

**Bacterial Communities during Heifer Development and their Impact on
the Uterine Environment**

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Doctor of Philosophy
Degree**

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DEDICATION

In loving memory of Makayla.

ABSTRACT

Uterine and ruminal bacterial communities are major contributors to reproductive success and feed efficiency by their impact on their respective environments. The first objective is to evaluate the effect of protein supplementation on uterine and ruminal bacterial communities during heifer development. Pre-pubertal Angus heifers were assigned to a level of crude protein supplementation: 10% control, 20%, or 40%. Every 56 days, uterine flushes and rumen content were collected for bacterial DNA extraction and amplification of the 16S gene. Bacterial analyses were performed in R 4.1 and statistical analyses in SAS 9.4. Observed amplicon sequence variants increased overtime and by pubertal status ($P < 0.05$), each indicating significant clustering by principal coordinate analyses ($P < 0.001$) in the uterus. Numerous bacterial taxa abundances differed by protein treatment, pubertal status, and day in the uterus and rumen ($P < 0.05$). The second objective was to evaluate the impact of lipopolysaccharide (LPS) on the bovine uterine endometrium during the follicular and luteal phases. Explants were collected from the endometrium of the contralateral and ipsilateral horns of uteri in mid-luteal or follicular phases. Explants were cultured at standard conditions for 8 hours in 1 mL complete media with or without LPS (1 $\mu\text{g/mL}$). Extraction of RNA from explants was reverse transcribed to cDNA for targeted RNA sequencing. Normalized total reads counts for gene expression were analyzed in Metaboanalyst 5.0 and SAS 9.4. Seven genes increased in LPS treatment compared to other groups ($P < 0.05$). Multiple genes were affected by estrous cycle phase and uterine horn, independent of treatment ($P < 0.05$). Clustering analysis indicated overall gene expression differences with similar clustering between luteal and follicular phase

explants of controls, but distinct separate clustering between phases with LPS treatment ($P = 0.001$). Mucins contributed the most to differences observed between phases by treatment. Therefore, estrous cycle phase resulted in differing overall endometrial gene expression profiles of immune response to LPS treatment. In conclusion, uterine bacterial communities develop through puberty and may be affected by nutrition. Differing immune responses of the endometrium to bacteria between estrous cycle phases may influence uterine environment and pregnancy success.

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CHAPTER I: LITERATURE REVIEW

Introduction

The world population is expected to increase to 10 billion people by the year 2050, challenging the agricultural industry to increase sustainable food production to provide for the increased demand (United Nations, 2017). However, the land and resources required to feed the growing population are limited (Davis et al., 2016). Therefore, production efficiency must improve for current agriculture practices and food production systems. Animal agriculture is a major contributor towards providing high quality amino acid, vitamin, and mineral quantities for the increased demand of the growing population. By improving the efficiency and sustainability of livestock production, researchers and producers will be able to provide more sustainable food production while maintaining profitability and preventing overconsumption of resources.

Goals have been set to achieve optimal beef production and increase efficiency. Efficient cattle growth is considered as gaining an average of two pounds per day, with optimal reproductive efficiency defined as a cow producing a calf every 365 days (Diskin et al., 2016). Ideally, the animal must achieve these goals with as few inputs as possible. However, these goals are often not met, with reproductive losses and reduced gains costing billions of dollars each year (Bellows et al., 2002). Replacement heifers provide a major opportunity to improve reproductive efficiency of cow-calf systems. By optimizing nutrition for early puberty attainment, replacement heifers can greatly contribute to improving overall production (Patterson et al., 1992).

Currently, researchers are evaluating factors that influence the efficiency of beef cattle production and developing methods to optimize animal agricultural practices. These factors include nutrition, host genetics, and the resident microbial communities in the body. Identification

of microbial communities throughout the body in multiple organ systems has revolutionized the understanding of the host-microbe relationship in humans and animals. The rumen and its microbiome have been well established as critical for converting indigestible feedstuffs into usable energy for the ruminant (Bergman, 1990). Previously, the reproductive tract was thought to be sterile with the presence of microbes indicating potential infection and ultimately resulting in negative impacts on reproductive success (Perez-Muñoz et al., 2017). However, the development of sequencing technologies has improved the ability to identify microbial-derived sequences, allowing for detection of the reproductive tract microbiome. Following the first characterization of the ruminant reproductive tract microbiome by Swartz et al. (2014), further studies have identified the unique microbiomes between the uterus and vagina in cattle during different stages of reproduction and life stages, and determined the importance of the reproductive tract microbiome towards reproductive success (Laguardia-Nascimento et al., 2015, Clemmons et al., 2017, Ault et al., 2019a,b).

The current review discusses the goals of heifer development, the importance of nutrition to achieve developmental milestones, and the role of bacterial communities in the rumen and uterus. Together, these serve to ultimately influence the uterine environment and optimize reproductive efficiency.

Heifer Development

The incorporation of proper heifer development practices for replacement heifers is essential for cow-calf operations. As the next generation of breeding animals for the producer's operation, heifers provide a major opportunity to improve the herd's reproductive efficiency. For heifers to reach maximal reproductive efficiency, various developmental goals have been

determined for heifers to successfully calve by two years of age (Patterson et al., 1992). Heifers that calve by two years of age are more likely to continue to breed and calve early (Perry and Cushman, 2013). Heifers that breed early tend to remain in the herd longer and increase herd productivity (Cushman et al., 2013). Together, attaining these goals has been shown to ultimately increase the reproductive efficiency and subsequent lifetime productivity of the animal with more and heavier calves weaned (Patterson et al., 1992). For these goals to be reached, two major milestones often targeted are early attainment of puberty and reaching a percentage of mature body weight by the breeding season.

Attainment of Puberty

The attainment of puberty is a complex physiological event, largely dependent on activity of the endocrine system. In the pre-pubertal heifer, the surge center of the hypothalamus is not yet active due to the strong sensitivity of low estradiol concentrations causing a negative feedback (Schillo et al., 1982). Therefore, there is no surge of gonadotropin releasing hormone (GnRH) and subsequent luteinizing hormone (LH) surge to induce ovulation. Approximately 50 days prior to the onset of puberty, the strong sensitivity to the estradiol negative feedback begins to be reduced (Day et al., 1987). It was first suggested in humans that a certain threshold of body condition must be met to stimulate the transition from negative to positive feedback of estradiol (Frisch, 1985). Although the exact signaling for this change is not fully understood, it is believed the kisspeptin neurons may play a role (Cardoso et al., 2020). Kisspeptin neurons synapse in the hypothalamus directly on the GnRH neurons of the surge center (Hassaneen et al., 2016). These kisspeptin neurons contain receptors for various metabolic signals such as leptin, IGF-1, insulin, and glucose (Castellano et al., 2010; Wahab et al., 2018). By activation of these receptors, kisspeptin neurons

may become active and indicate the growing heifer has reached a threshold of sufficient nutritional status and body condition to support reproductive function (D'Occhio et al., 2019). At the onset of puberty, the sensitivity to the negative feedback of estradiol has decreased, allowing for activity of the surge center (Day et al., 1987; Cardoso et al., 2020). Increased GnRH, LH, and follicle stimulating hormone (FSH) secretions stimulate the final follicular development and maturation of the dominant follicle. Eventually, the surge center activity will be great enough to initiate the first ovulation.

The first ovulation resulting in the presence of a corpus luteum is often believed to be the onset of puberty; however, this ovulation typically goes unnoticed as behavioral signs of standing heat may be minimal (Morrow, 1969). Similar to the first estrous cycle of postpartum cows after an anestrus period, the first estrous cycle of puberty is often considered a “short cycle” in which estrus expression is low and luteolysis occurs prematurely, preventing maintenance of a pregnancy (Perry et al., 1991; Rantala and Taponen, 2015). Stimulation of pubertal onset can be performed with the use of progestins by intravaginal releasing devices during estrous synchronization or consumption of melengestrol acetate in advance of the breeding season (Imwalle et al., 1998; Rodrigues et al., 2013). With removal of the progestin source, the inhibitory effect of estrogen on the surge center is decreased and the hypothalamus is sensitized to the positive feedback of estrogen, leading to an increase in LH secretion for the surge to stimulate ovulation (Anderson et al., 1996). However, using artificial stimulation of pubertal onset is not effective if body weight and body condition of the heifer is not sufficient and the reproductive tract has not matured. The use of these methods to jumpstart the estrous cycle ensures heifers are pubertal by breeding, reducing the likelihood of a short cycle and increasing fertility (Lamb et al., 2010). Heifers that

reach puberty early and have multiple estrous cycles prior to the breeding season have greater pregnancy rates than those bred at the first pubertal estrus (Byerley et al., 1987; Roberts et al., 2018). Additionally, heifers reaching puberty early should achieve a successful pregnancy with fewer inseminations, allowing them to calve by two years of age (Larson, 2007; Kuehn et al., 2011; Perry, 2016). Heifers that successfully breed early and maintain their pregnancy will calve early in the calving season, resulting in heavier calves at weaning and a longer postpartum period for the first calf heifer to resume estrous activity before the next breeding season (Patterson et al., 1992).

Nutrition during Development

The nutrition provided to heifers following weaning often determines their age at puberty, productivity as a breeding female, and longevity in the herd. Reproductive processes will suffer if nutrient requirements for basal metabolism, normal activity, and growth are not first met (Short and Adams, 1988). As discussed previously, metabolic signaling to the hypothalamus via kisspeptin neurons is an indicator of heifer body condition and crucial for the onset of puberty and hypothalamic activity (D'Occhio et al., 2019; Cardoso et al., 2020). Heifers attain puberty while still growing into a mature animal, therefore, the nutrients provided during this time period are critical for supporting proper growth and puberty in a timely manner.

Target Body Weight

Target body weights are often the first and major goal of heifer development to reach a body condition score (BCS) of approximately 5-6 (Utter et al., 1993). By reaching a moderate BCS of 5-6, heifers will be more likely to have adequate body fat reserves, without excess adipose, to

support reproductive function and the attainment of puberty (Wagner et al., 1988). A BCS of less than 5 had been associated with poor reproductive performance (Selk et al., 1987). Potential replacement heifers should be selected at weaning that are more likely to reach their targeted weight and attain puberty early; these selection criteria may include factors such as BCS, body weight, and born early in the calving season. These heifers should then be developed to weigh approximately 60% of their mature body weight by the breeding season, but the ideal percent of mature body weight has been debated. Typically, it is recommended heifers reach a minimum of 60-65% of their mature body weight. However, studies have indicated reproductive success in heifers reaching as low as 50-55% of their mature weight compared to 60-65% by the first breeding season (Funston and Deutscher, 2004; Martin et al., 2008, Funston et al., 2012). In a study comparing development to 55% vs 60% mature body weight, heifers developed to a lighter weight had reduced second calf calving difficulties and greater second calf performance (Funston and Deutscher, 2004). Additionally, the major benefit to a lighter development strategy is the reduction in cost of development per heifer while achieving similar reproductive success parameters (Martin et al., 2008, Funston et al., 2012). However, lighter developed heifers are less likely to have reached puberty by the beginning of the breeding season and require additional steps during estrous synchronization to stimulate the hypothalamic-pituitary-gonadal axis or require an extended breeding season (Martin et al., 2008). If a heifer conceives at a lighter weight, the heifer may also be lighter at calving. Due to the high nutrient demand also required by the growing fetus, especially during the final trimester, nutrition must be adjusted to support the heifer and fetal growth to maintain a positive plane of nutrition. Ideally, heifers should weigh approximately 80-85% of their mature weight by calving to minimize calving difficulties and support lactation and early

conception in the next breeding season (Engelken, 2008). Heifers that calved at a BCS of 6 had higher rebreeding pregnancy rates than heifers with a BCS of 5 or 4 (Spitzer et al., 1995). Therefore, a low BCS at calving may extend the postpartum period and result in difficulty to conceive the following breeding season (Lalman et al., 1997).

Although there are advantages and disadvantages to the different target body weight developmental strategies, these considerations must be weighed to determine the best choice for the operation and its herd. The ideal targeted percent of mature body weight largely depends on the breed or type of cattle (Larson, 2007; Perry, 2016). For example, *Bos indicus* breeds of cattle are larger framed and later maturing than most *Bos Taurus* breeds, therefore requiring a greater percent of their final body weight to attain puberty. Additionally, cost to the producer is another consideration when choosing whether to achieve the minimal target body weight or higher percent of mature weight (Funston et al., 2012). The cost of heifer development greatly increases with the producer decision to keep the heifer at weaning as a potential replacement (Hindman and Engleken, 2021). The majority of the incurred costs during development are related to feeding (Hindman and Engleken, 2021). The increased costs of producing heifers to a greater percent of mature body weight should be considered against the potential return to maximize heifer efficiency and operation profits.

Protein Supplementation

Breeding seasons are often implemented in cow-calf operations to confine the calving seasons. However, the use of breeding seasons may result in heifers being developed during periods of lower forage quality. Protein is one of the most crucial nutrients for development and reproduction, but is often the first limiting nutrient in dormant forages (Marston et al., 1995).

Protein resulting from the breakdown of ruminal microorganisms provides the majority of protein and amino acids (AA) to the ruminant; however, bacterial derived proteins may not be adequate to meet all individual AA requirements, especially for reproductive functions (Storm and Orskov, 1984). Therefore, supplemental feeds high in crude protein are often provided to heifers for continued growth and development and future reproductive success.

Crude protein in the diet consists of true protein and non-protein nitrogen (NPN). Non-protein nitrogen includes sources of nitrogen in the feed not incorporated into proteins such as individual amino acids, ammonia, or urea (Tadele and Amha, 2015). True protein can be further categorized as rumen undegradable protein (RUP) or rumen degradable protein (RDP). Rumen undegradable protein are proteins that are not able to be broken down by the rumen microbes and will then pass through the rumen for further digestion and absorption in the small intestine. Rumen degradable protein are feed proteins that are able to be broken down to AA by rumen microbes to build their own proteins, and eventually become the major supply of AA to the ruminant as microbial crude protein (Mathis, 2003; Rinehart, 2008). However, a greater abundance and diversity of AA than microbial sourced AA may be required, especially during lactation and pregnancy. Therefore, supplementation of rumen undegradable protein (RUP) and/or NPN is often included in the diet (Klopfenstein, 1996). Feeds with more RUP content will provide increased AA diversity that will reach the small intestine and be available to the animal without alteration from the microbes.

Previous studies have shown supplementing protein can be beneficial for development of replacement heifers (Lalman et al., 1993; Martin et al., 2007). Supplementing RUP resulted in heifers that were significantly heavier at puberty compared to other groups; however, there was no

difference in pregnancy rates (Lalman et al., 1993). Therefore, RUP may help to ensure adequate nutrient supply for reaching a target body weight. In contrast, other studies have indicated the potential for protein to benefit reproductive performance, with or without weight gains. The utilization of dried distiller's grains as a source of RUP during heifer development resulted in a greater artificial insemination (AI) pregnancy rate compared to an isocaloric control supplemented group, but body weights at puberty or breeding did not differ between groups (Martin et al., 2007). The use of protein supplementation has also indicated the potential to increase circulating leptin concentrations as well as glucose, insulin, and IGF-1 (Cappelozola et al., 2014). As previously discussed, leptin signaling is a major factor, in addition to glucose, insulin, and IGF-1, for the activation of kisspeptin neurons for the onset of puberty. However, conflicting results have been observed regarding protein supplementation affecting heifer age at puberty, with studies reporting decreased age at puberty (Granger et al., 1989), no difference (Martin et al., 2007), or delayed puberty attainment (Lalman et al., 1993). These differences are likely attributed to protein amount and type of protein supplied, and may be a result of complications from excess protein supplied. Protein supply is also critical post-breeding to support fetal development during gestation. As gestation progresses, the requirements for protein and other nutrients increase to supply enough protein and amino acids via the placental transport directly to the fetus for growth and development (National Academies of Sciences and Medicine, 2016). Protein supply during gestation can affect the calf's future performance, specifically in heifer calves. Providing protein supplementation during late gestation increased heifer calf body weights at breeding and decreased the age at puberty (Funston et al., 2010), indicating the potential for protein to impact fetal programming.

Bacterial Communities in the Body

The total number of microorganisms in the body have been suggested to outnumber or closely match the total number of cells in the body (Sender et al., 2016). With the development of laboratory technologies such as next generation sequencing, scientists are now better able to gain a deeper insight into the animal's microbiome, detect previously indiscernible microbial species, and evaluate their relationship with host processes. In cattle, the majority of microbiome research has focused on the segments of the gastrointestinal tract and the resident microbial community's role in digestion. Recently, researchers have begun to study other microbial communities outside the gastrointestinal tract, specifically in the reproductive organs, and their interactions with various physiological processes. The rumen and reproductive tract microbiomes have recognized importance in controlling animal feed efficiency and reproductive performance, respectively (Myer et al., 2015, Ault et al., 2019). These microbiomes contain microorganisms from all three domains of life and multiple kingdoms. However, bacteria are the largest portion of the total microbiome in abundance and contribute the greatest towards the host-microbe relationship (Hespell et al., 1997; Comtet-Marre et al., 2017); therefore, the following review focuses on the structure of bacterial communities and their relationship to the host processes in the rumen and reproductive tract. By gaining an understanding of the presence of these bacterial communities and the factors that influence their abundances, future studies may be able to manipulate these communities to optimize their respective environment and subsequent productivity of the animal.

Bacterial Communities of the Rumen

The ruminal microorganisms are the primary providers of nutrients (~70%) to the ruminant by digesting plant materials and other feeds that are not readily digested by the animal's own

enzymes (Bergman, 1990). The first goal of ruminant nutrition is to provide feeds with adequate nutrients to support the diversity of microorganisms present and optimize their fermentation. The diversity of bacteria in the rumen can be categorized based on their functional roles and major fermentation outputs, or their structure. One of the classical structural categories of bacteria, regardless of the environment they are located, is based on the structure of the bacterial cell wall determined by Gram staining: Gram-positive or Gram-negative (Coico, 2006). Gram-positive bacteria have an exterior thick layer of peptidoglycan surrounding a cytoplasmic membrane. Gram-negative bacteria are defined by their thin peptidoglycan layer in between an outer membrane and cytoplasmic membrane. Importantly, this outer membrane releases toxic substances, called endotoxins, when disrupted (Nagaraja et al., 1978; Heumann and Roger, 2002). The most common endotoxin associated with Gram-negative bacteria is lipopolysaccharide (LPS) which is present in the outer membrane (Dong et al., 2017). Due to the presence of LPS, Gram-negative bacteria can include common pathogens or opportunistic pathogens. These opportunistic pathogens are present in minor abundances in normal, healthy animals, but can greatly increase in abundance when provided the opportunity, potentially causing disease (Shen et al., 2019). Stressors, such as sudden nutritional changes or disease, can also result in an altered environment that favors opportunistic pathogen growth. For example, sudden increases in grain feeding leading to acidosis of the rumen increases Gram-positive and decreases Gram-negative bacteria (Li et al., 2012; Monteiro and Faciola, 2020). With the changing environment impact on Gram-negative bacteria, LPS is released at a greater rate that can elicit a significant immune response in the animal through the innate immune system via toll-like receptors (TLR), mainly TLR4, to produce pro-inflammatory mediators to increase immune cell responses (Nagaraja et al., 1978; Takuchi et al.,

1999; Huemann and Roger, 2002; Elson et al., 2006; Lu et al., 2008). Feed additives, such as ionophores, target Gram-positive bacteria to reduce their abundance and increase Gram-negative bacteria, resulting in a shift of volatile fatty acid (VFA) production to improve feed efficiency (Russel and Strobel, 1989). Therefore, the differences between Gram-positive and Gram-negative bacteria have a major effect on the health, fermentation outputs, and productivity of ruminants.

Beyond their potential pathogenicity and commensal relationships, the rumen bacteria are able to provide VFA and other intermediary metabolites via fermentation which can then be used by the ruminant for production of energy (Hespell et al., 1997; Mao et al., 2015). The production of VFA in the rumen is largely controlled by the dominant bacteria phyla, Bacteroidetes and Firmicutes. Bacteroidetes typically dominates in abundance within ruminants consuming a forage-based diet, while Firmicutes dominates with greater starch-based diets (Wang et al., 2020). Additionally, shifts between these bacterial communities due to the diet will also affect the resulting VFA proportions and rumen environment (Wang et al., 2020). Forage-based diets typically result in greater amounts of acetate production and greater rumen pH. In contrast, greater starch-based diets will typically increase the propionate production and lower the rumen pH. Therefore, the composition of the rumen microbiome and its corresponding metabolic output is largely impacted by diet composition.

In addition to the ruminal bacteria's major role of converting indigestible feedstuffs into energy and usable substrates for the ruminant, bacteria are also crucial for providing protein and amino acids (Clark et al., 1992). Rumen degradable protein is the portion of crude protein microbes are able to breakdown. Adequate supply of RDP in the diet, balanced with RUP, is crucial to support the ruminal microbes for their own protein synthesis (Harun and Sali, 2019). If RDP is

limited, microbial fermentation of all dietary components will be limited, reducing the total nutrients, vitamins, and energy available to the ruminant (Stokes et al., 1991, Clark et al., 1992; Harun and Sali, 2019). Additionally, RDP aids the ruminant by supporting microbial growth to provide MCP, which provides the majority of protein and AA supply to the ruminant (Clark et al., 1992). The fate of MCP is associated with the flushing of microorganisms from the rumen to the small intestine. Digestion of the microorganisms themselves releases proteins and AA that can be absorbed and utilized by the ruminant host. Providing excess RDP protein, however, can lead to issues due to increases in ammonia production and excretion, reducing overall efficiency (Hristov et al., 2004). Therefore, crude protein provided in the diet must be balanced between RDP and RUP to support microbial protein synthesis for their own functions, as well as supporting AA delivery to the ruminant (Klopfenstein, 1996; Mathis, 2003).

Bacterial Communities of the Reproductive Tract

The uterus was previously thought to be a sterile cavity in humans and animals, with the presence of bacteria indicating infection and harmful to reproductive performance and health (Perez-Muñoz et al., 2017). However, the transition from culture-dependent identification of bacteria to culture-independent sequencing methods has allowed for characterization of a normal, healthy reproductive tract microbiome of humans (NIH HMP Working Group et al., 2009), primates (Yildirim et al., 2014), and ruminants (Swartz et al., 2014; Clemmons et al., 2014). In 2007, the National Institutes of Health began the Human Microbiome Project to study the microbial communities throughout multiple body systems, including the reproductive tract. The discoveries of the human reproductive tract microbiome have led to research in other species, including the bovine, to identify differences and impact on fertility.

Healthy Bacterial Communities of the Reproductive Tract

The reproductive tract of humans has been found to contain approximately 9% of the total microbial communities in the human body (NIH HMP Working Group et al., 2009). In contrast to other microbial communities, such as in the gastrointestinal tract and nasal airways, the human reproductive tract has a very low diversity of microbes. The low diversity is attributed to the vaginal bacterial community that is dominated by *Lactobacillus* species, which is typically greater than 90% abundance in healthy subjects (The Human Microbiome Project Consortium, 2012). The importance of *Lactobacillus* species for health in humans is attributed to its influence on the reproductive tract environment. Estrogen stimulates the production of glycogen by vaginal epithelial cells which is then released into the environment. Glycogen serves as a substrate for fermentation of *Lactobacillus* for the production of lactic acid and hydrogen peroxide. Women with greater glycogen concentrations in the vagina have increased abundance of *Lactobacillus* and lower vaginal pH (Mirmonsef et al., 2014). The lactic acid and hydrogen peroxide produced contribute to the environment by maintaining a low pH which serves as a defense mechanism against pathogen colonization (Rönnqvist et al., 2006; Mirmonsef et al., 2014). The maintenance of the reproductive tract environment by *Lactobacillus* is associated with reproductive health. In a study involving women of various ages and ethnicities, five different core vaginal microbiomes were identified that each contained similar abundances of various bacterial taxa (Ravel et al., 2011). Out of the five core microbiomes, four were dominated by a different *Lactobacillus* species, but similar in overall health and acidic pH. Women in the fifth core microbiome group, however, did not have a vaginal bacterial community dominated by a single taxon, but contained a greater diversity of bacteria. Additionally, these women had a more basic pH and higher Nugent score; a

diagnosis for bacterial vaginosis that often requires treatment (Ravel et al., 2011). In the uterus, *Lactobacillus* is still the dominant genus; however, overall bacterial abundance is significantly reduced compared to the vagina (Mitchell et al., 2015; Moreno et al., 2016; Chen et al., 2018). Various theories have emerged for this difference between environments including the cervix acting as a barrier or the result of differences in immune responses between the two organs (Moreno et al., 2018). However, determination of the healthy uterine bacterial communities in women is challenging due to the difficulty of sampling with various methods utilized, and the majority of samples are collected from women with health complications, undergoing hysterectomy, or fertility issues.

The majority of previous research on bacteria in the bovine reproductive tract was associated with the presence of pathogens and the development of postpartum uterine diseases. The increased exposure of the reproductive tract to the external environment during parturition allows pathogenic bacteria to enter and colonize. Pathogens such as *Escherichia coli*, *Trueperella* (previously known as *Arcanobacterium*) *pyogenes*, and *Fusobacterium necrophorum* are commonly associated with the development and presence of uterine infections (Sheldon and Dobson, 2004; Machado et al., 2012; Karstrup et al., 2017; Sheldon and Owens, 2017; Galvao et al., 2019). Failure of the reproductive tract and resident immune cells to recognize and remove the pathogenic bacteria leads to the development of subclinical or clinical infections, most commonly endometritis or metritis. Signs of these diseases, including vaginal discharge, inflammation, and elevated temperature, can be identified within the first week of the postpartum period, and up to 50 days following parturition (Sheldon et al., 2009). The probability of infection is greater following complications from parturition such as retained fetal membranes or dystocia. Due to the

short duration of the postpartum period in order for the cow to be re-bred, these potential infections must be cleared quickly or treated. If an infection persists to the time of re-breeding, fertility is often compromised with delayed return to estrous and failure to establish a pregnancy (Bonnett et al., 1993; LeBlanc et al., 2002; Ribeiro et al., 2013). Therefore, understanding the bacteria of the uterus regarding disease is a major focus to reduce the effects of postpartum diseases and increase reproductive success.

