td>2 Bred 2 Open</td>
<td>0.77</td>
<td>2 Bred 2 Open</td>
<td>0.32</td>
</tr>
<tr>
<td>1 Bred 2 Bred</td>
<td>0.59</td>
<td>1 Bred 2 Bred</td>
<td>0.89</td>
</tr>
<tr>
<td>1 Open 2 Open</td>
<td>0.95</td>
<td>1 Open 2 Open</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Fecal samples were obtained from 32 beef heifers. Individuals that established a pregnancy (n=16) and those that did not (n=16) were sampled before breeding (stage 1) and during the first trimester (stage 2).

* Pair-wise comparisons between stage and pregnancy status were determined to be statistically significant at $P < 0.05$. 
Figure 1. Vaginal community alpha diversity comparisons between bred and open females by stage. Diversity was measured by Shannon index (A) and observed OTUs (richness) (B). The top and bottom boundaries of individual boxes represent the 75th and 25th quartile values, respectively. The median value are represented by the horizontal black lines within each box. Lines moving from box to box connect sample values for individual animals. Red and black lines correspond to bred and open females, respectively. Stages 1 through 4 represent pre-breeding and trimesters 1, 2 and 3 of gestation.
Figure 2. Fecal community alpha diversity comparisons between bred and open females by stage. Diversity was measured by Shannon index (A) and observed OTUs (richness) (B). The top and bottom boundaries of individual boxes represent the 75th and 25th quartile values, respectively. The median value are represented by the horizontal black lines within each box. Lines moving from box to box connect sample values for individual animals. Red and black lines correspond to bred and open females, respectively.
Figure 3. PCoA of community membership and structure. Community membership and structure distances were measured using Jaccard dissimilarity matrices for vaginal (A) and fecal (C) samples. The calculated Bray-Curtis dissimilarity matrices were used to visualize community membership in vaginal (B) and fecal (D) samples. Triangles and squares represent bred and open females, respectively. Stages are indicated by color: gold, blue, green and black represent pre breeding, and gestational trimesters 1 through 3 respectively.
Figure 4. Relative abundance of OTUs at the genus level in the vaginal microbiota of beef heifers. Each chart represents a stage, indicated on the right. Each bar represents a sample. Relative abundance is given on the y-axis.
Figure 5. Relative abundance of OTUs at the genus level in the fecal microbiota of beef heifers. Each chart represents a stage. Each bar represents a sample. Relative abundance is given on the y-axis.
Figure 6. OTUs predictive of pregnancy status from vaginal microbiota. Each feature (OTU) was ranked based on importance in predicting pregnancy status or time period and assigned a mean decrease accuracy. The top 3 features from each prediction are represented by the relative abundance of the OTU. Bred individuals are represented by the light green boxes, while open individuals are represented by the dark green boxes. (u) denotes an unclassified genus.
Figure 7. OTUs predictive of gestation from vaginal microbiota. Each feature (OTU) was ranked based on importance in predicting pregnancy status or time period and assigned a mean decrease accuracy. The top 3 features from each prediction are represented by the relative abundance of the OTU. Bred individuals are represented by the light green boxes, while open individuals are represented by the dark green boxes. (u) denotes an unclassified genus.
Figure 8. OTUs predictive of pregnancy status (A) and gestation (B) from fecal microbiota. Each feature (OTU) was ranked based on importance in predicting pregnancy status or time period and assigned a mean decrease accuracy. The top 3 features from each prediction are represented by the relative abundance of the OTU. Bred individuals are represented by the light green boxes, while open individuals are represented by the dark green boxes. (u) denotes an unclassified genus.
References


Campero, C.M., Conosciuto, G., Odriozola, E. 1992 Hallazgos clínicos, bacteriológicos e histopatológicos en vacas lecheras, asociados con problemas reproductivos. Revista de Medicina Veterinaria 73.


67


70


van der Burgt, G., Clark, W., Knight, R. 2007 Cattle fertility problems and Histophilus somni Vet Rec 160, 600.


MEMORANDUM

TO:                   Jiangchao Zhao
FROM:     Craig N. Coon, Chairman
DATE:   9/22/15
SUBJECT: IACUC Approval
Expiration Date:   Sep 21, 2018

The IACUC has approved your protocol #16024 "Characterization of the bovine respiratory and gastrointestinal tract microbiome". You may begin work immediately.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond the indicated expiration date, you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem
cc: Animal Welfare Veterinarian