Recently, researchers have discovered healthy communities of bacteria in the reproductive tract of cattle. One of the first studies to evaluate the ruminant reproductive tract bacterial communities was conducted in 2014, using vaginal swabs in ewes and cows of different ages and stages of production (Swartz et al., 2014). Contrasted to previous studies in humans and primates, the ruminant vaginal bacterial communities were highly diverse and contained distinctly different taxa (Swartz et al., 2014). Furthermore, specific comparison between non-pregnant and pregnant animals found reduced diversity in the vagina of pregnant heifers and cows than non-pregnant heifers and cows (Laguardia-Nascimento et al., 2015). Following these studies, discovery of the uterine bacterial communities was necessary. Comparison between the uterine and vaginal bacterial communities was conducted by collection of flushes from non-pregnant cows two days prior to AI. The uterus had a significantly reduced number of bacterial taxa (495 ± 208 operational taxonomic units [OTU]) compared to the vagina (838 ± 144 OTU). Phyla-level abundances were similar, but multiple genera-level differences were detected of bacteria between the two environments (Clemmons et al., 2017). Notable among these studies, *Lactobacillus* species were detected in the majority of samples, but at very low abundances corresponding with the greater pH observed in the vagina of cattle. Numerous studies are in agreement that the *Lactobacillus*

prevalence of the bovine reproductive tract is likely insignificant, contrasting that of the human reproductive tract (Laguardia-Nascimento et al., 2015; Clemmons et al., 2017; Ault et al., 2019a; Quereda et al., 2020). Overall, research has consistently recognized the bovine vagina, despite breed, production stage, and reproductive status, is dominated by three major phyla: Firmicutes, Bacteroidetes, and Proteobacteria (Swartz et al., 2014; Laguardia-Nascimento et al., 2015; Clemmons et al., 2017; Ault et al., 2019a), similar to the rumen microbiome. Other phyla, such as Fusobacteria, have also appeared among the most dominant phyla in some studies (Swartz et al., 2014; Quereda et al., 2020; Messman et al., 2020), which contain common pathogenic bacteria. Clemmons et al. (2017) suggested the vaginal bacterial communities were highly influenced by gastrointestinal bacteria due to its close proximity and exposure to the environment. The uterus has been less investigated, however, it shares similar taxa with the vagina with different proportions of dominant phyla (Clemmons et al., 2017; Moore et al., 2017; Ault et al., 2019b). Additionally, cluster analyses have indicated the uterus contains its own unique bacterial communities distinct from the vagina (Clemmons et al., 2017). The specific contributions of these bacteria in the uterus and vagina remain to be fully understood. However, these communities likely provide similar functional roles as in the gastrointestinal tract, such as providing nutrients and intermediate metabolites for physiological processes and influencing immunological environment of the reproductive tract. The family Ruminococcaceae has been found to be highly abundant across the uterus and vagina (Ault et al., 2019), and is a known major producer of the VFA butyrate in the gastrointestinal system (Esquivel-Elizondo et al., 2017; Fu et al., 2019). Butyrate is involved in the development of immune cells, specifically T regulatory cells, which help maintain an immunotolerant environment (Furusawa et al., 2013; Zhang et al., 2016). In the reproductive tract,

T regulatory cells are important during pregnancy establishment and maintenance to prevent maternal rejection of the developing fetus (Aluvihare et al., 2004; Shima et al., 2010). Therefore, the presence of the bacteria in this family, and many other throughout the reproductive tract, may be important for preventing inflammation to maintain an environment suitable for optimal reproductive success.

These studies discovered the bovine reproductive tract microbiome and set the stage for beginning to identify potential roles for bacterial communities in physiological processes of the reproductive tract. Limitations of these studies, however, involve only utilizing a single time point or samples were not controlled for similar estrous cycle stage or phase. Therefore, further studies are needed to fully understand the effects of estrous cycle stage, and other factors such as management practices, nutrition, or reproductive status that can influence the reproductive tract microbiome.

Reproductive Bacterial Communities during the Menstrual and Estrous Cycle

In humans, the menstrual cycle is characterized by menses at the beginning and end of the cycle, with ovulation in the middle. Approximately half of the cycle consists of the follicular phase (or proliferative phase) with rising estrogen during follicular development, resulting in ovulation. The remaining ~50% of the cycle is the luteal phase (or secretory phase) with increasing progesterone for the preparation of a pregnancy (Mihim et al., 2011). Menses occur when there is no developing pregnancy, leading to the shedding of the endometrial lining (Nair and Taylor, 2010). Due to this process, studies have evaluated the changes in the reproductive tract bacterial communities throughout the estrous cycle. The diversity of bacteria and non-*Lactobacillus* species abundance in the vagina was observed to be greater at menses, indicating lower stability of the

bacterial communities (Eschenbach et al., 2000). Similarly, Gajer et al. (2012) observed the microbiome was the least stable during menses and associated with hormones. The greatest stability was observed with peak estrogen secretion, followed by peak progesterone secretion (Gajer et al., 2012). Therefore, stability during ovulation and pregnancy preparation and establishment is likely critical for an optimal reproductive tract environment. Further studies have found differences in bacterial functional pathways between the proliferative and secretory phase (Chen et al., 2017). During the proliferative phase, pathways such as pyrimidine and purine metabolism were enriched, potentially indicating increased bacterial cell proliferation. Different pathways, including multiple amino acid metabolism pathways, were enriched during the secretory phase. Additionally, multiple bacterial species in the uterus and vagina were differentially abundant depending on menstrual cycle phase (Chen et al., 2017). Therefore, due to the physiological changes occurring, especially menses, bacterial communities and their respective functions are also likely shifting throughout the menstrual cycle.

The estrous cycle of the bovine differs from humans as it is characterized by ovulation at the beginning of the cycle, followed by a long luteal phase making up 80% of the cycle, until the next ovulation to start another cycle. Additionally, there is no shedding of the endometrial lining as occurs in humans. Studies are limited but have indicated differences in the reproductive tract bacterial communities depending on the stage of the estrous cycle. Based on heat detection, vaginal swabs were taken during estrus and diestrus to represent the follicular and luteal phase, respectively. Although diversity analyses did not detect significant differences, multiple taxa significantly differed and clustering of communities was observed by phase (Quereda et al., 2020). Reproductive tract bacterial communities have also been observed at multiple time points during

a 7-Day Co-Synch estrous synchronization protocol. Bacterial communities clustered by day of the protocol according to principal coordinate beta diversity analysis, and alpha diversity significantly decreased two days prior to TAI. Additionally, multiple phyla and genera abundances shifted between days of the protocol (Ault et al., 2019a,b). Therefore, the fluctuations in various hormones throughout the estrous cycle may influence the fluctuations observed in the uterine and vaginal microbiome. To determine the impact of specific hormonal status on the vaginal microbiome, Messman et al. (2020) classified cows into groups based on their circulating estradiol concentration at the time of breeding, following synchronization. No diversity or overall composition differences were detected, however multiple species level abundances differed between estradiol groups (Messman et al., 2020). Therefore, the bovine uterine and vaginal bacterial communities experience shifts in composition between phases of the estrous cycle, and some taxa may be impacted by specific hormone concentrations. Further research is needed to understand how these bacterial abundance and diversity shifts occurring during the estrous cycle may impact subsequent fertility.

Relationship between the Reproductive Bacterial Communities and Fertility

The bacterial communities of the reproductive tract have an important role in maintaining a healthy environment, likely influencing fertility. As previously mentioned, in humans, the dominance of *Lactobacillus* is critical for maintaining the proper reproductive tract environment and inhibiting the growth and colonization of pathogens. This dominance of *Lactobacillus* has also shown importance in reproductive outcomes and fertility issues. Women with varying causes of infertility were found to have greater microbial diversity and a different overall microbiome when compared with fertile women, largely attributed to differences in *Lactobacillus* species

composition (Campisciano et al., 2017). Furthermore, infertile women undergoing in-vitro fertilization had their vaginal and endometrial microbiomes classified as having either *Lactobacillus*-dominated reproductive tract bacterial communities, consisting of >90% abundance of *Lactobacillus* species, or non-*Lactobacillus*-dominated with >10% non-*Lactobacillus* species. Patients with non-*Lactobacillus*-dominated reproductive tract bacterial communities had significantly lower implantation and pregnancy rates (Moreno et al., 2016). In established pregnancies, microbial dysbiosis in the uterus or vagina that occurs during gestation can affect pregnancy maintenance. Influx of pathogenic bacteria into the uterus can ultimately result in preterm birth or miscarriage (Payne and Bayatibojakhi, 2014; Moreno et al., 2016; Al-Memar et al., 2020). Therefore, clinical studies in humans have indicated strong associations of the uterine and vaginal microbiome with reproductive success.

Limited research has been conducted to determine the influence of bacterial communities on reproductive performance and breeding outcomes in cattle. It has been well established that postpartum uterine infections can affect reproductive performance by increasing the time to conception (Bonnet et al., 1993; LeBlanc et al., 2002; Ribeiro et al., 2013). However, the influence of the healthy microbiome on reproductive performance is limited. Ault et al. (2019a,b) performed estrus synchronization in cows for uterine and vaginal flush collections to determine the bacterial community at multiple time points until two days prior to breeding via AI, with pregnancy determined 30 days after breeding. Beta diversity using principal coordinate analyses indicated clustering among bacterial samples with distinct separation of resulting pregnant and non-pregnant animals. Samples from non-pregnant animals clustered closely together indicating a similar overall bacterial community composition that was potentially influencing the animal's ability to conceive.

Multiple genera level differences were detected between resulting outcomes, especially at two days prior to TAI. Cows that became pregnant had potentially pathogenic bacteria present in abundances <1%, while cows that did not become pregnant had these same bacteria present with abundances ranging from 1-10%, although no signs of infection were observed (Ault et al., 2019a,b). Therefore, minor differences in bacterial abundances may affect the uterine environment and impact fertility. Further research is necessary to characterize the interaction of these bacteria with the uterus leading to reduced reproductive performance.

Uterine Environment

The uterine environment is crucial for preparation, development, and support of a pregnancy for optimal reproductive efficiency. As discussed previously, the uterine microbiome is a recently discovered but major component of the uterine environment that can impact reproductive success. Uterine histotroph is another major component of the uterine environment, resulting from secretions of endometrial glands. Histotroph contains nutrients, hormones, growth factors, and immune signaling molecules that can be produced from uterine tissues or originate from systemic circulation to support normal physiological functions (Bazer, 1975; Gao et al., 2009; Mullen et al., 2012). During early pregnancy, histotroph is especially vital for the survival of the developing embryo by providing all necessary nutrients until placental attachment and hemotrophic support (Bazer, 1975). Knockout models of the uterine glands have indicated reproductive failure by prevention of pregnancy or severe developmental delay (Gray et al., 2002). Therefore, the presence and of nutrients, specifically protein and amino acids, and cytokines in the uterine environment and their relationship with the uterine microbiome may contribute toward reproductive efficiency.

Proteins and Amino Acids

Protein is one of the most important nutrients for reproductive function. Amino acids from RDP, RUP, and NPN are a critical component of the histotroph. During early pregnancy, an influx of AA and total protein into the uterine environment has been observed (Groebner et al., 2011; Mullen et al., 2012; Forde et al., 2014; Crouse et al., 2019). Pathway analyses indicate proteins related to uterine remodeling, embryo development, and maternal immune function have greater abundance in the uterus during early pregnancy to facilitate pregnancy establishment (Mullen et al., 2012). As the early embryo is free-floating in the uterine environment for an extended period, it fully relies on the nutrients available in the histotroph for growth and development and successful establishment and maintenance of pregnancy (Bazer, 1975). Although providing adequate protein is highly important to the ruminant diet, especially to breeding cows, excess protein may cause negative effects on early pregnancy. Due to the known impact of uterine pH on sperm motility and survival, excess protein provided can increase the acidity of the uterine environment and inhibit pregnancy establishment (Elrod and Butler, 1993; Ocon and Hansen, 2003). Excess protein, especially in the form of NPN, cannot all be utilized by the rumen microbes, resulting in an increase of plasma urea and ammonia that can travel to the uterus and decrease pH (Dawuda et al., 2002). However, studies are conflicting on excess protein impacting the uterus and fertility, with no impact on uterine pH or reproductive functions (Amundson et al., 2016). Further research is needed to determine the exact factors leading to this effect in some cases. Therefore, protein is vital for reproductive functions but must be balanced to support microbial activity and create the proper uterine environment for optimal reproductive performance.

Immunological Environment by Immune Cells and Cytokines

The immunological environment of the uterus is dynamic for preventing infections and maintaining a healthy, stable environment conducive for pregnancy. Cytokines are some of the most important immunological factors in the reproductive tract environment and can be produced by endometrial cells or resident immune cells (Tabibzadeh, 1991). The endometrial epithelium is the first line of defense in the uterus, which also expresses Toll-like receptors to recognize pathogen associated molecular patterns (Cronin et al., 2012; Fu et al., 2013; Dadarwal et al., 2017). If the epithelial barrier is disrupted, the stromal layer containing resident immune cells of the endometrium is exposed. Neutrophils and macrophages are the most abundant innate immune cells of the bovine uterus, which also express Toll-like receptors including TLR4 to respond to LPS presence (Dadarwal et al., 2017). When LPS activates TLR4 on epithelial or innate immune cells, pro-inflammatory cytokines TNF α , IL-6, and IL-1 production is upregulated stimulating an inflammatory response (Cronin et al., 2012; Swangchan-Uthai et al., 2012). These cytokines will signal for activation of resident macrophages towards a M1, pro-inflammatory phenotype and recruit additional immune cells. To resolve the inflammation and return to a normal balance, anti-inflammatory cytokines, such as IL-10, TGF- β , and IL33 are secreted by epithelial and immune cells to shift macrophages activation to an M2 phenotype (Dadarwal et al., 2017).

Increases in pro-inflammatory cytokines are most often stimulated by the increasing presence of pathogenic bacteria, specifically Gram-negative bacteria expressing LPS, due to the activation of TLR4 on the uterine endometrium or resident immune cells (Lu et al., 2008; Davies et al., 2008; Yunhe et al., 2013). The expression of TLR4 has also shown to differ across the estrous cycle in humans and porcine, with greater expression during stages of higher estrogen

dominance (Hirata et al., 2007; Yoo et al., 2019). As previously mentioned, alterations in bacterial community composition can be caused by different diets or physiological changes from the estrous cycle. In the uterus, increases in Gram-negative pathogenic bacteria prior to breeding were observed to reduce pregnancy outcomes (Ault et al., 2019a). Nutritional changes resulting in increased LPS release from the rumen, have been linked with effects both systemically and in the reproductive tract (Zebeli et al., 2012; Bilal et al., 2016). Animals with ruminal acidosis had higher LPS detected in blood as well as increases in pro-inflammatory cytokine concentration in blood and gene expression in the endometrium (Bilal et al., 2016). Although the TLR4 response has been associated with negative outcomes, recently, it has been identified with a beneficial cytokine response following mating (Chan et al., 2021). In mice mated with wild-type TLR4 or females with a null mutation of TLR4, wild-type females had better reproductive success with greater pregnancy rates and improved placental and fetal development. Additionally, wild-type females had a greater cytokine response to mating than the null mutation TLR4 females, which was determined to benefit pregnancy establishment (Chan et al., 2021). Further research is warranted to understand how the changes in the uterine microbiome may be altering this response, shifting the presence of various cytokines, and impacting breeding success.

Regulation of inflammation is one of the most common functions of cytokines systemically and in the reproductive tract. Many cytokines have classically been categorized as pro-inflammatory (e.g. IL1 β , TNF α , IFN γ , IL6, etc.) or anti-inflammatory (e.g. IL4, IL10, TGF β , etc.). The balance between pro- and anti-inflammatory cytokines is necessary to maintain an environment conducive for pregnancy establishment, but also prepared to respond to inflammatory stimuli such as a developing infection. The actions of inflammatory cytokines are believed to be

important in providing the immunotolerant environment of the reproductive tract (Raghupathy, 2001). As the developing embryo and growing fetus in mammals is allogenic by expressing both maternal and paternal antigens, immunotolerance is crucial for the establishment and maintenance of pregnancy (Schjenken et al., 2012). Shifts in the balance of inflammatory cytokines towards greater pro-inflammatory cytokine abundance have been linked to failure of pregnancy establishment or pregnancy loss. Gene expression analysis of the endometrium indicated inflammation was identified to be a major differentially expressed pathway between heifers of differing future fertility, determined by success of multiple rounds of artificial insemination (Killeen et al., 2014). Greater levels of genes related to inflammation were observed in heifers of lesser fertility (Killeen et al., 2014). Uterine cytokine concentrations were different prior to AI between cows that became pregnant and those that did not (Poole et al., 2021), indicating an influence of the cytokines in the uterine environment on pregnancy establishment. Additionally, uterine cytokines such as TGF β , IL1 β , and IL6 were correlated to the abundance of various bacteria genera, suggesting a potential influence by the uterine bacterial communities (Poole et al., 2021). Therefore, imbalances in cytokine concentrations, specifically upregulation of pro-inflammatory signals, of the uterus and vagina may reduce reproductive efficiency.

Generally, cytokines are most commonly associated with their numerous immune system related functions including regulation of inflammation and immune cell recruitment (Dimberg, 2010). Specific to the reproductive tract, they are also involved in other non-immune system related processes such as ovulation (Espey et al., 2004), corpus luteum function and luteolysis (Penny et al., 1999), embryo development and implantation (Zolti et al., 1991; Simón et al., 1998; Ealy et al., 2021), angiogenesis, placental development, and immunotolerance to the growing fetus

(Raghupathy et al., 2001). Cytokines can also be considered embryokines, which are produced by the early embryo for development and signaling to maternal tissues, or produced by the maternal tissues to support early embryo development (Ealy et al., 2021). Similar to the reproductive tract microbiome, cytokines and chemokines can fluctuate overtime, including through the attainment of puberty (Ault-Seay et al., 2021), across the estrous cycle (Krakowski and Zdzisinska, 2007; Oliveira et al., 2013; Poole et al., 2021), and during pregnancy (Krakowski and Zdzisinska, 2007; Oliveira et al., 2013). In comparison, uterine cytokines were observed to fluctuate during estrous synchronization with minimal change in the vagina (Poole et al., 2021). Therefore, cytokines are pleiotropic and provide numerous functions for immune and non-immune related reproductive processes. Fluctuations throughout the reproductive cycle may help maintain the optimal uterine environment in preparation for pregnancy. However, dysregulation of cytokines in the reproductive tract may influence reproductive efficiency.

Conclusion

In conclusion, heifer development is a crucial period for replacement heifers in cow-calf operations to improve the reproductive efficiency of their herd (Patterson et al., 1992). Providing proper nutrition for heifers to reach a targeted body weight ensures adequate body reserves for metabolic signaling to hypothalamic neurons for the onset of puberty. Earlier puberty attainment and optimal body condition increases the heifer's potential for first breeding season success, better calf performance, reduced postpartum interval, and longevity in the breeding herd (Cushman et al., 1993; Perry and Cushman, 2013). Both reproductive success and nutritional feed efficiency are highly influenced by the bacterial communities present in the rumen and uterus. Ruminal bacterial communities are the major producers of nutrients by fermentation of

feedstuffs to produce VFA, and digestion of microbes themselves provide the majority of amino acids and proteins to the ruminant host (Bergman, 1990; Clark et al., 1992; Hespell et al., 1997; Mao et al., 2015). Recent developments have not only identified a uterine and vaginal microbiome of the bovine, but associated it with pregnancy outcomes (Clemmons et al., 2017; Ault et al., 2019a). Due to the importance of the uterine environment, especially during early pregnancy, the uterine bacterial communities may contribute to the immunological environment of the uterus (Poole et al., 2021). Further studies are necessary to determine the factors affecting the presence of bacterial species and the specific mechanisms bacteria utilize to interact with the uterine environment and influence reproductive outcomes. Further understanding of these roles may allow for manipulation of the uterine bacterial communities to benefit reproductive efficiency.

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CHAPTER II:
UTERINE AND RUMINAL BACTERIAL COMMUNITIES DURING
HEIFER DEVELOPMENT WITH PROTEIN SUPPLEMENTATION

Abstract

Bacterial communities play major roles in the rumen and uterus toward optimal animal performance and may be affected by changes occurring during heifer development such as nutritional supplementation for optimal growth and the attainment of puberty. The objective of the current study was to evaluate effects of different levels of protein supplementation on ruminal and uterine bacterial communities following weaning through the first breeding of heifers. Angus heifers (n=39) were blocked by body weight and randomly assigned to either control (10% CP, n=14), 20% CP (n=11), or 40% CP (n=14) treatment groups, with supplementation occurring for 140d. Growth and development was monitored by body weight, with blood samples collected every 14d to determine puberty by progesterone concentration. Uterine flush, via 20 mL of saline through a Foley catheter, and rumen content collections, via esophageal tubing, occurred on d 56, 112, and 163 relative to start of supplementation. Fluids were used for bacterial DNA extraction, amplification, and sequencing of the V1-V3 hypervariable regions of the 16S rRNA gene. Bacterial sequences were processed in R 4.1. Statistical analyses were performed in SAS 9.4 with a GLIMMIX procedure utilizing fixed effects of protein, month, pubertal status, and interactions, with random effects including BW, interaction of BW and protein, and heifer within the interaction, and repeated measures of day. In the uterus, pubertal status and day of supplementation affected observed amplicon sequence variants (ASV) and led to clustering of samples observed with principal coordinate analysis (PCoA) as a measure of beta diversity ($P < 0.05$), with no effects by protein supplementation. Ruminal samples clustered in PCoA ($P = 0.001$) and observed ASV were impacted overtime ($P < 0.0001$), but no effect of protein supplementation was detected. Protein supplementation, pubertal status, and day of supplementation affected the abundance of

multiple phyla and genera in the uterus and rumen ($P < 0.05$). The effects observed overtime and by pubertal status on bacterial communities of the heifer uterus potentially indicates progression of a maturing uterine microbiome through development. Protein supplementation altering the abundance of bacteria may provide the opportunity to influence bacterial community composition and maximize future productivity.

Introduction

The development of replacement heifers is crucial for incorporation into a breeding cow herd to benefit reproductive efficiency. The nutrition provided to heifers during the time of development often determines their age at puberty, future success as a breeding cow, and longevity in the herd (Bellows and Short, 1971). Therefore, it is crucial to provide the required nutrition to meet the demands of the growing heifer, in addition to supporting reproduction. Ideally, heifers should reach puberty early, attain approximately 60% of their mature body weight by the first breeding, and achieve a successful pregnancy with as few inseminations as possible to calve by two years of age (Larson, 2007; Kuehn et al., 2011; Perry, 2016). In many operations using defined breeding seasons, weaned heifers are often developed during a time of dormant forages with lower quality. Protein is one of the most vital nutrients for development and reproduction, but often the first limiting nutrient in dormant forages (Marston et al., 1995). Previous studies have shown supplementing heifers with protein increases their breeding success, muscle growth, and weight gain (Lalman et al., 1993; Martin et al., 2007; Dickinson et al., 2019). Therefore, supplemental feeds high in crude protein are often provided to heifers for continued growth and development for future reproductive success.

The rumen microbiome has been well established as the major producer of energy for the ruminant through the conversions of indigestible feed stuffs to provide volatile fatty acids (VFA), amino acids, metabolites, vitamins, and other useful nutrients (Bergman, 1990). As different feedstuffs vary in their amount and quality of protein, and other components such as fiber and starches, ruminal bacterial communities can be affected (Belanche et al., 2012). Shifts in the bacterial community composition may alter the nutrients metabolized and absorbed for use by the ruminant; these effects have been observed systemically and in the reproductive tract through immune system responses (Zebeli et al., 2012; Bilal et al., 2016). Additionally, providing supplemented protein in excess has been shown to potentially impact the pH of the reproductive tract by increased plasma urea and ammonia reaching the uterus (Elrod and Butler, 1993; Dawuda et al., 2002). These effects on the reproductive tract are primarily mediated through histotrophic secretions by the uterine endometrium. Histotroph contains a variety of nutrients and other factors that contribute to overall uterine health and embryo development (Bazer et al., 2015). Therefore, differences in the nutrient profiles of variable feedstuffs, which impact the rumen microbial communities, may potentially alter the composition of endometrial secretions and the uterine environment via nutrient transporters, impacting future reproductive success.

Recently, the uterine microbiome of ruminants has been determined to be an important contributor to the uterine environment. Although the reproductive tract was thought to be sterile, with the presence of bacteria indicating infection, the implementation of next-generation sequencing has provided the opportunity to recognize the reproductive tract as harboring its own distinct microbiome (Swartz et al., 2014; Laguardia-Nascimento et al., 2015; Clemmons et al., 2017). The composition of the uterine bacterial communities prior to breeding has been associated

with differences in breeding outcomes (Ault et al., 2019a, b). For maintenance of healthy bacterial communities in the uterus, the environment must be favorable for microorganisms to perform their metabolic processes, which can benefit reproductive success. However, there has been limited research on the factors that can impact the composition of the uterine bacterial community, potentially compromising the establishment and maintenance of pregnancy. Due to the observed impacts of diet on the uterine environment in cattle, nutrition may influence the bacterial communities in the uterus. Additionally, differences in bacterial communities have been detected between different phases of the estrous cycle and throughout estrus synchronization protocols (Quereda et al., 2019; Ault et al., 2019). Thus, the physiological changes that occur through the attainment of puberty and changes in hormone response in heifers may affect the uterine bacterial communities present. Therefore, through heifer development and attainment of puberty, the impact of nutrition on the uterine environment may provide the opportunity to influence uterine bacterial communities toward optimal reproductive health and efficiency.

The objective of the current study was to evaluate the effects of different levels of protein supplement on ruminal and uterine bacterial communities following weaning (pre-pubertal) through first breeding (pubertal) of beef heifers. We hypothesize 1) protein supplementation will affect developing heifer's ruminal and uterine bacterial communities during development, and 2) bacterial communities will differ between pre-pubertal and pubertal heifers.

Materials and Methods

All animal experimental procedures for the current study were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville.

Experimental Design

Commercial Angus heifers (n = 60) housed at the Middle Tennessee Research and Education Center were enrolled in the study 23 days (d) following weaning. Figure 1 depicts the following study timeline with sampling and estrus synchronization days. On d 0, heifers were evaluated for baseline measurements including body weight (BW), body condition scores (BCS), and blood samples via the jugular vein for determination of pubertal status. Heifers were blocked by BW and assigned to a treatment group (n = 20 heifers per treatment): control supplement (CON; 1.59 kg corn per day), 20% crude protein (CP) Supplement (P20; 1.59 kg per day: 75% dried distillers' grains 25% corn), or 40% CP Supplement (P40; 1.59 kg per day: 75% soybean meal 25% dried distillers' grains). Heifers were maintained in 1.21 hectare pens with five heifers per pen resulting in four pens per treatment group. Supplements were provided four times each week for 140 days, beginning in early October through late March. Body weight, BCS, and blood samples were collected once every two weeks to monitor development and pubertal onset. Blood samples were centrifuged at $5000 \times g$ and 4°C for 20 minutes to collect serum. Serum samples were stored at -80°C until further analysis. Circulating progesterone samples were quantified in serum using the Double Antibody RIA Kit (MP Biomedicals, Irvine, CA) validated previously by Pohler et al. (2016). Progesterone concentration of ≥ 1 ng/mL in two consecutive samplings that corresponded with a normal estrous cycle indicated a functional CL, representing puberty (Polat et al., 2009). The heifer was considered pubertal at the time of the second sample of ≥ 1 ng/mL progesterone. Serum progesterone concentrations at puberty, age at puberty, BW, and BCS for all heifers throughout development, with supplement and total diet feed analyses were reported in Brandt et al. (2021).

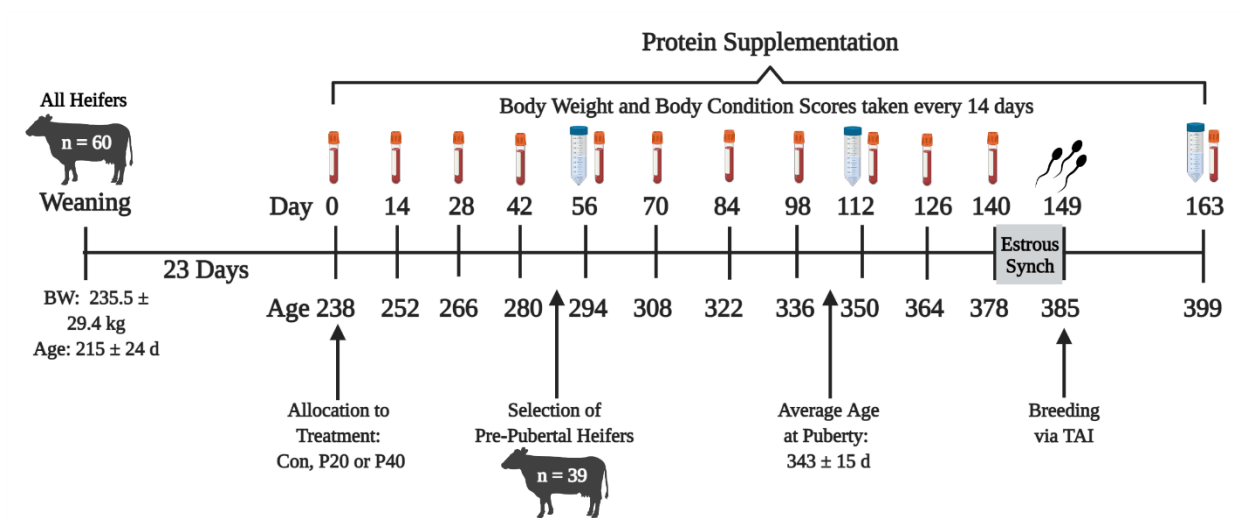


Figure 1. Timeline of sample collections and estrus synchronization.

Pre-pubertal heifers (n = 39 total, including n = 14 from the C and P40 treatment groups and n = 11 from the P20 treatment group) were selected to continue through the remainder of the study based on serum progesterone concentrations from the four blood collections between d 0 through 42 (Figure 1). Uterine flushes and rumen fluid were collected every other month on d 56, 112, and 163, relative to the start of protein supplementation, to evaluate the bacterial communities present. For uterine flushes, a Foley catheter was inserted through the vagina and cervix into the uterus to deliver 20 mL of sterile saline, manipulated throughout the uterus by rectal massage, then collected by syringe. Rumen fluid (~50 mL) was collected by oroesophageal tubing into the rumen attached to a hand pump with resulting fluid collected into a flask (Guan et al., 2008). Foley catheters and oroesophageal tubing were disinfected and washed between each heifer to eliminate contamination. Uterine flush and rumen fluid were immediately placed on ice for transport then stored at -80 °C until bacterial community analysis.

Prior to the final uterine flush and rumen fluid collections on d 163, heifers were subjected to estrus synchronization utilizing an industry standard 7-Day Co-Synch + CIDR protocol beginning on d 140. Controlled intravaginal drug release devices (CIDR; 1.38 g progesterone; Zoetis Animal Health, Florham Park, NJ) were inserted and GnRH (100 µg; Cystorelin®, Boehringer Ingelheim, Duluth, GA) was administered i.m. on d 140. An injection of PGF_{2α} (500 µg; Cloprostenol sodium; Synchsure™, Boehringer Ingelheim, Duluth, GA) was administered i.m. at CIDR removal on d 147. Approximately 52 hours after CIDR removal, heifers were administered GnRH, followed by artificial insemination on Day 149.

Bacterial DNA Extraction, Library Preparation, and Sequencing

Uterine flush bacterial DNA extraction was performed according to methods from Clemmons et al. (2017). Samples were thawed to room temperature (22°C) and 5 mL aliquots were placed in 15 mL conical tubes for centrifugation at $5000 \times g$ and 4°C for 10 minutes. The supernatant was removed and the resulting pellet was resuspended in enzymatic lysis buffer (4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA) for incubation at 37°C. Bacterial DNA was extracted using the DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to manufacturer protocol. Extracted DNA concentrations were determined by spectrophotometer (Denovix, Wilmington, Delaware), then stored at -20°C.

Bacterial DNA was extracted from rumen fluid using a modified method validated by Yu and Morrison (2004). Samples were thawed to room temperature (22°C) and approximately 0.2g of rumen fluid was transferred to a ZR BashingBead Lysis Tube (Zymo Research Corp., Santa Ana, CA, USA) with enzymatic lysis buffer for chemical lysis. Samples were placed in the TissueLyser II system (Qiagen, Hilden, Germany) for mechanical lysis at 21 Hz for 3 min. Cell debris was removed using 10M ammonium acetate, then nucleic acids were precipitated using isopropanol. Proteinase K and RNase were used to remove all proteins and RNA. Lastly, DNA samples were purified by centrifugation in QIAamp columns from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Resulting DNA concentrations were quantified via spectrophotometer (Denovix, Wilmington, Delaware), then stored at -20°C.

Library preparation and sequencing were performed at the US Meat Animal Research Center (Clay Center, NE, USA). Bacterial DNA was amplified using polymerase chain reaction (PCR) with Accuprime Taq high fidelity DNA polymerase (LifeTechnologies, Carlsbad, CA) for

library preparation. Primers 27F (5'-Adapter/Index/AGAGTTTGATCCTGGCTCAG) and 519R (5'-Adapter/Index/GTATTACCGCGGCTGCTG) with TruSeq indices were used to target the V1-V3 hypervariable region of the bacterial 16S rRNA gene (Myer et al., 2015). The resulting rumen and uterine bacterial DNA libraries were sequenced using the 2 × 300, v3 600-cycle kit on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, United States).

Sequence Processing and Statistical Analyses

Sequence quality filtering, trimming, and taxonomic assignment were performed in R 4.1 using the dada2 pipeline (Callahan et al., 2016). Forward sequences were filtered with standard dada2 parameters and a truncation length of 270. Resulting sequences were used to construct an amplicon sequence variant (ASV) table. Chimeric sequences were identified and removed then taxa were assigned using the SILVA database version 138. Metadata were imported and merged with the taxa table for alpha and beta diversity analyses using the phyloseq package (McMurdie and Holmes, 2013) in R 4.1. Unassigned reads and sequences assigned as Eukaryota or Cyanobacteria were removed for all downstream analyses. Observed ASV and Shannon Diversity Index were used to describe alpha diversity. Beta diversity analyses utilized the principle coordinate analysis (PCoA) method with Bray Curtis distances. Visualizations were generated with ggplot including ellipses representing a 95% confidence interval for each cluster. Relative abundance plots were generated for taxa using ggplot for the top 10 phyla and genera with a relative abundance $\geq 1\%$. Genera with a relative abundance $< 1\%$ were combined to one group for visualization.

For beta diversity, the vegan package with the adonis function in R 4.1 was used to perform a PERMANOVA to determine the significance of each PCoA. Statistical analyses for observed

ASV and bacterial taxa abundances were performed in SAS 9.4 (SAS Institute, Cary, NC). Normality of observed ASV and bacterial taxa raw abundance values were evaluated by the univariate procedure. Data were determined to be non-normal with a Shapiro-Wilk value of $W < 0.80$. Non-normal data were normalized by log transformation to achieve a normal distribution and Shapiro-Wilk value of $W > 0.85$. A completely randomized block design with repeated measures was utilized with the GLIMMIX procedure including fixed effects of day of sampling, protein supplementation treatment, and pubertal status, and interactions of day $\times$ protein and protein $\times$ pubertal status. Random effects included the block of BW, interaction of BW $\times$ protein, and heifer within the interaction, with repeated measures of day. For bacterial taxa abundance data, means are reported as the mean raw abundance value $\times 100$ to indicate percent relative abundance out of 100%. Significance was determined by $P \leq 0.05$ and trends by $0.05 \leq P \leq 0.10$ for all analyses.

Results

Beta diversity by PCoA among all samples indicated distinct separation between the uterus and rumen ($P = 0.001$, Figure 2). Rumen samples clustered tightly indicating a high degree of similarity of bacterial community composition among all rumen samples. Uterine samples clustered loosely indicating higher variation in overall bacterial community composition of uterine samples (Figure 2). A total of 39 phyla and 1,633 genera were detected among all uterine and ruminal samples. Figure 3 illustrates the overall relative abundance of the top 10 phyla detected among all samples between the uterus and rumen. Firmicutes and Bacteroidota were the most abundant phyla in the uterus and rumen, respectively, independent of protein supplementation treatment, pubertal status, or day of sampling (Figure 3). Figure 4 depicts genera with a relative abundance $\geq 1\%$ in the uterus and rumen, with all genera present in $<1\%$ grouped. Twenty-three

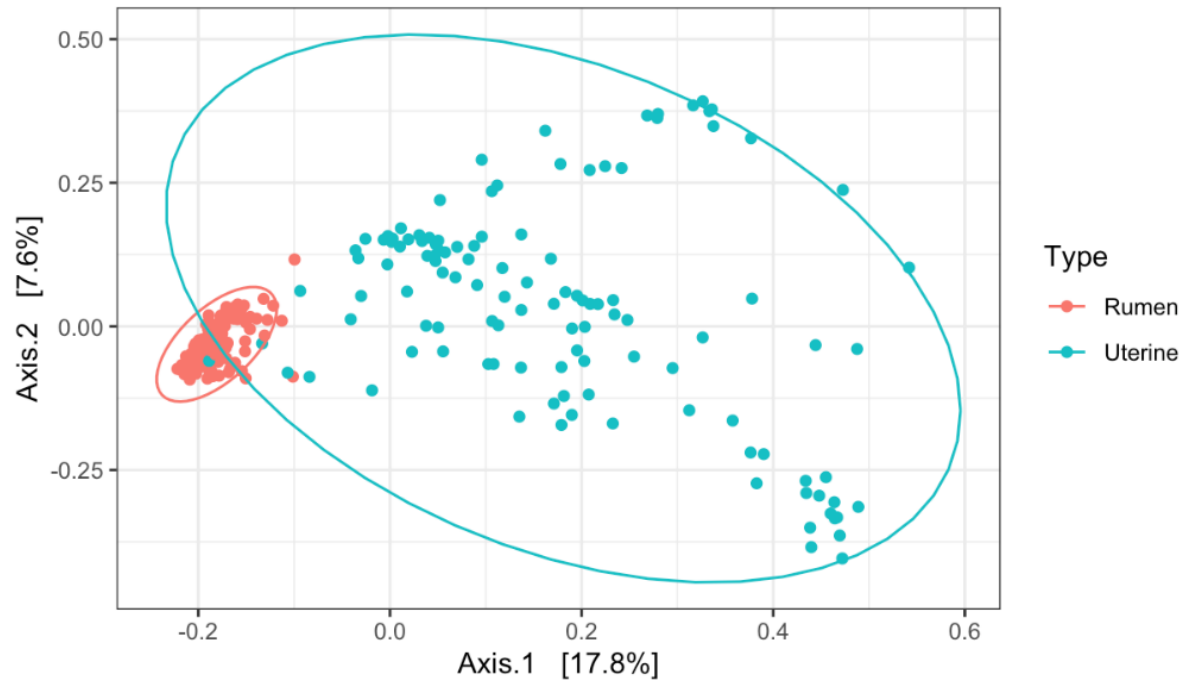


Figure 2. Beta diversity between all uterine and rumen samples ($P = 0.001$). Ellipses represent 95% confidence interval.

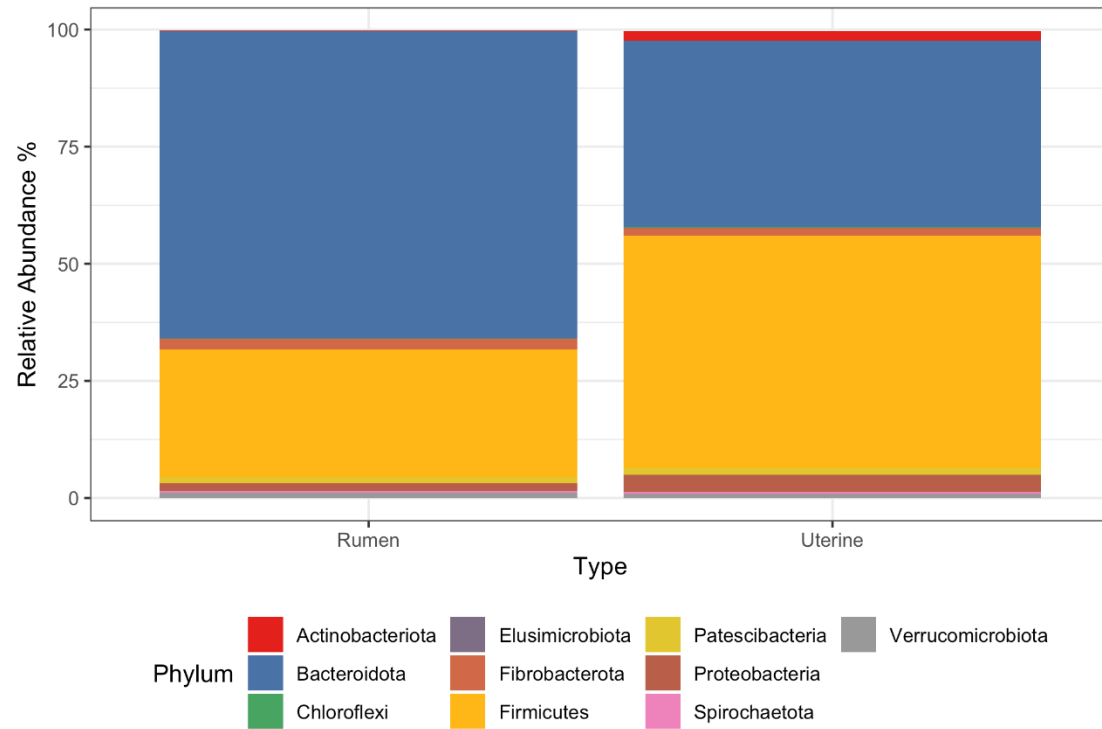


Figure 3. Relative abundance of the top 10 bacterial phyla in the uterus and rumen among all samples.

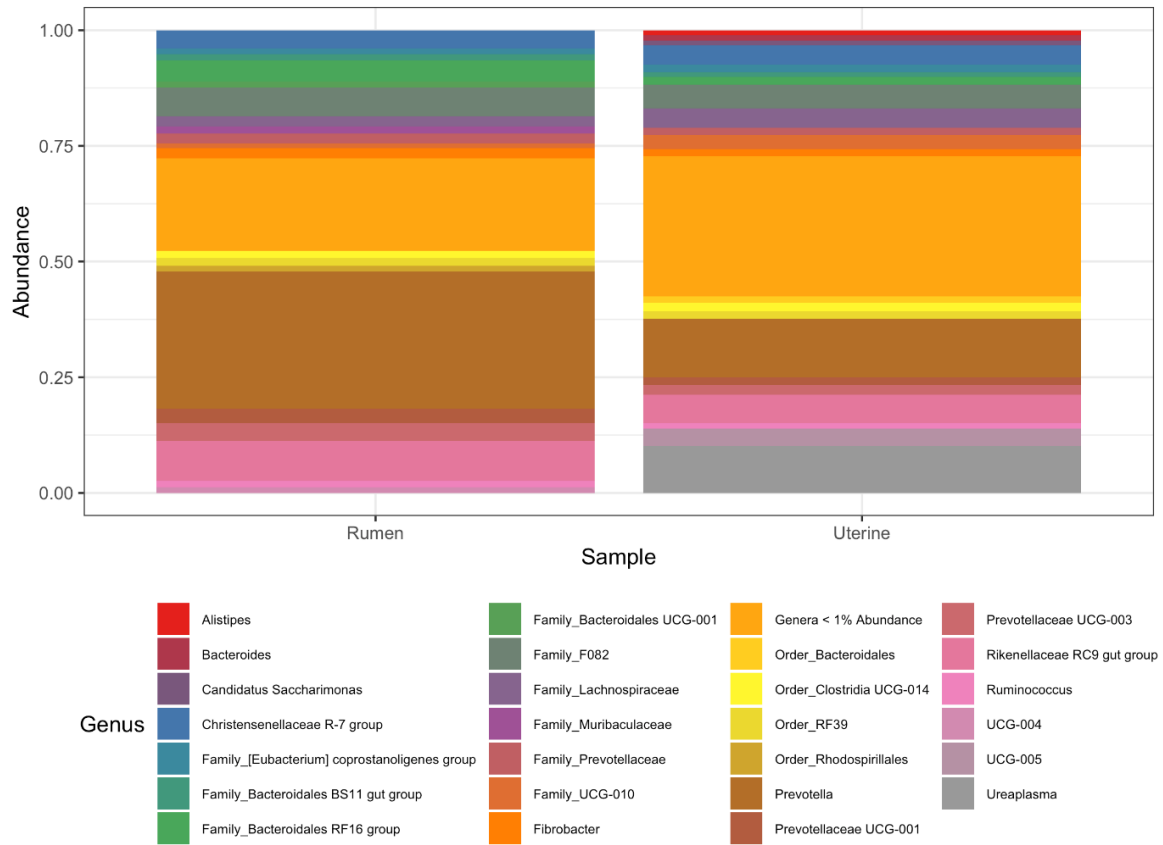


Figure 4. Bacterial genera relative abundances in the uterus and rumen among all samples.

Genera present in less than 1% relative abundance are combined to a single group.

genera in the uterus and 25 genera in the rumen were detected with relative abundances $\geq 1\%$. The total relative abundance of genera with $< 1\%$ abundance was 30.27% in the uterus, and 20.01% in the rumen. *Prevotella* was the most abundant genera in both uterine and ruminal samples across all groups (Figure 4).

Protein Supplementation Level

The level of protein supplementation did not affect alpha diversity metrics, observed ASV or Shannon's Diversity Index, in the uterus or rumen ($P > 0.05$). Similarly, no clear clustering pattern by protein supplementation level was observed by the beta diversity PCoA in uterine ($P = 0.06$, Figure 5) or ruminal ($P = 0.008$, Figure 6) samples, although significance was detected. However, multiple phyla and genera relative abundances were affected in the uterus and rumen by protein supplementation level.

In the uterus, the most abundant phyla, Firmicutes, was affected by protein treatment, with P20 having the greatest and P40 having the least abundance of the phylum ($P = 0.05$, Table 1). In addition to Firmicutes, Fibrobacterota and Proteobacteria abundances were affected by protein supplementation ($P < 0.05$) and tended to affect the abundance of Chloroflexi ($P = 0.09$; Table 1). In the rumen, Bacteroidota abundance increased with increasing crude protein as P40 heifers had the greatest abundance, and control heifers with the least ($P = 0.02$; Table 2). Actinobacteriota abundance in the rumen was also affected by protein supplementation, with P20 heifers having the greatest abundance ($P = 0.02$; Table 2). Additionally, Firmicutes, Proteobacteria, and Spirochaetota abundances tended to be affected by protein supplementation ($P \leq 0.10$; Table 2).

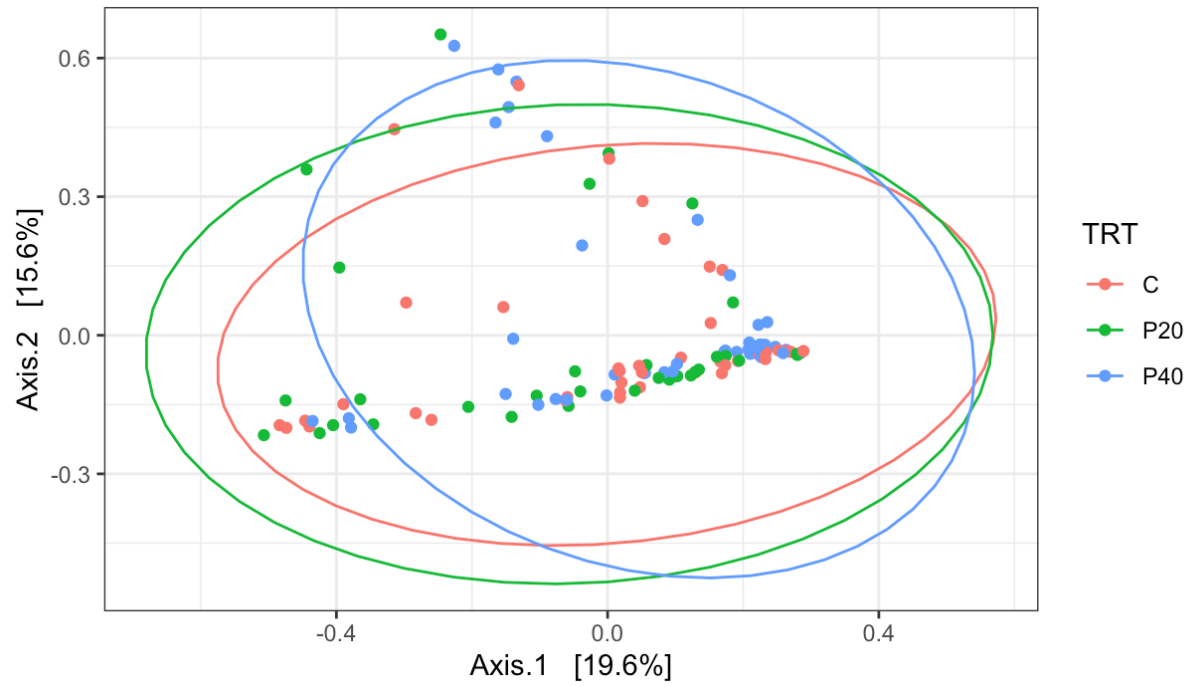


Figure 5. Beta diversity in the uterus by protein supplementation treatment ($P = 0.06$). Ellipses represent 95% confidence interval.

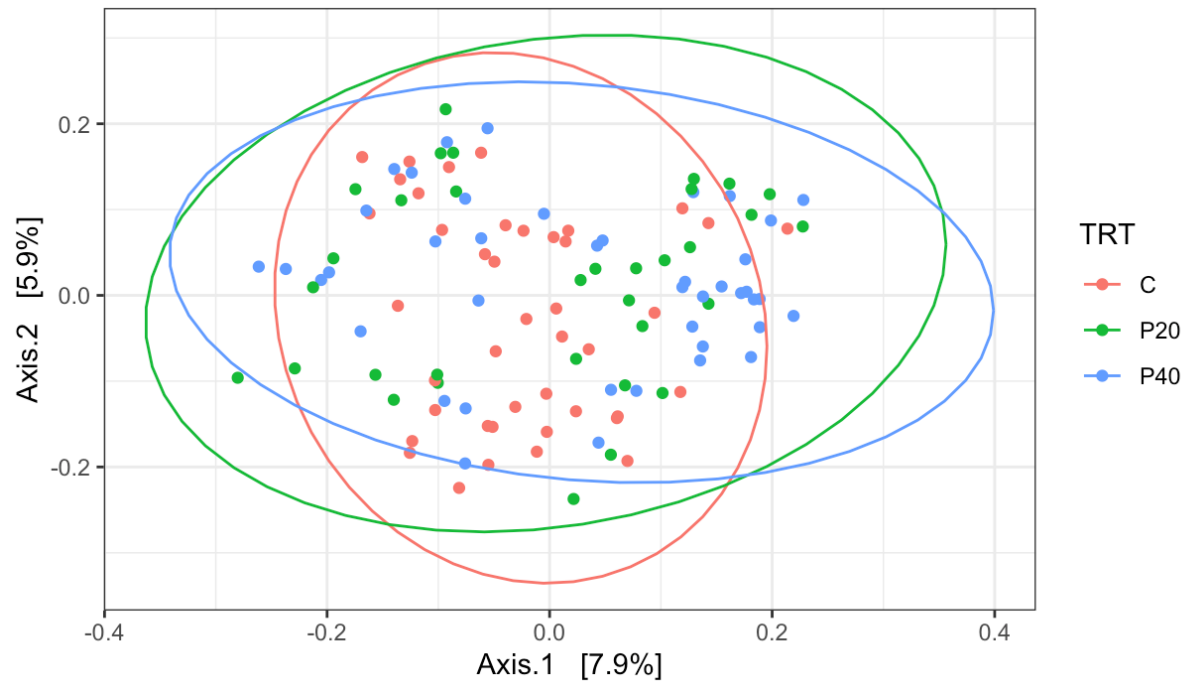


Figure 6. Beta diversity in the rumen by protein supplementation treatment ($P = 0.008$). Ellipses represent 95% confidence interval.

Table 1. Top 10 bacterial phyla in the uterus by protein supplementation treatment.

Phyla	Protein Supplementation Treatment ¹			<i>P</i> Value ²
	CON	P20	P40	
Actinobacteriota	2.55 ± 0.26	2.31 ± 0.27	2.15 ± 0.25	0.48
Bacteroidota	40.57 ± 2.29	37.55 ± 2.41	42.36 ± 2.22	0.42
Chloroflexi	0.12 ± 0.01	0.122 ± 0.01	0.09 ± 0.01	0.09
Elusimicrobiota	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.009	0.99
Fibrobacterota	1.40 ± 0.13 ^B	1.44 ± 0.14 ^B	1.92 ± 0.12 ^A	0.008
Firmicutes	48.92 ± 2.88 ^{AB}	52.72 ± 2.88 ^A	45.35 ± 2.61 ^B	0.05
Patescibacteria	1.37 ± 0.10	1.27 ± 0.10	1.38 ± 0.09	0.66
Proteobacteria	3.20 ± 0.59 ^{AB}	2.53 ± 0.62 ^B	4.99 ± 0.56 ^A	0.01
Spirochaetota	0.54 ± 0.04	0.47 ± 0.04	0.51 ± 0.04	0.60
Verrucomicrobiota	0.87 ± 0.06	0.89 ± 0.07	0.87 ± 0.06	0.94

¹ Relative abundances presented as Mean (%) ± SEM² ^{AB} represents $P \leq 0.05$

Table 2. Top 10 bacterial phyla in the rumen by protein supplementation treatment.

Phyla	Protein Supplementation Treatment ¹			<i>P</i> Value ²
	CON	P20	P40	
Actinobacteriota	0.074 ± 0.007 ^B	0.097 ± 0.007 ^A	0.069 ± 0.007 ^B	0.02
Bacteroidota	64.26 ± 0.77 ^B	65.60 ± 0.82 ^{AB}	67.39 ± 0.74 ^A	0.02
Chloroflexi	0.08 ± 0.006	0.07 ± .006	0.07 ± 0.005	0.25
Elusimicrobiota	0.13 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.15
Fibrobacterota	2.40 ± 0.18	2.12 ± 0.19	2.30 ± 0.17	0.55
Firmicutes	28.11 ± 0.77	27.32 ± 0.82	25.65 ± 0.74	0.07
Patescibacteria	1.20 ± 0.08	1.33 ± 0.09	1.23 ± 0.08	0.52
Proteobacteria	1.89 ± 0.14	1.46 ± 0.15	1.52 ± 0.14	0.06
Spirochaetota	0.52 ± 0.03	0.44 ± 0.03	0.41 ± 0.03	0.07
Verrucomicrobiota	1.04 ± 0.10	1.05 ± 0.11	1.00 ± 0.10	0.94

¹ Relative abundances presented as Mean (%) ± SEM² ^{AB} represents $P \leq 0.05$

Genera *Alistipes*, *Bacteroides*, *Fibrobacter*, *Prevotella*, *UCG-005*, and unclassified genera from Family Bacteroidales BS11 and Family Lachnospiraceae abundances were affected by protein supplementation in the uterus ($P \leq 0.05$; Table 3). The most abundant uterine genera, *Prevotella*, had the greatest abundance in the P40 supplement group, with lesser, similar abundances in the CON and P20 groups ($P = 0.02$; Table 3). Similarly in the rumen, the most abundant genera, *Prevotella*, increased in abundance with increasing crude protein level of the supplement ($P = 0.05$; Table 4). Protein supplementation level also affected ruminal relative abundances of the unclassified genera from Family Lachnospiraceae and Family UCG-010 ($P \leq 0.05$), and tended to affect *UCG-004* ($P = 0.10$; Table 4).

Pubertal Status

In the uterus, Observed ASV and Shannon's Diversity Index were affected by pubertal status. Pubertal heifers had a greater number of observed ASV ($P = 0.03$), and tended to have a higher Shannon Diversity Index value ($P = 0.08$), than pre-pubertal heifers (Table 5). Clustering was observed in the PCoA by pubertal status in uterine samples ($P = 0.001$), depicting high variation in pre-pubertal heifers and tighter clustering of pubertal heifers (Figure 7). However, pubertal status did not impact rumen alpha diversity metrics or beta diversity PCoA clustering ($P > 0.05$).

Pubertal status affected the abundance of the most abundant uterine bacterial phyla, Firmicutes, with pre-pubertal heifers having the greatest abundance compared to pubertal heifers ($P = 0.04$ Table 6). Although the abundance of Firmicutes was less in pubertal heifers, multiple phyla were greater or tended to be greater in pubertal heifers including Bacteroidota ($P = 0.06$), Elusimicrobiota ($P = 0.05$), Proteobacteria ($P = 0.04$), and Verrumicrobiota ($P = 0.07$; Table 6).

Table 3. Bacterial genera with relative abundance $\geq 1\%$ in the uterus by protein supplementation treatment.

Genera	Protein Supplementation Treatment ¹			P Value ²
	CON	P20	P40	
<i>Alistipes</i>	1.36 $\pm$ 0.23 ^A	1.27 $\pm$ 0.25 ^A	0.74 $\pm$ 0.22 ^B	0.04
<i>Bacteroides</i>	1.51 $\pm$ 0.22 ^A	1.31 $\pm$ 0.23 ^A	0.70 $\pm$ 0.21 ^B	0.006
<i>Candidatus</i> <i>Saccharimonas</i>	1.07 $\pm$ 0.08	0.98 $\pm$ 0.09	1.06 $\pm$ 0.08	0.71
<i>Christensenellaceae</i> <i>R-7 group</i>	4.15 $\pm$ 0.22	4.53 $\pm$ 0.23	4.09 $\pm$ 0.21	0.34
Family Eubacterium coprostanoligenes	1.56 $\pm$ 0.12	1.81 $\pm$ 0.13	1.50 $\pm$ 0.11	0.18
Family Bacteroidales BS11 gut group	1.04 $\pm$ 0.10 ^{AB}	0.88 $\pm$ 0.11 ^B	1.21 $\pm$ 0.10 ^A	0.03
Family Bacteroidales RF16 group	1.74 $\pm$ 0.22	1.66 $\pm$ 0.23	1.69 $\pm$ 0.21	0.71
Family F082	5.13 $\pm$ 0.41	4.69 $\pm$ 0.43	5.69 $\pm$ 0.39	0.17
Family Lachnospiraceae	5.12 $\pm$ 0.38 ^A	4.19 $\pm$ 0.40 ^A	3.22 $\pm$ 0.36 ^B	0.0008
Family Prevotellaceae	1.57 $\pm$ 0.13	1.39 $\pm$ 0.13	1.69 $\pm$ 0.12	0.24
Family UCG-010	2.95 $\pm$ 0.41	3.46 $\pm$ 0.43	2.58 $\pm$ 0.39	0.20
<i>Fibrobacter</i>	1.39 $\pm$ 0.13 ^B	1.43 $\pm$ 0.14 ^B	1.91 $\pm$ 0.19 ^A	0.008
Order Bacteroidales	1.47 $\pm$ 0.11	1.40 $\pm$ 0.12	1.23 $\pm$ 0.11	0.30
Order Clostridia UCG-014	1.93 $\pm$ 0.13	1.88 $\pm$ 0.14	1.87 $\pm$ 0.13	0.93
Order RF39	1.56 $\pm$ 0.10	1.58 $\pm$ 0.10	1.48 $\pm$ 0.09	0.73
<i>Prevotella</i>	12.08 $\pm$ 1.22 ^B	11.06 $\pm$ 1.30 ^B	15.09 $\pm$ 1.18 ^A	0.02
<i>Prevotellaceae</i> UCG- 001	1.61 $\pm$ 0.13	1.56 $\pm$ 0.14	1.82 $\pm$ 0.13	0.28
<i>Prevotellaceae</i> UCG- 003	2.13 $\pm$ 0.13	2.01 $\pm$ 0.14	2.12 $\pm$ 0.12	0.73
<i>Rikenellaceae</i> RC9 gut group	6.53 $\pm$ 0.35	6.11 $\pm$ 0.38	6.25 $\pm$ 0.34	0.66
<i>Ruminococcus</i> UCG-004	1.09 $\pm$ 0.08	1.17 $\pm$ 0.09	1.19 $\pm$ 0.08	0.53
UCG-005	0.89 $\pm$ 0.08	0.70 $\pm$ 0.08	0.63 $\pm$ 0.07	0.91
<i>Ureaplasma</i>	4.89 $\pm$ 0.61 ^A	4.45 $\pm$ 0.64 ^A	2.29 $\pm$ 0.58 ^B	0.006
	7.12 $\pm$ 3.50	9.30 $\pm$ 3.70	10.94 $\pm$ 3.32	0.92

¹ Relative abundances presented as Mean (%) $\pm$ SEM

² ^{AB} represents $P \leq 0.05$

Table 4. Bacterial genera with relative abundance $\geq 1\%$ in the rumen by protein supplementation treatment.

Genera	Protein Supplementation Treatment ¹			P Value ²
	CON	P20	P40	
<i>Candidatus</i>	0.82 $\pm$ 0.08	0.91 $\pm$ 0.09	0.83 $\pm$ 0.08	0.70
<i>Saccharimonas</i>				
<i>Christensenellaceae</i> R7 group	3.80 $\pm$ 0.17	4.11 $\pm$ 0.18	3.83 $\pm$ 0.16	0.42
Family Eubacterium coprostanoligenes	1.08 $\pm$ 0.07	1.26 $\pm$ 0.07	1.16 $\pm$ 0.07	0.28
Family Bacteroidales BS11 gut group	1.26 $\pm$ 0.16	1.18 $\pm$ 0.17	1.61 $\pm$ 0.15	0.17
Family Bacteroidales RF16 group	5.34 $\pm$ 0.56	4.87 $\pm$ 0.60	3.93 $\pm$ 0.54	0.28
Family Bacteroidales UCG-001	1.45 $\pm$ 0.07	1.35 $\pm$ 0.08	1.30 $\pm$ 0.07	0.36
Family F082	5.61 $\pm$ 0.60	6.02 $\pm$ 0.63	6.53 $\pm$ 0.59	0.56
Family Lachnospiraceae	2.43 $\pm$ 0.08 ^A	2.01 $\pm$ 0.08 ^B	1.99 $\pm$ 0.08 ^B	0.0002
Family Muribaculaceae	1.65 $\pm$ 0.21	1.59 $\pm$ 0.22	1.53 $\pm$ 0.21	0.91
Family p-251-o5	0.90 $\pm$ 0.09	1.02 $\pm$ 0.10	1.06 $\pm$ 0.09	0.45
Family Prevotellaceae	2.22 $\pm$ 0.10	2.21 $\pm$ 0.10	2.03 $\pm$ 0.09	0.28
Family UCG-010	0.97 $\pm$ 0.04 ^B	1.15 $\pm$ 0.04 ^A	1.00 $\pm$ 0.04 ^B	0.01
<i>Fibrobacter</i>	2.40 $\pm$ 0.18	2.11 $\pm$ 0.19	2.29 $\pm$ 0.17	0.58
<i>NK4A214</i> group	0.90 $\pm$ 0.08	1.03 $\pm$ 0.08	0.88 $\pm$ 0.08	0.39
Order Clostridia UCG-014	1.66 $\pm$ 0.24	1.56 $\pm$ 0.25	1.38 $\pm$ 0.22	0.61
Order RF39	1.84 $\pm$ 0.18	1.59 $\pm$ 0.19	1.54 $\pm$ 0.18	0.41
Order Rhodospirillales	1.37 $\pm$ 0.12	1.14 $\pm$ 0.13	1.25 $\pm$ 0.11	0.40
<i>Prevotella</i>	28.03 $\pm$ 1.18 ^B	29.38 $\pm$ 1.26 ^{AB}	31.95 $\pm$ 1.14 ^A	0.05
<i>Prevotellaceae</i> UCG-001	3.19 $\pm$ 0.12	3.27 $\pm$ 0.13	3.02 $\pm$ 0.12	0.34
<i>Prevotellaceae</i> UCG-003	3.58 $\pm$ 0.21	3.92 $\pm$ 0.22	3.86 $\pm$ 0.20	0.32
<i>Rikenellaceae</i> RC9 gut group	8.85 $\pm$ 0.39	8.63 $\pm$ 0.42	8.29 $\pm$ 0.37	0.57
<i>Ruminococcus</i>	1.42 $\pm$ 0.06	1.49 $\pm$ 0.07	1.30 $\pm$ 0.06	0.12
<i>Saccharofementans</i>	0.84 $\pm$ 0.07	0.94 $\pm$ 0.07	0.86 $\pm$ 0.07	0.60
<i>Succiniclasticum</i>	0.88 $\pm$ 0.09	0.96 $\pm$ 0.10	0.85 $\pm$ 0.09	0.74
<i>UCG-004</i>	1.36 $\pm$ 0.13	1.29 $\pm$ 0.14	0.95 $\pm$ 0.13	0.10

¹ Relative abundances presented as Mean (%) $\pm$ SEM

² ^{AB} represents $P \leq 0.05$

Table 5. Alpha diversity metrics of the rumen and uterus by pubertal status.

Environment	Alpha Diversity	Pubertal Status		<i>P</i> Value ¹
		No	Yes	
Rumen	Observed ASV	2709 ± 86	2662 ± 111	0.78
	Shannon	7.13 ± 0.04	7.10 ± 0.05	0.65
Uterine	Observed ASV	5499 ± 341 ^B	7013 ± 449 ^A	0.03
	Shannon	6.75 ± 0.23	7.54 ± 0.30	0.08

¹ ^{AB} represents $P \leq 0.05$

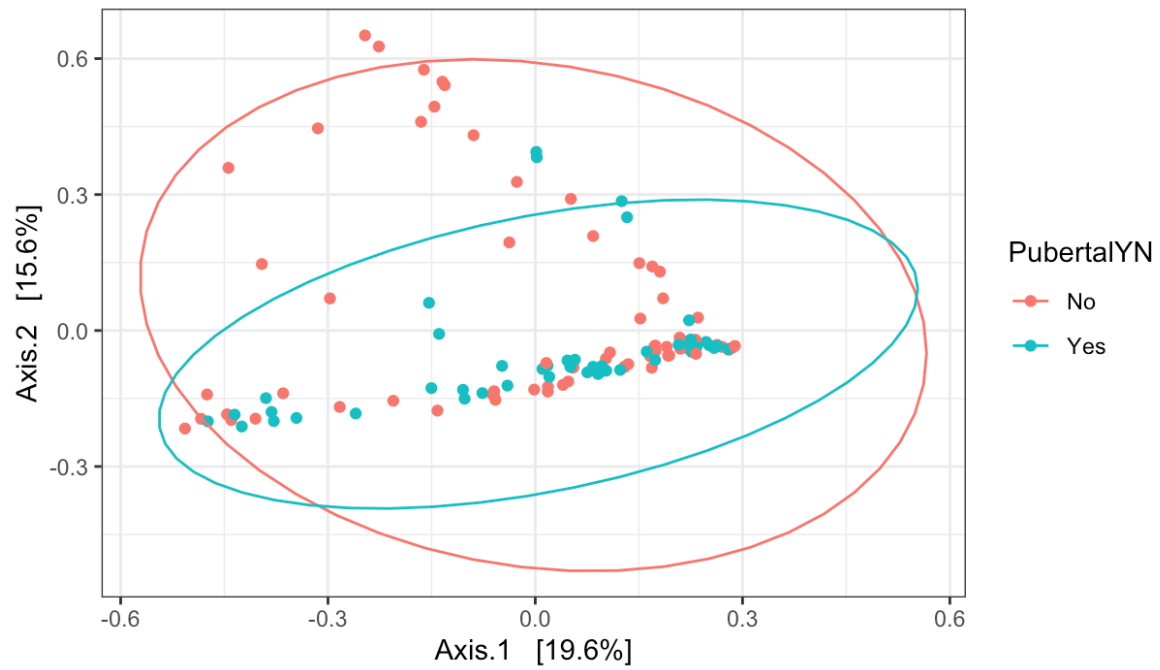


Figure 7. Beta diversity in the uterus and pubertal status ($P = 0.001$). Ellipses represent 95% confidence interval.

Table 6. Top 10 bacterial phyla in the uterus by pubertal status.

Phyla	Pubertal Status ¹		<i>P</i> Value ²
	Pre-Pubertal	Pubertal	
Actinobacteriota	2.02 ± 0.24	2.65 ± 0.31	0.15
Bacteroidota	37.19 ± 2.19	43.13 ± 2.66	0.06
Chloroflexi	0.10 ± 0.01	0.12 ± 0.01	0.26
Elusimicrobiota	0.10 ± 0.009 ^B	0.14 ± 0.01 ^A	0.05
Fibrobacterota	1.49 ± 0.12	1.69 ± 0.16	0.42
Firmicutes	53.85 ± 2.53 ^A	44.15 ± 3.21 ^B	0.04
Patescibacteria	1.24 ± 0.09	1.43 ± 0.12	0.29
Proteobacteria	2.51 ± 0.54 ^B	4.64 ± 0.72 ^A	0.04
Spirochaetota	0.49 ± 0.04	0.53 ± 0.05	0.62
Verrucomicrobiota	0.77 ± 0.06	0.98 ± 0.07	0.07

¹ Relative abundances presented as Mean (%) ± SEM

² ^{AB} represents $P \leq 0.05$

In the rumen, pubertal status did not affect the abundance of Bacteroidota ($P \geq 0.10$). However, pubertal status affected the ruminal abundance of Actinobacteria ($P = 0.03$), Firmicutes ($P = 0.01$), and Proteobacteria ($P = 0.02$; Table 7). Similar to the uterus, Firmicutes were decreased, while Proteobacteria was increased in the rumen of pubertal heifers compared to pre-pubertal heifers (Table 7).

The relative abundance of unclassified genera from Family F082 ($P = 0.04$) was affected in the uterus by pubertal status, and tended to affect *Prevotellaceae-UCG001*, *Ureaplasma*, and unclassified genera from Family Bacteroidales BS11 and Family Prevotellaceae ($P \leq 0.10$; Table 8). All of these genera, except *Ureaplasma*, had a greater relative abundance in pubertal heifers (Table 8). Puberty affected ruminal relative abundance of *NK4A214* and *Succiniclasicum*, which were greater in pre-pubertal heifers, and unclassified genera from Order Rhodospirillales which was greater in pubertal heifers ($P \leq 0.05$; Table 8).

Day of Supplementation

Observed ASV in the uterus were affected by day of supplementation. The least observed ASV was detected on d 56, then significantly increased to the greatest observed ASV by d 112 and remained similar on d 163 ($P = 0.0002$; Table 9). Shannon diversity index followed a similar trend but was not significant ($P = 0.81$; Table 9). Ruminal observed ASV and Shannon's Diversity were affected by day, with the greatest number detected on d 112 and less observed ASV on d 56 and d 163 ($P < 0.0001$; Table 9).

Beta diversity analysis by PCoA indicated uterine samples clustered by day with overlap ($P = 0.001$, Figure 8). Samples on d 56 had the greatest variation overlapping with d 112 and 163 uterine samples. However, d 112 and 163 samples clustered tightly and depicted the greatest

Table 7. Top 10 bacterial phyla in the rumen by pubertal status.

Phyla	Pubertal Status ¹		<i>P</i> Value ²
	Pre-Pubertal	Pubertal	
Actinobacteriota	0.09 ± 0.006 ^A	0.07 ± 0.008 ^B	0.03
Bacteroidota	64.57 ± 0.73	66.92 ± 0.98	0.12
Chloroflexi	0.08 ± 0.005	0.07 ± 0.007	0.14
Elusimicrobiota	0.10 ± 0.01	0.13 ± 0.01	0.17
Fibrobacterota	2.10 ± 0.17	2.45 ± 0.22	0.31
Firmicutes	28.90 ± 0.73 ^A	25.15 ± 0.97 ^B	0.01
Patescibacteria	1.25 ± 0.08	1.25 ± 0.10	0.96
Proteobacteria	1.33 ± 0.13 ^B	1.91 ± 0.17 ^A	0.02
Spirochaetota	0.43 ± 0.02	0.48 ± 0.03	0.20
Verrucomicrobiota	1.01 ± 0.08	1.05 ± 0.10	0.76

¹ Relative abundances presented as Mean (%) ± SEM² ^{AB} represents $P \leq 0.05$

Table 8. Bacterial genera with relative abundance $\geq 1\%$ in the uterus and rumen affected by pubertal status.

Uterine Genera	Pubertal Status ¹		<i>P</i> Value ²
	Pre-Pubertal	Pubertal	
Family Bacteroidales	0.90 $\pm$ 0.10	1.18 $\pm$ 0.11	0.07
BS11 gut group			
Family F082	4.45 $\pm$ 0.39 ^B	5.90 $\pm$ 0.49 ^A	0.04
Family Prevotellaceae	1.35 $\pm$ 0.12	1.75 $\pm$ 0.15	0.08
<i>Prevotellaceae</i> UCG-001	1.48 $\pm$ 0.13	1.85 $\pm$ 0.16	0.09
<i>Ureaplasma</i>	13.78 $\pm$ 3.19	4.46 $\pm$ 4.25	0.07
Rumen Genera			
<i>NK4A214</i> group	1.04 $\pm$ 0.06 ^A	0.83 $\pm$ 0.07 ^B	0.03
Order Rhodospirillales	0.98 $\pm$ 0.10 ^B	1.53 $\pm$ 0.13 ^A	0.006
<i>Succiniclasticum</i>	1.04 $\pm$ 0.07 ^A	0.76 $\pm$ 0.08 ^B	0.007

¹ Relative abundances presented as Mean (%) $\pm$ SEM

² ^{AB} represents $P \leq 0.05$

Table 9. Alpha diversity metrics of the rumen and uterus by day of supplementation.

Environment	Alpha Diversity	Day of Supplementation			<i>P</i> Value ¹
		56	112	163	
Rumen	Observed ASV	2161 ± 117 ^B	3367 ± 92 ^A	2529 ± 108 ^B	< 0.0001
	Shannon	6.88 ± 0.05 ^B	7.30 ± 0.04 ^A	7.17 ± 0.05 ^A	< 0.0001
Uterus	Observed ASV	4738 ± 490 ^B	7178 ± 381 ^A	6852 ± 455 ^A	0.002
	Shannon	7.10 ± 0.34	7.03 ± 0.26	7.31 ± 0.31	0.81

¹ ^{AB} represents $P \leq 0.05$

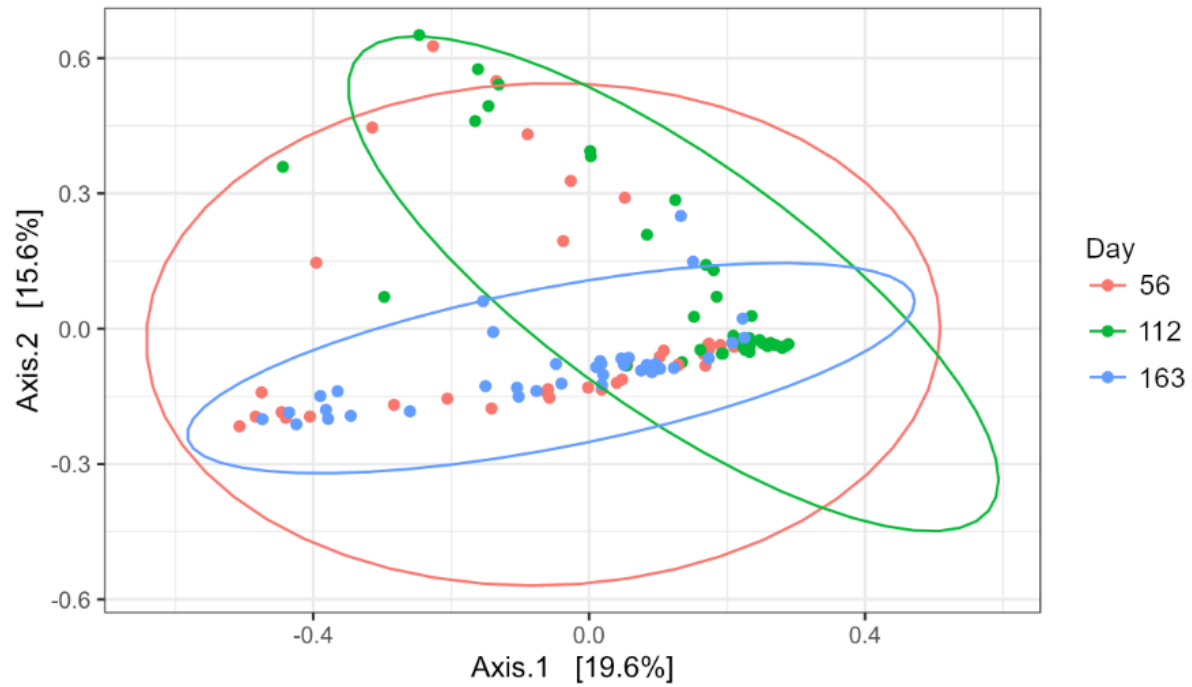


Figure 8. Beta diversity in the uterus by day of supplementation ($P = 0.001$). Ellipses represent 95% confidence interval.

separation between days, with slight overlap (Figure 8). Similar to the uterus, rumen samples also clustered by day with overlap ($P = 0.001$, Figure 9). The greatest separation was observed between d 56 and 112 indicating the greatest difference in overall community composition.

In the uterus, day of sampling tended to affect the abundance of Firmicutes ($P = 0.09$, Table 10). Multiple additional phyla also shifted over time including Actinobacteriota ($P = 0.004$), Chloroflexi ($P = 0.04$), Fibrobacterota ($P = 0.07$), and Patescibacteria ($P < 0.0001$; Table 10). Of the 23 genera detected in uterus with a relative abundance $\geq 1\%$, 16 shifted over time ($P \leq 0.10$; Table 11). All top 10 phyla detected in the rumen, except Fibrobacterota, were affected by the day of sampling ($P \leq 0.05$; Table 12). The most abundant phyla in the rumen, Bacteroidota, had the greatest abundance on d 56, with lesser, similar abundances on d 112 and 163 ($P < 0.0001$; Table 12). Additionally, all rumen genera detected with relative abundance $\geq 1\%$ shifted over time ($P \leq 0.10$; Table 13), except for unclassified genera from Family Prevotellaceae.

Interactions of Protein Supplementation Level, Day of Supplementation, and Pubertal Status

The triple interaction of protein supplementation level, day of supplementation, and pubertal status, and the interaction of day of supplementation $\times$ pubertal status, was not able to be analyzed in the current study due to all heifers were pre-pubertal at the first sampling on d 56. For the interaction of protein supplementation level $\times$ day of supplementation, no significance was detected for alpha diversity metrics in the uterus and rumen, or for any of the top 10 uterine bacterial phyla relative abundances ($P > 0.10$). For uterine genera, however, a protein supplementation $\times$ day interaction was observed for *UCG-005* ($P = 0.04$) and unclassified genera from Family Lachnospiraceae ($P = 0.009$). In the rumen, a supplement $\times$ day interaction was detected for phyla Bacteroidota ($P = 0.01$), Firmicutes ($P = 0.02$) and Spirochaetota ($P = 0.04$),

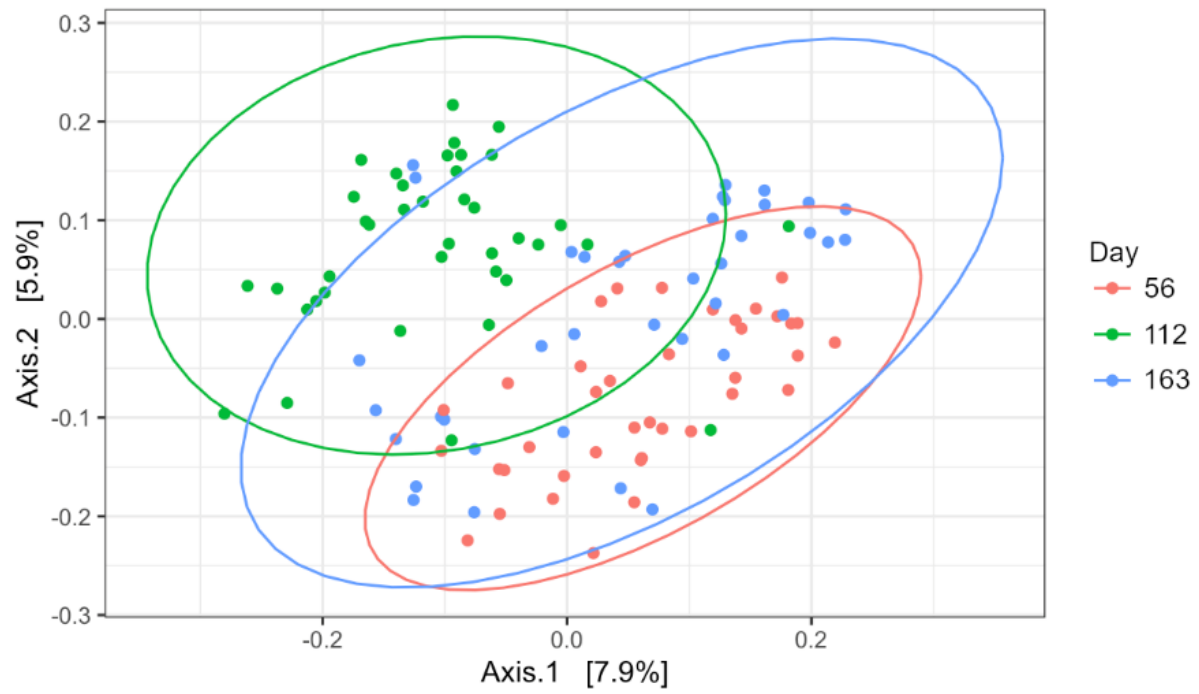


Figure 9. Beta diversity in the rumen by day of supplementation ($P = 0.001$). Ellipses represent 95% confidence interval.

Table 10. Top 10 bacterial phyla in the uterus by day of supplementation.

Phyla	Day of Supplementation ¹			<i>P</i> Value ²
	56	112	163	
Actinobacteriota	3.27 ± 0.35 ^A	2.37 ± 0.28 ^B	1.38 ± 0.33 ^C	0.004
Bacteroidota	41.44 ± 2.97	41.67 ± 2.41	37.36 ± 2.81	0.69
Chloroflexi	0.12 ± 0.01 ^{AB}	0.13 ± 0.01 ^A	0.09 ± 0.01 ^B	0.04
Elusimicrobiota	0.11 ± 0.01	0.14 ± 0.01	0.11 ± 0.01	0.19
Fibrobacterota	1.43 ± 0.18	1.85 ± 0.14	1.49 ± 0.17	0.07
Firmicutes	47.07 ± 3.61	45.81 ± 2.82	54.11 ± 0.03	0.09
Patescibacteria	1.04 ± 0.13 ^B	1.74 ± 0.10 ^A	1.23 ± 0.12 ^B	<0.0001
Proteobacteria	3.52 ± 0.82	4.41 ± 0.62	2.79 ± 0.76	0.21
Spirochaetota	0.57 ± 0.05	0.47 ± 0.04	0.48 ± 0.05	0.26
Verrucomicrobiota	0.92 ± 0.08	0.92 ± 0.06	0.78 ± 0.07	0.39

¹ Relative abundances presented as Mean (%) ± SEM² ^{AB} represents $P \leq 0.05$

Table 11. Bacterial genera with relative abundance $\geq 1\%$ in the uterus by day of supplementation.

Genera	Day of Supplementation ¹			P Value ²
	56	112	163	
<i>Alistipes</i>	1.46 $\pm$ 0.33 ^A	0.24 $\pm$ 0.25 ^B	1.68 $\pm$ 0.31 ^A	<0.0001
<i>Bacteroides</i>	1.43 $\pm$ 0.31 ^A	0.24 $\pm$ 0.24 ^B	1.85 $\pm$ 0.29 ^A	<0.0001
<i>Candidatus</i> <i>Saccharimonas</i>	0.79 $\pm$ 0.11 ^B	1.37 $\pm$ 0.08 ^A	0.95 $\pm$ 0.10 ^B	<0.0001
<i>Christensenellaceae</i> <i>R-7 group</i>	4.28 $\pm$ 0.30	4.54 $\pm$ 0.23	3.96 $\pm$ 0.27	0.28
Family Eubacterium coprostanoligenes	1.63 $\pm$ 0.14 ^{AB}	1.33 $\pm$ 0.11 ^B	1.92 $\pm$ 0.13	0.004
Family Bacteroidales BS11 gut group	1.13 $\pm$ 0.13 ^A	1.27 $\pm$ 0.10 ^A	0.73 $\pm$ 0.12 ^B	0.003
Family Bacteroidales RF16 group	1.47 $\pm$ 0.30	1.72 $\pm$ 0.23	1.91 $\pm$ 0.28	0.58
Family F082	4.89 $\pm$ 0.55 ^B	6.44 $\pm$ 0.43 ^A	4.18 $\pm$ 0.52 ^B	0.001
Family Lachnospiraceae	3.78 $\pm$ 0.56 ^B	2.48 $\pm$ 0.42 ^C	6.27 $\pm$ 0.52	<0.0001
Family Prevotellaceae	1.45 $\pm$ 0.17 ^B	1.95 $\pm$ 0.13 ^A	1.24 $\pm$ 0.16 ^B	0.0007
Family UCG010	2.97 $\pm$ 0.61 ^A	1.68 $\pm$ 0.46 ^B	4.35 $\pm$ 0.56 ^A	0.0009
<i>Fibrobacter</i>	1.42 $\pm$ 0.18	1.84 $\pm$ 0.14	1.48 $\pm$ 0.17	0.07
Order Bacteroidales	1.44 $\pm$ 0.15	1.20 $\pm$ 0.11	1.45 $\pm$ 0.14	0.17
Order Clostridia UCG014	1.52 $\pm$ 0.18 ^B	2.33 $\pm$ 0.14 ^A	1.83 $\pm$ 0.17 ^B	0.0005
Order RF39	1.25 $\pm$ 0.14 ^B	1.74 $\pm$ 0.10 ^A	1.64 $\pm$ 0.13 ^{AB}	0.01
<i>Prevotella</i>	14.14 $\pm$ 1.56	13.46 $\pm$ 1.27	10.62 $\pm$ 1.47	0.28
<i>Prevotellaceae</i> UCG- 001	1.66 $\pm$ 0.18 ^{AB}	1.93 $\pm$ 14 ^A	1.40 $\pm$ 0.17 ^B	0.04
<i>Prevotellaceae</i> UCG- 003	2.14 $\pm$ 0.18	2.24 $\pm$ 0.14	1.88 $\pm$ 0.17	0.30
<i>Rikenellaceae</i> RC9 gut group	5.93 $\pm$ 0.45	6.83 $\pm$ 0.36	6.12 $\pm$ 0.43	0.11
<i>Ruminococcus</i>	1.12 $\pm$ 0.11	1.23 $\pm$ 0.09	1.10 $\pm$ 0.11	0.51
UCG-004	0.63 $\pm$ 0.12 ^B	1.10 $\pm$ 0.09 ^A	0.90 $\pm$ 0.11 ^{AB}	0.003
UCG-005	4.25 $\pm$ 0.83 ^A	0.83 $\pm$ 0.64 ^B	6.55 $\pm$ 0.78	<0.0001
<i>Ureaplasma</i>	6.48 $\pm$ 4.81	14.00 $\pm$ 3.63	6.88 $\pm$ 4.45	0.07

¹ Relative abundances presented as Mean (%) $\pm$ SEM² ^{AB} represents $P \leq 0.05$

Table 12. Top 10 bacterial phyla in the rumen by day of supplementation.

Phyla	Day of Supplementation ¹			<i>P</i> Value ²
	56	112	163	
Actinobacteriota	0.045 ± 0.009 ^B	0.096 ± 0.007 ^A	0.098 ± 0.008 ^A	<0.0001
Bacteroidota	72.51 ± 1.18 ^A	61.22 ± 0.88 ^B	63.52 ± 1.10 ^B	<0.0001
Chloroflexi	0.06 ± 0.007 ^B	0.08 ± 0.006 ^A	0.09 ± 0.007 ^A	0.01
Elusimicrobiota	0.10 ± 0.01 ^B	0.15 ± 0.01 ^A	0.09 ± 0.01 ^B	0.0004
Fibrobacterota	1.86 ± 0.26 ^B	2.18 ± 0.20 ^{AB}	2.77 ± 0.24 ^A	0.11
Firmicutes	21.09 ± 1.15 ^B	30.07 ± 0.86 ^A	29.92 ± 1.07 ^A	<0.0001
Patescibacteria	0.57 ± 0.11 ^C	1.92 ± 0.09 ^A	1.26 ± 0.10 ^B	<0.0001
Proteobacteria	1.95 ± 0.19 ^A	2.10 ± 0.15 ^A	0.81 ± 0.17 ^B	<0.0001
Spirochaetota	0.46 ± 0.03 ^{AB}	0.50 ± 0.03 ^A	0.41 ± 0.03 ^B	0.05
Verrucomicrobiota	0.98 ± 0.10	1.17 ± 0.08	0.94 ± 0.10	0.06

¹ Relative abundances presented as Mean (%) ± SEM² ^{AB} represents $P \leq 0.05$

Table 13. Bacterial genera with relative abundance $\geq 1\%$ in the rumen by day of supplementation.

Genera	Day of Supplementation ¹			P Value ²
	56	112	163	
<i>Candidatus</i>	0.37 $\pm$ 0.10 ^C	1.45 $\pm$ 0.08 ^A	0.74 $\pm$ 0.09 ^B	<0.0001
<i>Saccharimonas</i>				
<i>Christensenellaceae</i> R7 group	3.30 $\pm$ 0.24 ^B	4.26 $\pm$ 0.19 ^A	4.19 $\pm$ 0.23 ^A	0.004
Family Eubacterium coprostanoligenes	0.81 $\pm$ 0.07 ^C	1.48 $\pm$ 0.06 ^A	1.20 $\pm$ 0.07 ^B	<0.0001
Family Bacteroidales BS11 gut group	1.30 $\pm$ 0.16	1.58 $\pm$ 0.13	1.18 $\pm$ 0.16	0.06
Family Bacteroidales RF16 group	6.31 $\pm$ 0.72 ^A	5.25 $\pm$ 0.55 ^A	2.58 $\pm$ 0.67 ^B	0.004
Family Bacteroidales UCG-001	1.51 $\pm$ 0.10 ^A	1.42 $\pm$ 0.08 ^{AB}	1.16 $\pm$ 0.09 ^B	0.10
Family F082	5.91 $\pm$ 0.57 ^{AB}	6.85 $\pm$ 0.47 ^A	5.40 $\pm$ 0.54 ^B	0.04
Family Lachnospiraceae	1.95 $\pm$ 0.11 ^B	2.40 $\pm$ 0.09 ^A	2.08 $\pm$ 0.10 ^B	0.001
Family Muribaculaceae	1.35 $\pm$ 0.22 ^B	1.06 $\pm$ 0.17 ^B	2.37 $\pm$ 0.21 ^A	<0.0001
Family p-251-o5	0.95 $\pm$ 0.12	0.84 $\pm$ 0.09	1.19 $\pm$ 0.11	0.06
Family Prevotellaceae	2.07 $\pm$ 0.13	2.05 $\pm$ 0.10	2.34 $\pm$ 0.12	0.26
Family UCG-010	0.74 $\pm$ 0.06 ^B	1.26 $\pm$ 0.05 ^A	1.12 $\pm$ 0.06 ^A	<0.0001
<i>Fibrobacter</i>	1.86 $\pm$ 0.26 ^B	2.17 $\pm$ 0.20 ^{AB}	2.77 $\pm$ 0.24 ^A	0.10
<i>NK4A214</i> group	0.66 $\pm$ 0.08 ^C	0.84 $\pm$ 0.07 ^B	1.30 $\pm$ 0.08 ^A	<0.0001
Order Clostridia UCG-014	0.78 $\pm$ 0.32 ^B	1.72 $\pm$ 0.25 ^A	2.09 $\pm$ 0.30 ^A	0.003
Order RF39	0.87 $\pm$ 0.20 ^B	2.11 $\pm$ 0.16 ^A	2.00 $\pm$ 0.19 ^A	<0.0001
Order Rhodospirillales	1.45 $\pm$ 0.14 ^A	1.71 $\pm$ 0.11 ^A	0.60 $\pm$ 0.13 ^B	<0.0001
<i>Prevotella</i>	36.45 $\pm$ 1.75 ^A	22.63 $\pm$ 1.33 ^C	30.27 $\pm$ 1.65 ^B	<0.0001
<i>Prevotellaceae</i> UCG-001	3.36 $\pm$ 0.16 ^A	2.89 $\pm$ 0.12 ^B	3.23 $\pm$ 0.14 ^{AB}	0.01
<i>Prevotellaceae</i> UCG-003	4.55 $\pm$ 0.25 ^A	3.97 $\pm$ 0.21 ^B	2.84 $\pm$ 0.24 ^C	<0.0001
<i>Rikenellaceae</i> RC9 gut group	6.84 $\pm$ 0.50 ^B	10.55 $\pm$ 0.38 ^A	8.38 $\pm$ 0.46 ^B	<0.0001
<i>Ruminococcus</i>	0.92 $\pm$ 0.09 ^B	1.54 $\pm$ 0.07 ^A	1.75 $\pm$ 0.08 ^A	<0.0001
<i>Saccharofementans</i>	0.65 $\pm$ 0.06 ^C	1.11 $\pm$ 0.05 ^A	0.88 $\pm$ 0.06 ^B	<0.0001
<i>Succiniclasticum</i>	0.66 $\pm$ 0.08 ^B	0.79 $\pm$ 0.07 ^B	1.24 $\pm$ 0.08 ^A	<0.0001
<i>UCG-004</i>	0.96 $\pm$ 0.15 ^B	1.49 $\pm$ 0.12 ^A	1.16 $\pm$ 0.14 ^{AB}	0.001

¹ Relative abundances presented as Mean (%) $\pm$ SEM

² ^{AB} represents $P \leq 0.05$

and tendencies for Actinobacteria ($P = 0.10$) and Chloroflexi ($P = 0.09$). Ruminal genera *Prevotella*, *UCG-004*, and unclassified genera from Family Lachnospiraceae ($P \leq 0.05$) were affected by the interaction. Similarly, for the interaction of protein supplementation level $\times$ pubertal status, no alpha diversity metrics or genera relative abundances in the uterus and rumen were affected ($P > 0.10$). The only significant interaction detected was the phyla Actinobacteria in the rumen ($P = 0.03$).

Discussion

During heifer development, management changes such as nutritional supplementation to support growth and development, and physiological changes such as the attainment of puberty are occurring. Ruminal and uterine bacterial communities have been established as playing important roles in feed efficiency and breeding outcome success, respectively (Myer et al., 2015; Ault et al., 2019a). Therefore, bacterial communities of these environments may be affected during heifer development and potentially influence long term productivity of the heifer. The first objective of the current study was to evaluate the impact of differing levels of supplemented crude protein throughout heifer development on ruminal and uterine bacterial communities. Ruminal bacterial communities are well known to shift with changes in dietary feedstuffs or nutrient composition (McCann et al., 2014; Clemmons et al., 2019a; Gruninger et al., 2019). As expected, ruminal bacterial communities were affected by differences in protein supplementation treatment. *Prevotella*, the most abundant genera detected in the rumen, and its respective phylum Bacteroidota, were observed to have greater abundances as supplement crude protein level increased. Previous studies agree *Prevotella* spp. are commonly the dominant genera in the rumen, although abundances may differ by diet type with increasing concentrates resulting in increased

Prevotella spp. (Stevenson and Weimer, 2007; Pitta et al., 2014). Additionally, abundance of *Prevotella* has been greater in heifers than mature cows (Liu et al., 2017), and different between cattle of differing feed efficiencies (Carberry et al., 2012; Myer et al., 2015). Although functionally diverse, *Prevotella* spp. are well established as major proteolytic bacterial species in the rumen and capable of proteolysis via a variety of mechanisms (Wallace, 1996; Griswold et al., 1999). Multiple studies have observed a greater abundance of *Prevotella* spp. in diets with greater crude protein content (Belanche et al., 2012; Pitta et al., 2014), as observed in the current study. Due to the high proteolytic activity of *Prevotella*, increases in its abundance potentially impact nitrogen utilization and amino acids reaching the lower gastrointestinal tract for absorption and use by the host (Bach et al., 2005). However, companion data from the current study did not observe changes in uterine pH based on protein supplementation treatment, and only two amino acid concentrations in the uterine luminal fluid were impacted (Brandt, 2020).

Previous studies have not evaluated the impact of nutrition on uterine bacterial communities in bovine. In the current study, only protein level of the supplement was modified between treatment groups, while the base diet was balanced. With the protein supplement, overall diet crude protein was 11%, 15%, and 19% among groups. Regardless, the relative abundance of multiple bacterial phyla and genera were affected in the uterus by protein supplementation differences. Although Firmicutes was the most abundant bacterial phyla detected in the uterus, *Prevotella* was the most abundant genera and increased in abundance with increasing supplemented crude protein, similar to ruminal *Prevotella*. Interestingly, increased *Prevotella* spp. in the uterus is typically associated with uterine disease in mature cows (Sheldon et al., 2009; Galvao et al., 2019). The relative abundance of *Prevotella* spp. in the uterus of healthy cows has

been previously observed to range between 12% to <2% on average (Clemmons et al., 2017; Ault et al., 2019a). Additionally, *Prevotella* was indicated as potentially negatively affecting breeding success, being one of eight bacteria that had an abundance >1% in resulting non-pregnant cows while <1% in resulting pregnant cows prior to breeding (Ault et al., 2019a). However, limited research exists on the uterine microbiome of heifers between weaning and breeding. Pubertal, virgin heifers have been associated with the presence of *Prevotella*, and other uterine disease associated bacteria, on endometrial biopsies from the uterus (Moore et al., 2017). Likewise, the current study found common uterine pathogen *Ureaplasma* to also be highly abundant in the uterus of heifers, but was not affected by protein supplementation level and did not shift over time. Other bacteria not associated as uterine pathogens were also affected by protein supplementation, such as *Alistipes* and *Bacteroides*, with the greatest abundance detected in control and P40 groups. However, these phyla have been associated with greater abundances in the lower gastrointestinal tract and feces of ruminants (Holman and Gyzl, 2019). These genera were detected in >1% relative abundance in uterine samples but not rumen samples of the current study. Vaginal bacterial communities commonly share genera with gastrointestinal and fecal bacteria, which can ascend into the uterus (Clemmons et al., 2017), or possibly enter the uterine environment through blood (Jeon et al., 2017). *Alistipes* and *Bacteroides* have been recognized as core vaginal bacteria (Rodrigues et al., 2015), then also identified as potential predictors of reproductive success based on abundance in fecal samples (McClure, 2018). Further research is needed to understand the role of potentially pathogenic bacteria from gastrointestinal sources, and their effects on the uterine environment and endometrial tissue. Additionally, despite the minimal differences in overall diet crude protein, differences in uterine bacterial communities by protein supplementation treatments

were detected. Greater differences in diet such as different forage to concentrate ratios, energy supply, or other overall nutrient levels may have a larger impact on additional bacteria in the uterus. Therefore, the ability of protein supplementation to affect the abundances of bacteria in the uterus may allow for manipulation of the microbiome toward an optimal uterine environment.

The attainment of puberty is a significant milestone in heifer development. The activity of endometrial tissues and the uterine environment changes with the onset of puberty including tissue gene expression, hormonal signal response, and uterine histotrophic secretions (Cánovas et al., 2014; Fortes et al., 2018). Therefore, an additional objective of the current study was to evaluate the impact of pubertal status on bacterial communities, beginning with pre-pubertal heifers and ending two weeks following the first breeding. Alpha diversity metrics indicated differences in the uterus, as observed ASV's increased over time and were greater in pubertal than pre-pubertal heifers. In a study of the vaginal microbiome between cows and heifers, cows had a greater number of observed bacterial species in than heifers (Laguardia-Nascimento et al., 2015). Additionally, pubertal heifers had increased similarity of their overall uterine microbiome than pre-pubertal heifers according to beta diversity analyses of the current study. When evaluating specific bacterial taxa in the uterus, five phyla, including the two most abundant of Firmicutes and Bacteroidota, were affected by pubertal status. Of these phyla, four were observed to have greater relative abundances in pubertal heifers compared to pre-pubertal heifers, but Firmicutes was greater in pre-pubertal than pubertal heifers. Similar results were observed previously in cows during estrous synchronization leading up to breeding (Ault et al., 2019a). Firmicutes was highly abundant and dominated the uterine bacterial communities, averaging 61-74% relative abundance, at the beginning of estrous synchronization during low progesterone concentrations. Firmicutes then

decreased to 36% on average, with other phyla significantly increasing, two days before breeding during higher progesterone concentrations (Ault et al., 2019a). Luteal function and resulting progesterone concentrations are the defining parameter of pubertal animals, indicative of their ability to successfully ovulate and establish and maintain a pregnancy (Kinder et al., 1995; Atkins et al., 2013; Fortes et al., 2018). Progesterone influences uterine function through changes in endometrial gene expression related to activities such as immune response, nutrient exchange to the lumen, and other processes related to the establishment of pregnancy (Forde et al., 2010; Forde et al., 2014; Fortes et al., 2018). Companion data from the current study reported by Brandt (2020), found 18 amino acids, regardless of protein supplementation treatment, reached their highest concentration in the uterus at d 163 indicating changes to the uterine environment through development to puberty attainment. The uterine environment and endometrial tissue changes occurring with pubertal onset from progesterone stimulation likely influence the bacterial communities of the uterus, leading to a more similar overall uterine microbiome of pubertal animals.

Furthermore, there was no difference in observed ASVs and fewer bacterial phyla and genera were affected by pubertal status in the rumen compared to the uterus. Progesterone receptors were previously undetected in the rumen, unlike other steroid hormone receptors associated with reproduction such as estrogens and androgens (Pfaffl et al., 2003). Therefore, minimal impact on the bacterial communities is expected in the rumen due to the changes specifically associated with puberty and increased progesterone secretion. However, temporal shifts in bacterial diversity and relative abundances of taxa were observed in the current study in the rumen and uterus. Alpha diversity analyses indicated changes in the number of ASV and beta

diversity indicated a slight shift in the rumen overall rumen bacterial community composition over time. Ruminal bacterial communities are well known for their shifts according to a change in diet (McCann et al., 2014; Clemmons et al., 2019a; Gruninger et al., 2019). However, previous research has indicated stability of the rumen microbiome by 9 weeks following a diet change (Clemmons et al., 2019b). Sampling of the rumen bacterial communities in the current study did not occur until 56 days following the beginning of supplementation. Therefore, the rumen microbiome was likely established following the addition of supplements to the heifer's diet. The development of the rumen microbiome from birth to adulthood is not an immediate process, with the number of species shown to increase from birth up to 2 years of age (Guo et al., 2020), along with shifts in bacterial taxa composition (Jami et al., 2013). The first few days of life and weaning are likely the two most influential periods on rumen microbiome development (Guo et al., 2020; Amin and Seifert, 2021). Therefore, the rumen microbiome of heifers in the current study was likely continuing to develop following weaning into its mature state.

In conclusion, weaning through breeding during heifer development is a crucial period to evaluate the uterine and ruminal bacterial communities and factors that may influence their composition, such as nutrition and puberty attainment. Despite minimal differences in overall diet crude protein, the current study was able to detect differences in the uterine and rumen bacterial communities with varying levels of protein supplementation. Pubertal status had a greater impact on uterine bacterial communities than in the rumen. The effects observed over time and by pubertal status on bacterial communities of the heifer uterus potentially indicates progression of a maturing uterine microbiome through development. Future studies are needed to understand how nutrition and the overall diet may be used to manipulate uterine bacterial communities, and how the

changing uterine microbiome through development may impact the uterine environment. Together, a greater understanding of the developing uterine bacterial communities and how they can be modified will provide insight of their effect on future reproductive success and lifetime reproductive efficiency.

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CHAPTER III:
EFFECT OF LIPOPOLYSACCHARIDE ON THE UTERINE
ENDOMETRIUM IN THE LUTEAL AND FOLLICULAR PHASES
BETWEEN CONTRALATERAL AND IPSILATERAL HORNS

Abstract

Uterine bacterial community abundances shift throughout the estrous cycle, potentially altering the immunological environment of the uterus and impacting subsequent fertility. The objective of the current study was to evaluate the immunological impact of lipopolysaccharide (LPS), as a model for potentially pathogenic bacteria, throughout the uterine endometrium between the luteal and follicular phase of the estrous cycle. Bovine uterine tracts were harvested in mid-luteal (n=7) or follicular (n=7) phase. Explants were collected from the contralateral and ipsilateral horn relative to the dominant follicle or corpus luteum, then subjected to one of three treatments: uncultured control, cultured control, or cultured with LPS (1 µg/mL). Explants were used for RNA extraction and targeted RNA sequencing for expression analyses of 40 immune response related genes. Sequencing reads were mapped to *Bos taurus* genome in CLC Genomics Workbench. Resulting total read counts were normalized by housekeeping gene GAPDH and analyzed for overall expression profile by Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) and Variable Importance in Projection (VIP) analyses in Metaboanalyst. Individual gene expression differences were determined by GLIMMIX procedure in SAS with fixed effects of treatment, estrous phase, uterine horn, and their interaction, with random effect of individual uterus. Expression of 29 genes were affected among treatment groups, with seven genes increased in LPS treatment compared to other groups ($P < 0.05$). Multiple genes were affected by estrous cycle phase and uterine horn, independent of treatment ($P < 0.05$). The OPLS-DA analyses indicated overall gene expression differences due to clustering by estrous cycle and treatment ($P < 0.001$), with no effect of uterine horn ($P > 0.10$). Similar clustering was observed between luteal and follicular phase explants of controls, but distinct separate clustering between phases with LPS

treatment ($P = 0.001$). According to VIP analyses, mucins were identified as contributing the most to differences observed between phase and treatment. In conclusion, estrous cycle phase resulted in differing overall endometrial gene expression profiles of immune response to LPS treatment. Therefore, altered immunological environment of the uterus in response to bacteria at different estrous cycle stages may influence reproductive success.

Introduction

Reproductive failures and pregnancy loss cost the beef industry millions of dollars annually (Bellows et al., 2002). In postpartum cows, bacterial infections in the uterus are a common cause of low rebreeding success (Bonnett et al., 1993; LeBlanc et al., 2002; Sheldon et al., 2009; Ribeiro et al., 2013). Through parturition and uterine involution, there is high risk for bacterial contamination and infection resulting in uterine diseases. Bacterial communities in healthy postpartum cattle have shown to influence rebreeding success (Ault et al., 2019). Cows that did not become pregnant had a greater abundance of uterine disease-associated bacterial genera in the uterus of cows, although no presence of infection was observed (Ault et al., 2019). Interestingly, many of these potentially pathogenic bacteria in postpartum cows with uterine disease have also been found in the healthy virgin heifer and pregnant uterus (Moore et al., 2017). Specific genera of these potentially pathogenic bacteria include *Acinetobacter*, *Fusobacteria*, *Proteus*, *Prevotella*, and *Peptostreptococcus*. These genera and numerous others associated with disease in the uterus are classified as Gram-negative bacteria. Gram-negative bacteria are defined by their cell wall composition with a thin peptidoglycan layer with an outer membrane. The outer membrane of Gram-negative bacteria contains lipopolysaccharides (LPS), which is considered an endotoxin that can elicit a significant immune response in the host (Dong et al., 2017). Through toll like receptor

signaling, LPS stimulates a pro-inflammatory reaction in various tissues, mainly through the production of cytokines and chemokines (Nagaraja et al., 1978; Takuchi et al., 1999; Huemann and Roger, 2002; Elson et al., 2006; Lu et al., 2008). Cytokines and chemokines are commonly known for regulating inflammation and immune cell recruitment (Dimberg, 2010), but are also involved in reproductive processes such as corpus luteum function, embryo development, placental attachment, and preventing rejection of the fetus (Penny et al., 1999, Zolti et al., 1991; Simón et al., 1998; Ealy et al., 2021). The presence of cytokines and chemokines have shown to fluctuate across the estrous cycle and pregnancy, and throughout puberty attainment (Krakowski and Zdzisinska, 2007; Oliveira et al., 2013; Ault-Seay et al., 2021; Poole et al., 2021). Due to the importance of inflammatory balance in the uterus (Raghupathy, 2001), minor shifts in bacterial communities may disrupt this balance and negatively influence reproductive success.

Similar to cytokines and chemokines, fluctuations in bacterial communities of the reproductive tract occur between the luteal and follicular phases of the estrous cycle. Changes in both diversity and taxonomic composition have been observed over natural estrous cycles, estrus synchronization, and differences in pregnancy status (Laguardia-Nascimento et al., 2015; Quereda et al., 2019; Ault et al., 2019). The concentration of hormones, such as progesterone and estrogen, locally and systemically is a major distinguishing factor between phases of the estrous cycle. The direct effect of these hormones on bacterial communities is not well understood. However, the effects of these hormones on the uterine endometrium may indirectly impact the uterine bacterial community composition. The majority of differences observed in the bacterial communities between resulting pregnant and non-pregnant cows occurred during estrus synchronization when CL regression would begin to occur with the transition from a progesterone dominated to estrogen

dominated environment (Ault et al., 2019). Evaluating the impact of bacteria on the uterus during different phases of the estrous cycle will contribute to the understanding of microbial impact on reproductive performance. Additionally, global gene expression analyses have indicated differences between the contralateral and ipsilateral uterine horns, relative to the dominant follicle or corpus luteum, as well as the presence of a hormone concentration gradient (Pope et al., 1982; Boos et al., 1986; Takahashi et al., 2016). Assessing the response of the contralateral and ipsilateral endometrium in each phase of the estrous cycle will determine the response to bacteria through the hormone concentration differences and varying responses across the uterus. Therefore, evaluating the impact of potentially pathogenic bacterial components on the uterus and uterine environment through the estrous cycle will provide insight for the influence of the reproductive tract microbiome on breeding success.

The objective of the current study was to evaluate the impact of potentially pathogenic bacteria on the endometrium throughout the uterus between the follicular and luteal phases of the estrous cycle. We hypothesized the immunological response in the endometrium to LPS, as model for potentially pathogenic bacteria, will differ between both the luteal phase and follicular phase, and the ipsilateral and contralateral uterine horns.

Materials and Methods

Collection and Selection of Reproductive Tracts

Reproductive tracts were collected in late spring from the local abattoir immediately following slaughter. Only reproductive tracts with both ovaries attached and no abnormalities present on the ovaries were considered for use. The reproductive tract and surrounding tissues

were required to have moderate adiposity corresponding to a moderate BCS; tracts with no signs of adipose or excessive adiposity were not utilized. Tracts were then selected in the mid- to late luteal phase (progesterone dominated tract; n = 7), and in the follicular phase (estrogen dominated tract; n = 7). To determine the phases, gross ovarian measurements were evaluated to determine reproductive tract estrous cycle phase using standards previously described by Ireland et al. (1980), Miyamota et al. (2000), and Arosh et al. (2002). Example images of the contralateral and ipsilateral ovaries, relative to the dominant follicle or CL, on luteal and follicular phase tracts utilized for the current study are shown in Figure 10A-F. For the follicular phase (Figure 10A and B), a large, dominant, pre-ovulatory follicle (≥ 13 mm diameter) must be present on one ovary, and its closest uterine horn was considered the ipsilateral horn (Figure 10A). The second ovary had a small, regressing CL and/or small, non-dominant follicle(s), in which the closest uterine horn would be the contralateral horn (Figure 10B). For the luteal phase (Figure 10C-F), tracts were characterized by a large current CL on one ovary, and a small follicle(s) on either ovary; the uterine horn closest to the ovary with the CL was considered the ipsilateral horn. Luteal phase tracts were utilized if the CL was tan to orange in color externally and internally, ~ 1.5 to 2 cm in diameter, with some vasculature over apex and on periphery (Figure 10C and E). The follicles on either ovary were < 10 mm in diameter, or not visible (Figure 10D and F). Reproductive tracts meeting these criteria for inclusion were transported on ice to the laboratory for processing.

Endometrial Explant Collection

Pinching the cervix closed, reproductive tracts were rinsed with ethanol and water. The uterus was then cut away from all other tissues and the contralateral and ipsilateral horns of the uterus were separated. Each horn was then opened longitudinally for collection of endometrial

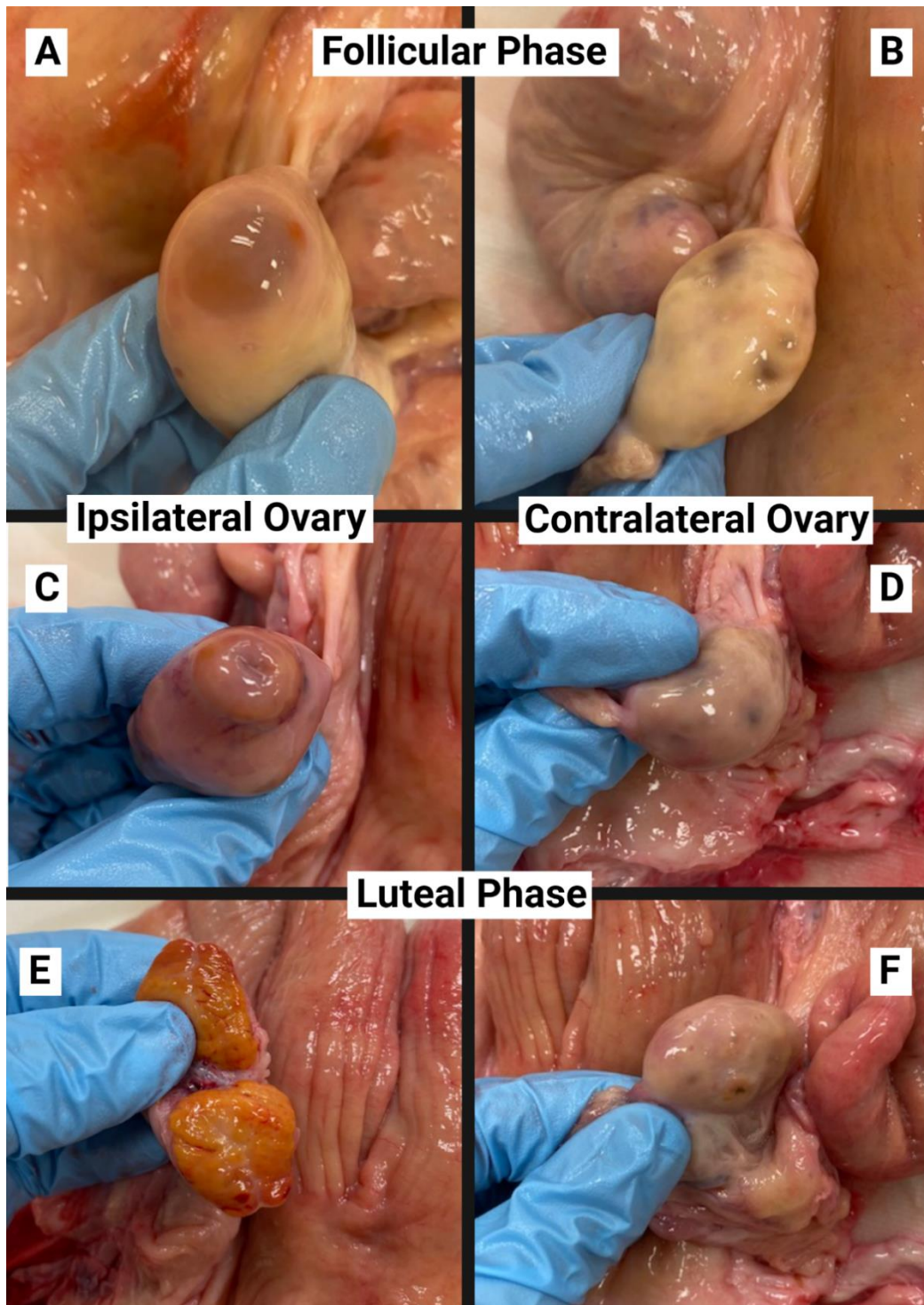


Figure 10. Examples ovaries for staging of the estrous cycle on follicular phase (A and B) and luteal phase (C-F) reproductive tracts utilized for explant collection from the ipsilateral (A, C, and E) and contralateral (B, D, and F) uterine horn.

explants. If any signs of placental membranes, infection, excessive mucus, or abnormalities were observed once either uterine horn was opened, the whole tract was discarded and excluded from the study.

The contralateral and ipsilateral horns were split into even halves, resulting in four sections per uterus. Endometrial explants were collected using an 8mm biopsy punch. Explants were taken from the intercaruncular area approximately 1-1/12" from the uterine body and approximately 1-1/12" past the uterine bifurcation towards the tip of the uterine horn of the contralateral and ipsilateral horns. Any visible myometrium obtained during the biopsy punch was trimmed off of the explant. Three explants were taken from each section for the three treatments (uncultured control, cultured control, and LPS) resulting in 12 endometrial explants per tract. Figure 11 depicts the sections of each uterine horn and the collected explants. The explants serving as uncultured controls treatment were immediately snap frozen in liquid nitrogen and stored at -80°C. Explants for culture treatments were placed in a 50 mL conical of Hank's Balanced Salt Solution (HBSS; Gibco, Waltham, Massachusetts, USA) until all explants were collected. Explants were transferred from the first HBSS media to a second HBSS media, then transferred to HBSS with 1% antibiotic and antimycotic media (ABAM; Gibco, Waltham, Massachusetts, USA) until plating for culture.

Explant Culture and Treatment

Explants were removed from HBSS with 1% ABAM and plated into individual wells with Roswell Park Memorial Institute 1640 medium (RPMI; Gibco, Waltham, Massachusetts, USA) with 1% ABAM. Plates with explants were incubated for 2 hours at 38.8°C with 5% CO₂ and air for the removal of cellular debris and red blood cells. After the first incubation, explants were transferred to a new plate into individual wells with 1 mL of fresh media and treatment. Cultured

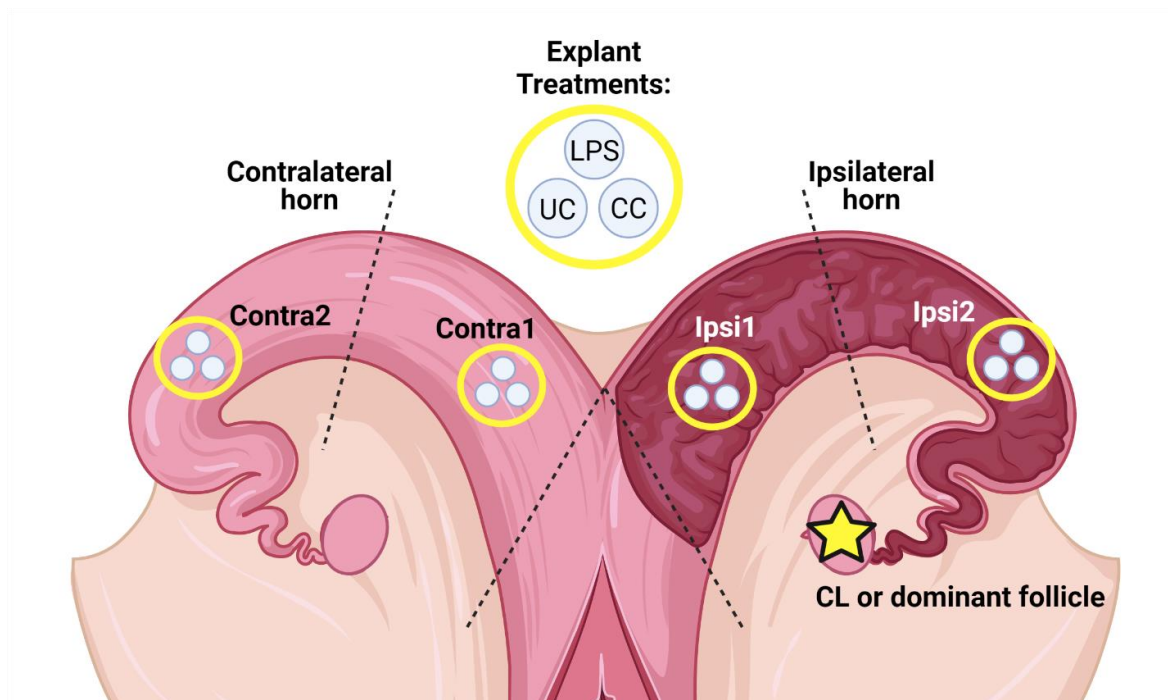


Figure 11. Sections of uterine horns with collected explants and their respective treatments.

LPS: Cultured with complete media + LPS. CC: Control cultured in complete media without LPS. UC: Uncultured control.

control explants were incubated in RPMI with 1% ABAM. Treated LPS explants were incubated in RPMI with 1% ABAM and 1 µg/mL LPS (Ultrapure LPS, *E. coli* 0111:B4; InvivoGen, San Diego, CA, USA). Following incubation at 38.8°C with 5% CO₂ and air for 8 hours, explants were removed and snap frozen in liquid nitrogen, then stored at -80°C.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from ~50 mg endometrial explants using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) with TRIzol (Invitrogen, Carlsbad, CA, USA) and chloroform (Thermo Scientific, USA). Explants were homogenized for 2 × 2 minutes with a 5 mm steel bead (Qiagen, Hilden, Germany) and TRIzol. Following homogenization, chloroform was added and centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous layer was transferred to a new tube with 1.5x volume of 100% ethanol. The resulting solution was added to a RNeasy column and proceeded with the Qiagen RNeasy kit according to manufacturer instructions. Quantity and quality of extracted RNA was evaluated via NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA) and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). Extracted RNA with a RIN value < 6 and concentration < 35 ng/uL were not utilized for cDNA synthesis and further analyses. Synthesis of cDNA from extracted RNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to manufacturer protocol with ~500 ng/mL of RNA.

Targeted RNA Sequencing

Genes of interest related to immune response and associated with reproductive processes were selected for targeted RNA sequencing and identified using the NCBI Gene database from the

Bos taurus genome. A total of 40 genes were selected including interleukin family genes (*IL-1 α* , *IL1 β* , *IL4*, *IL6*, *IL10*, *IL17A*, *IL36RA*, *LIF*), various cytokines (TGF β , TNF α , VEGFA), CXCL chemokines (*CXCL1*, *CXCL2*, *CXCL8*, *CXCL10*), CCL chemokines (*CCL2*, *CCL3*, *CCL4*), mucins (*MUC1*, *MUC4*, *MUC13*, *MUC15*), steroid receptors (*PGR*, *ESR1*, *ESR2*), interferon associated genes (*IFN γ* , *MX1*, *MX2*, *ISG15*, *OAS2*, *IRF1*, *IRF3*, *IRF8*), colony stimulating factors (*CSF2*, *CSF3*), prostaglandin pathway genes (*PTGES*, *PTGES2*, *PTGESR2*), and toll-like receptor pathway genes (*TLR4*, *NF κ BI*). For all genes of interest, primers were designed in NCBI Primer BLAST to span across an intron and result in amplicons of 90-110 base pairs. Quantification of housekeeping genes for sample normalization included *GAPDH*, *ACTB*, *UXT*, and *RSP9*. Table 14 lists all genes of interests and housekeeping genes with their respective accession numbers from the NCBI database and resulting primers. Amplification of cDNA was performed in duplicate via Hi-Plex approach optimized by Floodlight Genomics (Knoxville, TN, USA), followed by multiplex sequencing using a 2 $\times$ 150 kit on an Illumina MiSeq (San Diego, CA, USA). Resulting reads were mapped to the *Bos taurus* genome using CLC Genomics Workbench version 9.5.2 (Qiagen, Bethesda, MD, USA). Reads were standardized according to the total read count per sample to obtain the proportion of reads for each gene. Values for each sample duplicates were averaged among each gene within each sample for further analyses (Mihelic et al., 2020).

Statistical Analyses

Resulting gene expression values were imported to Metaboanalyst 5.0 and normalized according to the expression of the housekeeping gene *GAPDH*, which indicated stable expression among treatment, estrous cycle phase, and uterine horn. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were performed in Metaboanalyst 5.0 to evaluate

Table 14. Genes of interest and NCBI gene accession number with resulting forward and reverse primers for targeted RNA sequencing.

Gene	Accession #	Forward Primer	Reverse Primer
<i>TLR4</i>	NM_174198.6	CCTGAGAACCGAGAGTTGGG	TATGGGGATGTTGTCTGGGGA
<i>IL1α</i>	NM_174092.1	AGGTCCATACCTGACGGCTA	AGAGTCACAGGAAGCTGAGAA
<i>IL1β</i>	NM_174093.1	AAAAATCCCTGGTGTCTGGCT	GCACAAAGCTCATGCAGAACA
<i>TNFα</i>	NM_173966.3	TCACATACCCTGCCACAAGG	CCACATCCCGGATCATGCTT
<i>IFNγ</i>	NM_174086.1	TCTTATGGCCAGGGCCAATTT	AGGCCACCCTTAGCTACAT
<i>IL10</i>	NM_174088.1	GGCCTGACATCAAGGAGCAC	GCCTTGCTCTTGTTCGCA
<i>IL6</i>	NM_173923.2	AAGCGCATGGTCGACAAAAT	TTCTGCCAGTGTCTCCTTGC
<i>MUC15</i>	NM_176631.1	TCCCACCCTGAAATTCACCA	AGCACCTAAAATGGCCCCAA
<i>MUC13</i>	XM_002684796.6	AGCCAGAGAACCCCTACACA	ATGAGTTGAGGGGCTGTAGA
<i>MUC1</i>	NM_001372018.1	CAGTGCCCCATTTCCCTTCCT	ATAGATGATGGCCAGCGCAA
<i>MUC4</i>	XM_024994862.1	CCGATTCACAAGTCACCCCA	CCCGTAGGGGAAGAGGAGAA
<i>MX1</i>	NM_173940.2	AGATGGTTCATTCTGACTTGGGT	TTGGCTGTACAGGTTGCTCT
<i>MX2</i>	NM_173941.2	CAAGAGTTCGGTGCTGGAGG	CCCGCTTTGTCAGTTTCAGC
<i>ISG15</i>	NM_174366.1	AGGAGCGTGTACAAGCAGAC	CCCCTTCATGAGGCCGTATT
<i>OAS2</i>	NM_001024557.1	ACAGCGCAATTTTGTTTCGCT	GCAGAGACCCCTTTTGCTTC
<i>IRF1</i>	NM_001191261.2	CATGCCCTCCACCTCTGAAG	CCACCCTGTTTGCTCCAAGA
<i>IRF3</i>	NM_001029845.3	TGTGCAGCTGAGAGTTCGAG	GTCTGGCCTAAGTGTTGGGT
<i>IRF8</i>	NM_001083769.2	CGGACTGGTGGGTGCAG	GACCATCTGGGAGAAGGCTG
<i>PTGES2</i>	NM_001166554.1	TTATGGCCCAGGAAGGAGAGA	TGATGATGTCTGCCAGGGGT
<i>PTGER2</i>	NM_174588.2	ATGACCATCACCTTCGCCGT	CTAAGAGCTTGAGGTCCAC
<i>PTGES</i>	NM_174443.2	CCTCCAGTATTGCCGGAACG	AGACAAAGCCCAGGAACAGG
<i>NFκB</i>	NM_001076409.1	TTGGGAGGGCGTGAACAAAT	GGCCATCTGCTGTTGGTAGT
<i>CXCL8</i>	NM_173925.2	GACTTCCAAGCTGGCTGTTG	ATGCATTGGCATCGAAGTTCTG
<i>CCL2</i>	NM_174006.2	AGACCCCAAGCAGAAATGGG	TTGGGTTGTGGAGTGAGTGC
<i>TGFβ</i>	NM_001166068.1	AACCTGTGTTGCTCTCTCGG	GAGGTAGCGCCAGGAATTGT
<i>CCL3</i>	NM_174511.2	CAGCATCATGAAGGTCGCCG	TTGGGGTGTGAGCGCCAAAT
<i>CSF2</i>	NM_174027.2	ACTTCCTGTGGAACCCAGTT	TGGTTCCCAGCAGTCAAAGG
<i>CSF3</i>	NM_174028.1	TACACGGCGGCCTCTTTCTC	TCCAGCTGCAGTGTGTCCAAG
<i>CXCL1</i>	NM_175700.2	ACCGCCCCCATGGTTAAGAA	CACTGAGGCTGCTGGAGTATC
<i>CXCL2</i>	NM_174299.3	GCCGCTCCCATGGTTAAGAA	TGGAACAGCCATCCAAGAGC
<i>CXCL10</i>	NM_001046551.2	GGAATACACGCTGTTCTCTGC	CACGTGGGCAGGATTGACTT
<i>LIF</i>	NM_173931.1	AACTGCCGGCATCTAAGGTC	AGTGGAGAACCAGCAGCAAG
<i>IL4</i>	NM_173921.2	GCCACACGTGCTTGAACAAA	TGTGCTCGTCTTGCTTCAT
<i>IL17A</i>	NM_001008412.2	ACATCGTTAACCGGAGCACA	CTCTCAGGGTCCTCATTGCG
<i>IL36RN</i>	NM_001075775.1	CTCAAGATGGTCCTGAGCGG	TGTAGGCCTCCGGCTAGAAG
<i>CCL4</i>	NM_001075147.2	GCGGAAGATTCCTCGCAACT	CCTGCCCTTTTGGTCTGGA
<i>VEGFA</i>	NM_001316955.1	GGCGAGGCAGCTTGAGTTA	CTGGTTCCCGAAACCCTGAG

Table 14 continued.

Gene	Accession #	Forward Primer	Reverse Primer
<i>PGR</i>	NM_001205356.1	AGAAAGTGCTGTCAGGCTGG	GGAGGGCAACAGCATCTAGT
<i>ESR1</i>	NM_001001443.1	TCTGGAAAGACGCTCTTGATCC	GTCATGGTCATGATGAGCGG
<i>ESR2</i>	NM_174051.3	CGACGTAAAAGCTGTCAGGC	ATGCGGTACCCACACCTTTC
<i>GAPDH</i>	NM_001034034.2	TGGTGAAGGTCGGAGTGAAC	ATGGCGACGATGTCCACTTT
<i>ACTB</i>	NM_173979.3	GCAGGAGTACGATGAGTCCG	TGCGCAAGTTAGGTTTTGTCA
<i>UXT</i>	NM_001037471.2	AACACTGGCAGAAGCTCTCA	ACATGTGGATATGGGCCTTGA
<i>RPS9</i>	NM_001101152.2	CTGCTGACGCTGGATGAGAA	CCAGCTTCATCTTGCCCTCA

the overall gene expression differences between groups. Variable importance in projection (VIP) plots were generated to determine genes contributing to clustering differences between groups in the OPLS-DA. Resulting normalized expression values from Metaboanalyst 5.0 were also utilized for a GLIMMIX procedure in SAS 9.4 with effects of treatment (UC, CC, and LPS), estrous cycle phase (follicular and luteal), uterine horn (contralateral and ipsilateral), and their interaction, with random effect of uterus. Section of the uterine horn from which the explant was collected was evaluated but did not affect any individual gene expression or overall gene expression via OPLS-DA and was removed from all analyses. Normal distribution of gene expression values were evaluated by the UNIVARIATE procedure and genes with a Shapiro-Wilk score < 0.85 were log transformed. Significance for all analyses was determined by a $P < 0.05$, and tendencies with $P < 0.10$.

Results

Overall expression of targeted genes of interest was evaluated by OPLS-DA. Distinct clustering of samples was observed between luteal and follicular phase samples with slight overlap ($P < 0.001$; Figure 12). Clustering within each phase indicates similar overall expression of targeted genes within each phase. The overlap observed between phases indicates some similarity of gene expression not impacted by phase of the estrous cycle. The five most influential genes contributing to observed clustering by phase determined by VIP were *MUC13*, *MUC1*, *IFN γ* , *ISG15*, and *ESR1* (Figure 13). Of these genes, *MUC13*, *MUC1*, *ISG15*, and *ESR1* had greater expression in explants of luteal than follicular phase, while *IFN γ* had greater expression in the follicular than luteal phase (Figure 13). Treatment of explants indicated clustering, with complete separation of uncultured controls from cultured controls and LPS, which also clustered, but with

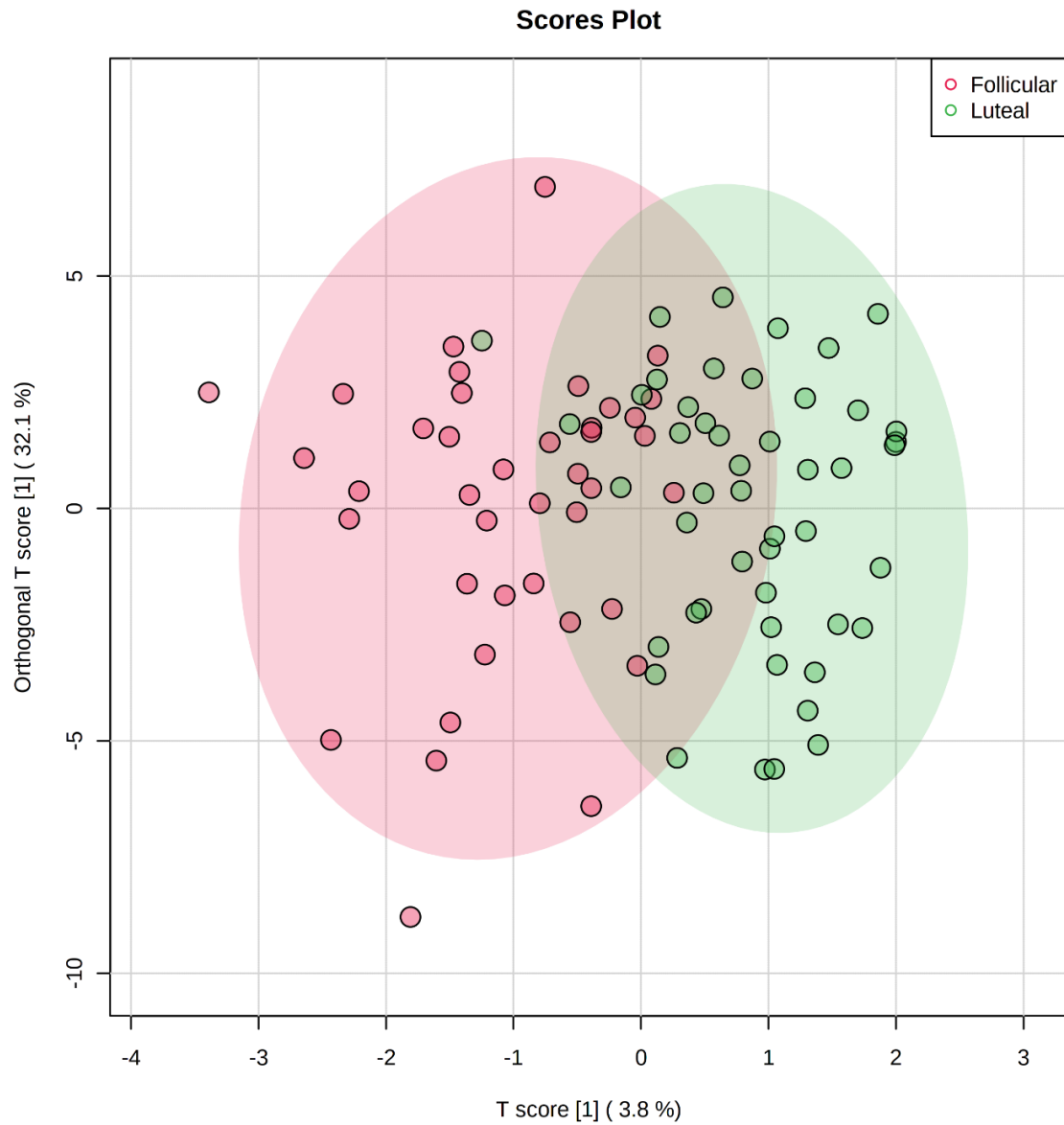


Figure 12. Overall gene expression clustering of explants between the estrous cycle phases ($P < 0.001$) by OPLS-DA.

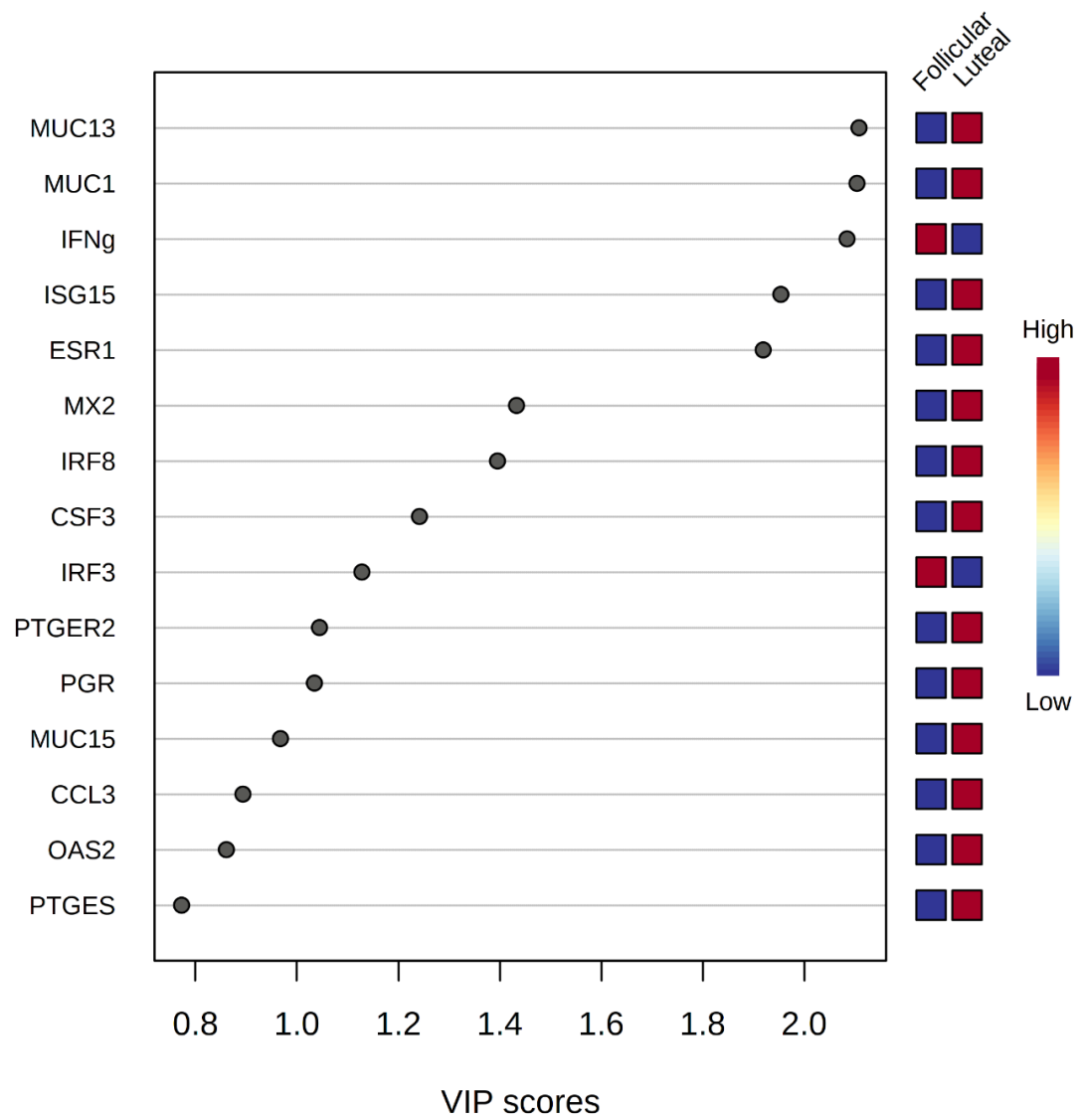


Figure 13. VIP plot for genes contributing to observed clustering in OPLS-DA by estrous cycle phase.

slight overlap ($P < 0.001$; Figure 14). Scores from VIP determined *CXCL8*, *IL6*, *IL1 β* , *CXCL2*, and *IL1 α* to contribute the most to differences between treatment groups (Figure 15). Cytokines *CXCL8*, *IL6*, *IL1 β* , and *CXCL2* had *IL1 α* greater expression in cultured control explants, with the least expression in uncultured control explants. Expression of was greatest in LPS treatment and the least in uncultured controls (Figure 15). Due to the observed difference between uncultured and cultured explants, cultured explants serve as a better control to determine the specific effect of LPS; therefore, uncultured controls were removed for further analysis. In comparison of cultured controls and LPS treated explants, clear separation of overall gene expression between the groups was observed (Figure 16). The VIP plot indicated CSF2, CCL3, LIF, IL10, and OAS2 to contribute the most to the separation of cultured controls and LPS treated explants (Figure 17). All genes had greater expression in LPS explants except LIF which was greater in cultured controls (Figure 17). The relationship between phase of the estrous cycle and treatment is depicted in Figure 18. Separation with slight overlap of follicular and luteal phase explants in uncultured ($P = 0.001$, Figure 18A) and cultured controls ($P = 0.01$, Figure 18B) was observed. However, distinct separation with no overlap of follicular and luteal phase explants in response to LPS treatment was observed ($P = 0.001$, Figure 18C), potentially indicating differing responses between estrous cycle phases to LPS than other treatments. Figure 19 indicates the VIP for the genes contributing to differences in observed clustering between phases for LPS treatment depicted in Figure 18C. Separation of overall gene expression was not observed by uterine horn ($P = 0.41$, Figure 20).

Differences in the expression of individual genes by treatment, estrous phase, and uterine horn was determined by ANOVA. The expression of 29 genes were affected by treatment ($P < 0.05$, Table 15). Of these genes, 15 were affected between uncultured and cultured explants, with

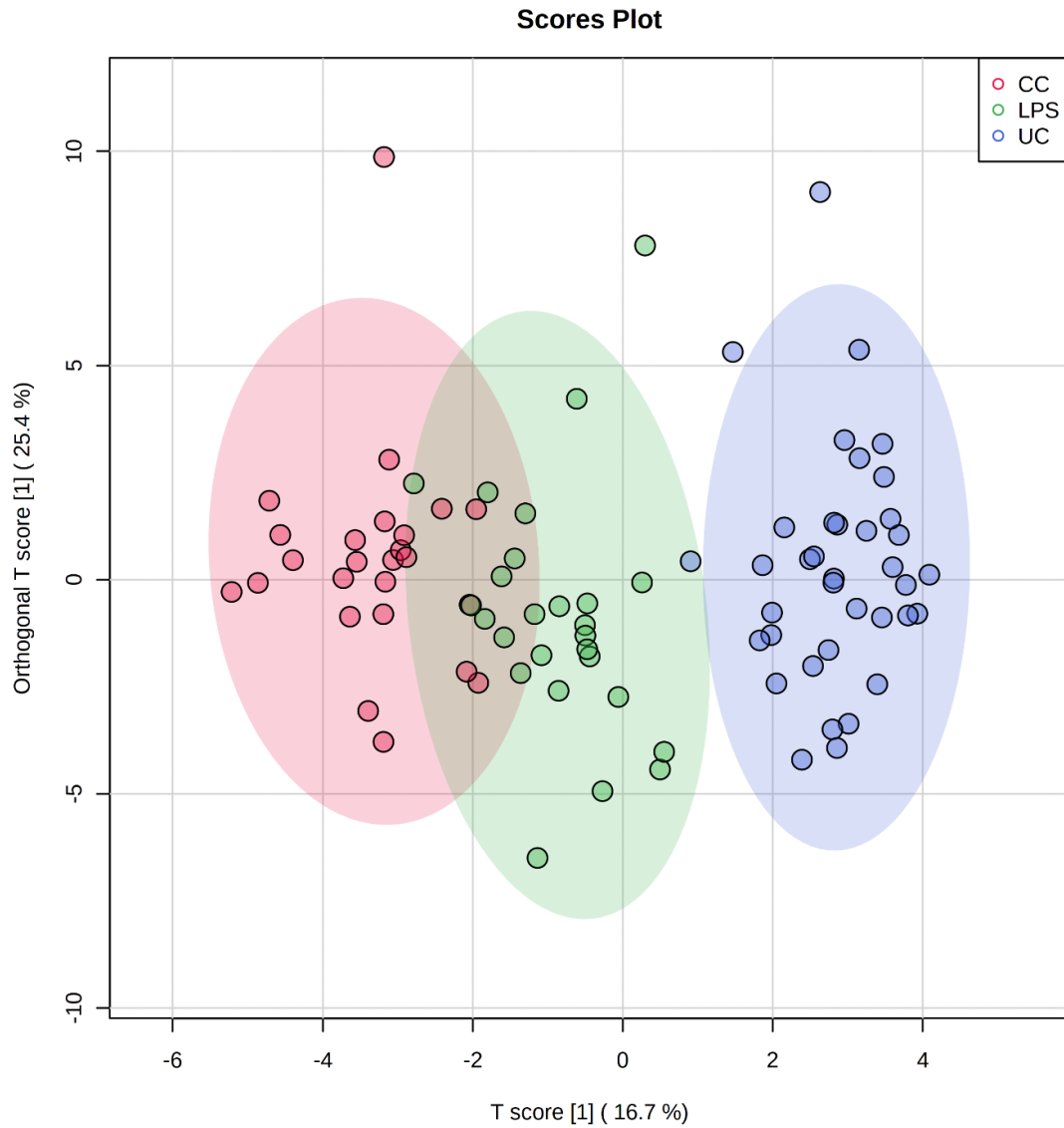


Figure 14. Overall gene expression clustering of explants between treatments ($P < 0.001$) by OPLS-DA. CC: Cultured Controls; LPS: Lipopolysaccharide; UC: Uncultured Controls

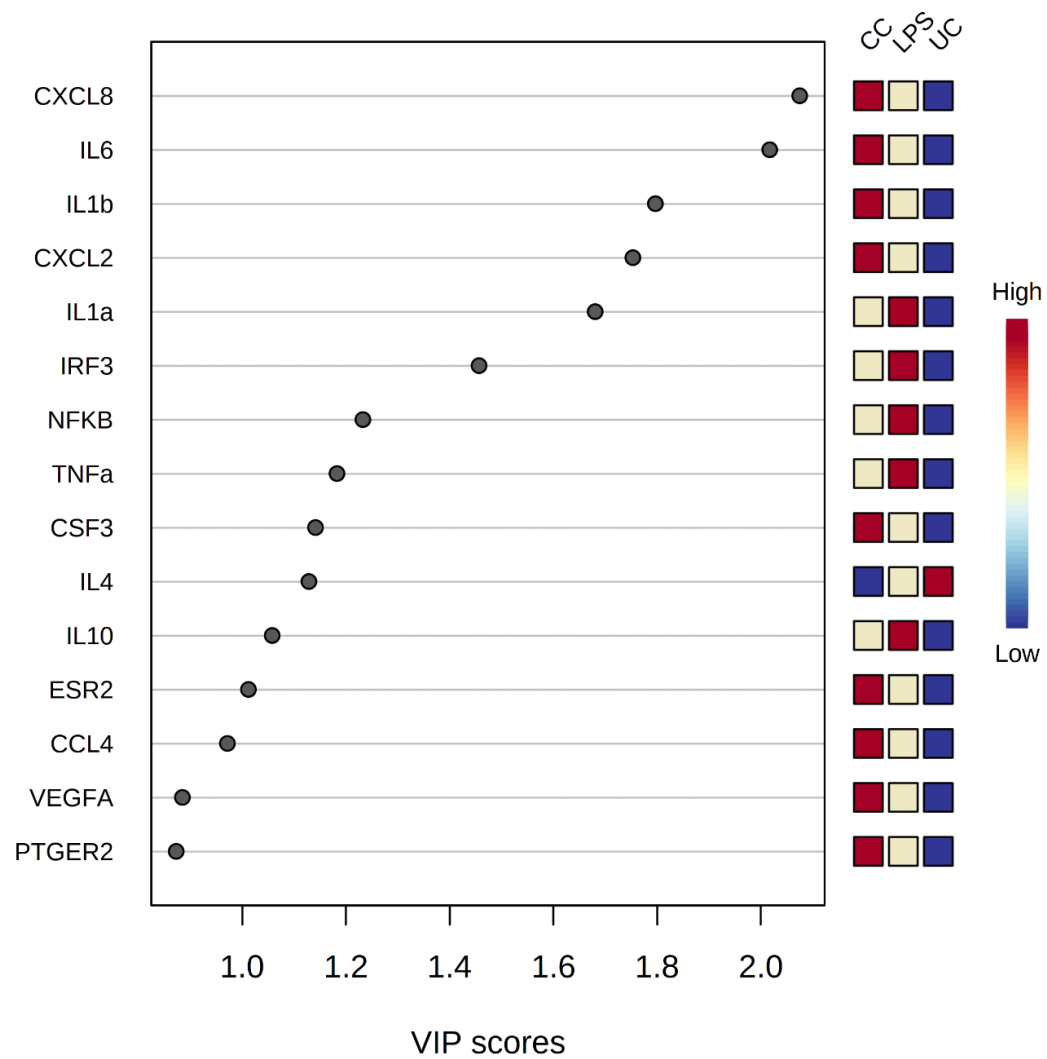


Figure 15. VIP plot for genes contributing to observed clustering in OPLS-DA by treatment.

CC: Cultured Controls; LPS: Lipopolysaccharide; UC: Uncultured Controls

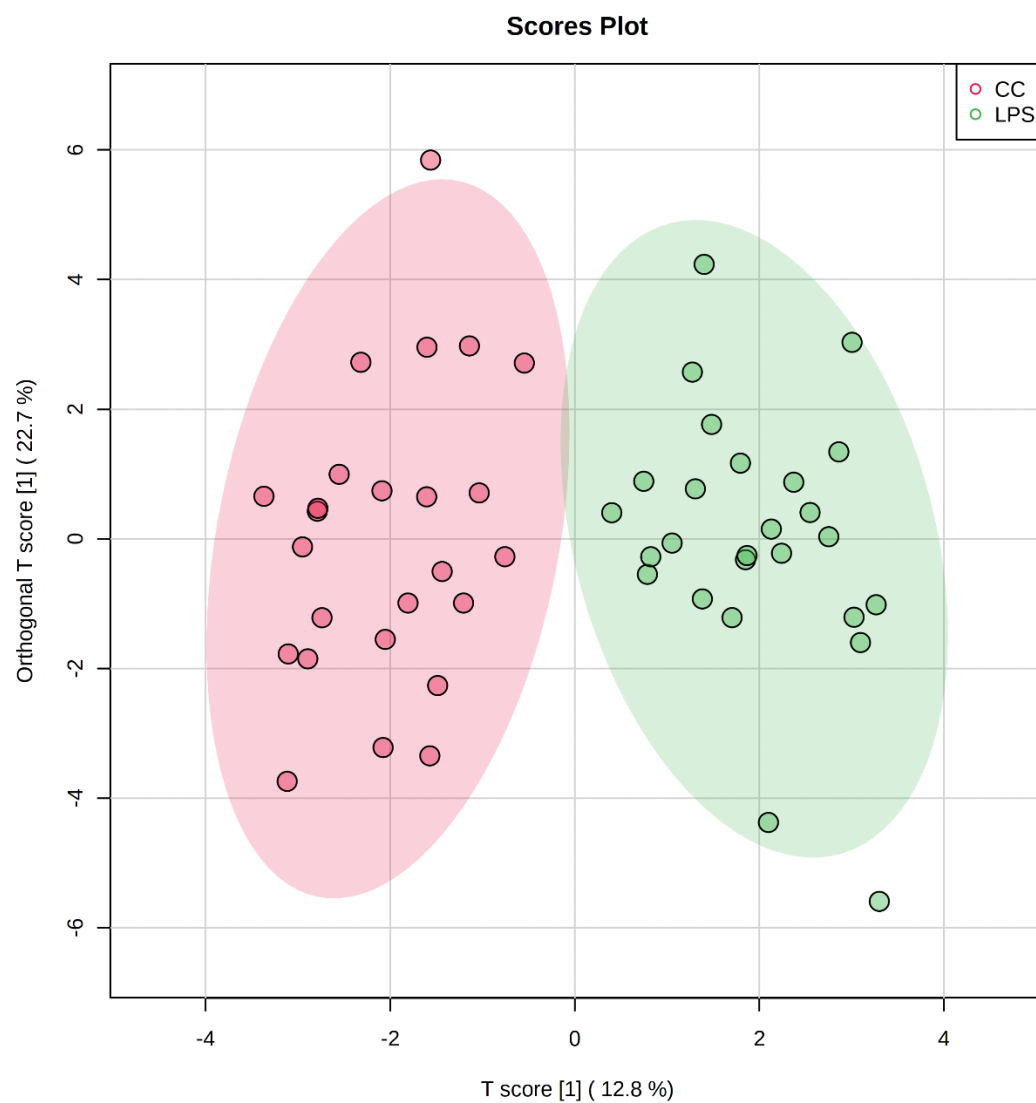


Figure 16. Overall gene expression clustering of explants between cultured controls (CC) and LPS explants ($P < 0.001$) by OPLS-DA.

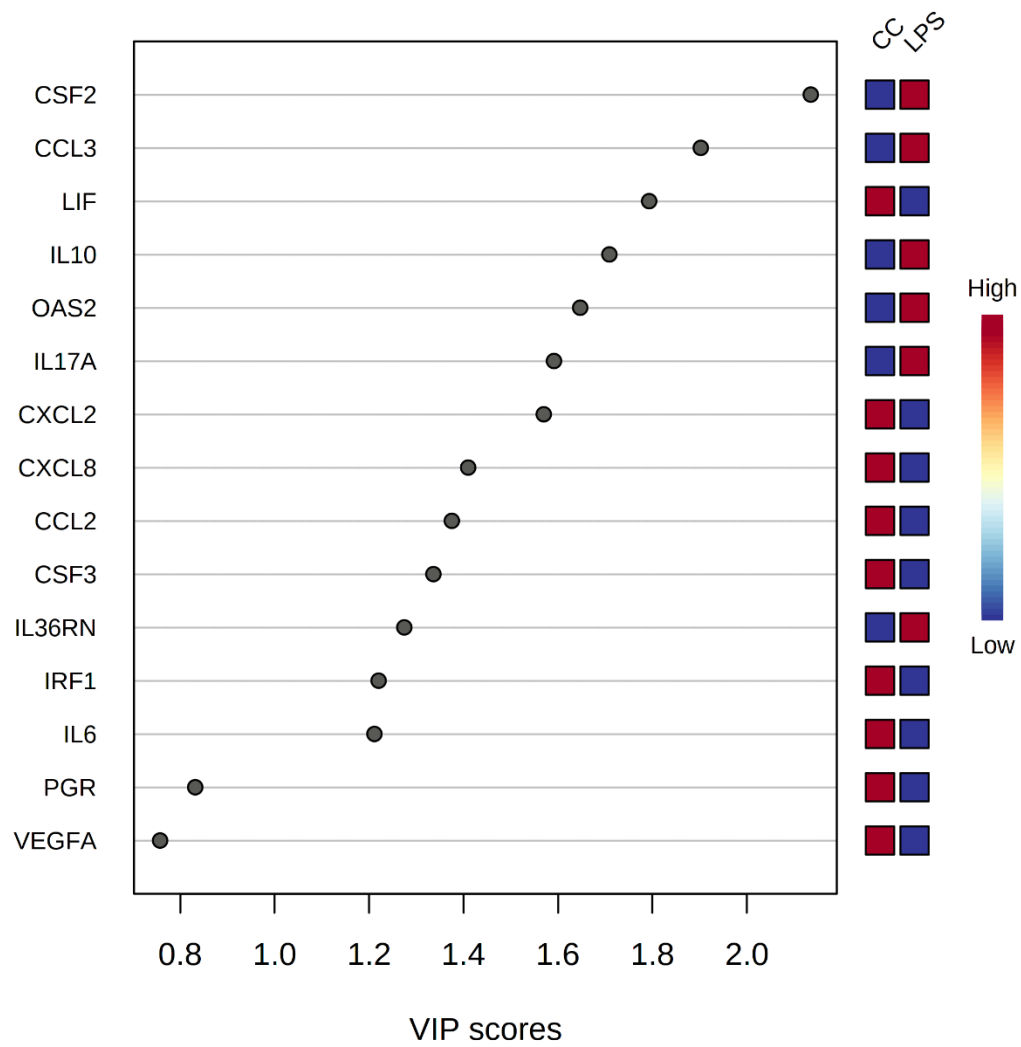


Figure 17. VIP plot for genes contributing to observed clustering in the OPLS-DA between cultured controls (CC) and LPS treated explants.

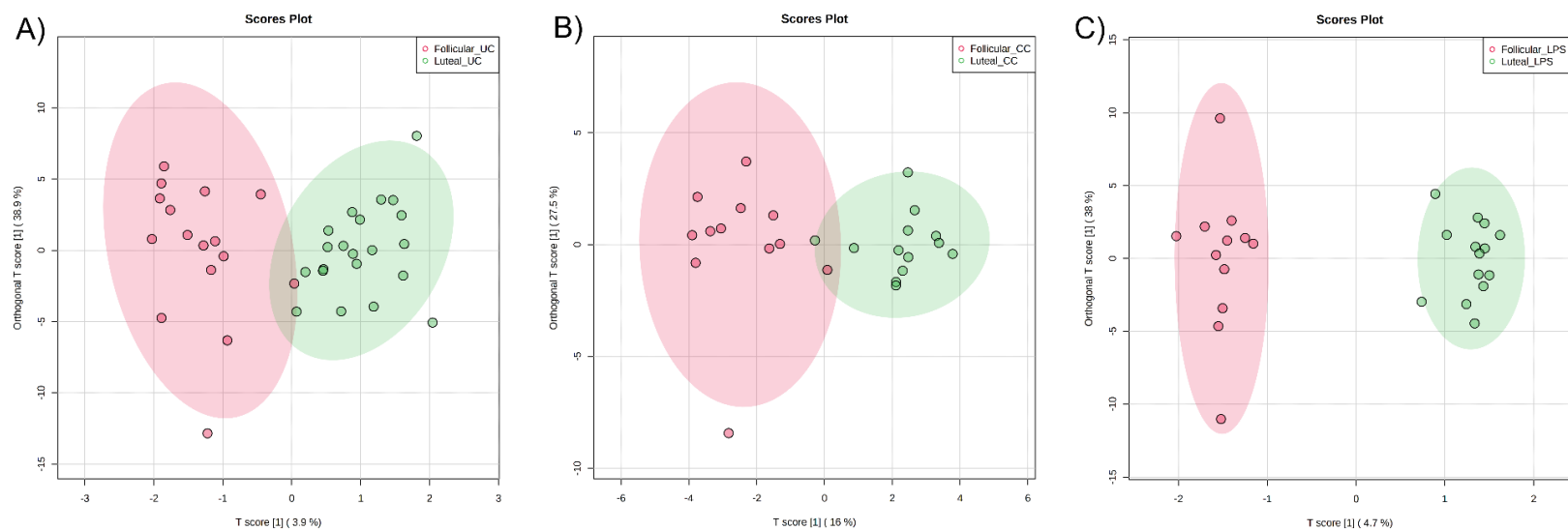


Figure 18. Overall gene expression clustering by OPLS-DA between follicular and luteal phase explants for UC (A; $P = 0.001$), CC (B; $P = 0.01$), and LPS (C; $P = 0.001$) treatments.

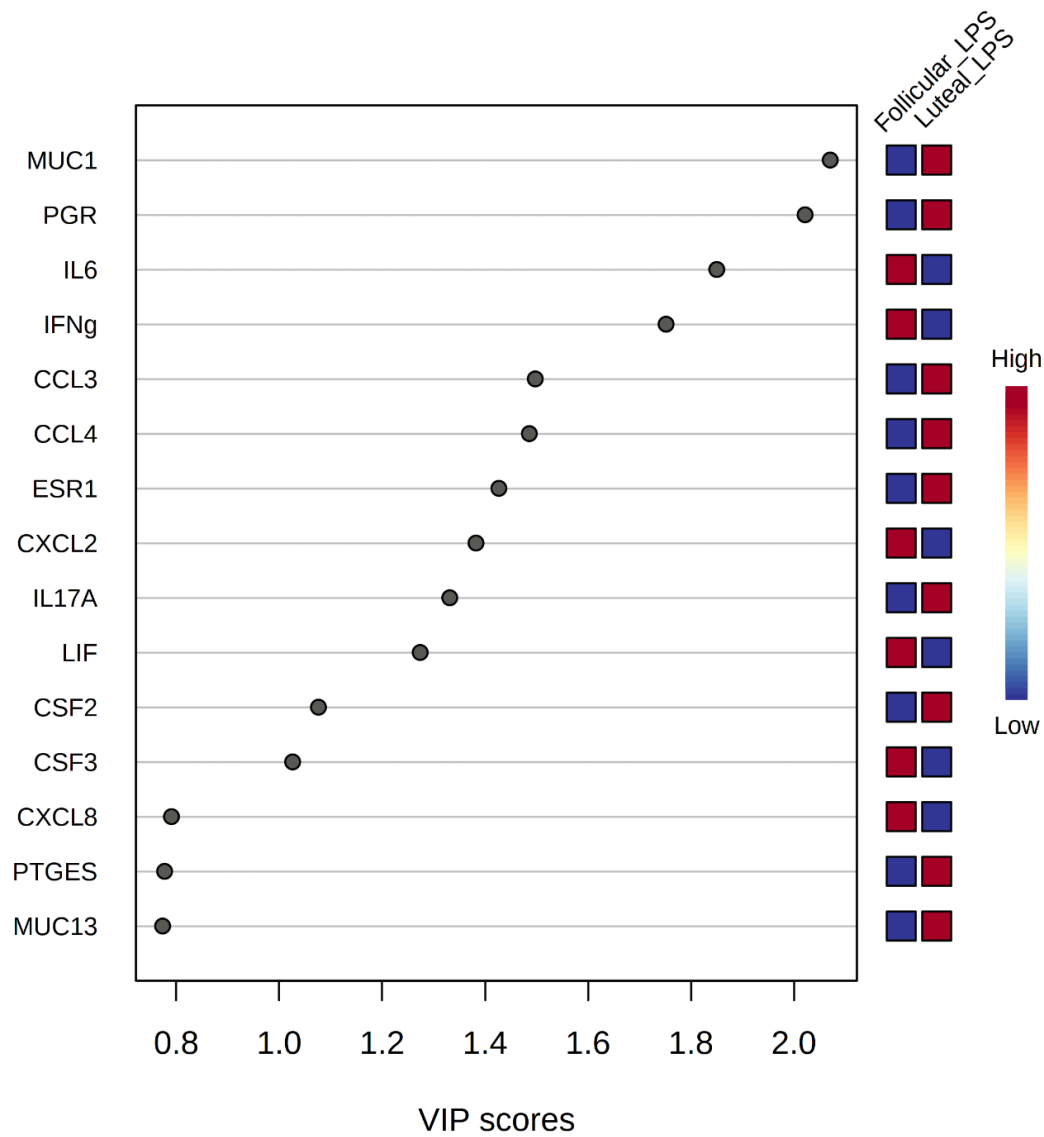


Figure 19. VIP plot for genes contributing to observed clustering in OPLS-DA between luteal and follicular explants in response to LPS treatment.

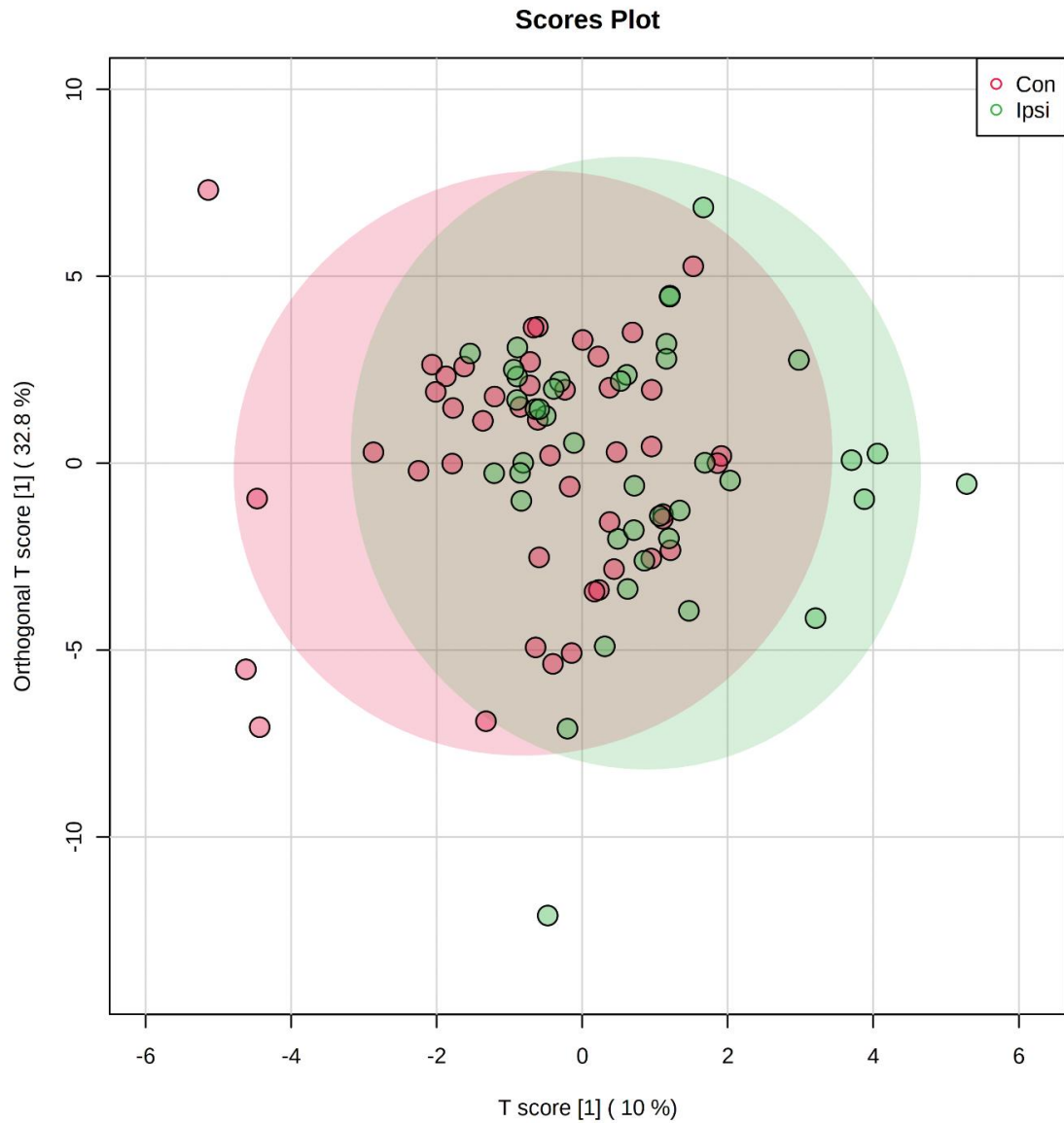


Figure 20. Overall gene expression clustering of explants between uterine horns ($P = 0.41$) by OPLS-DA. Con: Contralateral uterine horn; Ipsi: Ipsilateral uterine horn

Table 15. Expression of genes affected by treatment group.

Gene	Treatment ¹			P Value ²
	UC (n=37)	CC (n=25)	LPS (n=26)	
<i>CCL4</i>	1316.93 ± 43.79 ^B	1541.43 ± 50.57 ^A	1513.66 ± 50.06 ^A	0.0004
<i>TGFβ</i>	1299.32 ± 14.31 ^B	1378.4 ± 16.81 ^A	1338.86 ± 16.65 ^{AB}	0.001
<i>TNFα</i>	1123.45 ± 53.36 ^B	1460.42 ± 62.55 ^A	1548.96 ± 61.96 ^A	<0.0001
<i>ESR1</i>	1425.29 ± 29.82 ^B	1589.43 ± 35.54 ^A	1519.04 ± 35.25 ^A	0.002
<i>CXCL8</i>	187.64 ± 45.02 ^C	1465.8 ± 50.25 ^A	1320.29 ± 49.72 ^B	<0.0001
<i>IRF3</i>	1219.56 ± 14.96 ^B	1374.36 ± 17.43 ^A	1364.81 ± 17.26 ^A	<0.0001
<i>IL1β</i>	568.56 ± 47.49 ^B	1268.78 ± 54.87 ^A	1315.25 ± 54.31 ^A	<0.0001
<i>VEGFA</i>	1219.8 ± 13.22 ^B	1285.93 ± 14.83 ^A	1260.95 ± 14.67 ^A	0.0004
<i>IL6</i>	469.38 ± 37.98 ^B	1256.57 ± 43.1 ^A	1193.44 ± 42.65 ^A	<0.0001
<i>IRF1</i>	1213.49 ± 18.46 ^A	1175.54 ± 21.77 ^A	1102.33 ± 21.58 ^B	0.0005
<i>TLR4</i>	969.47 ± 12.84 ^B	1037.94 ± 15.2 ^A	1027.26 ± 15.06 ^A	0.0008
<i>CXCL2</i>	633.1 ± 45.37 ^B	1135.62 ± 50.74 ^A	1056.34 ± 50.21 ^A	<0.0001
<i>IL10</i>	581.36 ± 74.5 ^C	1009.59 ± 84 ^B	1648.08 ± 83.11 ^A	<0.0001
<i>CSF3</i>	1018.05 ± 62.36 ^C	1448.04 ± 72.3 ^A	1240.03 ± 71.58 ^B	<0.0001
<i>ISG15</i>	805.27 ± 44.96 ^B	974.86 ± 50.94 ^A	1002.29 ± 50.41 ^A	0.0014
<i>IL1A</i>	162.5 ± 36.76 ^C	846.36 ± 44.23 ^B	979.74 ± 43.92 ^A	<0.0001
<i>PTGER2</i>	895.92 ± 15.3 ^B	979.54 ± 18.26 ^A	936.94 ± 18.12 ^A	0.0009
<i>ESR2</i>	1161.76 ± 37.99 ^B	1395.44 ± 44.01 ^A	1327.15 ± 43.57 ^A	<0.0001
<i>PGR</i>	759.8 ± 14.6 ^C	854.47 ± 16.79 ^A	802.77 ± 16.62 ^B	<0.0001
<i>OAS2</i>	795.99 ± 67.59 ^B	533.07 ± 76.48 ^C	1021.34 ± 75.67 ^A	<0.0001
<i>CCL2</i>	868.76 ± 9.98 ^A	846.85 ± 11.74 ^A	785.06 ± 11.63 ^B	<0.0001
<i>NFκB</i>	701.78 ± 31.58 ^B	951.54 ± 38.13 ^A	973.68 ± 37.88 ^A	<0.0001
<i>IL17A</i>	74.76 ± 89.79 ^C	303.45 ± 95.31 ^B	771.9 ± 94.56 ^A	<0.0001
<i>MX1</i>	539.09 ± 13.67 ^B	621.27 ± 16.51 ^A	581.08 ± 16.4 ^{AB}	0.001
<i>CSF2</i>	16.77 ± 25.81 ^C	166.01 ± 28.93 ^B	554.05 ± 28.62 ^A	<0.0001
<i>CCL3</i>	3.99 ± 12.32 ^C	57.53 ± 13.32 ^B	186.43 ± 13.2 ^A	<0.0001
<i>IFNγ</i>	6.44 ± 1.15 ^A	1.96 ± 1.3 ^B	3.14 ± 1.29 ^B	0.003
<i>IL4</i>	19.75 ± 2.33 ^A	5.15 ± 2.59 ^B	7.84 ± 2.56 ^B	<0.0001
<i>IL36RN</i>	-0.21 ± 1.81 ^C	0.81 ± 2.07 ^B	11 ± 2.04 ^A	<0.0001

¹ UC: Uncultured Controls; CC: Cultured Controls; LPS: Lipopolysaccharide² ABC denotes P < 0.05 between groups within each gene

no additional effect of LPS. Expression of *CCL4*, *TNF α* , *ESR1*, *IRF3*, *IL1 β* , *VEGFA*, *IL6*, *TLR4*, *CXCL2*, *ISG15*, *PTGER2*, *ESR2*, and *NF κ B* was the least in uncultured explants, with greater expression that did not differ between cultured controls and LPS explants ($P < 0.05$, Table 15). In contrast, *IFN γ* ($P = 0.003$) and *IL4* ($P < 0.0001$) had the greatest expression in uncultured explants, with lesser similar expression between cultured controls and LPS explants. Explants from the cultured control treatment group had the greatest expression of *CXCL8*, *TGFB*, *CSF3*, *PGR*, and *MX1* relative to the LPS and uncultured control groups ($P < 0.05$, Table 15). Six genes had increased expression in response to LPS including *IL10*, *IL1 α* , *IL17A*, *CSF2*, *CCL3*, and *IL36RN*, having the least expression in uncultured explants and greatest expression in LPS explants, with intermediary expression in cultured control explants ($P < 0.05$, Table 15). Conversely, LPS explants had lower expression of *IRF1* ($P = 0.005$) and *CCL2* ($P < 0.0001$) compared to uncultured and cultured control explants having greater similar expression (Table 15).

Estrous cycle phase affected the expression of genes *ESR1*, *ISG15*, *IL1 α* , *PTGER2*, *PGR*, *MUC1*, *MUC13*, and *NF κ B* ($P < 0.05$), and tended to affect *CXCL8* and *IL1 β* ($P = 0.09$; Table 16). All genes impacted by estrous cycle phase had greater expression in explants from the luteal than follicular phase (Table 16). The interaction of treatment*phase was observed for *IL1 α* ($P = 0.005$) and *PGR* ($P = 0.05$), with tendencies for *ISG15* ($P = 0.10$) and *NF κ B1* ($P = 0.07$). For *IL1 α* , the least expression was observed for uncultured explants of luteal and follicular phase, with the greatest expression in LPS explants of luteal and follicular phase. Similarly, luteal and follicular uncultured explants had the least expression of *PGR*. However, follicular LPS and cultured control explant's *PGR* expression did not differ from uncultured controls, but expression was higher in luteal cultured control explants than all other groups.

Table 16. Expression of genes affected by phase of the estrous cycle.

Gene	Estrous Cycle Phase		<i>P</i> Value ¹
	Luteal (n=48)	Follicular (n=40)	
<i>ESR1</i>	1573.44 ± 27.92 ^A	1449.07 ± 29.84 ^B	0.003
<i>CXCL8</i>	1039.29 ± 50.79	943.19 ± 52.27	0.09
<i>IL1β</i>	1112.56 ± 48.93	989.17 ± 51.19	0.09
<i>ISG15</i>	1017.12 ± 48.84 ^A	837.82 ± 50.59 ^B	0.01
<i>IL1α</i>	718.29 ± 33.29 ^A	607.44 ± 35.87 ^B	0.03
<i>PTGER2</i>	969.21 ± 14.25 ^A	923.66 ± 15.25 ^B	0.03
<i>PGR</i>	838.42 ± 15.24 ^A	772.94 ± 15.9 ^B	0.004
<i>MUC1</i>	561.75 ± 45.93	430.07 ± 48.09	0.05
<i>MUC13</i>	21.66 ± 0.62 ^A	19.3 ± 0.66 ^B	0.01

¹ ^{AB} denotes *P* < 0.05 between groups within each gene

Expression of genes that differed by uterine horn included *ESR1*, *IRF8*, *IL10*, *OAS2*, *NFκB*, *MUC1*, *MUC13*, *IFNγ*, and *MUC15* ($P \leq 0.05$), and tended to affect expression of *CCL4*, *IRF8*, *OAS2*, *NFκB*, and *VEGFA* ($P \leq 0.10$; Table 17). The ipsilateral horn had the greatest expression of all affected genes (Table 17). No interactions between treatment*horn were observed, but the interaction of phase*horn was detected for *ESR1* and *MUC13* ($P = 0.05$). The observed expression of *ESR1* and *MUC13* followed the same pattern, with greatest expression detected in the ipsilateral horn of luteal phase explants, with similar expression in the luteal contralateral horn and follicular ipsilateral horn. However, less expression of *ESR1* and *MUC13* was observed in the follicular contralateral horn ($P = 0.05$). No genes were affected by the triple interaction of treatment, phase, and horn ($P > 0.10$).

Discussion

Potentially pathogenic bacterial species are present in multiple microbial communities, including the uterine microbiome, without the presence of disease (Karstrup et al., 2017). However, various stimuli such as diet and external environment can influence the composition of the microbiome and lead to greater abundances of potentially pathogenic bacteria (Li et al., 2012; Monteiro and Faciola, 2020). Recently, shifts in the abundance of common pathogens in a healthy uterus through the estrous cycle were associated with influencing breeding success (Ault et al., 2019). The interaction of these bacteria with the uterine environment leading to changes in reproductive outcomes must be explored to better understand their role in reproductive efficiency. Therefore, the current study utilized LPS as a model for potentially pathogenic bacteria to evaluate its effects on the immunological response in the uterine endometrium between phases of the estrous cycle and across the uterine horns. Differences in the expression of LPS binding pattern

Table 17. Expression of genes affected by uterine horn.

Gene	Uterine Horn		<i>P</i> Value ¹
	Ipsilateral (n=42)	Contralateral (n=46)	
<i>CCL4</i>	1498.72 ± 41.97	1415.97 ± 40.06	0.10
<i>ESR1</i>	1550.4 ± 28.6 ^A	1472.11 ± 26.94 ^B	0.04
<i>IRF8</i>	1347.87 ± 50.08	1231.23 ± 47.49	0.07
<i>IL10</i>	1174.49 ± 71.58 ^A	984.86 ± 68.95 ^B	0.02
<i>OAS2</i>	844.16 ± 64.91	722.78 ± 62.45	0.09
<i>NFκB</i>	914.79 ± 30.34	836.54 ± 28.4	0.06
<i>MUC1</i>	556.05 ± 42.86 ^A	435.77 ± 40.89 ^B	0.02
<i>MUC13</i>	21.47 ± 0.65 ^A	19.49 ± 0.61 ^B	0.03
<i>IFNγ</i>	4.88 ± 1.11 ^A	2.81 ± 1.07 ^B	0.05
<i>MUC15</i>	9.85 ± 4.62 ^A	8.46 ± 4.42 ^B	0.02
<i>VEGFA</i>	0.14 ± 0.01	0.10 ± 0.01	0.06

¹ ^{AB} denotes *P* < 0.05 between groups within each gene

recognition receptor TLR4 and its transcription factor NF κ B1 were not detected in LPS treated explants. Previous studies have reported the ability of LPS to increase expression of TLR4, with other studies reporting minimal to no difference between control and LPS challenged tissues (Cronin et al., 2012; Swangchan-Uthai et al., 2012; Fu et al., 2013; Ding et al., 2020). The TLR4 receptors are present on endometrial tissue, including epithelial and stromal layers, and resident immune cells, such as macrophages, without LPS challenge in preparation for bacterial response (Herath et al., 2006; Dadarwal et al., 2017). Although tissues were selected based on negative signs of pregnancy and infection, level of TLR4 expression may be influenced by the state of the uterine microbiome, previous infection, or reproductive status prior to tissue collection.

No differences in the individual cytokine gene expression were detected in commonly LPS-stimulated cytokines *TNF α* , *IL6*, and *IL1 β* of the current study. The LPS concentration used has been validated by previous studies and shown to elicit a response (Dohmen et al., 2000; Herath et al., 2006), with responses to LPS occurring as early as 3 hours, up to 24 hours (Borges et al., 2012; Swangchan-Uthai et al., 2012; Fu et al., 2013). However, the early response to LPS may result in various patterns of cytokine concentrations. Concentration of *TNF α* peaked by 1 hour in bovine endometrial culture with LPS, while *IL1 β* did not reach peak concentration until 3 to 6 hours post LPS, with both decreasing up to 24 hours (Swangchan-Uthai et al., 2012; Fu et al., 2013). Other pro-inflammatory cytokines *IL1 α* and *IL17A*, and chemokine *CCL3*, however, had greater expression in LPS treated explants of the current study. Bovine endometrial epithelial cells treated with LPS indicated *IL1 α* to be the most upregulated pro-inflammatory cytokine gene, and one of the most upregulated genes among all immune response genes (Oguejiofor et al., 2015). A greater response of *IL1 α* than *IL1 β* and *IL6* relative to controls was observed in LPS treated endometrial

cells, suggesting IL1 α aids in upregulating the inflammatory response to LPS (Healy et al., 2014). The IL17 signaling pathway was also identified as a major inflammatory pathway in LPS stimulation (Ding et al., 2020). In addition to these increases in pro-inflammatory cytokine expression, anti-inflammatory cytokine *IL10* also had greater expression in explants treated with LPS. Anti-inflammatory cytokine expression increases in response to elevated pro-inflammatory cytokine concentrations to balance the level of inflammation, typically due to a transition from M1 to M2 macrophage activation (Dadarwal et al., 2017). An increased ratio of IL1a and IL1b to IL10 concentration in postpartum cows with uterine disease was associated with failure to rebreed (Herath et al., 2009). Stromal cells have indicated greater production of IL10 when exposed to LPS compared to controls and epithelial cells treated with LPS (Koh et al., 2018). Therefore, the culture of explants in the current study resulted in increased expression of pro-inflammatory cytokines in response to LPS, as well as upregulation of anti-inflammatory cytokine IL10 in an attempt to balance the level of inflammation.

Dominance of steroid hormones estradiol and progesterone characterizes estrous cycle phases to prepare the uterus for reproductive events such as pregnancy development and maintenance. As expected, differences in the gene expression of *PGR* and *ESR1* were observed between phases, with *PGR* expression highest during the luteal phase of the current study. The abundance of progesterone receptors on the endometrium epithelial cells peaks during mid-luteal phase with increasing progesterone secretion (Robinson et al., 2001; Okumu et al., 2010). Following mid-luteal phase, the progesterone receptors begin to decrease on the endometrium epithelial cells to prepare other responses for pregnancy development, but remain present in the stromal cells (Okumu et al., 2010; Kimmins and MacLaren, 2001). Additionally, *PTGER2*

expression was greater in luteal phase explants. Secretion of PGE₂ and its receptor *PTGER2* expression has previously shown to be greater during mid-diestrus when progesterone secretion is greatest, then decreases around the time of luteolysis (Arosh et al., 2003; Zhang et al., 2017). Therefore, the greater expression of *PGR* and *PTGER2* of the current study, in addition to careful selection of reproductive tracts and staging the estrous cycle of ovaries, may indicate successful selection of uteri in mid-luteal phase at high progesterone secretion. Due to the importance of controlling the maternal immune system response to prevent rejection of the fetus, progesterone dominance is typically classified as an anti-inflammatory period (Raghupathy et al., 2001; Schjenken et al., 2012). However, *IL1 α* , *IL1 β* , and *CXCL8*, which are classically categorized as pro-inflammatory, were detected to have greater abundance in luteal phase explants in the current study. High progesterone supplementation on bovine endometrial epithelial cells has indicated increases in pro-inflammatory cytokine expression (Cui et al., 2020). By estrous cycle stage, concentrations of *IL1 α* , *IL1 β* , and *CXCL8* indicated higher concentrations in early to mid-diestrus, but decreased by late di-estrus in non-pregnant animals, suggesting preparation for pregnancy beginning around the time of maternal recognition (Oliveira et al., 2013). Additionally, activation of *PTGER2* receptors stimulated production of *CXCL8* for endometrial epithelial cell proliferation in bovine endometrial explant culture (Zhang et al., 2017). Therefore, fluctuations of individual inflammatory cytokines occur throughout the estrous cycle, reaching an anti-inflammatory state by the time of pregnancy establishment. However, these differences across the cycle may indicate a greater susceptibility when exposed to stressors such as pathogenic bacteria and their LPS membrane component.

The inflammatory response to a stressor is not based on the expression of a single immune response mediator, but the cohesive actions of multiple cytokines, chemokines, and other immune response related proteins. Therefore, the overall profile of immune-related gene expression may be a better representation of the response to LPS and differences between estrous cycle phases. Interestingly, profiles grouped by estrous cycle phases but with similar gene expression of both control treatment groups, but distinct profiles were observed in response to LPS treatment between the follicular and luteal phase. The mucin-family genes, *MUC1* and *MUC13*, were affected by estrous cycle phase with greater expression in the luteal than follicular phase, and determined by VIP as the top contributors to differences in overall gene expression between phases, and differences in phase response to LPS. Mucins are anti-adhesive proteins that serve as a defense mechanism for the innate immune system, especially in the reproductive tract (Brayman et al., 2004). Greater expression of mucins has been associated with uterine disease and infertility, potentially preventing trophoblast attachment (Horne et al., 2005; Kasimanickam et al., 2014). Decreases in endometrium epithelial *PGR* expression during the late luteal phase, especially before maternal recognition of pregnancy in pregnant animals, is associated with reductions in mucin expression to prepare the uterus to allow embryo attachment and implantation (Carson et al., 2000; Spencer et al., 2006). As mucins are a surface protein in the uterine lumen, the greater expression of *PGR*, *MUC1*, and *MUC13* in luteal phase explants of the current study indicate mid-luteal phase uteri prior to the loss of *PGR* on endometrial epithelial cells. Although individual mucin expression was not affected by LPS treatment, OLPS-DA analysis indicated *MUC1* to contribute the most to the difference between luteal and follicular phase response to LPS. Additionally, pro-inflammatory *IL6* and *IFN γ* were important contributors to phase response to LPS, being higher in follicular

phase explants, although they did not differ in individual expression by treatment. Due to the protective role of mucins to bind bacteria and prevent invasion into uterine tissue (Brayman et al., 2004; Sando et al., 2009), mucin expression differences between estrous phases may be a major determinant in the total cytokine and other immune factor responses.

Greater progesterone concentrations have been previously observed in the cranial portion of the ipsilateral horn closest to the CL, compared to the contralateral horn, suggesting a hormonal gradient throughout the uterus potentially impacting the uterine environment in response to progesterone actions (Pope et al., 1982; Weems et al., 1988; Takahashi et al., 2016). Global gene expression has indicated greater variation between the contralateral and ipsilateral horns during the early estrous cycle that decreased towards the period of uterine receptivity to pregnancy (Sanchez et al., 2014). All mucins evaluated in the current study had greater expression in the ipsilateral than contralateral horn. The strong influence of steroid hormones on the expression of mucins potentially contributed to their observed differences by uterine horn, although divisions of each horn did not impact expression in the current study. In addition to mucins, multiple other genes were affected by explants taken from the contralateral and ipsilateral horn. The major angiogenic cytokine *VEGFA* was increased in the ipsilateral horn compared to the contralateral horn. Blood flow to the ipsilateral horn, especially in preparation for pregnancy is crucial to supply the early embryo with nutrients for survival (Ford et al., 1979). This change in *VEGFA* suggests vascular development cytokines begin to stimulate these changes in the estrous cycle of non-pregnant animals. Additionally, anti-inflammatory cytokine *IL10* and pro-inflammatory *IFN γ* had greater expression in the ipsilateral horn. Previous studies indicated altered response of the ipsilateral or contralateral uterine tissue to culture with a conceptus (Bages-Arnal et al., 2020).

Due to the importance of a controlled immune response for pregnancy development (Raghupathy et al., 2001), the difference in cytokine expression between uterine horns indicates the regulation necessary in the ipsilateral horn for pregnancy. Therefore, the majority of genes that differed between the contralateral and ipsilateral uterine horns are important for pregnancy establishment and maintenance.

In conclusion, estrous cycle phase resulted in differing overall endometrial gene expression profiles of immune response to LPS treatment. Multiple immune response genes differed between the follicular and luteal phases. Mucins are greatly impacted by the dominant steroid hormone present of each estrous cycle phase and their protective role during high abundance of the luteal phase likely impacts the resulting immune response to a stimulus such as LPS. However, overall gene expression did not differ between uterine horns and did not influence response to LPS treatment. Further research is needed to understand how these differences in estrous cycle response to LPS correlate to changes in the uterine bacterial communities and their direct effects on pregnancy establishment.

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CHAPTER IV: CONCLUSION

Bacterial communities play crucial roles in the production efficiency of cattle through both nutrition and reproduction. Recent developments have indicated bacterial communities in the uterus can impact on breeding success. Therefore, it is important to gain an understanding of the factors that affect their presence in the uterus and how they interact with the uterine environment to potentially affect pregnancy.

The first objective was to evaluate the effects of protein supplementation level on ruminal and uterine bacterial communities in heifers through puberty attainment. Differences were detected in the uterine and rumen bacterial communities with varying levels of protein supplementation, despite the resulting overall diet crude protein difference between groups was minimal. Interestingly, pubertal status of heifers impacted uterine bacterial communities, with differences observed in phyla and genera abundances. Pubertal heifers also had less variation with a more similar overall uterine bacterial community than pre-pubertal heifers. These differences by pubertal status, as well as the shifts in the taxa abundances of bacterial communities overtime may indicate maturation of the uterine and ruminal bacterial communities through development. The second objective was to evaluate the impact of bacteria, as modeled by LPS, on the uterine endometrium between the estrous cycle phases and uterine horns. Expression of genes related to immune response differed between the phases of the estrous cycle according to individual gene expression differences and overall gene expression profiles. Additionally, greater differences were observed in the overall gene expression profile between the luteal and follicular phase when treated with LPS compared to uncultured and cultured controls. Mucins were identified as major contributors to differences in these gene expression profiles and are greatly impacted by the

dominant steroid hormone present of each estrous cycle phase. Their protective role during high abundance of the luteal phase likely impacts the resulting immune response to a stimulus such as LPS. Although a few individual gene expression differences were detected between the contralateral and ipsilateral uterine horn, gene expression profiles did not differ and were not affected in response to treatment.

Together, these studies indicate the interaction of 1) nutrition and differences in diet, 2) the estrous cycle and its respective steroidal hormones, and 3) the uterine bacterial communities. Figure 21 depicts the overall proposed model from the results of these studies. In summary, Differences in diet and phases of the estrous cycle influences the nutrient and hormone composition of the uterine environment as well as endometrial tissue activity. The altered uterine environment may positively or negatively impact the balance of the resident uterine microbiome. Additionally, through the highly steroid hormone involved event of puberty attainment, bacterial community abundances may fluctuate in response to changes in uterine environment stimulated by hormones. If potentially pathogenic bacteria increase in abundance, specifically the presence of LPS, resident endometrial immune cells may be activated and release pro-inflammatory cytokines. Furthermore, the specific bacterial components present, such as LPS, influence the uterine environment and immune response differently depending on stage of the estrous cycle and hormone concentrations. Imbalances in cytokines may lead to a greater overall inflammatory environment, potentially affecting pregnancy establishment. Future studies are needed to understand 1) how nutrition and the overall diet may be used to manipulate uterine bacterial communities to benefit the uterine environment and future reproductive success, and 2) how differences in estrous cycle response to other bacterial components in addition to LPS correlate to

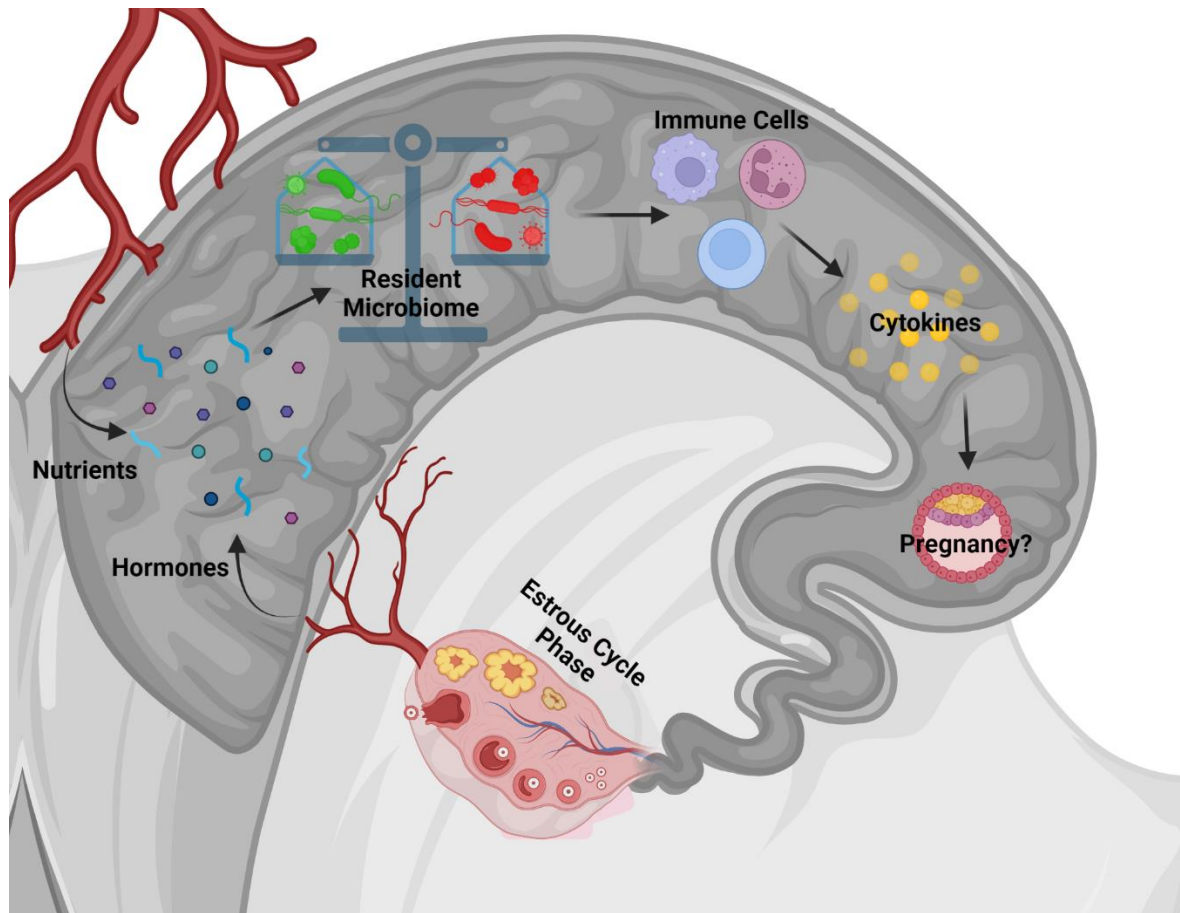


Figure 21. Overall proposed model of the interaction of nutrition and phases of the estrous cycle with uterine bacterial communities and immune response potentially impacting pregnancy establishment.

fluctuations in the uterine bacterial communities impacting pregnancy establishment. Therefore, enhanced understanding of the uterine bacterial communities can impact affect future reproductive success and lifetime reproductive efficiency of the bovine.

VITA

Taylor Brianne (Ault) Seay was born and raised in the Knoxville, TN area. Her passion for working with animals began with training and showing dogs at 12 years old. She had multiple accomplishments in Junior Handling and Breed Conformation rings as well as other dog performance events. Her love for working with dogs led her to the Animal Science program at the University of Tennessee, Knoxville. She loved the program and developed a passion for working with livestock animals. Taylor graduated Magna Cum Laude from the University of Tennessee, Knoxville with a Bachelor of Science in Animal Science and a Minor in Business Administration in May 2016. In August 2017, she began her Master's degree program in Animal Science with mentors Dr. Ky Pohler and Dr. Phillip Myer, focusing her research on animal reproductive physiology and completed all degree requirements in August 2018. In January 2019, she began her PhD studies at the University of Tennessee, Knoxville in Animal Science with mentors Dr. Kyle McLean and Dr. Phillip Myer to continue her research evaluating bacterial communities of the reproductive tract and rumen in beef heifers.